ARTICLE

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Stoichiometric Modeling of Oxidation of Reduced Inorganic Sulfur Compounds (Riscs) in *Acidithiobacillus thiooxidans*

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ABSTRACT: The prokaryotic oxidation of reduced inorganic sulfur compounds (RISCs) is a topic of utmost importance from a biogeochemical and industrial perspective. Despite sulfur oxidizing bacterial activity is largely known, no quantitative approaches to biological RISCs oxidation have been made, gathering all the complex abiotic and enzymatic stoichiometry involved. Even though in the case of neutrophilic bacteria such as *Paracoccus* and *Beggiatoa* species the RISCs oxidation systems are well described, there is a lack of knowledge for acidophilic microorganisms. Here, we present the first experimentally validated stoichiometric model able to assess RISCs oxidation quantitatively in *Acidithiobacillus thiooxidans* (strain DSM 17318), the archetype of the sulfur oxidizing acidophilic chemolithoautotrophs. This model was built based on literature and genomic analysis, considering a widespread mix of formerly proposed RISCs oxidation models combined and evaluated experimentally. Thiosulfate partial oxidation by the Sox system (SoxABXYZ) was placed as central step of sulfur oxidation model, along with abiotic reactions. This model was coupled with a detailed stoichiometry of biomass production, providing accurate bacterial growth predictions. In silico deletion/inactivation highlights the role of sulfur dioxygenase as the main catalyzer and a moderate function of tetrathionate hydrolase in elemental sulfur catabolism, demonstrating that this model constitutes an advanced instrument for the optimization of *At. thiooxidans* biomass production with potential use in biohydrometallurgical and environmental applications.

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KEYWORDS: *At. thiooxidans*; reduced Inorganic sulfur compounds (RISCs); chemolithoautotrophic oxidation

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Introduction

Sulfur bacteria were recognized more than a century ago (see Kelly et al., 1997, for a more comprehensive perspective) and different metabolic pathways used by prokaryotes to oxidize reduced inorganic sulfur compounds (RISCs) have been described. Oxidation of RISCs constitutes a fundamental aspect of the nutrient biogeochemistry of the sulfur cycle as well as biohydrometallurgical processes such as bioleaching of heavy metals (Rohwerder and Sand, 2007). In the last process, the mechanism of bioleaching of metal sulfides has been described with a concomitant attack of ferric ions and protons. The first coming from Iron(II)-oxidizing bacteria such as *Acidithiobacillus ferrooxidans* and the last coming from sulfur-compound oxidizing bacteria such as *At. ferrooxidans* and *At. thiooxidans* (Rohwerder et al., 2003). Moreover, it is widely recognized that the oxidation of RISCs to sulfuric acid is of great importance for biohydrometallurgical technologies. For example, the inhibition and promotion of biochemical steps in elemental sulfur oxidation pathways is highly relevant for

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bioleaching operations (Mohapatra et al., 2008; Rohwerder and Sand, 2007).

There is some agreement on two fundamental oxidation processes for sulfide, sulfur, and thiosulfate biooxidation: one mechanism not involving polythionates which is found mainly in neutrophilic microorganisms, and a second one present in neutrophilic as well as acidophilic chemolithotrophic sulfur bacteria and archaea, able to obtain energy to support growth from the oxidation of RISCs involving polythionates as pathway intermediates (reviewed in Friedrich et al., 2005; Ghosh and Dam, 2009; Mohapatra et al., 2008; Rohwerder and Sand, 2007).

In the case of neutrophilic bacteria, the role of the adenosine phosphosulfate (APS) pathway in *Beggiatoa* spp. strain MS-81-1c including intracellular sulfur globules (Hagen and Nelson, 1997) as well as the thiosulfate-oxidizing multi-enzyme system (TOMES) (Kelly et al., 1997) analogous to the so-called sulfur-oxidizing (Sox) system in *Paracoccus* sp. (reviewed in Friedrich et al., 2001), are well described. *Paracoccus pantotrophus* has seven thiosulfate-inducible genes (*soxXYZABCD*) coding for essential proteins for sulfur oxidation in vitro. However, incomplete sox genes clusters without the *soxCD* genes able to oxidize RISCs are present in the genomes of different bacteria such as *Aquifex aeolicus*, *Ralstonia solanacearum*, and the phototrophic sulfur bacteria *Chlorobium tepidum* and *Allochromatium vinosum* (Friedrich et al., 2005; Ogawa et al., 2008), among others. The *soxXYZABCD* system yields 8 mol electrons per mol of thiosulfate with no detectable intermediates, whereas the partial sox system without sulfur dehydrogenase soxCD yields only two, as the current mechanism proposes (Friedrich et al., 2001). This partial thiosulfate oxidation in *C. tepidum* and *A. vinosum* has been linked to the formation of protein-coated sulfur globules in the periplasm and the central role of the *dsr* gene cluster for oxidation of intracellular sulfur compounds (Frigaard and Dahl, 2009; Pott and Dahl, 1998; Prange et al., 1999).

