For publication

**Sulfur compound oxidation and carbon co-assimilation in the
haloalkaliphilic sulfur oxidizers *Thioalkalivibrio versutus* and
*Thioalkalimicrobium aerophilum***

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Abstract

Alkaliphilic sulfur-oxidizing bacteria are highly abundant in naturally occurring soda lakes, where they are found both in surface waters and sediments. Here we studied oxidation of sulfide and thiosulfate in batch cultures of *Thioalkalimicrobium aerophilum* and *Thioalkalivibrio versutus*, two species that represent different metabolic types, as indicated by the absence or presence of sulfur production during growth, respectively. With thiosulfate, both species showed the expected sulfur oxidation patterns; however, during growth on sulfide, both *T. aerophilum* and *T. versutus* produced sulfur as an intermediate. While *T. aerophilum* likely uses a Sox-type sulfur oxidation pathway, *T. versutus* appeared to use a combination of some Sox proteins with heterodisulfide reductase complexes, which is supported by gene expression data. Interestingly, intermediate sulfur production by *T. versutus* occurred when the sulfur source in the medium had been nearly exhausted, which is unlike what has been described for the well-studied Dsr/Sox pathway in phototrophic sulfur bacteria. Inclusion of some carbon sources (acetate, propionate, fructose) slightly enhanced growth of *T. versutus* and *T. aerophilum* in batch cultures, suggesting that carbon co-assimilation may be occurring. Our results indicate that sulfur oxidation processes in alkaliphilic sulfur oxidizers are more complex than previously assumed, and that the enzymes involved warrant further study.

Keywords: Chemolithoautotrophy; Sulfur oxidation; Haloalkaliphilic bacteria

1. Introduction

Alkaline soda lakes are high pH environments with, occasionally, very high salt concentrations, but despite this they are characterized by strong primary production. In addition to anaerobic phototrophic sulfur-oxidizing bacteria such as *Ectothiorhodospira* spp., these lakes are also the habitat of a large number of very diverse aerobic bacteria, many of which are able to oxidize inorganic sulfur compounds for energy generation [36, 29-31]. As a result of being able to grow in extreme environments, these bacteria have attracted a great deal of attention as potential sources of enzymes for industrial applications, and they have also been used in wastewater treatment processes for the removal of sulfide [23, 29].

Thioalkalimicrobium aerophilum and *Thioalkalivibrio versutus* are representatives of these alkaliphilic sulfur-oxidizing bacteria (ASOB), and were first isolated from two different soda lakes in Siberia in 1995 and 1989, respectively [32]. Both species are obligate chemolithoautotrophs, but they differ in both their growth profiles and ability to oxidize sulfur compounds.

T. aerophilum species are characterized by relatively high aerobic growth rates (0.33 h^{-1} in thiosulfate-limited chemostat culture) and are able to oxidize thiosulfate and sulfide, but not sulfite, tetrathionate, tri- or pentathionate [32]. Generally, during the oxidation of sulfur compounds, no formation of sulfur was observed. In contrast, *T. versutus* species grow much more slowly (0.18 h^{-1} in thiosulfate-limited chemostat culture), and produce sulfur as an intermediate during oxidation of a variety of compounds, including thiosulfate, sulfide, sulfite, tri-tetra and pentathionate [32].

This production of extracellularly deposited sulfur during growth is indicative of a sulfur oxidation mechanism similar to that of anaerobic phototrophic bacteria, particularly those of the family *Ectothiorhodospiraceae*, to which *Thioalkalivibrio* species also belong, and that occur in the same natural habitat [34, 35]. At present, there are two major bacterial

sulfur oxidation pathways, the Sox pathway and the Dsr/Sor pathway, that have been described in sulfur-oxidizing bacteria, with only the latter leading to formation of sulfur as an intermediate [5, 9, 8]. The two pathways differ in the type of *sox* genes found, with *soxCD* genes being absent in organisms that oxidize sulfur via the Dsr/Sox pathway [5].

Here we evaluated growth of both species of sulfur-oxidizing bacteria on thiosulfate and sulfide, with particular focus on the sulfur species present during batch growth. We also determined the effect of small organic acids and hexose sugars on growth of these ASOB, and found that inclusion of these compounds can increase the final culture density, although different compounds appeared to be preferred for assimilation by each species. Enzyme assays and qRT-PCR were used to analyze underlying changes in gene expression of sulfur-oxidizing enzymes.

