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Souhela Boughanemi, Pascale Infossi, Marie-Thérèse Giudici-Ortoni, Barbara Schoepp-Cothenet, Marianne Guiral. Sulfite oxidation by the quinone-reducing molybdenum sulfite dehydrogenase SoeABC from the bacterium *Aquifex aeolicus*. *Biochimica biophysica acta (BBA) - Bioenergetics*, 2020, 10.1016/j.bbabi.2020.148279 . hal-02936602

HAL Id: hal-02936602

<https://hal.science/hal-02936602>

Submitted on 11 Sep 2020

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Journal Pre-proof

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PII: S0005-2728(20)30129-8

DOI: <https://doi.org/10.1016/j.bbabbio.2020.148279>

Reference: BBABIO 148279

To appear in: *BBA - Bioenergetics*

Received date: 23 April 2020

Revised date: 3 July 2020

Accepted date: 10 July 2020

Please cite this article as: S. Boughanemi, P. Infossi, M.-T. Giudici-Orticoni, et al., Sulfite oxidation by the quinone-reducing molybdenum sulfite dehydrogenase SoeABC from the bacterium *Aquifex aeolicus*, *BBA - Bioenergetics* (2020), <https://doi.org/10.1016/j.bbabbio.2020.148279>

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Sulfite oxidation by the quinone-reducing molybdenum sulfite dehydrogenase SoeABC from the bacterium *Aquifex aeolicus*

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Abstract

The microaerophilic bacterium *Aquifex aeolicus* is a chemolithoautotroph that uses sulfur compounds as electron sources. The model of oxidation of the energetic sulfur compounds in this bacterium predicts that sulfite would probably be a metabolic intermediate released in the cytoplasm. In this work, we purified and characterized a membrane-bound sulfite dehydrogenase, identified as an SoeABC enzyme, that was previously described as a sulfur reductase. It is a member of the DMSO-reductase family of molybdenum enzymes. This type of enzyme was identified a few years ago but never purified, and biochemical data and kinetic properties were completely lacking. An enzyme catalyzing sulfite oxidation using Nitro-Blue Tetrazolium as artificial electron acceptor was extracted from the membrane fraction of *Aquifex aeolicus*. The purified enzyme is a dimer of trimer ($\alpha\beta\gamma$)₂ of about 390 kDa. The K_M for sulfite and k_{cat} values were 34 μ M and 567 s^{-1} respectively, at pH 8.3 and 55°C. We furthermore showed that SoeABC reduces a UQ₁₀ analogue, the decyl-ubiquinone, as well, with a K_M of 2.6 μ M and a k_{cat} of 52.9 s^{-1} . It seems to specifically oxidize sulfite but can work in the reverse direction, reduction of sulfur or tetrathionate, using reduced methyl viologen as electron donor. The close phylogenetic relationship of Soe with sulfur and tetrathionate reductases that we established, perfectly explains this enzymatic ability, although its bidirectionality *in vivo* still needs to be clarified. Oxygen-consumption measurements confirmed that electrons generated by sulfite oxidation in the cytoplasm enter the respiratory chain at the level of quinones.

Keywords: Sulfite dehydrogenase, Sulfite oxidation, DMSO reductase family of molybdenum enzymes, *Aquifex aeolicus*, Quinone, Phylogeny

Abbreviations

AMP: Adenosine monophosphate

APS: Adenosine-5'-phosphosulfate

ATP: Adenosine triphosphate

BN gel: Blue-Native gel

DB: Decyl-ubiquinone

DCPIP: 2,6-dichlorophenolindophenol

DDM: n-Dodecyl β -D-maltoside

DMK₇: 2-VI, VII-tetrahydromultiprenyl-1,4-naphthoquinone

DMSO: Dimethyl sulfoxide

EDTA: 2,2',2'',2'''-(Ethane-1,2-diyl)dinitrilo)tetraacetic acid

HQNO: 2-heptyl-4-hydroxyquinoline N-oxide

MES: 2-(N-morpholino)ethanesulfonic acid

MV: Methyl viologen

NBT: Nitro-blue tetrazolium

PAPS: 3'-Phosphoadenosine-5'-phosphosulfate

PGD: Pyranopterin Guanosine Dinucleotide

PIPES: Piperazine-N,N'-bis(2-ethanesulfonic acid)

Pmf: Proton motive force

PMS: Phenazine methosulfate

SO: Sulfite oxidase

Tris : 2-Amino-2-(hydroxymethyl)propane-1,3-diol

1. Introduction

Inorganic sulfur compound sulfite is mostly found as the sulfite anion (SO_3^{2-}) and hydrogen sulfite (HSO_3^-) at physiological pH (pK_a $\text{H}_2\text{SO}_3/\text{HSO}_3^-$ and $\text{HSO}_3^-/\text{SO}_3^{2-}$ are 1.80 and 6.97, respectively) and has a very low redox potential ($E_{\text{mpH}7}(\text{SO}_4^{2-}/\text{HSO}_3^-$ or $\text{SO}_4^{2-}/\text{SO}_3^{2-}) = -516$ mV or -480 mV [1-3]). Sulfite is highly reactive and toxic, and is endogenously produced by some bacterial and archaeal cells that use sulfur compounds as energy substrates or electron acceptors and some prokaryotes are able to use exogenous sulfite as sole electron donor for growth. Sulfite can also be generated during catabolism of sulfur-containing metabolites by numerous cells. This sulfur intermediate is usually rapidly converted, by various enzymes, into less toxic compounds or incorporated into molecules [4]. In bacteria and archaea, two main pathways of oxidation of sulfite into sulfate have been identified. The indirect AMP-dependent pathway requires the intervention of two soluble cytoplasmic enzymes APS reductase (EC 1.8.99.2) and ATP sulfurylase (Sat, EC 2.7.7.4) or adenylylsulfate:phosphate adenylyltransferase (APAT, EC 2.7.7.5). This indirect pathway consumes AMP and sulfite and releases ATP and sulfate. Another pathway directly oxidizes sulfite with water yielding sulfate (two-electron oxidation), in the presence of an appropriate electron acceptor. The enzymes responsible for this direct pathway are sulfite dehydrogenases (1.8.2.1 and 1.8.5.6). This pathway also exists in Eukaryotes performed by the sulfite oxidases (EC 1.8.3.1) whose deficiency in humans causes severe diseases and death. All three are molybdenum-coordinating enzymes, but not all use the same acceptor. Only sulfite oxidases can reduce oxygen, while sulfite dehydrogenases transfer electrons to cytochrome *c* or quinones. They belong either to the so-called Sulfite Oxidase (SO) family (for EC 1.8.3.1 and EC 1.8.2.1) or to the so-called DMSO-reductases or Complex Iron-Sulfur Molybdoenzymes (CISM) family (for EC 1.8.5.6). The structure of the pterin cofactor (called MoPTT for Molybdenum pyranopterin), a conserved cysteine residue as a ligand of the Mo, the architecture of the active site and the soluble character of the protein are common features of the members of the SO family [5]. One of the best characterized systems of the SO family in bacteria is the periplasmic heterodimeric sulfite dehydrogenase SorAB from *Starkeya novella*, composed of a large subunit carrying the molybdopterin cofactor and a small monoheme cytochrome *c* subunit [6,7]. The periplasmic enzyme SorT from the bacterium *Sinorhizobium (S.) meliloti*, another member of the SO family well characterized as well, is homodimeric using a cytochrome *c* (Sor U) as independent partner rather than subunit [8]. Few other sulfite dehydrogenases of this family have been purified from various bacterial sources but are less

described in details [reviewed in 5]. In the archaeon *Acidianus ambivalens*, a sulfite-oxidizing activity was detected in the membrane fraction but the corresponding enzyme, hypothesized to reduce the quinone pool, was neither identified nor purified [9]. A membrane-bound sulfite dehydrogenase was also purified from the bacterium *Acidithiobacillus (A.) thiooxidans* (formerly *Thiobacillus thiooxidans*), but not identified [10]. More recently, genes for a novel sulfite dehydrogenase called SoeABC (EC 1.8.5.6) were identified in bacterial species [11-15]. Unlike the soluble sulfite dehydrogenases described already (EC 1.8.2.1), this three-subunit enzyme is assumed to be membrane-bound and quinone reactive (*via* the SoeC subunit), facing the cytoplasm where SoeB (a module that carries Fe-S centers) and the SoeA (the Mo-carrying catalytic subunit) are exposed. Another striking structural difference between this new sulfite dehydrogenase and the already described enzymes is that Soe contains, in its active site, a molybdenum atom coordinated by two molecules of pyranopterin guanosine dinucleotide (therefore also named Mo-*bis*PGD enzymes [16]) rather than a single pyranopterin. It has been shown by a genetic approach (in a strain carrying a *soeA* gene deletion) that SoeABC is the major sulfite-oxidizing enzyme in the purple sulfur bacterium *Allochromatium (A.) vinosum* [12], but biochemical data and kinetic properties are still lacking.

In this paper, we focus on the sulfite oxidation in the marine hyperthermophilic bacterium *Aquifex (A.) aeolicus*, which has been extensively studied as a model for metabolism of inorganic sulfur compounds. It is a microaerophilic autotrophic chemolithoautotroph that uses molecular hydrogen (H_2) or inorganic sulfur compounds (elemental sulfur (S^0) or thiosulfate ($S_2O_3^{2-}$)) as electron sources and oxygen (O_2) as electron acceptor. Genome and biochemical analyses have led to the identification of several enzymes involved in the pathways for oxidation of sulfur compounds in *A. aeolicus* [17]. The current model of this oxidation in *A. aeolicus* predicts that sulfite is a metabolic intermediate released in the cytoplasm from the sulfur oxygenase reductase (SOR, Aq_455, entry O66762) which catalyzes disproportionation of S^0 , in the presence of O_2 , into hydrogen sulfide (H_2S) and sulfite [18] and also possibly from the heterodisulfide reductase (Hdr)-like complex (Aq_391 to Aq_400) [19]. It was genetically validated that this latter complex has a sulfur-oxidizing function in the dimethylsulfide-degrading bacterium *Hyphomicrobium denitrificans*, but the enzyme reaction and exact substrate(s) and product(s) of this enzyme are still unknown, although it is proposed in the literature that it could release sulfite [20]. In *A. aeolicus*, this Hdr-like complex faces the cytoplasm and is bound to the membrane [19]. Although the production of sulfite, which

should then be oxidized to sulfate, is very probable during the growth of *A. aeolicus* on sulfur compounds, the sulfite-oxidizing enzyme has never been described in this bacterium.

The objectives of this work were to identify, purify and characterize the enzyme that oxidizes sulfite in *A. aeolicus*. We have shown that it is a large membrane-bound complex, formerly called SreABC [21] and renamed SoeABC on account of its homology with other identified Soe representatives in the DMSO reductase superfamily of molybdenum enzymes. It oxidizes sulfite in the cytoplasm and electrons generated by sulfite oxidation enter the respiratory chain at the level of quinones.

2. Material and Methods

2.1. Growth and cell fractionation of *A. aeolicus* VF5

A. aeolicus was grown as previously described, at 85°C in the presence of thiosulfate (1g/L), with 68 mmol H₂ in each flask [21]. Cells were harvested by centrifugation and immediately used for oxygen-consumption assays or stored at -80°C if to be used further for protein purification. For membrane preparation, cells were lysed by two passages through a cell disrupter (Constant system Ltd) in 50 mM Tris-HCl pH 7.8, protease inhibitors mixtures and DNase I (10µg/mL) at a pressure of 1.6 kbar. Unlysed cells were removed by centrifugation at 10,000g for 15 min and the soluble and membrane parts were obtained by ultracentrifugation at 39,000g at 10°C for 1h (Beckman Coulter, Type 45Ti Rotor).

2.2. Solubilization of membranes with detergents

The membrane fraction was resuspended in 50 mM Tris-HCl pH 7.8, 5% (v/v) glycerol (Buffer A) at a protein concentration of 10mg/mL and solubilized with various non-ionic detergents : n-Dodecyl β-D-maltoside (DDM), Digitonin, Octylthioglucoside (OTG) or IGEPAL CA-630 (a substitute for Nonidet P-40; named IGEPAL throughout the manuscript) at a final concentration of 0.5%, 1% or 3% (w/v for DDM, digitonin and OTG and v/v for IGEPAL). Solubilization was carried out for 30 minutes at 37 °C and suspension was ultracentrifuged at 40000g for 1h to separate the solubilized membrane proteins (in the supernatant) from the insoluble material.

2.3. Protein purification

All steps were performed under argon at room temperature. All the buffers were degassed with argon. The purification of SoeABC was carried out from 40 to 50g of *A. aeolicus* cells.

After solubilization of membranes with 1% IGEPAL (see section 2.2), the supernatant was loaded onto a hydroxylapatite (HA) column (4 x 22cm, Bio-Gel; Bio-Rad) equilibrated with buffer A containing 0.02% (v/v) IGEPAL. The proteins were eluted by steps with potassium phosphate buffer pH 7.8 supplemented with IGEPAL. The sulfite oxidase activity was found in the fractions eluted at 40 mM potassium phosphate. This active fraction was diluted 3-fold in buffer A and applied directly on a Q Sepharose 26/10 column controlled by a fast protein liquid chromatography system (Akta pure, GE Healthcare) equilibrated with buffer A, 0.02% (v/v) IGEPAL. The absorbed proteins were eluted with a linear gradient from 0 to 500 mM NaCl in buffer A and 0.02% IGEPAL. The activity was found in fractions eluted between 250 and 300mM NaCl, which were concentrated on a Vivaspin centrifugal concentrator (membrane cut-off 100 kDa) and loaded onto a gel filtration S200 (10/300; GE Healthcare) column preequilibrated with buffer A containing 0.02% (v/v) IGEPAL and 150 mM NaCl. After concentration and dialysis, the pure sulfite dehydrogenase protein preparation was analyzed by native and SDS polyacrylamide gel electrophoreses and used for enzyme assays and characterization. The protein concentration was determined with the Bicinchoninic Acid Protein Assay Kit (Sigma) using bovine serum albumin as protein standard.

