Kinetic Enrichment of $^{34}$S during Proteobacterial Thiosulfate Oxidation and the Conserved Role of SoxB in S-S Bond Breaking

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During chemolithoautotrophic thiosulfate oxidation, the phylogenetically diverged proteobacteria Paracoccus pantotrophus, Tetrathiothietobacter kashmiensis, and Thiomicrospira crunogena rendered steady enrichment of $^{34}$S in the end product sulfate, with overall fractionation ranging between $-4.6\%$ and $+5.8\%$. The fractionation kinetics of T. crunogena was essentially similar to that of P. pantotrophus, albeit the former had a slightly higher magnitude and rate of $^{34}$S enrichment. In the case of T. kashmiensis, the only significant departure of its fractionation curve from that of P. pantotrophus was observed during the first 36 h of thiosulfate-dependent growth, in the course of which tetrathionate intermediate formation is completed and sulfate production starts. The almost-identical $^{34}$S enrichment rates observed during the peak sulfate-producing stage of all three processes indicated the potential involvement of identical S-S bond-breaking enzymes. Concurrent proteomic analyses detected the hydrolase SoxB (which is known to cleave terminal sulfone groups from SoxYZ-bound cysteine $S$-thiosulphonates, as well as cysteine $S$-sulphonates, in P. pantotrophus) in the actively sulfate-producing cells of all three species. The inductive expression of soxB during tetrathionate oxidation, as well as the second leg of thiosulfate oxidation, by T. kashmiensis is significant because the current Sox pathway does not accommodate tetrathionate as one of its substrates. Notably, however, no other Sox protein except SoxB could be detected upon matrix-assisted laser desorption ionization mass spectrometry analysis of all such T. kashmiensis proteins as appeared to be thiosulfate inducible in 2-dimensional gel electrophoresis. Instead, several other redox proteins were found to be at least 2-fold overexpressed during thiosulfate- or tetrathionate-dependent growth, thereby indicating that there is more to tetrathionate oxidation than SoxB alone.

Lithotrophic sulfur oxidation is an ancient metabolic process carried out by ecologically and taxonomically diverged prokaryotes via apparently distinct sets of enzymes and electron transport systems (1). Sulfur lithotrophs exhibit differential abilities to utilize different reduced sulfur compounds as substrates, in addition to which, the efficacy of energy conservation from the same sulfur substrates by different organisms (at their respective pH and temperature optima) also varies considerably (2). Thiosulfate ($S_{2}O_{3}^{2-}$/H$^{+}$) is the common substrate oxidized by almost all sulfur chemotrophs and a considerable percentage of phototrophs (1). Some chemotrophs additionally utilize tetrathionate ($S_{4}O_{6}^{2-}$) and other polythionates as energy and electron sources. Tetrathionate is also produced as an intermediate during the oxidation of thiosulfate by several beta- and gammaproteobacteria which follow the so-called tetrathionate intermediate ($S_{4}$J) pathway (2–5). Although a few alphaproteobacteria can oxidize tetrathionate as a starting substrate, none of them form tetrathionate during thiosulfate oxidation (6–8).

The mechanistic multiplicity of chemolithotrophic sulfur oxidation notwithstanding, sox gene homologs are phylogenetically widespread in the domain Bacteria, even though they are completely missing in Archaea (9, 10). Although the presence of sox genes in several species from a wide variety of bacterial lineages insinuates their ancient and conserved status, it is only in certain facultatively lithotrophic alphaproteobacteria that the Sox multi-enzyme system has been proven functionally fundamental (8, 11–13). As such, the model Sox complex of Paracoccus pantotrophus, comprised of SoxXA (both c type cytochromes), SoxYZ (a covalently sulfur-binding and a sulfur compound-chelating protein, respectively), SoxB (sulfate thiol esterase), and Sox(CD)$_2$ (a unique sulfur dehydrogenase), directly oxidizes thiosulfate, sulfite, sulfide, and elemental sulfur but not tetrathionate to sulfate ($SO_{4}^{2-}$) (2, 14). In all these oxidations, the Sox complex covalently binds the substrate via a cysteinyl residue and oxidizes sulfone, as well as sulfane, sulfan atoms to sulfate by transferring electrons to c-type cytochromes without the formation of any free intermediate (2, 11, 14). In the case of thiosulfate, the SoxXA heterodimer oxidatively couples the sulfane sulfur atom to a SoxY-cysteine-sulhydryl group of the SoxYZ complex from which the terminal sulfone group is released by SoxB (12, 15–17). The sulfane sulfur upon the residual SoxY-cysteine persulfide is then oxidized to cysteine-S-sulfonate by Sox(CD)$_2$, from where the sulfonate moiety is again hydrolyzed by SoxB, thereby regenerating SoxYZ (14).

