Intersection of Biotic and Abiotic Sulfur Chemistry Supports Extreme Microbial Life in Hot Acid

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Intersection of Biotic and Abiotic Sulfur Chemistry Supports Extreme Microbial Life in Hot Acid

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Abstract: Microbial life on Earth exists within wide ranges of temperature, pressure, pH, salinity, radiation, and water activity. Extreme thermoacidophiles, in particular, are microbes found in hot, acidic biotopes laden with heavy metals and reduced inorganic sulfur species. As chemolithoautotrophs, they thrive in the absence of organic carbon, instead using sulfur and metal oxidation to fuel their bioenergetic needs, while incorporating CO_2 as a carbon source. Metal oxidation by these microbes takes place extracellularly, mediated by membrane-associated oxidase complexes. In contrast, sulfur oxidation involves extracellular, membrane-associated and cytoplasmic biotransformations, which intersects with abiotic sulfur chemistry. This novel lifestyle has been examined in the context of early aerobic life on this planet, but it is also interesting when considering the prospects of life, now or previously, on other solar bodies. Here, extreme thermoacidophily (growth at pH below 4.0, temperature above 55°C), a characteristic of species in the archaeal order Sulfolobales, is considered from the perspective of sulfur chemistry, both biotic and abiotic, as it relates to microbial bioenergetics. Current understanding of the mechanisms involved are reviewed which are further expanded through recent experimental results focused on imparting sulfur oxidation capacity on a natively non-sulfur oxidizing extremely thermoacidophilic archaeon, *Sulfolobus acidocaldarius*, through metabolic engineering.

Introduction

Sulfur is among the most abundant elements on Earth, ranking even above carbon, making up 5.4% of Earth's mantle and crust[1]. Sulfur-rich environments have also been identified on Mars, leading to theories that the planet as a whole is more sulfur-rich than Earth[2]. Sulfur has a similar electronegativity to carbon and exhibits oxidation states anywhere from -2 to +6. Because of this, the element exists in numerous electron-dense species, ranging from metal-rich minerals and ores to gasses and fumes from hydrothermal vents. These diverse chemical species encompass a wide range of Gibbs free energies of formation (**Figure 1**), which are frequently exchanged through the sulfur cycle. Another key feature of sulfur chemistry is the polymeric structure arising from sulfur-sulfur bonds. These long chains exhibit an overall oxidation state of -2 and act as the site of attack for more nucleophilic species[3]. This enables chain-lengthening and chain-shortening reactions, thereby generating polysulfides, cyclized sulfur[4], polythionates, and sulfane monosulfonate intermediate species of varying chain length[5].

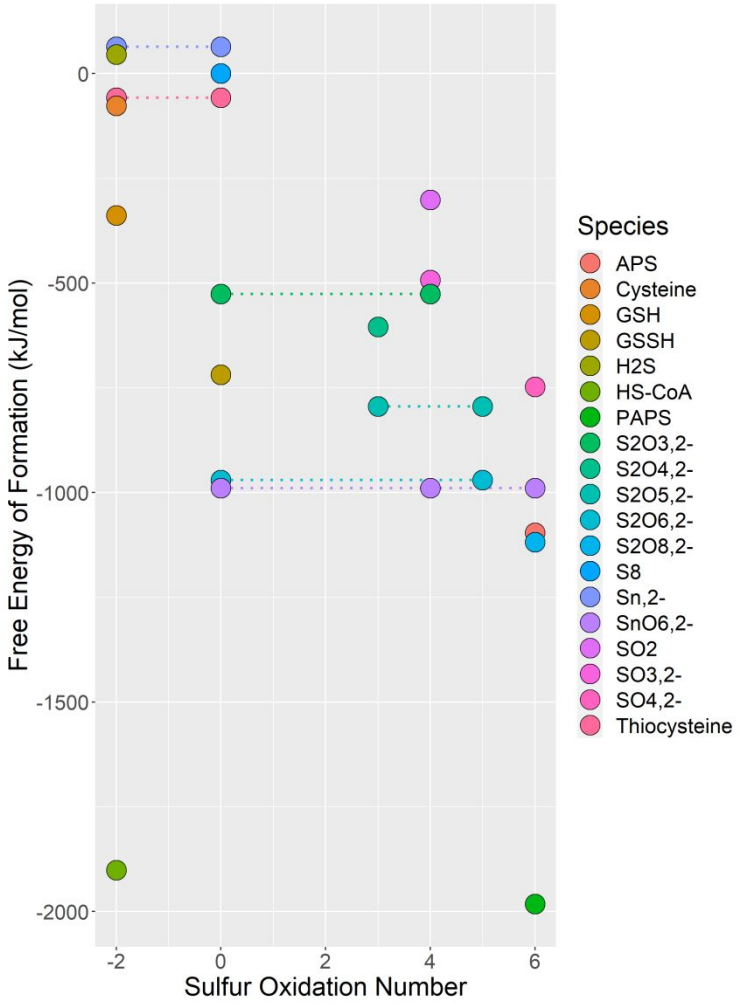

Figure 1: Distribution of oxidation states and ΔG_f^0 of various sulfur species; APS: adenylyl sulfate, GSH: glutathione, GSSH: glutathione disulfide, PAPS: phosphoadenylyl sulfate; dotted lines indicate multiple sulfur oxidation states within the same molecule

Sulfur's multiple oxidation states and stepwise depolymerization potential means that sulfur can be incrementally oxidized through intermediate species in order to maximize energy conservation, much like the stepwise degradation of glucose that is characteristic of cellular metabolism. The opportunity presented by these energy-rich sulfur species has not been overlooked in nature. Prokaryotes in domains Bacteria and Archaea oxidize reduced inorganic sulfur compounds (RISCs) to elemental sulfur (S^0) or sulfate for phototrophic or chemolithotrophic growth. Indeed, the biological oxidation of sulfur plays a major role in the sulfur cycle on Earth[6]. The diversity of sulfur species identified on Mars suggest the possibility that a similar mechanism of sulfur cycling occurred at some point on this planet, and recent thermodynamic analyses show that a chemolithoautotrophic metabolism could be supported even in the limited Martian atmosphere[7]. Thus, a deeper understanding of the primitive and more extreme terrestrial forms of life on Earth could provide clues towards the possibility of extraterrestrial life.

While sulfur-oxidizing Bacteria span a wide range of genera, the sulfur-oxidizing Archaea belong exclusively to the order Sulfolobales[6]. The order consists entirely of extremely thermoacidophilic species, with optimal temperatures greater than 55°C and pH optima less than 4. Not all extremely thermoacidophilic archaea oxidize sulfur[8]. In fact, these microbes exhibit physiologies ranging from facultative anaerobes, capable of sulfur oxidation and reduction (*Acidianus ambivalens*[9]), to aerobic chemolithautotrophs, leveraging sulfur oxidation and iron oxidation (*Sulfuracidifex metallicus* [10] and *Metallosphaera sedula* [11], respectively), to obligate heterotrophs (*Saccharolobus solfataricus*[12]). There is some evidence that certain Sulfolobales may even be able to oxidize vanadium and molybdenum for energetic benefit[13]. A single obligate anaerobe is now part of the order (*Stygiolobus azoricus*[14]), and recently a sulfur-inhibited *Sulfolobales* member has been described (*Sulfodiicoccus acidiphilus*[15]). In all, the order *Sulfolobales* now contains more than twenty distinct species, with some isolates still awaiting classification. Among the most studied organisms in the order is *Sulfolobus acidocaldarius*, the

first isolated species of this order[16]. While *S. acidocaldarius* was initially believed to be a sulfur oxidizer, the current lab-cultured strain does not have this capability[17]. It is, however, one of the few Sulfolobales with a tractable genetic toolkit that can be used for mutational analysis and metabolic engineering[18].

Because of the lack of genetic tools for the Sulfolobales, most efforts to understand sulfur oxidation in the order have been focused on characterization of individual enzyme activities[19-23] and comparative "omics" analyses to relate what is known about bacterial enzymes to the archaeal Sulfolobales[24-27]. These approaches offer snapshots of the overall landscape of biological sulfur oxidation that involves a complex web of both abiotic and enzymatic reactions. Recently, efforts to engineer some of these enzymes into *S. acidocaldarius* have begun[17]. Engineering *S. acidocaldarius* to become a sulfur-oxidizer demonstrates and validates an understanding of the sulfur oxidation mechanism in the Sulfolobales and also presents interesting opportunities for biotechnological application. Here, we examine the abiotic and enzymatic reactions implicated in sulfur oxidation and evaluate the prospects for energy conservation from these reactions. Furthermore, evidence of energy conservation in an engineered strain of *S. acidocaldarius* supports the prospect that energy conservation through the coordination of biotic and abiotic sulfur chemistry is indeed possible.

METHODS

Cultivation of S. acidocaldarius Strains

All strains of *S. acidocaldarius* were grown in 125 mL serum bottles containing 1 g/L NZ Amine and 0.01 g/L uracil in Brock Salts (DSM medium #88 without yeast extract), which contains on a per liter basis: 1.3 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 4.5 mg $Na_2B_4O_7 \cdot 7H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.22 mg $Na_2MoO_4 \cdot 2H_2O$, 0.05 mg $CuCl_2 \cdot 2H_2O$, 0.03 mg $VOSO_4 \cdot 2H_2O$, and 0.01 mg $CoSO_4 \cdot 7H_2O$. The pH of the Brock Salts

was adjusted to 3.0 using concentrated sulfuric acid. All cultures were incubated in Eppendorf air shakers at 75°C with agitation (150 rpm). Except for the cultures used in the growth curve measurements, *S. acidocaldarius* media also contained 2 g/L sucrose. Cultures used to evaluate *S. acidocaldarius* growth on sulfur were provided with 10 g/L elemental sulfur.

Transformation of Free Energy of Formation Data

Free energy of formation data were collected from several sources to cover the range of chemical species needed for analysis. All data were examined at 25°C for consistency between sources. For inorganic compounds and sulfur species, free energy of formation data were taken from Amend and Shock, with multiple protonation states where possible[28]. Polysulfide data for n = 2-8 was collected from Kamyshny *et al*., again using all protonation states of polysulfide chains[29]. Organic molecules, including biological energy carriers and intermediates of glucose metabolism, were calculated by the eQuilibrator online database at standard state. Again, all available protonation states for each compound were used[30]. Three energy carriers specific to *S. acidocaldarius* were used to evaluate redox coupling of sulfur reactions. Because of their uniqueness, free energy of formation data were not available in all cases. Instead, experimental reduction potential was used for caldariellaquinone[31] and the [3Fe-4S] and [4Fe-4S] ferredoxins[32] from *S. acidocaldarius*.

Free energy values were adjusted for ionic strength, pH, and protonation state according to the methods laid out by Alberty[33]. Briefly, free energy values for all protonation states were adjusted for ionic strength according to:

$$\Delta G_f^0(i,I) = \Delta G_f^0(i,I=0) - \frac{2.91482 z_i^2 I^{1/2}}{1 + BI^{1/2}} \qquad \text{[EQN 1]}$$

For these calculations, the adjusted ionic strength was I=0.338 (the ionic strength of the Brock Salts medium), z_i is the charge number of species *i*, and B = 1.6 $L^{1/2}mol^{-1/2}$. ΔG_f^0 is in units of

kJ/mol. The transformed free energy was then calculated at pH intervals of 0.5 from 0.5 to 14 according to:

$$\Delta G'^{0}_{f}(i) = \Delta G^{0}_{f}(i) - N_H(i)\{\Delta G^{0}_{f}(H^+) + RT\ln[H^+]\} \quad \text{[EQN 2]}$$

where $N_H(i)$ is the number of hydrogen atoms in species *i* and $\Delta G_f^0(H^+)$ = 0 kJ/mol, as given by Amend and Shock[28]. Finally, the various protonation states of a single species were aggregated as a 'pseudoisomer' group according to:

$$\Delta G'^{0}_{f}(iso) = -RT\ln\left\{\sum exp\left(\frac{-\Delta G'^{0}_{f}(i)}{RT}\right)\right\} \quad \text{[EQN 3]}$$

This aggregate transformed free energy accounts for the dominant protonation state of a species at a particular pH, and so the pseudoisomer group was calculated separately for each pH interval. Free energy of reaction was then evaluated using these transformed pseudoisomer groups as:

$$\Delta G'^{0}_{rxn}(pH, I = 0.338) = \sum \Delta G'^{0}_{f,prod}(iso,pH,I = 0.338) - \sum \Delta G'^{0}_{f,react}(iso,pH,I = 0.338) \quad \text{[EQN 4]}$$

To calculate reduction potential, half-reactions of sulfur transformations and energy carrier reduction were generated. Transformed free energy of reaction for these half-reactions was converted to transformed reduction potential by:

$$E'^{0}(pH,I = 0.338) = -\frac{\Delta G'^{0}_{rxn}(pH,I = 0.338)}{nF} \quad \text{[EQN 5]}$$

where *n* is the number of electrons, F is the Faraday constant as 96.485 kJ $(V\ mol)^{-1}$, and E'^0 has units of V. For each half-reaction, the equilibrium limits of the half-reaction were based on maximum and minimum physiological concentrations of 10 mM and 1 μM for reactants and products[34].