2. Material and methods

2.1. Bacterial strains and growth media

T. versutus strain AL2 (DSMZ 13738^T) [32] and *T. aerophilum* AL3 (DSMZ 13739^T) [32] were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Strains were routinely grown using DSMZ medium no. 925 with thiosulfate (40 mM (*T. versutus*), 80 mM (*T. aerophilum*)) or sulfide (40 mM (*T. versutus*), 20 mM (*T. aerophilum*)) at 30°C and shaking at 200 rpm. Standard growth conditions were microaerophilic, with 100 ml of medium in 250 ml shake flasks. Small-scale growth tests on media supplemented with different carbon sources (2 ml medium/sample) used sterile 24-well plates (Becton Dickinson), covered with a lid and sealed with parafilm. Media for these growth tests were supplemented with 2, 5, 10 or 20 mM fructose, glucose, acetate or propionate, or organosulfur compounds (10, 20, 40 or 60 mM DMSO or DMS; 2.5, 5 or 10 mM beta-mercaptoethanol) in addition to thiosulfate as the main energy source. Substrate growth tests were carried out at least three times.

Growth curves under microaerophilic conditions (see above) used cultures that were inoculated at OD₆₀₀ of 0.015 (thiosulfate) or 0.025 (sulfide). All growth curves were carried out at least twice.

2.2. Molecular biology and biochemistry methods

Standard methods were used throughout [2]. Genomic DNA was isolated using DNAzol (Life Technologies) or Qiagen Genomic-tip 100 according to the manufacturer's instructions. All standard PCRs used GoTaq green Mastermix (Promega); oligonucleotide primers (Table S1) were from IDT.

Cell extracts were prepared using Bug Buster Mastermix (Novagen), and protein determinations used the BCA-1 kit (Sigma). Whole cell protein samples (3 x 100 µL, growth

curves) were extracted in 1 ml ice-cold acetone/methanol (70:20) and pellets collected by centrifugation at 4°C, 22,000 x rcf, 15 min. Air-dried pellets were resuspended in 100 µL water before protein determination as in [19].

2.3. RNA isolation and qRT-PCR

RNA isolation, contamination testing and qPCR protocols were designed based on [33]; 10 mL exponentially growing culture were added to 500 µL 20x STOP solution (200 mM Tris-Cl pH 8, 20 mM EDTA, 20 mM sodium azide) and harvested by centrifugation (2,800 rcf, 4°C, 15 min). Cell pellets were immediately resuspended in 1mL TRIzol® Reagent (Life Technologies) and RNA isolated as per the manufacturer's instructions. RNA cleanup used the RNAspin mini kit (GE Healthcare); PCR (16S rRNA primers 27F and 1492R [10]) was used to test for gDNA contamination and samples were considered gDNA-free if PCR reactions did not yield any bands after 30 cycles of amplification, using between 50-100 ng of RNA as template. cDNA was synthesized using SuperscriptIII (Life Technologies) from 500 ng of gDNA-free RNA using random hexamer primers (Life Technologies) and diluted to 1:100 or 1:1000 for use in qRT-PCR. qRT-PCR was carried out using SYBRgreen Mastermix (Applied Biosystems), an epMotion 5075 automated pipetting system (Eppendorf) for PCR-plate setup and a 7900 or Quantstudio RT real-time PCR system (Applied Biosystems) at an in-house qRT-PCR facility. qRT-PCR primers (product size: 101bp; *T. aerophilum* > 60°C) were designed using Vector NTI advance (Life Technologies) (Table S1) and validated by standard PCR using first gDNA and then cDNA as template. Potential reference genes (*16S*, *rpoA*, *gyrA*) were evaluated using Normfinder [1]. Based on these analyses, data were normalized to expression of the *gyrA* gene as described in [18], with PCR efficiencies determined using LinReg [26].

2.4. Sulfur species analysis

One mL samples passed through a 0.22 μm PES filter to remove solids before preparation of dilutions for ion chromatography analysis [22] were prepared in 2 mL polypropylene vials (Sigma-Aldrich®) containing 500 μL preservative reagent [6], 1.4 mL degassed H_2O and 100 μL for samples from thiosulfate growth or 500 μL preservative reagent, 1.45 mL degassed H_2O and 50 μL (sulfide growth) sample.

Determination of elemental sulfur used pellets collected from 1 mL culture samples (10,000 x rcf, 10 min, RT) resuspended in 8.35 mL H_2O . 1.5 mL of 30% (w/v) Na_2SO_3 in 1 mM Na_2EDTA , and 150 μL of 1 M NaOH were added before incubation in a shaking water bath (Julabo, SW22) at 200 rpm, 60°C, overnight. Samples were cooled to RT before sulfur species analysis via ion chromatography [22] at the Advanced Water Management Center Analytical Facility at the University of Queensland.

2.5. Enzyme assays

Sulfide dehydrogenase activity assays used the method of [27]. One ml assays contained 0.02 mM horse heart cytochrome *c*, 0.02 mM sodium azide, variable amounts of cell extract and between 1 and 20 μM sulfide in 100 mM Tris-Cl buffer, pH 9. Activities were calculated using ϵ_{550} (red-ox) of 21.1 $\text{mM}^{-1}\text{cm}^{-1}$.

