

³⁴S enrichment as a signature of bacterial chemolithotrophic thiosulfate oxidation

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Abstract

Kinetics of thiosulfate oxidation, product and intermediate formation, and ³⁴S fractionation, were studied for the alphaproteobacteria *Paracoccus* sp. SMMA_5 and *Mesorhizobium thioanganeticum* SJT^T, the betaproteobacterium *Pusillimonas ginsengisoli* SBO3, and the gammaproteobacterium *Thermithiobacillus* sp. SMMA_2, during chemolithoautotrophic growth in minimal salts media supplemented with 20 mM thiosulfate. The two alphaproteobacteria oxidized thiosulfate directly to sulfate, progressively enriching the end-product with ³⁴S; $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values recorded at the end of the two processes (when no thiosulfate was oxidized any further) were -2.925 ‰ and -3.520 ‰ respectively. The beta- and gammaproteobacterium, on the other hand, oxidized thiosulfate to sulfate via tetrathionate intermediate formation, with progressive ³⁴S enrichment in the end-product sulfate throughout the incubation period; $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$, at the end of the two processes (when no further oxidation took place), reached -3.486 ‰ and -3.767 ‰ respectively. Based on similar ³⁴S fractionation patterns recorded previously during thiosulfate oxidation by strains of *Paracoccus pantotrophus*, *Advenella kashmirensis*, and *Thiomicrospira crunogena*, it was concluded that progressive reverse fractionation, enriching the end-product sulfate with ³⁴S, could be a characteristic signature of bacterial thiosulfate oxidation.

Key Words: chemolithotrophic sulfur oxidation, bacterial thiosulfate oxidation, stable sulfur isotope fractionation, ³⁴S enrichment, reverse fractionation.

Introduction

In all natural samples, relative abundance of the stable isotopes of any element differs from their averaged global ratio due to mass-dependent fractionations during biotic or abiotic transformations (Canfield 2001; West *et al.* 2006; Hoefs 2009). For the same reason, within a given system, stable isotope ratios also vary between coexisting phases of the same element. Generally, lighter isotopes of individual elements get enriched in reaction products because bonds involving such atomic species are broken down more easily as they partition most of the bond energy to vibrational, rather than translational, modes (Canfield 2001). In case of the element sulfur - which constitutes ~5 % of the Earth's iron core (Emsley 2001) and is also central to the potential geothermal origin of life/metabolism on Earth (Wächtershäuser, 1990; Wächtershäuser, 1992) - ^{32}S , globally, is the most abundant (95 %) isotope, followed by ^{34}S (4.2 %), ^{33}S (0.75 %) and ^{36}S (0.02 %). Most of the information available on microbial sulfur isotope fractionation comes from the metabolisms involving reductive transformations or disproportionation of sulfur species (Kaplan and Rittenberg 1964; Canfield and Thamdrup 1994; Smock *et al.* 1998; Surkov *et al.* 2000; Canfield 2001). In general, small amounts of fractionations ($\Delta^{34}\text{S}$ 0.9-2.8 ‰ VCDT) are associated with assimilatory sulfate reduction (Kaplan and Rittenberg 1964). In contrast, dissimilatory sulfate reduction by phylogenetically diverse microorganisms render 2 ‰ to 46 ‰ VCDT fractionations (Canfield 2001; Detmers *et al.* 2001). Fractionations of -0.6 ‰ to 37 ‰ have been recorded during disproportionation of S^0 , SO_3^{2-} or $\text{S}_2\text{O}_3^{2-}$ by a variety of pure, or enriched consortium, cultures (Canfield and Thamdrup 1994; Canfield *et al.* 1998; Habicht *et al.* 1998; Böttcher and Thamdrup 2001).

