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

Kathryn K. Hobart[1,2], Gabriel M. Walker[1], Joshua M. Feinberg[1,2], Jake V. Bailey[1], Daniel S. Jones[1,3,4]

[1]Dept. Of Earth & Environmental Sciences, University of Minnesota, Minneapolis, MN 55455, U.S.A.

[2]Institute for Rock Magnetism, University of Minnesota, Minneapolis, MN 55455, U.S.A.

[3]Dept. of Earth and Environmental Science, New Mexico Institute of Mining and Technology, Socorro, NM 87801, U.S.A

[4]National Cave and Karst Research Institute, Carlsbad, NM, 88220, U.S.A.

Correspondence: Kathryn Hobart (khobart@usgs.gov), Daniel Jones (daniel.s.jones@nmt.edu)

Current addresses:

Kathryn K. Hobart: United States Geological Survey, Geology, Energy, and Minerals Science Center, Reston, VA, USA

Gabriel M. Walker: Diagnostic Radiology Residency, Henry Ford Hospital, Detroit, MI

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

known from diverse sulfur-rich terrestrial environments, and relatives of *Sulfuriferula* in

metagenomic and 16S rRNA gene datasets have been found in metal- and sulfide- rich mine

tailings and drainage (18–26), sewer and groundwater environments (27–29), hot springs and

volcanic deposits (30–32), seawater-influenced sulfide caves (33), and low-temperature lake

bottom sediments (18, 34, 35).

Here we describe new strains of *Sulfuriferula* that were isolated from weathered rocks

from the sulfide mineral-bearing Duluth Complex in Northern Minnesota. The Duluth Complex

contains copper, nickel, and platinum group element (Cu-Ni-PGE) deposits that may represent

the world's largest undeveloped economic Cu-Ni-PGE resource (36). The most abundant sulfide

mineral in these rocks is pyrrhotite ($Fe_{1-x}S$, $0 < x < 0.125$), followed by chalcopyrite ($CuFeS_2$),

cubanite ($CuFe_2S_3$), and pentlandite ($(Fe, Ni)_9S_8$), with smaller amounts of other Cu, Ni, and

PGE-bearing sulfide minerals (37). Sulfur- and iron-oxidizing microorganisms are known to

accelerate the dissolution of sulfide minerals under highly acidic conditions (38). However, in

contrast to many other well-studied ore and mine-waste environments where extreme acidity is

generated, the relatively low sulfide mineral content (usually <1% total sulfur) and buffering

capacity of the surrounding silicate minerals means that leachate from Duluth Complex waste

rock and tailings is moderately acidic and rarely reaches pH values of less than 4 (39, 40). The

role of microorganisms in this mildly acidic environment is not as well understood as in

extremely acidic systems.

Based on rRNA gene and transcript sequencing, *Sulfuriferula* spp. were some of the most

abundant and active organisms in experimentally weathered Duluth Complex rocks and tailings

(18), and were also found (although at lower abundances) in naturally-weathered Duluth

Complex outcrops (41), suggesting that they are important members of the rock weathering

community and may also be valuable for developing biological solutions to managing mine

waste and water from proposed mines in the region. To learn more about these important

organisms and the role they play in the biogeochemistry of Duluth Complex mine waste and

water, we isolated and characterized four strains of *Sulfuriferula* from experimentally weathered

Duluth Complex materials. We describe the genomic and physiological properties of the strains

and connect their diverse metabolic capabilities to their ecophysiology in the rock weathering

environment.

RESULTS

Isolation and identification of *Sulfuriferula*

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

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

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 ($Fe_{1-x}S$, $0 \leq x$

≤ 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,

consistent with complete thiosulfate oxidation to sulfate (Figure S1). In addition to reduced

inorganic sulfur compounds, all strains grew on ferrous iron (Fe^{2+}) in gradient tubes

(Supplementary Figure S1). However, we did not observe anaerobic growth on either $H_2S(aq)$ or

$S_2O_3^{2-}$ with NO_3^- as an electron acceptor under the conditions tested here, or when only hydrogen

(H_2) was provided as an electron donor under aerobic or anaerobic conditions.

Growth was not observed for any strains exclusively on LB broth or 1:10 diluted LB

broth. No increase in growth was observed when 5mM glucose, acetate, or succinate was added

to the thiosulfate media, suggesting that these strains are obligate autotrophs and are sensitive to

the presence of organics. Consistent with this, growth rate decreased in media buffered with

organic buffers rather than phosphate. Growth occurred in MES- and HEPES- but not citrate-

buffered media. However, growth was enhanced by adding 0.1% w/v yeast extract was added to

the growth media.

Each strain grew at slightly different pH ranges. Strain AH1 had the widest range, which

grew between pH 4.5 and 8. HF6a was most acid tolerant than the other strains, growing between

pH 4 and 7, while strains GW1 and GW6 were less so, growing between pH 5 and 8 and 6 and 8

respectively. During growth on thiosulfate, pH consistently initially increased and then decreased

(e.g., Supplementary Figure S2).

Whole genome sequencing and assembly

The genomes of the four isolates were each assembled into a single large chromosome,

between 2.92 and 3.33 Mb. One 39.1 Kb plasmid was recovered for strain AH1, and two

plasmids, 23.8 and 39.5 Kb, were recovered from strain GW6. Table 1 contains an overview of

the properties of the assembled genomes, which range from 55-58.7 %G+C content and 97-98%

protein-coding genes. GW1, GW6, and HF6a each have two *rrn* operons, and AH1 encodes

three. A summary of genes associated with sulfur, nitrogen, carbon, and iron metabolisms and

genes associated with biofilm formation are presented in Figure 2.

Carbon metabolism. All four strains of *Sulfuriferula* encode complete Calvin cycles for

CO_2 fixation. Strain AH1 has two copies of RuBisCO form I and one copy of RuBisCO form II,

strain GW1 has two copies of RuBisCO form I, strain GW6 has three copies of RuBisCO form I,

and strain HF6a has one copy each of RuBisCO form I and II. All four strains have a full

complement of carboxysome and carbonic anhydrase-coding genes (Figure 2b). All four strains

possess complete glycolysis pathways and incomplete TCA cycles.

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

possess complete assimilatory nitrate reduction pathway genes, although all strains possess the

nasA gene. Additionally, the genomes of strains AH1 and GW6 contain genes *ureABC* that

encode urea hydrolysis, indicating an ability to utilize urea as a source of ammonia.

Sulfur metabolism: The four strains encode diverse pathways for the oxidation of

reduced inorganic sulfur compounds (Figure 2a). All four strains encode sulfide:quinone

oxidoreductase (SQR) homologs. SQR is used by all domains of life for sulfide oxidation and/or

detoxification. Six structural types of SQR are known; microorganisms encoding at least one

representative enzyme from each of type I, III, IV, V, and VI can use sulfide as an electron

donor, while type II has not been directly linked to metabolic oxidation of sulfide and may be

used for detoxification (43, 44). All four *Sulfuriferula* strains have genes identified as SQR type

I, with strains GW1 and HF6a having two copies. Strain GW6 also possesses two copies of the

SQR type III, as well as *fcc*, which encodes flavocytochrome-c sulfide dehydrogenase, a

structurally-related protein that can also be utilized for sulfide oxidation (45).