Sulfite dehydrogenase assays with ferricyanide were carried out as in [38], using 20 mM Tris-Cl buffer (pH 8 and 9) or 100 mM sodium carbonate buffer (pH 10). K_M determinations used sulfite concentrations of 5 μM -2 mM. All enzyme assays used a Varian Cary50 (Agilent) spectrophotometer; enzyme kinetic data were fitted using Prism 6 (Graph Pad).

2.6. Determination of thiosulfate-dependent respiration

Thiosulfate-dependent respiration of washed cells (wash solution: medium 925 mineral base without additions, final concentration 7-10 μg protein mL^{-1}) was determined using an oxygen electrode (Hansatech Oxygraph 1.1) at 30°C and pH 8-10 using buffers containing 0.4

M total Na^+ . For pH 8, freshly prepared NaHCO_3 was used and for pH 9-10 a combination of $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ was used. Thiosulfate was used at 25-200 μM .

2.7. *Bioinformatic analyses*

Homologues of known genes involved in sulfur oxidation or carbon assimilation processes were identified by BLASTP searches using sequences of known proteins of this function as templates (Table S2). For confident identification, homologues had to have at least 70-80 % query cover, similarly conserved domains as the search model and low e-values (e-values considered significant varied, as e-values change based on sequence length). Additionally, name searches for gene annotations were used in some cases for proteins with known high variability (e.g. SoxA and SoxX). Sequences identified by name search were then confirmed as positive by analysis of conserved domains present using the CDD database available at NCBI.

2.8. *Accession numbers*

The accession number for the *T. aerophilum* genome is NZ_CP007030.1. Sequence data for *T. versutus* AL2 was generated at the Joint Genome Institute (DOE, USA), but is currently under embargo. The unreleased genome data is accessible at the Integrated Microbial Genomes Database (IMG, img.jgi.doe.gov). With permission from the relevant program coordinators, we retrieved sequences for individual genes involved in sulfur oxidation and deposited them with Genbank. Accession numbers will be given upon acceptance.

3.Results

3.1. Sulfur compound oxidation in *T. versutus* and *T. aerophilum*

The presence and absence of sulfur formation during growth on thiosulfate is a clear indicator of different sulfur oxidation pathways being used by *T. aerophilum* and *T.versutus*, and this would normally be reflected in physiological parameters as well as sulfur speciation during growth.

As expected, both *T. aerophilum* and *T.versutus* showed thiosulfate-dependent respiration in oxygen electrode experiments at pH 8, 9 and 10, with optimum respiration observed at pH 10 with 100 and 200 μ M thiosulfate (Fig. 1). At all three pH values, the respiratory rates increased with increasing amounts of thiosulfate present (Fig. 1). Respiratory activity was approximately 10 times higher in *T. aerophilum* than in *T.versutus* (Fig. 1), and the data are in good agreement with averaged respiratory data for 25 *Thioalkalivibrio* and 17 *Thioalkalimicrobium* strains reported in the original strain description. [32].

3.2. Growth of *T. versutus* and *T. aerophilum* on thiosulfate-containing medium

We then carried out growth experiments with thiosulfate as the substrate and analyzed the sulfur species present in growth media. In experiments with *T. aerophilum*, thiosulfate was consumed at a steady rate until its depletion after 40 h. Sulfate concentrations and OD₆₀₀ increased steadily from the start of the experiment until thiosulfate had become depleted. Formation of sulfite (2-5 mM) was observed while thiosulfate was present (up to ~ 42 h); sulfur or sulfide were not detected (Fig. 2).

In contrast, *T.versutus* cultures showed slow thiosulfate consumption for the first ~8 h, after which the utilization rates increased until all thiosulfate had been consumed (Fig. 2). Again, sulfate formation mirrored the rates of thiosulfate consumption. Sulfur was formed, initially in small amounts (~0.3-0.5 mM); however, after 26 h of incubation, coinciding with a

reduction in thiosulfate concentrations to 4 mM, the amount of sulfur formed began to increase to a maximal concentration of 3.5 mM after 29 h. This sulfur then appeared to be oxidized to sulfate, with an intermediate formation of thiosulfate. Sulfite was detected between 17 and 27 h in amounts of 80 μ M or less.

Both growth curves essentially conformed to expectations for these ASOB, although sulfur formation in *T.versutus* occurred later than expected.