2.4. Electrophoresis and in-gel sulfite dehydrogenase activity

Proteins were separated by SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel) or by Blue-Native (BN) gels as described previously [22]. The activity of sulfite oxidation was detected directly in BN gel using nitro-blue tetrazolium (NBT, $E_{mpH7.2} = -50$ mV) as electron acceptor and sodium sulfite as substrate, under aerobic conditions at 60°C. The gel was first equilibrated at 60°C in a 50 mM Tris-HCl buffer, pH 7.8 for 5 minutes, then incubated in 50 mM Tris-HCl, pH 7.8, 500 μ M sulfite and 0.2% (w/v) NBT at 60°C. Formation of reduced NBT induces formation of a purple precipitate. The gel was rinsed with cold water.

The sulfite-oxidizing activity was also detected in BN gel in semi-anaerobic conditions, in identical conditions as above, except that the reaction mixture was degassed with argon gas before introducing the gel and the reaction was conducted in the presence of argon in a sealed closed flask.

2.5. Kinetic measurements

2.5.1. Oxidation of sulfur compounds

The oxidation of sulfite was assayed spectrophotometrically, at 55°C using NBT as electron acceptor. The reaction mixture (500µL) contained 50 mM Tris-HCl buffer adjusted either at pH 8.3 (for the measurements with the purified enzyme) or pH 7.8 (for the measurements with the membranes solubilized with the detergent), 5% (v/v) glycerol, 0.02% (v/v) IGEPAL, 100µM sodium sulfite and 1mM NBT. Sodium sulfite (25 mM stock solution) was freshly prepared in water and NBT (10 mM stock solution) was dissolved in water and stored at -20°C. The cuvette was filled with buffer, sulfite and NBT and left for two minutes in the spectrophotometer at 55°C (Cary 60 UV-Vis equipped with a Peltier temperature controller, Agilent) before introducing the enzyme sample. The NBT reduction was monitored at 605 nm for a few minutes. No reduction of NBT was recorded in absence of the enzyme. NBT was previously used as an artificial electron acceptor of various dehydrogenases and assumed to be a 2-electron acceptor [23,24]. The specific activity is expressed as the number of µmol of NBT reduced min⁻¹ mg of proteins⁻¹, using $\epsilon = 17.2 \text{ mM}^{-1} \text{ cm}^{-1}$. k_{cat} values are reported as the number of sulfite molecules oxidized per second. For determination of the K_{M} , sulfite was used from 2 to 120µM. Kinetic parameters (and standard errors) were derived by fitting of the data to the Michaelis–Menten equation using SigmaPlot 11.0 software. The error associated with the ratio $k_{\text{cat}}/K_{\text{M}}$ was calculated as described in [25]. The pH-dependence of the sulfite-oxidizing activity was determined at 55°C as above, in Tris-HCl buffer adjusted from pH 7 to pH 9, 5% glycerol and 0.02% IGEPAL, using 100 ng of enzyme in each measurement.

Oxidation of other sulfur compounds by SoeABC was assayed spectrophotometrically at 55°C in Tris-HCl buffer pH 8.3, 5% glycerol and 0.02% IGEPAL. Polysulfide was used at concentrations ranging from 50 to 100µM with 1 mM NBT as the electron acceptor, but the chemical reaction (without enzyme) was very strong and prevented activity measurements. Polysulfide was prepared according to Ikeda et al. [26] by mixing and incubating, for 1 hour at room temperature, 1.2g of sodium sulfide with 160 mg of sulfur flower in 3mL of argon-degassed water in a Hungate tube. The mixture was then anaerobically diluted with water to give a final volume of 10mL (polysulfide concentration of 0.5 M). Oxidation of sodium tetrathionate by SoeABC was tested, using DCPIP (2,6-dichlorophenolindophenol) in the presence or absence of phenazine methosulfate (PMS). The reaction mixture contained sodium tetrathionate at 100µM, 2 mM or 4mM, 100µM DCPIP and 6 µg/mL PMS when added. The reduction of DCPIP was followed at 600 nm. Sodium thiosulfate – NBT or sodium thiosulfate – decyl-ubiquinone (DB) oxidoreductase activity was measured by

following absorbance changes at 605 and 275 nm respectively. A concentration of 100 or 200 μM thiosulfate, 1 mM NBT and 80 μM DB were used.

2.5.2. Quinone reduction

The reduction of decyl-ubiquinone (DB) using sodium sulfite as electron acceptor was spectrophotometrically followed at 55°C in 50 mM Tris-HCl buffer at pH 8.3, 5% (v/v) glycerol, 0.02% (v/v) IGEPAL. Sodium sulfite (25 mM stock solution) was freshly prepared in water and DB was dissolved in absolute ethanol (3 mM stock solution) and stored at -20°C. The cuvette (500 μL) was filled with buffer, 100 μM sulfite and 80 μM DB and left for two minutes in the spectrophotometer at 55°C (Cary 60 UV-Vis equipped with a Peltier temperature controller, Agilent) before introducing the enzyme sample. The DB reduction was monitored at 275 nm for a few minutes. No reduction of DB was recorded in the absence of enzyme. The specific activity is expressed as the number of μmol of DB reduced min^{-1} mg of proteins $^{-1}$, using $\epsilon = 15 \text{ mM}^{-1} \text{ cm}^{-1}$. k_{cat} values are reported as the number of sulfite molecules oxidized per second. Kinetic parameters and standard errors were calculated by fitting the data (DB concentration from 2 to 70 μM) to the Michaelis–Menten equation using SigmaPlot 11.0 software. The error associated with the ratio $k_{\text{cat}}/K_{\text{M}}$ was calculated as described in [25].

Inhibition of DB reduction, with sodium sulfite as the electron donor, was measured under identical conditions as without inhibitors, using Antimycin A at 27 μM (stock solution at 4.5 mM) or HQNO (2-heptyl-4-hydroxyquinoline N-oxide) at 10 μM (stock solution at 40mM). Inhibitor was introduced in the cuvette before the enzyme.

2.5.3. Reduction of sulfur compounds

The reduction of sulfur compounds by SoeABC (reductase activity) was followed either spectrophotometrically or by sulfide determination, with reduced methyl viologen (MV) as the electron donor in both cases, in 50 mM potassium phosphate buffer pH 8.2.

The reduced MV – sodium thiosulfate or reduced MV – sodium tetrathionate activity was determined anaerobically at 55°C by following the oxidation of the MV at 604 nm. The reaction mixture (500 μL) contained the argon-degassed buffer, 1mM MV, sodium dithionite, 1-2 mM thiosulfate or 1 mM tetrathionate. The cuvette was sealed and the reaction mixture was degassed for five minutes. A solution of 100 mM sodium dithionite was prepared in 1M potassium phosphate pH 8.2 degassed with argon and was added into the sealed cuvette with a

syringe to reduce the MV and obtain an absorbance at 604 nm of 1-1.2 before introducing the enzyme. The specific activity is expressed as the number of nmol of MV reduced $\text{min}^{-1} \text{mg}$ of proteins⁻¹, using $\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

The reduction of sulfur compounds was also followed at 70°C by assaying the evolution of sulfide formed by the reduction of the sulfur compound using a colorimetric assay [21]. The reaction mixture, in 5mL sealed tube, contained the buffer, the sulfur compound (at 2 mM final concentration), MV 2mM and sodium dithionite (prepared as described above). The tubes were degassed with argon for 15 min. The reaction and H₂S concentration determination (measurement of methylene blue at 670 nm) were performed as previously described [21] except that the vials were not incubated under H₂ and that the reaction was performed at 70°C for 20 minutes. Controls were measured in absence of the sulfur compound or the enzyme or the reduced MV. When elemental sulfur was used as electron acceptor, it was prepared as dispersed sulfur as described in [27].

2.6. Sulfite-dependent oxygen consumption

Measurement of O₂ consumption rates were carried out using an oxygraph (Hansatech Instruments) thermo stated at 60°C using a water bath. The Clark-type electrode was calibrated at 60°C using sodium dithionite. A mixture of buffers (polybuffer) at pH 7.8 containing MES, PIPES, Tris and Glycine, each at a concentration of 50 mM, was pre-equilibrated at 60°C and used for all O₂ uptake experiments. Sulfite was freshly prepared at 0.1 M in water supplemented with 50 mM EDTA. The polybuffer at pH 7.8 and 2 mM sulfite /1 mM EDTA (final concentrations) were placed in the measurement chamber and incubated for two minutes (with the stopper in place) before introduction of the sample with a syringe (whole cells or membranes from *A. aeolicus*). Intact membranes from *A. aeolicus* (see section 2.1 for membrane obtention) were resuspended, after the ultracentrifugation, in the polybuffer pH 7.8 at a concentration of 20 mg/ml of proteins. 60, 100 or 160µg of proteins were used in the assays. The specific O₂ consumption rate was expressed in µmoles of O₂ consumed $\text{min}^{-1} \text{mg}$ of proteins⁻¹. To test the effect of the inhibitor potassium cyanide (KCN), it was introduced in the chamber before the addition of membranes at a concentration of 500µM or 2mM (from a 100mM stock solution). HQNO was prepared at 40 mM and used at a final concentration of 10µM. For the determination of the optimal pH, the pH of the polybuffer was adjusted at 6.0, 7.0, 7.8, 8.5, 9.0 and 9.5 and O₂ consumption was measured as specified above using 200µg of proteins (except for pH 6 for which 400µg were introduced in the

chamber). The cells were centrifuged at the end of growth and resuspended in the polybuffer at pH 7.8 at a concentration of 0.5g of cells (wet weight) / mL and 2.5 or 5 mg of cells were used in each measurement. The specific O₂ consumption rate was expressed in micromoles of O₂ consumed per minute per gram of cells ($\mu\text{mol min}^{-1} \text{g cells}^{-1}$). With fresh cells, KCN was used at a concentration of 1mM and added before cells. For each measurement, the rate of O₂ consumption obtained after addition of enzyme samples (cell-free extracts of entire cells) was corrected by subtracting the low non-enzymatic chemical O₂ reduction rate by sulfite.

2.7. Protein identification by tandem mass spectrometry

Proteins present in bands of gels were identified as previously described [19].

2.8. Bioinformatics

2.8.1. Analysis of the *A. aeolicus* genome sequence

Sequences of SO family members from the three major groups of enzymes constituting this family, as defined by Kappler in 2011 [5], were retrieved from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and used as queries for BLAST searches (blastp on *Aquifex aeolicus* VF5): YedY of *Escherichia coli* (ABJ01306; group 1A), protein PA4882 of *Pseudomonas aeruginosa* (NP_253569.1; group 1B), human sulfite oxidase (AAA74886.1; group 2A), plant sulfite oxidase (NP_186840 *Arabidopsis thaliana*; group 2A), plant nitrate reductase (BAE99256.1 *Arabidopsis thaliana*; group 2A), SoxC of the SoxCD sulfur dehydrogenase of *Starkeya novella* (AAF61449.1, group 2B), SorA of the SorAB sulfite dehydrogenase of *Starkeya novella* (AAF64400.1; group 2C), SorT of *Sinorhizobium meliloti* (AGG75421.1; group 2 other), protein SSO3201 of *Sulfolobus solfataricus* (AAK43298; group 3A), and Smb20584 of *Sinorhizobium meliloti* (AGG72506.1; group 3B). Sequence identity percentages were determined using ClustalW (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html).

2.8.2. Occurrence of genes involved in oxidation of sulfite in *Aquificales*

Genes were retrieved with a BLAST Search, against Aquificae (taxid:200783), using as queries SorA (AAF64400.1) and SorB (AAF64401.1) sequences from *Starkeya novella*. For a number of species in the *Aquificaceae* family, a *bona fine sorAB* diade was not retrieved but a gene encoding a monoheme cytochrome *c* was found adjacent to the *sorA* gene. Genes coding for the ATP sulfurylase were found using Sat from *A. vinosum* (AAC23622.1), the ones for APS reductase with AprAB of *A. vinosum* (adenylylsulfate reductase alpha subunit

AAC23621 and adenylylsulfate reductase beta subunit AAC23620.1) and the ones for SoeA with the *soeA* gene (ADC63403.1) from *A. vinosum*.

2.8.3. Phylogeny

Open reading frames coding for subunits homologous to Thiosulfate reductase Phs sequences were retrieved from the National Center for Biotechnology Information by using the PhsA sequence from *Salmonella enterica* (NP_461010.1). Sulfur reductase Sre sequences were retrieved by using the SreA sequence from *Acidianus ambivalens* (CAC86937.1) [28] but Sre sequences analyzed by Sorokin et al. [29] were also included in the tree reconstruction. Sulfite dehydrogenase Soe sequences were retrieved by using the SoeA from *A. vinosum* (ADC63403.1) [12]. Polysulfide reductase (Psr) sequences were retrieved by starting from PsrA/B/C sequences from *Wolinella (W.) succinogenes* and Tetrathionate reductase (Ttr) sequences were retrieved by starting from TtrB/C/A sequences from *Salmonella typhimurium*. Because a still unidentified clade named Unk in [30] and a new clade named Arn (for antimonate reductase) in [31] have been determined close to the Molybdo-enzymes of the sulfur metabolism, we added these clades to our study. Aio sequences, used as outgroup, had been sampled before [30].

Multiple sequence alignments of recognized subfamilies of Mo-*bis*PGD subunits were automatically produced using ClustalX [32]. The automatically generated alignments were subsequently refined using Seaview [33] with respect to functionally conserved residues, to structural alignments and to secondary structures. Phylogenetic trees were reconstructed from these alignments using either the Neighbor-Joining (NJ)- algorithm or the Maximum Likelihood method implemented in MEGA7.

3. Results

3.1. Genome survey for genes involved in sulfite oxidation in *A. aeolicus*

As stated in the Introduction, bacteria can oxidize sulfite by a combination of two (or sometimes three) soluble enzymes of the AMP-dependent pathway. The second enzyme of the pathway, the ATP sulfurylase releasing sulfate and ATP, from APS and pyrophosphate, has been characterized as a bifunctional enzyme that exhibits both ATP sulfurylase and adenosine-5'-phosphosulfate (APS) kinase activities in *A. aeolicus* [34]. However, no gene coding for an APS reductase, the first enzyme of the pathway, which produces APS from

sulfite and AMP, could be found in the genome of *A. aeolicus*, suggesting that sulfite is not oxidized by this route which seems to be incomplete in this bacterium [17] (Table 1). The bifunctional Sat/APS kinase might be involved in sulfate assimilation or provide PAPS for sulfate ester formation by sulfotransferases [34].