Strains AH1, GW1 and HF6a possess partial *sox* systems, with genes *soxAX*, *soxYZ*, and

soxB but not *soxCD*, while strain GW6 possesses a complete sox system including *soxCD*. In the

complete pathway, SoxYZ complexes with thiosulfate to form a SoxY-cysteine S-thiosulfate

derivative, which move to sequential reactions with SoxAX and SoxB that oxidize one of the

sulfur atoms in the thiosulfate to sulfate, with two electrons transferred to the electron transport

chain. SoxCD then oxidizes the remaining sulfane sulfur of the residual SoxY-cysteine-S to

sulfate, which is hydrolyzed by SoxB, ultimately generating sulfate and transferring six electrons

to the electron transport chain (46). The sox pathway is able to catalyze oxidation of reduced

sulfur species besides thiosulfate, including sulfide, elemental sulfur, sulfite, and tetrathionate

(47–50), and this ability seems to be facilitated by the modular nature of the reactions (51).

While SoxCD is not essential for complete thiosulfate or sulfide oxidation, partial *sox* systems

lacking SoxCD are thought to produce elemental sulfur or polysulfides (52). In some organisms

with a partial *sox* system, that sulfur is then completely oxidized by the DSR system (53, 54).

Only strain GW1 contains genes encoding DsrAB (dissimilatory (bi)sulfate reductase), however,

indicating that the other strains either produce S(0) as a final product or use another process to

completely oxidize the S(0).

All four strains possess *hdrABC*, which encodes heterodisulfide reductase that is linked to

the oxidation of zero-valent sulfur in some bacteria (55). All four strains also possess genes

coding for sulfur dioxygenases (*sdo*). Sdo oxidizes the sulfate sulfur in glutathione persulfide

(GSSH) to sulfite (56). Homologous genes are found in mitochondria, where mutations on this

gene are implicated in a rare hereditary human disease (57). Widely distributed through the

Proteobacteria, *sdo* genes are used both in sulfide detoxification and in primary metabolism. The

Sulfuriferula strains examined here have two types of *sdo* sequences: one set are most similar to

the mitochondrial sulfide detoxification ETHE1 genes, while the other set are most similar to the

sdoS genes found in acidophillic bacteria like *Acidithiobacillus* (Figure 3). Strains AH1, GW1,

and HF6a have one copy of each of the two types of *sdo*, while strain GW6 has one copy

homologous with ETHE1 and two distinct copies of genes within the *sdoS* group. The strains did

not encode other known enzymes involved in S(0) oxidation like sulfur oxygenase reductase

(SOR) (58).

All four strains also possess *tth* genes for tetrathionate hydrolase, which catalyzes the

oxidation of tetrathionate to thiosulfate, elemental sulfur, and sulfate (59), and *soeABC*, which

encodes a sulfite dehydrogenase that allows the transfer of electrons to the quinone pool by the

oxidation of sulfite to sulfate (60). Strain GW1 additionally possesses genes *doxDA* for

thiosulfate dehydrogenase, which catalyzes the oxidation of thiosulfate to tetrathionate (61), and

ttrABC for tetrathionate reductase, which catalyzes the oxidation of tetrathionate to thiosulfate

(62). Strains AH1 and GW6 possess *sir* genes which code for ferrodoxin:sulfite reductase,

catalyzing the assimilatory reduction of sulfite to sulfide (63), and GW6 possesses *sorA*, which

encodes the A subunit of sulfide dehydrogenase (64). Strains GW1, GW6 and HF6a possess *sat*

genes for sulfate adenylyltransferase and *aprAB* for adenylylsulfate reductase. Both of these

proteins typically catalyze the reduction of sulfate (65, 66), but like DsrAB they can run in

reverse, oxidizing intermediate organosulfur compounds to sulfate (67, 68).

— 0.1 mean substitutions/site

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).

Iron oxidation. All four strains of *Sulfuriferula* described here contain genes that envode

for iron-oxidizing cytochromes Cyc1 and Cyc2 (Figure 2d) Cyc1 is a c-type cytochrome likely

located in the periplasm and is implicated in iron oxidation in extreme acidophiles (49, 69, 70),

while Cyc2 is a candidate iron oxidase found in both neutrophilic and acidophilic iron oxidizers

(71, 72). Cyc2 is further divided into three phylogenetically distinct clusters: cluster 1, which has

not been experimentally verified but is found in most well-established neutrophilic iron oxidizing

organisms; cluster 2, found in *Acidithiobacillus ferrooxidans*, *Ferrovum* spp., *Thiomonas* spp.

and other acidophiles, and cluster 3, found in marine iron-oxidizing bacteria and acidophilic

Leptospirillum spp. (73). The *cyc2* genes in the *Sulfuriferula* strains analyzed here are most

similar to those in cluster 2 of the Cyc2 phylogeny of (73).

Biofilm formation. All four strains of *Sulfuriferula* contain genes identified as belonging

to the wsp system, implicated in biofilm formation (74) (Figure 2d). In *Pseudomonas*

aeruginosa, the wsp system senses surface contact and activates genes that promote biofilm

formation and regulation (75, 76). Homologs of the *wsp* system are found in the *Beta-* and

Gammaproteobacteria (77). The four strains are missing *wspE* (Fig. 4d), although studies in *P.*

aeruginosa found that mutations on this gene did not affect biofilm formation or morphology

(76) although a recent study implicated mutations on *wspE* to the formation of "wrinkly" type

biofilms in *Burkholderia* spp. that result in more severe human disease (78).

DISCUSSION

Metabolic and genomic diversity of *Sulfuriferula*

Despite being isolated from the same site and under similar conditions, the four strains of

Sulfuriferula spp. described here are phylogenetically distinct, with different growth behavior

and genomic capabilities for sulfur and nitrogen metabolism. For example, strain AH1, which

consistently to the highest density on thiosulfate media under the growth conditions tested here,

as well as most rapidly on pyrrhotite (79), also has the fewest genes involved in the oxidation of

reduced inorganic sulfur compounds. A schematic overview of the pathway of sulfur through the

cell in each strain is shown in Figure 4. In strain AH1, thiosulfate is oxidized to a mixture of

sulfate and elemental sulfur by the incomplete sox pathway. Elemental sulfur is thought to

accumulate in organisms that lack both *soxCD* and *dsr,* which oxidizes elemental sulfur to

sulfate (80, 81). In strain AH1, however, produced elemental sulfur could be oxidized to sulfite

(SO_3^-) by *sdo* or *hdr*.