3.3. Growth of *T. versutus* and *T. aerophilum* on sulfide-containing medium

We then investigated growth of both ASOB on sulfide. As both bacteria are strict aerobes, and sulfide can be air-oxidized, we initially carried out a control experiment with non-inoculated, sulfide-containing ASOB medium. In this control experiment, during the first 8 h, no net change in sulfide concentrations was observed, followed by abiotic oxidation of sulfide to thiosulfate (~ 17 mM final concentration after 34 h), with only small amounts of sulfate (up to 2.5 mM, conc. increasing after ~ 26 h) being formed. Sulfite (up to ~ 1 mM) was present throughout the process (Fig. S1). After 26 h, the remaining sulfide (2 mM) was oxidized very slowly until concentrations were close to zero after 40 h. Some thiosulfate (~ 4 mM) was detected in the growth medium at the start of the experiment, indicating air-oxidation of the sulfide crystals despite washing of the crystal surfaces. Thiosulfate formation in the growth medium would have occurred as a result of abiotic reactions between sulfite and sulfide [13].

T.versutus growth on sulfide lasted about 40 h, as indicated by OD₆₀₀ and protein concentrations in the growth medium, with sulfur formation being observed at two stages during growth, as indicated by the spikes in OD₆₀₀ readings (Fig. 3).

Sulfur speciation data revealed two growth phases – one based on sulfide oxidation to thiosulfate, sulfur and sulfate, and a second phase during which thiosulfate was oxidized to

sulfate with sulfur as an intermediate. During sulfide-based growth, sulfide concentrations initially decreased slowly, followed by rapid consumption after 4-6 h of incubation. Formation of thiosulfate (18 mM max. concentration at 18 h), sulfur (0.47 mM at 18 h) and sulfite (~ 1 mM) was observed. Sulfate concentrations increased only slowly during this phase of the growth, from 4 mM at the start of the experiment to 6 mM after 18 h. As some thiosulfate was already present at the start of the experiment, it is not entirely clear whether *T.versutus* was growing on thiosulfate or sulfide; however, the faster rate of sulfide oxidation, including formation of significant amounts of sulfur that was not observed in the abiotic control, indicates that sulfide was actively oxidized by the bacteria. The accumulation of thiosulfate in this growth phase would likely have occurred through abiotic formation [13] as in the control medium, although formation as a result of bacterial actions cannot be completely excluded.

After 18 h and the depletion of sulfide, thiosulfate concentrations ceased to increase, and from 22 h began to decrease steadily. Sulfate was then produced in significant amounts, increasing to 41 mM at 34 h. Sulfur formed between 16-22 h also decreased in concentration to levels around 0.12-0.15 mM before increasing again to 0.62 mM at 34 h, when thiosulfate had become exhausted.

Unexpectedly, a similar experiment carried out with *T. aerophilum* also showed a complex oxidation pattern with intermittent formation of sulfur (Fig. 3) and two growth phases that appeared to depend on sulfide and thiosulfate, respectively. OD₆₀₀ values increased steadily from the start of the experiment, but spiked up sharply at 10 h to values of about 0.6, before returning to ~0.17 at 16 h and remaining steady thereafter. During the initial sulfide-dependent growth phase, sulfide concentrations were stable between 0-6 h, followed by very rapid oxidation, with concentrations dropping to zero by 11 h. Thiosulfate was formed while sulfide was being oxidized (max conc. 7.6 mM at 10 h) and formation

mimicked the sulfide oxidation rates, with thiosulfate formation rates picking up after 8 h. Again, thiosulfate formation could have been caused by abiotic and/or biotic processes. Sulfite was present between 2 and 14 h at concentrations between 0.25 and 0.6 mM. Unexpectedly, as sulfide concentrations fell below 3 mM after 8 h, the formation of sulfur (up to 1.44 mM at 11 h) was observed, accompanied by the characteristic increase in the OD₆₀₀ values.

As sulfide became exhausted, again a second, thiosulfate-dependent growth phase began. Thiosulfate concentrations decreased from 11 h, with sulfate concentrations steadily increasing (up to ~30 mM after 16 h) as reported above for thiosulfate-containing media. The accumulated sulfur also quickly disappeared, dropping to 0.17 mM at 18 h, and further to 0.08 mM after 24 h.

3.4. Expression of sulfur oxidation genes in *T. versutus* and *T. aerophilum*

Genes involved in sulfur oxidation processes in both species were identified using database searches; detailed results including e-values and similarity values are provided in Table S2.