Outside the Alphaproteobacteria, only the truncated Sox systems (SoxXAYZB) of the photolithothrops belonging to members of the Chromatiaceae (18) and Chlorobi (19, 20) and, perhaps, also that of the chemolithothroph Thiobacillus denitrificans (21) unequivocally oxidize the sulfone sulfur but not the sulfane sulfur atom of thiosulfate. This partial handicap is apparently attributable to their want of soxCD genes (1). As such, these organisms employ reverse-acting sulfate-reducing systems to oxidize sulfane sulfur species (22, 23). For other nonalphaproteobacterial lithotrophs, inferences regarding the involvement of sox homologs in...
relevant sulfur oxidation processes are solely based on genomic identification of the coding sequences, homology analysis, and citation of reference to the roles of alphaproteobacterial prototypes (24). Beyond that, no mutational, proteomic, or transcriptomic data are available to authenticate such putative attributions, which become all the more questionable because many sox gene-containing bacteria have no sulfur oxidation phenotype at all (1, 10). Moreover, while appraising the potential roles of sox clusters in nonalphaproteobacterial sulfur lithotrophs, it would be pertinent to remember that many of these loci are products of horizontal gene transfer (9, 10, 25). Their components have not coevolved parallel to each other but have assembled from diverged lineages to form mosaic sox clusters (10). It should be noted that paralogous copies of genes like sox are unlikely to render their typical enzyme functions immediately upon incorporation into new genomes, because Sox proteins require extensive mutual coordination to render the modular Sox pathway (1, 10). As such, novel/modified gene functions in many of those mosaic sox homologs are not unexpected (10), because paralogous genes, during prolonged evolution in new genomic contexts, are known to accumulate large numbers of mutations which, under appropriate selection conditions, often give rise to diverged gene families (26).

In view of the uncertainties noted above, we decided to explore whether nonalphaproteobacterial sulfur chemolithotrophs possessing intact sox loci in their genomes do oxidize thiosulfate by mechanisms comparable to the Sox pathway (now referred to as the Kelly-Friedrich pathway [1, 17]), which is epitomized in Paracoccus pantotrophus (2, 14). Thus, for the current investigation, we selected two well-studied atypical sox-bearing organisms, viz., the betaproteobacterium Tethyobacter kashmirensis (recently reclassified as Advenella kashmirensis [27]) and the gammaproteobacterium Thiomicrospira crunogena. The former oxidizes tetrahydrothionate directly to sulfate even as its thiosulfate oxidation proceeds via tetrathionate formation (4, 28). T. crunogena, on the other hand, oxidizes thiosulfate to sulfate by depositing elemental sulfur globules outside the cell under low-pH and low-oxygen conditions, besides transiently accumulating sulfite and polythionates as products of thiosulfate and sulfur oxidation (29). The molecular bases of these unique physiologies are not fully resolved, and evidence for the involvement of sox genes in these processes does not go beyond genomic data (30, 31).

Any biotic or abiotic transformation of a natural substance has a measurable and characteristic effect on the isotopic composition of the elements (below mass number 66) constituting that substance (32). The relative abundance of the stable isotopes of such elements in the product(s) varies from that in the substrate due to mass-dependent fractionations during the transformation. Lighter isotopes are generally enriched in reaction products with associated fractionation because their bonds are more labile due to partitioning of more of the total bond energy into vibrational rather than translational modes (33). The composition or abundance of stable isotopes (δ) in any sample is expressed as the per mille (%) difference between the ratio of the rare (heavy) and the common (light) isotope in the sample and the same in the global standard. In the present study, we first utilized the stable sulfur isotope (34S and 32S) discrimination potentials of the thiosulfate-oxidizing enzyme systems of T. kashmirensis and T. crunogena in comparison with that of the prototypical Sox system of P. pantotrophus to verify whether the apparently distinct phenotypes had any mechanistic commonality at all. These experiences were followed up with proteomic investigations primarily aimed at the identification of potential Sox proteins during thiosulfate oxidation by T. kashmirensis and T. crunogena.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. P. pantotrophus LMG 4218 and T. kashmirensis WT0011 were grown chemolithoautotrophically in a modified basal and mineral salts (MS) solution (1 g NH4Cl, 4 g K2HPO4, 1.5 g KH2PO4, 0.5 g MgCl2, and 5.0 ml trace metals solution (distilled water) supplemented with thiosulfate equivalent to 40 μg S atoms ml⁻¹ (MST; containing 20 mM Na2S2O3 · 5H2O) (28). The pH of the medium was adjusted to 7.0. T. kashmirensis was additionally grown in MS solution supplemented with tetrathionate, also equivalent to 40 μg S atoms ml⁻¹ (MSTt; containing 10 mM K2S4O6). Chemoorganoheterotrophic growth was performed in MS medium supplemented with 4.0 g dextrose liter⁻¹ (MSD). T. crunogena DSM 12353 was grown chemolithoautotrophically in artificial seawater (ASW; containing 430 mM NaCl, 7.6 mM NH4Cl, 6.1 mM MgCl2, 3.1 mM K2HPO4, 2.4 mM NaHCO3, 2.0 mM CaCl2, and 20.0 mM Trizma hydrochloride) (34) supplemented with 1 ml liter⁻¹ trace metal solution and 10 mM Na2S2O3 · 5H2O (T-ASW). No sulfate salt was provided in the MS, ASW, or trace metal solutions so as to ensure that the sulfates recovered after chemolithotrophic growth were exclusively produced from the oxidation of thiosulfate or tetrathionate. All three cultures were incubated under shaking condition (150 rpm) at 28°C.

