Thiosulfate oxidation by *Thiomicrospira thermophila*: metabolic flexibility in response to ambient geochemistry

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Summary

Previous studies of the stoichiometry of thiosulfate oxidation by colorless sulfur bacteria have failed to demonstrate mass balance of sulfur, indicating that unidentified oxidized products must be present. Here the reaction stoichiometry and kinetics under variable pH conditions during the growth of *Thiomicrospira thermophila* strain EPR85, isolated from diffuse hydrothermal fluids at the East Pacific Rise, is presented. At pH 8.0, thiosulfate was stoichiometrically converted to sulfate. At lower pH, the products of thiosulfate oxidation were extracellular elemental sulfur and sulfate. We were able to replicate previous experiments and identify the missing sulfur as tetrathionate, consistent with previous reports of the activity of thiosulfate dehydrogenase. Tetrathionate was formed under slightly acidic conditions. Genomic DNA from *T. thermophila* strain EPR85 contains genes homologous to those in the Sox pathway (soxAXYZBCDL) as well as rhodanese and thiosulfate dehydrogenase. No other sulfur oxidizing bacteria containing sox(CD)2 genes have been reported to produce extracellular elemental sulfur. If the apparent modified Sox pathway we observed in *T. thermophila* is present in marine *Thiobacillus* and *Thiomicrospira* species, production of extracellular elemental sulfur may be biogeochemically important in marine sulfur cycling.

Introduction

Sulfide oxidation is a critical step in the biogeochemical sulfur cycle and occurs via several complex reaction pathways, some of which are biologically mediated (Jørgensen, 1990; Jørgensen and Bak, 1991; Jørgensen and Nelson, 2004). The stepwise oxidation of sulfide to sulfate can occur via several reactive chemical species of intermediate oxidation state. These intermediates, including elemental sulfur, thiosulfate and polythionates, can be biogeochemically important even when their steady-state concentrations are low (sub-µM) due to rapid, hidden (or "cryptic") recycling (Thamdrup et al., 1994; Canfield et al., 2010). Elemental sulfur and thiosulfate can be oxidized, reduced or disproportionated, resulting in multiple pathways and redox intermediates of the sulfur cycle (Troelsøn and Jørgensen, 1982; Jørgensen and Nelson, 2004). However, pathways involving intermediate oxidation states of sulfur have been difficult to quantify, with many complex processes taking place simultaneously in the presence of several available oxidants and reductants (Jørgensen, 1990; Jørgensen and Bak, 1991; Flynn et al., 2014).

Two known enzymatic pathways mediate thiosulfate oxidation: (i) the $S_4$ Intermediate pathway, which involves production and consumption of polythionates (Kelly et al., 1997), and (ii) the multienzyme Sox pathway (Sox = sulfide oxidation) (Friedrich et al., 2001). The $S_4$ Intermediate pathway appears to be restricted to obligate chemolithotrophic strains within the genus *Thiobacillus* and involves the production of tetrathionate from thiosulfate via tetrathionate synthase (Lu and Kelly, 1988; Meulenberg et al., 1992). This process is likely coupled to enzymes that subsequently split tetrathionate to thiosulfate, sulfate or elemental sulfur (Lu and Kelly, 1988; Pronk et al., 1990; Meulenberg et al., 1992).
The Sox pathway is versatile and has been shown to be active in physiologically diverse organisms, including green sulfur bacteria, purple sulfur bacteria and colorless sulfur bacteria (Friedrich et al., 2001; Sauvé et al., 2007; Welte et al., 2009). Complete thiosulfate oxidation via the Sox pathway results in sulfate as the sole product; however, incomplete pathways in which Sox(CD)2 is absent can result in accumulation of elemental sulfur, either intracellularly (Grimm et al., 2008) or extracellularly (Van Gemerden, 1984; Gregersen et al., 2011). The colorless sulfur bacterium Thiomicrospira crunogena is known to use the Sox pathway to perform thiosulfate oxidation to sulfite and elemental sulfur (Scott et al., 2006; Sievert et al., 2008), but also produces tetrathionate under acidic conditions (Javor et al., 1990). The environmental conditions under which these various oxidation states are produced and/or consumed are poorly constrained for Thiomicrospira thermophila.

Here, we report new genomic and physiological evidence for a modified Sox pathway functioning in T. thermophila; a sulfur-oxidizing gammaproteobacterium found in marine settings and identified as an obligate aerobic chemolithomixotroph that can oxidize sulfide, thiosulfate and elemental sulfur (Kuenen and Tuovinen, 1981; Takai et al., 2004). Thiobacillus and Thiomicrospira spp. are widespread in marine sediments (Kuenen et al., 1985; Robertson et al., 1992; Brinkhoff and Muyzer, 1997) and in marine geothermal environments (Sievert et al., 2000, 2008), particularly in diffuse flow hydrothermal vents (Brinkhoff and Muyzer, 1997; Huber et al., 2003; Brazelton and Baross, 2010). We investigated thiosulfate oxidation by T. thermophila strain EPR85, isolated from a seafloor hydrothermal diffuse flow vent on the East Pacific Rise at 9° N. T. thermophila is closely related to the well-studied strain T. crunogena XCL-2, which employs a pH-dependent Sox pathway for thiosulfate oxidation (Scott et al., 2006; Sievert et al., 2008) that has been shown to produce (at pH 6.3) elemental sulfur as well as consume it (at pH 8.5) (Javor et al., 1990). However, previous examination of T. crunogena could not account for all of the sulfur in the system, suggesting the production of unidentified sulfur products under all pH conditions studied (6.3–8.5). Problems with sulfur mass balance were also present in studies of two closely related strains of Thiobacillus that oxidize sulfide to elemental sulfur and sulfate (Stefess et al., 1996). In contrast, studies of T. thermophila have indicated that thiosulfate oxidation produced equal concentrations of elemental sulfur and sulfate, accounting for all sulfur in the system (Takai et al., 2004). These inconsistent results suggest that thiosulfate oxidation by the Sox pathway may be more complex than previously thought and may result in the production of as-yet unidentified sulfur products.

In this study we have identified the sulfur oxidation reactions mediated by T. thermophila in the context of specific sulfur cycling enzymes found in its genome. Under various culture conditions we replicated both the sulfur balance and imbalances in the studies discussed above, and identified the missing sulfur product as tetrathionate. We report data on the stoichiometry and kinetics of these reactions (Table 1) and provide a genetic context for the mechanisms potentially responsible for thiosulfate oxidation to multiple products in T. thermophila EPR85.

**Results**

**Stoichiometry and mass balance**

When buffered at the high end of its pH tolerance (8.0), T. thermophila mediates complete oxidation of thiosulfate to sulfate with no significant buildup of elemental sulfur in the media (Fig. 1a). Maximum accumulation of moderate amounts of free elemental sulfur (S8) in the media (<150 μM) and Tris-extractable solid elemental (polymeric) sulfur (<250 μM) (Supporting Information Table S1) is concurrent with the period of fastest thiosulfate consumption. Dissolved oxygen was not completely consumed during growth (Supporting Information Table S1).