In *T. Aerophilum*, the core *sox* genes *soxXYZA* (Thiae_RS01260-01245), *soxB* (Thiae_RS04690) and *soxCD* (Thiae_RS00340and00345) were found in three separate genomic loci, in addition to two genes encoding putative flavocytochrome-*c*-like sulfide dehydrogenases ('SoxF' -F1: Thiae_RS00020, F2: Thiae_RS08945) with a third gene (F3: Thiae_RS02750) having lower similarity to *soxF*, probably encoding another type of flavin-containing enzyme. Among the accessory Sox proteins (SoxSRT, SoxVW and SoxGH) described for *Paracoccus pantotrophus* GB17 [8], only *soxW* (Thiae_RS06460) and genes encoding *soxH* homologues (Thiae_RS08565 and 09100) were identified. The genome also

contained two putative sulfide:quinone reductase genes of the SqrB (Thiae_RS00415) and SqrF (Thiae_RS03155) type described in [11].

No homologues of dissimilatory sulfite reductase (Dsr), APS reductase, sulfite dehydrogenase, heterodisulfide reductases, DMSO reductase or DMS dehydrogenase-encoding genes were present. These data confirm that *T. aerophilum* oxidizes reduced sulfur compounds using the Sox pathway and has the ability to produce ‘sulfur’ from sulfide (as observed during growth experiments on sulfide) via either a flavocytochrome *c* or sulfide:quinone reductase. We also identified genes (Thiae_RS06510-06535, annotated as Rsx complex) encoding proteins related to the sodium-pumping NADH-oxidizing Rnf complex found in sulfate-reducing bacteria [25]. We did not find homologues of other membrane complexes such as Qmo-type heterodisulfide reductase complexes.

Expression of the sulfur oxidation genes in *T. aerophilum* was analyzed by qRT-PCR using RNA isolated from cultures grown on sulfide and thiosulfate-containing medium (Fig.4). The data clearly show that expression of key *sox* genes (*soxA*, *soxB* and *soxY*) was high and did not vary greatly in response to changes in the sulfur source present in the growth medium. However, the data also demonstrate that, of the three candidate *soxF*-encoding genes, only *soxF1* shows significant expression; similarly, of the two SQR-encoding genes, only *sqrB* showed relatively high expression, with gene expression in sulfide-containing medium nearly double that observed for thiosulfate-containing medium (Fig.4). These data thus confirm that *T. aerophilum* expresses all genes required for oxidation of thiosulfate and sulfide constitutively, including genes encoding enzymes that can lead to formation of sulfur from sulfide.

There is currently no published genome for *T. versutus* AL2, but preliminary genomic data is available via the Integrated Microbial Genomes tool (img.jgi.doe.gov, accessed Dec 5, 2016). We analyzed these data to identify candidate genes encoding sulfur-oxidizing enzymes

(Table S2). We were able to identify putative genes encoding components of the Sox pathway (*soxA*, *soxX*, *soxY*, *soxZ*, and *soxB*), but were unable to identify homologues of *soxC*, *soxD* or the *dsr* genes encoding the dissimilatory sulfide reductase. Instead, we found homologues of genes encoding a heterodisulfide reductase-like complex and an SqrB-type sulfide:quinone reductase, as well as genes encoding an Rnf-like complex and two molybdenum enzymes of the sulfite oxidase family (*sorT*-like sulfite dehydrogenase and *yedY*-like put. sulfoxide reductase) (Table S2). Most genes were present in at least two copies.

Heterdisulfide reductases were first discovered in methanogens, where they catalyze the reversible reduction of a heterodisulfide of the two sulfur-containing coenzymes M and coenzyme B via a special mechanism called electron bifurcation [12, 3]; however, it has since been recognized that many of the energy-generating complexes in sulfate-reducing and sulfur-oxidizing bacteria (*dsr*, *qmo*, *qrc*) contain subunits related to heterodisulfide reductase complexes [25]. It will thus be necessary to investigate the exact function of the Hdr-like proteins in *T.versutus*.

During growth on sulfide and thiosulfate of *T.versutus*, high expression of the *sox* genes and the two *hdr* complexes was observed (Fig.4 and Figs. S2, S3). Gene expression levels were similar on the two substrates; only *soxA* and *soxB* showed higher expression during growth on sulfide. The *sqr* genes, Mo-enzymes *yedY* and *sorT* and the *rnf* complex were also expressed, although expression levels for *rnf* were low. The data indicate that, as observed for *T. aerophilum*, genes essential to energy generation by chemolithoautotrophy do not undergo significant regulation in *T.versutus* in response to changing sulfur substrates.