Oxidative metabolisms of sulfur reportedly yield little (-4.3 to 2.5 ‰ VCDT) or no fractionation; these include (i) phototrophic conversion of S^{2-} to S^0 , and S^0 to sulfate, by *Chlorobaculum parvum*, *Chlorobium vibrioforme*, *Chromatium vinosum* (Fry *et al.* 1984; Fry *et al.* 1985; Fry *et al.* 1988; Kelly 2008a); (ii) chemotrophic oxidation of S^{2-} by *Thiobacillus concretivorus* where S^0 is the main, and SO_4^{2-} and $\text{S}_x\text{O}_6^{2-}$ are the minor, end-products (Kaplan and Rittenberg 1964); (iii) chemotrophic oxidation of S^{2-} to SO_4^{2-} via nitrate-respiration by *Thiobacillus denitrificans* and *Sulfurimonas denitrificans* (Poser *et al.* 2014). Overall fractionation is also quite low (0 to 2.9 ‰ VCDT) during chemolithotrophic oxidation of the widely used substrate thiosulfate by different bacteria (Fry *et al.* 1985; Fry *et al.* 1986; Kelly 2008b). However, a previous study focused on the kinetics of sulfur isotope fractionation, and comparison of such time-course trends recorded for the phenotypically distinct mechanisms of chemolithotrophic thiosulfate ($\text{S}_2\text{O}_3^{2-}$) oxidation by the phylogenetically-diverse proteobacteria *Paracoccus pantotrophus*, *Advenella*

kashmirensis and *Thiomicrospira crunogena*, had revealed that the sulfates produced over time were progressively enriched in ^{34}S in all these processes (Alam *et al.* 2013). In the present study we testify whether progressive enrichment of ^{34}S in the produced sulfate is a phenomenon characteristically associated with thiosulfate oxidation across mechanistic and taxonomic spectra. While the test organisms included the alphaproteobacteria *Paracoccus* sp. SMMA_5 and *Mesorhizobium thioanganeticum* SJT^T, the betaproteobacterium *Pusillimonas ginsengisoli* SBO3, and the gammaproteobacterium *Thermithiobacillus* sp. SMMA_2, the data indicated that steady reverse fractionation was indeed typical of bacterial thiosulfate oxidation.

Materials and methods

Test organisms and growth conditions.

Four laboratory isolates, which have distinct taxonomic affiliations (across the class *Proteobacteria*) and which oxidize thiosulfate to sulfate either directly or via formation of tetrathionate as an intermediate, were tested for their ^{34}S fractionation trends. Of the four test organisms, the alphaproteobacterium *Mesorhizobium thioanganeticum* SJT^T was isolated previously on MS-based, thiosulfate- and yeast-extract-containing, mixotrophic medium, from rhizosphere soil of an Indian tropical leguminous plant (Ghosh and Roy 2006), the other three strains tested in this study are all current isolates of our laboratory. The second alphaproteobacterial strain, *Paracoccus* sp. SMMA_5 was isolated on MS-based, thiosulfate-containing, autotrophic medium, from an 80°C water-sample of a Trans-Himalayan sulfur-borax spring called *Lotus Pond*, located in the Puga geothermal area of Ladakh, India. The third test organism, *Thermithiobacillus* sp. SMMA_2, is a gammaproteobacterium that was also isolated from *Lotus Pond* in the same medium as SMMA_5; however, SMMA_2 was obtained from water-samples collected from the 30-42°C interface that *Lotus Pond*'s spring-water outflow forms with the water of an adjacent river. The fourth test organism, the betaproteobacterium *Pusillimonas ginsengisoli* SBO3 was isolated on MS-based, thiosulfate- and yeast-extract-containing mixotrophic medium, from an Arabian Sea sediment sample collected from 275 cm below the sea-floor, at a water-depth of 530 m below the sea-level, off the west coast of India.

To test their thiosulfate oxidation kinetics and ^{34}S fractionation trends, all the four strains were grown in chemolithoautotrophic MST medium comprising basal mineral salts and thiosulfate (Davidson and Summers 1983; Ghosh *et al.* 2005; Alam *et al.* 2013). Each liter of MST (pH 7.0) contained 1 g NH_4Cl , 4 g K_2HPO_4 , 1.5 g KH_2PO_4 , 0.5 g MgCl_2 , 5.0 ml trace metals solution (Vishniac and Santer, 1957), 10 ml vitamin mixture (Himedia, India) and 20 mM $\text{Na}_2\text{S}_2\text{O}_3$. All the

sulfate salts that are parts of standard mineral salts and trace metals solution were replaced by their chloride counterparts so as to ensure that the sulfates recovered from spent medium samples were unambiguously the products of thiosulfate oxidation. Initially, all the bacteria were grown in Luria broth medium up to mid-log phase to prepare seed inocula. Washed cells were then inoculated into chemolithoautotrophic media. All the cultures under investigation were incubated in a rotary shaker (150 rpm) at 28°C.