For acidophilic archaea, the elemental sulfur oxidation has been proposed to start with sulfur disproportionation catalyzed by sulfur oxygenase reductase (SOR), well characterized from *Acidianus ambivalens* (Urich et al., 2006), with formation of sulfite and sulfide, further oxidized by sulfite:acceptor oxidoreductase (SAR) to sulfate and sulfide:quinone oxidoreductase (SQR) to sulfur, respectively (Rohwerder and Sand, 2007). A similar picture has been described in acidophilic chemolithotrophic proteobacteria like Acidithiobacilli, in which SOR is replaced by a glutathione-dependent sulfur dioxygenase (SDO) (EC 1.13.11.18) as demonstrated in different strains of *At. thiooxidans* (Rohwerder and Sand, 2003). Moreover, a recent study based on transcriptomic data for *Acidithiobacillus caldus* MTH-04, a model for elemental sulfur and tetrathionate oxidation was proposed, including both SDO and SOR enzymes (Chen et al., 2012). Finally, a comparative genomic study using the draft genome sequences of relevant biomining acidophiles has shown the presence of *soxABXYZ* genes in *At. thiooxidans* and *At. caldus* and its absence in *At. ferrooxidans*, indicating that even within acidophilic chemolitotrophs the different mechanisms for RISCs oxidation have evolved (Valdés et al., 2008, 2011).

However, cellular RISCs oxidation stoichiometry is a complex combination of enzymatic and abiotic steps since spontaneous nucleophilic and condensation reactions take place simultaneously with those catalyzed by enzymes. Particularly interesting are the abiotic formation of thiosulfate from sulfur and unstable sulfite, and the spontaneous reaction of polythionates and sulfide to form thiosulfate and elemental sulfur (Suzuki, 1999). These observations explain why an additional group of enzymes are involved in RISCs oxidation in acidophilic archaea and bacteria including thiosulfate:quinone oxidoreductase (TQO) (Janiczek et al., 2007; Nakamura et al., 2001) and tetrathionate hydrolase (TTH) (Bugaytsova and Lindström, 2004; Kanao et al., 2007), which explains the involvement of polythionates as intermediates of sulfur oxidation as previously mentioned.

Although several mechanistic models have been developed to explain microbial sulfur oxidation (Friedrich et al., 2001; Rohwerder and Sand, 2007), and despite some kinetic approaches (Ceskova et al., 2002; Gourdon and Funtowicz, 1998) as well as studies of the effect of inhibitors and uncouplers over RISCs metabolism in Acidithiobacilli (Kamimura et al., 2005; Masau et al., 2001) are available, to our knowledge no attempt to model microbial RISCs oxidation quantitatively has been reported, probably due to the methodological difficulty in determining transient intermediates and complex kinetic mechanisms involved. It is within this context that metabolic flux analysis (MFA) of underdetermined stoichiometric models can be a useful tool for giving insights into the metabolic pathways of sulfur oxidation coupled to central metabolism in metabolic models that consider the full stoichiometry from the energy source until the synthesis of biomass with a detailed description of the major catabolic and anabolic pathways.

Previously, stoichiometric metabolic models have been developed for the biomining bacteria *Acidithiobacillus ferrooxidans* (Hold et al., 2009) and *Leptospirillum ferrooxidans* (Merino et al., 2010). *L. ferrooxidans* is not a sulfur oxidizer and therefore this process was not included in the model. In the case of the *At. ferrooxidans* model, the focus was put on ferrous iron oxidation, only with a single reaction for RISCs oxidation that was not considered for calculations due to the difficulty in distinguishing between the different sulfur oxidation states.

The present study aims to develop the first stoichiometric model of *At. thiooxidans* with a particular focus on RISCs oxidation to provide an analytical tool for studying quantitatively the metabolic behavior of this chemolithotrophic acidophile of industrial and environmental importance.

Materials and Methods

Strains and Culture Conditions

At. thiooxidans DSM 17318 (strain patented by BioSigma 'S.A.' for its use in bioleaching processes) was grown in a 1 L bioreactor (Model BioFlo 110; New Brunswick, Edison, NJ) operated at 30°C and fed in batch cyclic mode (withdrawal of 500 mL of culture followed by replacement with the same volume of fresh medium every 48 h) with a based medium (pH 1.8) as described previously (Bobadilla et al., 2011) supplemented with 1% sulfur (99.5% purity; Sigma–Aldrich, St. Louis, MO). The culture was continuously aerated (1 L/min) and stirred (250 rpm). The pH was controlled by addition of 1 M NaOH solution. Alternatively, strain DSM 17318 was cultivated on the same medium in batch cultures at pH 2.5 with 0.1% w/v potassium tetrathionate (99% purity; Fluka, Steinheim, Germany) as single source of energy. All cultures were initially inoculated at 1% v/v with cultures pre-grown with the respective energy source.

Enumeration of Bacteria and Quantification of Biomass

Cells number was determined by direct microscopic count using a Thoma Chamber (depth 0.010 mm) and converted to cell mass dry weight assuming a conversion factor of 2.00×10^{-13} g/cell as described previoulsy (Bratbak, 1985). Culture purity and quantification was assessed from purified genomic DNA extracted by phenol:chloroform:isopropyl alcohol method by specific q-PCR determinations with primers designed for specific 16S rRNA gene of *At. thiooxidans* DSM 17318 and total bacteria as proposed previously (Bobadilla et al., 2011) with comparable values for both determinations in the same order of magnitude.

Determination of Thiosulfate

The formation of thiosulfate was determined based on a colorimetric method (Sorbo, 1957). Briefly, thiosulfate in solution was separated from elemental sulfur by centrifugation (8,000*g*) and the supernatant was brought to pH 9.0. Then the thiosulfate was converted to thiocyanate and determined colorimetrically with ferric ions by measuring absorbance at 460 nm.

