

Kinetic enrichment of ^{34}S during proteobacterial thiosulfate oxidation and the conserved role of SoxB in S-S bond breaking

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ABSTRACT

During chemolithoautotrophic thiosulfate oxidation the phylogenetically-diverged proteobacteria *Paracoccus pantotrophus*, *Tetrathioabacter kashmirensis* and *Thiomicrospira crunogena* rendered steady enrichment of ^{34}S in the end product sulfate, with overall fractionation ranging between -4.6‰ and $+5.8\text{‰}$. 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 case of *T. kashmirensis*, the only significant departure of its fractionation curve from that of *P. pantotrophus* was observed during the first 36 h of thiosulfate-dependent growth, over which tetrathionate-intermediate formation is completed and sulfate production starts. Almost identical ^{34}S enrichment rates observed during the peak sulfate-producing stage of all the 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-thiosulfonates as well as cysteine S-sulfonates in *P. pantotrophus*) in the actively sulfate-producing cells of all three species. Inducible expression of *soxB* during tetrathionate oxidation as well as the second leg of thiosulfate oxidation by *T. kashmirensis* 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 MALDI-MS/MS analysis of all such *T. kashmirensis* proteins which appeared to be thiosulfate-inducible in 2D gel electrophoresis. Instead, several other redox proteins were found to be at-least twofold over-expressed 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 as well as 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 ($S_2O_3^{2-}$) is the common substrate oxidized by almost all sulfur-chemotrophs and a considerable percentage of phototrophs (1). Some chemotrophs additionally utilize tetrathionate ($S_4O_6^{2-}$) and other polythionates as energy and electron source. 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_4I) 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 multienzyme 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 (covalently sulfur-binding, and sulfur compounds chelating, proteins 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 sulfur atoms to sulfate by transferring electrons to c-type cytochromes without the formation of any free intermediate (2, 11, 14). In case of thiosulfate, the SoxXA heterodimer oxidatively couples the sulfane sulfur atom to a SoxY-cysteine-sulfhydryl 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 *Alphaproteobacteria*, only the truncated Sox systems (SoxXAYZB) of the photolithotrophs belonging to *Chromatiaceae* (18) and *Chlorobi* (19, 20), and perhaps also that of the chemolithotroph *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 non-alphaproteobacterial 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 is 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 non-alphaproteobacterial 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 assembled from diverged

lineages to form mosaic *sox* clusters (10). Now, 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 is not unexpected (10) because paralogous genes, over prolonged evolution in new genomic contexts, are known to accumulate large number 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 non-alphaproteobacterial 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)] epitomized in *Paracoccus pantotrophus* (2, 14). As such, for the current investigation we selected two well-studied atypical *sox*-bearing organisms, viz., the betaproteobacterium *Tetrathiobacter kashmirensis* [recently reclassified as *Advenella kashmirensis* (27)] and the gammaproteobacterium *Thiomicrospira crunogena*. The former oxidizes tetrathionate 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 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). 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). Composition or abundance of stable isotopes (δ) in any sample is expressed as per mil (‰) 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 (^{34}S and ^{32}S) 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 at all had any mechanistic commonality. 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* WT001^T were grown chemolithoautotrophically in a modified basal and mineral salts (MS) solution [1 g NH_4Cl , 4 g K_2HPO_4 , 1.5 g KH_2PO_4 , 0.5 g MgCl_2 and 5.0 ml trace metals solution l⁻¹ distilled water] supplemented with thiosulfate equivalent to 40 μg atoms of S ml⁻¹ (MST, containing 20 mM $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) (28). The pH of the media was adjusted to 7.0. *T.*

kashmirensis was additionally grown in MS solution supplemented with tetrathionate also equivalent to 40 μg atoms of S ml^{-1} (MSTr, containing 10 mM $\text{K}_2\text{S}_4\text{O}_6$). Chemoorganoheterotrophic growth was performed in MS media supplemented with 4.0 g dextrose l^{-1} (MSD). *T. crunogena* DSM 12353 was grown chemolithoautotrophically in artificial seawater (ASW, containing 430 mM NaCl, 7.6 mM NH_4Cl , 6.1 mM MgCl_2 , 3.1 mM K_2HPO_4 , 2.4 mM NaHCO_3 , 2.0 mM CaCl_2 and 20.0 mM Trizma hydrochloride) (34) supplemented with 1 ml l^{-1} trace metals solution and 10 mM $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (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 growths were exclusively produced from the oxidation of thiosulfate or tetrathionate. All three cultures were incubated under shaking condition (150 rpm) at 28 $^{\circ}\text{C}$.

