Sulfuriferula spp. from sulfide mineral weathering environments have diverse sulfur- and iron-cycling capabilities

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Abstract

Microorganisms are important catalysts for the oxidation of reduced inorganic sulfur compounds. One environmentally important source of reduced sulfur is metal sulfide minerals that occur in economic mineral deposits and mine waste. Previous research found that *Sulfuriferula* spp. were abundant and active in long-term weathering experiments with simulated waste rock and tailings from the Duluth Complex, Northern Minnesota. We therefore isolated several strains of *Sulfuriferula* spp. from these long-term experiments and characterized their metabolic and genomic properties to provide insight into microbe-mineral interactions and the microbial biogeochemistry in these and other moderately acidic to circumneutral environments. The *Sulfuriferula* strains are all obligate chemolithoautotrophs capable of oxidizing inorganic sulfur compounds and ferrous iron. The strains grew over different pH ranges, but all grew between pH 4.5-7, matching the weathering conditions of the Duluth Complex rocks. All strains grew on the iron-sulfide mineral pyrrhotite ($Fe_{1-x}S$, $0 < x < 0.125$) as the sole energy source, as well as hydrogen sulfide and thiosulfate, which are products of sulfide mineral breakdown. Despite their metabolic similarities, each strain encodes a distinct pathway for the oxidation of reduced inorganic sulfur compounds as well as differences in nitrogen metabolism that reveal diverse genomic capabilities among the group. Our results show that *Sulfuriferula* spp. are primary producers that likely play a role in sulfide mineral breakdown in moderately acidic to circumneutral mine waste, and the metabolic diversity within the genus likely explains their success in sulfide mineral-rich and other sulfidic environments.

Importance

Metal sulfide minerals such as pyrite and pyrrhotite are one of the main sources of reduced sulfur in the global sulfur cycle. The chemolithotrophic microorganisms that break down these minerals in natural and engineered settings are catalysts for biogeochemical sulfur cycling and have important applications in biotechnological processes such as biomining or bioremediation. *Sulfuriferula* is a recently described genus of sulfur oxidizing bacteria that are abundant primary producers in diverse terrestrial environments, including waste rock and tailings from metal mining operations. In this study, we explored the genomic and metabolic properties of new isolates from this genus, and the implications for their ecophysiology and biotechnological potential in ore and waste from economic mineral deposits.

Introduction

The oxidation of reduced inorganic sulfur compounds is a crucial step in the global sulfur cycle. Sources of reduced sulfur include metal sulfide minerals that are present in sedimentary, igneous, and metamorphic rocks, dissolved and gaseous hydrogen sulfide species that are common in anoxic environments, and compounds with intermediate valences that include both short-lived compounds like thiosulfate ($S_2O_3^{2-}$) and tetrathionate ($S_4O_6^{2-}$) and more stable species like elemental sulfur. Reduced inorganic sulfur compounds are a dynamic source of energy for modern microorganisms that drive the oxidation of these compounds in diverse environmental conditions (1–6), and have been important throughout Earth's history (7, 8). Many of these inorganic-sulfur-oxidizing bacteria and archaea are primary producers that fix carbon and nitrogen, and are abundant in environments with a range of pH conditions (from pH less than 1 to above 10) and temperatures (below 10 to over 90°C) (9). Terrestrial environments are particularly diverse settings for sulfur cycling because sulfur sources can be heterogeneously distributed. Steep gradients in environmental conditions like pH, temperature, and redox potential provide many distinct niches for different sulfur cycling organisms to thrive (1, 10–13).

Sulfuriferula are a recently described genus of sulfur-oxidizing bacteria. The first *Sulfuriferula* strains were isolated from a uranium mine using galena (PbS) as the sole electron donor, and was originally named '*Thiobacillus plumbophilus*' (14). The genus *Sulfuriferula* was formally described in 2015 within the new family *Sulfuricellaceae*, which included members reclassified from the polyphyletic genus *Thiobacillus* (15). Members of the genus *Sulfuriferula* can grow autotrophically on inorganic sulfur compounds, and some can grow organoheterotrophically on organic substrates (15). Formally described members of the genus *Sulfuriferula* include *S. multivorans*, which was isolated from a freshwater lake in Hokkaido, Japan (15); *S. thiophila*, which was isolated from a storage tank at a public hot spring bath in Yamanashi Prefecture, Japan (16); *S. nivalis*, isolated from snow collected at the shore of a pond in Daisetsuzan National Park, Japan (17); and *S. plumbiphila*, the original "Thiobacillus plumbophilus" isolate that was formally described and later renamed (14–16). All species have been reported to grow aerobically on thiosulfate, tetrathionate, and elemental sulfur, while only *S. multivorans* can grow heterotrophically and anaerobically with nitrate as an electron acceptor (15) and *S. plumbiphila* can also grow autotrophically on H_2 and PbS (14). *Sulfuriferula* are now

87 known from diverse sulfur-rich terrestrial environments, and relatives of *Sulfuriferula* in
88 metagenomic and 16S rRNA gene datasets have been found in metal- and sulfide- rich mine
89 tailings and drainage (18–26), sewer and groundwater environments (27–29), hot springs and
90 volcanic deposits (30–32), seawater-influenced sulfide caves (33), and low-temperature lake
91 bottom sediments (18, 34, 35).

92 Here we describe new strains of *Sulfuriferula* that were isolated from weathered rocks
93 from the sulfide mineral-bearing Duluth Complex in Northern Minnesota. The Duluth Complex
94 contains copper, nickel, and platinum group element (Cu-Ni-PGE) deposits that may represent
95 the world's largest undeveloped economic Cu-Ni-PGE resource (36). The most abundant sulfide
96 mineral in these rocks is pyrrhotite ($Fe_{1-x}S$, $0 < x < 0.125$), followed by chalcopyrite ($CuFeS_2$),
97 cubanite ($CuFe_2S_3$), and pentlandite ($(Fe, Ni)_9S_8$), with smaller amounts of other Cu, Ni, and
98 PGE-bearing sulfide minerals (37). Sulfur- and iron-oxidizing microorganisms are known to
99 accelerate the dissolution of sulfide minerals under highly acidic conditions (38). However, in
100 contrast to many other well-studied ore and mine-waste environments where extreme acidity is
101 generated, the relatively low sulfide mineral content (usually <1% total sulfur) and buffering
102 capacity of the surrounding silicate minerals means that leachate from Duluth Complex waste
103 rock and tailings is moderately acidic and rarely reaches pH values of less than 4 (39, 40). The
104 role of microorganisms in this mildly acidic environment is not as well understood as in
105 extremely acidic systems.