In contrast, growth at pH buffered at the low end of tolerance (pH ~ 5.5) results in accumulation of free elemental sulfur and sulfate in the media, consistent with incomplete thiosulfate oxidation to sulfite. Experimental data (Fig. 1b) suggest that thiosulfate oxidation and elemental sulfur oxidation occur simultaneously, as described by the combination of the chemical reactions (ib) and (ii) from Table 1. Over the course of the whole 8 h experiment, total sulfur, as measured by the sum of known reaction products ([2 * [S2O3] + [SO4] + [S]), decreased from approximately 23 to 15 mM (Fig. 1b). Abiotic controls at low pH (3.5–5.6), in media buffered with both citrate buffer and Bis-Tris buffer, show no loss of thiosulfate and concentrations of possible products such as tetrathionate, sulfate, or elemental sulfur were negligible for up to 73 h (Supporting Information Table S2).

Growth in unbuffered media results in a complex combination of reactions that depends on pH. As elemental sulfur accumulates in the media, the stoichiometry is consistent with a combination of incomplete thiosulfate oxidation to sulfate [reactions (ib) and (ii) from Table 1]. During this initial phase, the rate of elemental sulfur oxidation [reaction (ii)] is less than elemental sulfur production [reaction (i)], causing an accumulation of elemental sulfur in the media and a decrease in pH from 7.5 to approximately 6. However, a pronounced change in reaction stoichiometry occurs with the onset of elemental sulfur consumption (t = 8 h, Fig. 1c), which corresponds to a threshold pH of 6. Here, total sulfur, as measured by the sum of known reaction products ([2 * [S2O3] + [SO4] + [S]),
Table 1. Chemical reactions observed in thiosulfate oxidation by T. thermophila.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Net chemical reactions</th>
<th>ΔG (kJ/mole rxn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) $S_2O_3^{2-} \rightarrow SO_3^{2-} + E \cdot S$</td>
<td>$S_2O_3^{2-} + rhd\cdot S$ $\rightarrow SO_3^{2-} + rhd \cdot S \cdot S$</td>
<td>36.6</td>
</tr>
<tr>
<td>(b) $S_2O_3^{2-} + 1/2O_2 \rightarrow SO_4^{2-}$</td>
<td>$S_2O_3^{2-} + SoxYZ$ $\rightarrow SoxYZ \cdot S \cdot S$ $O_3^{2-} + 2e^{-}$</td>
<td>-229.6</td>
</tr>
<tr>
<td>(c) $S_x^{-} \rightarrow S_{x-1} + S^{2-}$</td>
<td>$S_x^{-}$ $\rightarrow abS$ $S_{x-1} + S^{2-}$</td>
<td></td>
</tr>
<tr>
<td>(d) $S + H_2O + 3/2O_2 \rightarrow SO_4^{2-} + 2H^+$</td>
<td>$SO_4^{2-} + SoxXY$ $\rightarrow SoxXY \cdot S \cdot SO_3^{2-} + 2e^{-}$</td>
<td>-266.2</td>
</tr>
<tr>
<td>(iv) $2S_2O_3^{2-} + 2H^+ + 1/2O_2 \rightarrow SO_4^{2-} + H_2O$</td>
<td>$2S_2O_3^{2-} + Sox\cdot S$ $\rightarrow SO_4^{2-} + Sox\cdot SO_3^{2-} + 2e^{-}$</td>
<td>-242.5</td>
</tr>
</tbody>
</table>

Inferred enzymes involved in thiosulfate and elemental sulfur oxidation for this study include rhodanese (rhd S), SoxAX, SoxYZ, SoxB, Sox(CD)2 and thiosulfate dehydrogenase (tds) as indicated over each reaction. Free energy for the net chemical reaction is given in kJ/mole of reaction, with negative values indicating spontaneous reaction in the direction written.

decreased from approximately 11 to 6 mM over 6 h (Fig. 1c), suggesting production of an additional as-of-yet unaccounted for sulfur product. On the contrary, abiotic controls in unbuffered seawater at pH 6.0–7.9 indicate no change in thiosulfate or sulfate concentrations over the course of 144 h at both 35°C and 4°C (Supporting Information Table S2).

To address the issue of a missing sulfur product(s) in both buffered and unbuffered media, we looked for other sulfur phases that could plausibly be present, including Tris-extractable solid elemental (polymeric S) sulfur, sulfide, polysulfides (here referring to the charged dissolved species $S_x^{2-}$), and polythionates. Only minimal concentrations (<300 μM) of Tris-extractable solid polymeric sulfur were present and polysulfides were not detected. Sulfide concentrations never exceeded 20 μM (Supporting Information Table S1), with the maximum values coinciding with a peak in elemental sulfur concentration. However, polythionates, primarily in the form of tetrathion ate ($S_4O_6^{2-}$), were detected by cyanolysis at concentrations up to 1 mM, with continuous production taking place after pH stopped decreasing (Fig. 2a). The abundance of tetrathionate is sufficient to account for the apparent missing sulfur (5.5 mM total) in this experiment (Fig. 2a) and tetrathionate concentrations never decreased under any experimental conditions examined. Thus, once pH drops below 5.7, metastable tetrathionate is present, elemental sulfur consumption begins, and competition for thiosulfate takes place between reactions (i) and (iv). Similarly, production of tetrathionate occurs in experiments buffered at low pH (5.5) and accounts for the apparent missing sulfur (7.7 mM total) in those experiments (Fig. 2b).

Physiology and growth rate

Specific growth rate of T. thermophila correlates with pH throughout our experiments. At pH 8.0, where thiosulfate is oxidized completely to sulfate, the maximal specific growth rate of 0.49 h⁻¹ is attained (Fig. 3). Specific growth rate decreases with pH, corresponding to a change in reaction stoichiometry (Fig. 3). In unbuffered media, the progression of reaction (ii) (Table 1) resulted in a decrease in pH due to proton production. In every unbuffered experiment, pH eventually reaches a constant value during the thiosulfate oxidation phase of growth, which is coincident with the proton-consuming onset of tetrathionate production [reaction (iv), Table 1]. This growth phase is characterized by the slowest rates observed (Fig. 3, open symbols).

Sequence analysis of T. thermophila draft genome

The genome (estimated length of 2.65 mbp) was partially assembled into 40 contigs, with the largest 20 accounting for 97.6% of the bases of the scaffold assembly. The G + C content of the assembly is 50.3%. For comparison, the T. crunogena XCL-2 genome has a total length of 2.43 mbp with a G + C content of 43% (Scott et al., 2006). The lengths of the largest 19 contigs in T. thermophila are
indicated in Supporting Information Fig. S1 and are referred to here as nodes, numbered for reference. Sulfur oxidation genes of interest are annotated (Supporting Information Fig. S1).

The genome of *T. thermophila* has homologues for sox-AXZYBCDL, all of which are necessary for the Sox system to completely oxidize thiosulfate to sulfate. However, in contrast to the well-studied *Paracoccus pantotrophus* (Friedrich et al., 2001), which has all Sox genes clustered in the same region of the genome, *T. thermophila* has homologues of soxAXYZ in one cluster and soxB, soxL clustered in another.