S. acidocaldarius Growth in Batch Cultures

S. acidocaldarius cultures were started from freezer stocks and grown with sucrose present, as described above. Cultures were passaged twice into fresh media upon reaching an OD600 value of 0.5-0.8. Cultures were passaged a final time into media containing no sucrose and some containing 10 g/L elemental sulfur to measure growth. One mL samples were taken

periodically from serum bottles and transferred to plastic cuvettes. Samples were allowed to settle for one min prior to measuring OD600 spectrophotometrically.

RESULTS AND DISCUSSION

Intersection of RISC Biology and Chemistry

The role of abiotic sulfur chemistry in biological sulfur oxidation is clearly evident in mining environments, where the breakdown of sulfidic ore is facilitated by acidophilic iron- and sulfur-oxidizing microbes. The well-studied mechanisms of this process reveal that microbes do not directly act on the ores; instead, the ore undergoes abiotic attack by protons and ferric iron[35]. The role of microbes in this scenario is to regenerate protons and ferric iron through sulfur and iron oxidation, respectively. Here, abiotic and biotic reactions act synergistically; the abiotic degradation of ores provides the microbes with an energy source for growth, and the byproducts of the microbes' metabolism accelerates ore dissolution. At the same time, the sulfur liberated from this process undergoes numerous abiotic reactions to generate a diverse pool of sulfur species and drives acidification. While mesoacidophilic bacteria play the dominant role biomining environments[36], high temperature biomining applications are often comprised of *Acidianus* and *Metallosphaera* spp.[37] and the bioleaching capabilities of the Sulfolobales have been investigated in laboratory settings[38].

Biological Sulfur Oxidation in the Sulfolobales

Oxidation of RISCs is a complex process that spans the extracellular space, the cell membrane, and the cytoplasmic space **(Figure 2)** and, while it has been extensively studied in mesoacidophiles, the thermoacidophilic mechanism of sulfur oxidation is less clear. Recent sequencing of Sulfolobales' genomes has enabled a comparative genomic analysis of relevant sulfur oxidation genes in an effort to identify the core constituents of the thermoacidophilic variant of sulfur oxidation, based on the established phenotypes of the Sulfolobales **(Table 1)**[25, 39]. A

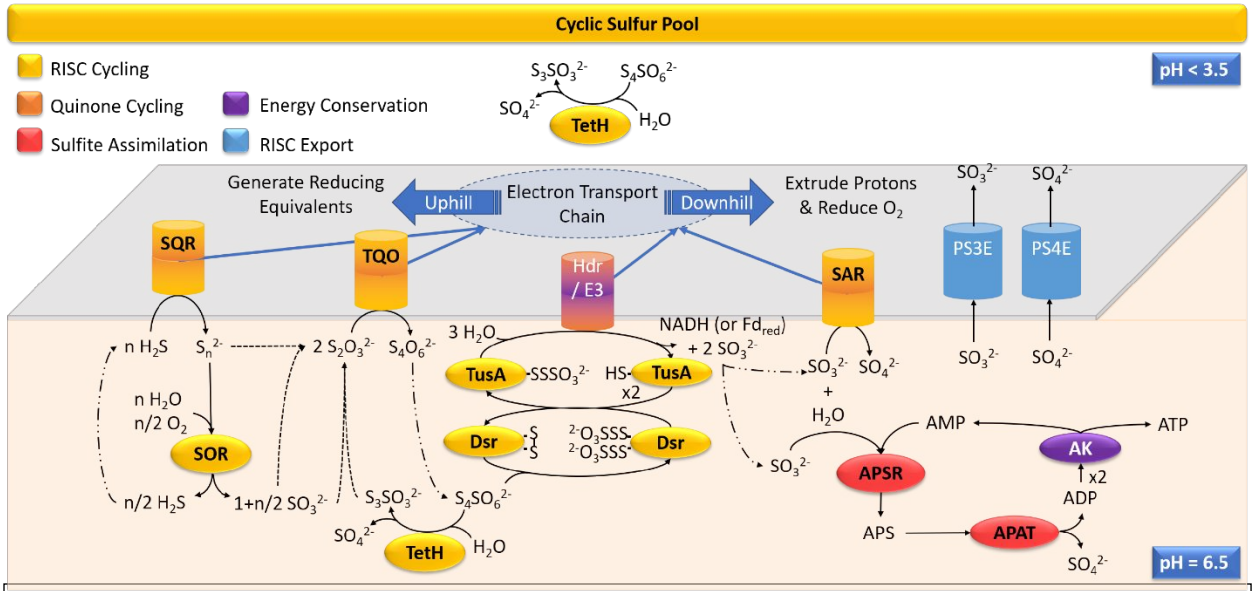

Figure 2: Schematic of Sulfolobales enzymes involved in sulfur oxidation; solid lines indicate enzymatic reactions, dashed lines indicate abiotic formation of thiosulfate, dashed-dotted lines indicate a shared sulfur species between reactions, blue arrows indicate the movement of quinones; Gray barrier represents the cell membrane, with cytoplasmic space below the barrier and extracellular space above; enzymes are colored according to their functional associations: cycling of RISCs (yellow), cycling of quinones (orange), assimilation of SO_3^{2-} (red), direct energy conservation through ATP or NAD(P)H (purple), and export of RISCs (blue); enzymes with multiple functional associations have a color gradient

major component of mesoacidophilic sulfur oxidation is the thiosulfate cycle catalyzed by a membrane-associated protein complex, SoxACBDXYZ [40]. However, this cycle is not present in thermoacidophilic organisms. Instead, the central enzyme in sulfur oxidation by the Sulfolobales is the sulfur oxygenase reductase (SOR), which disproportionates zero-valent sulfur into H_2S and SO_3^{2-} [22]. A 24-subunit homomeric cytoplasmic protein, SOR requires no cofactors and is inhibited by zinc ions [41]. An indirect product of this enzyme is thiosulfate ($S_2O_3^{2-}$), which is generated by an abiotic reaction of H_2S and SO_3^{2-} [42]. SOR is only expressed under aerobic conditions, possibly the result of its hypothesized oxygen-dependent reaction mechanism that involves polysulfide chains as the substrate for SOR rather than elemental sulfur [43].

The hot acidic environment of the Sulfolobales is particularly hostile towards secreted or surface-bound proteins. Therefore, it is notable that one such enzyme, tetrathionate hydrolase (TetH), is involved in sulfur oxidation. In the thermoacidophilic, sulfur-oxiding facultative anaerobe *Acidianus ambivalens*, the majority of TetH activity was located extracellularly, and the isolated

Table 1: Distribution of Sulfur Oxidation Genes in the Genome-Sequenced Sulfolobales

	Protein of Interest	Protein Cluster †	Protein Cluster Presence in Sequenced *Sulfolobales*																										
						Fox			Fox				Fox					Fox												
			Sred	Sred & Sox			Sox		Sred & Sox				Sox				Sred & Sox	Sox	‡										
			Autotrophy						Autotrophy & Heterotrophy								Heterotrophy							ND					
			Sazo	Aamb	Ainf	Asul	Smet	Abri	Acop	Aman	Ahos	Mcup	Mhak	Msed	Myel	Sohw	Stok	Ssol	Sisl HVE10/4	Sisl LD85	Sisl M162	Saci	Arsu	Ssp A20	Ssp E51F	Step	Sdac
Sulfur Enzymes with Direct Action	SQR	17768	X	XX	XX	XX	X	XX	X	XX	XX	X	X	X	X	X	X	X	X	X	X	-	X	X	X	X	X
	TQOa	17578	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	X	X	-	X	X
		17852	-	X	X	X	-	X	-	X	X	-	-	-	-	-	-	-	X	X	X	-	-	XX	-	X	-	-
	TQOb	17579	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	X	X	-	X	X
		17853	-	X	X	X	-	X	-	X	X	-	-	-	-	-	-	-	X	X	X	X	-	XX	-	X	-	-
	TetH	622	-	X	X	X	X	XX	XX	X	X	X	X	X	X	X	X	X	-	-	-	-	-	-	-	-	XX	-
	SOR	18472	-	X	X	X	X	X	X	X	X	-	-	-	-	X	X	-	-	-	-	-	X	-	-	-	X	-
	SAOR	1361	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	X	X	X	X	X
	PS3E	17110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	-	X	X	-	X	-
	APSRa																											
	APSRb	15	X	X	X	X	X	XX	X	XXX	X	XX	X	XX	X	XX	XX	X	XX	XX	XX	X	-	X	XX	X	X
	APAT AK ATPS																											
Sulfur Reduction	SreA	18679	X	X	X	X	-	X	-	X	-	-	-	-	X	X	-	X	X	X	X	-	X	-	X	-	-
	SreB	18973	X	X	X	XX	X	X	X	X	X	X	X	X	XX	XX	X	X	XX	XX	XX	X	X	X	X	-	-
	SreC	18251	X	X	X	X	-	X	-	X	-	-	-	-	X	X	-	X	X	X	X	-	XX	-	X	-	-
	SreD	17628	X	X	X	XX	X	X	X	X	X	X	X	X	XX	XX	X	X	XX	XX	XX	X	XX	X	-	-	-
Heterodisulfide Reduction System	HdrA	18047	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrB1	18048	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrC1	18049	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrB2	18044	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrC2	18045	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	TusA	18050	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	XXX	XX	-	XX	X	X	XX	XX	X	X
	DsrB(E2)	18051	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	DsrE3A	18052	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	DLD (E3)	18053	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	GSSH	18747	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LbpM1	18761	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LbpM2	18762	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LplA	18763	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LbpA	18765	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
Fox Cluster	FoxA	708	-	X	-	X	X	X	X	X	-	-	XXX	XX	XX	X	X	-	-	-	-	-	-	-	-	X	X
	FoxB	715	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxC	5690	-	-	-	X	-	-	X	X	-	-	X	X	X	-	X	-	-	-	-	-	-	-	-	X	X
	FoxD	717	-	X	-	X	X	X	X	X	-	-	X	X	-	X	X	-	-	-	-	-	-	-	-	X	X
	FoxE	710	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxF	709	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxG	707	-	X	-	X	X	X	-	X	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	X	-
	FoxH	706	-	X	-	X	X	X	X	X	-	X	X	X	X	X	X	-	-	-	-	-	-	-	-	X	-
	FoxI	22019	-	-	-	-	-	-	-	-	-	-	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-
	FoxJ	712	-	XX	X	X	X	X	X	X	X	-	X	X	X	XX	XX	-	X	X	X	-	-	X	X	X	X
	FoxV	711	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxW	716	-	X	-	X	X	X	X	X	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	X	X
	FoxY	714	-	X	-	X	X	X	-	X	-	-	X	X	-	X	-	-	-	-	-	-	-	-	-	X	X
	FoxZ	713	-	X	-	X	X	X	X	X	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	X	X

† Protein cluster numbers are based on comparative genomic analysis from Counts *et al.* (2020)[25]

‡ Phenotypes of Sulfolobales species are based on the assessment presented in Wheaton *et al.* (2015)[36]; species excluded from this assessment labeled as "ND"

"X" indicates that the genome contains a protein homologous to the particular protein cluster. Multiple "X's" indicate paralogs in that genome

protein had a pH optimum of 1[23]. In *A. ambivalens*, growth on tetrathionate increased transcription of the TetH gene compared to growth on elemental sulfur[17]. Homologous genes in a related species, *Acidianus brierleyi*, however, showed significant increase in transcription when grown on elemental sulfur compared to yeast extract[17]. While TetH is likely essential for growth on tetrathionate, it appears that it also plays a role in elemental sulfur oxidation.

