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Review

The Confluence of Heavy Metal Biooxidation and Heavy Metal Resistance: Implications for Bioleaching by Extreme Thermoacidophiles

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Abstract: Extreme thermoacidophiles (T_{opt} > 65 °C, pH_{opt} < 3.5) inhabit unique environments fraught with challenges, including extremely high temperatures, low pH, as well as high levels of soluble metal species. In fact, certain members of this group thrive by metabolizing heavy metals, creating a dynamic equilibrium between biooxidation to meet bioenergetic needs and mechanisms for tolerating and resisting the toxic effects of solubilized metals. Extremely thermoacidophilic archaea dominate bioleaching operations at elevated temperatures and have been considered for processing certain mineral types (e.g., chalcopyrite), some of which are recalcitrant to their mesophilic counterparts. A key issue to consider, in addition to temperature and pH, is the extent to which solid phase heavy metals are solubilized and the concomitant impact of these mobilized metals on the microorganism's growth physiology. Here, extreme thermoacidophiles are examined from the perspectives of biodiversity, heavy metal biooxidation, metal resistance mechanisms, microbe-solid interactions, and application of these archaea in biomining operations.

Keywords: extreme thermoacidophiles; bioleaching; heavy metal resistance; heavy metal biooxidation; archaea

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1. Introduction

The commercial application of microorganisms for the extraction of metals from sulfide ores, concentrates, low-grade ores and tailings, often referred to as bioleaching and biomining, falls within the discipline biohydrometallurgy [1,2]. Bioleaching leverages microbially-based conversion of insoluble metal sulfides (or oxides) to water-soluble metal sulfates. For example, conversion of insoluble chalcopyrite ($CuFeS_2$) to a soluble copper sulfate has become the basis for technologically important processes. Similarly, microorganisms have also been used as a pretreatment step for metals recovery. For example, the removal of sulfur from sulfidic gold ores can enhance downstream recovery and limit depletion of the cyanide extraction agent [3]. The development of bioleaching and biomining technologies has been ongoing for several decades, and more recently is finding increased interest in commercial application. Advances in molecular microbiology and genomic sciences present new opportunities for discovering, characterizing and implementing microbial systems for the recovery of base, precious and strategic metals.

The future of biomining was once declared to be "hot", owing to the recalcitrance of metal sulfides such as chalcopyrite at moderate temperatures, thereby requiring thermal conditions (65–80 °C) to obtain increased solubilization rates [4–6]. At higher temperatures, metal sulfide mobilizing consortia are dominated by archaea, mainly belonging to the genera *Acidianus*, *Metallosphaera*, and *Sulfolobus* [7–11]. The mechanisms by which metal biooxidation, metal resistance, and microbe-solid interactions take place in thermal, acidic environments are not well understood but, if elucidated, could provide valuable information necessary for the successful application and optimization of extremely thermoacidophilic bioleaching.

2. Biodiversity of Extremely Thermoacidophilic Microorganisms

To date, the only known extreme thermoacidophiles (as defined here, microorganisms with $T_{opt} > 65$ °C, $pH_{opt} < 3.5$) belong to the crenarchaeotal class of *Thermoprotei*, represented by the orders Desulfurococcales, Thermoproteales, Fervidococcales, Acidilobales, and Sulfolobales, with only certain species in the Sulfolobales thus far considered for bioleaching [12–14]. The Euryarchaeota order Thermoplasmatales, while containing extremely acidophilic genera, some found in bioleaching, are considered to be moderate thermophiles [15]. The Sulfolobales are comprised of the genera *Sulfolobus* (9 species), *Acidianus* (8 species), *Metallosphaera* (5 species), *Sulfurococcus* (2 species), *Stygioglobus* (one species), and *Sulfurisphaera* (one species). The following section highlights the physiology of the Sulfolobales, with an overview of isolation and sequencing chronology presented in Figure 1 and growth physiology in Table 1.

Table 1. Growth physiology information for the Sulfolobales. (Opt.) Optimum, (ED) Electron donor, (EA) Electron acceptor, (Seq.) Genome sequenced, (ND) Not determined, (COR) Complex organic compounds, (AA) Amino Acids.

Species	Isolated From	pH (Opt.)	*T* °C (Opt.)	ED	EA	Growth Modes	Seq.	Refs.
Sulfolobus acidocaldarius (98-3^T, DSM639)	Locomotive Spring, Yellowstone National Park, USA	1.0–5.9 (2.0–3.0)	55–85 (70–75)	H_2 ~, H_2S ?, S^0 ?, FeS ?, $K_2S_4O_6$?, COR, Sugars, AA	O_2	Heterotrophic	Y	[16–20]
Sulfolobus solfataricus (P2, DSM1617)	Hot spring, Pisciarelli Solfatara, Italy	2.0–4.0 (3.0)	65–87 (80)	H_2 ~, H_2S ?, S^0 ?, FeS ?, $K_2S_4O_6$?, COR, Sugars, AA	O_2	Heterotrophic	Y	[17–19, 21,22]
Sulfolobus shibatae (B12^T, DSM 5389)	Geothermal pool, Beppu, Kiushu Island, Japan	ND (3.0)	ND–86 (81)	H_2 ~, S^0, Sugars, AA	O_2	Heterotrophic Mixotrophic	N	[17,18,23]
Sulfolobus metallicus (Kra23^T, DSM 6482)	Continental solfataric fields, Iceland	1.0–4.5 (ND)	50–75 (65)	S^0, Fe^{2+}, FeS_2, $CuFeS_2$, ZnS, CdS	O_2	Chemolithoautotrophic	N	[18,24,25]
Sulfolobus tokodaii (7^T, DSM 16993)	Beppu Hot Springs, Kyushu Island, Japan	2.0–5.0 (2.5–3.0)	70–85 (80)	S^0, Fe^{2+}, COR, AA	O_2	Heterotrophic Mixotrophic	Y	[25–28]
Sulfolobus yangmingensis (YM1^T)	Acidic and muddy hot spring, Yang-Ming National Park, Taiwan	2.0–6.0 (4.0)	65–90 (80)	S^0, FeS, $K_2S_4O_6$, COR, Sugars, AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[29]
Sulfolobus tengchongensis (RT8-4^T)	Sulfur-rich hot spring, Tengchong, China	1.7–6.5 (3.5)	65–95 (85)	S^0, COR, Sugars, AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[30]
Sulfolobus islandicus (Ren1H1)	Solfataric fields, Iceland	ND	ND	ND	ND	Heterotrophic	N ^	[31]
Metallosphaera sedula (TH2^T, DSM 5348)	Thermal pond in Pisciarelli Solfatara, Italy	1.0–4.5 (2.0)	50–80 (75)	H_2, S^0, $K_2S_4O_6$, K_2SO_4, Fe^{2+}, FeS_2, $CuFeS_2$, CdS, SnS, ZnS, COR, Sugars, AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	Y	[18,32–34]
Metallosphaera prunae (Ron 12/II^T, DSM 10039)	Smoldering slag heap, uranium mine, Thüringen, Germany	1.0–4.5 (2.0)	55–80 (75)	H_2, S^o, FeS_2, $CuFeS_2$, ZnS, COR, Sugars , AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	Y %	[35,36]

Table 1. *Cont.*

Species	Isolated From	pH (Opt.)	*T* °C (Opt.)	ED	EA	Growth Modes	Seq.	Refs.
Metallosphaera hakonensis (HO1-1^T, DSM 7519)	Geothermal field, Hakone National Park, Japan	1.0–4.0 (3.0)	50–80 (70)	H_2S, S^0, $K_2S_4O_6$, Fe^{2+}, FeS, FeS_2, $CuFeS_2$, COR, Sugars, AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[19,37–40]
Metallosphaera cuprina (Ar-4^T)	Sulfuric hot spring in Tengchong, Yunnan, China	2.5–5.5 (3.5)	55–75 (65)	S^0, $K_2S_4O_6$, Fe^{2+}, FeS_2, $CuFeS_2$, COR, Sugars, AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	Y	[40,41]
Metallosphaera yellowstonensis (MK1^T)	Acidic iron mat, Yellowstone National Park, USA	1.0–4.5 (2.0–3.0)	45–85 (65–75)	S^0, Fe^{2+} sorbed, FeS, FeS_2, $CuFeS_2$, CuS, ZnS, COR	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	Y	[42,43]
Acidianus hospitalis (W1)	Acidic hot spring, Yellowstone National Park, USA	2.0 ?	85 ?	ND	ND	ND	Y	[44–46]
Candidatus Acidianus copahuensis (ALE1)	Copahue geothermal area, Argentina	1.0–5.0 (2.5–3.0)	55–80 (75)	H_2,S^0, $K_2S_4O_6$,Fe^{2+}, FeS_2, CuS, ZnS, COR, Sugars	Fe^{3+}, S^0, O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	Y	[47,48]
Acidianus infernus (So4a^T, DSM 3191)	Solfatara Crater and Pisciarelli Solfatara, Naples	1.0–5.5 (2.0)	65–96 (90)	H_2, H_2S, S^0	S^0, O_2, MO_4^{2-}	Mixotrophic Chemolithoautotrophic	N	[18,49,50]
Acidianus ambivalens (Lei 10^T, DSM 3772)	Solfatara, Iceland	1.0–3.5 (2.5)	70–87 (80)	H_2, H_2S, S^0	S^0, O_2	Mixotrophic Chemolithoautotrophic	N	[50–52]
Acidianus brierleyi (DSM 1651^T)	Acid hot spring, Yellowstone National Park	1.0–6.0 (1.5–2.0)	45–75 (70)	H_2 ?, H_2S, S^0, Fe^{2+},FeS_2, $CuFeS_2$, ZnS, MoS_2, COR	Fe^{3+}, S^0, O_2, MO_4^{2-}	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[14,18,21, 49,50,53–56]
Acidianus sulfidivorans (JPT^T, DSM 18786)	Solfatara on Lihir Island, Papua New Guinea	0.35–3.0 (0.8–1.4)	45–83 (74)	H_2S, S^0 ,Fe^{2+}, FeS_2,$CuFeS_s$, FeAsS	Fe^{3+}, S^0, O_2	Mixotrophic Chemolithoautotrophic	N	[50]
Acidianus tengchongensis (S5^T)	Acidothermal spring, Tengchong China	1.0–5.5 (1.5–2.0)	60–75 (70)	H_2, S^0, $S_2O_3^{2-}$	S^0, O_2	Chemolithoautotrophic	N	[57]

Table 1. *Cont.*

Species	Isolated From	pH (Opt.)	T °C (Opt.)	ED	EA	Growth Modes	Seq.	Refs.
Acidianus manzaensis (NA-1^T)	Fumarole in Manza, Japan	1.0–5.0 (1.2–1.5)	60–90 (80)	H_2, S^0, COR, Sugars	Fe^{3+}, O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[58]
Acidianus manzaensis (YN25)	Acidothermal spring, Yunnan China	1.0–6.0 (1.5–2.5)	50–85 (65)	H_2, S^0, $K_2S_4O_6$, Fe^{2+}, $CuFeS_2$,COR, Sugars, AA	S^0, O_2	Heterotrophic Mixotrophic	N	[59]
Sulfurisphaera ohwakuensis (TA-1^T, DSM 12421)	Acidic hot spring located in Ohwaku Valley, Hakone, Japan	1.0–5.0 (2.0)	63–92 (84)	H_2, S^0, COR	S^0, O_2	Heterotrophic Mixotrophic	N	[60]
Stygiolobus azoricus (FC6^T, DSM 6296)	Acidic geothermal spring (Furnas Caldeira), São Miguel Island, Azores	1.0–5.5 (2.5–3.0)	57–89 (80)	H_2	S^0	Mixotrophic Chemolithoautotrophic	N	[61]
Sulfurococcus yellowstonensis (Str6kar^T)	Thermal spring, Yellow Stone National Park, USA	1.0–5.5 (2.0–2.6)	40–80 (60)	S^0, FeS_2, ZnS, $CuFeS_2$, Fe^{2+}, COR, Sugars	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[13,62]
Sulfurococcus mirabilis (INMI AT-49^T)	Crater, Uzon volcano in Kamchatka, Russia	1.0–5.8 (2.0–2.6)	50–86 (70–75)	S^0, FeS_2, ZnS, $CuFeS_2$, COR, Sugars, AA	O_2	Heterotrophic Mixotrophic Chemolithoautotrophic	N	[13,62]

Notes: ^{T} Type strain; $^{\sim}$ Indicates poor growth; $^?$ Indicates conflicting evidence as growth substrate; $^{\wedge}$ Tentative type strain not sequenced but strains sequenced; $^{\%}$ Unpublished genome.