Analytical methods and isotope ratio mass spectrometry (IR-MS).

Thiosulfate, tetrathionate and sulfate concentrations were measured in spent medium samples by iodometric titration, cyanolytic method and gravimetric precipitation respectively (Kelly and Wood 1994; Alam *et al.* 2013). For this, 10 ml spent medium was collected at the incubation time-point of interest and centrifuged at 10,000 rpm for 5 minutes to pellet-down bacterial cells; the supernatant was used for the aforesaid analyses. For iodometric titration, 3.0 g KI was first dissolved in a solution containing 10 ml 0.01 N $K_2Cr_2O_7$, 10 ml HCl and 49 ml H_2O in a 1000 ml conical flask. After mixing the solution well, the flask was covered with a watch glass and incubated in the dark for 5-10 minutes. Subsequently, 230 ml H_2O and 1ml of 1 % starch solution was added to it and mixed thoroughly. When the solution became blue after adding starch, 1 ml sample was added and the mixture was titrated with 0.01 N sodium thiosulfate. Concentration of thiosulfate was calculated by plotting burette readings onto standard curve prepared using 0, 2, 4, 8, 12, 16 and 20 mM thiosulfate solutions.

To quantify tetrathionate in a spent medium sample via cyanolytic method, a reaction was carried out where 0.1 ml of a three-fold-diluted sample (containing $\leq 0.8 \mu\text{mol}$ tetrathionate) was added to 0.8 ml cyanolytic buffer (50 ml 0.2 M NaH_2PO_4 and 39 ml 0.2 M NaOH; pH 7.4) and the volume was adjusted to 2.0 ml using sterile double-distilled water. The mixture was chilled on ice for 20 minutes, following which 1 ml 0.1 M chilled potassium cyanide was added to it and mixed rapidly. This mixture was then incubated on ice for another 20 minutes. Finally, 0.6 ml ferric nitrate reagent was added to the mixture via uninterrupted agitation [ferric nitrate reagent contained 30.3 g $Fe(NO_3)_3 \cdot 9H_2O$ in 21.7 ml 72 % perchloric acid; final volume of the reagent was adjusted to 50 ml with double-distilled water]. The mixture was allowed to warm to room temperature until the white precipitate (if any) re-dissolved, its volume was made up to 5 ml, and absorbance of the ferric thiocyanate formed was measured at 460 nm using a Cary 100 UV-visible spectrophotometer (Agilent, USA). Concentration of tetrathionate in the samples was calculated from a standard curve prepared using 0.1 ml solutions containing 0, 50, 100, 200, 400, 600 and 800 and 1000 nmol tetrathionate.

Dissolved sulfate was precipitated from spent medium samples in the form of barium sulfate (BaSO_4). 5 ml medium aliquot was taken in a fresh tube and heated at 90°C for 20 minutes after acidification (to pH 2.0) with dilute HCl. Excess of preheated 0.05 M barium chloride (BaCl_2) was added to this, via continuous stirring. The BaSO_4 precipitate formed in this way was made free from other potential sulfate salts by digesting the mixture at 90°C for 2 h. Subsequent to this, BaSO_4 was collected by passing the entire mixture through fine-pore ash-less Whatman no. 42 filter paper; it was washed thrice with dilute HCl and re-filtered as stated above. The moist filter paper was folded and then subjected to prolonged dehydration in uncovered crucibles. Fully dry BaSO_4 powder was eventually collected and weighed for isotopic analysis after burning the ash-less filter paper away.

$\delta^{34}\text{S}$ value for a BaSO_4 sample (obtained as above), or the $\text{Na}_2\text{S}_2\text{O}_3$ stock used in the MST medium as chemolithotrophic substrate, was measured using a DeltaVPlus isotope ratio mass spectrometer (IR-MS) attached with an EA1112 elemental analyzer (both from Thermo Fisher Scientific, USA), as described previously (Alam *et al.*, 2013). Accuracy of all the $\delta^{34}\text{S}$ values reported was checked via three replica tests on the IR-MS; reproducibility in every case was better than ± 0.3 ‰. Fractionation (Δ) was expressed as the difference in isotope ratio between (A) the substrate thiosulfate and (B) the product sulfate ($\Delta_{\text{A-B}} = \delta^{34}\text{S}_{\text{A}} - \delta^{34}\text{S}_{\text{B}}$).