Analytical methods. The concentrations of dissolved thiosulfate, tetrathionate, and sulfate in the spent media were measured by iodometric titration, the cyanolytic method, and the gravimetric precipitation method, respectively (4, 28, 35). To precipitate dissolved sulfate as BaSO4, 5 ml of spent medium was collected periodically and centrifuged at 6,000 × g for 5 min to remove bacterial cells. The supernatants were collected in fresh tubes, acidified to pH 2.0 with dilute HCl, and heated at about 90°C for 20 min. An excess of preheated 0.05 M (0.1 N) barium chloride (BaCl2) solution was added to the acidified spent medium, stirring vigorously all the while (36). The precipitates formed after the addition of BaCl2 were digested for 2 h at 90°C and decanted through fine-pore ashless Whatman no. 42 filter paper. They were subsequently washed thrice by dilute HCl and refiltered as described above, and the moist filter papers folded into small cones and then allowed to dry for several days in uncovered crucibles. The dry white BaSO4 powders were recovered by burning away the filter papers and then weighed for subsequent experiments. Precipitates of elemental sulfur were filtered out from the spent culture medium of T. crunogena, dissolved in chloroform, and finally recovered in pure form after evaporating the solvent.

IR-MS. δ44S of sulfate precipitated from spent medium as BaSO4 or, for that matter, δ44S of thiosulfate (Na2S2O3) or tetrathionate (K2S4O6) salts used as chemolithothrophic substrates, was measured using a Thermo Delta V Plus isotope ratio mass spectrometer with continuous flow system with a Thermo EA1112 elemental analyzer attached. The sulfur salt under analysis was mixed with V2O5 and combusted at 1,150°C to produce SO2. The isotopic compositions were expressed as per mille (%) differences relative to the isotopic composition of the Vienna Canyon Diablo troilite (VCDT) standard and are presented in standard δ notation (33) as follows: δ44S = [(44S/32S)sample/(44S/32S)standard] - 1 × 1,000. To check the accuracy of all the reported δ44S values, every sulfur salt sample was run thrice in isotope ratio mass spectrometry (IR-MS), and they were found to have reproducibilities of better than ±0.3‰.

Fractionations were expressed as the isotope difference, Δ, between the two compounds of interest; viz., the reactant (substrate) A and the product B of the transformation in question (ΔA-B = δ44SA - δ44SB). IAEA standards SO-5 (BaSO4 [barium sulfate]: +0.5 ‰ VCDT), SO-6 (BaSO4: -34.1‰ VCDT), NBS-127 (BaSO4: +22.3‰ VCDT), S-1 (Ag2S [silver sulfide]: -0.3‰ VCDT), and S-2 (Ag2S: +22.7‰ VCDT) (http://nucleus.iaea.org/rpst/ReferenceProducts/Reference...
Materials/STable_Isotopes/34S32S/index.htm) were used to prepare calibration curves.

Isolation of protein, 2-dimensional (2-D) gel electrophoresis, and matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) analysis. Actively growing cells of *P. pantotrophus* (from both MST and MSD cultures) were harvested after 36 h of incubation, while for *T. crunogena*, cells (from T-ASW cultures) were recovered after 10 h and 14 h of growth. Total periplasmic proteins were isolated using the PeriPreps PeriPlasting kit (Epicentre Biotechnologies) following the manufacturer’s instructions. Subsequently, protein solutions were precipitated by incubating overnight at −20°C with ice-cold trichloroacetic acid (TCA) and ice-cold acetone added at a ratio of 1:1.8 (vol/vol/vol) protein solution-TCA-acetone. The suspensions were subsequently centrifuged at 16,000 × g for 20 min, following which protein pellets were washed thrice with ice-cold methanol and twice with ice-cold acetone. Pellets were finally air dried in a laminar airflow. In the case of (MALDI-MS) analysis.

45 min at 25°C. The gel pieces were then washed with digestion buffer (50 mM ammonium bicarbonate), and incubated overnight at 37°C. Peptides were then extracted from the gel matrices by incubating (along with vigorous vortexing) the gel pieces twice with extraction buffer (3% trifluoroacetic acid [TFA] plus 30% acetonitrile) and twice with 100% acetonitrile for 15 min at 25°C on each occasion. After each extraction cycle, the gel pieces were centrifuged at 6,000 × g for 1 min and the supernatant was recovered and combined with that from the previous extraction. The samples were dried to 10 to 20% of the original volumes and reacified with 40% (vol/vol) 2% TFA. Tandem mass spectrometric analysis of the extracted tryptic peptides was then performed using an AutoFlex II (Bruker Daltonics) MALDI-tandem time of flight (TOF/TOF) machine equipped with a pulsed N2 laser (λ = 337 nm, 50 Hz). MS or MS/MS analysis of extracted peptides was performed with flexAnalysis software version 2.4, and the processed peaks were transferred through the MS BioTools (version 3.0) program as inputs to MASCOT search engine version 2.2 (Matrix Science, Boston, MA, USA) for protein identification. The following specified parameters were applied for the database search: database (NCBI/GenBank); taxonomy (Other Proteobacteria); proteolytic enzyme (Trypsin); peptide mass tolerance (±0.5 Da); fragment mass tolerance (±1.0 Da); global modification (Carbamidomethyl, Cys); variable modification (Oxidation, Met); peptide charge state (1+); and maximum missed cleavages (1).