Determination of Intracellular Elemental Sulfur

Determination of intracellular elemental sulfur was performed spectrophotometrically as described in (Maurice, 1957). Culture samples were collected (10 mL) and centrifuged for 2 min at 1,000*g* in order to remove any remaining sulfur. The cell suspension was centrifuged at 8,000*g* and washed twice with $NaHSO_3$ 0.05 M solution to remove any elemental sulfur bound. Cells were finally resuspended in 0.5 mL of culture medium free of sulfur and lysed by sonication in an Omni-Rupter 250 equipment (Omni-International, Inc., Marietta, GA). The crude cell extract was directly extracted with analytical grade chloroform (1:10), or after centrifugation at 10,000*g* and measured spectrophotometrically at 265 nm.

Metabolic Modeling

Modeling and Simulation Software

The selection of the reactions included in our model was made using Pathways Tools 12.0 software (Karp et al., 2002) and manual curation. Flux balance analysis (FBA) was done using CellNetAnalyzer 7.0 software (Klamt et al., 2007). In the present work, FBA was performed to maximize biomass synthesis flux specific growth rate in all cases and setting flux constraints representing reaction reversibility.

Stoichiometric Model

The sequencing project of *At. thiooxidans* DSM 17318 performed by BioSigma "S.A." produced a 4.2 Mbp genome with 4,580 annotated coding sequences (CDS; unpublished data, available under demand). These data served to develop a metabolic reconstruction for this bacterium containing the smallest reaction set able to reproduce RISCs oxidation and major central metabolism pathways. The final model has 190 metabolites and 181 reactions mainly accounting for the central metabolism of *At. thiooxidans* DSM 17318, the synthesis of amino acids and nucleotides, and the oxidation of RISCs. Metabolic modeling for ATP production, Calvin cycle, pentose phosphate pathway, tricarboxylic acid cycle, DNA, RNA, amino acid, and protein biosynthesis follow the same criteria as described previously (Hold et al., 2009). The complete list of reactions and metabolites in the model can be found in the supplementary information. Specific pathways included in the model and their metabolic reconstruction is described below.

Catabolism

Metabolic Modeling of RISCs Oxidation in At. thiooxidans *DSM 17318*

To develop a full metabolic model of *At. thiooxidans* DSM 17318 the incorporation of a set of reactions for sulfur oxidation to sulfate was performed. Due to the lack of available information several assumptions were made. First, considering that thiol group-containing compounds (represented in the model as R-SH) have been proposed to be employed for sulfur transport, a non-specific sulfur transport reaction was taken into account as well as the thermodynamic stability of sulfur oxidation intermediates (Kelly, 1999), assuming an extracellular pH fluctuation between 0.8 and 1.8 and a periplasmic pH in the range of

2.5–3.0. Once in the periplasm, the oxidation of the oxidized thiol group (RSS^-) into sulfite was assumed to be carried out either by a SDO not yet identified in *At. thiooxidans* DSM 17318 due to the lack of a reference sequence (Rohwerder and Sand, 2003) or by disproportionation by SOR present in strain DSM 17318 (81% aminoacidic identity with SOR from *At. caldus* ATCC 51756). Further oxidation of sulfite to sulfate was not considered since a sulfite acceptor oxidoreductase (SAR) has so far not been identified on *At. thiooxidans* DSM 17318 genome. It was therefore assumed that all sulfite formed chemically reacts with elemental sulfur forming thiosulfate as previously described (Suzuki, 1999). Next, thiosulfate could be directly oxidized to sulfate by the incomplete sulfur-oxidizing (Sox) operon identified in *At. thiooxidans* DSM 17318 (genes *soxABXYZ*; Friedrich et al., 2005). This step involves the reaction of thiosulfate with the cysteine thiol group of SoxYZ heterodimeric protein (SoxYZ-S) and formation of the protein-cysteine-S complex (SoxYZ-S-S) (Quentmeier and Friedrich, 2001) that after interaction with the SoxAX and SoxB proteins leads to polysulfide (S_n^{2-}) that could be oxidized to elemental sulfur by the SQR recognized in the strain genome. Alternatively, thiosulfate could be oxidized to tetrathionate by the DoxDA protein identified in strain DSM 17318 (apparently a fusion protein sharing 72% amino acid identity with DoxD from *At. caldus* and 25% with DoxA-like protein from *Sulfolobus solfataricus* P2) and predicted to encode a subunit of thiosulfate:quinol oxidoreductase (Müller et al., 2004; Rohwerder and Sand, 2003). Next, the tetrathionate formed could be transformed by TTH forming sulfate, thiosulfate and elemental sulfur as previously reported (De Jong et al., 1997; Meulenberg et al., 1992) or chemically react with the polysulfide forming thiosulfate and elemental sulfur (Suzuki, 1999). The elemental sulfur formed could react chemically as mentioned above or be transformed enzymatically by SOR. Finally, considering the absence of the *dsr* genes in the genome of *At. thiooxidans* DSM 17318 as an indication of failure to oxidize sulfur globules (Friedrich et al., 2005), an extracellular sulfur storage step was included in order to prevent sulfur accumulation inside the cell that would lead to unreliable biomass optimization. The described model of sulfur oxidation in *At. thiooxidans* strain DSM 17318 is depicted schematically in Figure 1.