Analytical methods. Concentrations of dissolved thiosulfate, tetrathionate and sulfate in the spent media were measured by iodometric titration, cyanolytic method, and gravimetric precipitation method respectively (4, 28, 35). To precipitate dissolved sulfate as BaSO_4 , 5 ml of spent media were collected periodically and centrifuged at 6,000 g for 5 minutes to remove bacterial cells. The supernatants were taken in fresh tubes, acidified to pH 2.0 with dilute HCl, and heated at about 90 $^{\circ}\text{C}$ for 20 minutes. Excess of preheated 0.05 M (0.1 N) barium chloride (BaCl_2) solution was added to the acidified spent media, stirring vigorously all the while (36). The precipitates formed after addition of BaCl_2 were digested for two hours at 90 $^{\circ}\text{C}$ and decanted through fine-pore ash-less Whatman No. 42 filter paper. They were subsequently washed thrice by dilute HCl, re-filtered as above; and the moist filter papers folded into small cones and then allowed to dry for several days in uncovered crucibles. The dry white BaSO_4 powders were recovered by burning away the filter papers, and then weighed for subsequent

experiments. Precipitates of elemental sulfur were filtered out from spent culture media of *T. crunogena*, dissolved in chloroform, and finally recovered in pure form after evaporating the solvent.

Isotope ratio mass spectrometry (IRMS). $\delta^{34}\text{S}$ of sulfate precipitated from spent media as BaSO_4 , or for that matter $\delta^{34}\text{S}$ of thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) or tetrathionate ($\text{K}_2\text{S}_4\text{O}_6$) salts used as chemolithotrophic substrates, was measured by a Thermo Delta V Plus isotope ratio mass spectrometer with continuous flow system attached with a Thermo EA1112 elemental analyser. The sulfur salt under analysis was mixed with V_2O_5 and combusted at 1150°C to produce SO_2 . Isotopic compositions were expressed as permil (‰) differences relative to the isotopic composition of the Vienna Cañon Diablo Troilite (VCDT) standard, and presented in standard δ notation (33):

$$\delta^{34}\text{S} = \left[\left\{ \left(\frac{{}^{34}\text{S}}{{}^{32}\text{S}} \right)_{\text{sample}} / \left(\frac{{}^{34}\text{S}}{{}^{32}\text{S}} \right)_{\text{standard}} \right\} - 1 \right] \times 1000$$

To check the accuracy of all the reported $\delta^{34}\text{S}$ values, every sulfur salt sample was run thrice in IR-MS and found to have reproducibilities 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 [$\Delta_{\text{A-B}} = \delta^{34}\text{S}_\text{A} - \delta^{34}\text{S}_\text{B}$]. IAEA standards SO-5 (BaSO_4 : +0.5 ‰ VCDT), SO-6 (BaSO_4 : -34.1‰VCDT), NBS127 (BaSO_4 : +22.3‰ VCDT), S-1 (Ag_2S : -0.3‰ VCDT) and S-2 (Ag_2S : +22.7‰ VCDT) were used to prepare calibration curves.

Isolation of protein, 2-D gel electrophoresis and MALDI 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 manufacturer's instructions. Subsequently protein solutions were precipitated by incubating overnight at -20°C with ice cold TCA and ice cold acetone added at a ratio of 1:1:8 (v/v/v) 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 laminar airflow. In case of *T. kashmirensis*, total cellular protein was isolated and precipitated after 40 h of growth in MST, and 24 h of growth in both MSD and MSTr, by a modified version of the phenol extraction/methanol-ammonium acetate precipitation method described earlier for recalcitrant plant tissues (37). Dried protein pellets were resuspended in a ReadyPrep rehydration/sample buffer (BioRad) that contained 8M urea, 2% CHAPS and 50mM DTT, 0.2% (w/v) Bio-Lyte 3/10 ampholytes and bromophenol blue (trace).

All the protein preparations were quantified by Bradford method and applied to 11 cm linear IPG strips of different pH ranges at final concentrations of 250 μg of proteins in 180 μl rehydration buffer (rehydrated for 16h at 20°C). Isoelectric focusing was performed on a PROTEAN IEF cell (BioRad) maintaining the following conditions: linear ramping to 250 V for 20 min, linear ramping to 8000 V for 2.5 h, rapid ramping to 20000 Vhr, and eventual termination at 30,000 Vhr. The focused IPG strips were stored at -80°C until further use. Prior to putting onto SDS-PAGE the focused IPG strips were placed in equilibration buffer (0.375 M Tris-HCl, pH 8.8, 6 M Urea, 20% v/v glycerol and 2% w/v SDS) containing 2% w/v DTT for 10 min and soaked again in equilibration buffer containing 2.5% w/v iodoacetamide for 10 min. The strips were then placed on a 12.5% acrylamide:bis-acrylamide SDS-PAGE and sealed

with agarose sealing solution (0.5% w/v agarose, 25mM Tris, 192mM glycine, 0.1 w/v SDS and a trace of bromophenol blue). The second-dimension SDS-PAGE was run at 80 V for the first 30 min followed by 160 V until the dye front reached the bottom of the gel. The gels were stained with coomassie brilliant blue, documented using Versa Doc 4000 (BioRad), and analyzed using the software PDQuest Advanced version 8.0.1.