3.5. Activity of sulfide- and sulfite-converting enzymes in *T. aerophilum* and *T. versutus*

To confirm the data above, we determined enzymatic activities for different sulfide- and sulfite-converting enzymes. Cell extracts were prepared from cultures grown on

thiosulfate-containing medium. Sulfite oxidizing activity was present in *T.versutus*, with 0.429 ± 0.007 U/mg, compared to background levels of only 0.023 ± 0.006 U/mg in *T. aerophilum* at pH 9, providing an explanation for the accumulation of relatively high levels of sulfite observed in *T. aerophilum* cultures growing on thiosulfate, and matching the lack of genes encoding these enzymes in *T. aerophilum*. Sulfite-oxidizing activity in *T.versutus* was highest at pH 9 (pH 8: 0.336 ± 0.015 U/mg; pH 10: 0.404 ± 0.019 U/mg), with a $V_{\max}$ of 0.461 ± 0.022 U/mg and a K_{Msulfite} of 0.15 ± 0.023 mM (Fig. S4), which, interestingly, is an order of magnitude higher than the substrate affinity (K_{Msulfite} of 0.011 ± 0.001 mM) of the related SorT sulfite dehydrogenases from neutrophilic *Sinorhizobium meliloti* at the same pH [38, 20]. Activities of sulfide dehydrogenase (flavocytochrome *c*) were similar in both *T.versutus* and *T. Aerophilum*, with 69.8 ± 1.7 mU/mg and 86 ± 5.5 mU/mg, respectively, when assayed in the presence of 10 μ M sulfide at pH 9.

These results confirm data obtained from the growth curves and gene expression analyses.

3.6. Growth of ASOB on organo-sulfur compounds and in the presence of reduced carbon compounds

In addition to inorganic sulfur compounds, organosulfur and organic carbon compounds may be present in the natural habitats of ASOB species, and we tested the ability of *T. aerophilum* and *T.versutus* to metabolize either of these compounds. These experiments, which used small-scale cultures (2-20 ml), were repeated at least three times. No growth of either species was obtained with dimethylsulfide, dimethylsulfoxide or beta-mercaptoethanol.

Inclusion of organic carbon sources such as glucose, fructose, acetate and propionate in thiosulfate-based growth medium had little effect on growth; however, addition of propionate (10 and 20 mM) increased the final OD₆₀₀ of *T.versutus* cultures by ~ 50% (Fig. 5 and S5), and a similar effect was seen for 2 mM acetate, while high concentrations of fructose

inhibited growth. As, in this experiment OD₆₀₀ values were only recorded at 24 h intervals, the intermittent formation of sulfur for *T. versutus* was not observed in the OD₆₀₀ readings, as would have occurred between sampling times. For *T. aerophilum* only low concentrations of fructose (2 and 5 mM) led to an increase in the final OD₆₀₀ (Fig. 5 and S5). Although only limited carbon sources showed an effect on *T.versutus* and *T. aerophilum* growth, these results suggest that both ASOB species are probably capable of limited carbon co-assimilation, a phenomenon that has been documented for the also obligately autotrophic phototrophic green sulfur bacteria [14, 28, 24]. To the best of our knowledge, this is the first time this has been documented in detail for these two species of ASOB. If additional carbon is assimilated by chemolithoautotrophs, this can result in lower rates of sulfur oxidation and carbon dioxide fixation due to the lowered cellular energy demand resulting from the presence of reduced carbon [14]. While such an effect was not seen in *T. aerophilum* grown on thiosulfate medium supplemented with 2 mM fructose (Fig. 4), in *T.versutus* a clear reduction in expression of both sulfur oxidation genes and carbon dioxide fixation genes (*rbcL* encoding RuBisCo) was observed when the medium was supplemented with acetate (Fig. 4).

4. Discussion

In this work, we investigated the growth of two ASOB species on different sulfur and carbon compound combinations using physiological, molecular and physical data. The data reveal that, while *T. aerophilum* and *T. versutus* have distinct mechanisms for oxidizing sulfur compounds, as reflected in the type of sulfur-oxidizing enzyme systems present in each species and the clear differences in sulfur species formed during growth on thiosulfate, they showed very similar oxidation patterns for sulfide involving formation of elemental sulfur.

Our data also provide evidence for the co-assimilation of specific reduced carbon sources by both species of ASOB. The exact mechanism of co-assimilation will require further investigation; however, the combination of increases in culture density in the presence of specific carbon sources for both *T. aerophilum* and *T. versutus* with reduction in the expression of genes for carbon assimilation (*rbcL*) and sulfur oxidation (*soxA*, *soxB*, *soxY*) in *T. versutus* clearly suggests that both ASOB are able to assimilate small amounts of reduced carbon. A reduction in sulfur oxidation activity in the presence of acetate or propionate has previously been reported for species of green sulfur bacteria (GSB) grown in a bioreactor [14], and carbon co-assimilation is a well-known phenomenon in GSB [28]. An ability to assimilate small amounts of acetate or yeast extract is, in fact, referred to in the original species description of *T. aerophilum* as a property of some isolates [32], further supporting our results, although, for this specific strain, we were unable to find a positive effect of acetate on growth and instead found a positive effect of fructose.

The data presented form a coherent picture of sulfide and thiosulfate oxidation in the two species investigated, which allows generation of general models of sulfur oxidation in *T. aerophilum* and *T. versutus* (Fig. 6).