106 Based on rRNA gene and transcript sequencing, *Sulfuriferula* spp. were some of the most
107 abundant and active organisms in experimentally weathered Duluth Complex rocks and tailings
108 (18), and were also found (although at lower abundances) in naturally-weathered Duluth
109 Complex outcrops (41), suggesting that they are important members of the rock weathering
110 community and may also be valuable for developing biological solutions to managing mine
111 waste and water from proposed mines in the region. To learn more about these important
112 organisms and the role they play in the biogeochemistry of Duluth Complex mine waste and
113 water, we isolated and characterized four strains of *Sulfuriferula* from experimentally weathered
114 Duluth Complex materials. We describe the genomic and physiological properties of the strains
115 and connect their diverse metabolic capabilities to their ecophysiology in the rock weathering
116 environment.
117

118

119 **RESULTS**

120

121 **Isolation and identification of *Sulfuriferula***

122 Strains of *Sulfuriferula* were isolated from weathering experiments on mine tailings and
123 crushed rock from copper-nickel deposits in the Duluth Complex, northeastern Minnesota.
124 Strains AH1, GW1, and GW6 were isolated from tailings and crushed rock had been
125 experimentally weathered for more than 12 years using a laboratory humidity cell apparatus, and
126 strain HF6a was isolated from an experimental field pile that had been weathering outside for
127 more than 20 years (39, 40). Sixteen isolates were obtained, and four phylogenetically distinct
128 strains, referred to as AH1, GW1, GW6, and HF6a, were selected for further study. Strains AH1,
129 GW1, and HF6a were enriched and isolated on solid thiosulfate media that contained 6 mM
130 ammonium, while GW6 was enriched and isolated on media with urea as the sole provided N
131 source.

132 Phylogenetic analysis of 16S rRNA gene sequences from these isolates confirm that all
133 cluster with described *Sulfuriferula* species (Figure 1). Strains GW1 and HF6a are most closely
134 related to *S. multivorans* (15), strain GW6 is most closely related to *S. plumbiphila* (14, 15), and
135 strain AH1 is located in its own clade located at the base of the *Sulfuriferula* clade near *S.*
136 *thiophila* and *S. nivalis* (16, 17, 42). Strains had either two (GW1, GW6, and HF6a) or three
137 (AH1) *rrn* operons with identical 16S rRNA genes (Figure 1).

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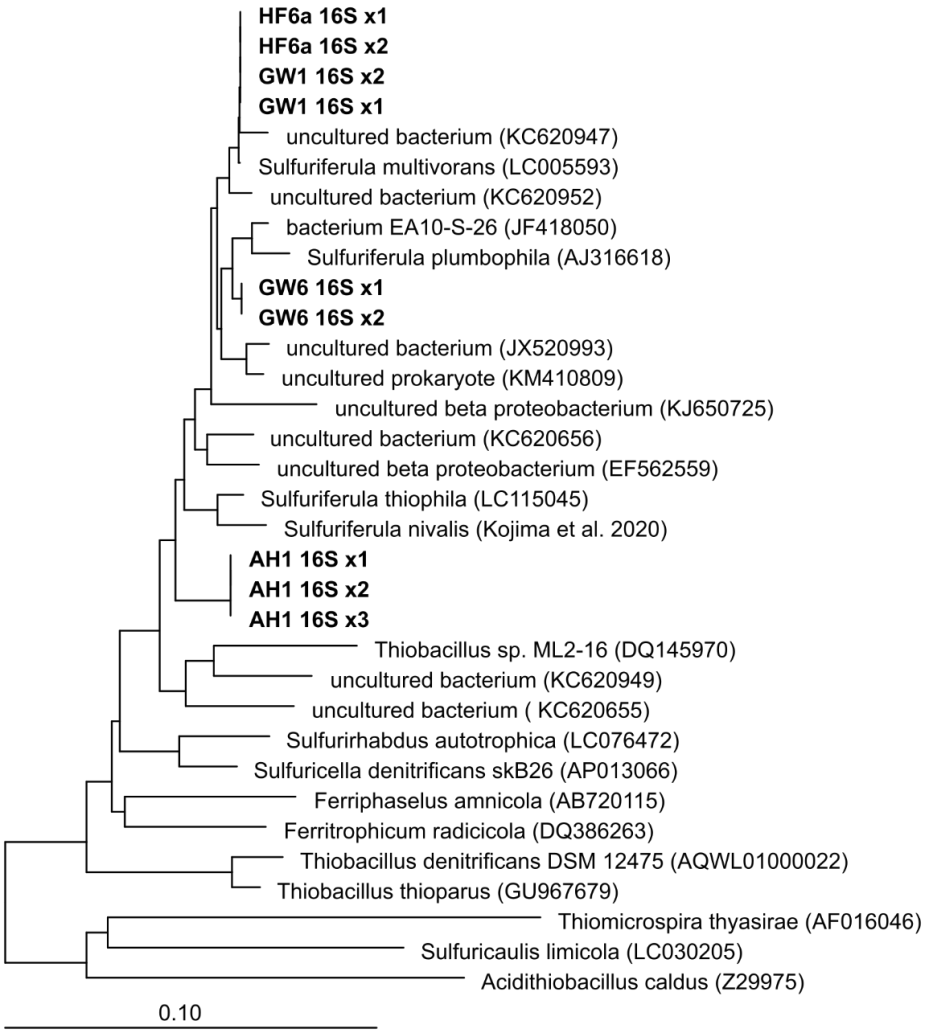

Figure 1. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences, showing the relationship of the four Sulfuriferula *isolates to other members of family* Sulfuricellaceae*. Names in bold font are sequences from this study, and the numbers after each name (x1, x2) indicate rRNA gene copies from multiple rrn operons in the genome.*

Growth characteristics

All four *Sulfuriferula* isolates grew on autotrophic media with thiosulfate ($S_2O_3^{2-}$) as the only available electron donor. No growth was detected on tetrathionate ($S_4O_6^{2-}$), sulfite (SO_3^{2-}), or elemental sulfur (S^0) on the media used here. All strains also grew on pyrrhotite ($Fe_{1-x}S$, $0 \leq x \leq 0.125$) as the sole electron donor, and on dissolved sulfide ($H_2S(aq)$) in gradient tubes. When grown on thiosulfate, strain AH1 accumulates sulfate and H^+ ions in the growth media,

