Received: 24 April 2024 | Accepted: 6 August 2024

DOI: 10.1111/1462-2920.16691

BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

Acquisition of elemental sulfur by sulfur-oxidising Sulfolobales

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Funding information
The Molecular Bioscience Program (MBSP); National Science Foundation, Grant/Award Number: EAR-1820658; Family of Beverly Ferguson

Abstract

Elemental sulfur (S_8^0)-oxidising Sulfolobales (Archaea) dominate high-temperature acidic hot springs (>80°C, pH <4). However, genomic analyses of S_8^0-oxidising members of the Sulfolobales reveal a patchy distribution of genes encoding sulfur oxygenase reductase (SOR), an S_8^0 disproportionating enzyme attributed to S_8^0 oxidation. Here, we report the S_8^0-dependent growth of two Sulfolobales strains previously isolated from acidic hot springs in Yellowstone National Park, one of which associated with bulk S_8^0 during growth and one that did not. The genomes of each strain encoded different sulfur metabolism enzymes, with only one encoding SOR. Dialysis membrane experiments showed that direct contact is not required for S_8^0 oxidation in the SOR-encoding strain. This is attributed to the generation of hydrogen sulfide (H_2S) from S_8^0 disproportionation that can diffuse out of the cell to solubilise bulk S_8^0 to form soluble polysulfides (S_x^{2-}) and/or S_8^0 nanoparticles that readily diffuse across dialysis membranes. The Sulfolobales strain lacking SOR required direct contact to oxidise S_8^0, which could be overcome by the addition of H_2S. High concentrations of S_8^0 inhibited the growth of both strains. These results implicate alternative strategies to acquire and metabolise sulfur in Sulfolobales and have implications for their distribution and ecology in their hot spring habitats.

INTRODUCTION

Members of the archaeal order Sulfolobales dominate acidic (pH <4.0) and high-temperature (>80°C) hot springs (Colman et al., 2018; Jiang et al., 2016; Urbieta et al., 2015; Ward et al., 2017). *Sulfolobus*, the first genus of Sulfolobales described, was shown to catalyse the oxygen (O_2)-dependent oxidation of orthorhombic elemental sulfur (S_8^0), generating sulfuric acid (H_2SO_4) as a product (Brock et al., 1972). This observation helped to explain the acidification of hot springs sourced by hydrogen sulfide (H_2S)-rich volcanic gas (Brock et al., 1972; Mosser et al., 1973). More specifically, the O_2-dependent oxidation of H_2S generates thiosulfate ($S_2O_3^{2-}$), which disproportionates at acidic pH to form S_8^0 and sulfite (SO_3^{2-}), the latter of which is also unstable in the presence of O_2 and oxidises to form SO_4^{2-}. However, these collective reactions do not generate net acidity (Fernandes-Martins et al., 2024; Nordstrom et al., 2005; Sims et al., 2023). Rather, it is the O_2-dependent oxidation of S_8^0 that generates net acidity in the form of H_2SO_4. Yet, S_8^0 is stable in the absence of microbial catalysts (Nordstrom et al., 2005; Xu et al., 1998). Members of the order Sulfolobales therefore became models to understand the oxidation of S_8^0 in acidic high-temperature hot springs (Brock et al., 1972; Colman et al., 2018; Mosser et al., 1973; Shivvers & Brock, 1973).

After >50 years of study of Sulfolobales, several themes have emerged of their ecology, physiology and evolution. For example, all cultivated members of Sulfolobales are thermoacidophiles that tend to be metabolically flexible, growing aerobically or anaerobically through chemoautotrophic, chemoheterotrophic or chemolithoheterotrophic pathways (Amenabar et al., 2018; Colman et al., 2018; Counts et al., 2021; Johnson, 1998; Johnson & Quatrini, 2020; Lewis et al., 2021; Liu et al., 2021). Further, recent phylogenomic analyses suggest that Sulfolobales diversified from their neutrophilic ancestors ∼1.0–0.6 Ma, coincident with the rise of atmospheric O_2 concentrations to near present-day levels (Colman et al., 2018). Yet, genomic analyses of Sulfolobales highlight many remaining unanswered questions of Sulfolobales physiology and ecology. For example, the majority of the proteins encoded by Sulfolobales have undescribed functions (Counts et al., 2021), and little is known about the feedbacks that allowed for the diversification of Sulfolobales into the acidic habitats that they helped create (Colman et al., 2018; Counts et al., 2021). Perhaps the largest gap in understanding is the apparent variation in the pathways of S_8^0 oxidation in Sulfolobales.

The only characterised pathway for S_8^0 oxidation in Sulfolobales starts with the O_2-dependent sulfur oxygenase reductase (SOR) enzyme that catalyses the disproportionation of S_8^0 to form H_2S, SO_3^{2-} and $S_2O_3^-$ in the cytoplasm of cells (Kletzin, 1989, 1992; Urich et al., 2004, 2006). Surprisingly, only members of the Sulfolobales genera *Acidianus*, *Sulfurisphaera*, *Stygiolobus* and *Sulfuricidiifex* encode SOR (Counts et al., 2021; Liu et al., 2021), while many other S_8^0-oxidising Sulfolobales genera, including *Metallosphaera*, *Sulfolobus* and *Saccharolobus* do not encode SOR (Counts et al., 2021; Jiang et al., 2014; Liu et al., 2021; Sakai & Kurosawa, 2018). Further, many Sulfolobales genomes have been assembled from metagenomic sequences that also do not encode SOR (Colman et al., 2022; Sims et al., 2023). However, without cultivation data, it cannot be assumed that they can oxidise S_8^0. Transcriptomic and comparative genomic studies have been used to suggest that sulfur dioxygenase (SDO), NADPH:sulfur oxidoreductase (NSR), or heterodisulfide reductase (HDR) may be involved in S_8^0-oxidation in members of the Sulfolobales that lack SOR (Colman et al., 2022; Jiang et al., 2014; Wang et al., 2020).