Conversely, strain GW6, which is the only strain with the complete sox pathway but

otherwise possesses an identical set of sulfur-oxidation genes to AH1, consistently showed the

lowest optical density (and therefore presumably the least growth) at all pH conditions tested,

suggesting that under these conditions there are other factors affecting growth rate. The addition

of organic carbon sources to the growth media (yeast extract, glucose, acetate, succinate) did not

increase the growth rate or maximum optical density of strain GW6, so its lower growth rate

when compared to other strains is not likely linked to organic carbon or specific nutrient

requirements. Strain GW6 does differ from strain AH1 in that it lacks a form II RuBisCO. Since

form II is higher efficiency (although lower specificity) than type I (82), the growth rate

differences between strains AH1 and GW6 could be a result of differences in efficiency of the

carbon fixation pathways.

Strains GW1 and HF6a share many similarities with their closest-related isolate, *S.*

multivorans (15), including the presence of *dsrAB* genes (in GW1) and genes for thiosulfate

oxidation and reduction (*tth*, *doxDA*). They also share a number of genes encoding proteins

involved in nitrogen cycling, including both denitrification and nitrogen fixation abilities.

Interestingly, *S. multivorans* lacks the *wsp* genes that are present in all the strains of *Sulfuriferula*

described here (Figure 2d), suggesting that biofilm formation is not an important part of its

metabolic capabilities. *S. multivorans* was initially enriched by anaerobic thiosulfate oxidation

linked to nitrate reduction (15), and so the similarities of the metabolic capabilities of *S.*

multivorans and strains GW1 and HF6a suggest that they may also have the ability to grow

anaerobically. Although we did not demonstrate anaerobic growth under the conditions tested

here, that may be due to specific nutrient requirements that were not present in the media used in

this study.

Strain GW6 is most closely related to *S. plumbiphila*, which was isolated on galena (PbS)

and was initially found to grow strictly aerobically on H_2S and H_2, but not on iron(II), elemental

sulfur, thiosulfate, tetrathionate, or other metal sulfides. However, recent work has described

growth of *S. plumbiphila* on tetrathionate, thiosulfate, and elemental sulfur (17). Strain GW6 and

S. plumbiphila both lack most of the genes affiliated with nitrogen cycling, although GW6

possesses genes encoding for a Mo-dependent nitrogenase complex. Strain GW6 is the only

isolate described here that possesses the complete Sox system; the only other isolate with genes

homologous to *soxCD* is *S. nivalis* (17).

Given the demonstrated growth of other *Sulfuriferula* spp. on elemental sulfur and

tetrathionate, we were surprised that all the strains described in this study could not grow on

these energy sources. This could be due to specific nutrient requirements not met in the media

used here, but it could also reflect specialization in conditions in the mine waste environment,

such as growth on acid-soluble sulfide minerals like pyrrhotite or chalcopyrite that are the most

abundant sulfides in the Duluth Complex. We were also surprised to find that the strains grew on

ferrous iron, given that iron oxidation capability has not been reported for other *Sulfuriferula*

strains (15–17, 42). However, it is consistent with the presence of *cyc2* in the genomes of all the

isolates. We also note that two of the strains described here, GW1 and HF6a, have the capacity to

partially reduce nitrate, with GW1 having a complete set of denitrification genes, showing even

more potential metabolic versatility among this genus.

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

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

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

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

Strain AH1 displays the most rapid thiosulfate oxidation rates and has also been shown to

oxidize sulfide minerals (specifically pyrrhotite, $Fe_{1-x}S$, $0 \leq x \leq 0.125$) much more rapidly than

other strains (79). This strain appears to have the simplest sulfur oxidation pathway and has the

smallest genome out of the four strains examined. This suggests that *Sulfuriferula* sp, strain AH1

is highly specialized for nutrient-poor sulfide mineral weathering environments, and indicate that

strain AH1 may have applicability in biomining and mine remediation. *Sulfuriferula* spp. are

sensitive to organic acids, and do not appear to be able to utilize organic carbon, and so their

activity in tailings or other mine waste environments could be controlled by the application or

removal of organic carbon compounds. If the desired goal is to prevent sulfide mineral oxidation,

adding organic compounds to the tailings pile would likely result in the chemolithoautotrophic

Sulfuriferula spp. being outcompeted by heterotrophic organisms (e.g., 41, 98–100). Conversely,

if the goal is to accelerate sulfide mineral dissolution, *Sulfuriferula* spp. could be enriched by

maintaining an environment depleted of organic carbon. Further experimentation will be

necessary to determine the biotechnological potential and explore specific applications for this

group of bacteria across the mining lifecycle.

METHODS

Isolation and identification of *Sulfuriferula* strains

Isolates AH1, GW1, and HF6a were isolated on solid thiosulfate media 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} $(NH_4)_2SO_4$, 0.4 g L^{-1}

$MgSO_4 \cdot 7H_2O$, 0.04 g $L^{-1}CaCl_2 \cdot 2H_2O$, trace element solution (101), and 1.5% agar. Strain GW6

was isolated similarly, but with urea provided as the sole N source.

Growth conditions

Growth of *Sulfuriferula* strains was assessed on liquid thiosulfate media, either buffered

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}

$(NH_4)_2SO_4$, 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

MES (reducing KH_2PO_4 and Na_2HPO_4 to 16 and 6 mg L^{-1}, respectively, and adding 20mM MES

buffer) to the appropriate pH. Growth was assessed qualitatively by measuring optical density

(OD) at 600nm and 450nm. Potential utilization of different carbon sources was tested by

growing the isolates on LB broth, 1:10 LB broth, or the phosphate-buffered thiosulfate media

with 1% yeast extract, 5mM glucose, 5mM acetate, or 5mM succinate. Growth experiments were

conducted in clear borosilicate test tubes or serum bottles, and growth at starting pH values of 4,

4.5, 6, 7, 7.5, and 8 was compared using MES buffered thiosulfate media. We did not test growth

below pH 4 because thiosulfate hydrolyzes abiotically and we could not identify an alternative

growth substrate, and because of precipitate in the media above pH 8. We do not report specific

growth rates because optical density measurements are impacted by the formation of S^0 during

hydrolysis of thiosulfate in the media, and by the formation of other precipitates. To track pH

and sulfate production during growth, pH was measured using a LAQUAtwin pH-22 handheld

pH meter (HORIBA, Kyoto, Japan) and sulfate was measured in duplicate by barium sulfate

($BaSO_4$) precipitation and absorbance at 450nm.

Growth on hydrogen sulfide and ferrous iron was evaluated with a gradient tube culturing

technique (102–104). Gradient tubes were prepared using the phosphate buffered media base as

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^{-1}

$^1MgSO_4 \cdot 7H_2O$, 0.04 g $L^{-1}CaCl_2 \cdot 2H_2O$, and trace elements). The plug was prepared with media

based, 1% agar, 2 mM $NaHCO_3$, and either 2 mM NaS_2 or 10 mM $FeSO_4$. The overlay was

prepared with media base and 0.25% agar. Tubes were inoculated after allowing the gradients to

develop for 5 days, and growth was evaluated based on the presence of a growth band as well as

cell visualization using DAPI (Supplementary Figure S2). We found that it was necessary to add

HCO_3^- to ensure cell growth, as well as high Fe^{2+} concentration to ensure effective gradient

development in the iron gradient tubes. Growth on pyrrhotite was evaluated by adding 0.5 g of

crushed pyrrhotite, prepared as in (79, 105), to thiosulfate-free growth media with both

phosphate and MES as buffers.