RESULTS

Kinetic enrichment of 34S during chemotrophic oxidation of thiosulfate and tetrathionate. During chemolithoautotrophic growth on thiosulfate, all three proteobacteria rendered progressive enrichment of 34S in the end product sulfate in comparison to the 34S value (3.9‰ VCDT) of the thiosulfate salt provided as the substrate. However, the range of fractionation and time course kinetics of enrichment varied to some extent from species to species. Overall, 34S fractionation (Δ 34Ssubstrate-sulfate) for the three thiosulfate oxidation processes ranged between −4.6‰ and +5.8‰.

When grown in MST medium, *P. pantotrophus* oxidized 80 to 85% of the supplied thiosulfate directly to sulfate (which was equivalent to ~32 µg S atoms ml−1) over an incubation period of 48 h. Cessation of growth coincided with decrease of the pH of the spent medium to 5.5, even as 15 to 20% of the thiosulfate remained unutilized (Fig. 1A1). In the first 8 h of incubation, there was no detectable oxidation of thiosulfate or, for that matter, acidification of the medium. Subsequently, when sulfate was first detected in the medium, it was found to be depleted in 34S, with a fractionation of +5.8‰ in comparison to the substrate thiosulfate (Fig. 1A2). However, on further incubation, the abundance of 34S increased in the end product sulfate, and eventually, when 80 to 85% of the supplied thiosulfate was completely oxidized, an enrichment of 34S corresponding to a fractionation of −1.8‰ was recorded in the sulfate produced (Fig. 1A2).

When grown in MST medium, *T. kashmirensis* also completely oxidized 80 to 85% of the supplied thiosulfate in more or less the same time as *P. pantotrophus* (Fig. 1B1). However, unlike the latter species, *T. kashmirensis* converted ~90% of the total thiosulfate (equivalent to ~36 µg S atoms ml−1) to a corresponding amount of tetrathionate in the first 16 h of incubation. At this time point, the pH of the spent medium went up to 8.5. Tetrathionate was subsequently oxidized to sulfate over the next 32 h of the incubation. In either phase of the process, 3 to 5 µg sulfur atoms ml−1

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medium remained unaccounted for. This could well be present in the form of sulfite, a possible intermediate of tetrathionate oxidation to sulfate (2, 4, 39). Apparent physiological and biochemical disparities notwithstanding, the 34S fractionation pattern observed during thiosulfate oxidation by T. kashmirensis (Fig. 1B2) was highly similar to the trends witnessed for P. pantotrophus. Quantitatively, however, the Δ34Sthiosulfate-sulfate values for T. kashmirensis ranged between +4.9‰ and +0.8‰, in contrast to the values of +5.8‰ to −1.8‰ in the case of P. pantotrophus. But the most significant deviation of the T. kashmirensis 34S fractionation curve was observed during the first 36 h of the process. It is during this phase of growth in MST that tetrathionate formation is completed and sulfate formation just starts picking up. The sulfates produced during these initial stages of growth (equivalent to only <5 μg sulfur atoms ml−1 medium in the first 24 h) were significantly depleted in 34S, but most remarkably, this level of depletion remained more or less unchanged until 36 h of incubation. Starting from after 36 h until the end of chemolithotrophic growth, there was steady enrichment of 34S in the end product sulfate in a manner almost identical to the fractionation kinetics of P. pantotrophus (Fig. 1B2). While the initial difference in the fractionation pattern of T. kashmirensis could be linked to its distinct step involving tetrathionate, the pronounced similarity observed in the second leg could be indicative of the involvement of Sox in the oxidation of the intermediary tetrathionate. It is further worthy of note that during growth in MSTr, T. kashmirensis also rendered a gradual enrichment of 34S in the product sulfate in comparison to the Δ34S value (3.2‰ VCDT) of the tetrathionate salt provided as the substrate. As such, the kinetic pattern of 34S fractionation (Δ34Ssulfate-sulfate) observed during tetrathionate oxidation (Fig. 1C2) was apparently similar (within the observed error limits of the experimental data) to the fractionation trend observed during the second leg of the thiosulfate oxidation process (Fig. 1B2).