S_8^0 has a low solubility (<500 nM at 80°C; Kamyshny, 2009), suggesting that cells must associate with the surface of the mineral to disproportionate or oxidise it (Weiss, 1973) or somehow otherwise promote its solubilisation. Interestingly, thermoacidophilic Archaea that reduce S_8^0, including a member of the Sulfolobales (*Acidianus* strain DS80) that encodes SOR, were shown to not associate with bulk S_8^0 during growth (Amenabar & Boyd, 2018; Boyd & Druschel, 2013). Rather, these cells reduced soluble nanoparticulate S_8^0 that formed when biologically produced H_2S reacted with bulk S_8^0, generating polysulfide (S_x^{2-}) that rapidly disproportionates at acidic pH to produce soluble molecular S_8 rings. Due to their hydrophobicity, these S_8 rings rapidly aggregate to form nanoparticulate S_8^0. These collective observations raise the question of whether a similar mechanism might be involved in the solubilisation of S_8^0 in SOR-encoding Sulfolobales strains and whether this might contribute differences to the respective ecologies of SOR- versus non-SOR-encoding strains, such as planktonic or mineral-surface associated growth.

We previously isolated two new Sulfolobales strains capable of oxidising S_8^0 from acidic hot springs in Yellowstone National Park (YNP), Wyoming, USA. One strain, *Stygiolobus* sp. RP85 encodes SOR, whereas the other strain, Sulfolobales sp. RB85, does not encode SOR. Microscopic analyses of the two cultures grown under S_8^0-oxidising conditions revealed that *Stygiolobus* sp. RP85 did not associate with S_8^0 during S_8^0-dependent growth, whereas Sulfolobales sp. RB85 was regularly associated with S_8^0 particles. Here, we hypothesise that SOR allows *Stygiolobus* sp. RP85 to grow without direct contact with S_8^0 since H_2S, a product of S_8^0 disproportionation, can initiate the production of S_x^{2-} and soluble nanoparticulate S_8^0, as described above. In contrast, we hypothesised that Sulfolobales sp. RB85 would require direct contact with S_8^0 to oxidise it. The results of experiments aimed at testing these hypotheses shed new light on relevant physiological differences among members of the Sulfolobales that likely contribute to the partitioning of the S_8^0 oxidation niche, thereby enabling their stable co-existence.

EXPERIMENTAL PROCEDURES

Strain selection

Stygiolobus sp. RP85 and Sulfolobales sp. RB85 were isolated from 'Realgar Pool' (RP; pH 3.9, *T* 85.8°C; 44.73558 N, 110.70705 W) and 'Red Bubbler' (RB; pH 3.0, *T* 90°C; 44.72650 N, 110.70900 W), respectively, both located at Norris Geyser Basin, YNP (Fernandes-Martins et al., 2024). *Stygiolobus* sp. RP85 was isolated under autotrophic and microaerophilic H_2S-oxidising conditions at 85°C and pH 4.0, and it can also oxidise S_8^0. Sulfolobales sp. RB85 was isolated under autotrophic and microaerophilic H_2S-oxidising conditions at 85°C and pH 3.0, and it can also oxidise S_8^0 and H_2 (Fernandes-Martins et al., 2024).

Culture conditions

Stygiolobus sp. RP85 and Sulfolobales sp. RB85 were cultivated in base salts medium amended with 20% filter-sterilised (0.22 μm) and autoclaved source water from each respective hot spring where the strains were originally isolated. For *Stygiolobus* sp. RP85, this was

'Realgar Pool' (pH 3.9, T 85.8°C) and for Sulfolobales sp. RB85, it was 'Red Bubbler' (pH 3.0, T 90°C). Additional details of the springs are reported elsewhere (Fernandes-Martins et al., 2024). Base salts medium contained: NH_4Cl (0.33 g L^{-1}), KCl (0.33 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.33 g L^{-1}), $MgCl_2 \cdot 6H_2O$ (0.33 g L^{-1}) and KH_2PO_4 (0.33 g L^{-1}) (Boyd et al., 2007). The pH of the base salts/filtered spring water medium was adjusted to the pH of the spring where the strain was isolated using 0.5 N HCl. The total Cl^- content of the base medium (~800 mg L^{-1}) is similar to the amount of Cl^- in the hot springs (~600 mg L^{-1}) where these organisms were originally isolated (Fernandes-Martins et al., 2024). Fifty-five millilitres of base salts/filtered spring water medium was dispensed into 160 mL serum bottles that were then sealed with black butyl rubber stoppers. Sealed serum bottles were autoclaved and then S_8^0 (0.16 or 1.6 g L^{-1}; sterilised at 100°C, 2 h) was added to the serum bottles once they cooled to below 100°C. Following the addition of S_8^0 and while still hot, vials and their contents were purged for 20 min with N_2 passed over heated (350°C) and H_2-reduced copper shavings. Next, the headspace was purged with a mixture of N_2:carbon dioxide (CO_2) (80:20) for 5 min, and vials were placed in an 80°C incubator. The headspace was equilibrated to atmospheric pressure after 2 h incubation, followed by the addition of anoxic and filter-sterilised (0.22 μm) solutions of Wolfe's vitamins (Atlas, 2004) and SL-10 metals (Widdel, 1983) to final concentrations of 1 mL L^{-1} each. Oxygen (O_2) (as air) was added to the headspace to a final concentration of 2% vol./vol. The final headspace contained approximately 78% N_2, 20% CO_2 and 2% O_2, as described above. Inoculum for use in S_8^0 oxidation experiments was grown with H_2S (added as Na_2S) as an electron donor to minimise the carryover of S_8^0, as previously described (Fernandes-Martins et al., 2024). Five millilitres (~1/10 dilution) of a log phase culture with depleted H_2S (below the limit of detection of 2 μM) was used as inoculum, and cultures were incubated on a shaking (50 rotations per min) platform incubator at a temperature of 80°C.

Monitoring of growth and activity

The production of cells was monitored by filtering subsamples of culture on black, 0.22 μm polycarbonate filters (Millipore Sigma, Billerica, MA), staining with 4′,6-diamidino-2-phenylindole (DAPI) (2 μg mL^{-1} final concentration) for 10 min, and enumeration on an Evos fluorescent microscope (ThermoFisher Scientific, Waltham, MA, USA). The concentration of total aqueous sulfide ($H_2S/HS^-/S^{2-}$ and acid volatile metal sulfides) in cultures was quantified using the methylene blue reduction assay (Fogo & Popowsky, 1949), while the production of sulfate (SO_4^{2-}) in cultures was quantified using a barium chloride turbidity assay (Kolmert et al., 2000).