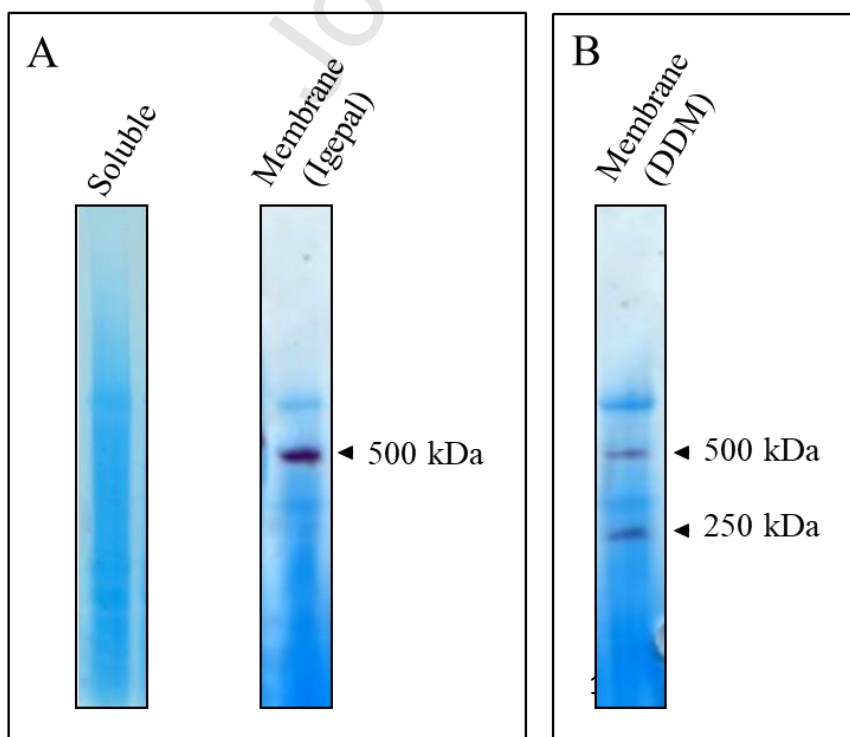
Genes coding for a sulfite dehydrogenase that directly oxidizes sulfite have then been looked for in the genome. Using sequences of SO family members from the three major groups of enzymes constituting this family (See [5] for enzymes classification and Material and Methods section) as queries for BLAST searches, the *aq_979* gene of *A. aeolicus* was retrieved in each case (except for the SorT protein from *S. meliloti* as query which did not match any gene). The sequence of the putative Aq_979 protein (accession AAC07069.1) indicates that it is a heme-free soluble cytoplasmic molybdenum enzyme of 23 kDa. It is predicted to be a member of the structural group 3B of SO family in which the putative oxidoreductase molybdopterin binding protein from *S. meliloti* (Accession AGG72506) [5], having about 41% sequence identity with Aq_979, belongs. In this group of enzymes, the conserved cysteine residue, coordinating the molybdenum, is included in the DFHCVTXWS motif sequence. However, members of this group are not characterized and they might catalyze reactions that are distinct from those usually associated with SO family enzymes [5]. From our survey, it is therefore predicted that *A. aeolicus* does not have any soluble periplasmic sulfite dehydrogenase with homology to SorAB or SorT (belonging to the group 2 of SO family; Table1), but it could potentially oxidize sulfite in the cytoplasm *via* a small soluble uncharacterized enzyme encoded by the *aq_979* gene. Finally, a search with as query the gene coding for the molybdenum subunit of the membrane-bound sulfite dehydrogenase (SoeA from *A. vinosum*, accession ADC63403.1, [12]) identified the *dmsA* (*aq_1234*) gene which codes for the large catalytic subunit of a complex purified from *A. aeolicus* membranes previously but characterized *in vitro* as a sulfur reductase and named Sre [21] (Table 1). *dmsA/sreA* is clustering with *dmsB1/sreB* (*aq_1232*) and *dmsC/sreC* (*aq_1231*) genes. *DmsA/SreA* from *A. aeolicus* and *SoeA* from *A. vinosum* share about 47% sequence identity. Sulfite oxidation in *A. aeolicus* might thus be mediated either by a small uncharacterized soluble cytoplasmic Aq_979 enzyme from the SO family and/or by an enzyme complex, with *Dms/SreABC* bound to the membrane and facing the cytoplasm (as suggested by the absence of TAT signal sequence in any of the three subunits), belonging to the large DMSO reductase family.

3.2. The sulfite-oxidizing entity of *A. aeolicus* is a large membrane-bound enzyme

With the aim of identifying and purifying the enzyme, we developed an activity-staining procedure to visualize the sulfite-oxidase activity in cell extracts directly in native gel. We first tried to display the activity on gel using sulfite as electron donor and ferricyanide, a common artificial electron acceptor for sulfite-oxidizing enzymes [5]. However, no sulfite-dependent reduction activity could be detected in this condition, neither with the soluble fraction nor with the membrane fraction of *A. aeolicus* (data not shown). This prompted us to test other electron acceptors. Of the various compounds tested, nitro-blue tetrazolium (NBT) was used as electron acceptor by the enzyme and resulted in a stained band of enzymatic activity for the membrane fraction, on Blue native (BN) gel (Figure 1A). The reduction of NBT (redox midpoint potential $E_0 = -50\text{mV}$ at pH 7.2 with respect to the normal hydrogen electrode, [35]) induces the formation of formazan, a purple insoluble precipitate. The apparent molecular size of the enzyme, indicated by the migration profile of the molecular mass markers, is about 500 kDa. This is probably an overestimate, because it corresponds to the size of the enzyme plus that of the detergent, lipids and Coomassie Blue G molecules bound to the enzyme. It is evident from this experiment that sulfite oxidation occurs in *A. aeolicus*, and that the activity appears to correspond to a large complex located in the bacterial membrane, as no band of activity was detected in the soluble fraction (Figure 1A). In agreement with this result, the sulfite-oxidizing specific activity (spectrophotometrically determined, see paragraph 3.4.1. for activity assay development) is more than five times higher in the membrane than in the soluble fraction.

Figure 1: Activity of sulfite oxidation detected on blue-native gel.

The total soluble fraction (100 μg of proteins, panel A) or the proteins solubilized from the membrane fraction (50 μg of proteins, panel A and panel B) were resolved by a 4-15% blue-native gel. The membranes were solubilized either with the detergent IGEPAL (Panel A) or with the detergent DDM (Panel B), both at a final concentration of 1%. The activity was detected using sulfite and NBT at pH 7.8 and 60°C. Arrows indicate bands in which the activity was detected. As appearance of activity in the IGEPAL solubilized membrane fraction is faster than the one using DDM, the incubation time for the enzyme activity detection is longer when using DDM compared to IGEPAL.



As the enzyme is associated with the membranes, several detergents were used at different concentrations to solubilize the *A. aeolicus* membranes, and the solubilized membrane proteins were resolved on a BN gel for sulfite-oxidizing activity detection. One intense band of activity was obtained with the IGEPAL detergent at both concentrations assayed whereas only a very faint band was visible with DDM and Digitonin at 3%, and no band appeared at all when the OTG was used (Supplemental Figure A1). This shows that the enzyme is well solubilized from the membrane with the detergent IGEPAL and that it retains its activity in this condition. It was thus chosen for the rest of the work. It is worth mentioning that when DDM was used to extract proteins from membranes, two bands of sulfite-oxidizing activity were usually observed, one at about 500 kDa and a second at about 250 kDa (Figure 1B, note that the incubation time for the enzyme activity detection in the experiment shown in Figure 1B is longer when using DDM compared to IGEPAL; for comparison with equal incubation time see Supplemental Figure A1). This could indicate the existence of a dimeric form of the enzyme at 500 kDa (the only one clearly visible when IGEPAL is used), which could be partly dissociated into a monomer, retaining the activity, in the presence of DDM.

3.3. Purification and identification of SoeABC as the sulfite dehydrogenase from *A. aeolicus*

The enzyme was purified starting from the membrane fraction of *A. aeolicus*, solubilized with 1% IGEPAL using several chromatographic steps (see section 2 for details). The enzyme activity was spectrophotometrically followed during the purification. The last purification step (size exclusion chromatography with a Superdex 200 column) seems to be detrimental for the enzyme as half the specific activity is lost after this step. The purification resulted in a 23-fold purification of sulfite-oxidizing activity as compared to the membrane fraction (58-fold purification before the last step) and a yield of 2%. Pure sulfite dehydrogenase had a specific activity of $84.2 \pm 7.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (at pH 8.3).

The enzyme activity detection of the purified fraction in BN gel displays two bands at roughly 500 and 250 kDa, which confirm that it tends to dissociate during purification (Figure 2A).

The protein fraction containing the target enzyme was also resolved by denaturing gel, which showed the presence of two main protein bands at about 100 and 32 kDa, in addition to two very faint bands (at 120 and 25 kDa) and a Coomassie blue staining corresponding to proteins unresolved by the gel migration (at the top of the gel) (Figure 2B). The proteins, from both

gels, were all identified by tandem mass spectrometry and correspond to subunits of the membrane-bound complex formed by Aq_1234 (DmsA/SreA), Aq_1232 (DmsB1/SreB) and Aq_1231 (DmsC/SreC) (Table 2), that was earlier shown to contain a molybdenum cofactor, in addition to several [Fe-S] clusters [21]. The enzyme extracted from *A. aeolicus* some time ago has been demonstrated to have a sulfur-reducing activity *in vitro* [21]. It is obvious from the present results that it possesses a sulfite-oxidizing activity as well. We propose to rename it SoeABC, like the enzyme from *A. vinosum* [12]. On the BN gel, the two bands contain the three proteins SoeA, SoeB and SoeC, which indicates that the same enzyme is present in these two bands but with a different oligomeric state (Figure 2A bands 1 and 2 and Table 2).

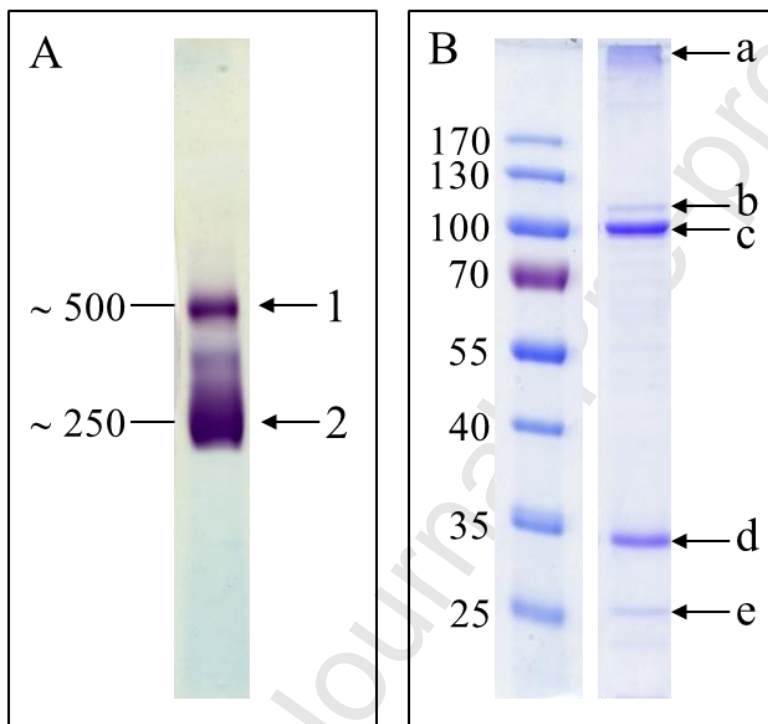


Figure 2: Migration profile of the purified complex on blue native and denaturing gel.

4.5 μg of proteins were loaded on a 4-15% BN gel and the activity of sulfite oxidation was revealed (panel A). The approximate molecular mass of the bands is indicated on the left in kDa. 4.5 μg of the complex were resolved on a 12% SDS gel and stained with Coomassie blue (panel B, right lane). Molecular mass markers were resolved (left lane) and their masses are indicated on the left in kDa. The complex composition was obtained after bands 1, 2, a, b, c, d and e were cut out from gels and analyzed by tandem mass spectrometry (see results in Table 2).

We therefore propose that this enzyme is organized as a dimer of the heterotrimer $(\alpha\beta\gamma)_2$ which tends to dissociate to give a monomer of the trimer $\alpha\beta\gamma$. On the denaturing gel, only the two proteins SoeA (Figure 2B band c) and SoeB (Figure 2B band d) are visible as a sharp band each. From sequence analyses, SoeC is probably composed of eight transmembrane helices and is thus highly hydrophobic [21]. It very likely precipitates in this denaturing condition and is consequently found, together with the two other subunits, at the top of the gel (Figure 2B band a and Table 2). The SoeC subunit seems to be present in significant amount in the purified enzyme because unique peptides and PSM numbers are relatively high for such a hydrophobic protein, especially when the enzyme is separated on BN gel (Table 2). However, it cannot be completely ruled out that SoeC may be under-represented in the preparation and not present in an equimolar quantity with respect to the other two subunits. The two substantially under-represented proteins on the SDS gel were identified as a protein annotated Aq_863 of 118 kDa with a probable function as an inorganic carbon pump (band b) [36] and an alkyl hydroperoxide reductase of 24 kDa (band e), both of which are probably contaminants.

The elution profile of SoeABC from a gel filtration chromatography (using a S200 column) showed the existence of two peaks (Figure A2), which we propose to correspond to the dimer and monomer of the enzyme. The molecular mass of the sulfite dehydrogenase was estimated to be 389 kDa for the dimer and 211 kDa for the monomer, which compare reasonably well with the theoretical ones (362 and 181 kDa for the dimer and the monomer, respectively).