Genome sequencing, assembly, and annotation

Cells of the strains investigated in this manuscript were collected from batch cultures

onto 0.2μm-pore filters and genomic DNA (gDNA) was isolated from the filters using the

PowerSoil DNA isolation kit (Qiagen). For strain AH1, SMRTbell libraries were prepared with

20μg of high molecular weight DNA, and sequenced on a Pacific Biosciences (PacBio) RSII

platform. For other strains, SMRTbell libraries were prepared with 2-3 ug of high molecular

weight DNA was prepared with a 10 Kb insert size at the University of Minnesota Genomics

Center (UMGC), and pooled and sequenced on a PacBio Sequel platform. Assembly of AH1 was

described in (106), and the other genomes were assembled using HGAP version 6 with default

parameters.

Sequences were uploaded to IMG/MER (107) and annotated using the JGI Integrated

Microbial Genomes (IMG) Pipeline (108). Presence and function of genes involved in carbon

and nitrogen metabolic pathways were determined using KEGG Ortholog annotation (109).

Genes involved in sulfur metabolism, iron metabolism, and biofilm formation were initially

determined using KEGG Orthology using GhostKOALA (109) and were confirmed using hidden

Markov models (HMM) with HMMer v. 3 (110). HMMs for genes in the *SoxB*, *SoxC*, and *SoxD*,

as well as *Sqr*/*Fcc* pathways were constructed by aligning a database of sequences using

TCOFFEE's Expresso aligner (111) and using HMMER to create the model out of the aligned

sequences. Databases for *soxB* were generated using sequence compilations from (112, 113),

databases for sqr were generated from (6, 43), and databases for sdo were generated from (56,

114). The isolate genomes were then converted from DNA to proteins using Prodigal v. 2.6.3

(115) and screened for matches using hmmsearch with an E-value cutoff of 10^{-7}. All other

HMMs used were part of the MagicCave package (116, 117).

Phylogenetic analysis

For phylogenetic analysis of 16S rRNA genes, sequences were aligned in ARB (118)

using the Silva database v. 132 (119). Sequences were filtered so that all *Sulfuriferula* sequences

were the same length and positions with more than 50% gaps removed (final alignment 1449

positions). Neighbor joining analyses were performed with the ARB-implementation of phylip

(120) with Jukes-Cantor-corrected distance. SDO phylogenies were created with maximum

likelihood analysis using RAxML v.8.0.24 (121) with the LG model of amino acid substitution

(122) with observed amino acid frequencies and the fraction of invariant sites and the shape

parameter (α) value estimated from the data. Phylogenetic analysis of SDO genes was performed

with representative sequences from (56, 114), and using clade names from those publications.

Accession numbers

The genomes of the *Sulfuriferula* strains in this study are available at the National Center

for Biotechnology under Bioproject accession PRJNA1160330. The specific accessions for each

strain and associated plasmids are CP021138-CP021139 (str. AH1), CP170197 (str. GW1),

CP170194-CP170196 (str. GW6), and CP170193 (str. HF6a).

ACKNOWLEDGEMENTS

We thank the Minnesota Department of Natural Resources Division of Lands and Minerals for

facilitating sampling of the weathered rock samples from which the strains were isolated,

especially Z. Wenz, K. Lapakko, M. Olson, S. Koski and P. Geiselman for access and assistance

with sample collection. Special thanks to A. Baloun and A. Hua for assistance with culturing,

and to B. Auch and the staff at the UMGC for assistance with genome sequencing. This work

was supported by University of Minnesota MnDRIVE initiative and the University of Minnesota

BioTechnology Institute. This work was supported by University of Minnesota MnDRIVE

initiative and the University of Minnesota BioTechnology Institute.

REFERENCES

1.   Holmer M, Storkholm P. 2001. Sulphate reduction and sulphur cycling in lake sediments: a review. Freshw Biol 46:431–451.

2.   Sharrar AM, Flood BE, Bailey JV, Jones DS, Biddanda BA, Ruberg SA, Marcus DN, Dick GJ. 2017. Novel large sulfur bacteria in the metagenomes of groundwater-fed chemosynthetic microbial mats in the Lake Huron basin. Front Microbiol 8.

3.   Baker BJ, Banfield JF. 2003. Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44:139–152.

4.   Macalady JL, Jones DS, Lyon EH. 2007. Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. Environ Microbiol 9:1402–1414.

5.   Hamilton TL, Welander PV, Albrecht HL, Fulton JM, Schaperdoth I, Bird LR, Summons RE, Freeman KH, Macalady JL. 2017. Microbial communities and organic biomarkers in a Proterozoic-analog sinkhole. Geobiology 15:784–797.

6.   Hamilton TL, Jones DS, Schaperdoth I, Macalady JL. 2014. Metagenomic insights into S(0) precipitation in a terrestrial subsurface lithoautotrophic ecosystem. Front Microbiol 5:1–16.

7.   Fike DA, Bradley AS, Rose CV. 2015. Rethinking the ancient sulfur cycle. Annu Rev Earth Planet Sci 43:593–622.

8.   Bontognali TRR, Sessions AL, Allwood AC, Fischer WW, Grotzinger JP, Summons RE, Eiler JM. 2012. Sulfur isotopes of organic matter preserved in 3.45-billion-year-old stromatolites reveal microbial metabolism. Proc Natl Acad Sci 109:15146–15151.

9.   Muyzer G, Kuenen JG, Robertson LA. 2013. Colorless Sulfur Bacteria, p. 555–588. *In* Rosenberg, E, DeLong, EF, Thompson, F, Lory, S, Stackebrandt, E (eds.), The Prokaryotes: Prokaryotic Physiology and Biochemistry. Springer Berlin Heidelberg.

10.   Klatt JM, Gomez-Saez GV, Meyer S, Ristova PP, Yilmaz P, Granitsiotis MS, Macalady JL, Lavik G, Polerecky L, Bühring SI. 2020. Versatile cyanobacteria control the timing and extent of sulfide production in a Proterozoic analog microbial mat. ISME J 14:3024–3037.

11.   Aronson HS, Monteverde DR, Barnes BD, Johnson BR, Zawaski MJ, Speth DR, Wang XT, Wu F, Webb SM, Trower EJ, Magyar JS, Sessions AL, Orphan VJ, Course 2017 G, Geobiology Course 2018, Fischer WW. 2022. Sulfur cycling at natural hydrocarbon and sulfur seeps in Santa Paula Creek, CA. Geobiology 20:707–725.

12.   Capone DG, Kiene RP. 1988. Comparison of microbial dynamics in marine and freshwater sediments: Contrasts in anaerobic carbon catabolism 1. Limnol Oceanogr 33:725–749.

13.   Diao M, Sinnige R, Kalbitz K, Huisman J, Muyzer G. 2017. Succession of bacterial communities in a seasonally stratified lake with an anoxic and sulfidic hypolimnion. Front Microbiol 8.