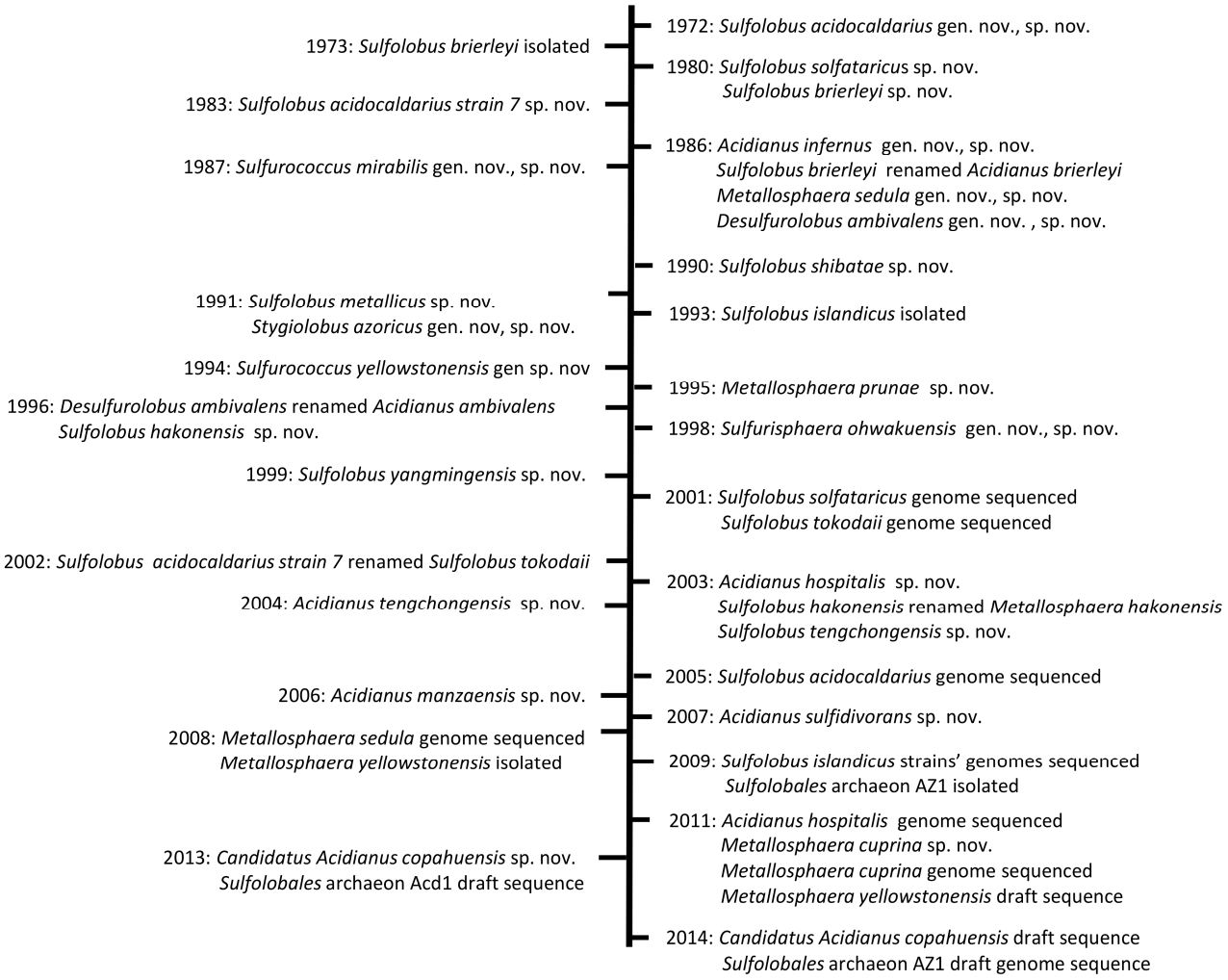

Figure 1. Chronology of microbiology advances within the *Sulfolobales*.

2.1. The Genus Sulfolobus

The genus *Sulfolobus* was defined in 1972 when the first species, *Sulfolobus acidocaldarius* (98-3), was isolated from Locomotive Spring in Yellowstone National Park, USA [16]. The genus is distributed globally, with species typically isolated from acidic geothermal hot springs. Only seven of the nine species comprising the genus have been characterized in any physiological detail. *Sulfolobus* species are strict aerobes, with metabolic features ranging from heterotrophy to obligate chemolithoautotrophy. The genus was initially defined by its members' ability to oxidize elemental sulfur (S^0), although subsequent work provided conflicting evidence on this physiological trait.

Heterotrophic growth represents a unifying trait among *Sulfolobus* species, except for *S. metallicus*, which is an obligate chemolithoautotroph. The range of substrates supporting heterotrophic growth varies markedly within the genus, though all species utilize complex organic substrates (e.g., yeast extract and tryptone). *S. acidocaldarius* grows on a limited set of monosaccharides (D-fucose, D-glucose), disaccharides (sucrose), and polysaccharides (maltotriose, dextrin, starch), along with certain amino acids (L-alanine, L-asparagine, L-aspartate, L-glutamate) [17]. *S. tengchongensis* utilizes more sugars and amino acids than *S. acidocaldarius*, with pentoses (D-arabinose, D-xylose), hexoses (D-galactose, D-fructose), disaccharides (maltose, sucrose), and amino acids (L-glutamic acid)

supporting growth [30]. Compared to *S. acidocaldarius*, *S. solfataricus* also grows on a broader range of sugars, including pentoses, hexoses, disaccharides, and polysaccharides [17]. *S. shibatae* shares many properties with *S. solfataricus*, but represents a distinct species based on genome content [17,23]. Heterotrophic growth of *Sulfolobus tokodaii* (7), formerly *S. acidocaldarius* strain 7, has only been determined for complex organic substrates and certain amino acids, and a complete survey of sugar utilization has not been done [26]. *S. yangmingensis* is capable of utilizing all sugars observed for other *Sulfolobus* species, in addition to L-rhamnose, and utilizes all 20 amino acids, except cysteine [29]. None of the tested complex organic substrates, sugars or amino acids supported growth of *S. metallicus* [24].

The use of inorganic substrates varies widely amongst *Sulfolobus* species. *S. acidocaldarius*, *S. solfataricus*, and *S. shibatae* can oxidize H_2, allowing for mixotrophic growth on H_2 and yeast extract, though growth was poor compared to growth of other *Sulfolobales* [18]. *S. metallicus*, the only obligate chemolithoautotroph within the genus, cannot grow on H_2 as an energy source [18]. H_2 utilization by *S. tokodaii, S. tengchongensis*, and *S. yangmingensis* was not determined at isolation, nor has it been reported to date [26,29,30]. There is conflicting evidence for the chemolithoautotrophic growth of the type strains of *S. acidocaldarius* Deutsche Sammlung von Mikroorganismen (DSM) 639 and *S. solfataricus* DSM 1616. At isolation, both *S. acidocaldarius* DSM 639 and *S. solfataricus* DSM 1616 were reported to grow autotrophically on S^0, but this observation was contradicted by subsequent work and several key reviews [32,62,63]. The consensus appears to be that these species mutated into obligately heterotrophic strains. However, strain analysis of *Metallosphaera hakonensis* (formerly *S. hakonensis*) and *S. tengchongensis*, which also included *S. acidocaldarius* DSM 639 and *S. solfataricus* DSM 1616, showed that these last two Sulfolobus species grew chemolithoautotrophically on S^0, FeS, potassium tetrathionate, and H_2S [19,30].

S. tokodaii was shown to be incapable of chemolithoautotrophic growth on S^0; limited growth was possible on S^0 when supplemented with organic carbon [26]. Further work revealed the capacity of *S. tokodaii* to oxidize Fe(II) with yeast extract supplementation, while weak growth and Fe(II) occurred for autotrophic conditions. [25]. *S. tokodaii*'s genome encodes genes related to hydrogen sulfide metabolism, though their connection to sulfur metabolism has not been determined. [28]. Chemolithoautotrophic growth of *S. yangmingensis* and *S. tengchongensis* by S^0 oxidation has been noted; the former utilizes FeS and potassium tetrathionate [29,30].

S. metallicus is distinctive among members of the genus *Sulfolobus* due to its inability to utilize organic carbon sources for growth, exhibiting obligate chemolithoautotrophy. Inorganic substrates supporting growth include: S^0, $FeSO_4$, FeS_2, $CuFeS_2$, ZnS, and CdS, but not H_2, FeAsS, Cu_5FeS_4, HgS, Cu_2S, CuS, FeS, MoS_2, Sb_2S_3, and SnS [18,24,25]. The ability of *S. metallicus* to oxidize S^0, reduced sulfur compounds, and Fe(II) makes it relevant to heavy metal mobilization applications, particularly bioleaching [64].

S. acidocaldarius and *S. solfataricus*, *S. tengchongensis*, and to a lesser extent *S. shibatae*, appear to be motile [17,23,30], while *S. metallicus* and *S. yangmingensis* are immotile [24,29]. It is not clear whether *S. tokodaii* is motile, but the genome contains the archaellum operon found in *S. acidocaldarius*, indicating *S. tokodaii* is likely motile [65]. To date, published genomes exist for *S. acidocaldarius* (98-3, N8, Ron12/I, SUSAZ), *S. solfataricus* (P2, 98/2), *S. tokodaii* (7), *Sulfolobus* Type II, and a myriad of *S. islandicus* strains [20,22,28,66–71].

S. acidocaldarius, *S. solfataricus*, and *S. islandicus* are the only *Sulfolobales* with genetic tools for molecular biology manipulation [72–76]. In the case of *S. acidocaldarius* and *S. solfataricus*, these genetic systems are dependent upon restoration of an engineered auxotrophy. The recent development of a uracil-auxotrophic *S. acidocaldarius* mutant MW001 has led to a tractable system for metabolic and genetic studies [72], as well as reliable inducible promoters [72,77] and a plasmid-based recombinant expression system [78]. While uracil selection has been utilized in *S. solfataricus*, more success has been achieved in the utilization of the lactose auxotrophic strain PBL2025 and the lactose/maltose auxotrophic strain PBL2069, which also allow for integration of linear DNA fragments [73,74]. In contrast, *S. islandicus* faced difficulties in the early stages of developing reliable auxotrophic selection [79]. However, the recent use of simvastatin affords a distinctly different route of selection based on antimicrobials [76,80]. These new genetic systems provide a means to further study and elucidate metabolic and genetic pathways involved in metal mobilization and resistance.

There is limited information on other Sulfolobus species that have been isolated (e.g., *S. neozealandicus*, [81,82]). *S. islandicus* strains have been isolated from around the world [31,68,83,84], although the growth physiology of the proposed type strain REN1H1 has not been studied to any significant extent [63]. *S. islandicus* is a model species for the study of clustered regularly interspaced short palindromic repeats (CRISPR) systems within the Sulfolobales [85,86].

2.2. The Genus Metallosphaera

The type strain of the genus, *Metallosphaera sedula* (TH2^T), was isolated from a thermal pond in Pisciarelli Solfatara (near Naples, Italy) [32]. To date, the genus *Metallosphaera* includes five reported species with diverse growth physiology. All *Metallosphaera* species are obligate aerobes, utilizing O_2 as their only terminal electron acceptor, and are capable of facultative chemolithoautotrophy, on a variety of inorganic substrates. Variations exists in degrees of heterotrophy in the genus *Metallosphaera*, with *M. cuprina* appearing to be the only member capable of significant growth on sugars [40]. *M. sedula* can grow on beef extract, casamino acids, peptone, trypone and yeast extract, but no utilization of sugars was noted at isolation [32]. However, recent work has indicated *M. sedula* can use D-mannose and individual amino acids (L-aspartic acid, L-glutamic acid, L-tryptophan, L-alanine), though growth is limited for D-mannose, L-aspartic acid, and L-glutamic acid [40]. Originally, *M. prunae* was reported to use the same heterotrophic substrates as *M. sedula*, except for casamino acids and tryptone which were not tested; no utilization of sugars was noted when isolated [35]. Recent work has shown that *M. prunae* can indeed utilize casamino acids and tryptone, along with D-mannose and individual amino acids (L-aspartic acid, L-glutamic acid, L-tryptophan, L-alanine) [40]. However, growth on D-mannose and L-tryptophan is limited compared to complex organic carbon sources. *M. hakonensis* exhibits strong heterotrophy on yeast extract, while has limited growth on maltose, L-glutamic acid and L-tryptophan [19]. However, subsequent analysis showed growth on beef extract, peptone, casamino acids, maltose, and L-glutamic acid, with weaker growth achieved on tryptone [40]. *M. cuprina* differentiates itself from other *Metallosphaera* species by its broader range of sugar utilization, as it is capable of growth on L-arabinose, D-xylose and D-glucose [40]. *M. yellowstonensis* growth on sugars and individual amino acids has not been determined, but the organism can grow on YE as the sole carbon and energy source [43].