**Fig. 1.** Time series data for (A) thiosulfate oxidation at high pH (8.0) buffered with Tris; (B) thiosulfate oxidation at low pH (5.5) buffered with Bis-Tris and (C) thiosulfate oxidation in unbuffered media. Error bars based on triplicate experiments are shown for all analyses.

**Fig. 2.** Stoichiometry of growth experiments including polythionate concentrations determined with the cyanolysis method. (A) Growth in unbuffered media follows reactions 1 and 2 until pH drops below 5.5, only below which tetrathionate production (reaction 4) begins. pH was adjusted back to 7.5 at 6 hrs (arrow), resulting in a return to reactions (i) and (ii). (B) Growth at low pH (5.5; Bis-Tris buffer) occurs with simultaneous production of elemental sulfur, sulfate and tetrathionate via reactions (i), (ii) and (iv) (Table 1). Error bars based on triplicate experiments are shown for all analyses.
buffer data are shown in green and (thiosulfate is completely oxidized to sulfate in Tris buffer (pH 8.0) will be mediated. Highest growth rates are observed when media. Start pH determines which thiosulfate-consuming reaction formation Fig. S1). Solid elemental sulfur to sulfate supports intermediate growth rates (open circles). Thiosulfate oxidation to elemental sulfur and sulfate with simultaneous oxidation of elemental sulfur to sulfate supports intermediate growth rates (solid diamonds). Specific growth rates in Bis-Tris buffer (pH 5.5) are shown in blue; unbuffered media data are shown in black; citrate buffer data are shown in green.

and sox(CD)2 in three separate regions (Supporting Information Fig. S1). T. crunogena shares this pattern of arrangement (Sievert et al., 2008) and the homologous Sox genes in T. thermophila are very closely related to those in T. crunogena XCL-2 (Table 2; Scott et al., 2006).

Also like T. crunogena, T. thermophila has two homologues of rhodanese, a thiosulfate sulfurtransferase specific to thiosulfate as a substrate that cleaves the sulfite moiety and transfers the outer sulfur to an acceptor substrate such as cyanide, lipoic acid or thioredoxin (Smith and Lascelles, 1966; Nandi et al., 2000). Thus, rhodanese could be an alternative enzyme scavenging thiosulfate in T. thermophila. The catalytic site in rhodanese binds to both the inner and outer sulfur atoms in thiosulfate in an orientation that polarizes the S—S bond, allowing enzymatic cleavage of the S—S bond (Glibichik et al., 1996). A copy of rhodanese with a completely conserved catalytic site (CRSGQR motif) exists in node 13 of the assembly (Supporting Information Fig. S1; closest relative T. crunogena (YP391186), Table 2).

Thiomicrospira thermophila contains additional homologues of genes involved in sulfur-cycling, including sulfide dehydrogenase, which is co-located with soxB on the genome, NADPH-dependent sulfite reductase and cytochrome c proteins, sulfide:quinone reductase, and thiosulfate dehydrogenase (Supporting Information Fig. S1). Flavocytochrome-c sulfide dehydrogenase (pfam09242) oxidizes hydrogen sulfide to elemental sulfur coupled to the reduction of cytochrome c (Chen et al., 1994; Visser et al., 1997). The highest-scoring matches for sulfide dehydrogenase are not T. crunogena, but Thioalkalivibrio sp. ALE20, Thiomicrospira halophila and Chlorobaculum parvum (Table 2). Sulfite reductase reduces sulfite to hydrogen sulfide coupled to the oxidation of cytochrome c (Crane and Getzoff, 1996). Closest matches for sulfite reductase are T. crunogena and Acinetobacter lwofii (Table 2). The sulfide:quinone reductase in T. thermophila is homologous to that of T. crunogena (Table 2; Supporting Information Fig. S1) that mediates tetrathionate production from thiosulfate (32.6% similarity to thiosulfate dehydrogenase from Allochromatium vinosum; Table 2; Denkmann et al., 2012).

No genes with significant homology to dissimilatory sulfite reductase (dsr) or sulfur reductase (sre) were identified in the T. thermophila genome. These genes could potentially code for enzymes mediating sulfite reduction to hydrogen sulfide and sulfur, or elemental sulfur reduction to hydrogen sulfide, respectively. Homologues for the sorAB genes that function as alternatives to the sox(CD)2 genes (Kappler et al., 2001) to oxidize sulfite to sulfate, were also not found. Homologous genes for thiosulfate reductase (dvu, phs; Aketagawa et al., 1985; Bang et al., 2000) that mediate the disproportionation of thiosulfate to sulfite and hydrogen sulfide were not found. Homologous genes for tetrathionate reductase (itr; Hensel et al., 1999) mediating the reduction of tetrathionate to thiosulfate were also not found. However, the failure to identify homologous sequences to these genes in T. thermophila does not unequivocally rule out their presence in unaligned portions of the genome (i.e., the genome was not closed).

Discussion

We have demonstrated that in T. thermophila strain EPR85 the rate of incomplete thiosulfate oxidation to elemental sulfur and sulfate (reaction ib in Table 1) is decoupled from the rate of elemental sulfur oxidation to sulfate (reaction (ii) in Table 1), with the rate of the latter being a strong function of pH. We have also confirmed that the stoichiometric imbalance of sulfur reported in this study is due to the production of tetrathionate at low pH (<6.0). The imbalance previously reported in Thiomicrospira and Thiobacillus strains is also likely due to tetrathionate production. Analysis of genomic DNA indicates the presence of enzymes for the complete Sox pathway. Based on these findings, we hypothesize that the observed pH