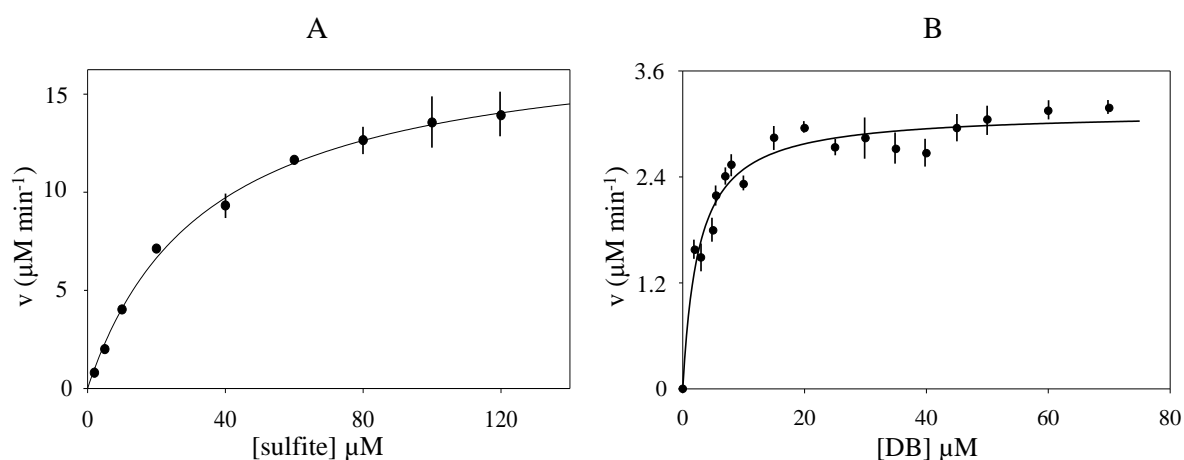
3.4. Kinetic properties of SoeABC from *A. aeolicus*

3.4.1. Optimization of kinetic assay conditions for sulfite oxidation and kinetic analysis

To measure the sulfite-oxidizing activity of the enzyme precisely we developed a spectrophotometric activity test assay. As stated above (section 3.2), sulfite dehydrogenases and sulfite oxidases previously characterized use ferricyanide as electron acceptor, as well as a cytochrome *c* in standard assays. None of them is reduced by the SoeABC enzyme in the presence of sulfite, whatever the concentration, pH or temperature conditions. On the other hand, the reduction of NBT is very efficient. The best measurement conditions were determined to be 55°C, pH 8.3, sulfite concentration of 100µM and NBT concentration of 1mM, in the presence of 5% (v/v) glycerol and 0.02% (v/v) IGEPAL. The maximal sulfite-oxidizing activity was observed at pH 8.3 and activities, in the same buffer at pH 7.0, 7.8, 8.0, 8.7 and 9.0 were 31, 67, 85, 93 and 70% of the maximum respectively (Supplemental Figure

A3 A). In absence of both glycerol and detergent, the initial reaction rate was not proportional to the amount of enzyme used, which suggests a possible denaturation or dissociation of the enzyme at low protein concentrations and a stabilization or protection of the complex by these two compounds (Figure A3 B). Many enzymes indeed suffer denaturation upon dilution.

The kinetic parameters of SoeABC were calculated from the Michaelis-Menten plot (using sulfite as electron donor and NBT as electron acceptor). At pH 8.3 and 55°C, the K_M for sulfite and k_{cat} values were $34.3 \pm 2.6 \mu\text{M}$ and $567 \pm 50 \text{ s}^{-1}$ respectively (Figure 3A and Table



3).

Figure 3: Steady-state kinetic data for the *A. aeolicus* Soe enzyme.

Initial velocities were plotted against substrate concentrations (sulfite in panel A, DB in panel B). Lines represent fits of the data to the Michaelis-Menten equation. See section 2.5.1 for details.

When the activity was detected on BN gel, the intensity of the band was much higher in semi-anaerobic compared to aerobic conditions. This was verified when using solubilized membranes as well as the purified enzyme (Supplemental Figure A4). However, this could not be reproduced using the spectrophotometric assay with which this activity of sulfite oxidation was found to be roughly the same in both conditions (data not shown). This may arise because of differences in experimental conditions in which the activity is measured (in solution *versus* in gel, anaerobiosis probably not fully comparable from one condition to another, substrates concentration, etc).

3.4.2. Reduction of quinones

SoeC, localized in the membrane, has been assumed to bind and reduce quinone [12]. We therefore tested its ability to reduce a commercially available UQ₁₀ analogue, the decyl-ubiquinone (DB), during sulfite oxidation. This enzyme can in fact use this quinone as electron acceptor *in vitro*, with a K_M for the DB of $2.6 \pm 0.35 \mu\text{M}$ and a k_{cat} of $52.9 \pm 4.1 \text{ s}^{-1}$ (at pH 8.3 and 55°C) (Figure 3B and Table 3). These values indicate that SoeABC has a high affinity for this quinone (although *A. aeolicus* contains in its membranes the 2-VI, VII-tetrahydromultiprenyl-1,4-naphthoquinone, a demethylmenaquinone, DMK₇ [37]), but the rate of catalysis is lower than with NBT.

The effect of well-known inhibitors affecting quinone binding sites in eukaryotic as well as prokaryotic oxidoreductases [16] was tested spectrophotometrically on the sulfite-dependent quinone reductase activity (Figure A5). HQNO and Antimycin A were found to be potent inhibitors of SoeABC (100% and 55% inhibition with HQNO at 10 μM and Antimycin A at 27 μM , respectively).

3.4.3. Sulfur electron donor specificity

SoeABC was assayed for its capacity of oxidation of other inorganic sulfur compounds with an appropriate electron acceptor (Table 4). In addition to sulfite, tetrathionate, polysulfide or thiosulfate were used to try to reduce various artificial electron acceptors. None of these compounds were found to be oxidized by the enzyme in our experimental conditions. It was not possible to determine whether polysulfide was oxidized by the enzyme, because of the strong reaction of this compound with the various electron acceptors. SoeABC seems therefore to oxidize sulfite specifically. Simple thermodynamics considerations (ΔG of the considered reaction; see Table 4) could be sufficient to rationalize the results obtained.

3.4.4. Capacity of SoeABC to reduce sulfur compounds

SoeABC was purified already and shown to be able to reduce tetrathionate and elemental sulfur (but not thiosulfate) in the presence of a hydrogenase, a menaquinone and hydrogen [21]. It was therefore verified that the purified enzyme in this work is well able to reduce these sulfur compounds using reduced MV as electron donor. This was shown by a spectrophotometric assay in which oxidation of MV was monitored as well as by a detection of the released sulfide using a colorimetric assay (Table 5).

As expected, the enzyme, in addition to being a sulfite dehydrogenase, can work in the other direction *in vitro*. As shown in Table 5, tetrathionate is reduced by the enzyme (63 nmol of MV oxidized $\text{min}^{-1} \text{mg}^{-1}$ at 55°C and pH 8.2) while thiosulfate is not able to act as an electron acceptor from MV. With the colorimetric assay for sulfide quantification, it was not possible to obtain a reliable value of activity for the reduction of tetrathionate for unknown reasons, although sulfide was most of the time produced (as assessed by the blue color obtained at the end of the reaction as well as the characteristic smell of sulfide). As predicted [21], elemental sulfur (prepared in the form of dispersed sulfur; [27]) was found to be an electron acceptor (119 nmol H_2S produced $\text{min}^{-1} \text{mg}^{-1}$). A thermodynamic reason could explain the absence of reduction of thiosulfate by SoeABC if we consider the reaction performed by the thiosulfate reductase Phs releasing sulfide and sulfite (in this case the redox couple to be considered is $\text{S}_2\text{O}_3^{2-} / \text{HS}^- + \text{HSO}_3^-$, Table 5). But this absence of reduction could hardly be explained by thermodynamics considering the redox couple $\text{S}_2\text{O}_3^{2-} / \text{HS}^-$ (Table 5), though no enzyme catalyzing such a reaction (reduction of thiosulfate in sulfide) is currently identified. Sulfite, which is rapidly oxidized by SoeABC, is not reduced to HS^- . It is to note, however, that this reaction (the redox couple to be considered here would be $\text{HSO}_3^- / \text{HS}^-$), uses a mechanism implying a direct transfer of $6e^-$ (including the MccA and DsrAB enzymes) (as reviewed in [4]). The strict reverse reaction of sulfite oxidation, *i.e.* reduction of sulfate to sulfite has not been assayed *in vitro*. The redox potentials of the available electron donors are inappropriate, the MV the best reducing agent, is still a stronger oxidant than sulfate.

3.5. Coupling of sulfite oxidation to cell respiration in *A. aeolicus*

SoeABC is bound to the membrane and very likely reduces the quinone pool *in vivo*. Electrons from sulfite could thus serve to reduce O₂ by O₂ reductases at the end of the respiratory chain. *A. aeolicus* contains genes for two cytochrome *c* oxidases, a putative *aa*₃-type (potentially composed of CoxA1 (AAC07899.1), CoxB (AAC07901.1) and CoxC (AAC07902.1)) and a *ba*₃ enzyme (composed of CoxA2 (AAC07900.1), CoxB2 (AEQ59231.1) and CoxIIa (CCA61232.1) [43]), as well as one *bd*-type quinol oxidase (formed by CydA (AAC07328.1) and CydB (AAC07329.1)) but it was shown that only the *ba*₃ cytochrome *c* oxidase (forming a supercomplex with the *bc*₁ complex) and the *bd* quinol oxidase were present in the membrane in the growth conditions employed in the present study [17,44]. To investigate whether electron transfer from sulfite to O₂ occurs, O₂ consumption by whole *A. aeolicus* fresh cells was measured by polarography in the presence of sulfite as the only electron donor, at pH 7.8 and 60°C. Oxygen was rapidly consumed after addition of cells in an air-equilibrated buffer supplemented in sulfite as shown in Supplemental Figure A6 A and the rate was proportional to the concentration of cells. No consumption was recorded when sulfite was omitted. The sulfite-dependent O₂ uptake rate was $3.9 \pm 0.2 \mu\text{mol O}_2 \text{ min}^{-1} \text{ g}$ of wet cells⁻¹. This is rather low but (i) in a same range of O₂ consumption activity determined for other sulfur oxidizing bacteria [45] and (ii) this value is probably largely underestimated because it corresponds to the oxidation of extracellularly added sulfite, probably not representative for the oxidation rate of intracellularly formed sulfite and also because measurements were made at 60°C, although the optimum temperature for growth of *A. aeolicus* is 85°C. This activity was completely inhibited by 40μM HQNO. These results demonstrate that sulfite feeds the electron transport chain up to O₂ reduction and that it involves at least one quinone-using complex.

Like cells suspensions, the fraction of intact membranes prepared from *A. aeolicus* were found to consume O₂ with sulfite as electron donor. The optimal pH for this activity is between 7.8 and 9 (Supplemental Figure A6 B). The rate was $0.19 \pm 0.04 \mu\text{mol O}_2 \text{ min}^{-1} \text{ mg}$ of proteins⁻¹ (at pH 7.8 and 60°C). This electron transport is completely inhibited by 10μM HQNO. KCN, an inhibitor of cytochrome *c* oxidases and quinol oxidases, had a strong effect on oxidation of sulfite at a concentration of 2mM (90% inhibition) while the inhibition was only of 47% at a concentration of 500μM. The *bd* quinol oxidase is known to be KCN-resistant in bacteria [46] and 500μM KCN are sufficient to completely inhibit the *A. aeolicus* *ba*₃ cytochrome *c* oxidase (Infossi P. and Guiral M., unpublished results). The *bd* quinol oxidase is therefore probably involved in the sulfite-dependent O₂ reduction in *A. aeolicus*. In

addition, an involvement of the *ba₃-bc₁* supercomplex cannot be excluded. Our results obtained on membrane fractions could be explained by the presence of the soluble cytochrome *c₅₅₅* trapped in the supercomplex (as suggested by results observed in purified supercomplex [44] or even in recycled invaginations (structures resembling membrane invaginations are in fact observed in *A. aeolicus* [17]). A membrane-bound *c₅₅₅*, not lost when intact membranes are prepared, could also be used as substrate. And lastly, an unusual quinol oxidase activity has been proposed for the *A. aeolicus ba₃* enzyme, in addition to its cytochrome oxidase activity [47]. This would support the participation of this oxidase in the membrane fraction.

Altogether, the results presented above support that electrons generated by sulfite oxidation enter the respiration chain at the level of the quinone pool from which the *bd* quinol oxidase or both the *ba₃-bc₁* supercomplex and *bd* oxidase, draw.

3.6. Phylogeny

The thermodynamics of the considered reactions is not sufficient to explain the selectivity of SoeABC in the oxidation and reduction of sulfur compounds. The observed results suggest that, in addition to the ΔG of the reaction considered, mechanistic constraints related to structural nature of the enzyme have to be considered to rationalize the results. As already described, SoeABC is a member of the so-called Mo-*bis*PGD family, which members are not structurally related to the various sulfite reductases (including the MccA and DsrAB enzymes) performing the direct reduction of sulfite to hydrogen sulfide by $6e^-$ (see [4]), or known to reduce thiosulfate to hydrogen sulfide only. However, several other Mo-*bis*PGD enzymes have been described as converting sulfur compounds. We therefore analyzed the closely homologous enzymes involved in sulfur reduction. No detailed phylogenetic analysis has been conducted trying to establish global relationships between all the enzymes, described in different organisms as thiosulfate reductase (named Phs in *Salmonella enterica*), sulfur reductase (named Sre in *Acidianus ambivalens* and *Haloarchaea*), polysulfide reductase (named Psr in *Wolinella succinogenes*), tetrathionate reductase (named Ttr in *Salmonella typhimurium*) and here sulfite dehydrogenase (named SoeABC in *A. vinosum*). We thus included in our analysis not only all the *bona fide* SoeA sequences (i.e. showing typical number and order of genes in *soe*-clusters, absence of TAT signals, and cofactor binding motifs) retrieved by using the sequence from *A. vinosum* as query but also all the others cited above. We also included in our tree the Unk family, presented by Duval et al. [30] from

which the function is still unknown and the Anr family, described by Abin and coworkers from which the antimonate reductase function has been established and which is very closely related to the *Mo-bis*PGD enzymes involved in sulfur metabolism [31]. For each of the sequence subfamilies, we have also taken special care to include sequences from the most diverse phylogenetic origins. The resulting reconstructed tree (Figure 4) therefore gives a more complete phylogenetic view of the family than the preceding one (see [48]). While the Ttr enzyme for example could be analyzed as a pre-LUCA enzyme showing a clear-cut separation of archaeal and bacterial sequences, this is not the case for the SoeABC enzymes. Almost respecting the phylogeny of their host, and thus appearing to have been mostly vertically inherited, the SoeABC seems to have been evolved in Bacteria only. The Soe shows the closest phylogenetic relationships with the Ttr and one clade of Sre. This could explain why the Soe possesses tetrathionate and sulfur reductase activities. The phylogeny even suggests Ttr and Soe to share a common ancestor. As already suggested by Ahn et al. [48], a sub-group of proteobacterial Soe sequences containing both the sequence of *A. vinosum* and *Thioalkalivibrio thiocyanodenitrificans* (noted A.v and T.t respectively in Figure 4) might be distinguished. The second clade, more disperse and diverse, contains the *A. aeolicus* sequence, together with the *Alkalilimnicola erlichii* and *Ectothiorhodospira* PHS-1 sequences standing in the the tree of Ahn et al. (noted A.a, A.e and E. PHS-1 on Figure 4). Ahn et al. suggested that this could reflect a distinct substrate specificity towards arsenic. To answer this question, we must wait for kinetic studies of all these enzymes using arsenic.