14.   Drobner E, Huber H, Rachel R, Stetter KO. 1991. *Thiobacillus plumbophilus* spec. nov., a novel galena and hydrogen oxidizer. Arch Microbiol 157

15.   Watanabe T, Kojima H, Fukui M. 2015. *Sulfuriferula multivorans* gen. nov., sp. nov., isolated from a freshwater lake, reclassification of 'Thiobacillus plumbophilus' as *Sulfuriferula plumbophilus* sp. nov., and description of *Sulfuricellaceae* fam. nov. and *Sulfuricellales* ord. nov. Int J Syst Evol Microbiol 65:1504–1508.

16.   Watanabe T, Kojima H, Fukui M. 2016. *Sulfuriferula thiophila* sp. nov., a
chemolithoautotrophic sulfur-oxidizing bacterium, and correction of the name
Sulfuriferula plumbophilus Watanabe, Kojima and Fukui 2015 to *Sulfuriferula*
plumbiphila corrig. Int J Syst Evol Microbiol 66:2041–2045.
17.   Kojima H, Mochizuki J, Fukui M. 2020. *Sulfuriferula nivalis* sp. nov., a sulfur oxidizer
isolated from snow and emended description of *Sulfuriferula plumbiphila*. Int J Syst Evol
Microbiol 70:3273–3277.
18.   Jones DS, Lapakko KA, Wenz ZJ, Olson MC, Roepke EW, Sadowsky MJ, Novak PJ,
Bailey JV. 2017. Novel microbial assemblages dominate weathered sulfide bearing rock
from copper-nickel deposits in the Duluth Complex, Minnesota, USA. Appl Environ
Microbiol 83:1–15.
19.   Laroche E, Casiot C, Fernandez-Rojo L, Desoeuvre A, Tardy V, Bruneel O, Battaglia-
Brunet F, Joulian C, Héry M. 2018. Dynamics of bacterial communities mediating the
treatment of an As-rich acid mine drainage in a field pilot. Front Microbiol 9:3169.
20.   Kadnikov VV, Gruzdev EV, Ivasenko DA, Beletsky AV, Mardanov AV, Danilova EV,
Karnachuk OV, Ravin NV. 2019. Selection of a microbial community in the course of
formation of acid mine drainage. Microbiology 88:292–299.
21.   Liu J, Yao J, Lu C, Li H, Li Z, Duran R, Sunahara G, Mihucz VG. 2019. Microbial
activity and biodiversity responding to contamination of metal(loid) in heterogeneous
nonferrous mining and smelting areas. Chemosphere 226:659–667.
22.   Liu J, Yao J, Wang F, Min N, Gu J, Li Z, Sunahara G, Duran R, Solevic-Knudsen T,
Hudson-Edwards KA, Alakangas L. 2019. Bacterial diversity in typical abandoned multi-
contaminated nonferrous metal(loid) tailings during natural attenuation. Environ Pollut
247:98–107.
23.   Liu J, Yin M, Zhang W, Tsang DCW, Wei X, Zhou Y, Xiao T, Wang J, Dong X, Sun Y,
Chen Y, Li H, Hou L. 2019. Response of microbial communities and interactions to
thallium in contaminated sediments near a pyrite mining area. Environ Pollut 248:916–
928.
24.   Gruzdev EV, Beletsky AV, Kadnikov VV, Mardanov AV, Ivanov MV, Karnachuk OV,
Ravin NV. 2020. Diversity of eukaryotic microorganisms in the drainage waters of a coal
open-cast mine. Microbiology 89:641–646.
25.   Pakostova E, Schmall AJ, Holland SP, White H, Ptacek CJ, Blowes DW. 2020.
Performance of a geosynthetic clay-liner cover system at a Cu/Zn mine tailings
impoundment: microbiological characterization. Appl Environ Microbiol 86:e02846-19.
https://doi.org/10.1128/AEM.02846-19.
26.   Sun X, Kong T, Häggblom MM, Kolton M, Li F, Dong Y, Huang Y, Li B, Sun W. 2020.
Chemolithoautotropic diazotrophy dominates the nitrogen fixation process in mine
tailings. Environ Sci Technol 54:6082–6093.
27.   Li X, Kappler U, Jiang G, Bond PL. 2017. The ecology of acidophilic microorganisms in
the corroding concrete sewer environment. Front Microbiol 8:683.
28.   Liang Y, Wei D, Hu J, Zhang J, Liu Z, Li A, Li R. 2020. Glyphosate and nutrients
removal from simulated agricultural runoff in a pilot pyrrhotite constructed wetland.
Water Res 168:115154.
29.   Novikov AP, Safonov AV, Babich TL, Boldyrev KA, Kryuchkov DV, Lavrinovich EA,
Kuzovkina EV, Emel'yanov AM, Goryachenkova TA. 2020. Biotransformation of
neptunium in model groundwaters. Geochem Int 58:182–188.

30.   Arce-Rodríguez A, Puente-Sánchez F, Avendaño R, Martínez-Cruz M, de Moor JM,
Pieper DH, Chavarría M. 2019. Thermoplasmatales and sulfur-oxidizing bacteria
dominate the microbial community at the surface water of a CO_2-rich hydrothermal spring
located in Tenorio Volcano National Park, Costa Rica. Extremophiles 23:177–187.
31.   Lathifah AN, Guo Y, Sakagami N, Suda W, Higuchi M, Nishizawa T, Prijambada ID,
Ohta H. 2019. Comparative Characterization of bacterial communities in moss-covered
and unvegetated volcanic deposits of Mount Merapi, Indonesia. Microbes Environ
34:268–277.
32.   Lopez Bedogni G, Massello FL, Giaveno A, Donati ER, Urbieta MS. 2019. A deeper look
into the biodiversity of the extremely acidic Copahue volcano-Río Agrio System in
Neuquén, Argentina. Microorganisms 8:58.
33.   D'Angeli IM, Ghezzi D, Leuko S, Firrincieli A, Parise M, Fiorucci A, Vigna B, Addesso
R, Baldantoni D, Carbone C, Miller AZ, Jurado V, Saiz-Jimenez C, De Waele J,
Cappelletti M. 2019. Geomicrobiology of a seawater-influenced active sulfuric acid cave.
PLoS ONE 14:e0220706.
34.   Shen L, Huang T, Chen Y, Chu Z, Xie Z. 2021. Diverse biotransformation of sulfur in
antarctic lake sediments. Preprint (version 1) available at Research Square
[https://doi.org/10.21203/rs.3.rs-230087/v1].
35.   Zakharyuk AG, Ryzhmanova YaV, Avtukh AN, Shcherbakova VA. 2019. Iron-reducing
microbial communities of the Lake Baikal low-temperature bottom sediments.
Microbiology 88:156–163.
36.   Miller JD Jr, Green JC, Severson MJ, Chandler VW, Hauck SA, Peterson DM, Wahl TE.
2002. *Geology and mineral potential of the Duluth Complex and related rocks of
northeastern Minnesota.* RI-58. Report of Investigations. St. Paul, MN: Minnesota
Geological Survey.
37.   Severson MJ, Miller JD, Peterson DM, Green JC, Hauck SA, Chandler VW, Wahl TE.
2002. Mineral potential of the Duluth Complex and related intrusions. Minn Geol Surv
Rep Investig 58:164–200.
38.   Nordstrom DK, Blowes DW, Ptacek CJ. 2015. Hydrogeochemistry and microbiology of
mine drainage: An update. Appl Geochem 57:3–16.
39.   Lapakko KA. 2015. Preoperational assessment of solute release from waste rock at
proposed mining operations. Appl Geochem 57:106–124.
40.   Lapakko KA, Antonson DA. 1994. Oxidation of sulfide minerals present in Duluth
Complex rock. In: Environmental geochemistry of sulfide oxidation. ACS Symposium
Series. Vol. 550. Washington, DC: American Chemical Society.
41.   Hobart KK, Greensky Z, Hernandez K, Feinberg JM, Bailey JV, Jones DS. 2023.
Microbial communities from weathered outcrops of a sulfide-rich ultramafic intrusion, and
implications for mine waste management. Environ Microbiol 25:3512–3526.
42.   Watanabe T, Kojima H, Kazuhiro U, Hori C, Takasuka T, Kato Y, Fukui M. 2019.
Genomes of Neutrophilic Sulfur-Oxidizing Chemolithoautotrophs Representing 9
Proteobacterial Species From 8 Genera. Front Microbiol 10:316.
https://doi.org/10.3389/fmicb.2019.00316
43.   Marcia M, Ermler U, Peng G, Michel H. 2010. A new structure-based classification of
sulfide:quinone oxidoreductases. Proteins Struct Funct Bioinforma 78:1073–1083.