Enzymes Coupled to the Electron Transport Chain

While SOR is central to the diversification of sulfur species within the cytoplasm, it is not directly coupled to energy conservation. Instead, the various RISCs associate with a range of membrane-bound quinone oxidoreductases to transfer electrons into the Electron Transport Chain (ETC). Thiosulfate:quinone oxidoreductase (TQO) was the first of these complexes identified in *A. ambivalens*, and it is responsible for oxidizing thiosulfate to tetrathionate [20]. By coupling sulfur oxidation to the ETC, the cell powers proton pumping and avoids the acidifying effect of SOR disproportionation. In fact, an engineered strain of *S. acidocaldarius* with a heterologous SOR was capable of oxidizing sulfur, but this ultimately proved to be toxic to the organism[17]. Upon the insertion of a gene encoding a heterologous TQO to the *S. acidocaldarius* mutant, normal growth was restored while maintaining the capacity to oxidize sulfur[17]. This *S. acidocaldarius* strain containing SOR and TQO was ultimately designated *Saci* RK34. TQO connects one product of the SOR disproportionation reaction to the ETC, and two other membrane-bound oxidoreductases serve similar functions for H_2S and SO_3^{2-}. The enzyme responsible for H_2S oxidation, sulfide:quinone oxidoreductase (SQR), was originally described as a novel type of NADH dehydrogenase[44]. However, further investigation revealed that NADH dehydrogenase activity was only possible with a truncated version of the enzyme. When the full protein sequence was intact, the additional amino acid chain on the C-terminus blocked the binding site for NADH. Instead, the enzyme assembles polysulfide chains from individual H_2S

monomers[19]. SQR reduces a quinone for each H_2S molecule added to the polysulfide chain, significantly increasing the energy conserved from a single elemental sulfur moiety. The proposed mechanism of SQR implicates two cysteine residues, which form a persulfide bond with incoming H_2S and build up a polysulfide chain between the two residues. Ultimately, an incoming H_2S molecule kicks out the assembled polysulfide chain and takes its place between the two cysteines, triggering the formation of a new polysulfide chain[45]. SO_3^{2-} is coupled to the quinone reduction by a putative sulfite:acceptor oxidoreductase (SAOR). While activity for this enzyme has been detected in aerobically grown *A. ambivalens*, it has not been linked to a coding region of the *A. ambivalens* genome[21]. Note that a similar enzyme, sulfite dehydrogenase, has been characterized in the mesoacidophile *Thiobacillus denitrificans*, although the electron acceptor in this case is cytochrome *c* [46].

Energy-Conserving Enzymatic Reactions

The final oxidation of SO_3^{2-} to SO_4^{2-} can occur in the cytoplasm as well, where it is directly coupled to the phosphorylation of ADP. Two routes of cytoplasmic sulfite oxidation exist, although they share a common first step. Initially, adenosine-5'-phosphosulfate reductase (APSR) catalyzes the reaction of sulfite with AMP to generate adenosine-5'-phosphosulfate (APS). The sulfate group of APS is then cleaved by either ATP sulfurylase with pyrophosphate to generate ATP or adenylylsulfate:phosphate adenylyltransferase (APAT) with phosphate to generate ADP. In the case of the latter reaction, two molecules of ADP are converted to ATP and AMP by an adenylate kinase (AK). A number of sulfur oxidizing organisms use both the cytoplasmic and membrane pathways for sulfite oxidation, and this is thought to increase the rate of generation of reducing equivalents [47]. Interestingly, the cytoplasmic path involving APS also operates in the reverse direction for the assimilatory reduction of sulfate [48]. It is only in this reductive function that the ATP sulfurylase and APAT/AK paths have been observed simultaneously. In oxidative organisms, the APAT/AK path dominates [47], although instances of the ATP sulfurylase have been

observed [49]. This observation is consistent with the limited work on sulfite oxidation in the *Sulfolobales*. Much like the membrane-bound SAOR, activity for the APAT/AK route has been observed in *A. ambivalens*, but no further characterization of the enzymes has been reported. ATP sulfurylase activity was investigated in the same experiment, but no activity was detected[21].

The most recent insights into *Sulfolobales* sulfur oxidation involves the highly conserved *hdr*/*dsr*/*tusA* locus. In mesoacidophilic sulfur oxidation, this complex has been linked to the oxidation of organic persulfides, namely glutathione, but also extended to sulfur-containing organic molecules, like dimethyl sulfide (DMS)[50, 51]. This complex is proposed to conserve energy through reduction of ETC electron carriers, although further experimentation is needed to confirm this function [52]. This is supported by the transmembrane domain contained in the HdrC subunit[51]. Comparative genomic analysis has identified homologs to the *hdr*/*dsr*/*tusA* complex in all genome-sequenced *Sulfolobales*[17]. However, characterization of the DsrE3A and TusA enzymes in *Metallosphaera cuprina* have shown activity on tetrathionate rather than organic persulfides [53]. As a result, the Dsr/TusA system serves to cycle tetrathionate and thiosulfate in conjunction with TQO, while also funneling polythionates towards total oxidation through formation of sulfite by Hdr. Despite these insights into substrate preference, the electron acceptor of the Hdr complex is still not clear. In the DMS-degrading *Hyphomicrobium denitrificans*, a lipoate-binding protein was found to be associated with the Hdr complex. The complex reduced the cyclic sulfur bond of lipoic acid, while oxidizing thiosulfate to sulfite. The reduced dihydrolipoic acid can power NAD^+ reduction with the E3 subunit of pyruvate dehydrogenase[24]. This newly proposed function of the *hdr*/*dsr*/*tusA* complex provides a direct route to reducing power for sulfur metabolism. However, the presence of this pathway in non-chemolithotrophic *Sulfolobales* (see **Table 1**) calls into question whether the complex serves to provide energy to the cell or detoxify cytoplasmic RISCs by oxidation. The synthesis of the lipoic acid cofactor could shed some light on Hdr function in the *Sulfolobales*. While homologs of the LipB-catalyzed lipoate synthesis mechanism were identified in a number of non-sulfur oxidizing *Sulfolobales*, *S. tokodaii* (the only sulfur oxidizer investigated)

appeared to only be capable of using exogenous lipoate scavenging [24]. Further understanding of lipoate synthesis in the *Sulfolobales* could clarify the role of the Hdr complex in thermoacidophilic sulfur oxidation.

Involvement of Abiotic RISC Reactions in Biology

Solid elemental sulfur is mainly orthorhombic and has limited solubility in water[54]. Ring-opening reactions typically involve nucleophilic attack by cyanide, bisulfide, or sulfite[5]. In the case of attack by bisulfide, polysulfide chains are generated[55]. Under alkaline conditions, polysulfides reach an equilibrium distribution of chain length[29]. As pH decreases, the reverse reaction takes place, with H_2S undergoing radical chemistry to build polysulfide chains; ultimately, the chain attacks itself, cleaving off a closed sulfur ring from the chain[56]. Similarly, sulfite attack on a sulfur ring creates a linearized chain of sulfur, this time in the form of sulfane monosulfonate ($S_nO_3^{2-}$)[5]. Further attack by sulfite leads to the stepwise release of thiosulfate from the chain, resulting in the total conversion of cyclic sulfur to thiosulfate[3]. Much like the polysulfide mechanism, as pH decreases, the reaction runs in reverse and acidified thiosulfate leads to the formation of sulfur rings[57].

Sulfane monosulfonates are highly unstable in water[5], but the combining of these chains, or direct oxidation of a single chain by diatomic oxygen, gives rise to polythionates[56, 58]. Polythionates are more stable than their sulfane monosulfonate precursors and are often present in fairly high concentration in native environments of the Sulfolobales[59]. However, polythionates are subject to hydrolysis, resulting in the release of sulfate from the chain and reformation of the sulfane monosulfonate species. Polythionates are also subject to attack by bisulfide, producing thiosulfate and polysulfide, even though polysulfide is often represented as elemental sulfur (S^0)[5, 56, 59]. While the exact reaction mechanism is not clear, a possible explanation is the sequential attack of bisulfide releasing thiosulfate from the polythionate chain, thereby forming sulfane monosulfonate first, before subsequent bisulfide attack forms just polysulfide.

Table 2: Summary of Key Abiotic RISC Reactions

Reaction #	Reaction	Representation in Reaction
[Rxn 1]	$H_2O + S_nO_6^{2-} \rightarrow S_{n-1}O_3^{2-} + SO_4^{2-} + 2\,H^+$	Yes[58-60]
[Rxn 2]	$H_2S + 2\,O_2 \rightarrow SO_4^{2-} + H^+$	Yes[60]
[Rxn 3]	$SO_3^{2-} + S_n^0 \rightarrow S_{n+1}O_3^{2-}$	Yes[3]
[Rxn 4]	$SO_3^{2-} + S_nO_3^{2-} \rightarrow S_{n-1}O_3^{2-} + S_2O_3^{2-}$	Yes[3]
[Rxn 5]	$HS^- + S_n^0 \rightarrow S_{n+1}^{2-} + H^+$	Yes[5, 35, 56, 60, 61]
[Rxn 6]	$SO_3^{2-} + S_n^{2-} \rightarrow S_{n-1}^{2-} + S_2O_3^{2-}$	Yes[3, 5]
[Rxn 7]	$SO_3^{2-} + S_2^{2-} + 2\,H^+ \rightarrow H_2S + S_2O_3^{2-}$	Yes[3, 5]
[Rxn 8]	$S_n^{2-} + {}^3/_2O_2 \rightarrow S_2O_3^{2-} + S_{n-2}^0$	Yes[56, 60]
[Rxn 9]	$S_nO_6^{2-} + SO_3^{2-} \rightarrow S_{n-2}O_3^{2-} + S_3O_6^{2-}$	Yes[3]
[Rxn 10]	$S_nO_6^{2-} + SO_3^{2-} \rightarrow S_2O_3^{2-} + S_{n-1}O_6^{2-}$	Yes[5, 59, 61, 62]
[Rxn 11]	$S_nO_3^{2-} + S_mO_6^{2-} \rightarrow S_{n-1}O_3^{2-} + S_{m+1}O_6^{2-}$	Yes[58]
[Rxn 12]	$2\,SO_3^{2-} + O_2 \rightarrow 2\,SO_4^{2-}$	Yes[63]
[Rxn 13]	$S_nO_3^{2-} + S_mO_3^{2-} + {}^1/_2O_2 + 2\,H^+ \rightarrow S_{n+m}O_6^{2-} + H_2O$	Yes[58, 59]
[Rxn 14]	$S_nO_3^{2-} + S_mO_3^{2-} + 2\,Fe^{3+} \rightarrow S_{n+m}O_6^{2-} + 2\,Fe^{2+}$	Yes[56, 58]
[Rxn 15]	$S_nO_3^{2-} + {}^3/_2O_2 \rightarrow S_nO_6^{2-}$	Yes[58]
[Rxn 16]	$S_nO_6^{2-} + HS^- \rightarrow S_{n-1}O_3^{2-} + S_2O_3^{2-} + H^+$	Yes*
[Rxn 17]	$S_nO_3^{2-} + HS^- \rightarrow S_{n-1}^{2-} + S_2O_3^{2-} + H^+$	Yes*
[Rxn 18]	$S_n^{2-} + H_2S + 2\,Fe^{3+} + 2\,H_2O \rightarrow S_{n+1}^{2-} + 2\,Fe^{2+} + 2\,H_3O^+$	Yes+
[Rxn 19]	$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 2\,SO_4^{2-} + 8\,Fe^{3+} + 10\,H^+$	Yes
[Rxn 20]	$S_n^{2-} + S_m^{2-} \rightarrow S_{n-1}^{2-} + S_{m-1}^{2-}$	Yes[4, 5, 56]
Overall Reactions Summarized by Rxns [1-20]	$S_nO_6^{2-} + (n-3)SO_3^{2-} \rightarrow S_3O_6^{2-} + (n-3)S_2O_3^{2-}$	[Rxn 9 + (n-4) Rxn 4] or [(n-2) Rxn 10][3, 5]
	$SO_3^{2-} + {}^1/_8S_8^0 \rightarrow S_2O_3^{2-}$	[Rxn 3 + Rxn 4 + Rxn 3r] [5, 59, 62, 64, 65]
	$S_nO_6^{2-} + HS^- \rightarrow 2\,S_2O_3^{2-} + (n-3)S^0 + H^+$	[Rxn 16 + Rxn 17 + Rxn] 3r][5]
	$S_nO_6^{2-} + HS^- + (n-3)SO_3^{2-} \rightarrow (n-1)S_2O_3^{2-} + H^+$	[Rxn 16 + (n-3) Rxn 4] [5]
	$S_nO_6^{2-} + S_mO_6^{2-} \rightarrow S_{n-1}O_6^{2-} + S_{m+1}O_6^{2-}$	[Rxn 10 + Rxn 10r] [5]
	$H_2S + Fe^{3+} \rightarrow Fe^{2+} + H_2S\cdot \rightarrow radical\ chain\ building$	[Component of Rxn 18] [35, 56]
	$S_nO_6^{2-} + 2\,HS^- \rightarrow 2\,S_2O_3^{2-} + (n-2)S_n^{2-} + 2\,H^+$	[Rxn 16 + Rxn 17] [56, 59]
	$4\,S_3O_3^{2-} \rightarrow S_8^0 + 4\,SO_3^{2-}$	[(4) Rxn 4r + (7) Rxn 4 + Rxn 3] [58]

Note: Reaction numbers followed by an "r" designate the reverse reaction corresponding to that number; numbers in parenthesis indicate multiple instances of that reaction occurring as part of the overall reaction

In addition to the exchange between polymeric sulfur species, chain-lengthening and chain-shortening reactions are possible. In particular, sulfite is capable of attacking each of the

three polymeric species discussed above, resulting in the liberation of thiosulfate[3, 5]. This reaction (combined with others discussed here) is often implicit in the overall reactions presented in sulfur biooxidation studies, such as the total conversion of polythionate to thiosulfate in the presence of bisulfide and sulfite[5] or the formation of cyclooctosulfur (S_8) from $S_3O_3^{2-}$[35]. A minimalist set of reactions (**Table 2**), most of which are described above, were identified such that they represent the broad array of overall reactions reported in literature. Select overall reactions are also presented, with a listing of the representative reactions that describe them.