Results and Discussion

Thiosulfate oxidation kinetics and ^{34}S fractionation trends in the alphaproteobacteria

Total amount of thiosulfate oxidized by the two alphaproteobacterial strains included in this study were found to be less than what is oxidized by other well-studied sulfur-chemolithotrophs belonging to this class (Alam *et al.* 2013; Lahiri *et al.* 2006). When grown in MST broth, *Paracoccus* sp. SMMA_5 and *Mesorhizobium thioangeticum* SJT^T could oxidize only 45 % (equivalent to 18 mM S) and 60 % (equivalent to 24 mM S) of the 40 mM S thiosulfate supplied to corresponding amounts of sulfate, over incubation periods of 48 h and 60 h, respectively. For both bacteria, the residual thiosulfate remained unutilized even after prolonged incubation beyond the aforesaid time periods (Figure 1 A and C).

The end-product sulfate harvested from the SMMA_5 and SJT^T cultures at different time-points of incubation in MST medium were found to be progressively enriched in ^{34}S ; corroboratively, negative $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values were observed throughout the oxidation period (Figure 1 B and D). For SMMA5 and SJT^T, $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values of the first-formed sulfate (detected and harvested after 6 and 12 h of incubation respectively) were -1.599 ‰ and -0.977 ‰ respectively. For both the alphaproteobacteria, $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values decreased steadily with

further incubation, and reached -2.925 ‰ and -3.520 ‰ at the end of the incubation, i.e. after 72 h and 84 h, respectively (Figure 1 B and D).

Thiosulfate oxidation kinetics and ^{34}S fractionation trends in the beta- and gammaproteobacteria

Pusillimonas ginsengisoli SBO3 and *Thermithiobacillus* sp. SMMA2 oxidized thiosulfate to sulfate via tetrathionate-intermediate formation; furthermore, in these two taxonomically distinct bacteria, the end-product sulfate and the intermediate tetrathionate both starts forming from the beginning of their incubations in MST. However, the betaproteobacterium SBO3 is distinguished from the gammaproteobacterium SMMA2 on account of having lower rate and extent of tetrathionate formation from thiosulfate. The strain SBO3, in the first 36 h of incubation in MST, converted 60 % (equivalent to 24 mM S) of the supplied 40 mM S thiosulfate to 14 mM S tetrathionate and 10 mM S sulfate. This was accompanied by a decrease in the pH of the medium to 6.2 from the initial level of 7.0 (Figure 1E). After a total incubation period of 72 h, pH of the spent medium came down to 5.5, and no thiosulfate was left in the medium while 36 mM S sulfate and 4 mM S tetrathionate were there (Figure 1E); this residual tetrathionate did not get oxidized even after prolonged incubation beyond 72 h. The strain SMMA2, on the other hand, converted the entire thiosulfate supplied in the MST medium, to 29 mM S tetrathionate and 11 mM S sulfate, in the first 24 h of incubation; this was accompanied by elevation of the pH of the spent medium to 8.0 from the initial level of 7.0 (Figure 1G). After total 48 h incubation, pH of the medium came down to 4.5, and 30 mM S sulfate formed alongside 10 mM S tetrathionate (Figure 1G); this residual tetrathionate remained unutilized even after prolonged incubation beyond 48 h. The above phenotypes illustrated that the thiosulfate oxidation process of *Pusillimonas ginsengisoli* SBO3 is likely to be mechanistically different from not only that of the *Thermithiobacillus* strain SMMA2, but also other tetrathionate-intermediate-forming betaproteobacteria such as *Advenella kashmirensis* (Ghosh *et al.* 2005) where sulfate formation during incubation in MST does not start until 50 % (20 mM S) of supplied 40 mM S thiosulfate is converted to tetrathionate (Pyne *et al.* 2017; Pyne *et al.* 2018).