Fig. 3. Specific growth rate as a function of starting pH of the media. Start pH determines which thiosulfate-consuming reaction will be mediated. Highest growth rates are observed when thiosulfate is completely oxidized to sulfate in Tris buffer (pH 8.0) (solid triangle) and lowest when thiosulfate is oxidized to tetrathionate (open squares), thiosulfate is oxidized to equal parts elemental sulfur and sulfate (open circles) or elemental sulfur is oxidized in the absence of thiosulfate (cross). Thiosulfate oxidation to elemental sulfur and sulfate with simultaneous oxidation of elemental sulfur to sulfate supports intermediate growth rates (solid diamonds). Specific growth rates in Bis-Tris buffer (pH 5.5) are shown in blue; unbuffered media data are shown in black; citrate buffer data are shown in green.
Table 2. Homologous genes for sulfur oxidation enzymes found on the \emph{T. thermophila} genome.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Node#/position in node</th>
<th>Highest sequence similarity</th>
<th>Nearest neighbor</th>
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<tbody>
<tr>
<td>\text{SoxA}</td>
<td>1 (241316-242146)</td>
<td>YP_390871.1 (1\text{e-}180/85.51%)</td>
<td>\text{T. crunogena XCL-2} Sulfur oxidation protein with signal peptide and conserved cytochrome c</td>
</tr>
<tr>
<td>\text{SoxZ}</td>
<td>1 (242251-242589)</td>
<td>YP_390872.1 (1\text{e-62}/89.81%)</td>
<td>\text{T. crunogena XCL-2} Sulfur oxidation protein (pfam08770)</td>
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<td>\text{SoxY}</td>
<td>1 (242629-243096)</td>
<td>YP_390873.1 (6\text{e-86}/91.61%)</td>
<td>\text{T. crunogena XCL-2} Sulfur oxidation protein with signal peptide</td>
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<tr>
<td>\text{SoxX}</td>
<td>1 (243134-243508)</td>
<td>YP_390874.1 (2\text{e-81}/93.55%)</td>
<td>\text{T. crunogena XCL-2} Cytochrome c with signal peptide</td>
</tr>
<tr>
<td>\text{Cytochrome c}</td>
<td>1 (267914-268273)</td>
<td>YP_390898.1 (3\text{e-57}/86.00%)</td>
<td>\text{T. crunogena XCL-2} Cytochrome c553 with signal peptide</td>
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<tr>
<td>\text{Hypothetical protein} (SoxA)</td>
<td>6 (119648-120526)</td>
<td>YP_003644808.1 (2\text{-}128/66.41%)</td>
<td>\text{Thiimonas intermedia} Sulfur oxidation with non-matching signal peptide (KEGG Adeh_2281; KEGG Anaek_1594)</td>
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<tr>
<td>\text{Hypothetical protein} (SoxX) rhodanese</td>
<td>6 (120852-121649)</td>
<td>YP_003644809.1 (4\text{-}38/61.50%)</td>
<td>\text{Thiimonas intermedia K12} Sulfur oxidation protein with cytochrome c sulfrtransferase</td>
</tr>
<tr>
<td>\text{SoxB}</td>
<td>5 (107062-108993)</td>
<td>YP_391815.1 (0.0/79.06%)</td>
<td>\text{T. crunogena XCL-2} Sulfate thiol esterase UshA with signal peptide, SoxB (cd07411) with metallophosphatase binding site, C-terminal domain (pfam204176)</td>
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<tr>
<td>\text{SoxC}</td>
<td>10 (68564-69925)</td>
<td>YP_390426.1 (0.0/94.48%)</td>
<td>\text{T. crunogena XCL-2} Sulfite oxidase with molydopterin-binding domain (cl00199)</td>
</tr>
<tr>
<td>\text{SoxD}</td>
<td>10 (69906-70994)</td>
<td>YP_390427.1 (0.0/88.69%)</td>
<td>\text{T. crunogena XCL-2} Hypothetical protein containing cytochrome c (pfam00034) Diheme cytochrome c SoxD (pfam00034)</td>
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<td>\text{Sulfide dehydrogenase}</td>
<td>5 (129755-131155)</td>
<td>WP_019591503.1 (3\text{-}139/52.18%)</td>
<td>\text{Thioalkalivibrio sp. ALE20} NAD(FAD) dependent dehydrogenase with NAD(P) binding domain (cl17500) and flavocytochrome c sulfide dehydrogenase (pfam204176)</td>
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<td>\text{Sulfide dehydrogenase}</td>
<td>9 (44242-45666)</td>
<td>WP_019896075.1 (0.0/79.64%)</td>
<td>\text{T. halophile} Hypothetical protein with pyridine nucleotide disulfide oxidoreductase (pfam07992) Flavocytochrome c sulfide dehydrogenase (pfam09242)</td>
</tr>
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<td>\text{Sulfite reductase}</td>
<td>3 (111050-112147)</td>
<td>YP_392157.1 (0.0/77.81%)</td>
<td>\text{T. crunogena XCL-2} Oxidoreductase containing FAD binding domain (pfam00667), cytochrome p450—sulfite reductase (NADPH dependent reduction of \text{SO}_2 to \text{H}_2\text{S})</td>
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<td>\text{Sulfite reductase}</td>
<td>4 (51414-53828)</td>
<td>WP_004280698.1 (0.0/39.79%)</td>
<td>\text{Acinetobacter lwoffii} Fe-regulated membrane protein PiuB containing CysJ sulfite reductase and flavoprotein with cytochrome p450—sulfite reductase</td>
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<td>\text{Sulfide-quinone reductase}</td>
<td>2 (33149-34273)</td>
<td>YP_391650.1 (0.0/85.29%)</td>
<td>\text{T. crunogena XCL-2} Sulfide-quinone reductase containing pyridine nucleotide—disulfide oxidoreductase (pfam07992)</td>
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Table 2. cont.

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<th>Protein</th>
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<td>T. arctica</td>
<td>Hypothetical protein—hem binding cytochrome c (pfam00034)</td>
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<td>rhodanese</td>
<td>13 (55570-55866)</td>
<td>YP_012969337.1 (9e-41/39.41%)</td>
<td>Aliochromatium vinosum DSM180</td>
<td>Thiosulfate dehydrogenase containing different signal peptide</td>
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<td>rhodanese (SoxL)</td>
<td>19 (25750-26430)</td>
<td>YP_003262911 (6e-63/95.88%)</td>
<td>T. crunigena</td>
<td>Rhodanese-like protein</td>
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<tr>
<td></td>
<td></td>
<td>YP_003444124 (7e-59/46.23%)</td>
<td>Halothiobacillus neapolitanus c2</td>
<td>Sulfurtransferase (SoxL)</td>
</tr>
</tbody>
</table>

Genbank accession number for sequence with highest similarity is shown with corresponding e-value/% similarity in brackets. Known function is included with references to protein families, if known.

dependence of elemental sulfur consumption is consistent with a modified Sox pathway that we outline below, which, if confirmed, represents the first report of elemental sulfur accumulation in bacteria that have genes for sox(CD)2.

**Thiosulfate consumption**

The Sox pathway is a common multienzyme pathway for thiosulfate oxidation found in both photosynthetic purple and green sulfur bacteria and neutrophilic lithoautotrophic sulfur bacteria (Friedrich et al., 2001; Kelly et al., 1997). This pathway requires seven essential proteins (SoxXY-ZABCD) to complete thiosulfate oxidation to sulfate (Friedrich et al., 2001). The combined activity of the periplasmic SoxYZ, SoxAX and SoxB proteins partially convert thiosulfate to one sulfate (Lu et al., 1985; Wodara et al., 1994; Friedrich et al., 2000, 2001; Welte et al., 2009; Fig. 4; Table 1, enzymatic reaction ib). The remaining enzyme-attached sulfane group is oxidized to a second sulfate by the combined activity of Sox(CD)2 and SoxB proteins (Fig. 4; Wodara et al., 1997; Denger et al., 2008; Quintmeier et al., 2000; Table 1 reaction (ii)). **T. thermophila** strain EPR85 has homologous genes encoding for all the Sox enzymes (soxAXYZB) needed to completely oxidize thiosulfate to sulfate. Abiotic controls performed at all experimental conditions indicate negligible chemical reaction rates over similar time periods (Supporting Information Table S2) and indicate that the observed differences in thiosulfate oxidation products (e.g., sulfate, elemental sulfur or tetrathionate) are due to microbiologically mediated reactions. However, our observations of incomplete thiosulfate oxidation to sulfate associated with formation of elemental sulfur at low pH conditions (Fig. 1a and b) suggest that the kinetic rate of thiosulfate oxidation to elemental sulfur (red path in Fig. 4) is enhanced compared with elemental sulfur oxidation to sulfate (blue path in Fig. 4).