The unifying trait among *Metallosphaera* species is their chemolithoautotrophy on S^0. H_2 oxidation has been determined only for *M. sedula* and *M. prunae* [18,35]. Beyond S^0 and H_2, *M. sedula* can grow chemolithoautotrophically with inorganic electron donors, including potassium tetrathionate, potassium sulfate, and metal sulfides (FeS_2, ZnS, $CuFeS_2$, CdS, SnS) and $FeSO_4$ [18,32,33,87]. However, *M. sedula* cannot utilize FeAsS, Cu_5FeS_4, HgS, Cu_2S, CuS, PbS, FeS, MoS_2 or Sb_2S_3 as growth substrates [32]. *M. prunae* is capable of chemolithoautotrophy with inorganic electron donors, including S^0, FeS_2, $CuFeS_2$, ZnS [35], but thiosulfate, potassium tetrathionate, $FeSO_4$, and various coal substrates do not serve as inorganic energy sources. *M. prunae*'s reduced capacity to utilize Fe(II) and potassium tetrathionate as an electron donor differentiates it from others in the genus. Growth on H_2S for *M. sedula* and *M. prunae* has not been reported [40]. *M. hakonensis* exhibits chemolithoautotrophy, with inorganic electron donors FeS, $FeSO_4$, $CuFeS_2$, potassium tetrathionate, H_2S [14,19,37]. Chemolithoautrophic growth for *M. cuprina* was supported by FeS, potassium tetrathionate, $FeSO_4$, FeS_2, $CuFeS_2$ [40]. Unlike *M. hakonensis*, *M. cuprina* was not able to utilize H_2S as an electron donor. Finally, a detailed analysis of *M. yellowstonensis* growth physiology revealed inorganic energy sources Fe(II) sorbed to ferrihydrite, FeS, FeS_2, $CuFeS_2$, CuS, and ZnS [42]. Growth was not supported by $FeCO_3$, Fe_3O_4, $FeSO_4$, potassium tetrathionate, or Na_2S. Autotrophic growth for *M. sedula*, like several other *Sulfolobales*, involves the 3-hydroxypropionate/4-hydroxybutyrate cycle to assimilate CO_2 [88]. This pathway is evident in the sequenced genomes of *M. cuprina* and *M. yellowstonensis* [41,43].

As indicated above, Fe(II) oxidation is associated with growth for *M. sedula*, *M. cuprina*, *M. hakonensis* and *M. yellowstonensis*, though for *M. yellowstonensis* only when Fe(II) is sorbed to ferrihydrite [42]. *M. prunae* cannot utilize Fe(II) for growth, perhaps because of a mutation [36]. As is the case with *S. metallicus*, the capability of *Metallosphaera* species to oxidize S^0, reduced sulfur compounds, and Fe(II) make these archaea relevant to heavy metal mobilization, particularly bioleaching [64].

Motility is a shared characteristic for *M. cuprina*, *M. sedula*, *M. prunae*, but not for *M. hakonensis*; motility has yet to be determined for *M. yellowstonensis*, though its genome contains the archaellum operon found in *S. acidocaldarius*, indicating the potential for motility [19,32,35,40,42,65]. To date, *M. sedula*, *M. cuprina*, and *M. yellowstonensis* have published genomes and an unpublished draft genome exists for *M. prunae* [34,36,41,43]. In fact, the nearly identical genomes of *M. sedula* and *M. prunae* suggest that these are strains and not different species [36].

2.3. The Genus Acidianus

Nine species currently comprise the genus *Acidianus*, all of which are facultative anaerobes capable of chemolithoautotrophy and, in some instances, facultative autotrophy. To date, only the genomes of *A. copahuensis* and *A. hospitalis* have been sequenced [46,48]. The common characteristic displayed by all *Acidianus* species is their ability to oxidize or reduce elemental sulfur, depending on oxygen availability. The only exception is *A. manzaensis* NA-1, which cannot use S^0 as an electron acceptor during anaerobic respiration. Under aerobic conditions, S^0 serves as an electron donor and is oxidized to sulfuric acid. While under anaerobic conditions, S^0 serves as an electron acceptor and is reduced to H_2S. Additionally, all characterized *Acidianus* species can utilize H_2 as an electron donor for aerobic respiration, except *A. sulfidivorans*.

Under anaerobic conditions, when S^0 serves as the electron acceptor, H_2 becomes the electron donor. *A. infernus*, *A. ambivalens*, *A. tengchongensis*, *A. manzaensis* YN25, and *A. copahuensis* are capable of utilizing H_2 as an electron donor, while *A. brierleyi* and *A. sulfidivorans* cannot [47,48,50,57,59]. This suggests that *A. brierleyi* and *A. sulfidivorans* lack the Ni-Fe-hydrogenase, an essential enzyme used by *A. ambivalens* for H_2 oxidation coupled to S^0 reduction [89]. There are conflicting reports as to whether *A. brierleyi* can utilize H_2 as an electron donor for S^0 reduction [18,49,50]. Other electron acceptors include Fe(III) coupled with electron donors S^0 and H_2 for *A. copahuensis* and *A. manzaensis* NA-1, and H_2S for *A. sulfidivorans* and *A. brierleyi* [47,50,58]. Furthermore, *A. brierleyi* and *A. infernus* can utilize MO_4^{2-} as an electron acceptor for S^0 oxidation [49].

A. brierleyi, *A. copahuensis*, *A. manzaensis* YN25, and *A. manzaensis* NA-1 are capable of heterotrophic growth, utilizing organic compounds as the sole energy source [47,49,58,59]. The former, along with *A. sulfidivorans*, are facultative autotrophs, capable of using either organic or inorganic carbon, while *A. infernus*, *A. ambivalens*, and *A. tengchongensis* are obligate autotrophs, solely reliant on inorganic carbon sources. *A. copahuensis*, *A. brierleyi*, *A. sulfidivorans*, and *A. manzaensis* YN25 can oxidize Fe(II) under aerobic conditions [47,49,50,53,59]. This trait, and the fact that *A. copahuensis*, *A. brierleyi*, *A. sulfidivorans,* and *A. manzaensis* YN25 utilize various metal sulfides, make them candidates for bioleaching applications [47,50,54–56,59].

A. hospitalis growth physiology has not been fully characterized, but genome analysis indicates that it can grow by facultative chemolithoautotrophy. As such, energy sources likely include complex organic compounds, S^0, hydrogen sulfide and other reduced inorganic sulphide compounds, but not Fe(II). No Ni–Fe-hydrogenase can be identified in this species, indicating that *A. hospitalis* does use H_2 as an electron donor for growth [46].

2.4. The Genera Sulfurisphaera, Stygiolobus and Sulfurococcus

The only current member of the genus *Sulfurisphaera*, *S. ohwakuensis* (TA-I), was isolated from an acidic hot spring located in Ohwaku Valley, Hakone, Japan [60]. *S. ohwakuensis* is a facultative anaerobe, utilizing S^0 and O_2 as electron acceptors, and H_2 along with complex organic compounds yeast extract and peptone as electron donors. The organism does not exhibit autotrophy, nor were pili- or flagella-like structures found.

Stygiolobus azoricus (FC6) was isolated from an acidic geothermal spring on São Miguel Island, Azores [61]. *St. azoricus* differentiates itself from all other *Sulfolobales* in being strictly anaerobic, but shares with *Acidianus* spp. the ability to grow chemolithoautotrophically by S^0 using H_2 [61]. The microorganism displays no flagella or motility, but is surrounded by pilus- or fimbria-like appendages.

The genus *Sulfurococcus* is represented by two species *S. yellowstonensis* and *S. mirabilis*, both facultative chemolithoautotrophs [13,62]. Both species use complex organic compounds and sugars for growth, but *S. mirabilis* can also utilize amino acids. While these *Sulfurococcus* species use S^0, FeS_2, ZnS, $CuFeS_2$, *S. yellowstonensis* also uses Fe(II). *S. yellowstonensis* capacity for growth on sulfur, mineral sulfides and Fe(II) makes it relevant to bioleaching, as is the case for other Sulfolobales noted above.

2.5. Sequenced and Unclassified Sulfolobales

One novel *Sulfolobales* archaeon, named Acd1, was isolated during a metagenomic study of nanoarchaeon from Obsidian Pool, Yellowstone National Park and has an available genome sequence [90]. Another, *Sulfolobales* archaeon AZ1 was isolated from a hot spring located at Los Azufres National Park, Mexico and has been proposed as Candidatus *Aramenus sulfurataquae*, representing a novel genus and species [91].

3. Biooxidation of Heavy Metals

Pioneering work by Ingledew and co-workers laid the framework for studies on microbial Fe(II) oxidation by the mesophilic bacterium *Acidithiobacillus ferrooxidans*, thereby forming a foundational basis to study Fe(II) oxidation pathways [15,92–96]. Extensively characterized, *A. ferrooxidans* has been the main microorganism considered for biohydrometallurical processes, but challenges with the recalcitrant nature of ores like chalcopyrite motivates the search for other bioleachers. Understanding the basic elements of energy metabolism in heavy metal mobilizing microorganisms is critical for future technological applications, especially when solutions tailored to specific ores are needed.

Initial efforts to understand the mechanisms driving the oxidation of metals by the *Sulfolobales* began more than 20 years ago, although it was only recently that significant progress has been reported in this regard. From the beginning, the respiratory clusters associated with Fe(II) oxidation of the extremely thermoacidophilic *Sulfolobales* could be differentiated from their mesophilic counterparts. Fe(II)-grown cells of *M. sedula* and *A. brierleyi* showed high expression of a novel membrane-bound yellow cytochrome, directly reduced by Fe(II), possibly representing a unique extremely thermoacidophilic redox-active enzyme associated with respiratory Fe(II) oxidation [97]. *Sulfolobus* strain BC, now *S. metallicus*, produced copious amounts of a similar novel acid-stable material during growth on Fe(II), revealing similarity among the Fe(II)-oxidizing archaea [97,98]. The results suggested phylogenetically distinct groups of Fe(II)-oxidizing organisms have characteristically unique acid-stable, redox-active biomolecule.

Until the early 2000s, most studies on crenarchaeotal respiratory chains focused on understanding the molecular properties of oxidases and associated electron transfer proteins [99–101]. The SoxABCD-SoxL complex, an aa_3 quinol oxidase [102–104], the SoxM supercomplex, a bb_3 terminal oxidase [105–108], and the CbsAB-SoxLN complex, a cytochrome *ba* [109] of *S. acidocaldarius*, later found in *A. ambivalens* [110], and the DoxBCE complex, an aa_3-type quinol oxidase [111–113] of *A. ambivalens* had encapsulated the current view of aerobic respiratory electron transfer in the *Sulfolobales*. Unfortunately, neither *S. acidocaldarius* nor *A. ambivalens* can grow well on metal sulfides, thus motivating study of Crenarchaeota capable of growth on metal sulfides.

M. sedula, capable of chemolithoautotrophy with metal sulfides (e.g., pyrite) or sulfur, and heterotrophy with complex organic substrates, is a prime candidate for investigation of Fe(II) oxidation mechanisms within the *Sulfolobales*. Prior to the availability of the *M. sedula* genome sequence, three gene clusters containing oxidases and cytochromes were observed to be differentially expressed, according to whether growth was chemolithoautotrophic (S^0 or FeS_2) or heterotrophic (yeast extract). *M. sedula*'s homologs to *soxB*, representative of the cluster, and *soxM* were highly expressed for

growth on S^0 and yeast extract, respectively [114]. The *soxL2N* transcriptional unit, separately located from *csbA*, exhibited high expression on either S^0 or yeast extract. Growth on S^0 and FeS_2 induced *csbA*, such that it was the highest transcribed gene for FeS_2, indicating the gene product's importance for chemolithoautotrophic growth. CsbA is a membrane-bound cytochrome $b_{566/588}$, implicated in electron shuttling across the pseudo-periplasmic space of *S. acidocaldarius* and speculated to be related to the previously mentioned novel yellow redox-active enzyme [97,114–116]. While a potential chemolithoautotrophic electron shuttle had been identified, the corresponding Fe(II)- and/or sulfur-oxidizing enzymes remained uncharacterized.