152 consistent with complete thiosulfate oxidation to sulfate (Figure S1). In addition to reduced
153 inorganic sulfur compounds, all strains grew on ferrous iron (Fe^{2+}) in gradient tubes
154 (Supplementary Figure S1). However, we did not observe anaerobic growth on either H_2S(*aq*) or
155 $S_2O_3^{2-}$ with NO_3^- as an electron acceptor under the conditions tested here, or when only hydrogen
156 (H_2) was provided as an electron donor under aerobic or anaerobic conditions.
157 Growth was not observed for any strains exclusively on LB broth or 1:10 diluted LB
158 broth. No increase in growth was observed when 5mM glucose, acetate, or succinate was added
159 to the thiosulfate media, suggesting that these strains are obligate autotrophs and are sensitive to
160 the presence of organics. Consistent with this, growth rate decreased in media buffered with
161 organic buffers rather than phosphate. Growth occurred in MES- and HEPES- but not citrate-
162 buffered media. However, growth was enhanced by adding 0.1% w/v yeast extract was added to
163 the growth media.
164 Each strain grew at slightly different pH ranges. Strain AH1 had the widest range, which
165 grew between pH 4.5 and 8. HF6a was most acid tolerant than the other strains, growing between
166 pH 4 and 7, while strains GW1 and GW6 were less so, growing between pH 5 and 8 and 6 and 8
167 respectively. During growth on thiosulfate, pH consistently initially increased and then decreased
168 (e.g., Supplementary Figure S2).
169
170 **Whole genome sequencing and assembly**
171 The genomes of the four isolates were each assembled into a single large chromosome,
172 between 2.92 and 3.33 Mb. One 39.1 Kb plasmid was recovered for strain AH1, and two
173 plasmids, 23.8 and 39.5 Kb, were recovered from strain GW6. Table 1 contains an overview of
174 the properties of the assembled genomes, which range from 55-58.7 %G+C content and 97-98%
175 protein-coding genes. GW1, GW6, and HF6a each have two *rrn* operons, and AH1 encodes
176 three. A summary of genes associated with sulfur, nitrogen, carbon, and iron metabolisms and
177 genes associated with biofilm formation are presented in Figure 2.
178
179 **Carbon metabolism.** All four strains of *Sulfuriferula* encode complete Calvin cycles for
180 CO_2 fixation. *S*train AH1 has two copies of RuBisCO form I and one copy of RuBisCO form II,
181 strain GW1 has two copies of RuBisCO form I, strain GW6 has three copies of RuBisCO form I,
182 and strain HF6a has one copy each of RuBisCO form I and II. All four strains have a full

183 complement of carboxysome and carbonic anhydrase-coding genes (Figure 2b). All four strains
184 possess complete glycolysis pathways and incomplete TCA cycles.
185

Table 1. Summary of genome properties.

	AH1	**GW1**	**GW6**	**HF6a**
Total # base pairs	2,916,145	3,363,341	3,329,881	3,261,505
G+C %	55.0%	57.1% (1,920,508)	58.7% (1,954,947)	57.1% (1,861,310)
# Contigs	1 circular contig	1 circular contig	1 circular contig	1 circular contig
Plasmids	1: 39,138 bp	Not present	2: 23,830 bp & 39,480 bp	Not present
Total # genes	2972	3369	3559	3319
Protein-coding genes	2906 (97.8%)	3301 (98.0%)	3450 (97.0%)	3252 (98.0%)
rRNAs	9 (0.30%)	6 (0.18%)	6 (0.17%)	6 (0.18%)
tRNAs	47 (1.58%)	50 (1.48%)	51 (1.43%)	48 (1.45%)
Protein-coding genes w/ functional prediction	2360 (79.4%)	2634 (78.2%)	2712 (76.2%)	2553 (76.9%)
Assigned to COGs	2042 (68.7%)	2705 (80.3%)	2786 (78.3%)	2624 (80.8%)
Assigned to KEGG Orthology (KO)	1659 (55.82%)	1810 (53.73%)	1835 (51.56%)	1722 (51.88%)