When $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values were determined for the sulfate harvested from the SBO3 and SMMA2 cultures at different time-points of incubation in MST, progressive enrichments of ^{34}S in the end-product sulfate with associated negative $\Delta^{34}\text{S}$ values were observed throughout the oxidation period (Figure 1 F and H). For SBO3 and SMMA2, $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values of the first-formed sulfate, detected and harvested after 12 h of incubation, were -0.555 ‰ and -0.235 ‰ respectively. For the two bacteria, $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values decreased steadily with further incubation, and reached

-3.486 ‰ and -3.767 ‰ at the end of the incubation, i.e. after 102 h and 48 h, respectively (Figure 1 F and H).

Concluding remarks

The extent and pattern of stable isotope fractionation in any biochemical process depend on the rate of the process, and the nature of the enzymes and the number of steps involved in it (Canfield 2001). A previous study of our laboratories corroborated that this principle was applicable to chemolithotrophic thiosulfate oxidation as the betaproteobacterium *Advenella kashmirensis* and the gammaproteobacterium *Thiomicrospira crunogena* shared a similar ³⁴S fractionation pattern with the alphaproteobacterium *Paracoccus pantotrophus*, and SoxB was the common enzyme responsible for S-S bond breaking in the three phylogenetically distinct bacteria (Alam *et al.* 2013).

The present study based on another *Paracoccus* strain SMMA5 (that is distantly related to *P. pantotrophus*), together with the alphaproteobacterium *Mesorhizobium thiogangeticum* SJT^T, the betaproteobacterium *Pusillimonas ginsengisoli* SBO3 and the gammaproteobacterium *Thermithiobacillus* sp. SMMA2, revealed that these four organisms, throughout their thiosulfate oxidation processes, exhibited the same pattern of ³⁴S fractionation as the one observed for *A. kashmirensis*, *T. crunogena* and *P. pantotrophus* (notably, however, the range of fractionation rendered over time was not same for the individual organisms tested). It, therefore, appears that progressive reverse fractionation enriching the end-product sulfate with ³⁴S is a universal signature of bacterial chemolithotrophic thiosulfate oxidation, irrespective of whether the pathway is via tetrathionate-intermediate formation or not; and in all such processes SoxB is apparently the key S-S bond hydrolyzing enzyme functioning in conjunction with other proteins.

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Conflict of interest

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1. Kinetics of thiosulfate oxidation and corresponding ^{34}S fractionation by *Paracoccus* sp. SMMA5 (A and B), *Mesorhizobium thiogangeticum* SJT^T (C and D), *Pusillimonas ginsengisoli* SBO3 (E and F) and *Thermithiobacillus* sp. SMMA2 (G and H) during growth in MST medium. —■—, —□— and —●— denote mM S present in the spent medium (at any time-point of incubation) in the form of thiosulfate, tetrathionate and sulfate respectively. —▼— denotes the pH of the spent medium at a given time-point of incubation. —◆— denotes ^{34}S fractionations ($\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$) observed in the sulfate produced over time.

