Bacterial sulfite dehydrogenases in organotrophic metabolism: separation and identification in *Cupriavidus necator* H16 and in *Delftia acidovorans* SPH-1

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The utilization of organosulfonates as carbon sources by aerobic or nitrate-reducing bacteria usually involves a measurable, uncharacterized sulfite dehydrogenase. This is tacitly assumed to be sulfite : ferricytochrome-*c* oxidoreductase [EC 1.8.2.1], despite negligible interaction with (eukaryotic) cytochrome *c*: the enzyme is assayed at high specific activity with ferricyanide as electron acceptor. Purified periplasmic sulfite dehydrogenases (SorAB, SoxCD) are known from chemoautotrophic growth and are termed 'sulfite oxidases' by bioinformatic services. The catalytic unit (SorA, SoxC; termed 'sulfite oxidases' cd02114 and cd02113, respectively) binds a molybdenum-cofactor (Moco), and involves a cytochrome *c* (SorB, SoxD) as electron acceptor. The genomes of several bacteria that express a sulfite dehydrogenase during heterotrophic growth contain neither *sorAB* nor *soxCD* genes; others contain at least four paralogues, for example *Cupriavidus necator* H16, which is known to express an inducible sulfite dehydrogenase during growth with taurine (2-aminoethanesulfonate). This soluble enzyme was enriched 320-fold in four steps. The 40 kDa protein (denatured) had an *N*-terminal amino acid sequence which started at position 42 of the deduced sequence of H16_B0860 (termed 'sulfite oxidase' cd02114), which we named SorA. The neighbouring gene is an orthologue of *sorB*, and the *sorAB* genes were co-transcribed. Cell fractionation showed SorA to be periplasmic. The corresponding enzyme in *Delftia acidovorans* SPH-1 was enriched 270-fold, identified as Daci_0055 (termed 'sulfite oxidase' cd02110) and has a cytochrome *c* encoded downstream. We presume, from genomic data for bacteria and archaea, that there are several subgroups of sulfite dehydrogenases, which all contain a Moco, and transfer electrons to a specific cytochrome *c*.

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INTRODUCTION

Sulfite dehydrogenases [EC 1.8.2.1] are prokaryotic enzymes (or sets of enzymes), which are usually associated with lithotrophic growth involving energy conservation from the oxidation of reduced inorganic sulfur species or

with the anoxic phototrophic oxidation of inorganic sulfur species. Two general chemistries seem to be involved: firstly the molybdenum chemistry of sulfite dehydrogenases [EC 1.8.2.1] (see below) or, secondly, the AMP-dependent pathway involving formation of adenylyl phosphosulfate (APS) (catalysed by adenylyl-sulfate reductase [EC 1.8.99.2]) and the pyrophosphate-dependent generation of ATP and sulfate (catalysed by sulfate adenylyltransferase [EC 2.7.7.4]); both chemistries can occur in one organism (Beller *et al.*, 2006; Chan *et al.*, 2007; Friedrich *et al.*, 2007; Fritz *et al.*, 2007; Kappler, 2007; Kelly *et al.*, 1997; Schiffer *et al.*, 2006). No evidence of the APS pathway was obtained in this work (see below), so it will not be considered further.

Many attempts have been made to purify sulfite dehydrogenase, but the first claim of purification was from

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Abbreviations: APS, adenylyl phosphosulfate; cd, conserved domain; CDD, conserved domain database; HIC, hydrophobic interaction chromatography; Moco, molybdenum cofactor; Sor, sulfite oxidoreductase; Sox, sulfur oxidation.

A supplementary table listing desulfonative organisms with sequenced genomes and candidate loci to encode enzymes that bind the molybdenum coenzyme is available with the online version of this paper.