The rate of thiosulfate oxidation in T. crunogena was much higher than the rate seen for P. pantotrophus or T. kashmirensis (Fig. 1D1). Batch cultures of this gammaproteobacterium took only 16 h to oxidize 50% of the supplied thiosulfate, following which growth and, hence, thiosulfate oxidation terminated. Extreme acidification (pH ~5.2) of the spent medium could be responsible for this abrupt expiry of the process. The 34S fractionation kinetics of T. crunogena was essentially similar to that of P. pantotrophus, even though a slightly higher magnitude and rate of 34S enrichment was observed in the former. As such, the Δ34Ssulfate-sulfate values recorded for T. crunogena ranged between +1.9‰ and −4.6‰ (Fig. 1D2), in comparison to +5.8‰ to −1.8‰ for P. pantotrophus. These discrepancies could well be attributable to the higher thiosulfate oxidation rate of the obligate chemolithotroph.

The atypical reverse fractionation, i.e., enrichment of the heavier isotope 34S, observed in the thiosulfate/tetrathionate oxidation processes studied could be attributable to the unique S-S bond-breaking step involved in these reactions. A close comparison of the 34S enrichment curves of P. pantotrophus, T. kashmirensis, and T. crunogena pointed out similar enrichment rates (slopes of the three curves) during the peak sulfate production phase of all the oxidation processes in question. Although P. pantotrophus rendered enrichment of 34S throughout its thiosulfate oxidation process, the rate of enrichment was maximal during the first 24 to 30 h of incubation, when >50% thiosulfate was converted to sulfate. The enrichment rates encountered during the peak sulfate-producing phases of T. kashmirensis and T. crunogena matched this part of the P. pantotrophus curve, thereby suggesting the involvement of identical enzymes in the S-S bond-breaking steps of all the studied chemolithotrophic processes.

Involvement of Sox in all chemolithotrophic processes. Currently, it is only for thiosulfate oxidation in P. pantotrophus that the hydrolase SoxB is definitively responsible for the hydrolytic cleavage of terminal sulfone (−SO3H) groups as sulfate from protein (SoxYZ)-bound cysteine S-thiosulfonates, as well as cysteine S-sulfonates (14, 16). However, in T. crunogena or T. kashmirensis, the molecular identity of the enzymes rendering the relevant S-S bond-breaking reactions is still unclear. As such, it was considered imperative to explore proteomically whether SoxB was expressed at all during the peak sulfate production phase of thiosulfate oxidation by the latter two species.

The Sox system of P. pantotrophus is known to oxidize thiosulfate in the periplasmic space (14). The translated sox gene products of T. crunogena (including the putative SoxB) could also be predicted (by using the Web-based software utilities CELLO, version 2.5 [http://cello.life.nctu.edu.tw/], and PSORTb, version 3.0.2 [http://www.psort.org/psortb/]) as localized in the periplasm. In contrast, there is no molecular information regarding the cellular localization of the enzymes rendering the different steps of thiosulfate oxidation in T. kashmirensis. Additionally, at least two putative soxB homologs, with locus tags TKWG_14010 and TKWG_08995, are present in the T. kashmirensis genome (CP003555), of which the former is plausibly nonperiplasmic. Hence, proteomic investigations for T. kashmirensis were performed upon total cellular protein extracts, even as we isolated the total periplasmic protein content of P. pantotrophus and T. crunogena to check the expression of soxB. The reproducibility of all proteomic data was checked by performing three independent experiments.

SoxB was detected in the system during the peak sulfate-producing phase of all three thiosulfate oxidation processes (Fig. 2). In the case of P. pantotrophus (Fig. 2A) and T. kashmirensis (Fig. 2B and 3), SoxB spots were unique to the MST-grown cells (i.e., absent during growth in MSD) recovered after 36 h and 40 h of incubation, respectively. This indicated that the soxB gene in either organism was thiosulfate inducible. In contrast to the two facultative sulfur oxidizers, a chemoorganoheterotrophic control was not possible for the obligate chemolithoautotroph T. crunogena, where sox gene expression could well be constitutive in nature (31). Therefore, SoxB was identified in T-ASW-grown cells...
alone, after 10 h and 14 h of incubation (Fig. 2C). This was the first direct experimental proof of the participation of Sox in the thiosulfate oxidation process of *T. crunogena*.

It has been reported previously (29) that, below pH 7, *T. crunogena* produces sulfur globules from thiosulfate, and above pH 7, it consumes those globules. This switch in metabolism is said to be immediate and reversible upon titration of the culture. Corroborating those reports, the present batch cultures of *T. crunogena* also deposited elemental sulfur with increasing acidification of the medium. The precipitate became visible to the naked eye after 12 h of growth and could be quantified from then on (Fig. 1D1). Since we did not titrate the batch cultures back to a pH of 7, the sulfur globules remained unutilized in the spent medium and could be extracted at the end of the growth. The accumulated sulfur was found to be twice as enriched in $^{34}$S (having a $\delta^{34}$S value of +17.8‰ VCDT) as the sulfate (having a $\delta^{34}$S value of +8.5‰ VCDT) produced at the end of the process. It has been suggested earlier that the sulfur deposited during thiosulfate oxidation by *T. crunogena* could be the sulfane sulfur atoms (13) that failed to be further oxidized due to the ineffective interaction of its discretely evolved SoxCD with SoxYZ and SoxB (10). However, there has so far been no experimental verification of whether SoxCD is synthesized at all during thiosulfate oxidation by *T. crunogena*. We found both SoxC and SoxD peptides to be present in the periplasm of *T. crunogena* (Fig. 2C) after 10 h and 14 h of growth in T-ASW, i.e., during as well as after the completion of the oxidation process. This observation in conjunction with the earlier data suggests that the supposed structural anomalies of the discretely evolved SoxCD of *T. crunogena* that purportedly obstruct its interaction with SoxYZ and/or SoxB (10) plausibly become accentuated at low pH.