Genomic and physiological analyses confirm that *T. aerophilum* uses a fairly typical Sox pathway for sulfur oxidation with all four major enzymes (SoxAX, SoxYZ, SoxB,

SoxCD) present, while many of the accessory components described for *P. pantotrophus* being absent. Also absent are typical sulfite-oxidizing enzymes such as SorAB and SorT-like sulfite dehydrogenases [20, 15, 16] or the alternative SoeABC enzyme [4], which, together with the absence of appreciable levels of enzyme activity in cell extracts, explains the accumulation of relatively high amounts of sulfite in the *T. aerophilum* growth medium. An interesting observation is the transient formation of sulfur in the growth medium during growth of *T. aerophilum* on sulfide. This had been previously observed for *Thioalkalimicrobium* ssp. cultures grown in the presence of polysulfides, or under oxygen limitation when both thiosulfate and sulfide were present [32]. The culture conditions used here were microaerophilic, the preferred growth condition for *T. aerophilum* strain AL3 [32], and some polysulfides may have been present in the sulfide stock solution that would explain ‘sulfur’ formation. However, while several enzymes that can participate in formation of ‘sulfur’ are present in *T. aerophilum* (Sqr, SoxF-type flavocytochromes), it is not entirely clear how the ‘sulfur’ that is formed is further oxidized. One can speculate that these ‘sulfur’ molecules may be fed back into the Sox pathway, becoming linked to SoxYZ and then oxidized; however, it is unclear as to what chain length such a mechanism would be effective and how the reaction linking the ‘sulfur’/polysulfide chains to the SoxYZ protein would take place. The SoxAX enzyme is generally known to catalyze formation of a heterodisulfide bond between thiosulfate and a conserved cysteine residue present in the SoxY protein [17], but it is not known whether polysulfides would also serve as substrates for SoxAX.

In contrast, *T. versutus* also contains genes encoding three of the core *sox* pathway proteins, SoxAX, SoxYZ and SoxB, but lacks genes encoding a SoxCD protein. This is similar to what has been described for sulfur-oxidizing phototrophs such as *Allochromatium vinosum*, that use the Dsr/Sox sulfur oxidation pathway to oxidize reduced sulfur compounds [5, 9]. However, the *T. versutus* genome lacks genes encoding the Dsr enzyme system that is

typically found in bacteria such as *A. vinosum*, leaving it unclear as to how the polysulfides produced by the incomplete Sox system are further oxidized. Based on our analysis, it seems possible that heterodisulfide reductase-like complexes may be taking over the function of the Dsr system in *T. versutus*. In fact, AvDsrA and the putative TvHdrA proteins share some homology, especially in the C-terminal regions of DsrA, as revealed by BLAST searches which also confirmed the general absence of genes encoding Dsr proteins in *Thioalkalivibrio* species.

We also briefly analyzed the recently released *T. versutus* D301 genome [21] for genes involved in sulfur oxidation, and did not identify *dsrA* or *aprA* genes, but found two copies of *soxB*, as well as *soxA*, *soxX*, *soxY*, *soxZ*, *soxF* and genes for two *hdrABC* complexes. In addition, homologues of RnfA, RsxE and QmoA of an additional membrane complex implicated in electron transfer [25] were present. This is in agreement with data retrieved from the draft *T. versutus* AL2 genome, and suggests that alkaliphilic sulfur oxidizers of the genus *Thioalkalivibrio* use sulfur oxidation pathways that involve currently unstudied enzyme systems and that differ from the well described Sox and Dsr/Sox pathways found mainly in neutrophilic sulfur-oxidizing bacteria. In future work, the exact function of the heterodisulfide reductase-like complexes in *T.versutus* species will need to be experimentally verified to confirm functional predictions made here.

An interesting difference between the well documented Dsr/Sox pathway and the sulfur oxidation pathway operating in *T.versutus* AL2 is the timing of sulfur formation. In the purple sulfur bacterium *Allochromatium vinosum*, which oxidizes sulfur compounds via the Dsr/Sox pathway, formation of elemental sulfur from sulfide and thiosulfate started immediately and concentrations increased until sulfide or thiosulfate had been used up [7]. In contrast, in *T.versutus*, accumulation of sulfur in the growth medium was only observed when sulfide or thiosulfate had been nearly used up. A similar sulfur production pattern was reported in 1983

for a species of *Ectothiorhodospira*, which is closely related to *T.versutus* [34], and provides some indication that the sulfur oxidation pathways in these two species are similar and do not involve a dissimilatory sulfite reductase.