Electron Transfer

Considering that electrons arising from RISCs oxidation allows reduction of $NAD(P)^+$ mediated by the quinone pool

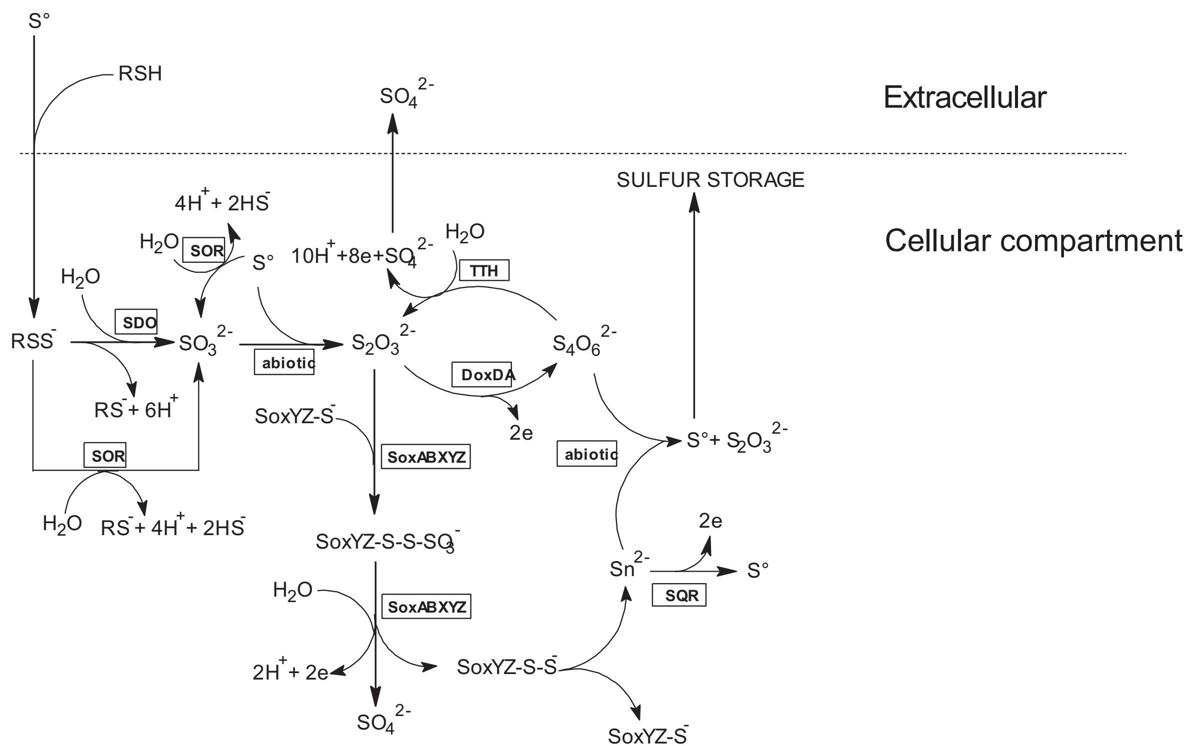

Figure 1. Schematic representation of RISCs oxidation model for *Acidithiobacillus thiooxidans* DSM 17318. SDO, sulfur dioxygenase; SOR, sulfur oxygenase reductase; TTH, tetrathionate hydrolase; DoxDA, Thiosulfate:quinol oxidoreductase; SoxABXYZ, sulfur-oxidizing operon; SQR, Sulfide:quinone oxidoreductase. Abiotic: spontaneous chemical reactions.

in chemolithoautotrophic bacteria (Brasseur et al., 2004), the incorporation of electrons obtained by the oxidation of RISCs was modeled including two reactions with oxygen and $NAD(P)^+$ as electron acceptors as previously reported (Cobley and Cox, 1983).

Anabolism

Biomass Equation

In order to simulate growth, a biomass equation was incorporated in the model using the biomass composition of *Acidihtiobacillus ferrooxidans* as described before (Hold et al., 2009). The contribution of main biomass macromolecules in the equation was considered in terms of their basic precursors (supplementary files) with the exception of nucleic acids and proteins, which were included directly. In the case of lipids, a previously reported fatty acid composition for *At. thiooxidans* was used to calculate the necessary precursors for their synthesis (Neidhardt et al., 1990).

Transport

Extracellular Transport

Reactions for the transport of CO_2 and O_2 from the extracellular medium were included in the model as well as those specified for RISCs. Additionally, a reaction for the uptake of ammonium ion was incorporated since the genes for an ammonium transporter were identified in the genome of strain DSM 17318 as lately reported (Levican et al., 2008).

Results and Discussion

The stoichiometric model approach developed for *At. thiooxidans* DSM 17318 was focused on the optimization of biomass production for selected RISCs as single sources of energy, taking into account the optimal mesoacidophilic environmental growth conditions for *At. thiooxidans*. Elemental sulfur was used first, considering that at an acidic pH below 3.0 and under moderate temperature and pressure it is the only thermodynamically stable reduced sulfur species, and therefore, the main source of RISCs in acidic environments. On the other hand, based on the RISCs oxidation pathway via polythionates postulated to occur in chemolithotrophic acidophilic bacteria and because of the chemical stability of tetrathionate under acidic conditions, it was selected as alternative energy source aside sulfur.