In case of *P. pantotrophus* and *T. kashmirensis*, all such protein spots that were either novel or more than twofold over-expressed in MST- or MSTr-grown proteomes (in comparison to MSD) were manually excised from the preparative 2-D gels and subjected to MALDI-based tandem mass spectrometric (MS/MS) identification. Since a non-induced heterotrophic growth condition was not possible for the obligate autotroph *T. crunogena*, all those protein spots in the T-ASW-grown proteome that matched the theoretical molecular weights and isoelectric points (pI values) of its predicted Sox proteins were selected for identification.

Tryptic digestion and subsequent extraction of peptides from gel matrices were carried out by methods improvised from earlier reports (38). In brief, excised spots were chopped into ~1x1 mm pieces, washed twice with a buffer containing 25 mM NH_4HCO_3 and 50% ethanol for 20 min in each occasion, and then dehydrated with 100% acetonitrile for 10 min at 25 °C. After drying for 5 min (or until the gel pieces were bouncing in the tube) the gel pieces were rehydrated with reduction buffer (10 mM DTT in 50 mM ammonium bi-carbonate) and incubated at 56 °C for one hour. Alkylation buffer containing 55 mM iodoacetamide was added after discarding the reduction buffer and incubated in the dark for 45 minutes at 25 °C. The gel pieces were then washed with digestion buffer (50mM ammonium bi-carbonate) for 20 minutes and dehydrated with 100% acetonitrile for 10 minutes. This washing and dehydration processes were repeated once with an additional dehydration step. The pieces were then dried

for 5 minutes (or until the gel pieces were bouncing in the tubes), rehydrated by inundating in trypsin solution (12.5 ng μl^{-1} trypsin in 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% 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 up to 10-20% of the original volumes, and re-acidified with 40% (v/v) 2% TFA. Tandem mass spectrometric analysis of the extracted tryptic peptides was then performed using an AutoFlex II (Bruker Daltonics) MALDI TOF TOF machine equipped with a pulsed N₂ laser ($\lambda=337$ nm, 50 Hz). MS or MS/MS of extracted peptides were analyzed by flexanalysis™ software version 2.4 and the processed peaks were transferred through the MS BioTools™ (version 3.0) program as inputs to MASCOT (<http://www.matrixscience.com>) search engine version 2.2 (Matrix Science, Boston, MA, USA) for protein identification. The following specified parameters were applied for database-search: database (NCBIInr); taxonomy (Other *Proteobacteria*); proteolytic enzyme (Trypsin); peptide mass tolerance ($\leq \pm 0.5$ Da); fragment mass tolerance ($\leq \pm 1.0$ Da); global modification (Carbamidomethyl, Cys); variable modification (Oxidation, Met); peptide charge state (1+) and maximum missed cleavage 1.

RESULTS

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

When grown in MST medium *P. pantotrophus* oxidized 80-85% of the supplied thiosulfate directly to sulfate (which was equivalent to $\sim 32 \mu\text{g atoms of S ml}^{-1}$) over an incubation period of 48 hrs. Cessation of growth coincided with decrease of pH of the spent medium to 5.5 even as 15-20% thiosulfate remained unutilized (Fig 1, panel A1). In the first 8 hrs 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 ^{34}S with a fractionation of +5.8‰ in comparison to the substrate thiosulfate (Fig 1, panel A2). However, on further incubation abundance of ^{34}S increased in the end product sulfate and eventually, when 80-85% of the supplied thiosulfate was completely oxidized, an enrichment of ^{34}S corresponding to a fractionation of -1.8‰ was recorded in the produced sulfate (Fig 1, panel A2).