Another interesting discovery was that sulfide oxidation mechanisms in both *T. aerophilum* and *T.versutus* appeared to be similar and were characterized by an initial lag in sulfide utilization, followed by rapid sulfide oxidation and formation of 'sulfur'. Very low levels of sulfate formation were observed in both cases, which is in keeping with incomplete oxidation of the sulfide to 'sulfur'. Thiosulfate was also formed during sulfide oxidation, but this was likely due to abiotic processes also occurring in the control experiment [13]. In both species, thiosulfate oxidation did not proceed before sulfide was completely exhausted, which could indicate an inhibitory effect of sulfide on the thiosulfate-oxidizing systems.

As no clear differential regulation of sulfur oxidation genes was observed for either *T. aerophilum* or *T.versutus*, it is at present not possible to clearly determine which enzyme systems are most important during this growth phase, although the likely candidates would be sulfide: quinone reductases or flavocytochromes, which showed slight increases in gene expression in the presence of sulfide.

Taken together, our results clearly indicate that alkaliphilic sulfur oxidation is a complex process, and several of the enzymes involved have not yet been unambiguously identified. Important questions for further investigation are how sulfur oxidation proceeds in *T. versutus* in the absence of the Dsr proteins, that have been studied in detail in *A. vinosum* [37], and how sulfur is formed and oxidized in *T. aerophilum* during growth on sulfide in the absence of proteins involved in sulfur mobilization from polysulfides.

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Conflict of interest

459 The authors declare no conflict of interest.

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577 **Fig. 1.** Average respiratory rates of *T. versutus* AL2 (**Panel A**) and *T. aerophilum* AL3
 578 (**Panel B**) with thiosulfate. Respiratory rates were determined using a Hansatech oxygen
 579 electrode; the bacteria were washed and resuspended in medium 925 mineral base without
 580 any added sulfur sources. Errors represent standard deviation of three repeat measurements.

581 **Fig.2.** Growth of *T. aerophilum* AL3 and *T. versutus* AL2 on thiosulfate. Panel A: *T.*
 582 *aerophilum*, 80 mM thiosulfate. Panel B: *T. versutus* 40 mM thiosulfate. Left: OD₆₀₀ values
 583 and protein content (shown as x10 for clarity), Right: sulfur speciation.

584 **Fig.3.** Growth of *T. aerophilum* AL3 and *T.versutus* AL2 on sulfide. Panel A: *T. aerophilum*,
 585 20 mM sulfide. Panel B: *T. versutus* 40 mM sulfide. Left: OD₆₀₀ values and protein content
 586 (shown as x10 for clarity), Right: sulfur speciation.

587 **Fig. 4.** Expression of genes involved in sulfur oxidation and carbon assimilation in
 588 *T.aerophilum* (Panel A) and *T. versutus* (Panel B). cDNA for use in these experiments was
 589 isolated from culture growing on thiosulfate, thiosulfate and fructose (*T. aerophilum*),
 590 thiosulfide and acetate (*T. versutus*) or sulfide. Gene expression data were normalized against
 591 *gyrA* expression. All samples were analyzed in triplicate; error bars show standard
 592 deviations of the measurements.

593 **Fig. 5** Normalized growth of *T. aerophilum* and *T. versutus* on thiosulfate-containing medium
 594 in batch cultures supplemented with either fructose (*T. aerophilum*) or propionate (*T.*
 595 *versutus*). Growth data were recorded as OD₆₀₀ and normalized to the maximum value
 596 obtained on medium containing only thiosulfate and no carbon source. The graph shows
 597 normalized, averaged data from experiments carried out on different days, with the standard
 598 error being shown for each datapoint.

599 **Fig. 6.** Model of sulfur oxidation in *T. aerophilum* and *T. versutus*. Only key reactants and
600 products are shown for each reaction. Protein names are marked; proteins marked with only a
601 single letter are Sox proteins (A- SoxA, B-SoxB etc.). M/UQ: mena-/ubiquinone; P =
602 periplasmic space, C = cytoplasm. Dots in proteins indicate the presence of metal centers;
603 dashes with square symbols represent heme groups.

Fig.

Fig. 1

614

A *Tm.aerophilum*

B *Tv. versutus*

sulfur species

sulfur species

615 ○ sulfide [mM] ■ thiosulfate [mM] ▼ sulfite [mM] ◆ sulfate [mM] × sulfur [mM]

616 Fig. 2

617

618

619

A *Tm.aerophilum* B *Tv. versutus*

620 ○ sulfide [mM] ■ thiosulfate [mM] ▼ sulfite [mM] ◇ sulfate [mM] × sulfur [mM]

621 Fig. 3

622

623

A *Tm.aerophilum*

B *Tv. versutus*

Fig. 4

Fig. 5

633

A *Tm. aerophilum*

B *Tv. versutus*

634

635 Fig. 6

636