Modeling and Simulation of Elemental Sulfur Oxidation

As described in the metabolic modeling of RISCs oxidation section, sulfur oxidation starts with extracellular elemental sulfur uptake. Many studies relate this transport with thiol groups able to transport it as persulfide sulfur, the only substrate transformed by SDO (Rohwerder and Sand, 2003). However, the genomic identification of the SOR enzyme raises the question whether the oxidation of the persulfide formed (RSS^-) could be carried out in parallel by both branches, the SDO and the SOR simultaneously. In silico deletion of SDO gave rise to an unfeasible scenario in which the model optimization did not converge and the resulting biomass yield was ten times higher (0.116 g-biomassDW mmol $S^{\circ\,-1}$) than the experimental observation (0.013 ± 0.002 g-biomassDW mmol $S^{\circ\,-1}$) which is similar to previously reported rates (Konishi et al., 1994). The experimental observations are attributable to the disproportionation of persulfide catalyzed by SOR and are considered to be the only source of sulfhydryl (HS^-) in the model. Sulfhydryl is a precursor for cysteine formation, which is an important biomass component. However, simulation with simultaneous SOR and SDO oxidative activities lead to a predicted biomass yield value of 0.012 g-biomassDW mmol $S^{\circ\,-1}$, very close when compared to the

Table I. Biomass, NAD(P)H and ATP yield coefficients in particular predicted scenarios for different RISCs substrates and comparison with experimental data.

	Model predictions				Experimental
Substrate	Deleted/modified reaction	Biomass yield [g DW/mmol substrate]	Maximum NAD(P)H yield [mmol NAD(P)H/mmol substrate]	Maximum ATP yield [mmol ATP/mmol substrate]	Biomass yield [g DW/mmol substrate]
Elemental sulfur (S°)	TTH with sulfur formation	0.012	0.837	1.503	0.013 ± 0.002
	TTH with sulfur formation and no abiotic tetrathionate reduction	0.012	0.837	1.503	
	TTH without sulfur formation	0.012	0.837	1.503	
Tetrathionate ($S_4O_6^{2-}$)	TTH with sulfur formation	0.018	1.279	2.296	0.041 ± 0.007
	TTH with sulfur formation and no abiotic tetrathionate reduction	0.012	0.852	1.531	
	TTH without sulfur formation	0.052	3,731	6,700	