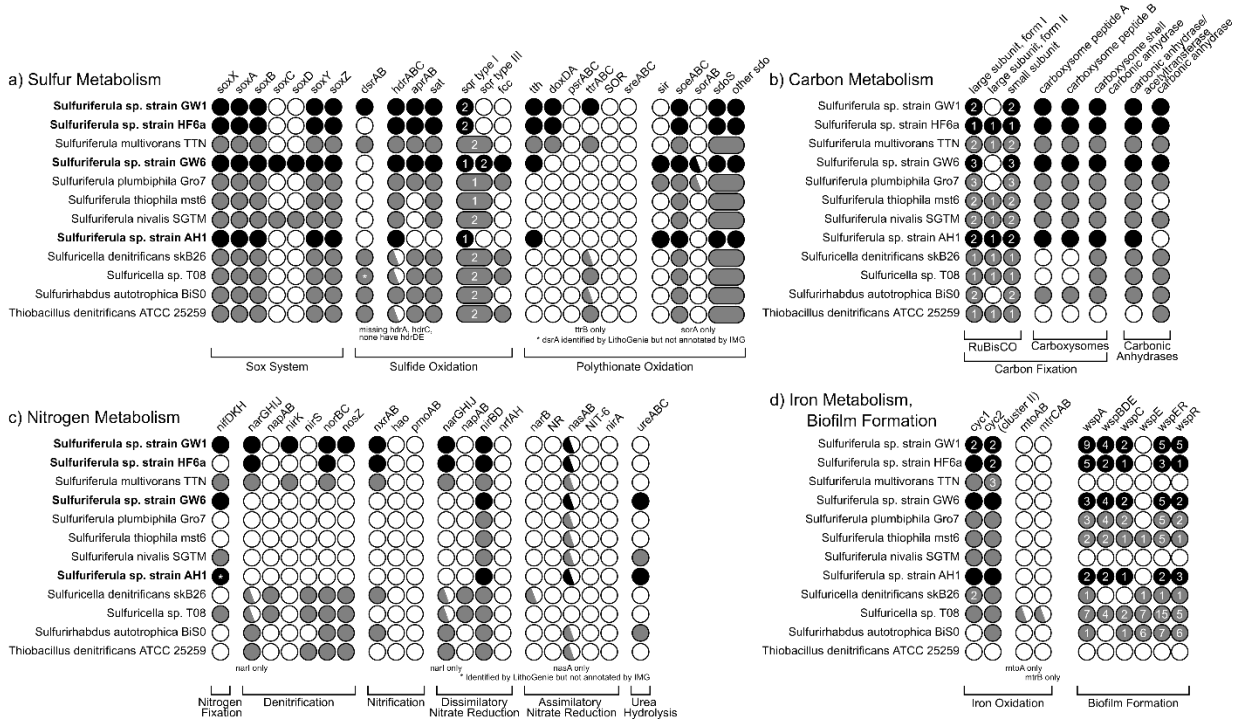

Figure 2. Overview of genes that encode for (a) sulfur, (b) carbon, (c) nitrogen, and (d) iron metabolism and biofilm formation. Filled circles indicate the presence of the gene as annotated by IMG and confirmed by hidden Markov models. A half-filled circle indicates the absence of one or more subunits. The number inside the filled circle indicates the number of copies of the gene found in the genome. Black circles are strains from this study. Larger ovals that overlap the sqr type I/type III and sdoS/other sdo indicate literature sources that did not report those gene clusters as individual genes.

Nitrogen metabolism. The four strains have diverse capabilities for nitrogen acquisition and dissimilatory nitrogen metabolism (Figure 2c). Strains AH1, GW1, and GW6 encode genes for a molybdenum-dependent nitrogenase typically associated with nitrogen fixation. Strains GW1 and HF6a possess genes *narGHIJ* and *norBC*, indicating that these strains can reduce nitrate and nitrous oxide, respectively. Strain GW1 additionally possesses *nirK* and *nosZ*, providing this strain with a complete set of genes necessary for denitrification. All four strains possess *nirBD* genes that encode dissimilatory nitrate reduction to ammonia. Strains GW1 and HF6a also possess the *nxrAB* genes, indicating the potential for nitrite oxidation. No strains

205 possess complete assimilatory nitrate reduction pathway genes, although all strains possess the
206 *nasA* gene. Additionally, the genomes of strains AH1 and GW6 contain genes *ureABC* that
207 encode urea hydrolysis, indicating an ability to utilize urea as a source of ammonia.
208
209 **Sulfur metabolism:** The four strains encode diverse pathways for the oxidation of
210 reduced inorganic sulfur compounds (Figure 2a). All four strains encode sulfide:quinone
211 oxidoreductase (SQR) homologs. SQR is used by all domains of life for sulfide oxidation and/or
212 detoxification. Six structural types of SQR are known; microorganisms encoding at least one
213 representative enzyme from each of type I, III, IV, V, and VI can use sulfide as an electron
214 donor, while type II has not been directly linked to metabolic oxidation of sulfide and may be
215 used for detoxification (43, 44). All four *Sulfuriferula* strains have genes identified as SQR type
216 I, with strains GW1 and HF6a having two copies. Strain GW6 also possesses two copies of the
217 SQR type III, as well as *fcc*, which encodes flavocytochrome-c sulfide dehydrogenase, a
218 structurally-related protein that can also be utilized for sulfide oxidation (45).
219 Strains AH1, GW1 and HF6a possess partial *sox* systems, with genes *soxAX*, *soxYZ*, and
220 *soxB* but not *soxCD*, while strain GW6 possesses a complete sox system including *soxCD*. In the
221 complete pathway, SoxYZ complexes with thiosulfate to form a SoxY-cysteine S-thiosulfate
222 derivative, which move to sequential reactions with SoxAX and SoxB that oxidize one of the
223 sulfur atoms in the thiosulfate to sulfate, with two electrons transferred to the electron transport
224 chain. SoxCD then oxidizes the remaining sulfane sulfur of the residual SoxY-cysteine-S to
225 sulfate, which is hydrolyzed by SoxB, ultimately generating sulfate and transferring six electrons
226 to the electron transport chain (46). The sox pathway is able to catalyze oxidation of reduced
227 sulfur species besides thiosulfate, including sulfide, elemental sulfur, sulfite, and tetrathionate
228 (47–50), and this ability seems to be facilitated by the modular nature of the reactions (51).
229 While SoxCD is not essential for complete thiosulfate or sulfide oxidation, partial *sox* systems
230 lacking SoxCD are thought to produce elemental sulfur or polysulfides (52). In some organisms
231 with a partial *sox* system, that sulfur is then completely oxidized by the DSR system (53, 54).
232 Only strain GW1 contains genes encoding DsrAB (dissimilatory (bi)sulfate reductase), however,
233 indicating that the other strains either produce S(0) as a final product or use another process to
234 completely oxidize the S(0).

235 All four strains possess *hdrABC*, which encodes heterodisulfide reductase that is linked to
236 the oxidation of zero-valent sulfur in some bacteria (55). All four strains also possess genes
237 coding for sulfur dioxygenases (*sdo*). Sdo oxidizes the sulfate sulfur in glutathione persulfide
238 (GSSH) to sulfite (56). Homologous genes are found in mitochondria, where mutations on this
239 gene are implicated in a rare hereditary human disease (57). Widely distributed through the
240 Proteobacteria, *sdo* genes are used both in sulfide detoxification and in primary metabolism. The
241 *Sulfuriferula* strains examined here have two types of *sdo* sequences: one set are most similar to
242 the mitochondrial sulfide detoxification ETHE1 genes, while the other set are most similar to the
243 *sdoS* genes found in acidophillic bacteria like *Acidithiobacillus* (Figure 3). Strains AH1, GW1,
244 and HF6a have one copy of each of the two types of *sdo*, while strain GW6 has one copy
245 homologous with ETHE1 and two distinct copies of genes within the *sdoS* group. The strains did
246 not encode other known enzymes involved in S(0) oxidation like sulfur oxygenase reductase
247 (SOR) (58).
248 All four strains also possess *tth* genes for tetrathionate hydrolase, which catalyzes the
249 oxidation of tetrathionate to thiosulfate, elemental sulfur, and sulfate (59), and *soeABC*, which
250 encodes a sulfite dehydrogenase that allows the transfer of electrons to the quinone pool by the
251 oxidation of sulfite to sulfate (60). Strain GW1 additionally possesses genes *doxDA* for
252 thiosulfate dehydrogenase, which catalyzes the oxidation of thiosulfate to tetrathionate (61), and
253 *ttrABC* for tetrathionate reductase, which catalyzes the oxidation of tetrathionate to thiosulfate
254 (62). Strains AH1 and GW6 possess *sir* genes which code for ferrodoxin:sulfite reductase,
255 catalyzing the assimilatory reduction of sulfite to sulfide (63), and GW6 possesses *sorA*, which
256 encodes the A subunit of sulfide dehydrogenase (64). Strains GW1, GW6 and HF6a possess *sat*
257 genes for sulfate adenylyltransferase and *aprAB* for adenylylsulfate reductase. Both of these
258 proteins typically catalyze the reduction of sulfate (65, 66), but like DsrAB they can run in
259 reverse, oxidizing intermediate organosulfur compounds to sulfate (67, 68).
260