Evaluating the requirement for direct contact to S_8^0 mineral

The requirement for direct contact of *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85 cells with S_8^0 (0.16 g L^{-1}) to catalyse the oxidation of the mineral with O_2 as the electron acceptor was investigated using dialysis membranes with 3.5 kDa pore sizes (Spectrum Laboratories, Gardena, CA). Briefly, dialysis membranes and clips were cleaned with autoclaved Milli-Q water and 50% ethanol incubation steps, as previously described (Amenabar & Boyd, 2018; Payne et al., 2021). After cleaning, dialysis membranes were kept moist and manipulated inside a UV-treated laminar flow hood. One end of each dialysis membrane was sealed with a clip so that sterilised S_8^0 could be added, followed by addition of 1 mL of sterile base salt medium (at the appropriate pH for each strain). Then, the other end was also sealed with a clip, and dialysis membranes were again rinsed with autoclaved Milli-Q to minimise potential S_8^0 contamination on the outside of the membranes.

The effect of H_2S (~15 μM added as Na_2S) amendment on the requirement for direct contact with S_8^0 to catalyse the oxidation of the mineral with O_2 as the electron acceptor was evaluated by sequestering mineral in dialysis membranes in cultures of Sulfolobales sp. RB85. Uninoculated abiotic controls and positive controls that allowed for direct contact with the mineral were included. Dialysis membranes and clips were included in both sets of controls.

Phylogenomic and genomic characterisation

The draft genome sequences of *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85 were generated as previously described and are deposited under BioProject PRJNA1019763, except for the translated protein content for Sulfolobales sp. RB85, which was provided in Table S2 (Fernandes-Martins et al., 2024). The two draft genomes of the isolates, along with 19 type strains of the Sulfolobales order, and outgroup taxa (*Desulfurococcus amylolyticus*, *Desulfuroccocus mucosus*, *Thermogladius calderae* and *Thermosphaera aggregans*) were subjected to marker gene ($n = 30$) identification, alignment and concatenation using Markerfinder (https://github.com/faylward/markerfinder#markerfinder). The resultant alignment block was subjected to phylogenomic reconstruction using the software IQ-Tree (v.1.6.11) (Nguyen et al., 2015) with the le and gascuel (LG) model specification and 1000

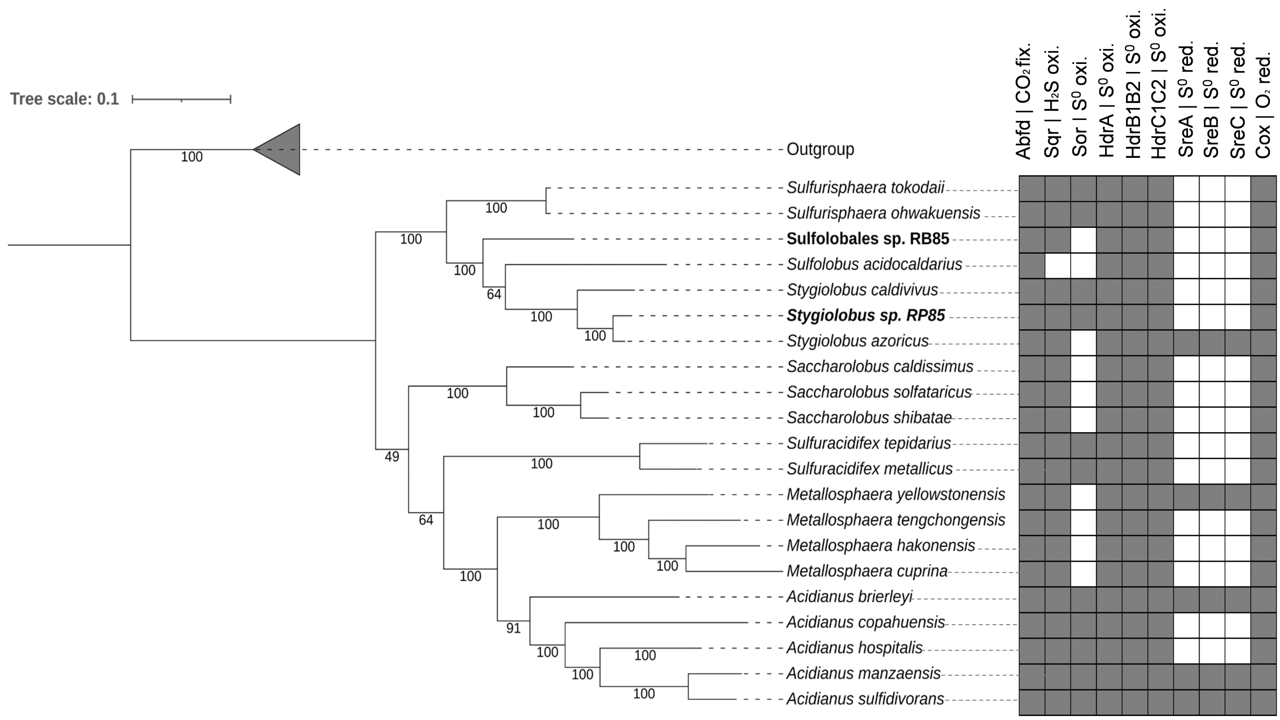

FIGURE 1 Phylogenomic reconstruction of cultivated Sulfolobales strains ($n = 19$) from previous studies and Sulfolobales isolates used in this study (Sulfolobales sp. RB85 and *Stygiolobus* sp. RP85). The Maximum-Likelihood phylogeny was constructed using an alignment of marker genes ($n = 30$) and the LG substitution model, adapted from (Fernandes-Martins et al., 2024). Numbers on edges indicate bootstrap values (out of 1000 replicates). All representative strains have the demonstrated ability to oxidise orthorhombic elemental sulfur (S_8^0), except for *Stygiolobus azoricus* and *Saccharolobus caldissimus* (references in Table S1). The presence of homologues of key sulfur-metabolising enzymes is mapped to each metagenome-assembled genome or genome (grey shade indicates presence). Abfd, 4-hydroxybutanoyl-CoA dehydratase; Cox, cytochrome *c* oxidase subunit I; HdrAB1B2C1C2, heterodisulfide reductase; SOR, sulfur oxidoreductase:reductase; SQR, sulfide:quinone oxidoreductase; SreABC, sulfur/polysulfide reductase.

'ultrafast' bootstrap replicates, as previously described (Fernandes-Martins et al., 2024).