44.   Lencina AM, Ding Z, Schurig-Briccio LA, Gennis RB. 2013. Characterization of the Type
III sulfide:quinone oxidoreductase from *Caldivirga maquilingensis* and its membrane
binding. Biochim Biophys Acta BBA - Bioenerg 1827:266–275.

45.   Tikhonova TV, Lilina AV, Osipov EM, Shipkov NS, Dergousova NI, Kulikova OG,
Popov VO. 2021. Catalytic properties of flavocytochrome c sulfide dehydrogenase from
haloalkaliphilic bacterium *Thioalkalivibrio paradoxus*. Biochem Biokhimiia 86:361–369.

46.   Friedrich CG, Rother D, Bardischewsky F, Quentmeier A, Fischer J. 2001. Oxidation of
reduced inorganic sulfur compounds by bacteria: Emergence of a common mechanism?
Appl Environ Microbiol 67:2873–2882.

47.   Wodara C, Bardischewsky F, Friedrich CG. 1997. Cloning and characterization of sulfite
dehydrogenase, two c-type cytochromes, and a flavoprotein of *Paracoccus denitrificans*
GB17: essential role of sulfite dehydrogenase in lithotrophic sulfur oxidation. J Bacteriol
179:5014–5023.

48.   Mukhopadhyaya PN, Deb C, Lahiri C, Roy P. 2000. A *soxA* Gene, encoding a diheme
cytochrome c, and a sox locus, essential for sulfur oxidation in a new sulfur lithotrophic
bacterium. J Bacteriol 182:4278–4287.

49.   Appia-Ayme C, Little PJ, Matsumoto Y, Leech AP, Berks BC. 2001. Cytochrome
complex essential for photosynthetic oxidation of both thiosulfate and sulfide in
Rhodovulum sulfidophilum. J Bacteriol 183:6107–6118.

50.   Lahiri C, Mandal S, Ghosh W, Dam B, Roy P. 2006. A novel gene cluster *soxSRT* is
essential for the chemolithotrophic oxidation of thiosulfate and tetrathionate by
Pseudaminobacter salicylatoxidans KCT001. Curr Microbiol 52:267–273.

51.   Sauvé V, Bruno S, Berks BC, Hemmings AM. 2007. The SoxYZ complex carries sulfur
cycle intermediates on a peptide swinging arm. J Biol Chem 282:23194–23204.

52.   Friedrich CG, Bardischewsky F, Rother D, Quentmeier A, Fischer J. 2005. Prokaryotic
sulfur oxidation. Curr Opin Microbiol 8:253–259.

53.   Beller HR, Chain PSG, Letain TE, Chakicherla A, Larimer FW, Richardson PM, Coleman
MA, Wood AP, Kelly DP. 2006. The genome sequence of the obligately
chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. J
Bacteriol 188:1473–1488.

54.   Dahl C, Franz B, Hensen D, Kesselheim A, Zigann R. 2013. Sulfite oxidation in the
purple sulfur bacterium *Allochromatium vinosum*: identification of SoeABC as a major
player and relevance of SoxYZ in the process. Microbiology 159:2626–2638.

55.   Boughanemi S, Lyonnet J, Infossi P, Bauzan M, Kosta A, Lignon S, Giudici-Orticoni M-
T, Guiral M. 2016. Microbial oxidative sulfur metabolism: biochemical evidence of the
membrane-bound heterodisulfide reductase-like complex of the bacterium *Aquifex*
aeolicus. FEMS Microbiol Lett 363:fnw156.

56.   Liu H, Xin Y, Xun L. 2014. Distribution, diversity, and activities of sulfur dioxygenases in
heterotrophic bacteria. Appl Environ Microbiol 80:1799–1806.

57.   Tiranti V, Briem E, Lamantea E, Mineri R, Papaleo E, Gioia LD, Forlani F, Rinaldo P,
Dickson P, Abu-Libdeh B, Cindro-Heberle L, Owaidha M, Jack RM, Christensen E,
Burlina A, Zeviani M. 2006. *ETHE1* mutations are specific to ethylmalonic
encephalopathy. J Med Genet 43:340.

58.   Kletzin A. 1989. Coupled enzymatic production of sulfite, thiosulfate, and hydrogen
sulfide from sulfur: purification and properties of a sulfur oxygenase reductase from the

facultatively anaerobic archaebacterium *Desulfurolobus ambivalens*. J Bacteriol
171:1638–1643.

59. Kanao T, Onishi M, Kajitani Y, Hashimoto Y, Toge T, Kikukawa H, Kamimura K. 2018.
Characterization of tetrathionate hydrolase from the marine acidophilic sulfur-oxidizing
bacterium, *Acidithiobacillus thiooxidans* strain SH. Biosci Biotechnol Biochem 82:152–
160.

60. Boughanemi S, Infossi P, Giudici-Orticoni M-T, Schoepp-Cothenet B, Guiral M. 2020.
Sulfite oxidation by the quinone-reducing molybdenum sulfite dehydrogenase SoeABC
from the bacterium *Aquifex aeolicus*. Biochim Biophys Acta BBA - Bioenerg
1861:148279.

61. Müller FH, Bandeiras TM, Urich T, Teixeira M, Gomes CM, Kletzin A. 2004. Coupling
of the pathway of sulphur oxidation to dioxygen reduction: characterization of a novel
membrane-bound thiosulphate:quinone oxidoreductase: Thiosulphate oxidoreductase from
Acidianus ambivalens. Mol Microbiol 53:1147–1160.