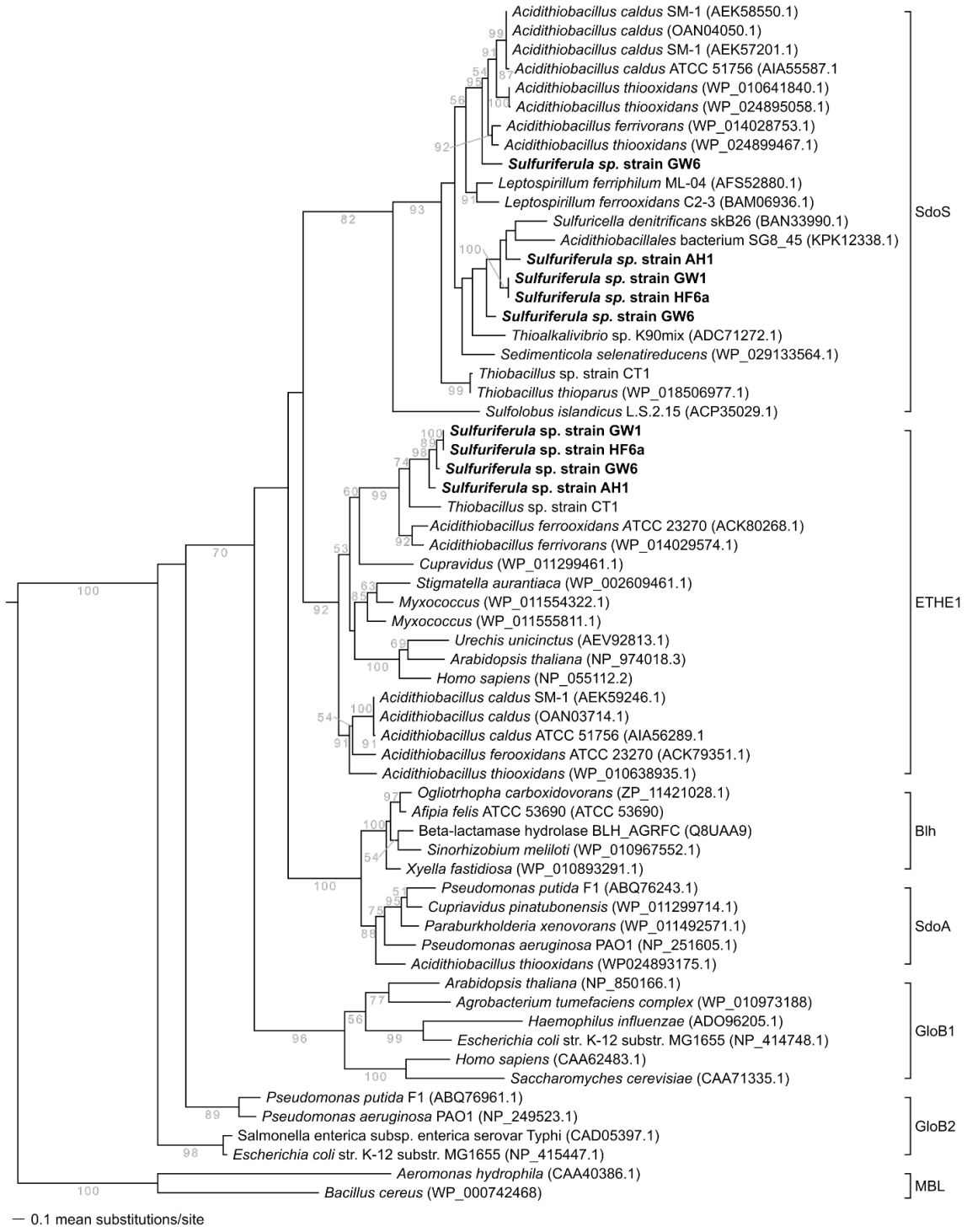

Figure 3. Maximum likelihood analysis of full-length Sdo from isolate organisms, with the genes from the isolates in this study highlighted in bold. The clade of metallo-beta-lactamase (MBL) sequences was used as the outgroup after Liu et al. (56).

266 **Iron oxidation.** All four strains of *Sulfuriferula* described here contain genes that envode
267 for iron-oxidizing cytochromes Cyc1 and Cyc2 (Figure 2d) Cyc1 is a c-type cytochrome likely
268 located in the periplasm and is implicated in iron oxidation in extreme acidophiles (49, 69, 70),
269 while Cyc2 is a candidate iron oxidase found in both neutrophilic and acidophilic iron oxidizers
270 (71, 72). Cyc2 is further divided into three phylogenetically distinct clusters: cluster 1, which has
271 not been experimentally verified but is found in most well-established neutrophilic iron oxidizing
272 organisms; cluster 2, found in *Acidithiobacillus ferrooxidans*, *Ferrovum* spp., *Thiomonas* spp.
273 and other acidophiles, and cluster 3, found in marine iron-oxidizing bacteria and acidophilic
274 *Leptospirillum* spp. (73). The *cyc2* genes in the *Sulfuriferula* strains analyzed here are most
275 similar to those in cluster 2 of the Cyc2 phylogeny of (73).
276
277 **Biofilm formation.** All four strains of *Sulfuriferula* contain genes identified as belonging
278 to the wsp system, implicated in biofilm formation (74) (Figure 2d). In *Pseudomonas*
279 *aeruginosa*, the wsp system senses surface contact and activates genes that promote biofilm
280 formation and regulation (75, 76). Homologs of the *wsp* system are found in the *Beta*- and
281 *Gammaproteobacteria* (77). The four strains are missing *wspE* (Fig. 4d), although studies in *P.*
282 *aeruginosa* found that mutations on this gene did not affect biofilm formation or morphology
283 (76) although a recent study implicated mutations on *wspE* to the formation of "wrinkly" type
284 biofilms in *Burkholderia* spp. that result in more severe human disease (78).
285
286
287 **DISCUSSION**
288
289 **Metabolic and genomic diversity of *Sulfuriferula***
290 Despite being isolated from the same site and under similar conditions, the four strains of
291 *Sulfuriferula* spp. described here are phylogenetically distinct, with different growth behavior
292 and genomic capabilities for sulfur and nitrogen metabolism. For example, strain AH1, which
293 consistently to the highest density on thiosulfate media under the growth conditions tested here,
294 as well as most rapidly on pyrrhotite (79), also has the fewest genes involved in the oxidation of
295 reduced inorganic sulfur compounds. A schematic overview of the pathway of sulfur through the
296 cell in each strain is shown in Figure 4. In strain AH1, thiosulfate is oxidized to a mixture of

297 sulfate and elemental sulfur by the incomplete sox pathway. Elemental sulfur is thought to
298 accumulate in organisms that lack both *soxCD* and *dsr*, which oxidizes elemental sulfur to
299 sulfate (80, 81). In strain AH1, however, produced elemental sulfur could be oxidized to sulfite
300 (SO_3^-) by *sdo* or *hdr*.