The Basic Local Alignment Search Tool (BLASTp) (Boratyn et al., 2012) was used to identify homologues involved (or proposed to be) in the steps of dissimilatory sulfur metabolism, including: sulfide:quinone oxidoreductase (SQR); sulfur oxygenase:reductase (SOR); SDO; HDR, HdrAB1B2C1C2; sulfur/polysulfide reductase (SreABC); cytochrome *c* oxidase subunit I (Cox). Query sequences for use in BLASTp were homologues from the genomes of strains with demonstrated metabolic activity (i.e., *Acidianus ambivalens*, *Acidianus brierleyi* and *Metallosphaera prunae*). An *E*-value cutoff of $1.0e^{-50}$, an amino acid identity of >50%, and a coverage of >60% of the query sequence were used to identify homologues (Tables S1; Fernandes-Martins et al., 2024).

RESULTS AND DISCUSSION

Phylogenomic analyses and genomic characterisation of Sulfolobales strains

A phylogenomic reconstruction of *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85, along with cultivated Sulfolobales strains ($n = 19$), was constructed and annotated with experimental data compiled from previous studies that report whether the organism could oxidise S_8^0 (Figure 1). Similarly, the presence and absence of sulfur oxidoreductase (SOR) homologues was overlaid on the phylogeny, among other protein homologues involved in sulfur metabolism. Only 2 of the 21 Sulfolobales included in the phylogeny have not been experimentally shown to oxidise S_8^0: *Stygiolobus azoricus* and *Saccharolobus caldissimus*. *S. azoricus* was initially reported as a strict anaerobe that coupled H_2 oxidation with S_8^0 reduction (Segerer et al., 1991), although more recent genomic sequencing data revealed the presence of Cox protein homologues indicative of an ability to respire aerobically (Counts et al., 2021). In addition, recently isolated *Stygiolobus* strains were shown to aerobically oxidise S_8^0 (Fernandes-Martins et al., 2024; Sakai et al., 2022). Thus, it cannot be ruled out that *S. azoricus* can oxidise S_8^0. On the other hand, *S. caldissimus* is a facultatively anaerobic iron reducer that was experimentally shown not to oxidise S_8^0 when provided with O_2 (Sakai & Kurosawa, 2018).

The phylogenetic distribution of SOR, the most common enzyme attributed to S_8^0 oxidation in the Sulfolobales (Counts et al., 2021; Ferreira et al., 2022; Liu et al., 2021), among the 21 Sulfolobales genomes is patchy and does not fully overlap with experimental data indicating an ability to oxidise S_8^0. Of the 19 genomes from Sulfolobales that can oxidise S_8^0, 11 encoded homologues of SOR and these belonged to only four Sulfolobales genera (*Sulfurisphaera*, *Stygiolobus*, *Sulfuracidifex* and *Acidianus*) (Figure 1). The most well-characterised SOR is from *A. ambivalens* (Kletzin, 1989, 1992; Urich et al., 2004, 2006) and this shares 67.4% sequence identities with SOR from *Stygiolobus* sp. RP85. Importantly, the key catalytic residues including Cys^{31}, His^{86}, His^{90}, Cys^{101}, Cys^{104} and Glu^{114} in *A. ambivalens* SOR (Uniprot P29082) are conserved in *Stygiolobus* sp. RP85 SOR. Notably, genomes affiliated with the genus *Sulfodiicoccus* were not included in this analysis since members were reported to be inhibited by S_8^0 (Sakai & Kurosawa, 2017). Similarly, members of the *Sulfurococcus* genus were not included since partial or complete genomes are not available for these strains (Liu et al., 2021) (Figure 1). Nonetheless, these results are consistent with previous studies that have shown that nearly half of characterised Sulfolobales do not encode homologues of SOR (Counts et al., 2021; Liu et al., 2021).

The absence of SOR in Sulfolobales strains demonstrated to oxidise S_8^0 has prompted transcriptomic, comparative genomic and mutagenesis studies to identify alternative mechanisms (Ai et al., 2019; Auernik & Kelly, 2008; Jiang et al., 2014; Zeldes et al., 2019). These studies have identified a complement of protein-encoding genes that appear to be necessary for dissimilatory oxidative sulfur metabolism in Sulfolobales, with the presence/absence of SOR standing out among them. These studies also identified a potential role for SDO in S_8^0 oxidation. Homologues of this enzyme tend to be present in organisms with the ability to oxidise S_8^0 but that lack SOR, with only *Metallosphaera cuprina*, *Saccharolobus solfataricus*, *S. azoricus* and *Sulfolobus acidocaldarius* lacking homologues of both SOR and SDO (Table S1). To the extent that SDO may participate in S_8^0 oxidation, the observed distribution of SOR and SDO, including their near-ubiquitous distribution among certain Sulfolobales genera, suggests that they differentially contribute to the physiology and thus ecology of these organisms.

Growth and activity of *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85 with S_8^0

Both *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85 were grown autotrophically with 2% O_2 vol./vol. and with S_8^0 at concentrations of 0.16 g L^{-1} (5 mM if fully solubilised) or 1.6 g L^{-1} (50 mM if fully solubilised). For the SOR-encoding *Stygiolobus* sp. RP85, S_8^0 oxidation was coupled to growth (Figure 2A,B). Interestingly, the growth rate and S_8^0-oxidation activity were greater in cultures provided with 0.16 g L^{-1} S_8^0 than those provided with 1.6 g L^{-1} S_8^0. Cultures provided with 0.16 g L^{-1} S_8^0 had no observed lag phase and achieved a higher cell density ($3.8 \pm 0.7 \times 10^6$ cells mL^{-1}) and a higher SO_4^{2-} concentration (1.5 ± 0.03 mM produced) than those provided with 1.6 g L^{-1} S_8^0. In cultures provided with 1.6 g L^{-1} S_8^0, the lag phase ended between 24 and 48 h, and the cultures achieved lower cell densities ($1.1 \pm 0.02 \times 10^6$ cells mL^{-1}) and SO_4^{2-} concentrations (0.2 ± 0.06 mM). The metabolic coupling efficiency (i.e., cells produced per mole of product produced) calculated during log phase growth in cultures provided with 0.16 g L^{-1} S_8^0 was 0.09 cells $pmol^{-1}$ SO_4^{2-} and in cultures provided with 1.6 g L^{-1} S_8^0 was 0.07 cells $pmol^{-1}$ SO_4^{2-}.