When grown in MST medium *T. kashmirensis* also completely oxidized 80-85% of the supplied thiosulfate in more or less the same time as *P. pantotrophus* (Fig 1, panel B1). But, unlike the latter species, *T. kashmirensis* converted $\sim 90\%$ of the total thiosulfate (equivalent to $\sim 36 \mu\text{g atoms of S ml}^{-1}$) to corresponding amount of tetrathionate in the first 16 hrs of incubation. At this time point, pH of the spent media went up to 8.5. Tetrathionate was

subsequently oxidized to sulfate over the next 32 hrs of the incubation. In either phase of the process 3-5 μg atoms of sulfur ml^{-1} of 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 ^{34}S fractionation pattern observed during thiosulfate oxidation by *T. kashmirensis* (Fig 1, panel B2) was just about similar to the trends witnessed for *P. pantotrophus*. Quantitatively, however, $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values for *T. kashmirensis* ranged between +4.9‰ to +0.8‰ in contrast to +5.8‰ to -1.8‰ in case of *P. pantotrophus*. But the most significant deviation of the *T. kashmirensis* ^{34}S 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. Sulfates produced during these initial stages of growth (equivalent to only <5 μg atoms of sulfur ml^{-1} of medium in the first 24 h) were significantly depleted in ^{34}S ; but most remarkably this level of depletion remained more or less unchanged until 36 h of incubation. Starting from after 36 h till the end of chemolithotrophic growth there was steady enrichment of ^{34}S in the end product sulfate in a manner almost identical to the fractionation kinetics of *P. pantotrophus* (Fig 1, panel B2). 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 noteworthy that during growth in MSTr, *T. kashmirensis* also rendered a gradual enrichment of ^{34}S in the product sulfate in comparison to the $\delta^{34}\text{S}$ value (3.2‰ VCDT) of the tetrathionate salt provided as substrate. As such, the kinetic pattern of ^{34}S fractionation ($\Delta_{\text{tetrathionate-sulfate}}$) observed during tetrathionate oxidation (Fig 1, panel C2) 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 1, panel B2).

Rate of thiosulfate oxidation in *T. crunogena* was much faster than *P. pantotrophus* or *T. kashmirensis* (Fig 1, panel D1). 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 (~5.2) of the spent medium could be responsible for this abrupt expiry of the process. The ^{34}S fractionation kinetics of *T. crunogena* was essentially similar to that of *P. pantotrophus*, even though a slightly higher magnitude and rate of ^{34}S enrichment was observed in the former. As such, $\Delta_{\text{thiosulfate-sulfate}}$ values recorded for *T. crunogena* ranged between +1.9‰ and -4.6‰ (Fig 1, panel D2) in comparison to +5.8‰ to -1.8‰ for *P. pantotrophus*. These discrepancies could well be attributable to the faster thiosulfate oxidation rate of the obligate chemolithotroph.

Atypical reverse fractionation, i.e., enrichment of the heavier isotope ^{34}S , observed in the studied thiosulfate/tetrathionate oxidation processes could be attributable to the unique S-S bond breaking step involved in these reactions. A close comparison of the ^{34}S 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 ^{34}S all along its thiosulfate oxidation process, the rate of enrichment was maximal during the first 24-30 h of incubation when >50% thiosulfate was converted to sulfate. 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. Now, it is only for thiosulfate oxidation in *P. pantotrophus* that the hydrolase SoxB is definitively responsible for the hydrolytic cleavage of terminal sulfone ($-\text{SO}_3^-$) groups as sulfate from protein (SoxYZ)-bound cysteine S-thiosulfonates as well as cysteine S-sulfonates (14, 16). But 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 proteomically explore whether SoxB was at all expressed 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 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. On the contrary, there is no molecular information regarding the cellular localization of the enzymes rendering the different steps of thiosulfate oxidization in *T. kashmirensis*. On top of this, at-least two putative *soxB* homologs, locus tags TKWG_14010 and TKWG_08995, are present in the *T. kashmirensis* genome (CP003555), out of which the former is plausibly non-periplasmic. 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*. Reproducibility of all proteomic data was checked by three independent experiments.

SoxB was detected in the system during the peak sulfate producing phase of all the three thiosulfate oxidation processes (Fig. 2). In case of *P. pantotrophus* (Fig. 2A) and *T.*

kashmirensis (Fig. 2B & Fig. 3) SoxB spots were unique to MST-grown cells (i.e., absent during growth in MSD) recovered after 36h and 40h 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). As such SoxB was identified in T-ASW-grown cells alone, after 10h as well as 14h 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 earlier (29) that below pH 7, *T. crunogena* produces sulfur globules from thiosulfate, and consume those globules above pH 7. This switch in metabolism is said to be immediate and reversible upon titration of the culture. Corroborating those reports, 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 then on (Fig 1, panel D1). Since we did not titrate the batch cultures back to pH >7, the sulfur globules remained unutilized in the spent media and could be extracted at the end of the growth. The accumulated sulfur was found to be twice more enriched in ^{34}S (having a $\delta^{34}\text{S}$ value of +17.8‰ VCDT) than the sulfate (having a $\delta^{34}\text{S}$ 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) which failed to get 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 whether SoxCD is at all synthesized 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 10h as well as 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 obstructs its interaction with SoxYZ and/or SoxB (10) plausibly get accentuated at low pH.