301 Conversely, strain GW6, which is the only strain with the complete sox pathway but
302 otherwise possesses an identical set of sulfur-oxidation genes to AH1, consistently showed the
303 lowest optical density (and therefore presumably the least growth) at all pH conditions tested,
304 suggesting that under these conditions there are other factors affecting growth rate. The addition
305 of organic carbon sources to the growth media (yeast extract, glucose, acetate, succinate) did not
306 increase the growth rate or maximum optical density of strain GW6, so its lower growth rate
307 when compared to other strains is not likely linked to organic carbon or specific nutrient
308 requirements. Strain GW6 does differ from strain AH1 in that it lacks a form II RuBisCO. Since
309 form II is higher efficiency (although lower specificity) than type I (82), the growth rate
310 differences between strains AH1 and GW6 could be a result of differences in efficiency of the
311 carbon fixation pathways.

312 Strains GW1 and HF6a share many similarities with their closest-related isolate, *S.*
313 *multivorans* (15), including the presence of *dsrAB* genes (in GW1) and genes for thiosulfate
314 oxidation and reduction (*tth*, *doxDA*). They also share a number of genes encoding proteins
315 involved in nitrogen cycling, including both denitrification and nitrogen fixation abilities.
316 Interestingly, *S. multivorans* lacks the *wsp* genes that are present in all the strains of *Sulfuriferula*
317 described here (Figure 2d), suggesting that biofilm formation is not an important part of its
318 metabolic capabilities. *S. multivorans* was initially enriched by anaerobic thiosulfate oxidation
319 linked to nitrate reduction (15), and so the similarities of the metabolic capabilities of *S.*
320 *multivorans* and strains GW1 and HF6a suggest that they may also have the ability to grow
321 anaerobically. Although we did not demonstrate anaerobic growth under the conditions tested
322 here, that may be due to specific nutrient requirements that were not present in the media used in
323 this study.

324 Strain GW6 is most closely related to *S. plumbiphila*, which was isolated on galena (PbS)
325 and was initially found to grow strictly aerobically on H_2S and H_2, but not on iron(II), elemental
326 sulfur, thiosulfate, tetrathionate, or other metal sulfides. However, recent work has described
327 growth of *S. plumbiphila* on tetrathionate, thiosulfate, and elemental sulfur (17). Strain GW6 and

328 *S. plumbiphila* both lack most of the genes affiliated with nitrogen cycling, although GW6
329 possesses genes encoding for a Mo-dependent nitrogenase complex. Strain GW6 is the only
330 isolate described here that possesses the complete Sox system; the only other isolate with genes
331 homologous to *soxCD* is *S. nivalis* (17).
332 Given the demonstrated growth of other *Sulfuriferula* spp. on elemental sulfur and
333 tetrathionate, we were surprised that all the strains described in this study could not grow on
334 these energy sources. This could be due to specific nutrient requirements not met in the media
335 used here, but it could also reflect specialization in conditions in the mine waste environment,
336 such as growth on acid-soluble sulfide minerals like pyrrhotite or chalcopyrite that are the most
337 abundant sulfides in the Duluth Complex. We were also surprised to find that the strains grew on
338 ferrous iron, given that iron oxidation capability has not been reported for other *Sulfuriferula*
339 strains (15–17, 42). However, it is consistent with the presence of *cyc2* in the genomes of all the
340 isolates. We also note that two of the strains described here, GW1 and HF6a, have the capacity to
341 partially reduce nitrate, with GW1 having a complete set of denitrification genes, showing even
342 more potential metabolic versatility among this genus.
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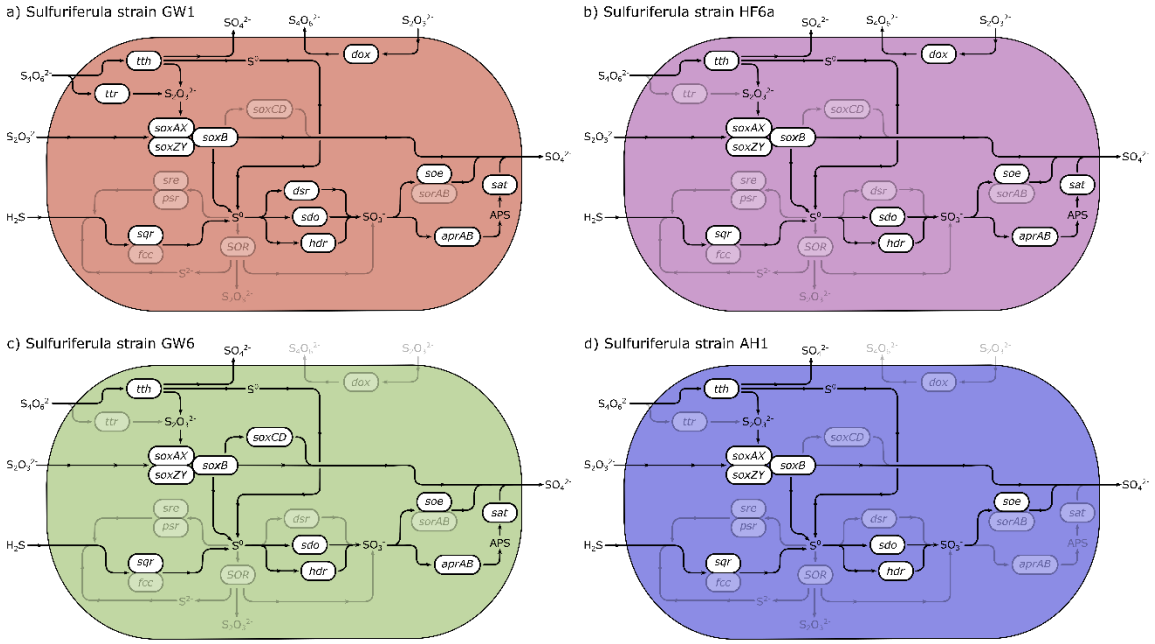