Also of note, inducible expression of *soxB* during the second leg of thiosulfate oxidation by *T. kashmirensis* essentially implicated tetrathionate as a Sox substrate. As such, to verify the contention that SoxB could be involved in tetrathionate oxidation by this bacterium, we recovered MSTr-grown cells after 24 h of incubation and subjected the total protein extract to 2-D gel electrophoresis and MALDI-MS/MS analysis. As expected, SoxB synthesis was found to be induced during growth on MST relative to its induction on MSD (Fig. 3), thereby reiterating that SoxB was involved in tetrathionate oxidation. To unequivocally illustrate the differential synthesis of SoxB in *T. kashmirensis*, images of SoxB-specific regions of the gels obtained from three separate sets of growth experiments on MSD, MST, and MSTr are presented in Figure 3.

All the MALDI-based protein identification data discussed above are presented in Table 1, where the number of matched peptides, percentage of the putative amino acid sequence length covered in peptide mass fingerprinting (MS), corresponding specific NCBI protein accession numbers, and significance scores are also indicated. In all of the above-described cases of protein iden-

![FIG 2](http://aem.asm.org/figures/2.png)

**FIG 2** 2-D peptide maps of the total periplasmic proteins isolated from thiosulfate-grown cells of *P. pantotrophus* (A) and *T. crunogena* (C), and total cellular proteins isolated from thiosulfate-grown cells of *T. kashmirensis* (B). Sox proteins relevant to this study are indicated by arrows. The inset within panel A depicts the particular vacant region of the periplasmic peptide map of MSD-grown *P. pantotrophus* that corresponds to the SoxB-containing region of the MST-grown map of this bacterium. The region demarcated by a dotted rectangle in panel B has been specifically focused in **Figure 3** to show the differential synthesis of SoxB in MSD-, MST-, and MSTr-grown cells of *T. kashmirensis*.

![FIG 3](http://aem.asm.org/figures/3.png)

**FIG 3** Differential synthesis of SoxB by MSD-, MST-, and MSTr-grown cells of *T. kashmirensis*. The horizontal rows R1 through R3 show three different sets of 2-D gels of the total cellular protein of *T. kashmirensis* obtained from three separate sets of growth experiments on MSD, MST, and MSTr. All nine gel images correspond to the SoxB-containing gel region of **Figure 2B** that is demarcated by a dotted rectangle.
There is an acute dearth of stable isotope-related information, with as the oxidative half of inorganic sulfur metabolism is concerned, biological dynamics of sulfide ore formation, etc. However, as far been successfully extrapolated on natural (geological) samples to Moreover, the few studies that were attempted earlier did not tent, disproportionation reactions (45,46). Those data have also length.

**DISCUSSION**

Most of our knowledge of microbial sulfur isotope fractionation pertains to the reductive processes (33, 42–44) and, to some extent, disproportionation reactions (45, 46). Those data have also been successfully extrapolated on natural (geological) samples to elucidate topics such as the ancient biogeochemical history of the Earth and its atmosphere, sulfur cycles of anoxic sediments, geo- biological dynamics of sulfide ore formation, etc. However, as far as the oxidative half of inorganic sulfur metabolism is concerned, there is an acute dearth of stable isotope-related information, with very few organisms having been investigated so far (Table 2). Moreover, the few studies that were attempted earlier did not reveal substantial fractionation of stable sulfur isotopes. For example, phototrophic conversion of $S^{2−}$ to $S^{0}$ and $S^{0}$ to $SO_{4}^{−2}$ was reported to yield little or no fractionation (33); similar observations were also reported for certain cases of chemotrophic oxidation of $S^{2−}$ to $SO_{4}^{−2}$ (47, 48) or of other reduced intermediates to $SO_{4}^{−2}$ (44, 47). Again, in partial chemotrophic oxidations, where $S^{2−}$ is mainly oxidized to $S^{0}$ and $SO_{4}^{−2}$ and $SO_{3}^{−2}$ are minor end products, significant $Δ^{34}S$ depletion and enriched $SO_{4}^{−2}$ and $SO_{3}^{−2}$ yields, respectively, have been reported (44). Notably, however, in such processes, the main product, $S^{0}$, allegedly showed negligible fractionation compared to that of the original $S^{2−}$ (33, 44).