Analysis of *S. metallicus* and *S. tokodaii* grown on Fe(II) finally revealed a suspected genetic basis for Fe(II) oxidation among the *Sulfolobales*. The *f*errous iron *ox*idation (*fox*) genes, encoding a novel terminal oxidase complex first characterized in *S. metallicus*, were highly induced during Fe(II) oxidation, with homologs present in *S. tokodaii*, capable of Fe(II) oxidation, but not *S. acidocaldarius*, incapable of Fe(II) oxidation [25]. The significant involvement of the *fox* genes in Fe(II) oxidation was further supported by the observation that pyrite-grown cells, but not sulfur-grown cells, exhibited a dominant membrane-bound protein corresponding to FoxA. Additionally, the observations suggest that one of the *fox* genes is a more likely candidate, than *csbA*, for the previously noted redox-active enzyme, associated with Fe(II) oxidizing *Sulfolobales* [25,97,98,114]. The availability of genome sequence data for the *Sulfolobales* has established the presence of the *fox* gene cluster in Fe(II)-oxidizing species, and the absence of this cluster in non-Fe(II)-oxidizing species, except for *M. cuprina* (Table 2). The genome of *M. prunae* contains the *fox* cluster, but with a mutated foxA', possibly impacting activity of the cluster as indicated by a reduced capacity for uranium and Fe(II) oxidation [36]. The presence of the *fox* cluster appears to correlate to Fe(II)-oxidizing capacity. However, the fact that the *M. cuprina* appears not to encode this cluster, but oxidizes Fe(II), needs to be resolved.

Results from transcriptomic studies continue to support the hypothesis of significant involvement of the *fox* gene cluster in Fe(II) oxidation, though in the absence of biochemical characterization other candidates cannot be completely ruled out [33,43]. As previously noted, the *cbsA-soxLN2* complex exhibited high expression levels in *M. sedula* when grown on Fe(II) and S^0, with further work revealing preferential differential up-regulation for Fe(II) [33,114]. In contrast, analysis of *M. yellowstonensis* showed that *cbsA* expression was the same in the presence and absence of Fe(II) [43]. Furthermore, *csbA-soxNL2* homologs are present in non-Fe(II)-oxidizing *Sulfolobus* species. In light of the previous observations, the cluster cannot be ruled out for Fe(II) oxidation.

Other likely Fe(II) oxidation candidates include the other three terminal oxidase clusters, namely SoxABCD, SoxM supercomplex and DoxBCE. However, gene expression and physiological analysis point to functions of sulfur oxidation for SoxABCD and DoxBCE, and heterotrophy for the SoxM super-complex [33,34,43,114]. As with *csbA-soxNL2*, homologs are present in non-Fe(II)-oxidizing *Sulfolobus* species. The function of rusticyanin, sulfocyanin, and other novel multi-copper oxidases has yet to be determined for extremely thermoacidophilic Fe(II)-oxidizers, though they have been hypothesized to function as electron shuttles to the terminal oxidase. Rus-like blue copper proteins in *Metallosphaera spp.*, with plastocyanin type I domain, were highly transcribed on growth on chalcopyrite and triuranium octaoxide, begging the question about the cellular localization of Rus and its role in the electron transport chain for Fe(II) oxidation in *Metallosphaera spp.* [33,36].

Table 2. Complete terminal oxidase clusters and associated proteins identifiable in sequenced extreme thermoacidophile genomes. (x) and (-) indicate presence and absence, respectively, with (y) being used when a secondary candidate exists. Species in bold are known to oxidize Fe(II). Blank boxes indicate absence of sequence data for those species. Homology searches were completed using previously identified *M. sedula* proteins as search queries with The National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) against the Sulfolobales (taxid:2281) database.

Terminal Oxidase	Fox ABCDEFG	Sox ABCDL	Sox EFGHIM	DoxBCE	CbsAB-SoxL2N	Rusticyanin	Sulfocyanin
Metallosphaera sedula	x	x	x	x	x	x, y	x, y
Metallosphaera prunae	x	x	x	x	x	x, y	x, y
Metallosphaera cuprina	-	x	x	x	x	-	x, y
Metallosphaera yellowstonensis	x	x	x	x	x	x, y	x, y
Sulfolobus solfataricus	-	x	x	x	x	x	x, y
Sulfolobus acidocaldarius	-	x	x	x	x	-	x, y
Sulfolobus tokodaii	x	x	x	x	x	-	x, y
Sulfolobus metallicus	x						
Sulfolobus islandicus	-	x	x	x	x	x, y	x, y
Acidianus brierleyi		x					
Acidianus ambivalens				x	x		
Acidianus hospitalis	-	-	-	x	x	-	x
Candidatus Acidianus copahuensis	x	x	-	x	x	x	x, y

Notes: Gene IDs used for BLAST queries: FoxA (Msed_0484), FoxB (Msed_0480), FoxC (Msed_0478), FoxD (Msed_0477), FoxE (Msed_0475), FoxF (Msed_0474), FoxD (Msed_0469); SoxA (Msed_0289), SoxB (Msed0290), SoxC (Msed_0288), SoxD (Msed_0285), SoxL (Msed_0287); SoxE (Msed_0323), SoxF (Msed_0322), SoxG (Msed_0321), SoxH (Msed_0320), SoxI (Msed_0219), SoxM (Msed_0324); DoxB (Msed_2032), DoxC (Msed_2031), DoxE (Msed_2030); CbsA (Msed_0504), CbsB (Msed_0503), SoxL2 (Msed_0501), SoxN (Msed_0500); Rusticyanin 1 (Msed_0966), Rusticyanin 2 (Msed_1206); Sulfocyanin 1 (Msed_0323), Sulfocyanin 2 (Msed_0826).

There are additional membrane bound redox complexes which may respond to different organic and inorganic substrates: Nicotinamide adenine dinucleotide reduced (NADH):quinone oxidoreductase from *A. ambivalens* and *S. metallicus* is proposed to transfers electron from NADH to quinones [117], while the succinate:quinone oxidoreductase SdhABCD from *A. ambivalens* and *S. tokodaii* is proposed to transfer electrons from succinate to quinones [118,119]. The thiosulfate:quinone oxidoreductase, tetrathionate hydrolase TetH from *A. ferrooxidans* and *Acidithiobacillus caldus* [120,121] functions in S^0 oxidation, in agreement with the *M. sedula* model [33,96]. The DoxDA, aa_3 type quinol oxidase, has been annotated as a thiosulfate:quinone oxidoreducatase (TQO) in *A. ambivalens* [111], with likely involvement in sulfur oxidation. It has been shown that TQO can oxidize thiosulphate to tetrathionate, using ferricyanide or decyl ubiquinone (DQ) as electron acceptors [122].

As noted above, biooxidation of Fe(II) has been most widely and extensively studied in *A. ferrooxidans* and a brief overview of the current model is warranted prior to discussion of models within the *Sulfolobales*. The overall organization of Fe(II) oxidation components, mainly the vertical topography where Fe(II) is kept outside the cell, appears to be conserved, but significant diversity exists among the redox proteins [15]. For *A. ferrooxidans*, the transfer of electron from Fe(II) to oxygen involves a super-complex connecting the outer and inner membranes. The super-complex consists of an outer membrane high molecular-weight cytochrome *c*, encoded by *cyc*2, where Fe(II) oxidation occurs [123,124], a gene of unknown function (ORF1), a periplasmic soluble blue copper protein rusticyanin encoded by *rus* believed to responsible of uphill/downhill bifurcation [125,126], and a periplasmic membrane-bound di-hemic cytochrome c_4 encoded by *cyc*1 [127]. Downhill flow proceeds to a terminal aa_3-type cytochrome oxidase encoded by the *coxBACD* gene cluster [128,129]. The uphill components flow proceeds to a cytochrome bc_1 complex (complex III, ubiquinol-cytochrome *c* reductase) through the quinone pool, and finally to a NADH1 dehydrogenase complex [130–132]. The bc_1 complex is part of a five-gene operon, termed the *petI* operon, which is adjacent to the *resBC* operon, suspected to be involved in the construction of the c_1 cytochrome [96]. Elements remaining to be determined in the electron transport chain are the specific interactions between certain complexes, assembly proteins, and the mechanisms of regulation for modulating uphill or downhill flux [96].

To date, hypothetical models for Fe(II) oxidation by the Fox cluster has been proposed for *Metallosphaera* species, based on expression, modeling and comparative genomic analysis (Figure 2) [33,43]. The Sulfolobales do not have the initial electron acceptor from Fe(II) found in *A. ferrooxidans*, a *c*-type cytochrome, supporting the notion that the *Sulfolobales* Fe(II) oxidation pathway is evolutionarily distinct [15]. Initially, electrons are extracted from Fe(II) by FoxCD, cytochrome *b*, and shuttled by a multi-copper oxidase either uphill or downhill. FoxA1 and FoxA2 are annotated as cytochrome c-oxidases (subunit I), forming a complex with FoxB, annotated as cytochrome c-oxidase (subunit II), and receive electrons proceeding in the downhill direction from the multi-copper oxidase. FoxG has been annotated as a 4Fe–4S polyferredoxin-like protein, and can form a complex with FoxCD for uphill electron flow through the multi-copper oxidase to the cytochrome *ba* complex, CbsAB-SoxLN. Electrons then pass through the quinone pool, finally to a NADH dehydrogenase. FoxH has been annotated as a signal transduction protein and its location in the *fox* cluster suggests an Fe(II)-sensing role.

Recently, *M. sedula* was found to oxidize uranium trioxide, with the *fox* cluster likely mediating the oxidation process [36]. The conclusion was supported by *M. prunae*'s inability to transform the oxide

and a corresponding frame shift in *foxA*', possibly also exerting a polar effect on the *fox* cluster. Prior to this report, three microorganisms possessed the ability to oxidize tetravalent U(IV) to hexavalent U(VI), namely the aerobic acidophilic chemolithotroph *A. ferrooxidans*, previously discussed, the anaerobic chemoorganoheterotroph *Geobacter metallireducens*, and the anaerobic obligate chemolithotroph *Thiobacillus denitrificans* [133–135]. The latter two catalyze the nitrate-dependent oxidation of U(IV). Two di-heme, *c*-type cytochromes, putatively c_4 and c_5 cytochromes, have been found to play a major role in the nitrate-dependent U(IV) oxidation by *T. denitrificans* [136]. The two cytochromes are membrane-associated and may be periplasmic, based on homology to characterized c_4 and c_5 cytochromes in *Pseudomonas stutzeri*. The fact that periplasmic, rather than outer membrane, proteins are involved in the oxidation of UO_2 suggests that U(IV) dissolution occurs before U(IV) oxidation, because it is unknown how periplasmic proteins would interact with a solid mineral substrate. Siderophores could enhance the solubility of U(IV), making it more bioavailable to the periplasmic cytochromes, or perhaps some yet undetermined outer membrane protein directly contacts the UO_2. The biological oxidation of uranous sulfate, a soluble U(IV) species, by *A. ferrooxidans* has been demonstrated [133]. The authors hypothesized that rusticyanin was the first protein in the electron transport chain for the uranous ion. Subsequent electron transfer involved a yet unidentified electron acceptor between rusticyanin and cytochrome *c*. Based on more recent evidence for Fe(II) oxidation, the initial electron acceptor could be Cyc2, as opposed to rusticyanin. This is supported by the fact that the uranous ion has been found to be a competitive inhibitor of Fe(II) oxidation, which would implicate use of the same cytochrome *c* [137].