A visualization combining this reaction network with the known enzymatic sulfur reactions of the Sulfolobales highlights points of synergy or antagonism (**Figure 3**). While the Sulfolobales possess several enzymes equipped to cycle thiosulfate and tetrathionate, no enzymatic step has been identified to facilitate the formation of thiosulfate from polysulfide or H_2S. It appears that the

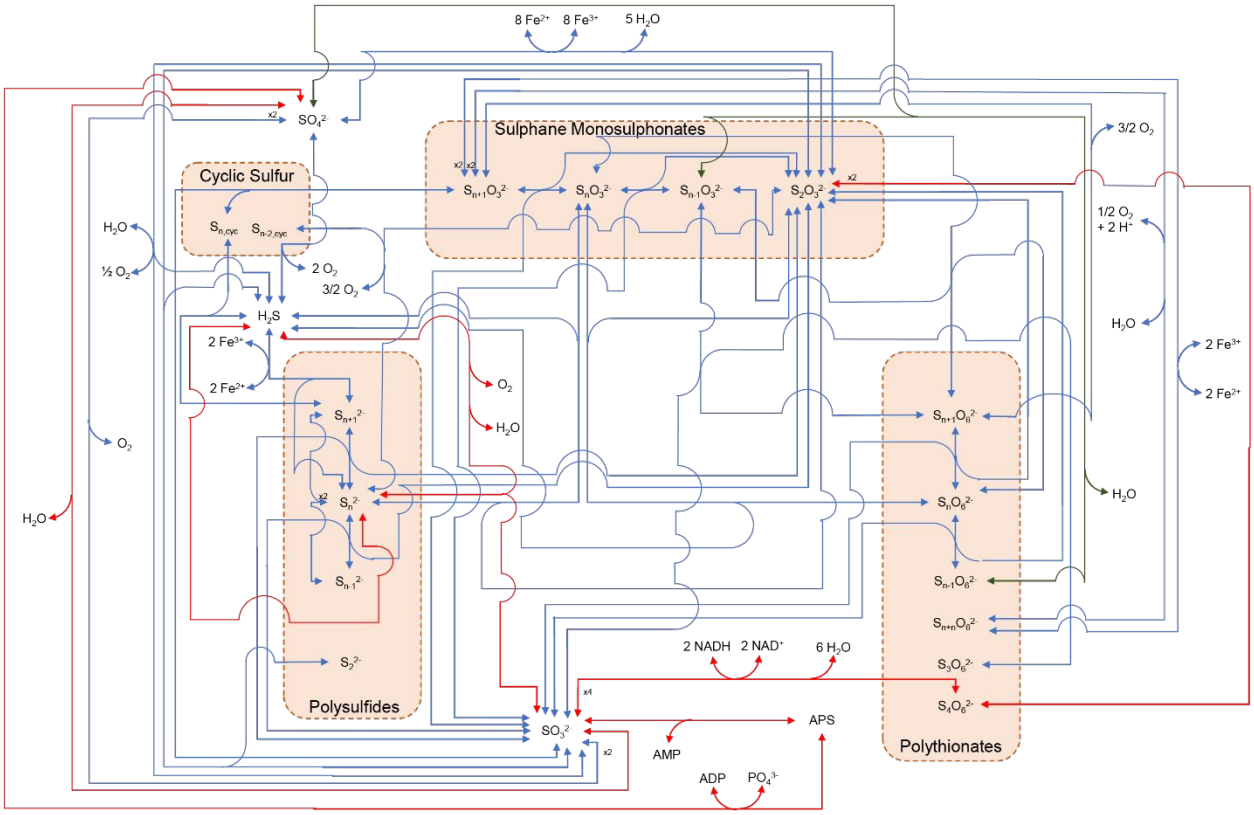

Figure 3: Minimalist representation of RISC reactions involved in Sulfolobales sulfur oxidation; Blue lines indicate abiotic reactions; Red lines indicate enzymatic reactions; Green lines indicate abiotic reactions also catalyzed by enzymes. Tan boxes indicate a class of sulfur species with varying chain length

cell instead relies on the abiotic formation of thiosulfate from polysulfide degradation by sulfite. This reaction is often accounted for in models of sulfur oxidation in thermoacidophiles and mesoacidophiles alike[41, 66]. Other abiotic paths to thiosulfate include degradation of polythionates by sulfite (Reaction 10), degradation of polythionate by H_2S (Reactions 16 and 17), and hydrolysis of polythionates (Reaction 1 plus *n*-2 Reaction 4).

Direct competition between abiotic and enzymatic reactions also exist, notably in the case of all three enzymes directly linked to the ETC. Conversion of H_2S to polysulfide (SQR), thiosulfate to tetrathionate (TQO), and sulfite to sulfate (SAOR) all occur abiotically. Therefore, only a portion of the available energy from these reactions is actually captured by the ETC and made available to the cell. Accounting for this partial energy conservation is key in any model of the sulfur oxidation metabolism, although the extent of energy loss to abiotic reactions necessitates a more detailed kinetic understanding of the enzymatic steps. This consideration is only pertinent to enzymatic steps that conserve energy. TetH, for instance, catalyzes the hydrolysis of polythionates, namely tetrathionate, and this reaction also occurs abiotically. However, there is no energy conserved by TetH, so the distinction between enzymatic and abiotic hydrolysis is not significant.

Finally, some abiotic reactions may be directly antagonistic to the Sulfolobales. H_2S forms cyclic sulfur through sequential chain-building of polysulfides via radical chemistry, where the oxidizing agent is often Fe^{3+} [4]. This reaction leads to the generation of Fe^{2+}, which when combined with peroxide byproducts of the ETC can lead to the generation of hydroxyl radicals through Fenton chemistry and subsequent oxidative stress in the cell. Notably, all sulfur-oxidizing Sulfolobales possess the *fox* cluster of genes, which are linked to biological iron oxidation[27]. While some of these species rely on iron oxidation for energy, this gene cluster may also be providing a way for the sulfur-oxidizers to deal with the toxic byproducts of H_2S radical chemistry.

Accessing Extracellular Sulfur Substrates

In contrast to the extracellular localization of sulfur reduction, sulfur oxidation occurs largely on the cytoplasm side of the cell membrane where the pH is more circumneutral. Transport of sulfur across the cell membrane is a particularly difficult process, considering that elemental sulfur dominates the distribution of sulfur species at low pH and is largely insoluble in water [54]. Sulfur transport has been studied in mesophilic sulfur oxidizers, and multiple mechanisms have been proposed. In the mesophilic, photoautotrophic purple sulfur bacterium *Chromatium vinosum*, vesicles encapsulating extracellular sulfur are formed. Proteins encoded by *dsrE* and t*usA* then cleave individual sulfide atoms within the vesicle and transport them into the cytoplasm[67]. Mesoacidophilic *Acidithiobacillus* spp. exhibit a similar mechanism, where proteins in the cell membrane form persulfide bonds, which are then cleaved on the cytoplasmic side of the membrane [68]. No such transport mechanism has been identified in the *Sulfolobales* to date. However, hydrogen sulfide appears to be capable of crossing the cell membrane. Because of its structural similarity to water, H_2S appears to be capable of passing through aquaporins into the cytoplasmic space [69], where the near-neutral cytoplasm enables the abiotic redistribution of RISCs. In fact, H_2S may not even need the aid of a transporter to cross into the cytoplasm; rather it is capable of passive diffusion across the membrane [70]. It has been postulated that cyclic S_8 could diffuse across the cell membrane in a similar manner due to its hydrophobic character an neutral charge[71]. However, transmembrane diffusion of S_8 has not yet been demonstrated experimentally.

Recently, a study of the extremely thermoacidophilic archaeon *Acidianus* DS80 showed that, while sulfur reduction can occur even when sulfur was sequestered away from the organism, sulfur oxidation required direct contact between the cells and solid sulfur substrate[72]. In the case of sulfur reduction, organism growth was dependent on the pore size of the dialysis bag, indicating that the particle size distribution of sulfur influenced sulfur reduction[72]. Whether this is indicative of mass transfer-limited growth or reaction-limited growth is unclear, but it is possible that for

acidophiles, nanocrystalline S_8 is the substrate for sulfur reducers and is formed by ring-opening of H_2S and subsequent ring closure by the reverse reaction. This independence of direct contact for sulfur reduction has previously been explained in neutrophilic Archaea by polysulfide chains acting as the actual substrate for the cell[73]. This would require extracellular cleavage of cyclic sulfur to generate polysulfide in the first place, and the thermodynamic equilibrium of cleavage by H_2S at acidic extracellular pH is highly unfavorable (see **Figure 4 bottom**). However, cleavage by SO_3^{2-} has a more favorable equilibrium. In the presence of oxygen, extracellular sulfite would be rapidly oxidized to sulfate according to Reaction 12 (see **Table 2**). This instability of sulfite in aerobic conditions could explain the need for direct interaction with (or at least proximity to) the sulfur substrate for oxidation, while it would not be required for sulfur reduction under anaerobic conditions. Notably, all sulfur-oxidizing species in the order Sulfolobales contain a putative sulfite exporter, which could provide the nucleophile necessary for ring cleavage (see **Table 1**).

Influence of pH on Reaction Directionality

How pH influences reactions involving RISCs is particularly pertinent when considering thermoacidophilic biooxidation. While these organisms do thrive at acidic pHs, they maintain a near-neutral cytoplasmic pH of ~6.5[74, 75]. The cell membrane acts as a discrete barrier to this large pH gradient, which causes a drastic shift in reaction equilibria as RISCs cross the cell membrane. ΔG_f^0 values from literature were transformed to account for ionic strength of the standard Brock Salts medium and the protonation state of the species, and the equilibrium constant of abiotic reactions of RISCs was evaluated over a range of pH values **(Figure 4)**. The equilibrium between polysulfide chains of varying lengths has been well-studied under alkaline conditions[29]. However, the instability of polysulfides in acid makes the direct examination of this equilibria at low pH challenging. In general terms, the ΔG_{rxn} appears to favor combining shorter chains into longer polysulfide chains at pH below 7, even at the expense of liberating H_2S **(Figure 4 top)**. However, the presence of an oxidant, like ferric iron, enables the radical chemistry necessary to assemble