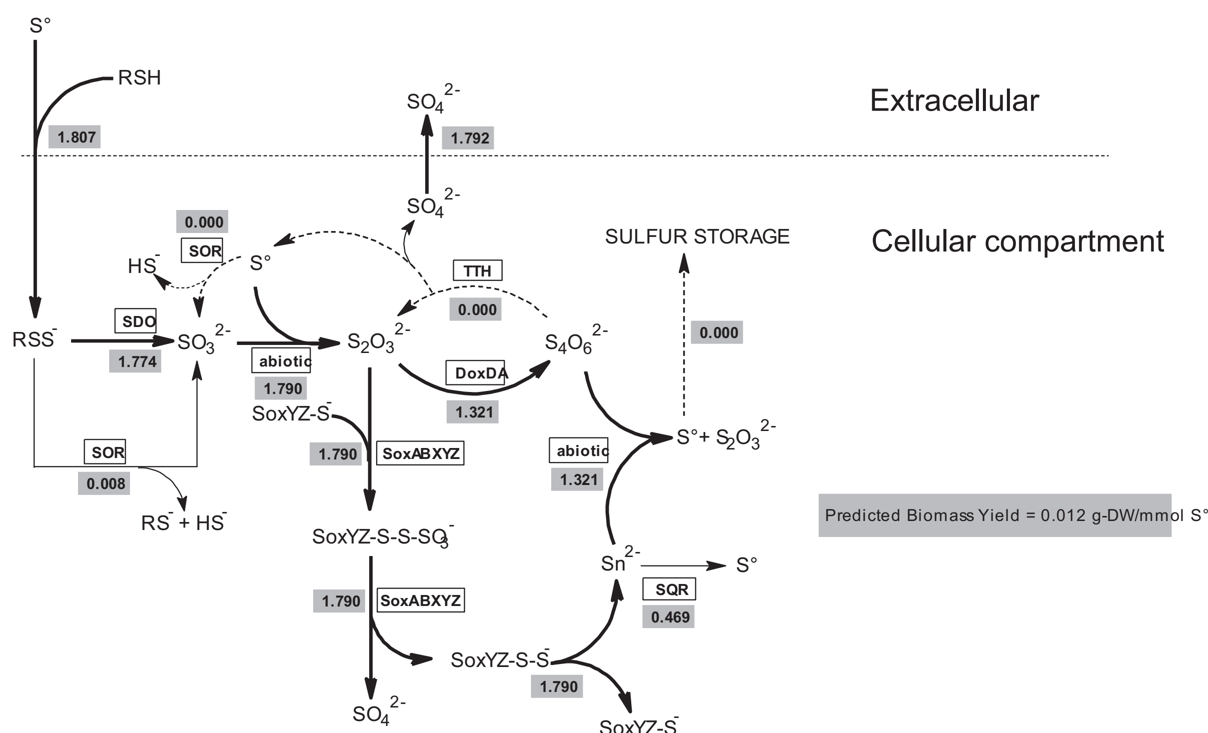

Figure 2. Representation scheme of stoichiometric model predictions of elemental sulfur oxidation by *Acidithiobacillus thiooxidans* DSM 17318. Arrow thickness represents stoichiometric favored reactions (flux values in gray boxes are expressed in mmol · g-biomass^{-1} h^{-1}). Dotted arrows represent reactions with no flux. SDO, sulfur dioxygenase; SOR, sulfur oxygenase reductase; TTH, tetrathionate hydrolase; DoxDA, thiosulfate:quinol oxidoreductase; SoxABXYZ, sulfur-oxidizing operon; SQR, Sulfide:quinone oxidoreductase. Abiotic: spontaneous chemical reactions. Experimental biomass yield equals 0.013 ± 0.002 g-biomassDW · mmol S°$^{-1}$.

experimental observations (Table I) and showing a preferred flux via SDO (99% of total flux, as shown in Fig. 2). A possible explanation for such behavior could be physiological substrate availability due to the different locations of SDO and SOR in the cell. SDO is reported to be a periplasmic enzyme (Rohwerder and Sand, 2003) whereas isolated and characterized SORs have so far been located in the cytoplasm (Sun et al., 2003; Tian et al., 2003). Table I shows as well the ATP and NAD(P)H yields obtained per unit substrate. Our model predictions indicate a yield of 1,503 mmol of ATP per mmol of elemental sulfur, which compared to previously reported predictions for *At. ferrooxidans* grown in ferrous ion (0.25 mmol ATP/mmol Fe(II)) (Hold et al., 2009) clearly shows the more favorable thermodynamics of sulfur, which are comparable to anaerobic *Escherichia coli* cultures that obtain between 2.75 and 1.24 mmol ATP per mmol of glucose (Hempfling and Mainzer, 1975; Nielsen et al., 2003).

Interestingly, based on the experimentally determined oxidation rate of elemental sulfur in *At. thiooxidans* DSM 17318 cyclic batch culture (1.807 ± 0.014 mmol S° g-biomassDW^{-1} h^{-1}) used here as the fixed flux for MFA calculations, the model predicted no sulfur storage and no flux through TTH, indicating that the tetrathionate formed is rapidly reduced to thiosulfate in a chemical reaction which is stoichiometrically favored. Nevertheless, both elemental sulfur and thiosulfate were found accumulated in the cultures. In the first case, elemental sulfur was found intracellularly at a concentration of 4.13×10^{-15} and 1.19×10^{-14} g S° per cell, accounting between 4% and 12% of the cell dry weight. Extracellular thiosulfate was determined in a concentration of 0.257 ± 0.104 g/L. One possible explanation for the lack of accumulation in the model predictions is that this behavior may occur at non-exponential growth phases and therefore, is not captured by the model.

Several parameters are key factors for RISCs oxidation. Among them, oxygen and carbon dioxide availability are essential. The above mentioned simulations of elemental sulfur oxidation consider an unconstrained flux of oxygen and carbon dioxide. However, when the experimentally determined flux of elemental sulfur (1.807 mmol S° g-biomassDW^{-1} h^{-1}) was used for MFA calculations under limited oxygen or carbon dioxide fluxes, the predicted biomass yield varied proportionally and optimal fluxes of 1.039 mmol O_2 g-biomassDW^{-1} h^{-1} and 0.7106 mmol CO_2 g-biomassDW^{-1} h^{-1} were obtained (Fig. 3). This values are one order of magnitude lower than those used for *At. ferrooxidans* model (Hold et al., 2009), indicating major differences in oxygen and carbon dioxide requirements between iron and elemental sulfur oxidation in acidophilic strains.

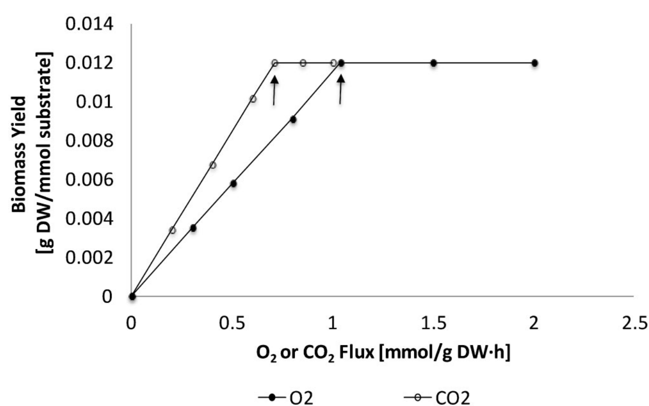

Figure 3. Predicted biomass yield as a function of oxygen or carbon dioxide fluxes under a fixed flux of elemental sulfur (1.807 mmol S° g-biomassDW^{-1} · h^{-1}), optimal fluxes for oxygen (1.039 mmol O_2 g-biomassDW^{-1} · h^{-1}) and carbon dioxide (0.7106 mmol CO_2 · g-biomassDW^{-1} h^{-1}) are indicated with arrows.