In this scenario, the present study is the first report of significant $Δ^{34}S$ fractionation by model chemolithotrophic bacteria, which render complete oxidation of reduced sulfur species, such as thiosulfate or tetrathionate, to sulfate. The overall isotope enrichment pattern emanating from the different time course experiments (Fig. 1) unambiguously reflected kinetic fractionation. All the oxidation processes studied started with positive $Δ^{34}S$ values, which subsequently followed the path of Rayleigh fractionation and approached zero when $δ^{34}S$ of the produced sulfate became close to $δ^{34}S$ of the initial substrate thiosulfate or tetrathionate. Specifically, in the case of *P. pantotrophus* and *T. crunogena*, as the reactions progressed, the $Δ^{34}S_{thiosulfate−sulfate}$ values became negative because of continued $δ^{34}S$ enrichment in the sulfate product. Even though thiosulfate oxidation and the corresponding growth ceased abruptly in *T. crunogena* after 16 h of incubation, the quantity and the $Δ^{34}S_{thiosulfate−sulfate}$ value of the end product sulfate remained unchanged within the spent medium well beyond 16 h (Fig. 1D1 and D2). This observation clearly pointed to the absence of any exchange reaction with respect to sulfate ions and demonstrated the existence of a proportionality between the reaction

**TABLE 2 Sulfur isotope fractions recorded earlier during oxidation of inorganic sulfur compounds by phototrophic as well as chemotrophic bacteria**

<table>
<thead>
<tr>
<th>Type of reaction, microorganism</th>
<th>Reaction</th>
<th>Fractionation ($‰$)$^{a}$</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Phototrophic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorobaculum parvum</em></td>
<td>$S^{2−}$ to $S^{0}$</td>
<td>0$^{b}$</td>
<td>50</td>
</tr>
<tr>
<td><em>Chlorobium vibrioforme</em></td>
<td>$S^{2−}$ to $S^{0}$</td>
<td>−2.4</td>
<td>51</td>
</tr>
<tr>
<td><em>Chromatium vinosum</em></td>
<td>$S^{2−}$ to $SO_{4}^{−2}$</td>
<td>−2.5</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>$SO_{3}^{−2}$ to $SO_{4}^{−2}$</td>
<td>0$^{b}$</td>
<td></td>
</tr>
<tr>
<td><em>Chromatium sp.</em></td>
<td>$S^{2−}$ to $S^{0}$</td>
<td>+3.6 to +10</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>$S^{2−}$ to $SO_{4}^{−2}$</td>
<td>−0.9 to +2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$S^{2−}$ to $SO_{3}^{−2}$</td>
<td>−4.9 to −11.2</td>
<td></td>
</tr>
<tr>
<td>Chemotrophic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thiobacillus concretivus</em></td>
<td>$S^{2−}$ to $S^{0}$</td>
<td>−1.2 to +2.5</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>$S^{2−}$ to $SO_{4}^{−2}$</td>
<td>+10.5 to +18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$S^{2−}$ to $SO_{3}^{−2}$</td>
<td>−0.6 to −19</td>
<td></td>
</tr>
<tr>
<td><em>Halothiobacillus neapolitanus</em></td>
<td>$SO_{3}^{−2}$ to $SO_{4}^{−2}$</td>
<td>+1.2 to +2.9</td>
<td>41</td>
</tr>
<tr>
<td><em>Thiobacillus versutus</em></td>
<td>$SO_{3}^{−2}$ to $SO_{4}^{−2}$</td>
<td>0$^{c}$</td>
<td>47</td>
</tr>
</tbody>
</table>

$a$ Fractionation ($‰$) expressed as $Δ_{A→B}$ or $ε_{A→B}$ (where A is the substrate and B is the product) (33).

$b$ $SO_{3}^{−2}$ produced in the first half of the reaction showed fractionation in the range of −4 to −5, but in the second half of the process, fractionation of around +5 was noted; thus, the overall fractionation was considered to be nil.

$c$ Under phototrophic conditions, sulfide is only converted to elemental sulfur.

$d$ Under chemotrophic conditions, the intermediary elemental sulfur is converted to sulfate.

*Thiobacillus concretivus* was once listed as a distinct species in *Bergey’s Manual of Determinative Bacteriology* but was later recognized as a member of *Thiobacillus thiooxidans* (1).

Minor product of the same reaction where sulfide is mainly converted to elemental sulfur.

*Thiobacillus versutus* is now known as *Paracoccus versutus* (1).
rates and overall fractionations achieved. However, it is undeniable that more time course fractionation experiments with phylogenetically diverse sulfur-oxidizing bacteria need to be carried out to fully elucidate these fractionation trends and their relationships with reaction kinetics.