Notably again, 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 24h 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 MStr relative to 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 have been documented in Fig. 3.

All the above discussed MALDI-based protein identification data are given 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 IDs and significance scores have also been indicated. In all the above cases of protein identification at-least two matched peptides from the relevant MS spectra were cross-checked by MS/MS ion search.

DISCUSSION

Most of our knowledge on 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 like the ancient biogeochemical history of the Earth and its atmosphere, sulfur cycles of anoxic sediments, geobiological dynamics of sulfide-ore formation etc. But so 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 being 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-} , were reported to yield little or no fractionation (33); similar observations were also reported for certain cases of chemotrophic oxidation of S^{2-} to S^0 (47, 48), or other reduced intermediates to SO_4^{2-} (44, 47). Again, partial chemotrophic oxidations where S^{2-} is mainly oxidized to S^0 , and SO_4^{2-} and $S_xO_6^{2-}$ are minor end products, significant ^{34}S depletion and enrichment in the yielded SO_4^{2-} and $S_xO_6^{2-}$ respectively have been reported (44). Notably however, in such processes, the main product S^0 allegedly showed negligible fractionation compared to the original S^{2-} (33, 44).

In this scenario, the present study is the first report of significant ^{34}S fractionation by model chemolithotrophic bacteria, which render complete oxidation of reduced sulfur species like 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 studied oxidation processes started with positive $\Delta^{34}S$ values, which subsequently followed the path of Rayleigh fractionation and approached zero when $\delta^{34}S$ of the produced sulfate became close to $\delta^{34}S$ of the initial substrate thiosulfate or tetrathionate.

Specifically in case of *P. pantotrophus* and *T. crunogena*, as the reactions progressed, the $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values became negative owing to continued ^{34}S enrichment in the product sulfate. Even though thiosulfate oxidation and corresponding growth in *T. crunogena* abruptly ceased after 16 h of incubation, the quantity as well as the $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ value of the end product sulfate remained unchanged within the spent media well beyond 16 h (Fig. 1, panels D1 & D2). This observation clearly pointed out the absence of any exchange reaction with respect to sulfate ions and demonstrated the existence of a proportionality between reaction 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. Positive $\Delta^{34}\text{S}$ values obtained during the initial phases of all the three thiosulfate oxidation processes implied that ^{32}S -enriched sulfur atoms got incorporated in the sulfate molecules produced in the corresponding periods of growth. On the other hand, negative $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values obtained in the later phases denoted that the produced sulfates got incrementally enriched in ^{34}S (in comparison to the starting substrate thiosulfate) owing to Rayleigh fractionation in 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 ^{32}S than the sulfone sulfur, and differences in the $\delta^{34}\text{S}$ values of the sulfane and sulfone components range from +4‰ to +15‰ VCDT (40, 41). Taking these facts into consideration, the pattern of ^{34}S enrichment witnessed in the current experiments can only be explained by invoking a scenario where the two sulfur atoms do not contribute to sulfate production uniformly 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 produced sulfate, is most pronounced in 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 ^{32}S and ^{34}S 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 multi-step process. ^{34}S 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 $\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ values observed between 24 and 36 h (representing depletion of ^{34}S 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 produced sulfate seems to begin

just after 36 hrs of growth, as indicated by the sharp enrichment of ^{34}S in the product. It is most likely that the conversion of sulfite to sulfate via hydrolysis causes this enrichment of ^{34}S in the end product. Concurrently, during tetrathionate oxidation 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*. 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 *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- 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 over-expressed (at-least twofold) or appeared *de novo* in MST- and/or MSTr-grown cells in comparison to those grown in MSD. Instead, several other proteins like (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 interchange protein DsbA precursor (YP_006378075), (vi) a dioxygenase superfamily protein (YP_006381572), (vii) a S-(hydroxymethyl)glutathione dehydrogenase (YP_006380164), (viii) a family 1 extracellular

solute-binding protein (YP_006381398) etc. were found to be at-least twofold over-expressed in these chemoautotrophic cultures (Alam et al., unpublished observation). 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 these thiosulfate- and/or tetrathionate-induced proteins, and 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 knock-out mutants for all these genes and follow the resultant sulfur oxidation phenotypes.

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Table 1. Details of the relevant Sox proteins identified by peptide mass fingerprinting during the study.