Figure 4. Sulfur oxidation pathways in the four Sulfuriferula *strains highlighting differences in metabolic capabilities based on genome reconstruction. Enzymes are as follows: tth, tetrathionate hydrolase (59); dox, thiosulfate oxidoreductase, tetrathionate-forming (61); ttr, tetrathionate reductase (62), sox, multicomponent sulfur oxidation pathway (46); sre, sulfur reductase (83); psr, polysulfide reductase (84); sqr, sulfide:quinone oxidureductase (43); fcc, flavocytochrome c sulfide dehydrogenase (85); SOR, sulfur oxygenase reductase (58); dsr, dissimilatory sulfite reductase (86); sdo, sulfur dioxygenase (56); hdr, heterodisulfide reductase (60); soe, sulfite dehydrogenase (54); sorAB, sulfite dehydrogenase (87); aprAB, adenylylsulfate reductase (88), sat, dissimilatory sulfate adenylyltransferase (89).*

Implications for the biogeochemistry of Duluth Complex mine waste

All strains in this study grew on the iron-sulfide mineral pyrrhotite as a sole energy source, which is the most abundant sulfide in the weathered rock from which they were isolated. Microbial consortia in biomining or other sulfide mineral-rich environments include organisms that play the role of “oxidant manufacturers”, “acid generators”, and “janitors”, which recycle ferric iron, produce sulfuric acid, and consume low molecular weight organics, respectively (90). All the *Sulfuriferula* spp. characterized here all have the potential to oxidize inorganic sulfur

366 compounds and ferrous iron, indicating that they could either play the role of oxidant
367 manufacturer by generating ferric iron, acid generator by oxidizing intermediate sulfur
368 compounds released during sulfide mineral oxidation, or both, depending on the conditions.
369 Furthermore, pyrrhotite is an acid soluble sulfide that can be oxidized by ferric iron or oxygen,
370 and can also be broken down non-oxidatively by sulfuric acid (91, 92). It is therefore possible
371 that *Sulfuriferula* spp. could directly contribute to the breakdown of pyrrhotite through either its
372 iron or sulfur metabolisms. However, the oxidation of pyrrhotite above pH 4 is not well
373 understood (91, 93–96), so further experimentation is necessary to determine biological
374 pathways of sulfide mineral breakdown in these deposits.

375 The metabolic diversity of *Sulfuriferula* strains could impact their fitness for, and
376 distribution in, different rock and mineral weathering environments. Previous work found that
377 the *Sulfuriferula* OTUs found in humidity cell experiments on Duluth Complex ore tailings were
378 distinct (<97% 16S sequence similarity) from *Sulfuriferula* OTUs found in sediment from
379 naturally weathered outcrops of ore-bearing Duluth Complex rock (41). In addition to mining-
380 influenced environments (14, 18, 20, 22–24, 53, 97), *Sulfuriferula* spp. have also been found in
381 Antarctic lakes (34), constructed wetlands (28, 29) and hot springs (30, 32, 42), as well as
382 hydrogen sulfide-rich aquatic environments (15–17, 35, 42) The diversity of sulfur oxidation and
383 nitrogen metabolic capabilities in *Sulfuriferula* spp. could result in different strains being more
384 successful in different environments.

385 For example, humidity cell experiments are typically conducted indoors and have
386 negligible inputs of organic carbon or nitrogen, so *Sulfuriferula* strains with the capability to fix
387 nitrogen (strains AH1, GW1, GW6) will have a competitive advantage over strains that cannot
388 (e.g., strain HF6a). Conversely, in a natural weathering environment, where organic carbon and
389 nitrogen species will be available from plants or soil bacteria, the ability to metabolize oxidized
390 or reduced nitrogen species (like strain GW1, which possesses a complete denitrification
391 pathway) could provide a competitive advantage. Similarly, *S. multivorans* or other organisms
392 which are able to grow heterotrophically or mixotrophically on organic carbon would have an
393 advantage over the obligate chemoautotrophic strains studied here. Although the pH of leachate
394 from experimentally-generated Duluth Complex mine waste remains near-neutral (39, 40), the
395 ability to oxidize iron, particularly if conditions in a waste rock pile or tailings basin became
396 microoxic, could provide an additional source of metabolic energy to these strains.

397 Strain AH1 displays the most rapid thiosulfate oxidation rates and has also been shown to
398 oxidize sulfide minerals (specifically pyrrhotite, $Fe_{1-x}S$, $0 \leq x \leq 0.125$) much more rapidly than
399 other strains (79). This strain appears to have the simplest sulfur oxidation pathway and has the
400 smallest genome out of the four strains examined. This suggests that *Sulfuriferula* sp, strain AH1
401 is highly specialized for nutrient-poor sulfide mineral weathering environments, and indicate that
402 strain AH1 may have applicability in biomining and mine remediation. *Sulfuriferula* spp. are
403 sensitive to organic acids, and do not appear to be able to utilize organic carbon, and so their
404 activity in tailings or other mine waste environments could be controlled by the application or
405 removal of organic carbon compounds. If the desired goal is to prevent sulfide mineral oxidation,
406 adding organic compounds to the tailings pile would likely result in the chemolithoautotrophic
407 *Sulfuriferula* spp. being outcompeted by heterotrophic organisms (e.g., 41, 98–100). Conversely,
408 if the goal is to accelerate sulfide mineral dissolution, *Sulfuriferula* spp. could be enriched by
409 maintaining an environment depleted of organic carbon. Further experimentation will be
410 necessary to determine the biotechnological potential and explore specific applications for this
411 group of bacteria across the mining lifecycle.
412
413
414 **METHODS**
415
416 **Isolation and identification of *Sulfuriferula* strains**
417 Isolates AH1, GW1, and HF6a were isolated on solid thiosulfate media containing 10 g
418 L^{-1} $NaS_2O_3 \cdot 5H_2O$, 1.5 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.4 g L^{-1} $(NH_4)_2SO_4$, 0.4 g L^{-1}
419 $MgSO_4 \cdot 7H_2O$, 0.04 g $L^{-1}CaCl_2 \cdot 2H_2O$, trace element solution (101), and 1.5% agar. Strain GW6
420 was isolated similarly, but with urea provided as the sole N source.
421
422 **Growth conditions**
423 Growth of *Sulfuriferula* strains was assessed on liquid thiosulfate media, either buffered
424 with phosphate (containing 10 g L^{-1} $NaS_2O_3 \cdot 5H_2O$, 1.5 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.4 g L^{-1}
425 $(NH_4)_2SO_4$, 0.4 g $L^{-1}MgSO_4 \cdot 7H_2O$, 0.04 g $L^{-1}CaCl_2 \cdot 2H_2O$, and a trace element solution (101)) or
426 MES (reducing KH_2PO_4 and Na_2HPO_4 to 16 and 6 mg L^{-1}, respectively, and adding 20mM MES
427 buffer) to the appropriate pH. Growth was assessed qualitatively by measuring optical density