The same pattern of activity was observed for the non-SOR encoding Sulfolobales sp. RB85 strain, where the concentration of S_8^0 influenced growth and activity (Figure 2C,D). Specifically, no lag phase was observed in cultures provided with 0.16 g L^{-1} S_8^0. Similar to *Stygiolobus* sp. RP85, cultures of Sulfolobales sp. RB85 achieved higher cell densities ($3.6 \pm 0.9 \times 10^6$ cells mL^{-1}) and SO_4^{2-} concentrations (1.7 ± 0.28 mM) than those provided with 1.6 g L^{-1} S_8^0 ($1.1 \pm 0.05 \times 10^6$ cells mL^{-1} and 0.7 ± 0.12 mM, respectively). The calculated metabolic coupling efficiency during log phase growth in cultures provided with 0.16 g L^{-1} S_8^0 was 0.05 cells $pmol^{-1}$ SO_4^{2-} and 0.03 cells $pmol^{-1}$ SO_4^{2-} in cultures provided with 1.6 g L^{-1} S_8^0. Unfortunately, the pathway of S_8^0 oxidation in Sulfolobales that do not encode SOR has yet to be determined. However, recent investigations suggest that SDO, NSR and/or the HDR complex could be responsible for S_8^0 oxidation in these strains (Colman et al., 2022; Jiang et al., 2014; Quatrini et al., 2009; Rohwerder & Sand, 2003; Wang et al., 2014).

Interestingly, for both *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85, an increase in the concentration of S_8^0 inhibited growth and activity, as evidenced by a longer lag phase, slower growth rate, slower SO_4^{2-} production rate and lower metabolic coupling efficiencies (Figure 2). Previous studies have shown that S_8^0 can negatively influence the growth of yeast and bacteria (Cetkauskaite et al., 2004; Chen & Lin, 2004; Libenson et al., 1953; Wang et al., 2022). While the mechanisms of toxicity are not well known, one of the prevailing hypotheses is that S_8^0, which is uncharged and is thought to freely diffuse into the cell (Boyd & Druschel, 2013), can generate oxidative stress once in the cytoplasm (Libenson et al., 1953; Wang et al., 2022). In this role, S_8^0 is thought to oxidise thiol (-SH) compounds (that can have antioxidant properties), leaving the cells unable to balance the redox state of the cytoplasm (Libenson et al., 1953; Wang et al., 2022). This would be particularly detrimental for a

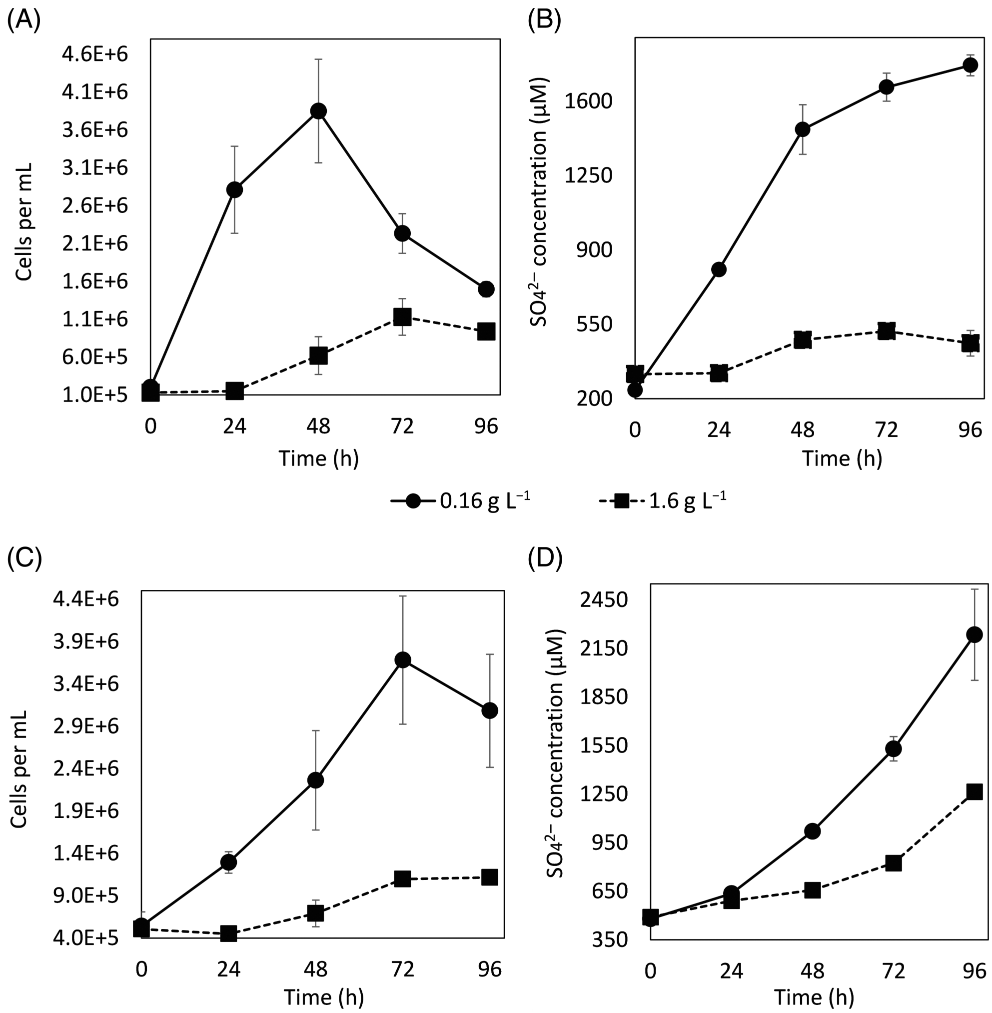

FIGURE 2 Production of cells and sulfate (SO_4^{2-}) in cultures of *Stygiolobus* sp. RP85 (A, B) and Sulfolobales sp. RB85 (C, D) provided with different starting amounts of orthorhombic elemental sulfur (S_8^0). Oxygen (2% headspace vol./vol.) was the electron acceptor and carbon dioxide (20% vol./vol.) was the carbon source. Cultures were incubated on a shaker (50 rotations per min) at 80°C.

thermoacidophile considering that the combination of acidic pH and high temperature imparts significant oxidative stress (Maaty et al., 2009). It is thus possible that the higher amount of S_8^0 used herein imposed additional oxidative stress on cells, resulting in slower growth rates and lower metabolic coupling efficiencies than cultures grown with less S_8^0. Importantly, however, for this to be true, an active mechanism of promoting S_8^0 solubilisation must be taking place, as discussed below. All further experiments were conducted using the lower concentration of S_8^0 (0.16 g L^{-1}).