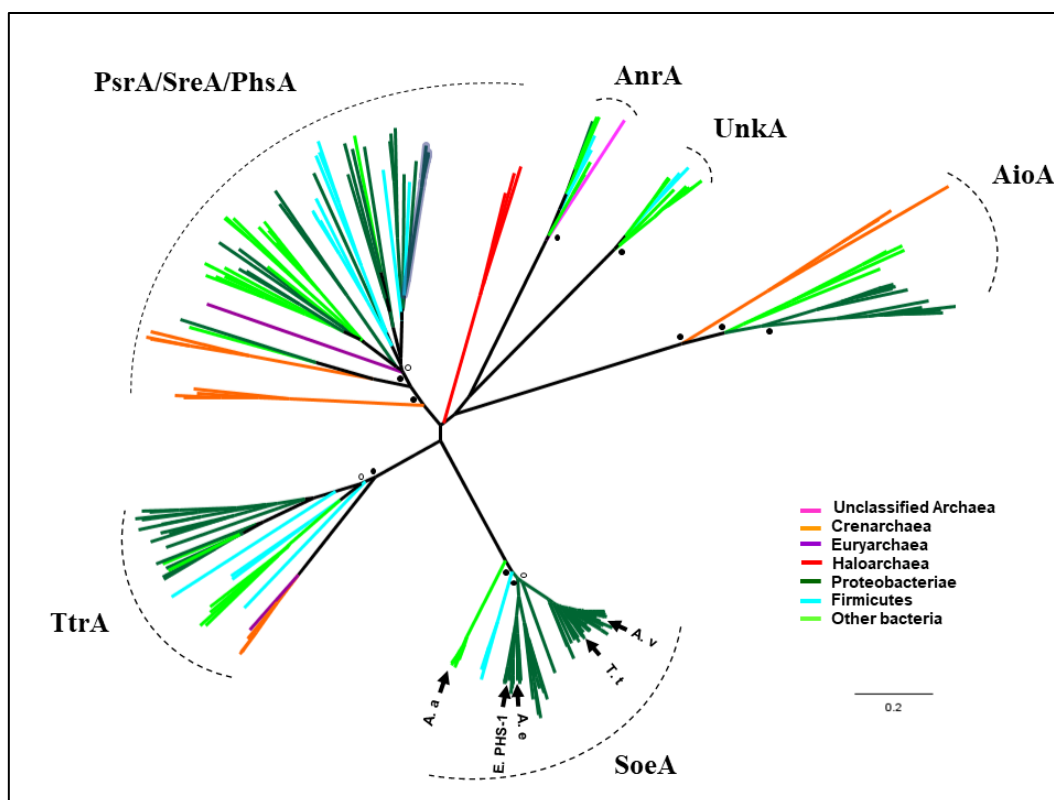


Figure 4: Neighbor-Joining phylogenetic tree of Mo-bisPGD enzymes involved in sulfur compounds conversion.

Sequences of the catalytic subunit from sulfur compounds converting enzymes, SreA, PhsA, PsrA, TtrA and SoeA, have been retrieved either from literature or from Blast searches as detailed in the Material and Methods Section. Sequences of the catalytic subunits from antimonite reductase (AnrA) as well as from a still unidentified Unk enzyme (UnkA) have been added to the tree due to their close relationship to sulfur metabolism. The AioA sequences, of the catalytic subunit from the arsenite oxidase, come from previous published results. These later sequences are used as outgroup. Violet, orange, red and pink denote eury-, cren-, halo- and unclassified archaeal branches, dark green, light blue and light green stand for Proteobacteria, Firmicutes and other Bacteria, respectively. Filled dots indicate bootstrap values for deep branching nodes exceeding 90, open dots indicate bootstrap values for deep branching nodes between 60 and 90.

3.7. Genes for sulfite oxidation in *Aquificales* members

The order *Aquificales* is divided in two families [49]. Most of the members of the *Aquificaceae* (to which *A. aeolicus* belongs) and *Hydrogenothermaceae*, which are (hyper)thermophiles and chemolithoautotrophs, can use sulfur compounds as substrate for growth (thiosulfate and elemental sulfur for most of them). A search for *soeABC* and *sorAB* genes as well as for genes for the APS reductase and ATP sulfurylase in the genome of the sequenced species of these families indicated that in members of the *Hydrogenothermaceae*

family, sulfite oxidation may proceed only *via* the periplasmic SorAB system, without any involvement of SoeABC as *soe* genes are completely missing (Table 1). SoeABC is, however, almost universally present in the *Aquificaceae* family (except in *Hydrogenobaculum* species, while being a sulfur-oxidizer) irrespective of the occurrence of *sorAB*-like genes, denoting a potential important role for this cytoplasmic facing sulfite dehydrogenase in this family (Table 1). In a number of species of this family, SoeABC seems to be the sole enzyme known to oxidize sulfite, as both SorAB and the APS reductase/ATP sulfurlyase pathway are lacking. SoeABC is thus likely to be an essential enzyme in these bacteria.

4. Discussion

The mode of oxidation of the intermediate sulfite was unresolved in the sulfur-oxidizing bacterium *A. aeolicus*. In this work, we have identified, purified and characterized the molybdoenzyme sulfite dehydrogenase SoeABC from this bacterium. This new type of enzyme was earlier proven, using deletant strains, to be a major player catalyzing direct oxidation of sulfite to sulfate in *A. vinosum* [12], but it was never purified from any bacterium.

Unlike the sulfite dehydrogenases well described so far that are members of the SO family, SoeABC belongs to the DMSO reductase family (Table 6), which gather catalytically and functionally very diverse enzymes possessing a Mo-*bis*PGD cofactor [16]. SoeABC from *A. aeolicus* was already shown to contain a molybdenum cofactor in a previous study [21] and all genes involved in the Mo-*bis*PGD cofactor synthesis are present in the genome of the bacterium.

According to its sequence (not shown), the Mo ion is supposedly coordinated by a sulfur from a Cys residue as in nitrate reductases Nap [52], arsenate reductases Arr [53] or polysulfide reductases Psr [54]. This enzyme does not share any feature of sulfite dehydrogenases from the SO family: the cellular localization, subunit composition, molecular mass and electron acceptor are all different. Indeed, SoeABC, bound to the cytoplasmic membrane, is a large complex (about 390 kDa) presumably facing the cytoplasm as suggested by the absence of TAT signal on any of the three subunits. In the *A. aeolicus* membrane, SoeABC is likely a dimer of trimer ($\alpha\beta\gamma$)₂, like other Mo-*bis*PGD enzymes like PsrABC from *Thermus thermophilus* [54] or the nitrate reductase NarGHI from *Escherichia coli* [55]. The purified

enzyme efficiently oxidizes sulfite and reduces quinone (or NBT as artificial electron acceptor). Determination of the kinetic parameters indicates that it has a high affinity for sulfite, in the micromolar range, and a turnover number in the same range as those determined for bacterial sulfite dehydrogenases SorAB and SorT. In the case of Sor from *Thermus thermophilus*, a certainly erroneous value of 53318 s^{-1} has been published. In-depth analysis of the other data presented in the same publication suggests that 53.3 s^{-1} should have been read [5]. The value determined for *A. aeolicus* enzyme is therefore in line with other sulfite dehydrogenases although not members from the same enzyme family (Table 6). A sulfite dehydrogenase has been purified from *A. thiooxidans*, located in the cell membrane with a large molecular mass (400 kDa). It consists of three subunits, like SoeABC, but with very divergent sizes, and also has enzymatic properties distinct from those established here. The enzyme from *A. thiooxidans* was shown to reduce ferricyanide and did not use oxygen as electron acceptor (as sulfite oxidases from the SO family do), more similar to sulfite dehydrogenases from the SO family. It was specific for sulfite (no oxidation of thiosulfate or tetrathionate), like the Soe from *A. aeolicus* but with a very high K_M for sulfite (1.95 mM) [10]. Although the *A. thiooxidans* enzyme and SoeABC from *Aquifex aeolicus* have common properties, it is difficult to predict whether the *A. thiooxidans* enzyme is also a SoeABC.

SoeABC from *A. aeolicus* was originally characterized as a sulfur reductase and called Sre [21,56]. The enzyme, purified at that time with a hydrogenase, could reduce sulfur and tetrathionate *in vitro* in the presence of hydrogen, with a menaquinone reacting as shuttle between the hydrogenase and the sulfur reductase. In the present work, the SoeABC enzyme was purified separately from the hydrogenase and we confirmed that it reduces these two sulfur compounds using reduced methyl viologen as electron donor and that thiosulfate is, as found earlier, not reduced, as is sulfite. From these present results, it can be proposed that, SoeABC can work in both directions (oxidation and reduction) *in vitro*. A number of members of the DMSO reductase family were shown to be bidirectional [16]. We can mention like closely related examples the isolated PsrABC from *Wolinella succinogenes* that catalyzes the reduction of polysulfide but also oxidation of sulfide by a menaquinone *in vitro* [57], the arsenite oxidase ArxABC (preliminarily called arsenate reductase) from *A. erlichii* working in the oxidative way but also reductive way *in vitro* [58] or the thiosulfate reductase PhsABC from *Salmonella enterica* forming thiosulfate from sulfite and sulfide [59]. The observation of the reactivity of SoeABC with sulfur and tetrathionate is directly related to the close relationship of the enzyme with homologous Sre and Ttr enzyme belonging to the same Mo-

*bis*PGD family. The fact that thiosulfate is not reduced *in vitro* by Soe, despite the close homology of Phs with Sre, Ttr and Soe, could be explained by the highly unfavorable thermodynamics of the reaction. Even the Phs is not able to reduce thiosulfate without consumption of proton motive force (*pmf*) [59].

SoeABC reduces sulfur *in vitro* with reduced MV (this work) or in the presence of hydrogen and a menaquinone [21]. Considering this reduction reaction in the cell, DMK₇ is theoretically not sufficiently electronegative to reduce elemental sulfur or polysulfide ($E_{\text{mpH}7} \text{S}^0/\text{HS}^- = -270$ mV and $E_{\text{mpH}7} \text{polysulfide}/\text{HS}^- = -260$ mV [57]). Consequently, this reduction reaction would be thus highly endergonic *in vivo* (as the reduction of thiosulfate is, see above) if not driven by hydrogen (but possible *in vitro* given the very low redox potential of methyl viologen). Tetrathionate, with its higher midpoint potential, could possibly serve as electron acceptor. However, tetrathionate is not a known sulfur intermediate in *A. aeolicus*, in which no enzyme known to convert or release it was found. It cannot be completely excluded at present that SoeABC might also function as a reductase *in vivo* depending on the conditions. *A. aeolicus* uses hydrogen, elemental sulfur or thiosulfate as energy sources in the presence of O₂. Sulfide was found to be released in the external medium at the late exponential growth phase when *A. aeolicus* grows with hydrogen, elemental sulfur and oxygen in batch conditions [21].

In vivo, SoeABC certainly functions as a sulfite-oxidizing enzyme when growing by sulfur compounds oxidation, especially since no other sulfite dehydrogenase was biochemically identified. The putative Aq_979 protein predicted to be a member of the structural group 3B of SO family (see paragraph 3.1.) is either not produced in *A. aeolicus* cells in the growth conditions used or might not be a sulfite dehydrogenase (or not active in the experimental conditions employed in this work). During sulfite oxidation at the catalytic site in SoeA, electrons are putatively transferred *via* five [Fe-S] clusters (one situated in SoeA and four in SoeB) to ultimately reduce the quinone DMK₇ in SoeC. Given standard redox potential values of the sulfate/sulfite couple (-480 or -516 mV, [1-3]) and of the DMK₇/DMK₇H₂ couple (-9 mV, [37]), the reaction catalyzed by SoeABC is supposed to be thermodynamically favorable *in vivo*. However, the oxidation of sulfite is not predicted to necessarily generate a *pmf*. Indeed, the « NrfD-type » membrane-integrated subunit SoeC is homologous to PsrC [54,60] or ActC of the alternative complex III [61-63] whose three-dimensional structure resolution indicate a quinone binding site on the periplasmic side of the inner membrane. Equivalent enzyme architecture for Soe would imply the quinone binding site close to the cytoplasm (opposite orientation compared to Psr or Act) and thus the proton release from

oxidation of hydrogen sulfite and proton uptake from quinone reduction, both in the cytoplasm (Figure 5 and Figure A7 proposition 1). This hypothetical functioning of SoeABC is based on the classical case where protons come from the compartment near the quinone binding site (see Figure 2 in [60]) and enzyme functioning in this case would be therefore electroneutral (i.e. no productive of *pmf*) [4]. SoeC is also homologous to QrcD, a 10-transmembrane helices containing subunit of the periplasmically-oriented Qrc complex involved in sulfate reduction pathway, belonging to the “NrfD family”. This complex was recently demonstrated to be electrogenic, but does not act as a proton pump. Instead, QrcD, with a quinone binding site on the periplasmic side, takes protons up from the cytoplasm *via* a putative proton channel [63]. If we consider a similar mechanism of action between SoeC and QrcD, then the reaction of the SoeABC enzyme would consume *pmf* as protons would be consumed in the periplasm and released in the cytoplasm (Figure 5 and Figure A7 proposition 2). Residues putatively involved in protons translocation in QrcD are not conserved in ActC. The NrfD/PsrC/QrcD protein family is quite diverse and a different mechanism may be used by different enzymes of the family [63]. A third possibility cannot be at present discarded, in which SoeABC would be a true proton pump (as complex I) and protons would be translocated by SoeC from the cytoplasm to the periplasmic compartment (Figure 5 and Figure A7 proposition 3) [54,62,63]. In this case, the reaction of the enzyme could be electrogenic and contribute to the *pmf*. Regardless of whether SoeABC contributes or consumes *pmf* by itself, the entire electron transport chain results overall in positive charges being translocated across the membrane and in the generation of a *pmf* (Figure 5).