62. Hensel M, Hinsley AP, Nikolaus T, Sawers G, Berks BC. 1999. The genetic basis of
tetrathionate respiration in *Salmonella typhimurium*. Mol Microbiol 32:275–287.

63. Gisselmann G, Klausmeier P, Schwenn JD. 1993. The ferredoxin:sulphite reductase gene
from *Synechococcus* PCC7942. Biochim Biophys Acta BBA - Bioenerg 1144:102–106.

64. Kappler U, Bennett B, Rethmeier J, Schwarz G, Deutzmann R, McEwan AG, Dahl C.
2000. Sulfite:cytochrome *c* oxidoreductase from *Thiobacillus novellus*: Purification,
character, and molecular biology of a heterodimeric member of the sulfide oxidase family.
J Biol Chem 275:13202–13212.

65. Lampreia J, Moura I, Teixeira M, PECK Jr HD, Legall J, Huynh BH, Moura JJG. 1990.
The active centers of adenylylsulfate reductase from *Desulfovibrio gigas*. Eur J Biochem
188:653–664.

66. Carlson HK, Youngblut MD, Redford SA, Williamson AJ, Coates JD. 2021. Sulfate
adenylyl transferase kinetics and mechanisms of metabolic inhibitors of microbial sulfate
respiration. ISME Commun 1:1–4.

67. Yamamoto M, Takai K. 2011. Sulfur metabolisms in epsilon- and gamma-*Proteobacteria*
in deep-sea hydrothermal fields. Front Microbiol 2:192.

68. Kappler U, Dahl C. 2001. Enzymology and molecular biology of prokaryotic sulfite
oxidation. FEMS Microbiol Lett 203:1–9.

69. Bonnefoy V, Holmes DS. 2012. Genomic insights into microbial iron oxidation and iron
uptake strategies in extremely acidic environments: Iron oxidation and iron homeostasis in
acidophiles. Environ Microbiol 14:1597–1611.

70. Peng Z, Liu Z, Jiang Y, Dong Y, Shi L. 2022. In vivo interactions between Cyc2 and Rus
as well as Rus and Cyc1 of *Acidithiobacillus ferrooxidans* during extracellular oxidization
of ferrous iron. Int Biodeterior Biodegrad 173:105453.

71. Barco RA, Emerson D, Sylvan JB, Orcutt BN, Jacobson Meyers ME, Ramírez GA, Zhong
JD, Edwards KJ. 2015. New insight into microbial iron oxidation as revealed by the
proteomic profile of an obligate iron-oxidizing chemolithoautotroph. Appl Environ
Microbiol 81:5927–5937.

72. Castelle C, Guiral M, Malarte G, Ledgham F, Leroy G, Brugna M, Giudici-Orticoni M-T.
2008. A new iron-oxidizing/O_2-reducing supercomplex spanning both inner and outer
membranes, isolated from the extreme acidophile *Acidithiobacillus ferrooxidans*. J Biol
Chem 283:25803–25811.

73.   McAllister SM, Polson SW, Butterfield DA, Glazer BT, Sylvan JB, Chan CS. 2020.
Validating the Cyc2 neutrophilic iron oxidation pathway using meta-omics of
Zetaproteobacteria iron mats at marine hydrothermal vents. mSystems 5:e00553-19.
74.   Garber AI, Ramirez GA, Merino N, Pavia MJ, McAllister SM. 2020. MagicLamp: toolkit
for annotation of genomic data using discreet and curated HMM sets. 2023: MagicLamp,
GitHub repository: https://github.com/Arkadiy-Garber/MagicLamp.
75.   Armbruster CR, Lee CK, Parker-Gilham J, de Anda J, Xia A, Zhao K, Murakami K,
Tseng BS, Hoffman LR, Jin F. 2019. Heterogeneity in surface sensing suggests a division
of labor in *Pseudomonas aeruginosa* populations. eLife 8:e45084.
76.   Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates
biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci
102:14422–14427.
77.   Kessler C, Mhatre E, Cooper V, Kim W. 2021. Evolutionary divergence of the Wsp signal
transduction systems in Beta- and Gammaproteobacteria. Appl Environ Microbiol
87:e01306-21.
78.   Cooper VS, Staples RK, Traverse CC, Ellis CN. 2014. Parallel evolution of small colony
variants in *Burkholderia cenocepacia* biofilms. Genomics 104:447–452.
79.   Hobart KK. 2022. Understanding microbially-mediated pyrrhotite dissolution in the
Duluth Complex, Northern MN. University of Minnesota, Minneapolis, MN, USA.
80.   Dahl C, Kredich NM, Deutzmann R, Trlfper HGY 1993. 1993. Dissimilatory sulphite
reductase from *Archaeoglobus fulgidus*: physico-chemical properties of the enzyme and
cloning, sequencing and analysis of the reductase genes. Microbiology 139:1817–1828.
81.   Dahl C, Prange A, Steudel R. 2005. Metabolism of Natural Polymeric Sulfur Compounds.
In Biopolymers Online, A. Steinbüchel (Ed.).
https://doi.org/10.1002/3527600035.bpol9002
82.   Berg IA. 2011. Ecological aspects of the distribution of different autotrophic CO_2 fixation
pathways. Appl Environ Microbiol 77:1925–1936.
83.   Laska S, Lottspeich F, Kletzin A. 2003. Membrane-bound hydrogenase and sulfur
reductase of the hyperthermophilic and acidophilic archaeon Acidianus ambivalens.
Microbiology 149:2357–2371.
84.   Dietrich W, Klimmek O. 2002. The function of methyl-menaquinone-6 and polysulfide
reductase membrane anchor (PsrC) in polysulfide respiration of *Wolinella succinogenes*.
Eur J Biochem 269:1086–1095.
85.   Kusai K, Yamanaka T. 1973. The oxidation mechanisms of thiosulphate and sulphide in
Chlorobium thiosulphatophilum: roles of cytochrome c-551 and cytochrome c-553.
Biochim Biophys Acta 325:304–314.
86.   Pott AS, Dahl C. 1998. Sirohaem sulfite reductase and other proteins encoded by genes at
the dsr locus of *Chromatium vinosum* are involved in the oxidation of intracellular sulfur.
Microbiol Read Engl 144 (Pt 7):1881–1894.
87.   Lyric RM, Suzuki I. 1970. Enzymes involved in the metabolism of thiosulfate by
Thiobacillus thioparus. II. Properties of adenosine-5′-phosphosulfate reductase. Can J
Biochem 48:344–354.
88.   Fritz G, Roth A, Schiffer A, Buchert T, Bourenkov G, Bartunik HD, Huber H, Stetter KO,
Kroneck PMH, Ermler U. 2002. Structure of adenylylsulfate reductase from the
hyperthermophilic *Archaeoglobus fulgidus* at 1.6-A resolution. Proc Natl Acad Sci
99:1836–1841.