428 (OD) at 600nm and 450nm. Potential utilization of different carbon sources was tested by
429 growing the isolates on LB broth, 1:10 LB broth, or the phosphate-buffered thiosulfate media
430 with 1% yeast extract, 5mM glucose, 5mM acetate, or 5mM succinate. Growth experiments were
431 conducted in clear borosilicate test tubes or serum bottles, and growth at starting pH values of 4,
432 4.5, 6, 7, 7.5, and 8 was compared using MES buffered thiosulfate media. We did not test growth
433 below pH 4 because thiosulfate hydrolyzes abiotically and we could not identify an alternative
434 growth substrate, and because of precipitate in the media above pH 8. We do not report specific
435 growth rates because optical density measurements are impacted by the formation of S^0 during
436 hydrolysis of thiosulfate in the media, and by the formation of other precipitates. To track pH
437 and sulfate production during growth, pH was measured using a LAQUAtwin pH-22 handheld
438 pH meter (HORIBA, Kyoto, Japan) and sulfate was measured in duplicate by barium sulfate
439 ($BaSO_4$) precipitation and absorbance at 450nm.
440 Growth on hydrogen sulfide and ferrous iron was evaluated with a gradient tube culturing
441 technique (102–104). Gradient tubes were prepared using the phosphate buffered media base as
442 described above (1.5 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.4 g L^{-1} $(NH_4)_2SO_4$, 0.4 g L^{-}
443 $^{1}MgSO_4 \cdot 7H_2O$, 0.04 g $L^{-1}CaCl_2 \cdot 2H_2O$, and trace elements). The plug was prepared with media
444 based, 1% agar, 2 mM $NaHCO_3$, and either 2 mM NaS_2 or 10 mM $FeSO_4$. The overlay was
445 prepared with media base and 0.25% agar. Tubes were inoculated after allowing the gradients to
446 develop for 5 days, and growth was evaluated based on the presence of a growth band as well as
447 cell visualization using DAPI (Supplementary Figure S2). We found that it was necessary to add
448 HCO_3^- to ensure cell growth, as well as high Fe^{2+} concentration to ensure effective gradient
449 development in the iron gradient tubes. Growth on pyrrhotite was evaluated by adding 0.5 g of
450 crushed pyrrhotite, prepared as in (79, 105), to thiosulfate-free growth media with both
451 phosphate and MES as buffers.
452
453 **Genome sequencing, assembly, and annotation**
454 Cells of the strains investigated in this manuscript were collected from batch cultures
455 onto 0.2µm-pore filters and genomic DNA (gDNA) was isolated from the filters using the
456 PowerSoil DNA isolation kit (Qiagen). For strain AH1, SMRTbell libraries were prepared with
457 20µg of high molecular weight DNA, and sequenced on a Pacific Biosciences (PacBio) RSII
458 platform. For other strains, SMRTbell libraries were prepared with 2-3 ug of high molecular

459 weight DNA was prepared with a 10 Kb insert size at the University of Minnesota Genomics
460 Center (UMGC), and pooled and sequenced on a PacBio Sequel platform. Assembly of AH1 was
461 described in (106), and the other genomes were assembled using HGAP version 6 with default
462 parameters.
463 Sequences were uploaded to IMG/MER (107) and annotated using the JGI Integrated
464 Microbial Genomes (IMG) Pipeline (108). Presence and function of genes involved in carbon
465 and nitrogen metabolic pathways were determined using KEGG Ortholog annotation (109).
466 Genes involved in sulfur metabolism, iron metabolism, and biofilm formation were initially
467 determined using KEGG Orthology using GhostKOALA (109) and were confirmed using hidden
468 Markov models (HMM) with HMMer v. 3 (110). HMMs for genes in the *SoxB*, *SoxC*, and *SoxD*,
469 as well as *Sqr*/*Fcc* pathways were constructed by aligning a database of sequences using
470 TCOFFEE's Expresso aligner (111) and using HMMER to create the model out of the aligned
471 sequences. Databases for *soxB* were generated using sequence compilations from (112, 113),
472 databases for sqr were generated from (6, 43), and databases for sdo were generated from (56,
473 114). The isolate genomes were then converted from DNA to proteins using Prodigal v. 2.6.3
474 (115) and screened for matches using hmmsearch with an E-value cutoff of 10^{-7}. All other
475 HMMs used were part of the MagicCave package (116, 117).
476
477 **Phylogenetic analysis**
478 For phylogenetic analysis of 16S rRNA genes, sequences were aligned in ARB (118)
479 using the Silva database v. 132 (119). Sequences were filtered so that all *Sulfuriferula* sequences
480 were the same length and positions with more than 50% gaps removed (final alignment 1449
481 positions). Neighbor joining analyses were performed with the ARB-implementation of phylip
482 (120) with Jukes-Cantor-corrected distance. SDO phylogenies were created with maximum
483 likelihood analysis using RAxML v.8.0.24 (121) with the LG model of amino acid substitution
484 (122) with observed amino acid frequencies and the fraction of invariant sites and the shape
485 parameter (α) value estimated from the data. Phylogenetic analysis of SDO genes was performed
486 with representative sequences from (56, 114), and using clade names from those publications.
487

488 **Accession numbers**

489 The genomes of the *Sulfuriferula* strains in this study are available at the National Center
490 for Biotechnology under Bioproject accession PRJNA1160330. The specific accessions for each
491 strain and associated plasmids are CP021138-CP021139 (str. AH1), CP170197 (str. GW1),
492 CP170194-CP170196 (str. GW6), and CP170193 (str. HF6a).
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494
495
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497

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