Organism	Protein	NCBI accession number	Score/ Coverage[@]	No. of peptides matched / No. searched[§]
<i>P. pantotrophus</i>	SoxB	CAA55824	116/37	18/87
<i>T. kashmirensis</i>	SoxB	YP_006379123	198/61	33/169
<i>T. crunogena</i>	SoxB	YP_391815	107/33	16/56
	SoxC	YP_390426	122/46	27/72
	SoxD	YP_390427	123/58	16/55

[@] MASCOT score obtained during the NCBI non-redundant database search and sequence coverage of the matched peptides expressed as percentage of total sequence length.

[§] Total number of mass values matched in the peptide mass fingerprints and the total number of mass values searched.

Table 2. Sulfur isotope fractionations recorded earlier during oxidation of inorganic sulfur compounds by phototrophic as well as chemotrophic bacteria.

Microorganism	Reaction	Fractionation (‰) [‡]	Reference	
Phototrophic reactions				[‡] Fractionation (‰) expressed as Δ_{A-B} or ϵ_{A-B} (where A is the substrate and B is the product). * In these cases SO_4^{2-} produced in the first half of the reaction showed fractionation in
<i>Chlorobaculum parvum</i>	S^{2-} to S^0	0*	(50)	
<i>Chlorobium vibrioforme</i>	S^{2-} to S^0	-2.4	(51)	
<i>Chromatium vinosum</i>	S^{2-} to S^0 [#]	-2.5	(48)	
	S^0 to SO_4^{2-} [§]	0		
<i>Chromatium vinosum</i>	SO_3^{2-} to SO_4^{2-}	0*	(52)	
	$\text{S}_2\text{O}_3^{2-}$ to SO_4^{2-}	0		
<i>Chromatium sp.</i>	S^{2-} to S^0	+3.6 to +10	(44)	
	S^{2-} to SO_4^{2-}	-0.9 to +2.9		
	S^{2-} to $\text{S}_x\text{O}_6^{2-}$	-4.9 to -11.2		
Chemotrophic reactions				
<i>Thiobacillus concretivorus</i> **	S^{2-} to S^0	-1.2 to +2.5	(44)	
	S^{2-} to SO_4^{2-} [©]	+10.5 to +18		
	S^{2-} to $\text{S}_x\text{O}_6^{2-}$ [©]	-0.6 to -19		
<i>Halothiobacillus neapolitanus</i>	$\text{S}_2\text{O}_3^{2-}$ to SO_4^{2-}	+1.2 to +2.9	(41)	
<i>Thiobacillus versutus</i> ^Δ	$\text{S}_2\text{O}_3^{2-}$ to SO_4^{2-}	0	(47)	

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.

[#] Under photoheterotrophic conditions sulfide is only converted to elemental sulfur.

[§] But under photoautotrophic conditions the intermediary elemental sulfur is converted to sulfate.

** *Thiobacillus concretivorus* 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 products of the same reaction where sulfide is mainly converted to elemental sulfur.

^Δ *Thiobacillus versutus* is now known as *Paracoccus versutus* (1).