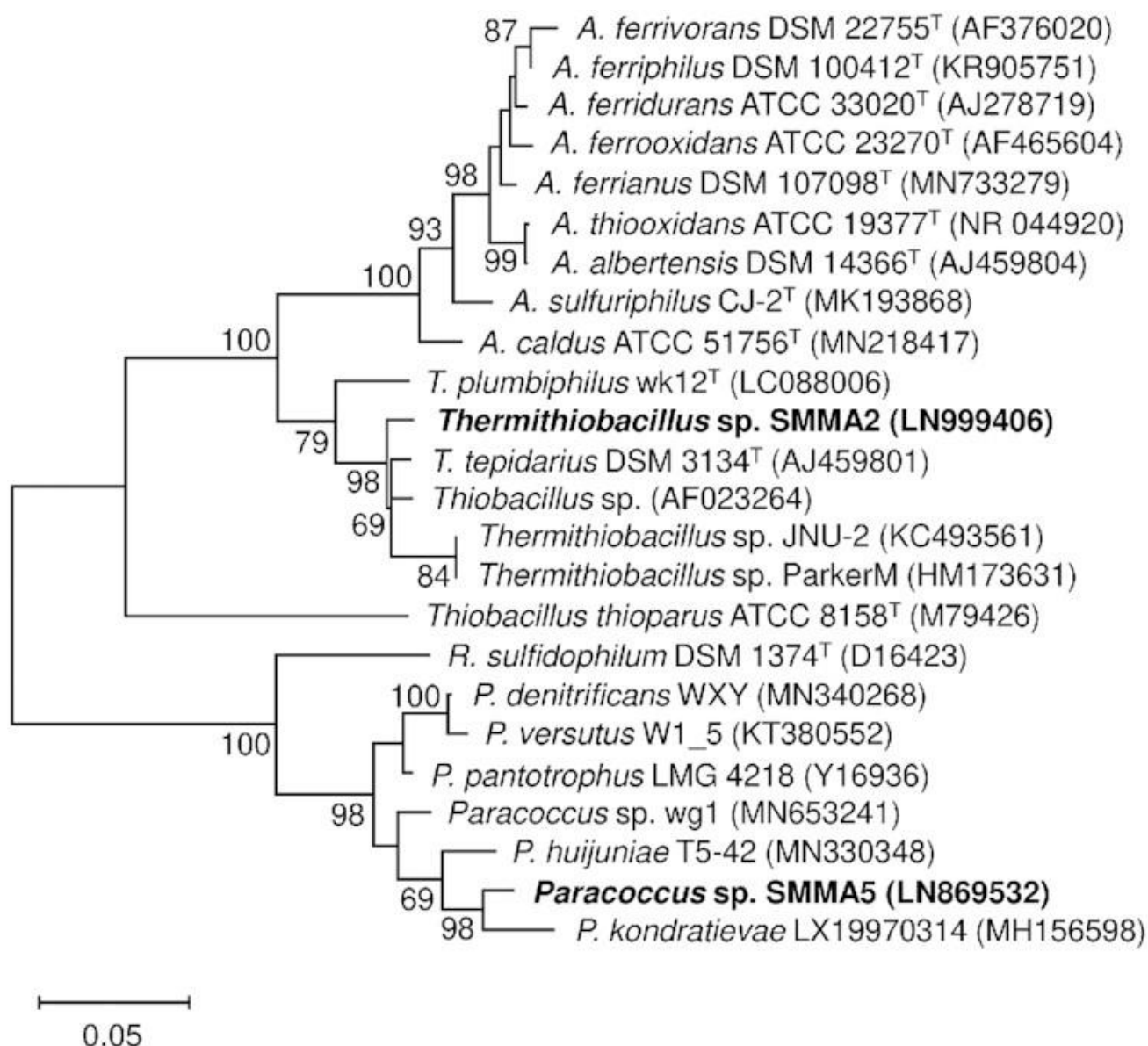


Table 1. Details of the strains used in this study with their $\delta^{34}\text{S}$ values recorded when sulfate was first detected as well as at the end of the incubation period in MST medium.

Name of the strain tested	SMMA5 = MTCC 12601	SJT ^T = MTCC 7001 ^T	SBO3 = MTCC12559	SMMA2
Genus/species level identification of the strain tested	<i>Paracoccus</i> sp.	<i>Mesorhizobium thioangeticum</i>	<i>Pusillimonas ginsengisoli</i>	<i>Thermithiobacillus</i> sp.
Phylum/class level affiliation of the strain tested	<i>Alphaproteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Betaproteobacteria</i>	<i>Gammaproteobacteria</i>
16S rRNA gene sequence accession number of the representative strain	LN869532	AJ864462	LN999404	LN999406
Broad description of thiosulfate oxidation phenotype	Thiosulfate to sulfate with no intermediate formation	Thiosulfate to sulfate with no intermediate formation	Thiosulfate to sulfate via tetrathionate as an intermediate	Thiosulfate to sulfate via tetrathionate as an intermediate
$\delta^{34}\text{S}$ of the first batch of sulfate detected and harvested during growth in MST[#]	9.157	8.534	8.112	7.792
$\delta^{34}\text{S}$ of the final sulfate harvested during growth in MST	10.482	11.077	11.043	11.324

[#] $\delta^{34}\text{S}$ value of the thiosulfate which was used as substrate was recorded as 7.557