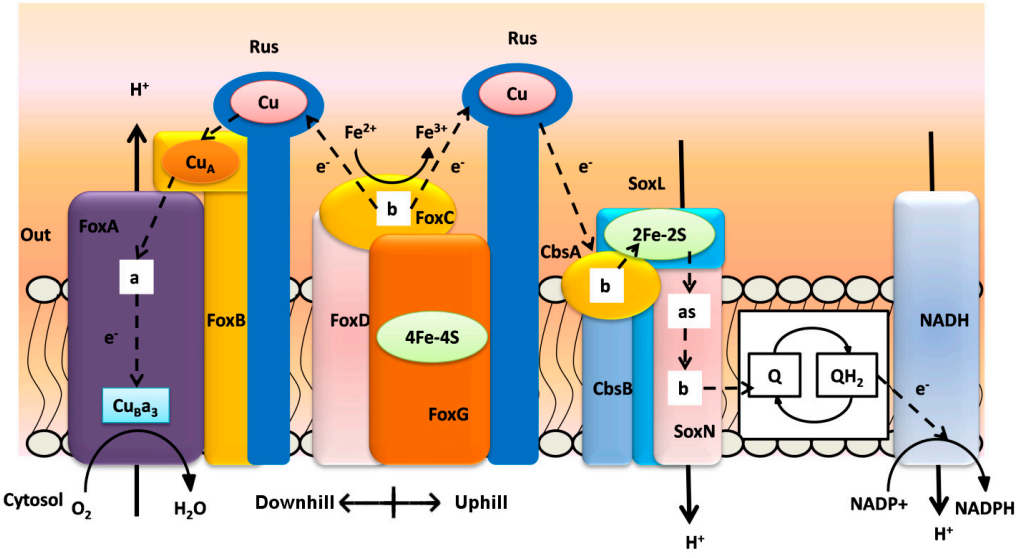

Figure 2. Proposed model for Fe(II) oxidation in *M. sedula*, based on transcriptional response experiments and bioinformatics analysis [33,43]. FoxC is believed to be the primary electron acceptor from metal ions and transfer the electrons to a blue copper protein (Rus—rusticyanin), which can follow an uphill electron flow to $NADP^+$ or a downhill electron flow to O_2. The dotted line shows the direction of electron flow. (**a**) heme a center in FoxA, (Cu_Ba_3) binuclear center in FoxA where O_2 is reduced to H_2O; (**b**) heme b in FoxC and soxN, (Cu_A) copper center in FoxB, (Fe–S) iron sulfur clusters in FoxG and SoxL, (Q) ubiquinone, (QH_2) hydroquinone.

4. Heavy Metal Resistance Systems in Extreme Thermoacidophiles

The dichotomy between metabolic requirements for metals by microorganisms and the potential associated toxicity has created an interesting and broad set of metal homeostasis and resistance systems required to maintain a delicate balance [138]. The concern of metal toxicity is particularly pertinent to metal mobilizing microbes, which must resist toxic heavy metals released into the environment as a result of their energy metabolism. In general, there are seven broadly defined categories of postulated mechanisms for metal resistance/tolerance in microorganisms (Figure 3): (1) passive tolerance; (2) metal exclusion by permeability barrier; (3) active transport of the metal; (4) intracellular sequestration of the metal by protein/chelator binding; (5) extracellular sequestration of the metal by protein/chelator binding; (6) enzymatic detoxification of the metal to a less toxic form, and (7) reduction in metal sensitivity of cellular targets to metal ions [139]. Microorganisms may contain one or more combinations of the above resistance mechanisms, but the primary mechanism for regulating intracellular metal concentrations under normal growth conditions involves membrane transport. However, exposure to higher toxic concentrations can elicit other more stringent responses that reduce non-specific uptake or induce specific metal resistance mechanisms, for example efflux [140]. The following section describes the current knowledge of metal resistance mechanisms with an emphasis on extremely thermoacidophilic microorganisms, useful for bioleaching applications. Comprehensive reviews covering microbial metal resistance exist, but few specifically target extreme thermoacidophiles [141–146].

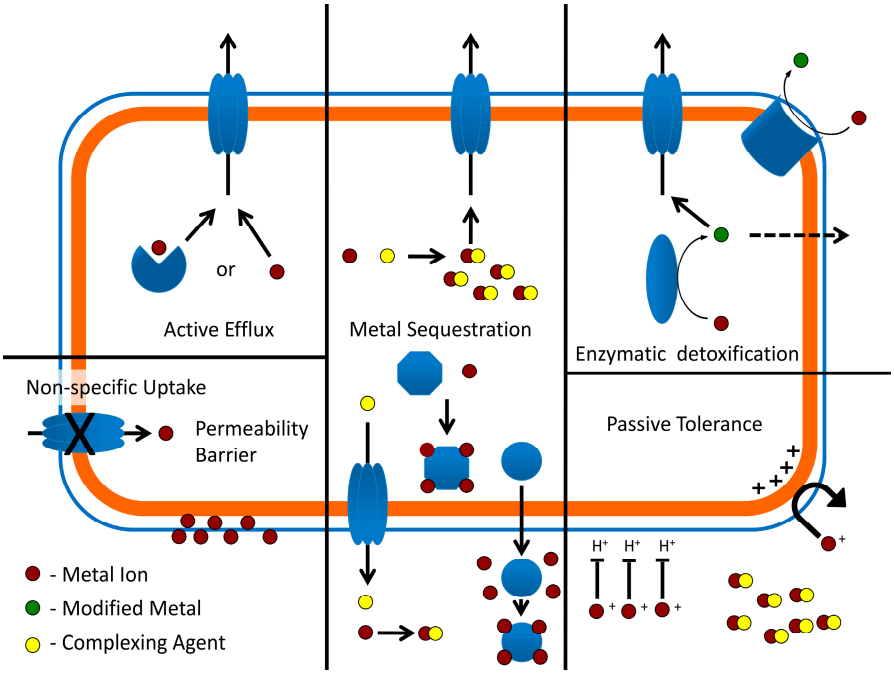

Figure 3. Overview of metal resistance mechanisms for acidophiles. Energy for transporters can be provided by ATP (P-type ATPase), proton gradient (RND), or chemiosmotic (CDF). Metal sequestration can occur through small molecule complexing agents (e.g., phosphate) or metal-chelating proteins. The exterior blue barrier represents some external permeability barrier (e.g., S-layer or biofilm). The figure does not including reduction in metal sensitivity of cellular targets.

4.1. Passive Tolerance and Metal Exclusion

Recent literature concerning the high capacity of acidophiles to tolerate significantly higher levels of metal ions than their neutrophilic counterparts has revealed a potentially important set of passive tolerance features [141,147]. The first passive mechanism relates to metal ion chelation by sulfate ions, which are typically high in acidophile habitats and are coupled to metal concentrations. Chelation significantly reduces the availability of free ions, which are much more toxic [147,148]. However, soluble complexes can still exert significant toxicity, as found for zinc phosphate with the neutrophile *Arthrobacter* sp. [149]. Unlike neutrophiles, acidophiles maintain an inside positive cytoplasmic transmembrane potential, thus generating a chemiosmotic gradient inhibiting proton and metal cation passage across the membrane [147,150,151]. At lower pH, a greater competition exists between protons and metal cations for metal-binding sites [152,153], which has been hypothesized to account for decreased toxicity of zinc at lower pH values for acidophiles [154]. However, for more toxic metals, the importance of the above passive systems might be limited [147]. For example, the capacity of an *M. sedula* strain, deficient in an active efflux system for copper, failed to mobilize chalcopyrite, despite the concomitant increase of sulfate during mineral dissolution [155].

Metal exclusion represents another general defense against toxic metal effects and involves alterations in the cell wall, membrane, envelope, or surface layer (S-layer) in an attempt to prevent damage to intracellular or cytoplasmic targets [139]. Arsenate can be taken up by phosphate transport systems in bacteria, but enhanced resistance can be achieved with highly specific phosphate transporters, excluding arsenate. Specifically, for *E. coli*, this involves use of the Pst system as opposed to the less specific Pit system [156]. A loss of function for the Pit system in *M. sedula* increased resistance compared to a spontaneous mutant harboring a restored version, but found to reduce resistance to copper [157]. The result is consistent with mutations of low-affinity, high velocity transporters *pit* and *corA* being more tolerant to arsenate and cobalt, respectively [158–161]. Therefore, a potential system of defense relies on the mutation of non-specific systems or use of more specific uptake systems for essential nutrients in an attempt to exclude toxic metals and avoid the "open gate" issue [140,162]. Limitations exist for this strategy, as use of more specific uptake systems generate tolerant mutants that are less robust than the wild-type [144]. Another resistance strategy exists for metals, such as nickel and cobalt, where repression of permeases responsible for their uptake can prevent associated toxic effects when extracellular concentrations become physiologically dangerous [163].

Non-specific binding of metals by the outer membrane, envelope, S-layer, extracellular polymeric substances (EPS), and/or lipopolysaccharide (LPS) offers yet another means of metal exclusion. Biofilms, generally composed of extracellular polymeric substances, are capable of enhancing metal tolerance of attached communities since they sorb metals [164–166]. The extracellular matrix and S-layer, known to contain many functional groups capable of interacting and trapping metals, have in the case of *Bacillus sphaericus* been shown to act as a protective uranium immobilizing matrix, resulting in a local detoxification [167–170]. This offers limited metal protection due to saturation of binding sites, but regeneration of binding sites by enzymes, such as phosphatases, can extend the effectiveness of the system [169].

4.2. Copper

Among the metals studied in extreme thermoacidophiles, copper has received the most attention because of the potential of these archaea for extraction of copper from primary ores like chalcopyrite. The known copper resistance strategies employed by these microorganisms includes active transport and metal sequestration. The active transport system utilizes members of the P-type ATPase superfamily, which includes many members responsible for pumping cations against steep electrochemical gradients by exploiting the energy from ATP hydrolysis [171]. A myriad of microorganisms, including the widely studied *S. solfataricus*, employ single-unit membrane class IB heavy metal translocating P-type ATPases [171–174], used for metal (Cu^{2+}, Cu^{+}, Ag^{+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}) homeostasis and resistance [175–178]. The metal sequestration system is associated with inorganic phosphate metabolism, known to be involved in bacterial stress responses [179–182]. The role of polyP metabolism in metal stress response among extremophiles has received limited attention, despite the potential of this system to confer significantly higher levels of copper resistance [183,184].

The *S. solfataricus* genome exhibits only two P-type ATPases, both belonging to class 1B, CopA and CopB encoded by Sso_2651 and Sso_2896, respectively [22]. CopA and CopB are known to impart copper resistance to *S. solfataricus*, with CopA being an effective copper pump at low and high copper concentrations, and CopB apparently functioning as a low-affinity copper export ATPase extending resistance at higher concentrations [178]. Similar roles have been observed for the homologous CopA and CopB of *Thermus thermophilus*, with stimulation of each occurring most in the presence of Cu(I) and Cu(II), respectively [185]. Interestingly, P_{IB}-type copper pumps of the CopA-2 subclass have been implicated in the assembly of metalloproteins, such as the copper-containing cbb_3-type respiratory oxygen reductases [186–188]. As a result, CopA and/or CopB may function together with periplasmic copper chaperons in the assembly of the ba_3-type and caa_3-type copper-containing respiratory oxygen reductases present in *T. thermophilus* [185]. However, Völlmecke *et al*. (2012) demonstrated neither P_{IB}-type ATPase, both of the CopA-1 subclass, found in *S. solfataricus* play an essential role in cytochrome oxidase biosynthesis.

The CopA operon occurs as the gene cluster *copRTA*, encoding the copper-responsive regulator CopR [189], the copper-binding protein CopT containing the metal coordinating ligands within the so-called trafficking, resistance and sensing of heavy metals (TRASH) domain [190], and the Cu(I) transporting P_{1B}-type ATPase, which are induced under the presence of excess copper and represent the general structure of the operon found in archaea [191,192]. The *copB* gene cluster is organized in a different region, with the transcriptional regulator *copY* and a small copper chaperone of the heavy metal associated (HMA) group *copZ* arranged in the opposite orientation of the Cu(II) transporting P_{1B}-type ATPase, *copYZ*/*copB* [178]. The catalytic ATP-binding/phosphorylation domain of CopB was shown to be active in the presence of Cu(II), but not Cu(I), and is believed to play a role in the transport of Cu(II) [193]. In the hyperthermophilic, sulfate-reducing archaeon *Archaeoglobus fulgidus*, two copper-transporting ATPases, CopA and CopB, were isolated, characterized and found to activated on Cu(I) and Cu(II), respectively [194,195]. The CopA of *A. fulgidus* has been extensively studied with regards to its metal binding, actuator, ATP binding domains, and interaction with chaperones [196–202].