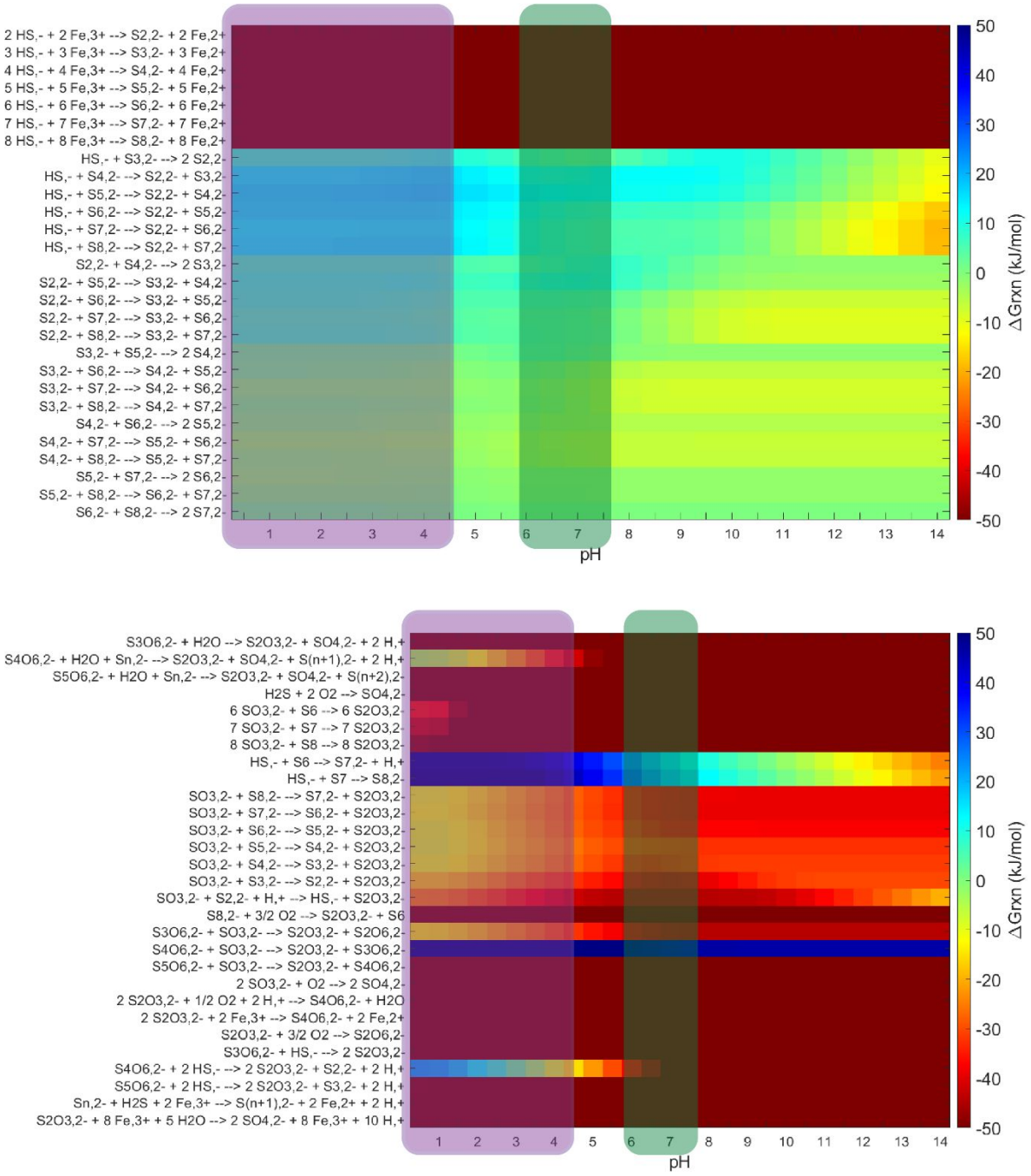

Figure 4: ΔG_{rxn} (kJ/mol) for RISC reactions at pH increments of 0.5; Colorscale boundaries are 50 and -50 kJ/mol, and any ΔG_{rxn} exceeding these values are shown at the bounds of the color scale; Purple shading indicates extracellular pH conditions; Green shading indicates cytoplasmic pH conditions; (Top): polysulfide chain-sizing from Reaction 20; (Bottom): RISC Reactions 1-19, excluding Reaction 9.

n H_2S into a polysulfide chain S_n^{2-} [56] and is favorable, independent of pH. Ultimately, this chain-building effect results in the formation of insoluble cyclic sulfur and release of H_2S.

There is a stark contrast between the nucleophilic attack of sulfur rings by H_2S and by sulfite **(Figure 4 bottom)**. At low pH, the reaction involving H_2S is favored in the reverse direction, forming sulfur rings from polysulfide chains by releasing H_2S. However, at near-neutral cytoplasmic pH, an equilibrium between reactants and products persists. In contrast, nucleophilic attack by sulfite (and the subsequent degradation of polysulfide chains by sulfite) is favored in the forward direction, even at low pH, and becomes more favorable as pH increases. This mechanism has implications for making sulfur accessible to the cell, as discussed below. It is somewhat complicated by the stability of SO_3^{2-}. In aerobic conditions, SO_3^{2-} will rapidly oxidize to SO_4^{2-} [63], and SO_3^{2-} is degraded in acid even in an anoxic environment, although measurable quantities of SO_3^{2-} were still detectable after 24 h of incubation[76].

Reaction equilibria of polythionates also appear to vary with chain length, but with a free energy minimum at n = 4 as tetrathionate. While the hydrolysis of polythionates is favored at cytoplasmic pH, hydrolysis of tetrathionate specifically approaches an equilibrium in the extracellular space. The stability of tetrathionate outside of the cell represents a possible bottleneck in the interchange of RISCs and may explain why the Sulfolobales produce an extracellular tetrathionate hydrolase, which catalyzes a reaction that is normally spontaneous at higher pH.

Energetics of the Sulfur Oxidation Metabolic Pathway

The comprehensive energetics of sulfur oxidation have previously been examined in a biological context[65]. However, these overall oxidation reactions represent only a maximum potential for energy conservation. To evaluate the metabolic potential of a pathway, the energetics of individual steps of the pathway must be assessed. To this end, energy conservation of sulfur oxidation in Sulfolobales can be compared to the primary heterotrophic pathway in the Sulfolobales, the non-phosphorylative Entner-Doudoroff Pathway[77, 78].

For the purposes of overall energetic comparison, a pathway was constructed for the total oxidation of H_2S to sulfate in the Sulfolobales **(Figure 5)**. Note that a number of recycle steps and branch points normally exist for the sulfur pathway, as outlined earlier **(Figure 2)**. However, this representation is intended to include all known enzymatic sulfur reactions of the Sulfolobales and to ensure total oxidation of the number of H_2S molecules considered. In this case, the overall biological oxidation of H_2S to sulfate is represented as:

$$5\ H_2S + 14\ Q + 2\ NAD^+ + AMP + 2\ P_i + 14\ H_2O + O_2$$

$$\rightarrow 5\ SO_4^{2-} + 14\ QH_2 + 2\ NADH + ATP \quad \text{[EQN 6]}$$

The electron acceptor for the sulfite:acceptor oxidoreductase and APS reductase reactions are unknown (shown as "R"/"RH_2" in **Figure 5**) and were assumed to be quinones for the purpose of pathway energetic calculations. Notably, the sulfur oxidation pathway reduces significantly more quinones than the NPED pathway **(Figure 5a, 5b)**, indicating that sulfur-oxidizing Sulfolobales rely much more heavily on the electron transport chain for energy than their heterotrophic

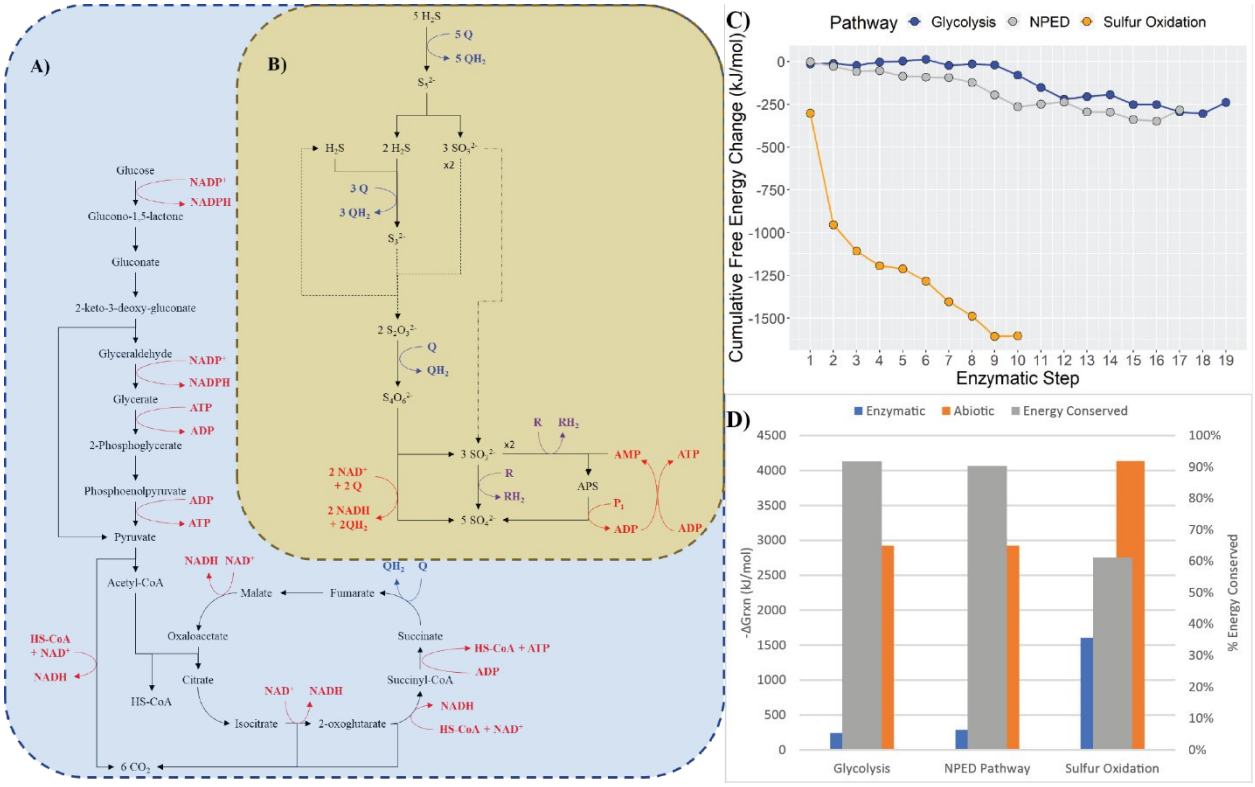

Figure 5: a) Non-phosphorylative Entner-Doudoroff pathway (NPED); b) Representative sulfur oxidation pathway; c) Cumulative free energy change by reaction step for glycolysis (blue), NPED (gray), and sulfur oxidation (orange); d) Overall percent energy conservation of pathways (gray) based on free energy change of total combustion or oxidation of substrate (orange) and free energy change of enzymatic pathway (blue).

counterparts. This may also be a means of dealing with the liberation of protons that occurs during cytoplasmic sulfur oxidation and the consequential acidification.

Total energy conservation of the sulfur oxidation pathway was compared against the NPED pathway of the Sulfolobales and glycolysis **(Figure 5c)**. The magnitude of the abiotic $\Delta G_{rxn}'^{0}$ for oxidation of four H_2S molecules is comparable to $\Delta G_{rxn}'^{0}$ for complete combustion of glucose. The sulfur oxidation pathway falls short of the >90% energy conservation from the two heterotrophic pathways, but it does still conserve greater than 60% of the available energy. However, this calculated energy conservation is assuming all molecules of H_2S proceed to SO_4^{2-} through enzymatic steps wherever possible. As discussed earlier, competitive abiotic sulfur reactions may cut into this energy conservation, making the practical energy conservation even lower. The extent of this interference requires more detailed kinetic enzymatic understanding.

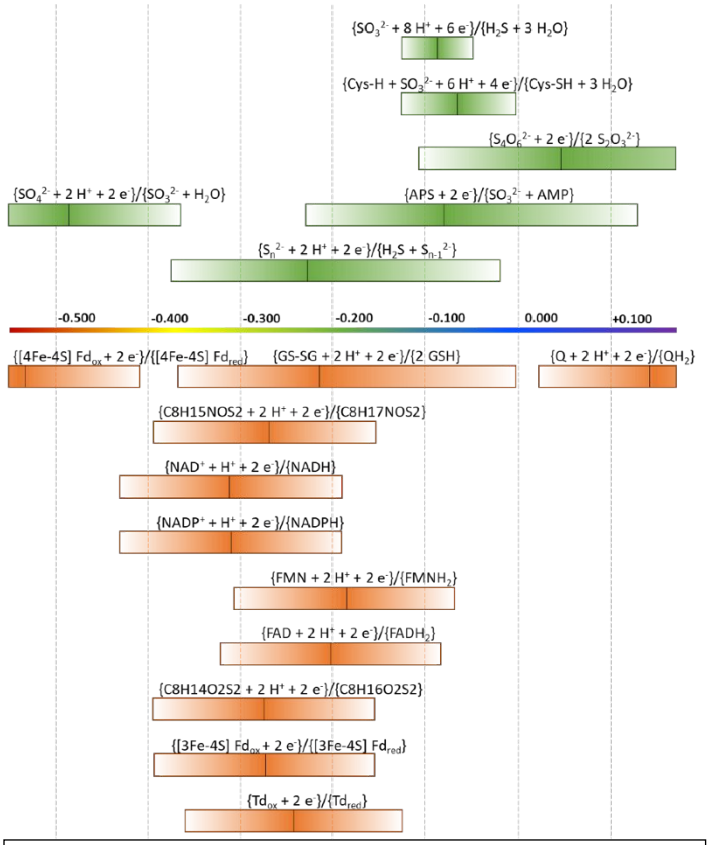

Figure 6: Reduction potential of enzymatic sulfur half-reactions (green) and energy carrier half-reactions (orange); bars represent the physiological range of reactant/product ratios; vertical lines in each bar represent the equimolar transformed reduction potential of the half-reaction.

A breakdown of the cumulative free energy change through each step of sulfur oxidation **(Figure 5d)** reveals that the first two steps of the pathway, H_2S to S^0 by sulfide:quinone oxidoreductase and the disproportionation of S^0 by sulfur oxygenase reductase, result in the most significant energy loss. This is not surprising for the SOR reaction, considering that it is not coupled to any biological energy carrier. Downstream of the SOR

reaction, the rate of free energy change is not too dissimilar from heterotrophic pathways, indicating a high degree of energy conservation in these steps.