Attempts to grow *A. aeolicus* with 5 mM exogenous sulfite (instead of thiosulfate or elemental sulfur) were all unsuccessful (data not shown). The cytoplasmic exposition of SoeABC is consistent with the intracellular sulfite release during sulfur compound oxidation in *A. aeolicus*. However, the periplasmic sulfur substrate-binding protein SoxYZ, which respective genes occur in *A. aeolicus* genome [17], is needed in parallel to the cytoplasmic SoeABC for effective sulfite oxidation in *A. vinosum*, suggesting some kind of interplay between these systems despite their localization in two different cellular compartments [12]. In *A. aeolicus*, sulfite is putatively released in the cytoplasm by the Hdr-like complex [19] and also the large soluble SOR enzyme [18]. In the current work, results obtained on entire cells and membrane fraction support that there is a coupling between sulfite oxidation and cell respiration and that reduction of oxygen is carried out by a quinol oxidase, and also potentially by a cytochrome *c* oxidase. *A. aeolicus* has a branched respiratory chain [17]. It has earlier been proposed that the *ba*₃ cytochrome *c* oxidase seems to be the major oxidase in

the standard growth conditions whereas the putative aa_3 -type cytochrome c oxidase is not detected in the membrane fraction [22,44]. We thus propose that the quinol pool fueled by SoeABC would be drawn by the bd -type quinol oxidase or this bd quinol oxidase together with the bc_1 complex- ba_3 cytochrome c oxidase supercomplex as shown Figure 5.

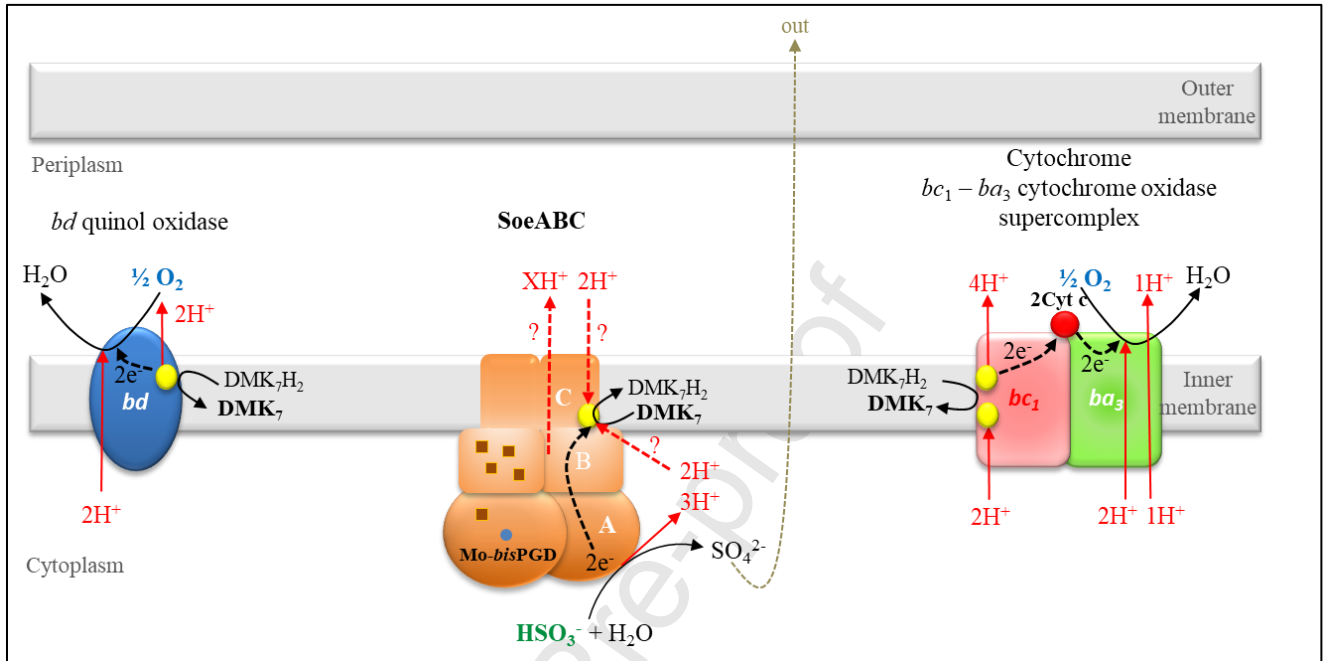


Figure 5: Schematic representation of cytoplasmic sulfite oxidation and oxygen reduction in *A. aeolicus*.

SoeABC is depicted in orange, the quinol oxidase in blue, the bc_1 - ba_3 supercomplex in light red and green and the periplasmic cytochrome c_{555} in dark red, with quinol/quinone reacting sites in yellow in all complexes. In SoeABC, the blue dot and the brown squares symbolize the Mo cofactor and the [Fe-S] clusters, respectively. Electron transfers are shown as black dotted arrows. For the bd quinol oxidase, bc_1 complex and ba_3 oxidase, proton movements are indicated by red arrows as described in [46,60,64]. For SoeABC, the proton movement is unknown and several hypotheses are shown with red dotted arrows (and detailed in Figure A7). The two forms HSO_3^- and SO_3^{2-} are both present in the cytoplasm, but for the sake of clarity only the protonated form is shown here.

5. Conclusion

The cytoplasmically-oriented SoeABC sulfite dehydrogenase purified from the sulfur-compounds oxidizing *A. aeolicus* is a large membrane-bound complex with a high affinity for sulfite and quinone. Based on this high affinity for sulfite and the established function of SoeABC in sulfite oxidation to sulfate in *A. vinosum* [12], we could propose an equivalent function for the enzyme in *A. aeolicus*. In this hypothesis, our results suggest the electrons to

be funneled from sulfite, an intermediate in sulfur compounds oxidation pathway, to the quinone pool from in which the respiratory chains draws and the *bd*-type quinol oxidase appears to be involved in respiration, as might also be the case for the *bc₁-ba₃* respiratory supercomplex. However, an ambivalent function as a sulfur reductase in addition to a sulfite oxidase in *A. aeolicus* is not yet completely discarded, as the enzyme is bidirectional and reduces sulfur *in vitro* and the bacterium can release sulfide in particular growing conditions [21]. This remaining ambiguity for the role of SoeABC in the cells is reminiscent to the one observed for Arr/Arx enzymes. The enzyme purified from *A. ehrlichii* was initially named arsenate reductase, due to its homology to Arr, shown as bidirectional *in vitro* [58] but working as arsenite oxidase in the host cells [65]. Only characterization of multiple host and enzymes allowed identification of two very closely related phylogenetic clades: Arr and Arx, the first working in arsenate reduction in the host, the second working in arsenite oxidation in the host [66]. In the Soe group, it is to note that two sub-groups have been proposed (in line with our more extended phylogenetic work): one containing the sequence from *A. vinosum*, the other one more closely related to the sequence of *A. aeolicus*. It has been proposed that the two types of enzyme could have different affinity for arsenicals [48]. But it could also be that the two types of enzyme have different features regarding their directionality *in vivo*. Characterization of diverse SoeABC, as well as production and activity of the enzyme in various growth conditions in *A. aeolicus*, will help to solve this ambiguity.

Acknowledgements

Ievgen Mazurenko (Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, Marseille) is gratefully acknowledged for redox midpoint potentials calculations, Régine Lebrun and Rémy Puppò (Proteomic Platform of the Mediterranean Institute of Microbiology, FR3479, CNRS, Marseille Proteomique -IBiSA and -Aix Marseille Univ labeled) for mass spectrometry analyses, Marielle Bauzan (Biomass and Biohydrogen Platform of the Mediterranean Institute of Microbiology, FR3479, CNRS, Marseille) for *Aquifex aeolicus* cells growth, Wolfgang Nitschke (Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, Marseille) for helpful discussions and María Luz Cárdenas and Athel Cornish-Bowden (Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, Marseille) for helpful discussions and correcting the English. S. Boughanemi was supported by a PhD fellowship from the French Ministry of Research. This research was funded by the CNRS.

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References

- [1] R.K. Thauer, K. Jungermann, K. Decker, Energy Conservation in Chemotrophic Anaerobic Bacteria, *Bacteriol. Rev.* 41 (1977) 100-180.
- [2] E.L. Barrett, M.A. Clark, Tetrathionate reduction and production of hydrogen sulfide from thiosulfate, *Microbiol. Rev.* 51 (1987) 192-205.
- [3] M. Bouroushian, Electrochemistry of the Chalcogens, in: *Electrochemistry of Metal Chalcogenides*, Monographs in Electrochemistry, Springer-Verlag Berlin Heidelberg, 2010, pp. 57-75. doi 10.1007/978-3-642-03967-6_2.
- [4] J. Simon, P.M. Kroneck, Microbial sulfite respiration, *Adv. Microb. Physiol.* 62 (2013) 45-117. doi: 10.1016/B978-0-12-410515-7.00002-0.
- [5] U. Kappler, Bacterial sulfite-oxidizing enzymes, *Biochim. Biophys. Acta* 1807 (2011) 1-10. doi: 10.1016/j.bbabi.2010.09.004.
- [6] U. Kappler, B. Bennett, J. Rethmeier, G. Schwarz, R. Deutzmann, A.G. McEwan, C. Dahl, Sulfite:Cytochrome *c* oxidoreductase from *Thiobacillus novellus*. Purification, characterization, and molecular biology of a heterodimeric member of the sulfite oxidase family, *J. Biol. Chem.* 275 (2000) 13202-13212. doi: 10.1074/jbc.275.18.13202.
- [7] U. Kappler, S. Bailey, Molecular basis of intramolecular electron transfer in sulfite-oxidizing enzymes is revealed by high resolution structure of a heterodimeric complex of the catalytic molybdopterin subunit and a *c*-type cytochrome subunit, *J. Biol. Chem.* 280 (2005) 24999-25007. doi: 10.1074/jbc.M503237200.
- [8] A.P. McGrath, E.L. Laming, G.P. Casas Garcia, M. Kvensakul, J.M. Guss, J. Trewheella, B. Calmes, P.V. Bernhardt, G.R. Hanson, U. Kappler, M.J. Maher, (2015). Structural basis of interprotein electron transfer in bacterial sulfite oxidation, *Elife* 4, e09066. doi: 10.7554/eLife.09066.
- [9] A. Kletzin, T. Urich, F. Müller, T.M. Bandejas, C.M. Gomes, Dissimilatory oxidation and reduction of elemental sulfur in thermophilic archaea, *J. Bioenerg. Biomembr.* 36 (2004) 77-91. doi:10.1023/b:jobb.0000019600.36757.8c.
- [10] K. Nakamura, H. Yoshikawa, S. Okubo, H. Kurosawa, Y. Amano, Purification and properties of membrane-bound sulfite dehydrogenase from *Thiobacillus thiooxidans* JCM7814, *Biosci. Biotechnol. Biochem.* 59 (1995) 11-15. doi: 10.1271/bbb.59.11.
- [11] S. Lenk, C. Moraru, S. Hahnke, J. Arnds, M. Richter, M. Kube, R. Reinhardt, T. Brinkhoff, J. Harder, R. Amann, M. Mußmann, *Roseobacter* clade bacteria are abundant in coastal sediments and encode a novel combination of sulfur oxidation genes, *ISME J.* 6 (2012) 2178-2187. doi: 10.1038/ismej.2012.66.
- [12] C. Dahl, B. Franz, D. Hensen, A. Kesselheim, R. Zigann, Sulfite oxidation in the purple sulfur bacterium *Allochromatium vinosum*: identification of SoeABC as a major player and

relevance of SoxYZ in the process, *Microbiology* 159 (2013) 2626-2638. doi: 10.1099/mic.0.071019-0.

[13] T. Berben, L. Overmars, D.Y. Sorokin, G. Muyzer, Diversity and Distribution of Sulfur Oxidation-Related Genes in *Thioalkalivibrio*, a Genus of Chemolithoautotrophic and Haloalkaliphilic Sulfur-Oxidizing Bacteria, *Front. Microbiol.* 10 (2019) 160. doi: 10.3389/fmicb.2019.00160.

[14] T. Watanabe, H. Kojima, K. Umezawa, C. Hori, T.E. Takasuka, Y. Kato, M. Fukui, Genomes of Neutrophilic Sulfur-Oxidizing Chemolithoautotrophs Representing 9 Proteobacterial Species From 8 Genera, *Front. Microbiol.* 10 (2019) 316. doi: 10.3389/fmicb.2019.00316.

[15] M. Oshiki, T. Fukushima, S. Kawano, Y. Kasahara, J. Nakagawa, Thiocyanate Degradation by a Highly Enriched Culture of the Neutrophilic Halophile *Thiohalobacter thiocyanaticus* FOKN1 from Activated Sludge and Genomic Insights into Thiocyanate Metabolism, *Microbes Environ.* 34 (2019) 402-412. doi: 10.1264/jsme2.ME19068.

[16] S. Grimaldi, B. Schoepp-Cothenet, P. Ceccaldi, B. Guigliarelli, A. Magalon, The prokaryotic Mo/W-bisPGD enzymes family: a catalytic workhorse in bioenergetic, *Biochim. Biophys. Acta* 1827 (2013) 1048-1085. doi: 10.1016/j.bbabi.2013.01.011.

[17] M. Guiral, L. Prunetti, C. Aussignargues, A. Ciaccafava, P. Infossi, M. Ilbert, E. Lojou, M.T. Giudici-Ortoni, The hyperthermophilic bacterium *Aquifex aeolicus*: from respiratory pathways to extremely resistant enzymes and biotechnological applications, *Adv. Microb. Physiol.* 61 (2012) 125-194. doi: 10.1016/B978-0-12-394423-8.00004-4.

[18] N. Pelletier, G. Leroy, M. Guiral, M.T. Giudici-Ortoni, C. Aubert, First characterisation of the active oligomer form of sulfur oxygenase reductase from the bacterium *Aquifex aeolicus*, *Extremophiles* 12 (2008) 205-215. doi: 10.1007/s00792-007-0119-5.

[19] S. Boughanemi, J. Lyonnet, P. Infossi, M. Bauzan, A. Kosta, S. Lignon, M.T. Giudici-Ortoni, M. Guiral, 2016. Microbial oxidative sulfur metabolism: biochemical evidence of the membrane-bound heterodisulfide reductase-like complex of the bacterium *Aquifex aeolicus*, *FEMS Microbiol. Lett.* 63(15). pii: fnw156. doi: 10.1093/femsle/fnw156.