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Figure 1

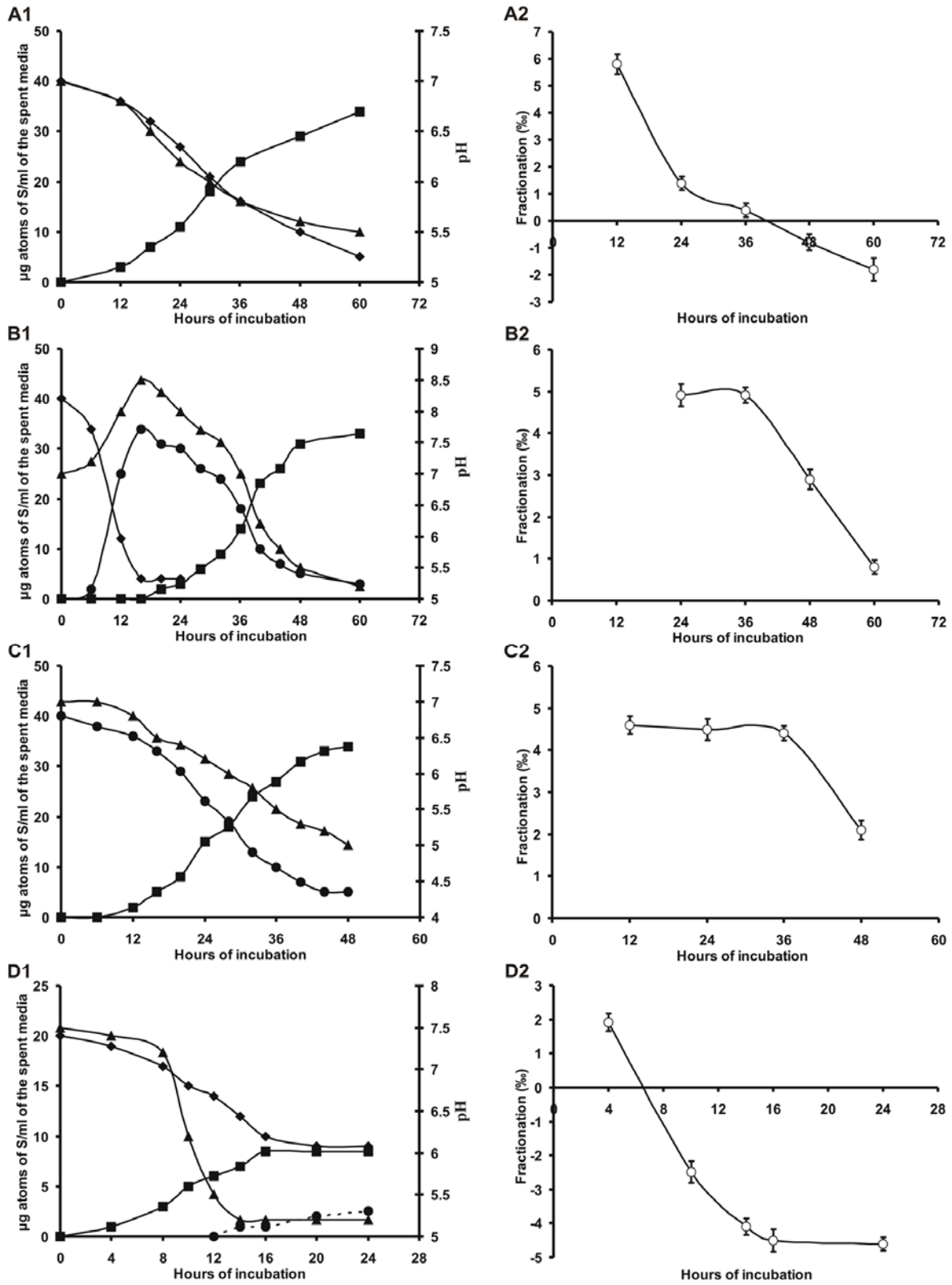


Figure 2

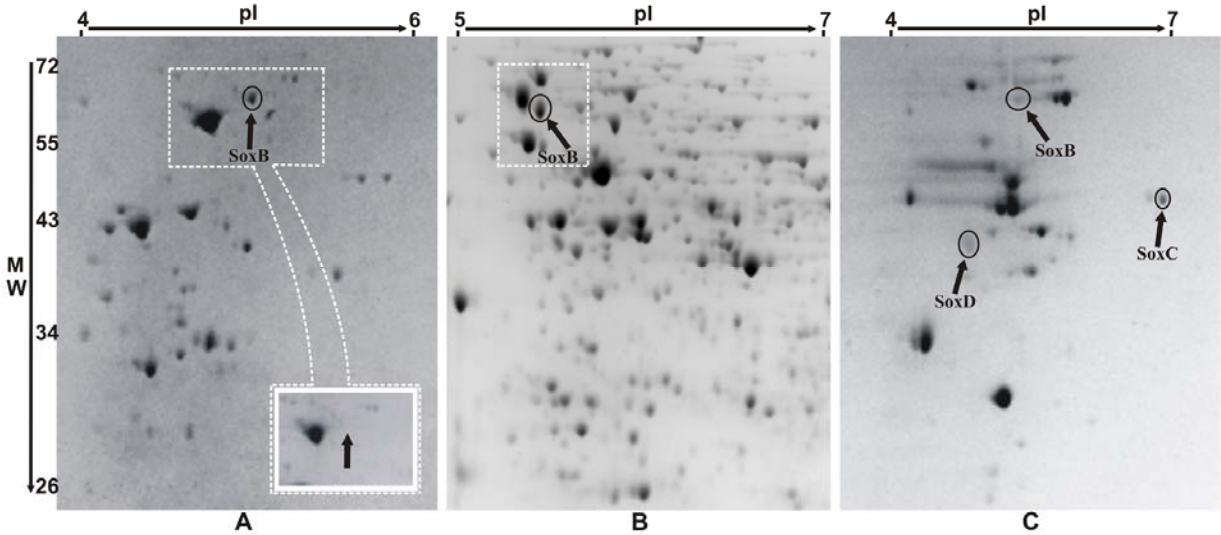
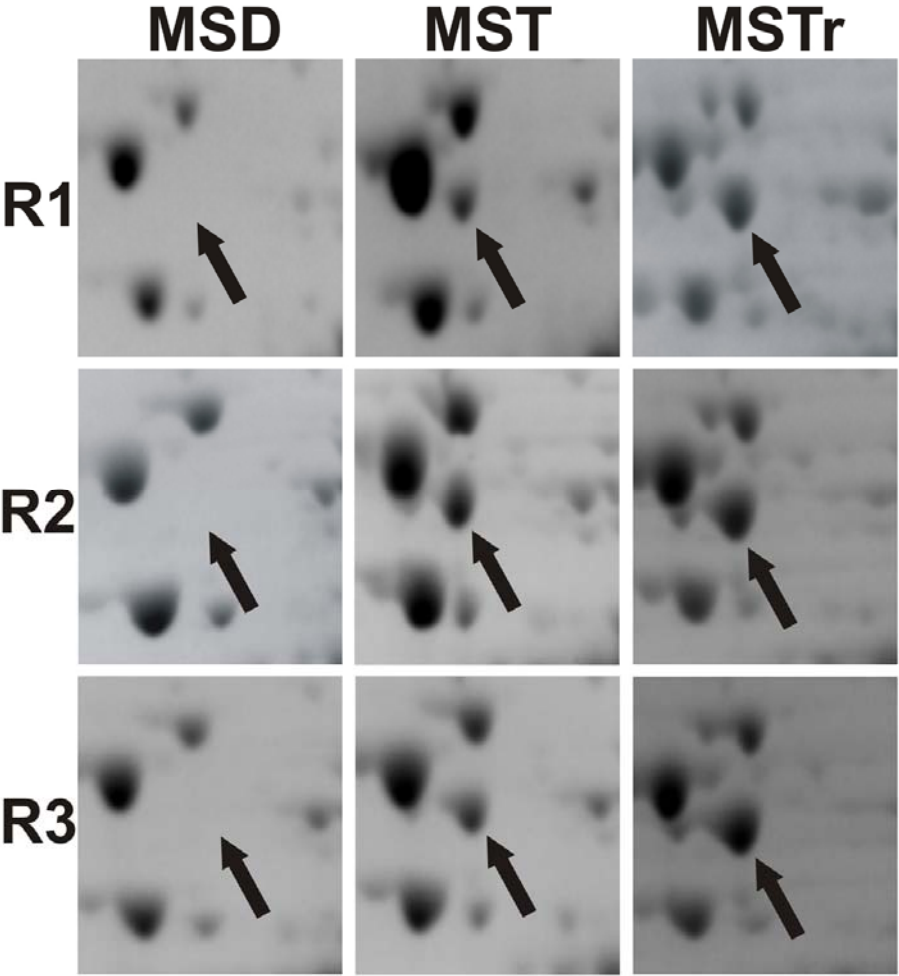