Modeling and Simulation of Tetrathionate Oxidation

Taking tetrathionate as a model substrate for RISCs oxidation in *At. thiooxidans* DSM 17318, the stoichiometric model proposes simple diffusion of tetrathionate from the medium into the periplasm without a change in its oxidation state. In the periplasm the tetrathionate could be reduced chemically with sulfide and/or be hydrolyzed enzymatically to thiosulfate, elemental sulfur and sulfate by TTH as previously described (De Jong et al., 1997; Meulenberg et al., 1992). In this case SDO does not play any role since no persulfide sulfur is formed, but in the model the disproportionation of elemental sulfur performed by the SOR enzyme is essential for biomass production. The model prediction using the experimental maximal specific substrate consumption rate (2.105 ± 0.240 mmol $S_4O_6^{2-}$ g-biomassDW^{-1} h^{-1}) showed that 49% of the sulfur-mole uptake goes to sulfur storage in the cellular compartment (see Fig. 4, panel A). In this respect, recent descriptions based on genome sequence analysis and biochemical evidence of the oxidative sulfur metabolism in phototrophic sulfur bacteria account for the formation of sulfur globules in the periplasm (Frigaard and Dahl, 2009; Ghosh and Dam, 2009).

In this sulfur storage scenario it was noted that the flux of TTH was activated only at very low substrate influx (>0.15 mmol $S_4O_6^{2-}$ g-biomassDW^{-1} h^{-1}). In silico inactivation of the abiotic reduction of tetrathionate resulted in a very slight increase in sulfur storage (0.4%) and a lower biomass yield (0.012 g-biomassDW mmol $S_4O_6^{2--1}$). This yield was only a third less than the average obtained experimentally, indicating that TTH plays a minor role in the metabolism of tetrathionate in *At. thiooxidans* DSM 17318. However, when the stoichiometry of the reaction catalyzed by TTH considered in the model is changed in order to do not produce elemental sulfur as previously reported (Tano et al., 1996), a major change in the flux distribution is observed, with a close prediction of the biomass yield (0.052 g-biomassDW mmol $S_4O_6^{2--1}$) and with no sulfur storage (see Fig. 4, panel B). Under this scenario, TTH plays a central role. This change does not affect the flux distribution in case of the modeling and simulation of elemental sulfur oxidation (yield values summarized in Table I). Interestingly, the maximum biomass yield reported in tetrathionate for *At. ferrooxidans* ATCC 19859 (0.013 g-biomassDW mmol $S_4O_6^{2--1}$) (Hazeu et al., 1986) is close to the value predicted in the model including sulfur formation on TTH reaction and no abiotic tetrathionate reduction, an indication that differences may exist for this particular enzyme within Acidithiobacilli. With respect to ATP yield, our model predictions show higher values compared to elemental sulfur as expected, based on the higher biomass yield experimentally observed. The metabolic scenario with a TTH reaction without sulfur formation predicts a 6.700 mmol ATP per mmol of tetrathionate, four times higher than the sulfur case, indicating an optimized metabolic condition. Previous reports on *Hallothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*) grown on thiosulfate as single source of energy have reported yields of 4.52 mmol ATP per mmol of thiosulfate, showing that substrate-level phosphorylation largely contributes to ATP formation in chemoautotrophs compared to *E. coli* heterotrophic glucose oxidation through the Embden–Meyerhof–Parnas pathway (Hempfling and Vishniac, 1967).

In view of the results shown in this study for the metabolic flux through TTH arising from our stoichiometric model predictions, it is important to emphasize that the mere existence of an enzymatic activity in vitro does not allow the inference of its physiological role. Such is the case for rhodanese, previously related to RISCs metabolism despite its physiological function not having yet been established (Friedrich, 1998). Sulfur transferases are widely distributed in bacteria, plants and animals, and experiments have demonstrated that rhodanese is not required for thiosulfate oxidation in vitro as shown in *Thiobacillus versutus* (now *Paracoccus versutus*) (Lu and Kelly, 1983). In contrast, the rhodanese-like protein SoxL has been recently proposed as an important factor for SoxY recycling since it increases thiosulfate oxidation rate in vitro (Welte et al., 2009). Based on this consideration, we included in the model only enzymatic activities previously reported to be directly involved in RISCs metabolism.

Conclusions

In this study, a first quantitative RISCs oxidation model taking into account all the complex enzymatic and abiotic reactions involved by means of a stoichiometric model for the archetype of the sulfur oxidizing acidophilic chemolithoautotroph *At. thiooxidans* is presented and validated