[20] T. Koch, C. Dahl, A novel bacterial sulfur oxidation pathway provides a new link between the cycles of organic and inorganic sulfur compounds, *ISME J.* 12 (2018) 2479-2491. doi: 10.1038/s41396-018-0209-7.

[21] M. Guiral, P. Tron, C. Aubert, A. Gloter, C. Iobbi-Nivol, M.T. Giudici-Ortoni, A membrane-bound multienzyme, hydrogen-oxidizing, and sulfur-reducing complex from the hyperthermophilic bacterium *Aquifex aeolicus*, *J. Biol. Chem.* 280 (2005) 42004-42015. doi: 10.1074/jbc.M508034200.

- [22] M. Guiral, L. Prunetti, S. Lignon, R. Lebrun, D. Moinier, M.T. Giudici-Orticoni, New insights into the respiratory chains of the chemolithoautotrophic and hyperthermophilic bacterium *Aquifex aeolicus*, *J. Proteome Res.* 8 (2009) 1717-1730. doi: 10.1021/pr8007946.
- [23] K. Hensley, Q.N. Pye, M.L. Maitt, C.A. Stewart, K.A. Robinson, F. Jaffrey, R.A. Floyd, Interaction of alpha-phenyl-N-tert-butyl nitron and alternative electron acceptors with complex I indicates a substrate reduction site upstream from the rotenone binding site, *J. Neurochem.* 71 (1998) 2549-2557. doi: 10.1046/j.1471-4159.1998.71062549.x.
- [24] C. Schäfer, B. Friedrich, O. Lenz, Novel, oxygen-insensitive group 5 [NiFe]-hydrogenase in *Ralstonia eutropha*, *Appl. Environ. Microbiol.* 79 (2013) 5137-5145. doi: 10.1128/AEM.01576-13.
- [25] R.A. Copeland, Propagation of uncertainties in experimental measurements, in: Wiley-Blackwell, *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists*, Second Edition, John Wiley & Sons, Inc., 2013, pp. 493-495. doi:10.1002/9781118540398.
- [26] S. Ikeda, H. Satake, T. Hisano, T. Terazawa, Potentiometric argentimetric method for the successive titration of sulphide and dissolved sulphur in polysulphide solutions, *Talanta* 19 (1972) 1650-1654. doi:10.1016/0039-9140(72)80240-6.
- [27] T. Rohwerder, W. Sand, The sulfane sulfur of persulfides is the actual substrate of the sulfur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp., *Microbiology* 149 (2003) 1699-1710. doi: 10.1099/mic.0.26212-0.
- [28] S. Laska, F. Lottspeich, A. Kletzin, Membrane-bound hydrogenase and sulfur reductase of the hyperthermophilic and acidophilic archaeon *Acidianus ambivalens*, *Microbiology* 149 (2003) 2357-2371. doi: 10.1099/mic.0.26455-0.
- [29] D.Y. Sorokin, I.V. Kublanov, S.N. Gavrillov, D. Rojo, P. Roman, P.N. Golyshin, V.Z. Slepak, F. Smedile, M. Ferrer, E. Messina, V. La Cono, M.M. Yakimov, Elemental sulfur and acetate can support life of a novel strictly anaerobic haloarchaeon, *ISME J.* 10 (2016) 240-252. doi: 10.1038/ismej.2015.79.
- [30] S. Duval, A.L. Ducluzeau, W. Nitschke, B. Schoepp-Cothenet, Enzyme phylogenies as markers for the oxidation state of the environment: the case of respiratory arsenate reductase and related enzymes, *BMC Evol. Biol.* 8 (2008) 206. doi: 10.1186/1471-2148-8-206.
- [31] C.A. Abin, J. T. Hollibaugh, Transcriptional response of the obligate anaerobe *Desulfuribacillus stibiiarsenatis* MLFW-2T to growth on antimonate and other terminal electron acceptors, *Environ. Microbiol.* 21 (2019) 618-630. doi:10.1111/1462-2920.14503.
- [32] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic acids research*, 25 (1997) 4876-4882. doi: 10.1093/nar/25.24.4876.

- [33] N. Galtier, M. Gouy, C. Gautier, SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny, *Computer applications in the biosciences*, CABIOS, 12 (1996) 543-548. doi: 10.1093/bioinformatics/12.6.543.
- [34] Z. Yu, E.B. Lansdon, I.H. Segel, A.J. Fisher, Crystal structure of the bifunctional ATP sulfurylase-APS kinase from the chemolithotrophic thermophile *Aquifex aeolicus*, *J. Mol. Biol.* 365 (2007) 732-743. doi: 10.1016/j.jmb.2006.10.035.
- [35] F.P. Altman, Tetrazolium salts: a consumer's guide, *Histochem. J.* 8 (1976) 471-485. doi: 10.1007/bf01003837.
- [36] J.J. Desmarais, A.I. Flamholz, C. Blikstad, E.J. Dugan, T.G. Laughlin, L.M. Oltrogge, A.W. Chen, K. Wetmore, S. Diamond, J.Y. Wang, D.F. Savage, DABs are inorganic carbon pumps found throughout prokaryotic phyla, *Nat. Microbiol.* 4 (2019) 2204-2215. doi: 10.1038/s41564-019-0520-8.
- [37] P. Infossi, E. Lojou, J.P. Chauvin, G. Herbette, M. Brugna, M.T. Giudici-Ortoni, *Aquifex aeolicus* membrane hydrogenase for hydrogen biooxidation: Role of lipids and physiological partners in enzyme stability and activity, *Int. J. Hydrog. Energy* 35 (2010) 10778-10789. doi: 10.1016/j.ijhydene.2010.02.054.
- [38] R. Covian, E.B. Gutierrez-Cirlos, B.L. Trumpower, Anti-cooperative oxidation of ubiquinol by the yeast cytochrome *bc₁* complex, *J. Biol. Chem.* 279 (2004) 15040-15049. doi: 10.1074/jbc.M400193200.
- [39] Y. Zhang, A. Qadri, J.H. Weiner, The quinone-binding site of *Acidithiobacillus ferrooxidans* sulfide: quinone oxidoreductase controls both sulfide oxidation and quinone reduction, *Biochem. Cell. Biol.* 94 (2016) 159-166. doi: 10.1139/bcb-2015-0097.
- [40] P.G. Tratnyek, T.E. Reilkoff, A.W. Lemon, M.M. Scherer, B.A. Balko, L.M. Feik, B.D. Henegar, Visualizing Redox Chemistry: Probing Environmental Oxidation-Reduction Reactions with Indicator Dyes, *Chem. Educator* 6 (2001) 172-179. doi: 10.1007/s00897010471a.
- [41] A.B. Hua, R. Justiniano, J. Perer, S.L. Park, H. Li, C.M. Cabello, G.T. Wondrak, 2019. Repurposing the Electron Transfer Reactant Phenazine Methosulfate (PMS) for the Apoptotic Elimination of Malignant Melanoma Cells through Induction of Lethal Oxidative and Mitochondriotoxic Stress. *Cancers (Basel)*. 11(5). pii: E590. doi: 10.3390/cancers11050590.
- [42] J.M. Kurth, C. Dahl, J.N. Butt, Catalytic Protein Film Electrochemistry Provides a Direct Measure of the Tetrathionate/Thiosulfate Reduction Potential, *J. Am. Chem. Soc.* 137 (2015) 13232-13235. doi: 10.1021/jacs.5b08291.
- [43] L. Prunetti, M. Brugna, R. Lebrun, M.T. Giudici-Ortoni, M. Guiral, 2011. The elusive third subunit IIa of the bacterial B-type oxidases: the enzyme from the hyperthermophile *Aquifex aeolicus*, *PLoS One* 6(6):e21616. doi: 10.1371/journal.pone.0021616.

- [44] L. Prunetti, P. Infossi, M. Brugna, C. Ebel, M.T. Giudici-Ortoni, M. Guiral, New functional sulfide oxidase-oxygen reductase supercomplex in the membrane of the hyperthermophilic bacterium *Aquifex aeolicus*, *J. Biol. Chem.* 285 (2010) 41815-41826. doi: 10.1074/jbc.M110.167841.
- [45] R.J.Y. Masau, J.K. Oh, I. Suzuki, Mechanism of oxidation of inorganic sulfur compounds by thiosulfate-grown *Thiobacillus thiooxidans*, *Can. J. Microbiol.* 47 (2001) 348–358. doi:10.1139/w01-015.
- [46] V.B. Borisov, R.B. Gennis, J. Hemp, M.I. Verkhovsky, The cytochrome *bd* respiratory oxygen reductases, *Biochim. Biophys. Acta* 1807 (2011) 1398-1413. doi: 10.1016/j.bbabi.2011.06.016.
- [47] Y. Gao, B. Meyer, L. Sokolova, K. Zwicker, M. Karas, B. Brutschy, G. Peng, H. Michel, Heme-copper terminal oxidase using both cytochrome *c* and ubiquinol as electron donors, *Proc. Natl. Acad. Sci. U S A* 109 (2012) 3275-3280. doi: 10.1073/pnas.1121040109.
- [48] A.C. Ahn, L. Cavalca, M. Colombo, J.M. Schuurmans, D.Y. Sorokin, G. Muyzer, Transcriptomic analysis of two *Thioalkalivibrio* species under arsenite stress revealed a potential candidate gene for an alternative arsenite oxidation pathway, *Front. Microbiol.* 10 (2019) 1514. doi: 10.3389/fmicb.2019.01514.
- [49] R.S. Gupta, R. Lali, Molecular signatures for the phylum *Aquificae* and its different clades: proposal for division of the phylum *Aquificae* into the emended order *Aquificales*, containing the families *Aquificaceae* and *Hydrogenothermaceae*, and a new order *Desulfurobacteriales* ord. nov., containing the family *Desulfurobacteriaceae*, *Antonie Van Leeuwenhoek* 104 (2013) 349-368. doi: 10.1007/s10482-013-9957-6.
- [50] A. Di Salle, G. D'Errico, F. La Cara, R. Cannio, M. Rossi, A novel thermostable sulfite oxidase from *Thermus thermophilus*: characterization of the enzyme, gene cloning and expression in *Escherichia coli*, *Extremophiles* 10 (2006) 587-598. doi: 10.1007/s00792-006-0534-z.
- [51] S. Robin, M. Arese, E. Forte, P. Sarti, A. Giuffrè, T. Soulimane, A sulfite respiration pathway from *Thermus thermophilus* and the key role of newly identified cytochrome c_{550} , *J. Bacteriol.* 193 (2011) 3988-3997. doi: 10.1128/JB.05186-11.
- [52] P. Arnoux, M. Sabaty, J. Alric, B. Frangioni, B. Guigliarelli, J.M. Adriano, D. Pignol, Structural and redox plasticity in the heterodimeric periplasmic nitrate reductase, *Nat. Struct. Biol.* 10 (2003) 928-934. doi:10.1038/nsb994.
- [53] N.R. Glasser, P.H. Oyala, T.H. Osborne, J.M. Santini, D.K. Newman, Structural and mechanistic analysis of the arsenate respiratory reductase provides insight into environmental arsenic transformations, *Proc. Natl. Acad. Sci. U S A* 115 (2018) E8614-E8623. doi: 10.1073/pnas.1807984115.

- [54] M. Jormakka, K. Yokoyama, T. Yano, M. Tamakoshi, S. Akimoto, T. Shimamura, P. Curmi, S. Iwata, Molecular mechanism of energy conservation in polysulfide respiration, *Nat. Struct. Mol. Biol.* 15 (2008) 730-737. doi: 10.1038/nsmb.1434.
- [55] M.G. Bertero, R.A. Rothery, M. Palak, C. Hou, D. Lim, F. Blasco, J.H. Weiner, N.C. Strynadka, Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A, *Nat. Struct. Biol.* 10 (2003) 681-687. doi: 10.1038/nsb969.
- [56] C. Aussignargues, M.C. Giuliani, P. Infossi, E. Lojou, M. Guiral, M.T. Giudici-Ortoni, M. Ilbert, Rhodanese functions as sulfur supplier for key enzymes in sulfur energy metabolism, *J. Biol. Chem.* 287 (2012) 19936-19948. doi: 10.1074/jbc.M111.324863.
- [57] W. Dietrich, O. Klimmek, The function of methyl-menaquinone-6 and polysulfide reductase membrane anchor (PsrC) in polysulfide respiration of *Wolinella succinogenes*, *Eur. J. Biochem.* 269 (2002) 1086-1095. doi: 10.1046/j.0014-2956.2001.02662.x.
- [58] C. Richey, P. Chovanec, S.E. Hoefft, R.S. Oremland, P. Basu, J.F. Stolz, Respiratory arsenate reductase as a bidirectional enzyme, *Biochem. Biophys. Res. Commun.* 382 (2009) 298-302. doi: 10.1016/j.bbrc.2009.03.045.
- [59] L. Stoffels, M. Krehenbrink, B.C. Berks, G. Udden, Thiosulfate reduction in *Salmonella enterica* is driven by the proton motive force, *J. Bacteriol.* 194 (2012) 475-485. doi: 10.1128/JB.06014-11.
- [60] J. Simon, R.J. van Spanning, D.J. Richardson, The organisation of proton motive and non-proton motive redox loops in prokaryotic respiratory systems, *Biochim. Biophys. Acta* 1777 (2008) 1480-90. doi: 10.1016/j.bbabi.2008.09.008.
- [61] C. Sun, S. Benlekber, P. Venkatakrishnan, Y. Wang, S. Hong, J. Hosler, E. Tajkhorshid, J.L. Rubinstein, R.B. Gennis, Structure of the alternative complex III in a supercomplex with cytochrome oxidase, *Nature* 557 (2018) 123-126. doi: 10.1038/s41586-018-0061-y.
- [62] J.S. Sousa, F. Calisto, J.D. Langer, D.J. Mills, P.N. Refojo, M. Teixeira, W. Kühlbrandt, J. Vonck, M.M. Pereira, Structural basis for energy transduction by respiratory alternative complex III, *Nat Commun.* 9 (2018) 1728. doi: 10.1038/s41467-018-04141-8.
- [63] A.G. Duarte, T. Catarino, G.F. White, D. Lousa, S. Neukirchen, C.M. Soares, F.L. Sousa, T.A. Clarke, I.A.C. Pereira, An electrogenic redox loop in sulfate reduction reveals a likely widespread mechanism of energy conservation, *Nat Commun.* 9 (2018) 5448. doi: 10.1038/s41467-018-07839-x.
- [64] C. von Ballmoos, P. Adelroth, R.B. Gennis, P. Brzezinski, Proton transfer in *ba₃* cytochrome *c* oxidase from *Thermus thermophilus*, *Biochim. Biophys. Acta* 1817 (2012) 650-657. doi: 10.1016/j.bbabi.2011.11.015.
- [65] S.E. Hoefft, J.S. Blum, J.F. Stolz, F.R. Tabita, B. Witte, G.M. King, J.M. Santini, R.S. Oremland, *Alkalilimnicola ehrlichii* sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or

oxygen as the electron acceptor, *Int. J. Syst. Evol. Microbiol.* 57 (2007) 504-512. doi: 10.1099/ijs.0.64576-0.