Here, we consider a kinetic rate law that describes the net chemical effect of microbial growth via a multienzyme pathway. The rate of thiosulfate consumption was fitted to the following equation:

\[ r = k C^q \]  \hspace{1cm} (1)

where \( r \) is the rate in mmol/h calculated for each timestep, \( k \) is the rate constant and \( q \) is a constant in h^{-1} (Walas, 1999). The power constant (\( q \)) is determined by fitting the rate data for \( S_2O_3 \) consumption as a linear function of the concentration of elemental sulfur. In the case where \( q \) is not equal to one, the integrated form of Eq. 1 is:

\[ k = \frac{C_{0}^{q-1}}{(q-1)(q-1)} \left[ \frac{(C_{0})^{q-1}}{C} \right] \]  \hspace{1cm} (2)

which can be linearized in the following form:

\[ \frac{1}{C}^{q-1} = \frac{1}{C_{0}^{q-1}} + k(q-1)(t-t_{0}) \]  \hspace{1cm} (3)

Rate data for reaction (i) (Table 1) from four datasets during which thiosulfate reaction to elemental sulfur occurs simultaneously with elemental sulfur oxidation, while mass balance is conserved, were used to determine this constant (\( q = 0.421 \); Fig. 5a). Thus, the rate of thiosulfate oxidation at all pH conditions tested is not first order, but rather a function of thiosulfate concentration as a power of the concentration of elemental sulfur. In other words, the mechanism by which thiosulfate is incorporated into the sulfur oxidizing pathway is the rate-limiting step in thiosulfate consumption for **T. thermophila**.
Elemental sulfur formation

It is thought that the accumulation of elemental sulfur as an intermediate in the Sox pathway only takes place in organisms that lack both Sox(CD)2, which converts the inner sulfur of thiosulfate directly to sulfate, and the dsr operon, which oxidizes elemental sulfur to sulfate (Dahl et al., 1993, 2002). In *Allochromatium vinosum*, a rhodanese-like SoxL protein has been implicated in the formation of intracellular sulfur globules, cleaving long chains of polysulfides from SoxYZ (Welte et al., 2009; Table 1 reaction (ib)). In the case of *T. thermophila* strain EPR85, elemental sulfur is extracellular (Fig. 6). Formation of sulfur globules via a partial Sox pathway has also been observed in colorless sulfur bacteria such as *Beggiaota* (Mußmann et al., 2007) and members of the *Chlorobiaceae* family of green sulfur bacteria (Gregersen et al., 2011). *T. thermophila* has a homologue of a rhodanese-like enzyme SoxL, but apparently no homologues for the dsr genes that would oxidize elemental sulfur (Table 2).

Formation of elemental sulfur by an organism that has genes for the Sox(CD)2 enzyme is unexpected given that pH-dependent inhibition of the activity of Sox(CD)2, responsible for converting the inner sulfur in thiosulfate to sulfate, has not been previously documented. However, it has been shown that the optimal pH for sulfite dehydrogenase activity of the Sox(CD)2 complex is 8.0, with a

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**Fig. 4.** Variations on the Sox pathway inferred to exist in *T. thermophila* based on this study. When *T. thermophila* is grown buffered at high pH (8.0), sulfur oxidation is consistent with the classic Sox pathway of *P. pantotrophus* (Friedrich et al., 2001), shown here annotated with the oxidation state of each sulfur atom in blue. Charged species are indicated in purple. In unbuffered experiments once tetrathionate formation begins, the thiosulfate substrate would have to be divided between SoxAX and thiosulfate dehydrogenase to explain the observed chemistry (tsd; orange arrows). When grown buffered at low pH (5.5), sulfur oxidation by *T. thermophila*, appears to mediate the second part of the pathway (blue arrows) at a slower rate than the first part (red arrows), allowing elemental sulfur to accumulate in the media (see text).

**Fig. 5.** Rate data during thiosulfate oxidation to elemental sulfur and sulfate. (A) The power constant of the rate law is determined by the linear relationship between the log of the rate of thiosulfate consumption and the log of the normalized elemental sulfur concentration, giving a q of 0.421. Initial thiosulfate oxidation rate data [via the simultaneous mediation of reactions (i–iii) in Table 1] from experiments ranging in pH from 7.5 to 5.0, each run in triplicate, were used to determine q. (B) The rate constant k for a power law is determined by the linear relationship between 1/[S2O3] q 2 1 over time. The slope of the linear relationship in this plot corresponds to k(q – 1) and the rate constant k for reaction (i) is 0.207.
10% decline at pH 7.3 and 8.5 (Lu and Kelly, 1984). In addition, the crystal structure of Sox(CD)\textsubscript{2} in Paracoccus pantotrophus indicates that after the addition of the third oxygen atom onto the SoxY-bound sulfane, the newly created SoxY-S-SO\textsubscript{3}\textsuperscript{3} complex is displaced by OH\textsuperscript{-}, regenerating the oxidized molybdenum cofactor (Zander et al., 2011). At low pH, this displacement reaction could be inhibited. In addition, given that Sox(CD)\textsubscript{2} releases 6 H\textsuperscript{+}, its activity may be inhibited when pH is already low. Our observations suggest a pH-dependent effect on Sox(CD)\textsubscript{2} activity that could also explain the observed elemental sulfur accumulation at low pH observed in \textit{T. crunogena} (Javor et al., 1990) and \textit{Thiobacillus} (Stefess et al., 1996).

In \textit{T. thermophilia}, the production of large quantities of extracellular elemental sulfur coincides with low pH and decreased growth rates (Fig. 3). In media buffered at high pH (8.0), the rate of production is inferred to equal consumption such that elemental sulfur does not appreciably accumulate in the media (Fig. 7a). In media buffered at low pH (5.5), elemental sulfur consumption occurs simultaneously with production that always exceeds consumption (Fig. 7b). In unbuffered media initially at pH 7.5, elemental sulfur production and consumption occur simultaneously until pH decreases below 5.5, at which point the rate of consumption exceeds production corresponding to the onset of stationary phase (Fig. 7c). In hydrothermal diffuse flow settings and on chimney surfaces bathed in