The reduction potential of the enzymatic half-reactions of sulfur were evaluated against the half-reaction reduction potential of major biological energy carriers **(Figure 6)**. The majority of sulfur half-reactions have moderately high reduction potential and so are only capable of coupling to quinone reduction. The lowest reduction potential of any enzymatic step is the oxidation of sulfite to sulfate, associated with sulfur:acceptor oxidoreductase. Notably, the electron acceptor of this enzyme remains unknown, but it has the energetic capability to reduce even the [4Fe-4S] ferredoxin, the energy carrier with the lowest known reduction potential in the Sulfolobales[32]. The Sulfolobales also possess a rather unusual [3Fe-4S] ferredoxin, which has a standard reduction potential of -0.275 V[32]. Interestingly, this energy carrier sits squarely in the range of the [H_2S,S_{n-1}^{2-}/S_n^{2-}] reduction potential and suggests that it may be a ferredoxin uniquely suited to the reduction potential of sulfur oxidation.

Supporting Evidence of Sulfur Oxidation in an Engineered *S. acidocaldarius* Strain

The engineered strain of *S. acidocaldarius* (RK34)[17] and the parent strain *S. acidocaldarius* MW001 were grown with limited heterotrophic nutrients, with and without elemental sulfur present **(Figure 7)**. The resulting growth data were fit to logistic equation of the form:

$$N_t = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e^{-rt}} \qquad \text{[EQN 7]}$$

where K is the carrying capacity of the population, N_0 is the initial population and r is the intrinsic growth rate of the population. The data were also analyzed by Principle Component Analysis (PCA) to evaluate differences in behavior between the growth conditions. When grown with sulfur, the carrying capacity of MW001 somewhat decreased compared to growth on just amino acids

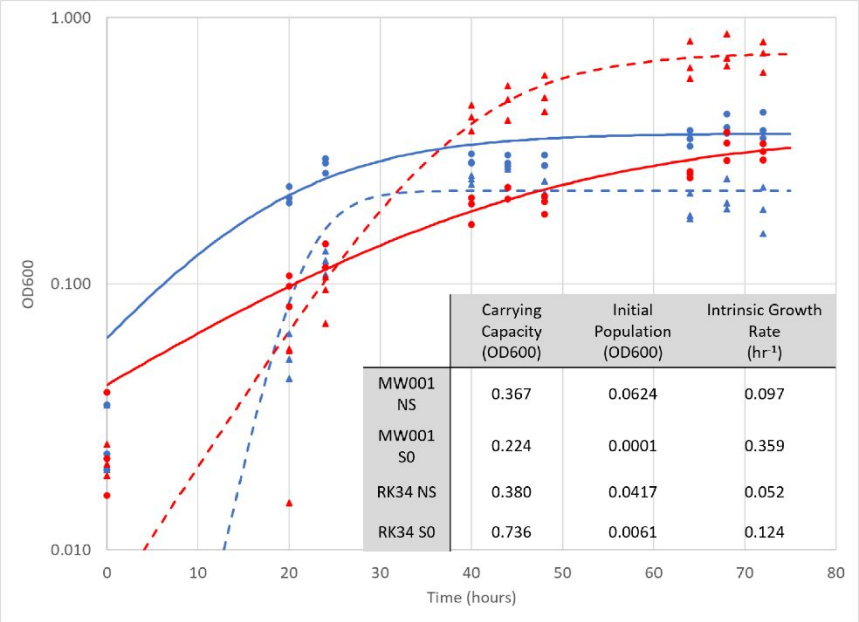

Figure 7: Growth curves of *Saci* MW001 (blue) and RK34 (red) on amino acids without sulfur (circles) and with sulfur (triangles); Logistic equation models for the data are shown as solid lines (without sulfur) or dotted lines (with sulfur); values for the logistic equation parameters are shown in the insert, where "NS" indicates the condition without sulfur and "S0" indicates the condition with sulfur

(NZ-Amine). In contrast, the RK34 strain nearly doubled, indicating that the presence of sulfur improved growth from amino acids alone. In the PCA analysis, minimal difference between MW001 and RK34 was observed in the first two components when grown without sulfur. Addition of sulfur to the cultures resulted in a shift in the second principle component that was similar between the two strains. However, the first principle component showed a divergence between MW001 and RK34 in the presence of sulfur. This implies that the RK34 strain gains an energetic advantage from sulfur that enables more of the amino acids to be allocated as a carbon source for biomass generation and not used as an energy source.

Notably, in the non-sulfur condition, batch cultures of both MW001 and RK34 had a final pH greater than 5 **(Figure 8)**. This was also the case for batch cultures of MW001 with sulfur. However, the RK34 strain grown with sulfur ended with a pH between 2 and 3. Maintaining the acidic environment in these cultures is likely a consequence of sulfur oxidation by RK34. Naturally one might assume that a lower extracellular pH, and therefore a larger transmembrane proton gradient, might enable the cell to generate more energy from protonmotive force. However, acidophilic microbes have been shown to throttle proton influx to levels similar to neutrophilic microbes due to a positive membrane potential[79]. In fact, acidophilic microbes have even been shown to adjust this membrane potential in response to changes in extracellular pH in order to

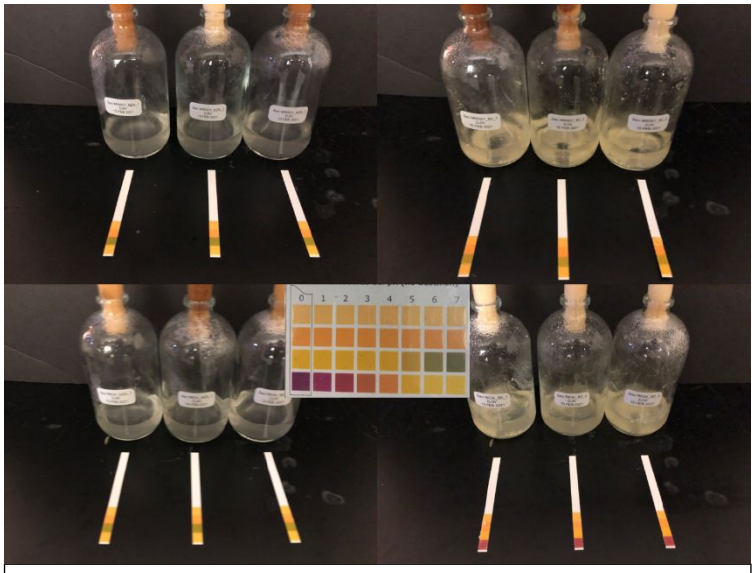

Figure 8: Final pH measurement of serum bottles for *Saci* MW001 without sulfur (pH 6, top left), *Saci* MW001 with sulfur (pH 5.5, top right), *Saci* RK34 without sulfur (pH 5.5, bottom left), and *Saci* RK34 with sulfur (pH 2, bottom right).

maintain a constant proton flux[74]. In the Sulfolobales, increasing the number of cyclopentyl rings attached to the tetraether lipid membrane reduces proton permeability in the membrane[80]. Therefore, it is possible that the difference in extracellular pH does not affect the energy conservation from protonmotive force in RK34. However, the microbe would have to change its membrane composition to deal with higher pH, therefore increasing the energy demand of the cell. As such, it remains to be seen if RK34 generates cellular energy directly from sulfur oxidation or as an indirect consequence of the maintained pH gradient.

CONCLUSION

The complexity of abiotic sulfur chemistry adds a challenging dimension to sulfur oxidation that is unique among inorganic metabolisms. As such, to fully understand the energetic potential of sulfur as a metabolic substrate, the effect of abiotic reactions on energy conservation must be considered. Some reactions, such as hydrolysis of polythionates and formation of thiosulfate, create synergy with enzymatic steps. Others impede energy conservation through direct competition with enzymatic steps or by creating cellular stress. The function of these abiotic reactions between the acidic extracellular space and the neutral cytoplasm may even provide insight into mechanisms of sulfur transport in the Sulfolobales.

The stepwise oxidation of sulfur offers numerous opportunities to conserve energy through enzymatic coupling to energy carriers, theoretically capturing over 60% of the available energy

from sulfur oxidation. Matching the oxidation step with an energy carrier of similar reduction potential is critical to minimizing energy loss while providing some clues as to the role of enzymes in sulfur metabolism. Even introducing only a few of these enzymes into a non-sulfur oxidizer provides an energetic advantage to the engineered strain.

Another consideration for the role of sulfur chemistry, biotic and abiotic, is the role that this element may have played in establishing life in an emerging aerobic system on Earth or elsewhere. The stepwise oxidation of sulfur creates an opportunity for efficient energy conservation, and the varied reduction potential of sulfur half-reactions enables reduction of varied biological energy carriers. Further understanding of how biological sulfur oxidation influences and responds to the distribution of RISCs in an environment may provide clues towards the presence or potential for life beyond the planet Earth.

SUPPORTING INFORMATION

Free energy of formation data for all chemical species and protonation states used for calculations, including source of the data.

ACKNOWLEDGMENTS

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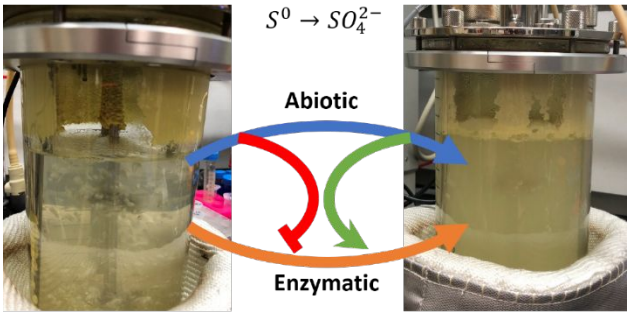
TOC Graphic



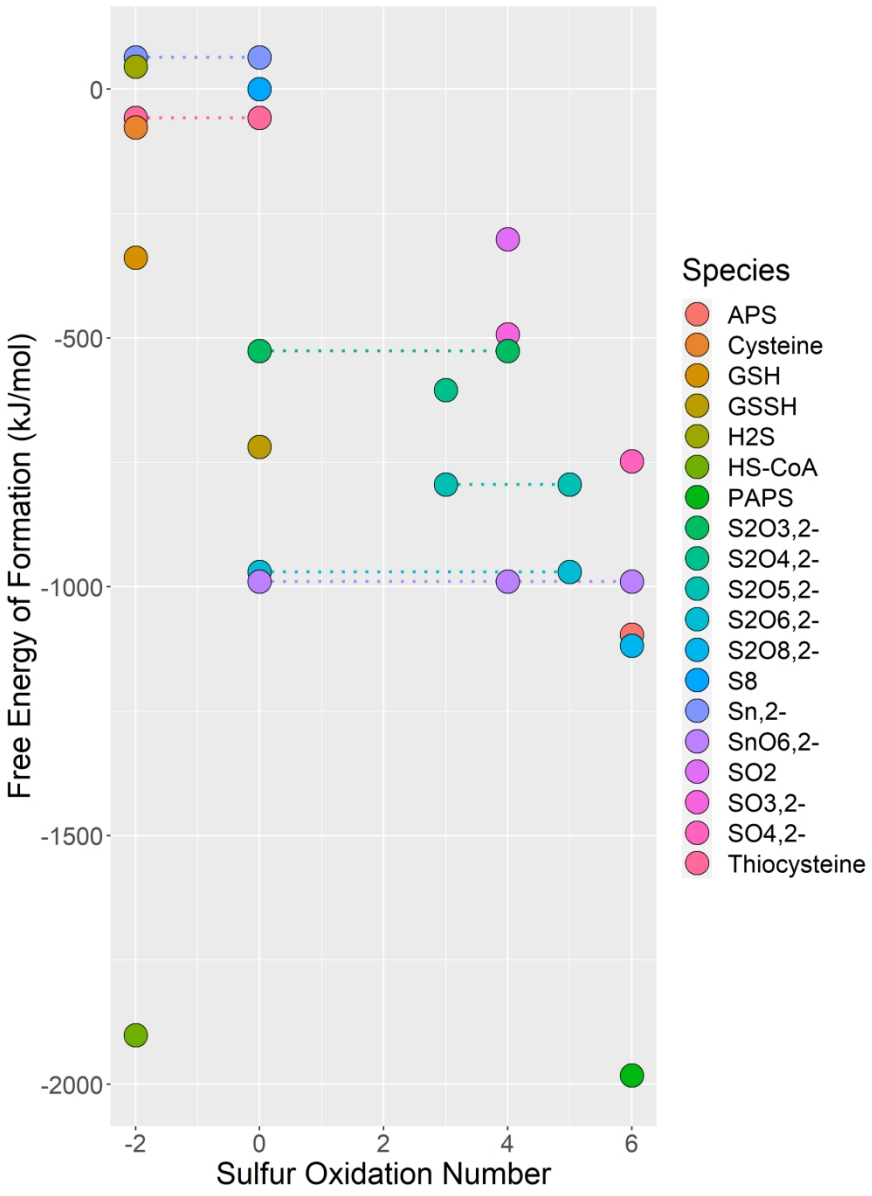


Figure 1: Distribution of oxidation states and ΔGf0 of various sulfur species; APS: adenylyl sulfate, GSH: glutathione, GSSH: glutathione disulfide, PAPS: phosphoadenylyl sulfate; dotted lines indicate multiple sulfur oxidation states within the same molecule