While the kinetics of growth and the metabolic coupling efficiencies were similar in cultures of *Stygiolobus* sp. RP85 and Sulfolobales sp. RB85, differences were noted in the association of cells with S_8^0 mineral regardless of the amount of S_8^0 provided. Whereas Sulfolobales sp. RB85 cells were regularly observed adhering and forming biofilms with S_8^0 mineral (Figure S1), as previously reported for Sulfolobales (Liu et al., 2018; Weiss, 1973; Zhang et al., 2019; Zhang, Neu, Bellenberg, et al., 2015; Zhang, Neu, Zhang, et al., 2015), *Stygiolobus* sp. RP85 cells were rarely observed in association with S_8^0. Interestingly, while both strains encoded homologues of the proposed key transcription regulators of biofilm formation in Sulfolobales (i.e., leucine responsive regulator of *Sulfolobus*, Lrs14) (Koerdt et al., 2011; Orell et al., 2013), only Sulfolobales sp. RB85 encoded homologues of the two main components required for the assembly of the archaeal type IV adhesive pili (Aap), AapE and AppF, which are suggested to encode for an ATPase and a transmembrane protein that anchors the pilus to the cell membrane, respectively (Henche et al., 2012; Pohlschroder & Esquivel, 2015) (Table S1). Since the solubility of S_8^0 is low (<500 nM at 80°C; Kamyshny, 2009), and oxidation of S_8^0 is thought to occur inside of the cell, this suggested differences in the mechanisms of accessing S_8^0 to support the energy metabolism of the two strains.

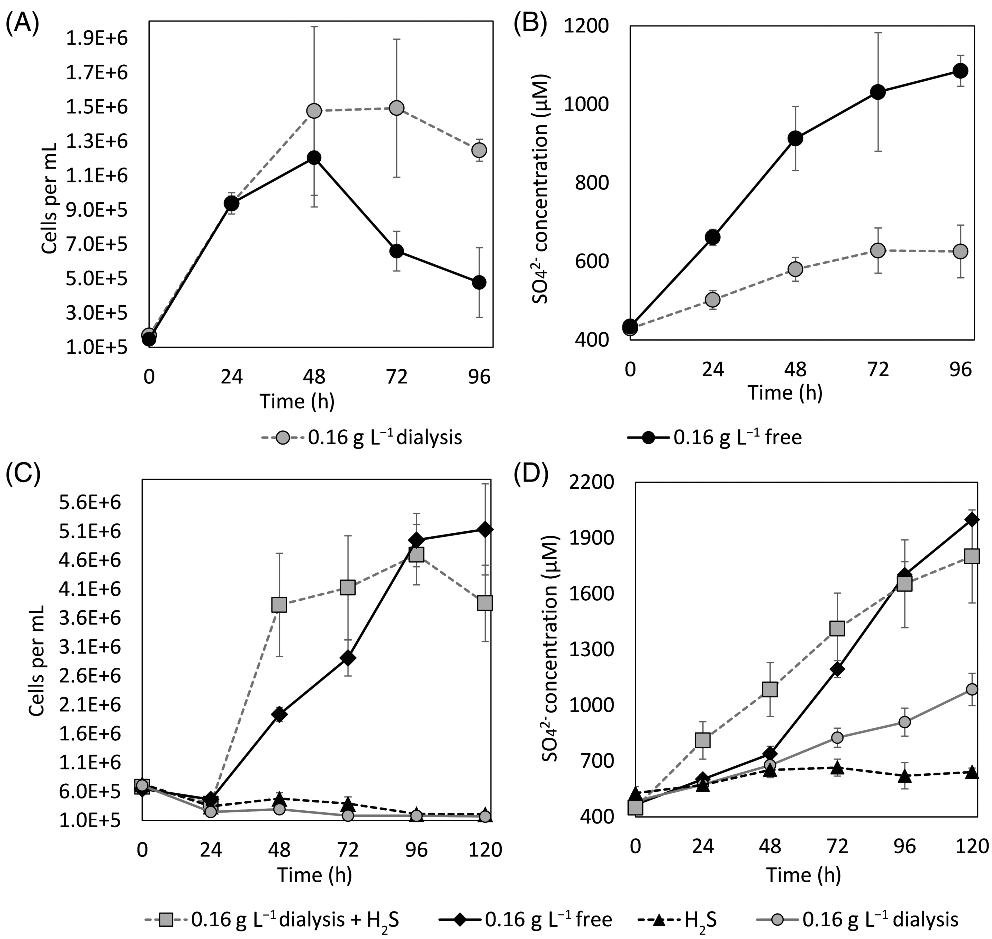

FIGURE 3 Production of cells and sulfate (SO_4^{2-}) in cultures of *Stygiolobus* sp. RP85 (A, B) and in cultures of Sulfolobales sp. RB85 (C, D). Oxygen (2% headspace vol./vol.) was the electron acceptor and carbon dioxide (20% vol./vol.) was the carbon source. Cultures were incubated on a shaker (50 rotations per min) at 80°C. *Stygiolobus* sp. RP85 cultures were provided with 0.16 g L^{-1} orthorhombic elemental sulfur (S_8^0) that was either sequestered in dialysis membranes with 3.5 kDa pore sizes or that was free in solution. Sulfolobales sp. RB85 cultures were provided with 15 μM hydrogen sulfide (H_2S) only, 0.16 g L^{-1} elemental sulfur (S_8^0) only, or 15 μM H_2S and 0.16 g L^{-1} elemental sulfur (S_8^0) as electron donors, as indicated. S_8^0 was either sequestered in dialysis membranes with 3.5 kDa pore sizes or was free in solution, as indicated. Where indicated, cultures were amended with 15 μM H_2S (as Na_2S) every 24 h.