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**Fig. 6.** TEM images of cell pellets with elemental sulfur from the media. (A) Small amounts of amorphous elemental sulfur (arrow) in the media when grown in Tris buffer at pH 8.0. The smooth edges above and below are the edges of the copper grid on the holder. No biofilm was visible. Scale bar represents 500 nm. (B) Crystalline monoclinic elemental sulfur exhibiting a characteristic coffin shape (arrow) formed during growth in citrate buffer at pH 5.5. No biofilm was present. Scale bar represents 500 nm. (C) Sheets of biofilm trapped in the grid holder, containing nucleating amorphous elemental sulfur (arrow), formed after 4 h of growth in unbuffered media. Biofilm (bold arrow) appears to effectively trap sulfur and allow growth of larger globules without leading to crystal formation, presumably aiding the cells in re-harvesting the trapped sulfur as other substrates become limiting. Scale bar represents 500 nm. (D) A single vibrioid cell (dark shape on the left of the image marked with arrow) surrounded by sulfur and imbedded in degrading biofilm network. Note the different scale bar (200 nm). Image taken after 22 h of growth in unbuffered media when sulfur is actively scavenged and tetrahionate is being produced.
hydrothermal fluids diluted with oxygenated seawater where *T. thermophila* is commonly found, these neutral pH values are common, particularly in fluids below 45°C (Foustoukos et al., 2009, Ding et al., 2005). However, in these environments, turbulent advective flow near chimney orifices frequently results in the outer surfaces of chimneys being subjected to higher temperature and more acidic fluids. Under these conditions, *T. thermophila* can oxidize thiosulfate to elemental sulfur and then maintain cell biomass by utilizing that sulfur.

It is significant that the activity of SoxL is thought to produce polysulfides (Welte et al., 2009). Small concentrations (<40 μM) of sulfide are observed to form and remain at constant levels in the media throughout growth experiments buffered at pH 5.5 (Supporting Information Table S1). A mechanism for elemental sulfur formation via a polysulfide precursor may play a role in the creation of extracellular elemental sulfur in a variety of thiosulfate-utilizing bacteria. The exact mechanism for transferring polysulfide to extracellular sulfur globules like those produced by *T. thermophila* is currently unknown. Polysulfides, as charged dissolved species, would be more likely to be excreted extracellularly and rapidly convert to elemental sulfur (initially solid polymeric sulfur and then further to stable S₈ rings) and sulfide (Steudel, 1996). Alternatively, it is possible that polysulfide degrades to elemental sulfur and sulfide in the periplasm and nanocrystalline uncharged elemental sulfur diffuses across the cell membrane as in acidophilic Archaea (Boyd and Druschel, 2013). Our observations in this study are consistent with the nature of either of these mechanisms; however, further study is needed to confirm this.

Tetrathionate production in *T. thermophila*

Previous studies on thiosulfate oxidation by *Thiobacillus* and *Thiomicrospira* strains have demonstrated variable reaction stoichiometry and mass imbalances suggesting that these bacteria have complex strategies for maintaining growth during variable environmental conditions (Javor et al., 1990; Stefess et al., 1996). In addition to thiosulfate oxidation, for example, *T. crunogena* changes from elemental sulfur production at pH 6.3 to consumption at pH 8.5 (Javor et al., 1990). The sum of products in batch growth of *T. crunogena* was less than the sum of reactants, indicating the presence of additional undetermined sulfur products in previous experiments (Javor et al., 1990). Similar discrepancies in sulfur cycling mass balance were observed for two closely related strains of *Thiobacillus* that oxidize sulfide to elemental sulfur and sulfate (Stefess et al., 1996). Multiple oxidation pathways of thiosulfate have also been documented in *Allochromatium vinosum*, which variably produces elemental sulfur and sulfate via the Sox pathway or produces tetrathionate at low pH (Hensen et al., 2006). Given the similarities in observed stoichiometry and enzymes utilized in thiosulfate oxidation between *A. vinosum* and *T. thermophila*, we investigated the occurrence of

Fig. 7. Schematic depicting the relative degree to which reactions (i), (ii) and (iv) (Table 1) occur over time at various pH conditions (indicated above each figure). Time series progression at high (A), low (B), and variable (C) pH conditions.
tetrahionate to determine if the sulfur imbalance was a result of polythionate formation in *T. thermophila*.

The tetrahionate-producing thiosulfate dehydrogenase of *T. thermophila* closely matches that of *A. vinosum* (Table 2). The purple sulfur bacterium *A. vinosum* has also been shown to oxidize thiosulfate to tetrahionate under neutral to acidic pH conditions using a thiosulfate dehydrogenase (Pott and Dahl, 1998; Hensen et al., 2006). In addition, previous studies of *Thiobacillus tepidarius* reported fast rates of tetrahionate production via thiosulfate dehydrogenase (Lu and Kelly, 1988). More importantly, though, a recent review of thiosulfate dehydrogenase genes identified two conserved heme-binding motifs: the necessary catalytic cysteine, and conserved distal ligands that assist in heme-binding (Denkmann et al., 2012). The homologous gene in *T. thermophila* shares all conserved regions with only minor substitutions in the heme-binding motifs (Supporting Information Fig. S2).

Below pH 6, *T. thermophila* abruptly switches to tetrahionate production from thiosulfate [Table 1 reaction (iv)], elemental sulfur consumption from the media, and continued processing of thiosulfate to sulfate (Fig. 2a). Comparison of experiments, both with unbuffered and buffered pH (5.5 and 8.0), indicates that this shift in tetrahionate formation correlates with pH (Fig. 8). The stoichiometry of sulfate production indicates that the elemental sulfur lost after this switch cannot account for all sulfate produced (about 1 mM excess SO₄; Fig. 1c); rather, some fraction of thiosulfate consumed must still be processed, albeit at a slow rate. This switch coincides with a change from decreasing to nearly invariant pH in the medium, possibly as a result of simultaneous and comparable reaction rates of elemental sulfur oxidation that consumes H⁺ and of tetrahionate production that produces H⁺ (reactions (ii) and (iv), Table 1). Given the similarities in reported stoichiometry of thiosulfate oxidation and tetrahionate production in other colorless sulfur bacteria such as *T. crunogena* (Javor et al., 1990; Scott et al., 2006), *Sulfurimonas denitrificans* (Sievert et al., 2008) and *Thiobacillus* strains (Stefess et al., 1986), the genetic mechanism responsible for our observations of extracellular elemental sulfur formation and simultaneous tetrahionate production may be more widespread than previously thought.

**Conclusions**

*Thiomicrospira thermophila* strain EPR85 has a wide range of metabolic options for coping with the temporal variability in fluid composition that characterizes seafloor diffuse-flow hydrothermal environments. In particular, pH is the dominant control over the type of reactions that are mediated and pH below 5.5 can cause the onset of stationary phase growth, even when thiosulfate is abundant. At pH below 6.0, thiosulfate is oxidized to tetrahionate, accounting for the missing sulfur observed in previous experiments. Thiosulfate is converted to elemental sulfur and sulfate at pH 5.5–8, with the amount of elemental sulfur accumulating in the media increasing as pH decreases.

The observed sulfur oxidation chemistry is consistent with the Sox pathway, for which homologues of all genes necessary were found in the genome of *T. thermophila* strain EPR85. The pH dependency of elemental sulfur accumulation suggests a pH-dependent limitation on the activity of Sox(CD)₂, resulting in incomplete thiosulfate oxidation. This is the first report of obligate formation of elemental sulfur as an intermediate in an organism that does not contain the *dsr* genes (Grimm et al., 2008; Dahl et al., 2002).