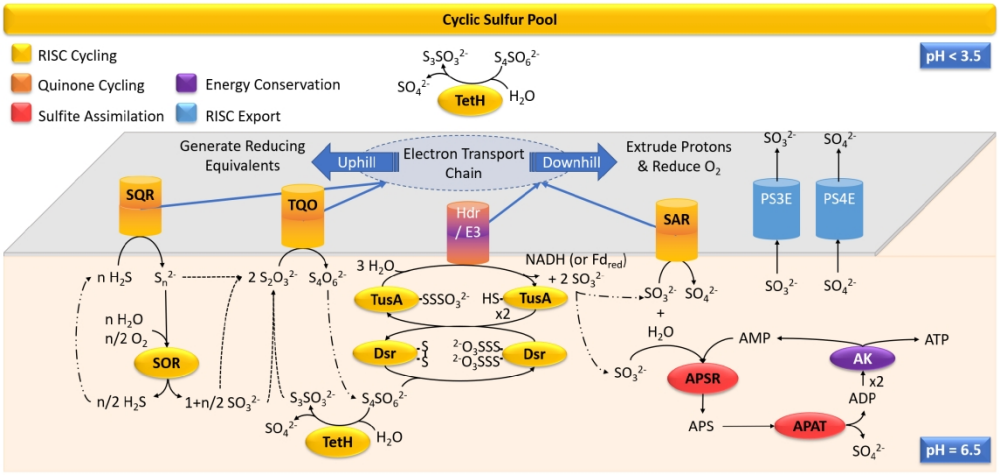


Figure 2: Schematic of Sulfolobales enzymes involved in sulfur oxidation; solid lines indicate enzymatic reactions, dashed lines indicate abiotic formation of thiosulfate, dashed-dotted lines indicate a shared sulfur species between reactions, blue arrows indicate the movement of quinones; Gray barrier represents the cell membrane, with cytoplasmic space below the barrier and extracellular space above; enzymes are colored according to their functional associations: cycling of RISCs (yellow), cycling of quinones (orange), assimilation of SO32- (red), direct energy conservation through ATP or NAD(P)H (purple), and export of RISCs (blue); enzymes with multiple functional associations have a color gradient

	Protein of Interest	Protein Cluster †	Protein Cluster Presence in Sequenced *Sulfolobales*																										
						Fox			Fox				Fox					Fox												
			Sred	Sred & Sox			Sox		Sred & Sox				Sox				Sred & Sox	Sox ‡											
			Autotrophy						Autotrophy & Heterotrophy								Heterotrophy								ND				
			Sazo	Aamb	Ainf	Asul	Smet	Abri	Acop	Aman	Ahos	Mcup	Mhak	Msed	Myel	Sohw	Stok	Ssol	Sisl HVE10/4	Sisl LD85	Sisl M162	Saci	Arsu	Ssp A20	Ssp E51F	Step	Sdac
Sulfur Enzymes with Direct Action	SQR	17768	X	XX	XX	XX	X	XX	X	XX	XX	X	X	X	X	X	X	X	X	X	X	-	X	X	X	X	X
	TQOa	17578	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	X	X	-	X	X
		17852	-	X	X	X	-	X	-	X	X	-	-	-	-	-	-	-	X	X	X	-	-	XX	-	X	-	-
	TQOb	17579	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	X	X	-	X	X
		17853	-	X	X	X	-	X	-	X	X	-	-	-	-	-	-	-	X	X	X	X	-	XX	-	X	-	-
	TetH	622	-	X	X	X	X	XX	XX	X	X	X	X	X	X	X	X	X	-	-	-	-	-	-	-	-	XX	-
	SOR	18472	-	X	X	X	X	X	X	X	X	-	-	-	-	X	X	-	-	-	-	-	X	-	-	X	-
	SAOR	1361	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	X	X	X	X
	PS3E	17110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	-	X	X	-	X	-
	APSRa																											
	APSRb	15	X	X	X	X	X	XX	X	XXX	X	XX	X	XX	X	XX	XX	X	XX	XX	XX	XX	X	-	X	XX	X	X
	APAT																											
	AK																											
	ATPS																											
Sulfur Reduction	SreA	18679	X	X	X	X	-	X	-	X	-	-	-	-	X	X	-	X	X	X	X	X	-	X	-	X	-	-
	SreB	18973	X	X	X	XX	X	X	X	X	X	X	X	X	XX	XX	X	X	XX	XX	XX	X	X	X	X	X	-	-
	SreC	18251	X	X	X	X	-	X	-	X	-	-	-	-	X	X	-	X	X	X	X	-	XX	-	X	-	-
	SreD	17628	X	X	X	XX	X	X	X	X	X	X	X	X	XX	XX	X	X	XX	XX	XX	X	XX	X	-	-	-
Heterodisulfide Reduction System	HdrA	18047	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrB1	18048	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrC1	18049	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrB2	18044	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	HdrC2	18045	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	TusA	18050	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	XXX	XX	-	XX	X	X	XX	XX	X	X
	DsrB(E2)	18051	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	DsrE3A	18052	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	DLD (E3)	18053	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	GSSH	18747	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LbpM1	18761	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LbpM2	18762	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LplA	18763	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
	LbpA	18765	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	-
Fox Cluster	FoxA	708	-	X	-	X	X	X	X	X	-	-	XXX	XX	XX	X	X	-	-	-	-	-	-	-	-	X	X
	FoxB	715	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxC	5690	-	-	-	X	-	-	X	X	-	-	X	X	X	-	X	-	-	-	-	-	-	-	-	X	X
	FoxD	717	-	X	-	X	X	X	X	X	-	-	X	X	-	X	X	-	-	-	-	-	-	-	-	X	X
	FoxE	710	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxF	709	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxG	707	-	X	-	X	X	X	-	X	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	X	-
	FoxH	706	-	X	-	X	X	X	X	X	-	X	X	X	X	X	X	-	-	-	-	-	-	-	-	X	-
	FoxI	22019	-	-	-	-	-	-	-	-	-	-	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-
	FoxJ	712	-	XX	X	X	X	X	X	X	X	-	X	X	X	XX	XX	-	X	X	X	-	-	X	X	X	X
	FoxV	711	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X
	FoxW	716	-	X	-	X	X	X	X	X	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	X	X
	FoxY	714	-	X	-	X	X	X	-	X	-	-	X	X	-	X	-	-	-	-	-	-	-	-	-	X	X
	FoxZ	713	-	X	-	X	X	X	X	X	-	-	X	X	X	X	X	-	-	-	-	-	-	-	-	X	X

† Protein cluster numbers are based on comparative genomic analysis from Counts *et al.* (2020)[25]

‡ Phenotypes of Sulfolobales species are based on the assessment presented in Wheaton *et al.* (2015)[36]; species excluded from this assessment labeled as "ND"

"X" indicates that the genome contains a protein homologous to the particular protein cluster. Multiple "X's" indicate paralogs in that genome

Table 1: Distribution of Sulfur Oxidation Genes in the Genome-Sequenced Sulfolobales

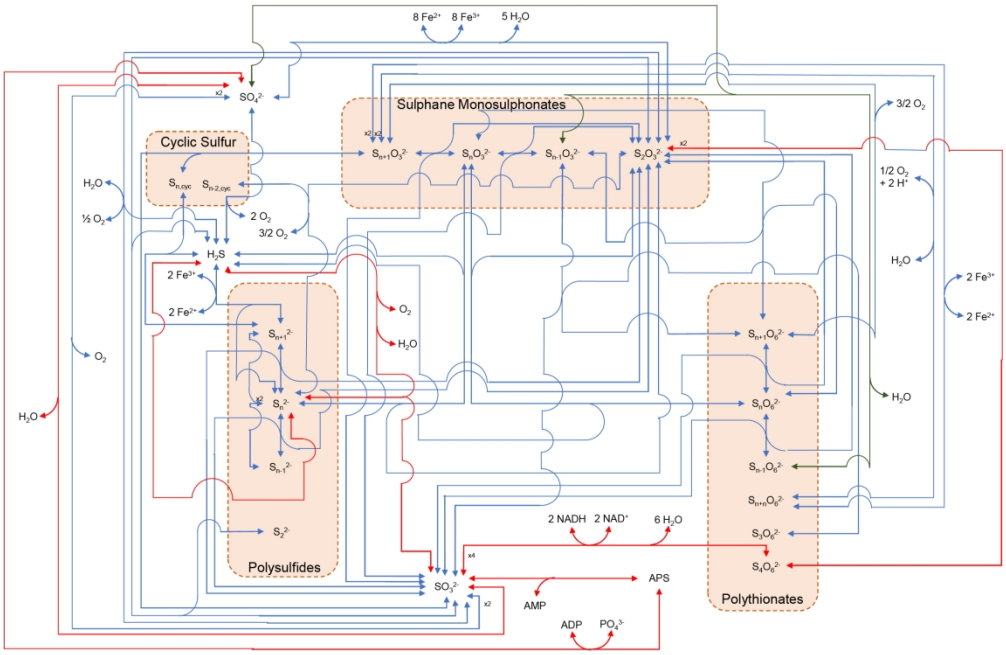


Figure 3: Minimalist representation of RISC reactions involved in Sulfolobales sulfur oxidation; Blue lines indicate abiotic reactions; Red lines indicate enzymatic reactions; Green lines indicate abiotic reactions also catalyzed by enzymes. Tan boxes indicate a class of sulfur species with varying chain length

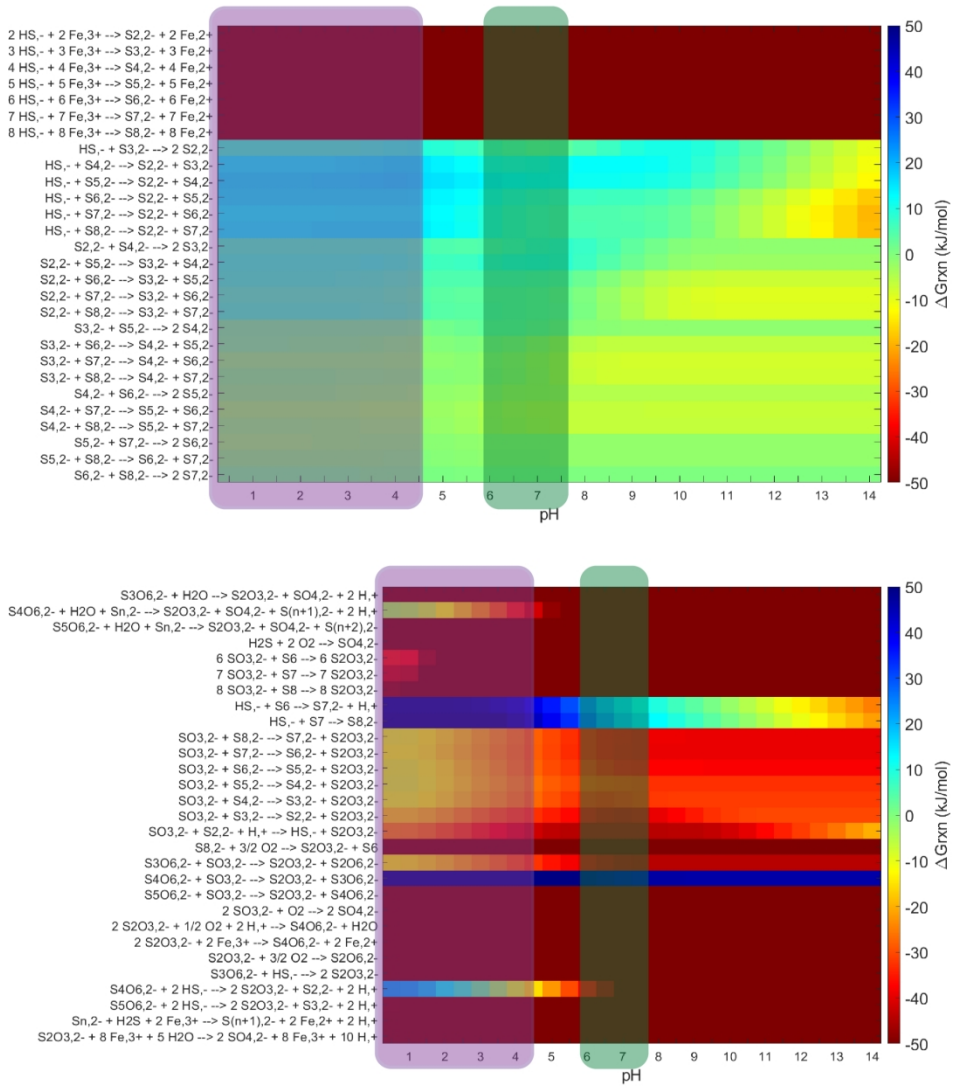


Figure 4: ΔGrxn (kJ/mol) for RISC reactions at pH increments of 0.5; Colorscale boundaries are 50 and -50 kJ/mol, and any ΔGrxn exceeding these values are shown at the bounds of the color scale; Purple shading indicates extracellular pH conditions; Green shading indicates cytoplasmic pH conditions; (Top): polysulfide chain-sizing from Reaction 20; (Bottom): RISC Reactions 1-19, excluding Reaction 9.