Requirement for direct contact for S_8^0 oxidation

The qualitative observation of a difference in the association of non-SOR-encoding Sulfolobales sp. RB85 and SOR-encoding *Stygiolobus* sp. RP85 with S_8^0 during growth, combined with differences in the encoded proteins putatively involved in promoting biofilm and pili formation, prompted quantitative experiments to determine the requirement of access to the mineral to catalyse its oxidation. This was achieved by sequestering bulk S_8^0 (0.16 g L^{-1}) in dialysis membranes with pore sizes of 3.5 kDa. The SOR-encoding *Stygiolobus* sp. RP85 grew when physical access to bulk S_8^0 was restricted (Figure 3A). Interestingly, although there was no difference in the kinetics of growth during the first 24 h of incubation in cultures provided access to S_8^0 or when S_8^0 was physically sequestered in dialysis membranes, cell viability was higher in the latter condition, with nearly twice the number of cells remaining at the end of 96 h incubation ($4.7 \pm 0.2 \times 10^5$ vs. $1.2 \pm 0.06 \times 10^6$ cells mL^{-1}). Moreover, production of SO_4^{2-} was much higher in cultures provided with direct access to S_8^0 for the duration of the experiment (Figure 3B). This is reflected in metabolic coupling efficiencies of 0.07 and 0.22 cells $pmol^{-1}$ SO_4^{2-} produced in cultures provided with direct access to S_8^0 versus those where direct access was restricted, respectively. This may point to a role for the dialysis membrane in limiting the flux of S_8^0 nanoparticles, which are known to rapidly aggregate due to their hydrophobicity once they are formed and solubilised (Boyd & Druschel, 2013). In this role, the limited flux of nanoparticles may have minimised oxidative stress, increased metabolic coupling efficiencies and minimised cell death.

For non-SOR-encoding Sulfolobales sp. RB85, S_8^0 oxidation (as assessed via SO_4^{2-} production) was only observed in cultures when cells were allowed direct access to S_8^0, resulting in the production of cells (Figure 3C). Interestingly, while cultures of Sulfolobales sp. RB85 did not grow when S_8^0 was sequestered in dialysis membranes, they generated ~600 μM SO_4^{2-} over the 120 h incubation period (Figure 3D). It is possible that the production of SO_4^{2-} was due to abiotic hydrolysis of S_8^0 ($4S + 4H_2O \rightarrow 3H_2S + H_2SO_4$; Ellis & Giggenbach, 1971), which can generate SO_4^{2-} and (in the presence of O_2) sulfur intermediates such as $S_2O_3^{2-}$ and $S_4O_6^{2-}$ (Xu et al., 1998), which could be soluble electron donors supporting cell metabolism. However, S_8^0 hydrolysis occurs at temperatures above the melting point of S_8^0 (~114.5°C; Steudel, 2003) and is of neglectable importance at temperatures <105°C (Figure S2) (Xu et al., 1998). As such, S_8^0 hydrolysis cannot account for the ~600 μM SO_4^{2-} generated at 80°C. Further, if S_8^0 hydrolysis was readily occurring and intermediates like $S_2O_3^{2-}$ and $S_4O_6^{2-}$ were being generated abiotically by O_2, then Sulfolobales sp. RB85 would not need direct contact with S_8^0 to grow. Instead, the observation that Sulfolobales sp. RB85 does appear to need direct access to S_8^0 to grow but not to metabolise S_8^0 is interpreted to reflect the solubility of S_8^0, which, while low (<500 nM at 80°C; Kamyshny, 2009), is not insoluble. In this model, limited S_8^0 diffused outside the membrane, but the amount/flux was not sufficient to support the production of cells. Consistent with this interpretation, the amount of SO_4^{2-} produced when cells were not provided direct contact with S_8^0 was ~33% of when direct contact was permitted (~1550 μM SO_4^{2-} produced after 120 h incubation).

Collectively, the microscopic observation that SOR-encoding *Stygiolobus* sp. RP85 does not associate with the surface of S_8^0 and does not require direct access to the mineral during S_8^0-dependent growth and that non-SOR-encoding Sulfolobales sp. RB85 strain does associate with the surface of S_8^0 and requires direct access to the mineral during S_8^0-dependent growth points to different mechanisms of acquiring S_8^0 between the two strains. In other words, Sulfolobales that disproportionate S_8^0 via SOR appear to indirectly oxidise S_8^0 and couple this to growth, while non-SOR-encoding Sulfolobales require direct contact with the S_8^0 mineral to oxidise it and couple this to growth. In support of this hypothesis, the SOR-encoding *Acidianus* strain DS80 was previously shown to grow via indirect contact while disproportioning or reducing S_8^0, presumably due to the role of H_2S in solubilising S_8^0 as S_x^{2-} that then disproportionated to soluble S_8^0 rings that ultimately aggregated as S_8^0 nanoparticles (Amenabar & Boyd, 2018). However, when cells were grown under S_8^0 oxidising conditions with Fe(III) ions as electron acceptors, direct contact was required to oxidise the mineral, presumably due to Fe(III) ions spontaneously oxidising H_2S, thereby preventing indirect S_8^0 solubilisation (Amenabar & Boyd, 2018; Fernandes-Martins et al., 2024).

H_2S solubilises S_8^0 permitting indirect disproportionation/oxidation

SOR disproportionates S_8^0 to generate H_2S/HS^-, SO_3^{2-} and $S_2O_3^-$ (Urich, 2005; Urich et al., 2004, 2006; Veith et al., 2011). While the actual substrate for SOR has yet to be fully resolved, it has been suggested that S_x^{2-} is the actual substrate. We were unable to detect SO_3^{2-} and $S_2O_3^-$ intermediates in culture medium in our studies, which is likely due to SOR being intracellular and these products also being generated in the cytoplasm. Further, both SO_3^{2-} and $S_2O_3^-$ are unstable at acidic pH (<4.0) and in the presence of O_2 (Colman et al., 2020; Nordstrom et al., 2005; Sims et al., 2023). On the other hand, H_2S/HS^- (pKa = 6.4 at 80°C (Amend & Shock, 2001)) is likely to be protonated and uncharged/volatile at the cytoplasmic pH of ~5.6 measured for *S. acidocaldarius* (Sulfolobales) (Lübben & Schäfer, 1989) and thus could freely diffuse out of the cell (Urschel et al., 2015) once it is produced and prior to its consumption via the activity of SQR (Fernandes-Martins et al., 2024).