**Implications of Rayleigh fractionation.** The positive Δ34S values obtained during the initial phases of all three thiosulfate oxidation processes implied that 32S-enriched sulfur atoms were incorporated in the sulfate molecules produced in the corresponding periods of growth. On the other hand, the negative Δ34Sthiosulfate-sulfate values obtained in the later phases indicated that the sulfates produced were enriched incrementally in 34S (in comparison to the starting substrate thiosulfate) due to Rayleigh fractionation in the course of the chemolithotrophic processes. In this context, it is imperative to remember that thiosulfate oxidation, in contrast to processes like sulfate reduction or sulfide oxidation, involves the participation of two distinct sulfur atoms that have different valence states, plus contrasting isotope ratios in any given sample. While the valence of the sulfane (S-) sulfur atom of thiosulfate is −1, that of the sulfone (−SO3) sulfur species is +5. At the same time, in all commercially available sodium thiosulfate salts, the sulfane sulfur atom is significantly more enriched in 32S than the sulfone sulfur, and differences in the δ34S values of the sulfane and sulfone components range from +4‰ to +15‰ VCDT (40, 41). Taking these facts into consideration, the pattern of 34S enrichment witnessed in the current experiments can only be explained by invoking a scenario where the two sulfur atoms do not contribute uniformly to sulfate production throughout the processes in question. In the initial stages, sulfane sulfur atoms apparently oxidize to sulfate in much greater proportions than the sulfone atoms, whereas the reverse happens in the later phases. The second event, i.e., the preferential contribution of more sulfone atoms to the sulfate produced, is most pronounced in the case of *T. crunogena*. This last observation concurs with the molecular biological prediction that this gammaproteobacterium may have an impaired sulfane sulfur-oxidizing machinery (13).

However, for a clear understanding of how sulfane and sulfone sulfur species (or for that matter, 32S and 34S isotopes) partition into different phases during thiosulfate/tetrathionate oxidation, it remains absolutely essential to measure the sulfur isotope ratios of the unutilized substrates, as well as the different intermediates formed during these processes.

**Thiosulfate oxidation by *T. kashmirensis* is a multistep process.** 34S enrichment (in sulfate) during thiosulfate oxidation by *T. kashmirensis* was found to take place in apparently distinct steps, which could be reflective of important junctures in the overall biochemical pathway. The plateau in the Δ34Sthiosulfate-sulfate values observed between 24 and 36 h (representing depletion of 34S in the produced sulfate) possibly reflects a predominant contribution of sulfane sulfur atoms in sulfate production and relatively less isotopic fractionation. As such, the dominance of sulfone sulfurs in the sulfate produced seems to begin just after 36 h of growth, as indicated by the sharp enrichment of 34S in the product. It is most likely that the conversion of sulfite to sulfate via hydrolysis causes this enrichment of 34S in the end product. Concurrently, during tetrathionate oxidation, the dominant contribution of sulfone sulfur atoms in the end product sulfate also seems to start after 36 h of growth.

**Partial involvement of Sox in thiosulfate or tetrathionate oxidation by *T. kashmirensis*.** The inducible production of SoxB during tetrathionate oxidation by *T. kashmirensis* is particularly interesting because the currently understood Sox mechanism does not accommodate tetrathionate as a substrate (14), even as a SoxB mutation in the alphaproteobacterium *Pseudaminobacter salicylatoxidans* KCT001 reportedly abolished tetrathionate oxidation (6, 8). In the present study, it was again interesting to observe that for MST-grown, as well as MSTr-grown, cells, no spot approximating the theoretical molecular weight and pI values of the other predicted sox gene products of *T. kashmirensis* appeared in the 2-D peptide maps. At the same time, no other Sox constituent except SoxB was detected even after MALDI-based MS/MS analysis of more than 100 such 2-D gel spots that were either overexpressed (at least 2-fold) or appeared de novo in MST- and/or MSTr-grown cells when compared to those grown in MSD. Instead, several other proteins, including (with GenBank sequence accession numbers given in parentheses) (i) a putative sulfite acceptor oxidoreductase homolog constituted of a sulfite oxidase (YP_006378720) and a cytochrome c (YP_006378719) subunit, (ii) a pyrrolo-quinoline quinone (YP_006380150), (iii) a short-chain dehydrogenase/reductase (YP_006381187), (iv) a disulfide isomerase or thiol-disulfide oxidase (YP_006379827), (v) a thiol-disulfide interconvert protein DsbA precursor (YP_006378075), (vi) a dioxygenase superfamily protein (YP_006381572), (vii) an S-(hydroxymethyl) glutathione dehydrogenase (YP_006380164), (viii) a family 1 extracellular solute-binding protein (YP_006381398), etc., were found to be at least 2-fold overexpressed in these chemoautotrophic cultures (M. Alam, P. Pyne, A. Sar, and W. Ghosh, unpublished observations). These results clearly indicated that there is more to tetrathionate oxidation than SoxB alone. Classical genetic data would be crucial in ascertaining the functions of all of these thiosulfate- and/or tetrathionate-induced proteins and of SoxB as well. Now that a well-developed shuttle vector system for complementation study is in place for *T. kashmirensis* (49), the logical next step would be to generate knockout mutants for all these genes and follow the resultant sulfur oxidation phenotypes.

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**REFERENCES**