89. Laue BE, Nelson DC. 1994. Characterization of the gene encoding the autotrophic ATP
sulfurylase from the bacterial endosymbiont of the hydrothermal vent tubeworm *Riftia*
pachyptila. J Bacteriol 176:3723–3729.
90. Johnson D. 2018. The evolution, current status, and future prospects of using
biotechnologies in the mineral extraction and metal recovery sectors. Minerals 8:343.
91. Belzile N, Chen YW, Cai MF, Li Y. 2004. A review on pyrrhotite oxidation. J Geochem
Explor 84:65–76.
92. Chiriță P. 2016. Aqueous oxidation of iron monosulfide (FeS) by molecular oxygen.
Miner Process Extr Metall Rev 37:305–310.
93. Bădică CE, Chiriță P. 2015. An electrochemical study of the oxidative dissolution of iron
monosulfide (FeS) in air-equilibrated solutions. Electrochimica Acta 178:786–796.
94. Chiriță P, Rimstidt JD. 2014. Pyrrhotite dissolution in acidic media. Appl Geochem 41:1–
10.
95. Harries D, Pollok K, Langenhorst F. 2013. Oxidative dissolution of 4C- and NC-
pyrrhotite: Intrinsic reactivity differences, pH dependence, and the effect of anisotropy.
Geochim Cosmochim Acta 102:23–44.
96. Bugajski J, Gamsjäger H. 1982. The kinetics of the dissolution of monoclinic pyrrhotite in
aqueous acidic solutions. Monatshefte Chem Chem Mon 113:1087–1092.
97. Pakostova E, Johnson DB, Bao Z, MacKenzie PM, Ptacek CJ, Blowes DW. 2020.
Bacterial and archaeal diversity in sulfide-bearing waste rock at Faro Mine Complex,
Yukon Territory, Canada. Geomicrobiol J 37:511–519.
98. Rawlings DE, Johnson DB. 2007. The microbiology of biomining: development and
optimization of mineral-oxidizing microbial consortia. Microbiology 153:315–324.
99. Johnson DB, Yajie L, Okibe N. 2008. "Bioshrouding"—a novel approach for securing
reactive mineral tailings. Biotechnol Lett 30:445–449.
100. Bwapwa JK, Jaiyeola AT, Chetty R. 2017. Bioremediation of acid mine drainage using
algae strains: A review. South Afr J Chem Eng 24:62–70.
101. Flood BE, Jones DS, Bailey JV. 2015. *Sedimenticola thiotaurini* sp. nov., a sulfur-
oxidizing bacterium isolated from salt marsh sediments, and emended descriptions of the
genus *Sedimenticola* and *Sedimenticola selenatireducens*. Int J Syst Evol Microbiol
65:2522–2530.
102. Emerson D, Merrill Floyd M. 2005. Enrichment and isolation of iron-oxidizing bacteria at
neutral pH, p. 112–123. *In* Methods in Enzymology. Academic Press.
103. Emerson D, Moyer C. 1997. Isolation and characterization of novel iron-oxidizing bacteria
that grow at circumneutral pH. Appl Environ Microbiol 63:4784–4792.
104. Lueder U, Druschel G, Emerson D, Kappler A, Schmidt C. 2018. Quantitative analysis of
O_2 and Fe^{2+} profiles in gradient tubes for cultivation of microaerophilic iron(II)-oxidizing
bacteria. FEMS Microbiol Ecol 94:fix177.
105. Hobart KK, Feinberg JM, Volk MWR, Jones DS. 2021. The importance of temperature-
dependent diffraction data in understanding magnetic changes across the pyrrhotite λ-
transition. Earth Space Sci Open Arch https://doi.org/10.1002/essoar.10507692.1.
106. Jones DS, Roepke EW, Hua AA, Flood BE, Bailey JV. 2017. Complete genome sequence
of *Sulfuriferula* sp. strain AH1, a sulfur- oxidizing autotroph isolated from weathered mine
tailings from the Duluth Complex in Minnesota. Genome Announc 5:17–18.
107. Chen I-MA, Chu K, Palaniappan K, Ratner A, Huang J, Huntemann M, Hajek P, Ritter S,
Varghese N, Seshadri R, Roux S, Woyke T, Eloe-Fadrosh EA, Ivanova NN, Kyrpides NC.

2021. The IMG/M data management and analysis system v.6.0: new tools and advanced
capabilities. Nucleic Acids Res 49:D751–D763.

108. Markowitz VM, Mavromatis K, Ivanova NN, Chen I-MA, Chu K, Kyrpides NC. 2009.
IMG ER: a system for microbial genome annotation expert review and curation.
Bioinformatics 25:2271–2278.

109. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. 2016. KEGG as a reference
resource for gene and protein annotation. Nucleic Acids Res 44:D457-462.

110. Johnson LS, Eddy SR, Portugaly E. 2010. Hidden Markov model speed heuristic and
iterative HMM search procedure. BMC Bioinformatics 11:431.

111. Notredame C, Higgins DG, Heringa J. 2000. T-coffee: a novel method for fast and
accurate multiple sequence alignment. J Mol Biol 302:205–217.

112. Meyer B, Imhoff JF, Kuever J. 2007. Molecular analysis of the distribution and phylogeny
of the soxB gene among sulfur-oxidizing bacteria - Evolution of the Sox sulfur oxidation
enzyme system. Environ Microbiol 9:2957–2977.

113. Edwardson CF, Hollibaugh JT. 2017. Metatranscriptomic analysis of prokaryotic
communities active in sulfur and arsenic cycling in Mono Lake, California, USA. ISME J
11:2195–2208.

114. Wu W, Pang X, Lin J, Liu X, Wang R, Lin J, Chen L. 2017. Discovery of a new subgroup
of sulfur dioxygenases and characterization of sulfur dioxygenases in the sulfur metabolic
network of *Acidithiobacillus caldus*. PLOS ONE 12:e0183668.

115. Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. 2010. Prodigal:
prokaryotic gene recognition and translation initiation site identification. BMC
Bioinformatics 11:119.

116. Garber AI, Nealson KH, Okamoto A, McAllister SM, Chan CS, Barco RA, Merino N.
2020. FeGenie: A comprehensive tool for the identification of iron genes and iron gene
neighborhoods in genome and metagenome assemblies. Front Microbiol 11.

117. Garber AI, Merino N, Pavia MJ, McAllister SM. 2020. MagicLamp: toolkit for annotation
of 'omics datasets using curated HMM sets. https://github.com/Arkadiy-
Garber/MagicLamp

118. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T,
Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S,
Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B,
Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A,
Schleifer K. 2004. ARB: a software environment for sequence data. Nucleic Acids Res
32:1363–1371.

119. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.
2013. The SILVA ribosomal RNA gene database project: improved data processing and
web-based tools. Nucleic Acids Res 41:D590–D596.

120. Felsenstein J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood
approach. J Mol Evol 17:368–376.

121. Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses
with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

122. Le SQ, Gascuel O. 2008. An improved general amino acid replacement matrix. Mol Biol
Evol 25:1307–1320.