The genome of the extreme acidophilic archaeon *Ferroplasma acidarmanus* contains a *cop* operon encoding a putative transcriptional regulator (*copY*), a putative metal-binding chaperone (*copZ*), and a putative copper-transporting P-type ATPase (*copB*) [190]. Transcript levels of the co-transcribed *copZB* were found to increase in response to exposure to high levels of Cu^{2+} [203]. Recently, a similar *copA* operon to the one in *S. solfatariucus* was studied in *M. sedula*, which has 20 times greater resistance to Cu^{2+} [32,34,155,189]. A genetics-based investigation proved the functional role of the P_{IB}-type ATPase operon *copRTA* found in the *M. sedula* genome [155]. Further, targeted recombination of *copA* compromised both metal resistance and eliminated chalcopyrite bioleaching. Two non-identical *cop* loci in *S. metallicus* have been identified to respond to both copper and cadmium, implicating functionality in resistance response [204].

Despite the possibility of polyP to impart broad metal resistance, study of polyP related to stress responses has largely been ignored in extremophiles, especially those best suited for biomining applications, except for the bacterium *A. ferrooxidans* and the archaeon *S. metallicus* [182,205,206]. Several acidophilic organisms, including *A. ferrooxidans*, *S. metallicus*, *S. acidocaldarius*, *A. thiooxidans*, *M. sedula*, *A. caldus* and to a lesser extent *S. solfataricus*, accumulate polyP [146,183,184]. Comparison of polyP production in *S. solfataricus* to that in *S. metallicus* showed that *S. metallicus* had significantly higher polyP synthesis and could tolerate up to 200 mM copper sulfate, while *S. solfataricus* could not resist more than 1–5 mM copper sulfate, suggesting a relationship between Cu-resistance and polyP levels [184]. Also, a study on the transcriptional and functional genes related to survival in the presence of copper for *A. ferrooxidans* identified polyP as contributing to copper resistance [207]. Another system found in *A. ferrooxidans*, but not found in the *Sulfolobales*, is the proton-driven Cus CBA-transport system, studied extensively in *E. coli* [146,208]. Both the Cop and Cus mechanisms are believed to be key determinants in the copper resistance of *A. ferrooxidans* [207].

Enzymes essential to polyP metabolism are the polyphosphate kinase (PPK) that catalyzes the reversible conversion of ATP's gamma phosphate into polyP and the exopolyphosphatase (PPX) that hydrolyzes terminal residues of polyP [206]. Interestingly, production of polyP has been proven to occur in several archaeal species, but only PPX proteins and genes have been described, in particular for *S. solfataricus* [209]. Further, analysis of extremophilic archaea has shown that Crenarchaeota possess *ppx* genes but lack *ppk* genes, while Euryarchaeota possess *ppk* genes, but no *ppx* genes. Most extremophilic bacteria included in the analysis contained both genes [206]. The role of polyP in metal resistance in extremophiles encompasses both its role as an energy source and metal chelating agent. Through the action of PPX, the microorganism can generate organic phosphate for metal chelation, or the reversible reaction catalyzed by PPK can generate additional ATP for heavy-metal efflux systems or other cellular metabolism associated with metal challenge [142]. The importance of phosphate metabolism, specifically import via an archaeal Pit system, to enhanced copper resistance for an *M. sedula* mutant has been established and indicates phosphate plays a key role in supernormal copper resistance [157].

4.3. Mercury

Currently, the most pervasive and generally employed mercury resistance strategy among bacteria and archaea occurs through volatilization [210–214]. Mercury methylation or reduction can lead to

volatilization, but only the latter is believed to operate as a resistance mechanism, because organomercurials are highly toxic [144]. Bacterial mediated mercury methylation occurs anaerobically, is coupled to dissimilatory reduction of various electron acceptors, mostly sulfate [215], and has been extensively studied and recently reviewed [216,217]. There exists a limited understanding of mercury methylation in archaea, but it has been shown to occur in certain methanogens [218]. The known mercury resistance strategy in extreme thermoacidophiles is based on reduction of Hg^{2+} to the volatile Hg^{0+} by the Hg-reductase MerA and is a homolog of the thoroughly investigated bacterial mercury resistance system (*mer*) encoded by the *mer* operon [144,210,213,219,220]. The extreme thermoacidophile mechanism is based on genes encoded by the *merRAHI* operon, which has been studied in detail for *S. solfataricus* [221,222]. The gene *merA* encodes a protein that is homologous to the bacterial mercury reductase MerA. MerR acts as a negative regulator inducing transcription without leaving the *merA* promoter. MerH contains the conserved metal binding TRASH domain and is suspected to chaperone mercury for mobilization. MerI has a yet undetermined functional role [221,222]. Like other early evolving microbial lineages, *Aquifica* [223] and *Thermus/Deinococcus* [224], *S. solfataricus*'s (Crenarchaeota) *mer* operon encodes fewer functional genes than the operons found in *Proteobacteria*, *Firmicutes*, and *Actinobacteria* [211]. Another avenue for mercury reduction beyond the *mer* system has been discovered in *A. ferrooxidans*, where a cytochrome *c* oxidase was found to detoxify mercurial compounds [225,226].

4.4. Other Metals (Arsenic, Cadmium, Nickel, Uranium)

Although most work to date centers on copper and mercury resistance, other metals have been studied. Arsenic resistance systems include arsenate reduction followed by arsenite efflux, complexation by metallothioneins, and methylation [227–229]. The most pervasive system employs an arsenic resistance (*ars*) operon encoding an As^{3+} responsive transcriptional repressor (ArsR) [230], an arsenate reductase (ArsC) responsible for extending resistance to As^{5+} by mediating As(V) reduction to As(III) [231], which is then extruded by the ArsB antiporter, catalyzing exchange of $As(OH)_3$ for protons and thus conferring resistance [232]. Additionally, some *ars* operons contain an ArsB complexing As^{3+}-translocating ATPase (ArsA), enhancing resistance [233], and an arsenic metallochaperone (ArsD) that transfers As^{3+} to ArsA, increasing its ability to extrude arsenite [234,235]. Other genes associated with *ars* operons include the putative thioredoxin reductase (*arsT*) [236,237] or thioredoxin system (*arsTX*) [238] required for As(V) reduction using NADPH reducing power, and two genes of unknown function with weak homology to oxidoreductases (*arsO* and *arsH*) [236,239,240]. The ArsH protein from *Shigella flexneri* was shown to have NADPH-dependent FMN reductase activity [241].

Several archaeal genomes contain homologs of the ArsC [162], but a sub-section that contain other *ars* operon components, or are known to be resistant to arsenate, appear to lack ArsC. The extreme arsenite resistance of *F. acidarmanus* has been attributed to the established *ars* operon, which for this organism is only represented by the arsenite inducible operon homologous to *arsRB* [242]. Despite the absence of a homolog to the arsenate reductase (*arsC*), the inability to reduce arsenate and accumulation of intracellular arsenate, *F. acidarmanus* still possesses extreme arsenate resistance [242,243]. Since ArsB can only extrude As(III), an unknown and novel arsenate resistance mechanism likely exists in *F. acidarmanus*, with modes including direct efflux of As(V), intracellular sequestration, resistance of

cellular components to As(V), or high levels of intracellular phosphate [242]. *F. acidarmanus* genome does contain a homolog to *arsA*, but due to the lack of a promoter and an N-terminal domain it is likely a pseudogene [242]. A similar arrangement can be found in other extreme acidophilic archaea, such as *Thermoplasma acidophilum* and *Picrophilus torridus*, with the addition of either a partial or complete separately encoded *arsA*, respectively [244,245]. The genomes of both *S. solfataricus* and *M. sedula* encode a stand-alone arsenite transporter ArsB [34]. Transcriptional analysis of the archaea *Pyrobaculum aerophilum* utilizing arsenate as the terminal electron acceptor revealed the up-regulation of a putative *arsR* homolog, but not the up-regulation of an annotated arsenical pump-driving ATPase and arsenite permease [246]. In contrast to acidophilic archaea, *arsC* containing operons are present in other acidophilic bacteria, such as *Acidithiobacillus ferrooxidans* [239], *Acidithiobacillus caldus* [247], *Acidiphilium multivorum* [248], and *Leptospirillium ferriphilum* [249].

Beyond *F. acidarmanus*, *ars*-based arsenic resistance in archaea has only been extensively characterized in *Halobacterium* sp. strain NRC-1. The megaplasmid pNRC100 encodes the gene clusters *arsADRC* and *arsR2M*, while *arsB* occurs on the chromosome [250]. Deletion of the *arsADRC* cluster resulted in increased sensitivity to arsenite and antimonite, while deletion of *arsB* caused no change in sensitivity to either arsenate or arsenite, indicating *Halobacterium* sp. strain NRC-1 contains a novel arsenite/antimonite extrusion system vastly different from bacterial counterparts [250]. The *arsM* gene was determined to be a putative methyltransferase, known to exist in mammals, and knockout of the gene produced sensitivity to arsenite, possibly indicating a novel detoxification stragtegy [250]. Analysis of microbial genomes identified 125 bacterial and 16 archaeal homologs of *arsM* genes, with a subset located downstream of an *arsR* gene, suggesting these ArsMs confer arsenic resistance [251]. The system now represents an established arsenic resistance system for certain archaea and bacteria [162].

Arsenite oxidation could represent an alternative or enhancing strategy to other known arsenic resistance systems [227,252]. The membrane fraction *S. metallicus* (formerly *Sulfolobus acidocaldarius* strain BC) has been shown to oxidize arsenite to the less toxic arsenate using an unknown oxidase [253]. Recently, *A. brierleyi* was shown to oxidize arsenite from refinery wastewater by an undetermined mechanism, presumably an arsenite oxidase [254]. During the bioleaching of arsenic containing ores and concentrates, considerable care must be exercised as mineral dissolution releases arsenite and unless sufficient Fe(III) is present to oxidize As(III), toxicity ensues [254]. The capacity of extremely thermoacidophilic archaea, involved in biomining, to oxidize arsenite differentiates them from the majority of their mesophilic counterparts [255].Many phylogenetically distinct bacteria are known to oxidize As^{3+} [256,257] using the heterodimeric enzyme Aio (formerly Aox, Aro or Aso; see [258]), comprised of the AioA (molybdopterin) and AioB (Rieske) subunits. Among archaea with sequenced genomes, which does not include *S. metallicus* or *A. brierleyi*, several including *Pyrobaculum calidifontis*, *Sulfolobus tokodaii*, and *Aeropyrum pernix*, were found to harbor *aio* clusters, indicating these as putative As^{3+}-oxidizing archaea [256]. The *aio* gene cluster appears to be linked to an "ancient" bioenergetic pathway [257].

In general, few studies exist exploring cadmium resistance mechanisms among acidophiles, especially extreme thermoacidophiles. The common detoxification mechanism in neutrophiles employs a variety of active efflux systems [144,145]. Although not elucidated, analysis of sequenced acidophile genomes indicates cadmium efflux, mediated by CadA, might be a common resistance mechanism amongst

acidophiles [259]. Exposure of *S. metallicus* to cadmium revealed the response of two *cop* loci, suggesting the locus not only functions for copper detoxification, but cadmium as well [204]. Additionally, the cadmium response, along with copper, elicited a defensive stress response including proteins related production and conversion of energy, amino acids biosynthesis, stress responses, and transcription regulation. The results of a general defensive response are consistent with previous characterization and appear to represent a general cellular response to metal challenge [203,242,260,261].

No determinants of nickel resistance have been experimentally identified in acidophilic archaea, despite a detoxification system, based on efflux, existing for bacteria [144,145]. The nickel resistance determinant has been identified for acidophilic bacteria *Leptospirillum ferriphilum*, which was attributed to a nickel–cobalt resistance operon (NCR) [262,263]. However, the only study to date in acidophilic archaea identified redox stress proteins involved in the adaptation response of *S. solfataricus* to nickel challenge [260].