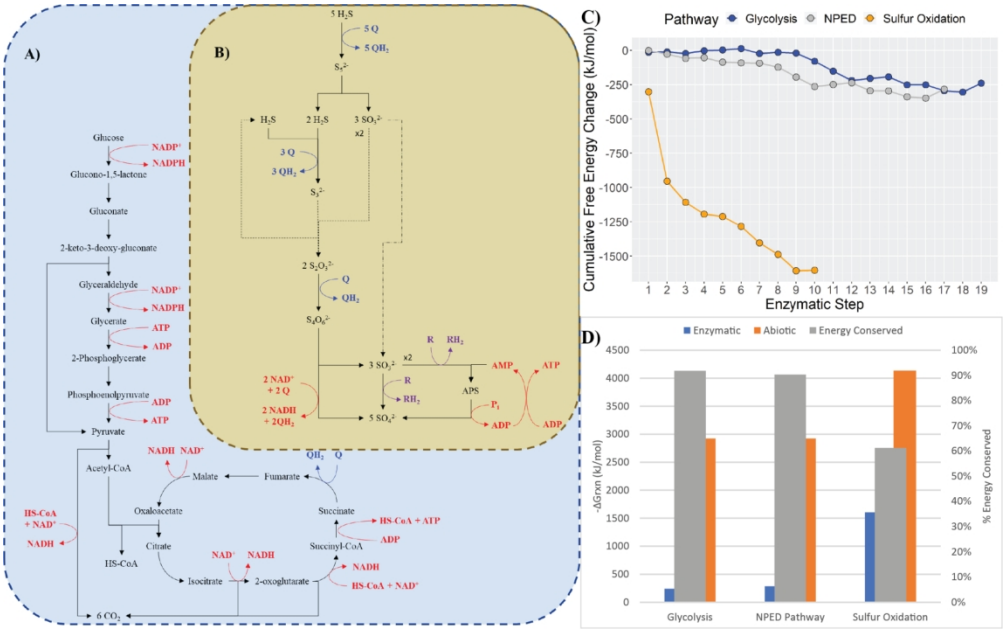


Figure 5: a) Non-phosphorylative Entner-Doudoroff pathway (NPED); b) Representative sulfur oxidation pathway; c) Cumulative free energy change by reaction step for glycolysis (blue), NPED (gray), and sulfur oxidation (orange); d) Overall percent energy conservation of pathways (gray) based on free energy change of total combustion or oxidation of substrate (orange) and free energy change of enzymatic pathway (blue).

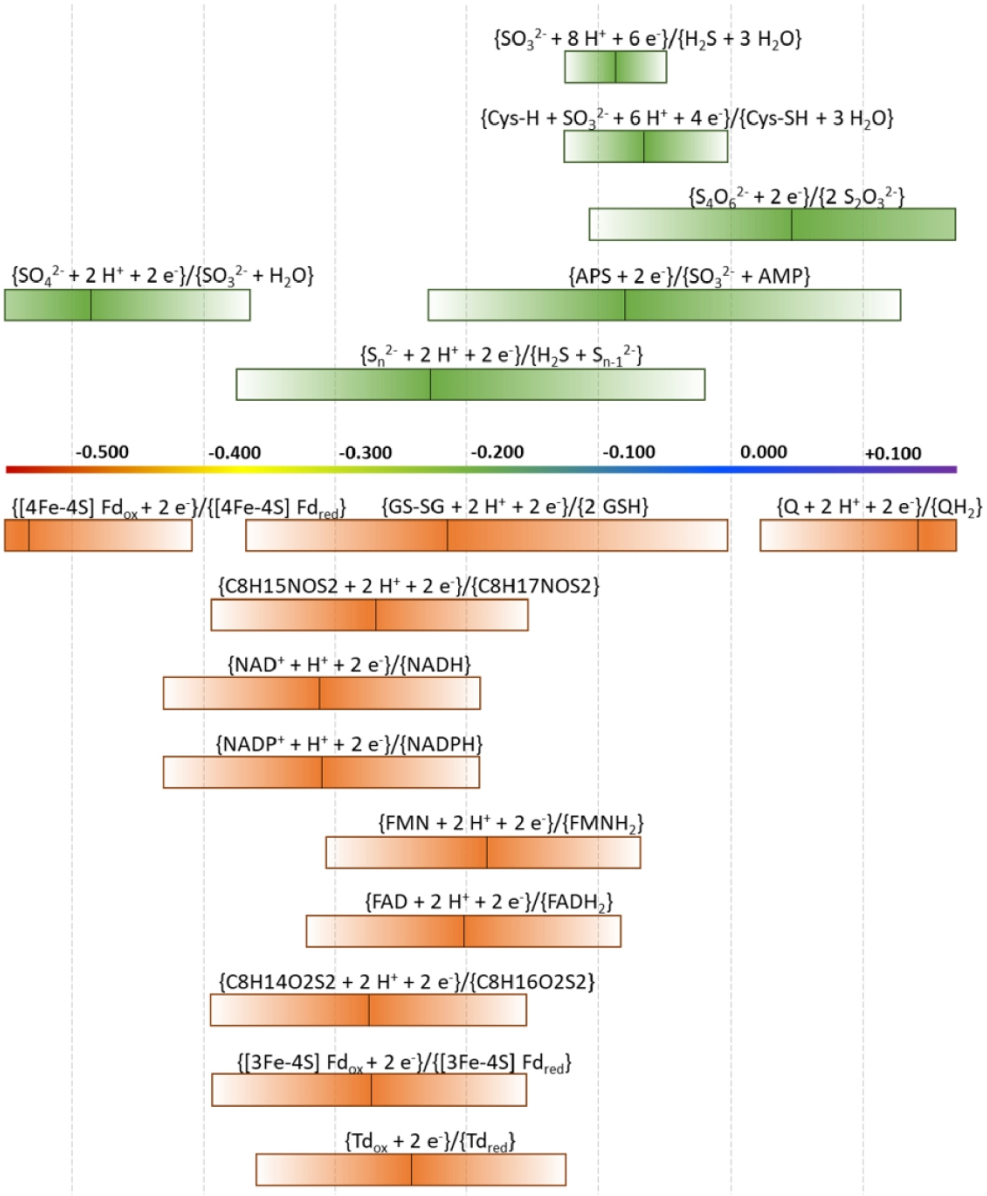
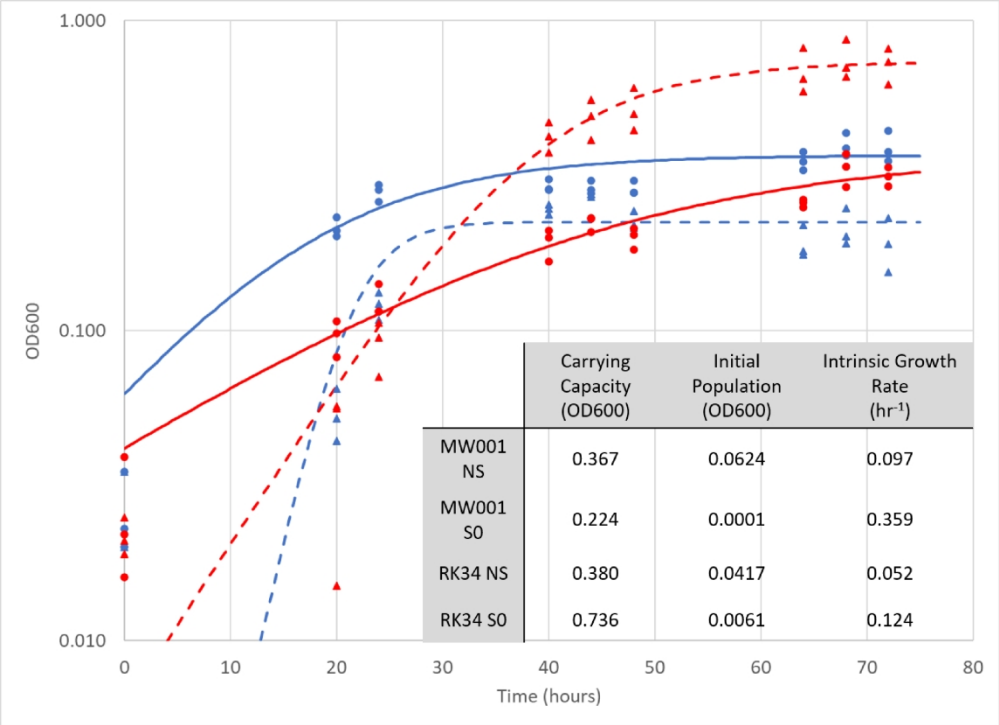


Figure 6: Reduction potential of enzymatic sulfur half-reactions (green) and energy carrier half-reactions (orange); bars represent the physiological range of reactant/product ratios; vertical lines in each bar represent the equimolar transformed reduction potential of the half-reaction.



	Carrying Capacity (OD600)	Initial Population (OD600)	Intrinsic Growth Rate (hr^{-1})
MW001 NS	0.367	0.0624	0.097
MW001 S0	0.224	0.0001	0.359
RK34 NS	0.380	0.0417	0.052
RK34 S0	0.736	0.0061	0.124

Figure 7: Growth curves of Saci MW001 (blue) and RK34 (red) on amino acids without sulfur (circles) and with sulfur (triangles); Logistic equation models for the data are shown as solid lines (without sulfur) or dotted lines (with sulfur); values for the logistic equation parameters are shown in the insert, where "NS" indicates the condition without sulfur and "S0" indicates the condition with sulfur

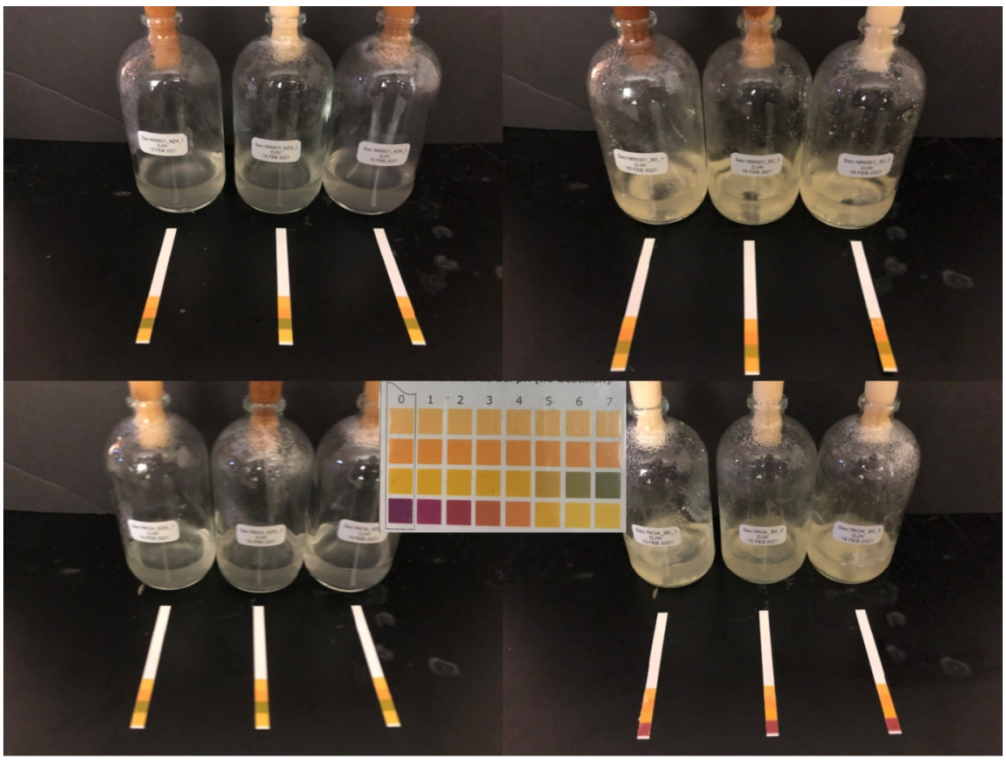


Figure 8: Final pH measurement of serum bottles for Saci MW001 without sulfur (pH 6, top left), Saci MW001 with sulfur (pH 5.5, top right), Saci RK34 without sulfur (pH 5.5, bottom left), and Saci RK34 with sulfur (pH 2, bottom right).