[66] K. Zargar, A. Conrad, D.L. Bernick, T.M. Lowe, V. Stolc, S. Hoefl, R.S. Oremland, J. Stolz, C.W. Saltikov, ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases, *Environ. Microbiol.* 14 (2012) 1635-1645. doi: 10.1111/j.1462-2920.2012.02722.x.

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Table 1: Overview of genes for sulfite oxidation in some members of the *Aquificales* order

species	SoeABC	SorAB	APS red/Sat
<i>Aquificaceae</i> family			
<i>Aquifex aeolicus</i> VF5	+	-	-/+
<i>Hydrogenivirga caldilitoris</i>	+	-	-/+
<i>Hydrogenivirga</i> sp. 128-5-R1-1	+	-	-/+
<i>Thermocrinis albus</i> DSM 14484	+	+	-/+
<i>Thermocrinis ruber</i> DSM 12173	+	-	-/+
<i>Thermocrinis minervae</i>	+	+	-/-
<i>Hydrogenobacter thermophilus</i> TK-6	+	+	-/+
<i>Hydrogenobacter hydrogenophilus</i>	+	+	-/+
<i>Hydrogenobaculum</i> sp. Y04AAS1	-	-	-/+
<i>Hydrogenothermaceae</i> family			
<i>Hydrogenothermus marinus</i>	-	+	-/+
<i>Persephonella marina</i> EX-H1	-	-	-/+
<i>Persephonella hydrogeniphila</i>	-	-	-/+
<i>Sulfurihydrogenibium</i> sp. YO3AOP1	-	+	-/-
<i>Sulfurihydrogenibium azorense</i> Az-Fu1	-	+	-/-
<i>Sulfurihydrogenibium subterraneum</i>	-	+	-/-

+ and - indicate that a gene or group of genes is found or not found in a genome sequence, respectively. APS red is for APS reductase, Sat for ATP sulfurylase.

Table 2: Identification of proteins from BN and SDS gels by tandem mass spectrometry

Gel band	Exp. MM (kDa)	Protein name	Gene, Locus tag	Accession	PS M	Unique peptides	Coverage (%)	Score	MM (kDa)
1 (blue-native gel)	500	DMSO reductase chain A	<i>dmsA, aq_123_4</i>	O67280	119	43	48	140	112.6
		DMSO reductase chain B	<i>dmsB1, aq_123_2</i>	O67279	44	18	84	54	29
		Protein Aq_863	<i>aq_863</i>	O67026	40	35	35	37	118.1
		DMSO reductase chain C	<i>dmsC, aq_123_1</i>	O67278	16	4	13	31	38.6
2 (blue-native gel)	250	DMSO reductase chain A	<i>dmsA, aq_123_4</i>	O67280	146	55	53	176	112.6
		DMSO reductase chain C	<i>dmsC, aq_123_1</i>	O67278	21	5	15	47	38.6
		DMSO reductase chain B	<i>dmsB1, aq_123_2</i>	O67279	47	19	84	44	29
a (SDS gel)	-	DMSO reductase chain A	<i>dmsA, aq_123_4</i>	O67280	339	57	57	772	112.6
		Protein Aq_863	<i>aq_863</i>	O67026	88	59	50	185	118.1
		Sulfide-quinone reductase	<i>sqr, aq_218_6</i>	O67931	74	26	66	171	47.4
		DMSO reductase chain C	<i>dmsC, aq_123_1</i>	O67278	59	8	18	170	38.6
b (SDS)	110	Protein Aq_863	<i>aq_863</i>	O67026	729	101	76	1648	118.1

gel)		DMSO reductase chain A	<i>dmsA</i> , <i>aq_123</i> 4	O67280	183	54	52	435	112.6
c (SDS gel)	100	DMSO reductase chain A	<i>dmsA</i> , <i>aq_123</i> 4	O67280	900	76	68	1821	112.6
d (SDS gel)	32	Uncharacterized protein	<i>aq_814</i>	O66996	216	15	71	564	32.6
		DMSO reductase chain B	<i>dmsB1</i> , <i>aq_123</i> 2	O67279	207	20	77	531	29
e (SDS gel)	25	Alkyl hydroperoxide reductase	<i>ahpC1</i> , <i>aq_486</i>	O66779	120	21	81	321	23.8

Only most abundant proteins identified in the gel bands are reported. Table heading: Gel band, letters refer to the blue-native or SDS gels in Figure 2; Exp. MM, experimental molecular mass of the band estimated by gel; Protein name, name in UniProtKB; Accession, accession number in UniProt database; PSM, number of peptide spectral matches given by the algorithm corresponding to the total number of identified peptide sequences for the protein, including those redundantly identified; Unique peptides, number of distinct peptides matching to protein sequence and unique to this protein; Coverage, percent protein sequence coverage by the matching peptides; Score, protein score given by Sequest algorithm; MM, theoretical molecular mass of the identified protein (given by Sequest algorithm).

Table 3: Kinetic parameters of SoeABC

Substrates	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Sulfite-NBT	34.3 ± 2.6	567 ± 50	$16.5 \times 10^6 \pm 1.9 \times 10^6$
Sulfite-DB	2.60 ± 0.35	52.9 ± 4.1	$20.3 \times 10^6 \pm 3.1 \times 10^6$

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Table 4: Oxidation of sulfur compounds by SoeABC

Electron donor	Redox potential of electron donor, mV	Electron acceptor	Redox potential of electron acceptor, mV	Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
Sulfite (SO_3^{2-})	- 480 ^a , - 516 ^a	NBT	- 50 ^b	84.2 \pm 7.4
Sulfite (SO_3^{2-})	- 480 ^a , - 516 ^a	DB	+ 90 ^c / \sim + 110 ^d	8.38 \pm 0.66
Polysulfide (S_n^{2-})	Negative ^e	NBT	- 50 ^b	non measurable ^f
Tetrathionate ($\text{S}_4\text{O}_6^{2-}$)	+ 29 ^g	DCPIP (+/- PMS)	+ 228 ^h (+ 63 ⁱ)	0
Thiosulfate ($\text{S}_2\text{O}_3^{2-}$)	- 245 ^j , + 77 ^k	NBT or DB	- 50 ^b or + 90 ^c / \sim + 110 ^d	0

All redox potentials are vs NHE, at pH 7 except otherwise specified. Spectrophotometric reduction of NBT, DB or DCIP was followed at 55°C and pH 8.3 using various inorganic sulfur electron donors.

^aCouple ($\text{SO}_4^{2-} / \text{HSO}_3^-$) [1-3].

^bAt pH 7.2 [35].

^c[38].

^d[39]; [1].

^ePolysulfide species are unknown in our preparation, however redox potentials of all couples ($\text{S}_n^{2-} / \text{S}_{n-1}^{2-}$, sulfur/ S_n^{2-} or $\text{S}_2\text{O}_3^{2-} / \text{S}_5^{2-}$) are negative (ranging roughly from -500 to -190 mV, [3]).

^fSpectrophotometric reduction of NBT using polysulfide as electron donor was not measurable because of the extremely high non-enzymatic, direct chemical reduction of the electron acceptor by the polysulfide.

^gCouple ($\text{S}_4\text{O}_6^{2-} / \text{HSO}_3^-$) calculated at pH 7 from the E^0 value [3].

^h[40].

ⁱ[41].

^jCouple ($2 \text{SO}_4^{2-} / \text{S}_2\text{O}_3^{2-}$) [1].

^kCouple ($\text{HSO}_3^- / \text{S}_2\text{O}_3^{2-}$) calculated at pH 7 from the E^0 value [3].

Table 5: Reduction of sulfur compounds by SoeABC, measured by spectrophotometry or by determination of produced sulfide

Electron donor	Redox potential of electron donor, mV	Electron acceptor	Redox potential E_0' of electron acceptor, mV	Specific activity (nmol MV ^a or H ₂ S ^b min ⁻¹ mg ⁻¹)
Reduced MV	- 446 ^c	Thiosulfate (S ₂ O ₃ ²⁻)	- 402 / - 420 / - 214 ^c	0 ^a
		Tetrathionate (S ₄ O ₆ ²⁻)	+ 198 / - 81 ^d	63 ^a
		Dispersed sulfur (S ⁰)	- 270 ^f	119 ^b
		Thiosulfate (S ₂ O ₃ ²⁻)	- 402 / - 420 / - 214 ^c	0 ^b
		Tetrathionate (S ₄ O ₆ ²⁻)	+ 198 / - 81 ^d	Not determined ^{b, g}
		Sulfite (SO ₃ ²⁻)	- 116 ^h	0 ^b

All redox potentials are vs NHE.

^aThe spectrophotometric reduction of thiosulfate and tetrathionate (both at 1mM) was followed at 55°C in potassium phosphate buffer pH 8.2 using reduced methyl viologen (MV) as electron donor.

^bReduction of sulfur compounds (at 2mM) was determined at 70°C, in potassium phosphate buffer pH 8.2, by sulfide (H₂S) determination using a colorimetric assay.

^c- 402 and - 420 mV are for the couple (S₂O₃²⁻ / HS⁻ + HSO₃⁻) [2,1]; - 214 mV is for the couple (S₂O₃²⁻ / HS⁻) calculated at pH 7 from the E⁰ value [3].

^dThe redox potential of the couple (S₄O₆²⁻ / 2 S₂O₃²⁻) is + 198 mV [42] but reduction may proceed up to sulfur and then sulfide. In this case the redox potential of the couple (S₄O₆²⁻ / S) is calculated to be - 81 mV at pH 7 from the E⁰ value [3].

^e[40]

^fCouple (S⁰ / HS⁻) [1]

^gAn accurate value could not be determined; however, a typical odor of sulfide was occasionally detected at the end of the reaction.

^hCouple (HSO₃⁻ / HS⁻) [1]

Table 6: Properties of some bacterial sulfite dehydrogenases

	SorT ^a	SorAB ^b	Sor ^c	SoeABC ^d
	<i>Sinorhizobium meliloti</i>	<i>Starkeya novella</i>	<i>Thermus thermophilus</i>	<i>Aquifex aeolicus</i>
Family	SO	SO	SO	DMSO reductase
Subunit composition	homodimeric	heterodimeric	homodimeric	Dimer of trimer
Molecular mass	78 000 Da	49 000 Da	80 000 Da	390 000 Da
Cellular location	Periplasm, soluble	Periplasm, soluble	Periplasm, soluble	Cytoplasm, membrane-bound
Redox centers	Mo	Mo, heme <i>c</i>	Mo	Mo, 5 [4Fe-4S] clusters
Electron acceptors	Cytochrome <i>c</i> , ferricyanide	Cytochrome <i>c</i> , ferricyanide	Cytochrome <i>c</i> , ferricyanide	Quinone, NBT
Turnover number	343 s ⁻¹ (pH 8, 25°C)	345 s ⁻¹ (pH8, 25°C)	53318 s ⁻¹ (pH8, 60°C)	567 s ⁻¹ (pH 8.3, 55°C)
K _M sulfite	15.5 μM (pH 8, 25°C)	27 μM (pH 8, 25°C)	10.7 μM (pH 8, 60°C)	34.3 μM (pH 8.3, 55°C)

Adapted from Kappler [5]

^a[8]^b[6]

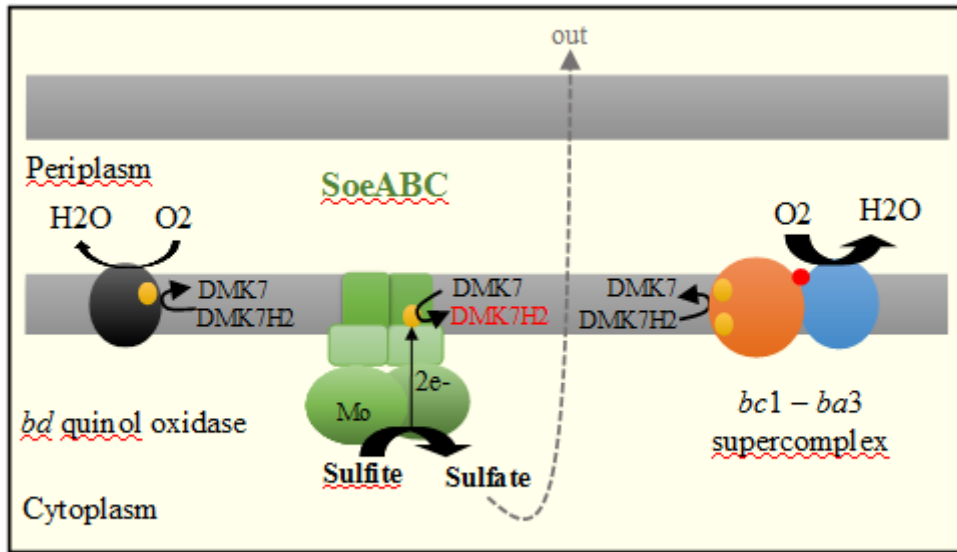
^c[50,51]. The turnover number indicated in [50] is probably erroneous as emphasized by Kappler et al. [5]. The purification table and K_M/k_{cat} ratio indicated in the publication suggest 53.3 s⁻¹ to be read for the turnover number.

^dThis work

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Graphical abstract

Highlights

- The membrane-bound sulfite dehydrogenase SoeABC is purified for the first time
- This large cytoplasmic enzyme is a member of the DMSO reductases family of Mo-enzymes
- It shows a high affinity for sulfite and quinone
- It is bidirectional *in vitro* and phylogenetically related to Sre and Ttr reductases
- Electrons generated by Soe fuel the quinones pool where O₂ reductase(s) draw on

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