Despite numerous attempts to measure H_2S/HS^- during the S_8^0-dependent growth of *Stygiolobus* sp. RP85, it was never detected (detection limit of 2 μM) in the spent medium of cultures. Nonetheless, in cultures of *Stygiolobus* sp. RP85, which did not require physical access to the S_8^0 mineral to oxidise it, it was hypothesised that a nominal amount of H_2S generated by SOR in the cytoplasm diffused out of the cell and reacted with the S_8^0 inside the dialysis membranes, solubilising it as S_x^{2-}. However, at the acidic pH of the growth medium (3.0–4.0, pending strain), S_x^{2-} is unstable and disproportionates to reform H_2S and S_8^0 rings that rapidly as S_8^0 nanoparticles. The S_8^0 nanoparticles are small during their initial formation (<20 nm within 2 min of S_x^{2-} acidification) (Boyd & Druschel, 2013), and because of that, can diffuse out of the dialysis membranes. As a consequence, growth and activity of SOR-encoding *Stygiolobus* sp. RP85 can be supported, whereas that does not occur for the non-SOR-encoding Sulfolobales sp. RB85 since these reactions do not take place.

If the model of S_8^0 solubilisation that is proposed here is correct, then the addition of small amounts of H_2S should promote the growth of the non-SOR-encoding Sulfolobales sp. RB85. To test this hypothesis, the non-SOR-encoding Sulfolobales sp. RB85 strain was grown with S_8^0 sequestered in dialysis membranes (3.5 kDa pore size), and culture vials were amended with 15 μM H_2S (added as Na_2S) every 24 h. This concentration of H_2S does not support the growth of Sulfolobales sp. RB85 (Figures S3 and

3C). In cultures with sequestered S_8^0 amended with H_2S, S_8^0-dependent growth was observed but was not observed in cultures not amended with H_2S (Figure 3C). In cultures with direct access to S_8^0 prevented, amendment with H_2S increased the rates of cell and SO_4^{2-} production (Figure 3C,D), presumably because the bioavailability of S_8^0 had increased through the series of chemical reactions described above. However, both conditions achieved the same cell density, ~5.1 ± 0.1 × 10^6 cells mL^{-1} by the end of the log phase at 96 h. The production of SO_4^{2-} corresponded to cell growth, and both conditions achieved similar final concentrations of ~2 mM.

CONCLUSIONS

Sulfolobales are facultative anaerobic thermoacidophiles that tend to inhabit sulfur-rich hot springs globally (Huber & Prangishvili, 2006). Despite being remarked as organisms that oxidise S_8^0 and contribute to the formation of acidic hot spring ecosystems (Brock et al., 1972; Colman et al., 2018; Mosser et al., 1973; Shivvers & Brock, 1973), fundamental gaps in our understanding of S_8^0 oxidation in these organisms remain, including disparities in the distribution of SOR. The present study aimed to begin to fill this gap by identifying phenotypic and ecological differences in SOR-(*Stygiolobus* sp. RP85) and non-SOR-(Sulfolobales sp. RB85) encoding members. When grown with direct access to S_8^0, both strains exhibited similar metabolic coupling efficiencies. However, SOR-encoding *Stygiolobus* sp. RP85 did not require direct contact with S_8^0 to oxidise the mineral, while the non-SOR-encoding Sulfolobales sp. RB85 required direct contact. This was attributed to SOR generating H_2S as a product of S_8^0 disproportionation that could diffuse out of the cell and react with sequestered bulk S_8^0. The nucleophilic attack of S_8^0 by H_2S releases S_x^2, which at acidic pH disproportionates to reform H_2S and S_8^0 rings that rapidly aggregate as nanoparticulate S_8^0. It is suggested that the latter supports the S_8^0-dependent growth of SOR-encoding strains, since S_8^0 nanoparticles are small and hydrophobic in nature allowing them to diffuse across the cell membrane (Boyd & Druschel, 2013). The requirement for direct contact with the mineral in the non-SOR-encoding Sulfolobales sp. RB85 could be overcome by the addition of small amounts of H_2S through artificial initiation of the aforementioned reactions that increase the solubility of S_8^0. Thus, non-SOR-encoding Sulfolobales inhabiting H_2S-rich springs likely do not require direct contact with S_8^0 to grow via its oxidation. Importantly, both strains appeared to metabolise the intermediate species of sulfur (i.e., S_8^0 nanoparticles) better than bulk S_8^0.

The collective observations herein highlight the need for additional investigation of S_8^0 oxidation in non-SOR-encoding Sulfolobales as well as an investigation into the potential impacts on the distribution and ecology of SOR- versus non-SOR-encoding Sulfolobales across hot springs and within-spring niche partitioning. For example, it is reasonable that the distribution and abundance of Sulfolobales in planktonic versus sediment communities can be influenced based on the requirement for direct contact (non-SOR-encoding strains) or not (SOR-encoding strains). To this end, this phenotypic difference could allow for the S_8^0 oxidation niche to be partitioned to minimise overlap and enable the co-existence of SOR- and non-SOR-encoding strains, such as is observed in acidic hot springs in YNP (Colman et al., 2021, 2022). This relationship may become less pronounced in hot springs that have both S_8^0 and H_2S, where the feedback between these chemical species can increase the bioavailability of S_8^0 and decrease the need to directly associate with the mineral. At the same time, springs with both S_8^0 and H_2S may increase the concentration of solubilised S_8^0 to the point that it induces oxidative stress, thereby decreasing the fitness of Sulfolobales. Such hypotheses should be tested in future metagenomic/metatranscriptomic analyses of planktonic and sediment-associated communities in acid high-temperature hot springs dominated by Sulfolobales.

AUTHOR CONTRIBUTIONS

Maria C. Fernandes-Martins: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; project administration; formal analysis; data curation; supervision. **Carli Springer:** Investigation; writing – review and editing. **Daniel R. Colman:** Funding acquisition; writing – review and editing; conceptualization. **Eric S. Boyd:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; methodology; validation; project administration; supervision; resources.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation (EAR-1820658) to Daniel R. Colman and Eric S. Boyd. Maria C. Fernandes-Martins is grateful to the family of Beverly Ferguson and the Molecular Bioscience Program (MBSP) for support of her graduate studies at Montana State University (MSU).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All sequencing data generated previously and used in this study are available under NCBI BioProject accession number PRJNA1019763. All supplemental data can be found under Figshare Project (https://figshare.

com/projects/Acquisition_of_elemental_sulfur_by_sulfur-oxidizing_Sulfolobales/216850).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Fernandes-Martins, M.C., Springer, C., Colman, D.R. & Boyd, E.S. (2024) Acquisition of elemental sulfur by sulfur-oxidising Sulfolobales. *Environmental Microbiology*, 26(9), e16691. Available from: https://doi.org/10.1111/1462-2920.16691