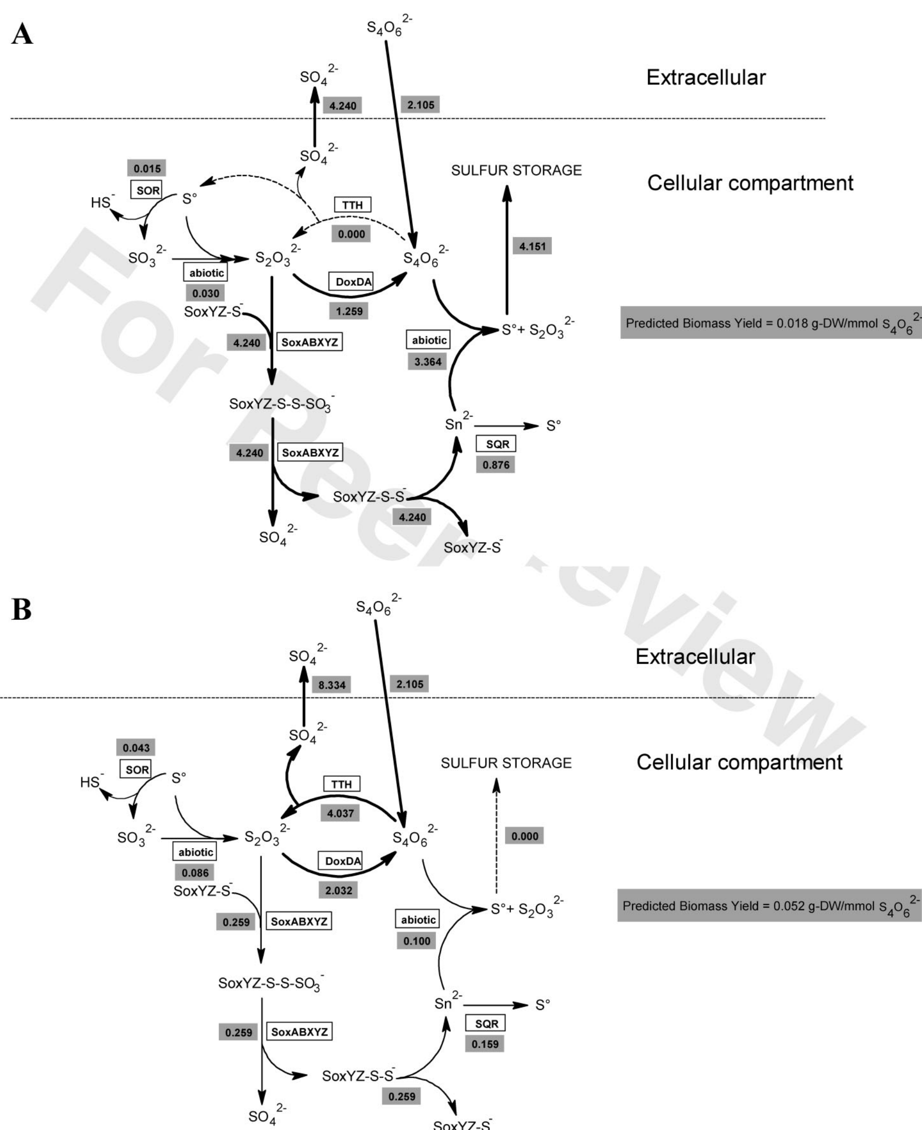

Figure 4. Representation scheme of stoichiometric model predictions of tetrathionate oxidation by *Acidithiobacillus thiooxidans* DSM 17318 assuming the presence (**A**) and absence (**B**) of production of elemental sulfur in the TTH reaction. Arrow thickness represents stoichiometric favored reactions (flux values in gray boxes are expressed in mmol g-biomass^{-1} h^{-1}). Dotted arrows represent reactions with no flux. SOR, sulfur oxygenase reductase; TTH, tetrathionate hydrolase; DoxDA, thiosulfate:quinol oxidoreductase; SoxABXYZ, sulfur-oxidizing operon; SQR, sulfide:quinone oxidoreductase. Abiotic: spontaneous chemical reactions. Experimental biomass yield equals 0.041 ± 0.007 g-biomassDW mmol $S_4O_6^{2-}$$^{-1}$.

with experimental values obtained for two relevant substrates, elemental sulfur and tetrathionate. The model includes sulfur oxygenase reductase gene, newly identified for chemolithotrophic bacteria but well known for archaea, and confirms the presence of the partial sulfur-oxidizing operon Sox, recently reported in *At. thiooxidans* (Valdés et al., 2008). Based on model predictions and in silico deletion/inactivation, the role of SDO as the major persulfide oxidative catalyzer, a possible role for simultaneous SOR activity and a diminished function of TTH are proposed in connection with growth on elemental sulfur. Afterwards, for an extremely important intermediate of RISCs oxidation in acidophiles as is tetrathionate, model adjustments to experimental growth rates gave the interesting finding of sulfur storage when elemental sulfur formation is considered as product of TTH reaction (De Jong et al., 1997; Meulenberg et al., 1992). However, subsequent adjustments of the experimental growth rates in the model changing the stoichiometry of TTH reaction with no elemental sulfur formation (Tano et al., 1996) led to the elimination of sulfur storage, highlighting the importance of TTH in RISCs oxidation in acidophiles, which identifies a key aspect of chemolithoautotrophic growth of *At. thiooxidans*. The stoichiometric model resulting from the

foregoing considerations constitutes a useful tool for understanding the metabolic behavior of *At. thiooxidans* under environmental conditions and/or industrial applications.

The replacement of the SAR as the central reaction to oxidize RISCs to sulfate as usually proposed in the literature dealing with acidophilic bacteria (Mohapatra et al., 2008; Rohwerder and Sand, 2007) with thiosulfate oxidation by the Sox system constitutes an important contribution and a paradigm shift to the understanding of RISCs oxidation in *At. thiooxidans*. The former together with the incorporation of a combination of enzymatic and abiotic reactions coupled to central metabolism summarizing the stoichiometry of *At. thiooxidans*, represents a new proposal and the core of the model presented here. Future model refinement should include exopolysaccharide production that was neglected in this study since it accounted for $<1\%$ of the total carbon produced, but certainly influences the oxidation of insoluble substrates such as elemental sulfur and sulfide minerals. Finally, we are certain that this model will become a useful prediction tool for the optimization of *At. thiooxidans* biomass production under different metabolic conditions.

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