Figure 3



Legends of Figures

Figure 1

Legend of Figure 1. Kinetics of thiosulfate or tetrathionate oxidation by *P. pantotrophus*, *T. kashmirensis* and *T. crunogena* in conjunction with corresponding ^{34}S fractionations in the end product sulfate. A1 and A2 respectively shows the kinetics of thiosulfate oxidation and corresponding ^{34}S fractionation by *P. pantotrophus*; B1 and B2 respectively shows the kinetics of thiosulfate oxidation and corresponding ^{34}S fractionation by *T. kashmirensis*; C1 and C2 respectively shows the kinetics of tetrathionate oxidation and corresponding ^{34}S fractionation by *T. kashmirensis*; D1 and D2 respectively shows the kinetics of thiosulfate oxidation and corresponding ^{34}S fractionation by *T. crunogena*.

‘—◆—’, ‘—●—’, ‘—■—’, ‘---●---’ denote μg atoms of S present ml^{-1} of the spent media in the form of $\text{S}_2\text{O}_3^{2-}$, $\text{S}_4\text{O}_6^{2-}$, SO_4^{2-} and S^0 respectively. ‘—▲—’ denotes the pH of the spent media recorded at the different time points. ‘—○—’ denotes ^{34}S fractionations ($\Delta^{34}\text{S}_{\text{thiosulfate-sulfate}}$ or $\Delta^{34}\text{S}_{\text{tetrathionate-sulfate}}$) observed in the end product sulfate during chemotrophic oxidation of thiosulfate or tetrathionate by the bacteria in question.

Figure 2

Legend of Figure 2. 2D 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 arrow marks. The inset within A depicts that particular vacant region of the periplasmic peptide map of MSD-grown *P. pantotrophus* which corresponds to the SoxB-

containing region of the MST-grown map of this bacterium. The region demarcated by a dotted rectangle in B has been specifically focused in Fig. 3 to show the differential synthesis of SoxB in MSD-, MST- and MStr-grown cells of *T. kashmirensis*.

Figure 3

Legend of Figure 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 2D 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 that SoxB-containing gel region of Fig. 2B which is demarcated by a dotted rectangle.