When pH is lower than 5.5, tetrahionate production is upregulated. *T. thermophila* strain EPR85 has a homologous gene for thiosulfate dehydrogenase, which can mediate tetrahionate production while consuming H⁺, partially counteracting the decrease of pH in the medium. Simultaneous with tetrahionate production is the shift to elemental sulfur consumption exceeding production, even if thiosulfate is still present in the media. Thus, *T. thermophila* strain EPR85 can mediate several sulfur oxidation reactions simultaneously, resulting in large geochemical changes with very little increase in biomass.

**Experimental procedures**

**Bacterial strain and growth conditions**

*Thiomicrospira thermophila* strain EPR85 was isolated from fluids collected from a diffuse flow vent on the East Pacific...
Rise (EPF; 9°50’N, 104°17’W), at a depth of approximately 2500 m in May 2005 (Cruise AT 11-26, Alvin dive 4103). Primary enrichment cultures were initiated shipboard by adding 1 ml of fluids to 10 ml of growth medium designed to enrich for chemolithoautotrophic thiosulfate oxidizing bacteria. The medium used was 142-A (Crespo-Medina et al., 2009). Cultures were incubated at 30°C in the dark. Growth was initially determined by the observation of a change in color of the pH indicator in the medium and then confirmed by direct microscopic counts after staining the cells with acridine orange. For isolation of single colonies, liquid cultures of strain EPR 85 were inoculated on Petri dishes containing medium 142-A solidified with 15 g L⁻¹ of noble agar (Sigma). Stocks for long-term storage were prepared by adding 150 μL of 100% sterile glycerol (Fisher Scientific) to 850 μL of overnight cultures, and were stored at −80°C.

In this study, T. thermophila strain EPR85 was grown routinely in batch culture with air in the headspace at 35°C on a shaker table. Unbuffered seawater salinity medium contained the following (L⁻¹): 25 g NaCl, 0.2 g NH₄Cl, 1.23 g MgCl₂·6H₂O, 0.4 g CaCl₂·2H₂O. After autoclaving, the following filter-sterilized solutions were added (by volume): 10 wt% K₂HPO₄ (0.25 g in 500 ml), 10 wt% NaHCO₃ (2.52 g in 500 ml), 1 wt% trace metal solution SL-10, and approximately 6 wt% Na₂SO₄·5H₂O (16 g in 500 ml). The trace metal solution SL-10 is made as follows (in 1 l): 1.5 g FeCl₂·4H₂O dissolved in 10 ml 25% HCl, 70 mg ZnCl₂, 100 mg MnCl₂·4H₂O, 6 mg H₂BO₃, 190 mg CaCl₂·6H₂O, 2 mg CuCl₂·2H₂O, 24 mg NiCl₂·6H₂O, 36 mg Na₂MoO₄·2H₂O. The thiosulfate stock solution was made fresh often and was not added to media until just prior to inoculation to prevent potential degradation in the media. Bicarbonate, phosphate and trace metals were added just prior to inoculation as well. For the citrate buffer experiments, the salts were added to a buffer base made by mixing 115 ml 0.1 M citric acid with 885 ml 0.1 M sodium citrate. The Bis-Tris buffer was made at 0.1 M and pH was set to the desired value with HCl. The Tris buffer was made by mixing 557 ml 0.1 M Tris-HCl with 443 ml 0.1 M Tris base. The pH was measured prior to inoculation.

Culture stock stored at −80°C was routinely pre-grown in gas-tight serum bottles containing 15 ml unbuffered medium and transferred to 50 ml volumes twice prior to the start of each experiment. The headspace volume in the gas-tight bottles was at least two-thirds of the total volume to ensure that oxygen gas would not be limiting. The inoculum culture was transferred in exponential growth phase at the start of each experiment to limit the lag phase. Serum bottles were constantly agitated on a Thermo Forma 420 orbital shaker table. Unbuffered seawater salinity medium contained 15 ml unbuffered medium and was transferred to 50 ml volumes twice prior to the start of each experiment to limit the lag phase. Serum bottles were constantly agitated on a Thermo Forma 420 orbital shaker table.

Inoculated bottles were incubated at 30°C in the dark. Growth was initially determined by the observation of a change in color of the pH indicator in the medium and then confirmed by direct microscopic counts after staining the cells with acridine orange. For isolation of single colonies, liquid cultures of strain EPR 85 were inoculated on Petri dishes containing medium 142-A solidified with 15 g L⁻¹ of noble agar (Sigma). Stocks for long-term storage were prepared by adding 150 μL of 100% sterile glycerol (Fisher Scientific) to 850 μL of overnight cultures, and were stored at −80°C.

Analytical techniques

Thiosulfate and sulfate concentrations were monitored by ion chromatography (Metrohm 881) after filtering out biomass and elemental sulfur using an anion column (Metrosep ASupp5-100) with suppression (0.1 M sulfuric acid). Conductivity and UV–Vis detectors (Metrohm 887) were employed in series. The eluent solution was 3.2 mM Na₂CO₃/1 mM NaHCO₃ with 2.5 vol% acetone as a modifier. The UV–Vis detector (215 nm) was used exclusively for thiosulfate analysis. Sulfate is not UV-detectable and dissolved species concentrations were analyzed by conductivity detector. The pH was monitored using a Mettler Toledo Ag/AgCl electrode. Sulfide was measured using the method of Cline (1969) as follows: 1 ml sample was fixed with excess Zn acetate (1 ml, 0.3 M); 1 ml of that solution was added to 0.08 ml of mixed diamine reagent, and allowed to sit for up to 1 h; after the color change this solution was diluted to a volume of 12.5 ml with deionized water, transferred to a plastic cuvette and absorbance at 664 nm was analyzed on a UV–Vis spectrometer (Thermo Evolution 60). A blank of sulfide-free media was also fixed in Zn acetate and processed in the same manner. Polythionates and thiosulfate were analyzed by the cyanalysis method (modified from Kelly and Wood, 1994) described in the Supporting Information.

Elemental sulfur (Sₐ) was extracted from water samples using dichloromethane in glass tubes without letting solvent touch the caps. Three sequential extractions were combined, each with two times the volume of the water sample, to ensure complete extraction. All transfers were made with glass pipettes and quartz cuvettes were used to allow detection in the UV range. The extracted DCM was then measured on a UV–Vis spectrometer (Thermo Evolution 60) by scanning from 200 to 700 nm. Elemental sulfur has a distinctive double peak (Meyer, 1976), which in DCM occurs at 270 and 285 nm. Both peaks are linear with respect to sulfur concentration. The 270 nm peak was chosen because at very low concentrations the 285 nm peak is sometimes indistinct. The scan of a blank (sulfur-free DCM) was subtracted from each sample and the peak height at 270 nm was normalized to the background at 550 nm, as there were differences in baseline in samples analyzed at different times. Polymeric sulfur is insoluble in organic solvents (Steudel and Eckert, 2003). We developed a protocol to measure polymeric sulfur by converting it to DCM-soluble Sₐ rings. Based on the stability of commercially available polymeric sulfur (Crystex, Eastman Chemical Company), after the first triple extraction we treated the remaining sample with Tris base (pH 9.4) to raise the pH and provide excess amines in solution. The sample was then autoclaved for 30 min and allowed to sit overnight to promote conversion of polymeric sulfur to stable Sₐ. Elemental sulfur (Sₐ) was then quantified after a second triple extraction in DCM. Although we cannot quantify the yield of this process, no insoluble residue was visible after this treatment.