A number of processes have been investigated for the bioaccumulation of uranium, which includes biosorption [264], bioreduction [265], and biominerlization [266–268]. These studies have largely focused on bacteria with the mechanisms of uranium accumulation and the resulting uranium complexes being poorly understood in archaea. Given the differences in cell wall structures between archaea and bacteria, differences in interaction mechanisms can be expected [269]. The anaerobic hyperthermophile, *Pyrobaculum islandicum*, has been shown to reduce U(VI) to the insoluble U(IV) mineral uraninite leading to the formation of extracellular deposits [270]. Dense uranium deposits were observed at the cell surface in the halophilic archaeon *Halobacterium halobium*, with complexation of uranium predominantly via cellular inorganic phosphate (uranyl phosphate) [271]. More recently, the interaction of *S. acidocaldarius* with U(VI) was studied under highly acidic (pH 1.5–3.0) and moderately acidic (pH 4.5) conditions, relevant to the physiological growth optimum of this organism and uranium polluted environments [269,272]. For the highly acidic conditions, U(VI) was demonstrated to complex with organic phosphate groups, while under moderately acid conditions carboxylic groups were also involved in U(VI) complexation. Intracellular deposits associated with the inner side of the cytoplasmic membrane represented the majority of U(VI) accumulation, with a small amount biomineralized extracellularly [269]. In contrast to the use of organic phosphate groups, neutrophilic bacteria are known to secrete orthophosphate (via polyphosphate metabolism) and form inorganic uranium precipitates that serve to protect bacterial cells from uranium toxicity [266,267,273]. The pH dependence of uranium complexation in *S. acidocaldarius* differs from the pH independent process of the acidophilic bacterium *A. ferrooxidans*, where uranium complexation occurred solely via organic phosphate groups between pH 2–4.5 [274,275]. Further, in contrast to U(VI) biosorption in *Chryseomonas* sp. [276], *Bacillus sphaericus* ATCC 14577 [277], *Pseudomonas fluorescence* ATCC 55241 [271], and *H. halobium* [271] under corresponding experimental conditions, *S. acidocaldarius* has a significantly lower capability [269]. As noted above, bacteria have significantly different cell wall structures from archaea, which contain a large number of uranium binding ligands, such as carboxylic and phosphate groups [169,278]. Although the cell wall of *H. halobium* only consists of a S-layer protein, in contrast to *S. acidocaldarius* this S-layer is enriched in carboxylic amino acid residues and can explain the higher uranium binding capacity [269,279]. A few microorganisms, such as like *Bacillus sphaericus* JG-A12, possess a phosphorylated S-layer allowing for binding of large amounts of uranium [169]. Taken together, *S. acidocaldarius* appears to interact

with and detoxify U(VI) differently than other acidophilic and non-acidophilic bacteria. Recently, a preliminary investigation into the uranium resistance of *M. prunae* compared to *M. sedula* indicated a novel role of a toxin-antitoxin in resistance [36].

5. Attachment of Extreme Thermoacidophiles to Surfaces

The vast majority of leaching bacteria adhere to the mineral sulfide surface, generally mediated by an exopolysaccharide (EPS) surrounding the cells [280–285]. The EPS provides an essential micro-environment and reaction space for organisms leaching mineral sulfides [286–289]. Certain species, like *Acidithiobacillus caldus*, cannot adhere and requires co-culture with EPS-forming acidophiles [290]. Curiously, if an organism is capable of mineral sulfide attachment, the space for attachment must be non-limiting [291,292]. The formation of EPS is known to be stimulated by attachment or surface contact [285,293]. The composition of EPS consists of sugars, fatty acids, glucuronic acid, and Fe(III) ions [39,281,294]. Adherence is mainly attributed to electrostatic interactions, but hydrophobic interactions do contribute and the magnitude of the adhesion force has been determined [281,295–299]. The EPS does display adaptability depending on whether the substrate is a metal sulfide or sulfur [281], though the molecular mechanisms used to adapt composition and amount of EPS according to growth substrate are still unknown [291]. While the site of attachment and mechanisms for specific site detection are still unknown, the process does not appear to be random, with cells attaching to areas of surface imperfection or low-degree of crystallinity [281,291,294,300–304]. Additional elements mediating adherence to surfaces include pili and S-layer proteins [170,305–307].

L. ferrooxidans and *A. ferrooxidans* possess chemotaxis systems for sensing Fe(II), which might function to identify specific sites on pyrite surfaces for attachment [308,309]. Quorum sensing functions in bioleaching bacterium allow for swarming behavior on metal sulfides and play a key role in biofilm formation, enhancing dissolution of the mineral substrate [310–315]. Early biofilm formation involves capsular polysaccharide production (CPS), up-regulation of genes related to pili and EPS production, motility and quorum sensing, synthesis of cell wall structures, specific proteases, stress response chaperons, and mixed acid fermentation [316–319]. Proteomic analysis revealed similar results with the addition of increased production of osmolarity sensing, outer-membrane efflux, iron uptake, sulfate uptake and assimilation, glutathione/coenzyme/cofactor biosynthesis, lipoproteins, and nucleotidases [320].

As discussed above, chalcopyrite bioleaching is more effective at temperatures above 65 °C, requiring the use of extremely thermoacidophilic microorganisms [4,321–323]. The majority of studies, related to mineral sulfides, focused on attachment parameters of temperature and culture history, the influence of planktonic and attached cells on the dissolution process, visualizing pyrite leaching, and biofilm development [324–327]. As is the case for other acidophilic metal mobilizers, EPS plays an important role in the adhesion to solid mineral substrates for extreme thermoacidophiles [34,39,283,328]. The EPS for *S. acidocaldarius*, *S. solfataricus*, and *S. tokodaii*, contains mannose, galactose, and N-acetylglucosamine [328,329]. Thicknesses of EPS for *M. hakonensis* grown on pyrite and chalcopyrite have been determined to be 8–12 and 4–8 μm, respectively [283]. Elemental analysis of EPS produced by *M. hakonensis*, grown on chalcopyrite, showed iron levels

below the detection limit, preventing assessment of the presence or absence of iron in the EPS [39]. The result suggests differences in Fe(II)-oxidation enzymes could be more important for dissolution than iron levels in the EPS. For *S. solfataricus* and *S. acidocaldarius* and likely other extremely thermoacidophilic archaea, initial attachment to solid substrates involves pili, and additionally flagella for *S. solfataricus* [328,330,331].

A proteomic/transcriptomic study of *S. acidocaldarius*, *S. solfataricus*, and *S. tokodaii* adjustment to biofilm lifestyle was strain specific [329]. As noted above for other acidophiles, these changes were largely associated with energy production and conversion, amino acid metabolism, lipid and carbohydrate metabolism, transport related functions, and cell surface modifications. Interestingly, very few changes were shared across the species, which included a family of Lrs14-like transcriptional regulators, several significantly influencing biofilm formation or cell motility [332]. No quorum sensing (QS)-phenomena were detected in the biofilm formation of *S. acidocaldarius*, *S. solfataricus*, and *S. tokodaii* nor for *F. acidarmanus Fer*1, leaving the significance of cell signaling and communication unknown [329,333]. The biofilms formed by *F. acidarmanus* rely on EPS and involve shifts in metabolism towards anaerobic growth. Further, the biofilms are monolayer, and like acidophilic bacteria, appear to preferentially occur at cracks/defects on pyrite surfaces [284,333]. Recently, a methodology for investigating archaeal biofilms was developed using fluorescence lectin-binding analysis. Results showed variations in EPS glycoconjugates for three archaeal species and that various substrates induce different EPS glycoconjugates, similar to the flexibility of bacteria [281,334]. For more information on the aspects of other archaeal biofilms, informative reviews are available [331,335–338].

In general, there exist three models describing microbe-mineral electron transfer: (i) direct; (ii) electron shuttle; (iii) nanowire [339,340]. For metal-reducing *Shewanella* and *Geobacter* species, several strategies have been proposed to mediate interfacial electron transport from the cell to the external solid-phase electron sink, though intense debate still exits concerning molecular details. For short distances, <2 nm, electron tunneling could play a critical role in electron transfer, whereby direct electron transfer occurs between the extracellular substrate and redox-active enzyme [341–343]. However, dramatically longer distances of electron transfer have been reported, ranging from nanometers to centimeters, requiring long-distance electron transport models [344]. A model for diffusive shuttling of electrons involves flavin-mediated transfer of electrons between the extracellular substrate and redox-active enzymes, multi-haem cytochromes, on the cell surface [345–347]. A model based on extracellular appendages, commonly called nanowires, involves electron transfer along these nanowires, believed to be either membrane- or pilin-based, between the solid substrate and cell [265,348–352]. Interestingly, the occurrence of nanowires coincides with formation of separate or attached redox-active membrane vesicles [352,353]. Further, bacterial biofilms incorporating nanowires or outer membrane cytochromes and multicellular bacterial cables can transfer electrons over long distances [354,355]. Electron conductance is proposed to occur from either metallic-like band transport or multi-step redox hopping mechanisms, though the former remains controversial [356–360].

A tentative "contact" model for metal sulfide dissolution (e.g., FeS_2), requiring an oxidizing attack by Fe(III), has been proposed [291]. The model postulates that Fe(III), complexed to glucuronic acid in the EPS, performs the oxidizing attack of the metal sulfide. The Fe(II) produced by the cathodic electron transfer is then released from EPS chelators and diffuses toward the outer membrane where (re)oxidation occurs, thus the cycle repeats [291]. The model is similar to the flavin-mediated

shuttling, noted above, with Fe(II) serving as the electron shuttle. The metal sulfide model rests on four assumptions: (i) oxidizing attack by Fe(III) is required; (ii) EPS-complexed Fe(III) fulfills this function; (iii) electron tunneling effects explain transfer, and (iv) Fe(II) ion-glucuronic acid complexes are less stable than Fe(III). The first assumption rests on the assertion that direct electron transfer from the metal sulfide to the attached cell does not occur, since no enzymes or nanowires have been demonstrated for metal sulfide attached cells [291]. This assumption seems tenuous, given the recent work on metal reducing species showing usage of nanowires for metal reduction, discussed above. Additionally, type IV pili in *A. ferrooxidans* are highly conductive and might function as nanowires, directly transferring electrons from the external substrate [361]. The second assumption is based on experimental evidence where an *A. ferrooxidans* strain with high Fe(III) in the EPS had a higher bioleaching capacity compared to strains with low Fe(III) concentration [289,294]. A similar phenomenon has been seen for *L. ferrooxidans*, but the results only revealed the importance of the local concentration of the corrosion promotor Fe(III) in the biofilm environment [362]. The complexation "probably" occurs with glucuronic acid residues, but conclusive evidence along with Fe(II)/Fe(III) binding constants has not been presented [288,289,294]. Electron tunneling over distances <2 nm is widely accepted [341,363] and would allow both EPS complexed and solution Fe(III) to be reduced. However, given the thickness of the EPS is 10–100 nm wide, not all Fe(III) would be within range, requiring diffusion towards the surface [286,364].

The above model does not incorporate the potential for mineral sulfide destabilizers that could help initiate release of Fe(II). Cysteine is known to accelerate FeS_2 dissolution, possibly by disrupting the FeS_2 surface, causing release of iron-sulfur species [365,366]. Further, *A. ferrooxidans*' aporusticyanin was suspected to function as a receptor for initial adhesion to mineral sulfides, in which the protein could destabilize the mineral surface, leading to Fe(II) dissolution [367].

As mentioned above, the occurrence of nanowires coincides with formation of separate or attached redox-active membrane vesicles for the metal-reducing *Shewanella* species [352,353]. In bacteria, vesicles have roles in colonization and cell co-aggregation, both critical to biofilm formation [368]. Gram-negative bacteria, along with the extremely thermoacidophilic archaea *S. acidocaldarius*, *S. solfataricus*, *S. tokodaii* and *S. islandicus*, release membrane vesicles [369–372]. The presence of archaeal homologous of the eukaryotic endosomal sorting complex required for transport-I (ESCRT) proteins in the crenarchaeal vesicles, suggests vesicle formation occurs through an outward budding process, similar to inward budding of the endosomal compartment in eukaryotes [372,373]. The archaeal vesicles could serve a homologous function in electron transfer from the solid substrate to the cell, as for the metal reducers.

The possibility that multiple mechanisms of interaction occur throughout different stages of mineral oxidation seems possible [374]. Initially, cells localize to non-random sites on the mineral sulfide surface, through an unknown mechanism, and attachment proceeds by CPS, pili, flagella, S-layer, mineral receptors (e.g., aporusticyanin), or a combination. Once attached mineral destabilizers cause an initial release of iron-sulfur species and cells switch to a sessile growth mode. EPS production occurs, providing an essential micro-environment and reaction space, where the corrosion promoter Fe(III) is entrapped and accelerates mineral dissolution. Though not experimentally observed to date, the implications of conductive pili and redox-active vesicles should not be ruled out.