Samples taken for cell counts were fixed in 4 vol% formaldehyde in phosphate buffered saline (PBS: 10.9 g K₂HPO₄, 3.2 g NaH₂PO₄, 90 g NaCl in 1 l DIW) to a dilution factor of 2. Due to the production of elemental sulfur solids in the media, optical density could not be used to determine biomass abundance. Cells were stained with DAPI (2 μl of 1 ppb DAPI in PBS per ml of sample) and allowed to sit for 10 min prior to filtering through 13 mm black polycarbonate 0.22 μm filters (Millipore). Variable volumes (100–400 μl) were carefully pipetted onto a single spot on the filter to concentrate the biomass for imaging. By varying the volume used, the optimal cell density for counting was achieved (<100 cells/field of view). The filters were imaged on a Nikon Eclipse 80i autofluorescence microscope at 1000× magnification. Five to ten representative fields of view were counted and averaged. The cell density...
V. chemostat shifted during an experiment. Any sequences with a (FastQC  
Illumina data. Replicate sequences were removed and the fur through a 5
期间的元素硫浓度，如图S3所示。具体生长速率，

50 ml unbuffered media and harvested during exponential phase by filtering out excess elemental sulfur started forming, as the cells begin to attach and form clumps with the sulfur. As a result the error of counting increases with elemental sulfur concentration, as shown in Supporting Information Fig. S3. Specific growth rates, reported in h⁻¹, were calculated according to:

$$\mu = \ln \left( \frac{X}{X_0} \right) / (t - t_0)$$  

Separate growth rates were calculated if the reaction stoichiometry shifted during an experiment.

Nucleic acid analysis

Cells were grown in 50 ml unbuffered media and harvested during exponential phase by filtering out excess elemental sulfur through a 5 μm filter before pelleting by centrifugation. Genomic DNA was extracted using the Promega Wizard Genome DNA Purification Kit, following the manufacturer’s instructions. Quality and concentration of extracted DNA was verified on a Nanodrop 2000c and by a 2 wt% agar gel to confirm the size and purity of the product.

From the extracted genomic DNA, the 16S DNA sequence was amplified by PCR (Phusion) using the universal bacterial primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTGGTACGACTT) on an Eppendorf Mastercycler. PCR product was cleaned using the Wizard SV Gel and PCR Cleanup kit (Promega) and the quality of PCR product was verified on a Nanodrop 2000c and by a 2 wt% agar gel to confirm the size and purity of the product.

Sequences were aligned to release 117 of the SILVA SSU refdatabase. Phylogenetic analysis

We obtained a nearly full-length sequence (1514 bp) of the 16S rRNA gene from T. thermofila strain EPR85 and, for comparison, T. crunogena XCL-2 (Scott et al., 2006). These sequences were aligned to release 117 of the SILVA SSU reference database (Quast et al., 2013) using the SILVA Incremental Aligner (SINA) (Pruesse et al., 2012). This method utilizes a combination of k-mer searching and partial-order alignment to optimize the accuracy of alignment. We then constructed a phylogenetic tree in ARB (Ludwig et al., 2004) of these sequences and other Gammaproteobacteria using the “All-Species Living Tree” Project (LTP) (Yarza et al., 2008; Münß et al., 2011) as a template. 16S rRNA gene sequences in the LTP are derived from type strains of all classified strains of Bacteria and Archaea. Tree construction was done using the maximum likelihood algorithm RAxML (Stamatakis, 2006). T. thermofila strain EPR85 and T. crunogena XCL-2 were inserted into the tree using the ARB parsimony algorithm.

Accession numbers in Genbank

The sequence for the 16S rRNA gene was submitted to GenBank under the accession number KP749905, Sequences for the SoxC and SoxD genes that code for proteins involved in the Sox sulfur oxidation pathway were submitted to GenBank under accession numbers KP749906 and KP749907, respectively.

Sulfur oxidation in Thiomicrospira thermofila XXX
TEM imaging

To investigate the nature of elemental sulfur produced by *T. thermophila* under different pH conditions, we imaged the sulfur-biomass clusters with a Tecnai Spirit TEM operated at 120 kV with a 2 K 4-megapixel CCD TEM camera. Culture samples at different stages of growth were pipetted (100 μl) onto copper grids, allowing excess water to wick onto a kimwipe. The loaded grid was then stained with methylene blue. Only material that attached to the edge of the grid was visible with this treatment and samples with a high abundance of EPS produced better images as a result.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Genome scaffold lengths labeled by node # referenced in the text. Locations of specific homologous genes are indicated by boxes in their order on the scaffold (cyt-c: cytochrome c; sqr: sulfide quinone reductase; sr: sulfite reductase; sd: sulfide dehydrogenase; rhd: rhodanese; tsd: thiosulfate dehydrogenase; SoxABCDLXY: enzymes involved in the Sox pathway). Genes indicated by parentheses are second copies of genes with weaker affinities.

**Fig. S2.** Sequence alignment of thiosulfate dehydrogenase (tsd) homologous proteins. Included is the *A. vinosum* and *Tint. 2892* genes of the alignment in Denkmann et al. (2012), the closest match with the *T. thermophila* gene annotated as a hypothetical protein in *T. arctica*, and the gene from *T. thermophila* from this study. The heme-binding motifs are highlighted in grey; distal ligands are in blue, and the catalytic cysteine in red.

**Fig. S3.** Phylogenetic tree of the Gammaproteobacteria created in ARB based on a 16S rRNA gene alignment from the SILVA “All-Species Living Tree” Project (Munoz et al., 2011; Yarza et al., 2008). Sequences from Thiomicrospira thermophila strain EPR85 and Thiomicrospira crunogena XCL-2 were inserted using the ARB parsimony algorithm.

**Table S1.** Stoichiometry of all growth experiments. Polythionates (S_2O_3 and S_2O_5) were determined using cyanolysis. *Indicates S_2O_3 concentrations determined with the cyanolysis method. All other S_2O_3 concentrations were determined by ion chromatography.

**Table S2.** Stoichiometry of all abiotic control experiments. Polythionates (S_2O_3) were determined using cyanolysis. *Indicates S_2O_3 concentrations determined with the cyanolysis method. All other S_2O_3 concentrations were determined by ion chromatography. All experiments were performed at 35°C unless otherwise noted. Measurements below detection are indicated as b.d. and as n.a. if not detected.