6. Bioleaching

Over the course of the past few decades, biomining has centered on the development of technologies to recover precious metals contained within ore-bearing matrices. In the recent past, numerous industrial processes have matured, primarily those involving the recovery of gold from refractory ores or the recovery of nickel or copper from base metal sulfides. In fact, some estimates suggest that as much as 15% of copper and 5% of gold production (on a global scale) utilize microbial-assisted extraction technologies [375]. Further, as the relative availability of higher-grade ores diminishes and environmental regulation increases, it is likely that interest in biomining will increase in an attempt to improve metal selectivity and yield, while minimizing the release of toxic pollutants.

Recent reviews have emphasized the importance of mesophilic and moderately thermophilic acidophiles involved in bioleaching, ranging from industrial prospects [292,323,375] to specific uses for secondary copper ores [376] and polymetallic ores [377]. There are fewer details on the successful development of bioleaching applications using extremely thermoacidophilic microorganisms [378]. Extremely thermoacidophilic bioleaching, as it exists, is dominated by the genera *Acidianus*, *Metallosphaera*, and *Sulfolobus* for copper recovery from recalcitrant ores [379,380] or for sulfur oxidation to improve gold recovery from biooxidation, e.g., BIOPRO^{TM} [381].

6.1. Current Biooxidation/Bioleaching Practices at Elevated Temperatures

The treatment of primary copper ores, such as chalcopyrite ($CuFeS_2$), has been the main driver of extremely thermoacidophilic bioleaching developments, see Figure 4. Under mesophilic conditions, heap bioleaching of chalcopyrite tends to achieve low copper yields, often attributed to passivation, or the formation of deposited layers of iron complexes or polysulfides on the mineral surface [323,375,376]. These passivation effects do not appear to be as severe in extremely thermoacidophilic cultures, based on laboratory evaluations and pilot plant testing demonstrating high copper dissolution [9,379,380,382]. Although kinetics may be a driving factor in the dissolution process, evidence has emerged that redox potential can play a role in mitigating passivation for some circumstances [383,384]. In fact, it may be possible to greatly improve the metal dissolution of mesophilic and moderately thermophilic organisms by redox controlling strategies [384] or by the addition of silver, which forms a galvanic couple in the presence of chalcopyrite [385]. However, this result may support a more recent hypothesis that accounts for lowered dissolution of chalcopyrite, due to electronic and interfacial structure that more closely resembles a semiconductor [386]. Given that iron precipitates and polysulfides are so commonplace in both successful and unsuccessful leaching operations, more research is needed to understand if the intrinsic difference in dissolution rates is related to temperature/kinetics or perhaps a yet to be discovered dissolution mechanism among the extreme thermoacidophiles.

Extremely thermoacidophilic archaea have some niche advantages in copper biomining. In the case of chalcopyrite-bearing molybdenite, extreme thermoacidophiles achieve selective copper dissolution, leading to improvement of molybdenum flotation concentrates, with minimal (<10%) molybdenum dissolution [387,388]. Further, there is evidence that extremely thermoacidophilic archaea may be more adept than mesophilic bacteria at extracting copper from other copper-sulfide ores, based on column bioleaching involving covellite (CuS) and enargite (Cu_3AsS_4) [6]. This suggests their potential

applications in processing mixed copper-bearing ores at elevated temperatures. Also, a study involving *A. brierley* bioleaching of enargite showed that the species can selectively mobilize copper, while simultaneously precipitating arsenic in the form of arsenate [389]. This approach could limit the deleterious effects of mining ores containing arsenic.

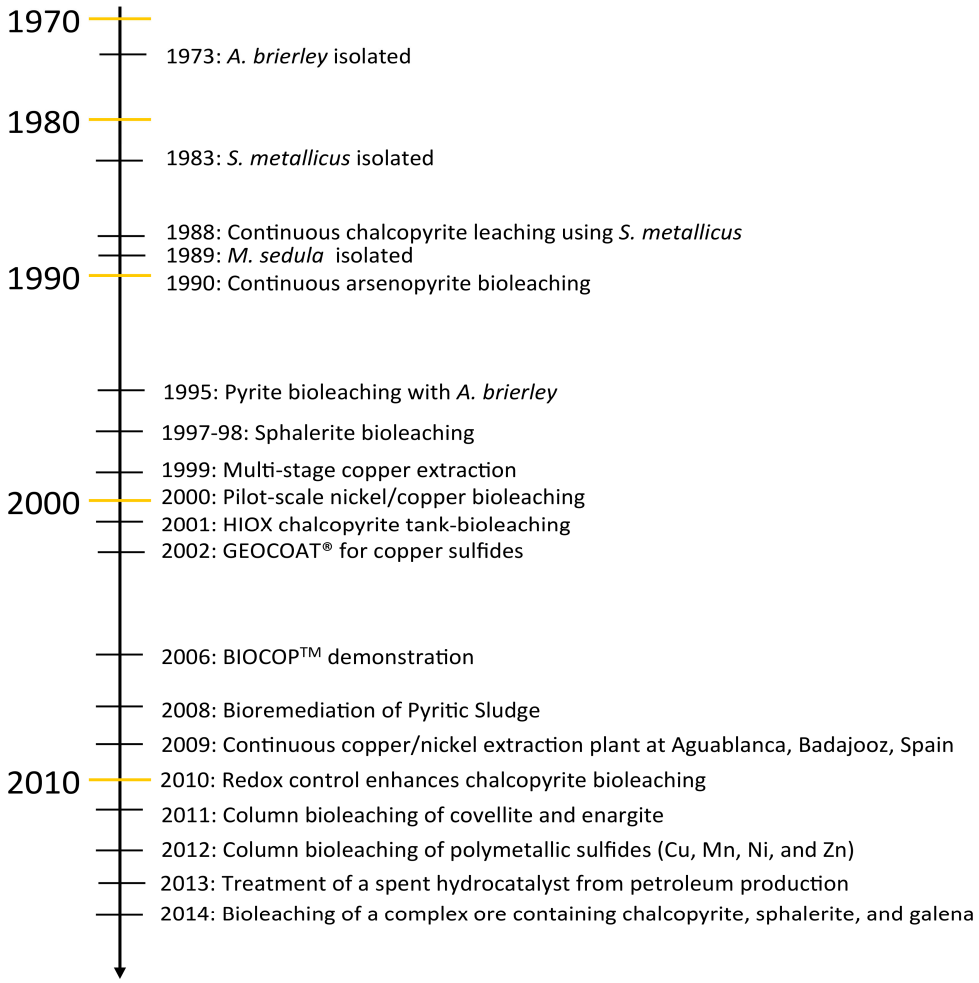

Figure 4. Chronology of selected developments in extreme thermoacidophilic biotechnology [6,9,32,53,54,379,380,382,384,390–400].

Issues with sulfur oxidation have emerged in some biooxidation/bioleaching processes. In particular, gold and copper biomining of ores containing high concentrations of pyrite/pyrrhotite presents a challenge in mesophilic bioleaching processes. In the case of gold biomining, sulfur is often retained in a partially oxidized form (such as elemental sulfur). This residual sulfur is then capable of reacting with cyanide in downstream processing, severely impacting recoveries and increasing operating costs [381,401]. A consortium of mesophilic, moderately thermophilic, and extremely thermoacidophilic microbes has shown promise in improving sulfur oxidation and subsequent metal recoveries [381,401]. In the case of pyrite/pyrrhotite-rich copper deposits, heap bioleaching generates large amounts of heat [401,402]. In some instances, the inability to control temperature in the heap, due to the exothermic nature of sulfur oxidation, can result in issues with population succession and dissolution [401,402]. This issue might be mitigated by the use of extremely thermoacidophilic archaea. However, column

leaching experiments involving copper ores have revealed a tendency to form percolation channels [6,9]. This is possibly due to iron precipitation as oxyhydroxysulfates at higher temperatures [10]. To improve the efficacy of extremely thermoacidophilic organisms bioleaching copper in heaps, one technology, GEOCOAT®, utilizes ground copper-ore concentrate coated onto a barren rock surface to increase surface area and, as a consequence, achieve increased rates and overall dissolution of copper [397,403].

6.2. Extreme Thermoacidophile Process Challenges

Currently, extremely thermoacidophilic bioleaching presents certain process dynamic challenges. One potential concern is the delicate nature of the archaeal cell envelope, which lacks the bacterial peptidoglycan outer-membrane. This potentially places a limit on agitation rates that the microbes can endure in tank bioleaching conditions and may facilitate the need for highly specialized turbine/agitator designs [378–380]. Another issue is oxygen demand in high temperature environments. A well-known consequence of higher temperatures is decreased dissolved oxygen content, requiring the use of enriched oxygen sources (at a much higher operating costs than air) [378–380]. Compounding this issue is the production of reactive oxygen species, especially in the presence of finely ground mineral stocks and low pH, conditions which typically optimize leaching [404,405]. In fact, this result may be a critical issue that prevents higher solids loading in extremely thermoacidophilic bioreactors [405]. However, in all of these cases, the concerns raised for extreme thermoacidophiles as bioleachers have not been confirmed, but should be assessed as related technologies move forward.

6.3. Polymetallic Ores and Industrial Waste

A common issue in modern mining is the need to maximize yields of numerous metals of varying values from complex polymetallic ores. Several studies over the past few decades have suggested the potential value of bioleaching complex deposits with extreme thermoacidophiles. In the case of zinc, high recovery has been observed from complex sulfides, containing sphalerite [54,396]. In this instance, the extreme thermophiles appear to outperform moderate thermophiles and mesophiles. More recent studies continue to highlight the ability of extreme thermoacidophiles to leach a variety of metals from complex ores. In the case of a black shale (containing Mn, Fe, Zn, Ni, Cu, and Co), over 90% dissolution of manganese, copper, zinc, and nickel was achieved with extreme thermoacidophilic cultures [406]. In the case of ores containing chalcopyrite, sphalerite, and galena, greater than 90% dissolution of copper and zinc were observed in the leachate, with more than 90% recovery of lead following brine precipitation [400]. Thus, the potential for improved kinetics of bioleaching by extreme thermoacidophiles is not limited to the current narrowly applied fields of sulfur oxidation and copper dissolution.

Another area of growing interest is the treatment of industrial and consumer waste [407,408]. *A. brierleyi* has been used to treat spent hydrocatalysts from petroleum processing in order to remove the molybdenum and nickel from the catalyst prior to disposal [399]. Another study focused on remediation of mining spillage composed primarily of pyrite, with some sphalerite and arsenopyrite. In this instance, extreme thermoacidophiles showed much faster kinetics for the dissolution of iron, zinc, and arsenic [398].

7. Conclusions

In general, bioleaching is likely to remain an important avenue for recovery of base, precious and strategic metals from mining operations. This processing approach reflects a trend toward more stringent environmental regulations which are incentivizing the use of sustainable industrial practices. In addition, the depletion of high-grade ores and the need to process increasing amounts of heavy metal waste will inevitably create a processing bottleneck, if only conventional chemical/physical metal extraction techniques are considered. Looking further into the future, implications for using extremely thermoacidophiles in asteroid mining creates yet another technological dimension [409].

Given the potential advantages and challenges associated with high temperature bioleaching operations, efforts to further understand the underlying metabolic, physiological and genetic mechanisms characteristic of extreme thermoacidophiles need to continue. In particular, as molecular genetics tools become more tractable and allow for metabolic engineering of biomining microorganisms to improve their efficacy, the corresponding issues with release of genetically modified organisms (GMOs) also arise. However, the unique aspects of extreme thermoacidophiles, and the inhospitable nature of biomining sites, may mitigate some of the concerns normally associated with release of GMOs. These concerns will need to be addressed from the perspective of microbial ecology of hot acid biotopes.

A new and exciting frontier, of strategic importance where bioleaching microorganisms could play a significant role, is in the extraction and recovery of rare earth elements. Microorganisms and certain fungi can accumulate and absorb rare earth elements, providing a fundamental framework to build novel extraction and recovery processes [410–413]. Interestingly, an extremely acidophilic methanotrophic microorganism requires certain rare earth elements for survival [414]. Given that microbial metalloproteomes are largely uncharacterized, the potential for discovering novel rare earth element binding proteins among extreme thermoacidophiles is promising [415].

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Author Contributions

Garrett Wheaton, James Counts, Arpan Mukherjee, Jessica Kruh and Robert Kelly contributed to the writing and analysis of the material included in this review.

Conflicts of Interest

The authors declare no conflict of interest.

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