Thiobacillus (now *Paracoccus*) *versutus* (Lu & Kelly, 1984). A continuation of this work in *Paracoccus denitrificans* (now *Paracoccus pantotrophus* GB17) involves the current nomenclature of one sulfite dehydrogenase in lithotrophic sulfur oxidation, namely SoxCD (Wodara *et al.*, 1997). Mature SoxC (44 kDa) is periplasmic and has a bound molybdenum cofactor (Moco) which is subject to oxidation by a periplasmic cytochrome *c* (SoxD). This Sox system does not seem to involve free intermediates (Friedrich *et al.*, 2007). SoxC was found to have sequence similarity to eukaryotic sulfite oxidases [EC 1.8.3.1], which interact directly with molecular oxygen, and current bioinformatic nomenclature in the Conserved Domain Database (CDD; Marchler-Bauer *et al.*, 2007) at the National Center for Biotechnology Information (NCBI) classifies SoxC as a sulfite oxidase (cd02113). Similar chemistry is found in another sulfite dehydrogenase, sulfite: cytochrome c oxido-reductase (SorAB), which is involved in lithotrophic sulfur metabolism in *Starkeya novella* (Kappler *et al.*, 2000). SorAB, a heterodimer, is periplasmic, and the reaction involves a sulfite-oxidase-type Moco (Doonan *et al.*, 2006; Metzler, 2001) associated with SorA (40 kDa), while SorB is a *c*-type cytochrome (cf. Fig. 1). The conserved 'sulfite oxidase' domain in SorA is cd02114.

General recognition of the need for sulfite dehydrogenases in bacterial organotrophic growth came when sulfite was shown to be a transient intermediate during the catabolism of arylsulfonates, alkylsulfonates and taurine (2-aminoethanesulfonate) (Johnston *et al.*, 1975; Kondo & Ishimoto, 1972; Thyse & Wanders, 1974), and inducible sulfite dehydrogenases were subsequently found in a desulfonative *Burkholderia* (King *et al.*, 1997; King & Quinn, 1997; Ruff

et al., 2003). The specific activities of the inducible sulfite dehydrogenases assayed by Quinn's group are low, and Reichenbecher *et al.* (1999), working with *Delftia acidovorans* P53, found negligible reduction of cytochrome *c* in the presence of sulfite, whereas ferricyanide was a very effective electron acceptor. This inducible enzyme was termed an 'atypical' sulfite dehydrogenase, and it is found at high specific activity in many organisms (e.g. *Cupriavidus necator* H16, *D. acidovorans* NAT and *Ruegeria pomeroyi* DSS-3; Gorzynska *et al.*, 2006; Mayer *et al.*, 2006; Weinitschke *et al.*, 2007; Yi *et al.*, 2007). The enzyme from *D. acidovorans* P53 was enriched some 230-fold, but it was unclear which of the five bands on SDS-PAGE gels represented the sulfite dehydrogenase; the native molecular mass was 67 kDa (Reichenbecher *et al.*, 1999). The novelty is that the electron acceptor in enzyme assays is not cytochrome *c*, routinely a eukaryotic cytochrome *c*, but the artificial acceptor, ferricyanide, and we wondered which natural electron acceptor it represented.

We now report that the 'atypical' sulfite dehydrogenase in *C. necator* H16, for which genome sequence data are available, is an orthologue of SorA. The gene neighbouring *sorA* is *sorB*, which encodes a cytochrome *c* presumed to interact with SorA. Analogous data were obtained from *D. acidovorans* SPH-1.

METHODS

Materials. Sodium sulfite ($\geq 98\%$ pure) and sodium sulfate ($\geq 99\%$ pure) were from Merck. Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). *Taq* DNA polymerase and M-MuLV reverse transcriptase were from Fermentas and they were used as specified by the supplier. RNase-free DNase was from Fermentas. Chromosomal DNA was isolated from bacteria as described by Desomer *et al.* (1991). Total RNA was isolated using the E.Z.N.A. bacterial RNA kit (Omega Bio-Tek). A 50 bp DNA ladder (Fermentas) was used.

Organisms, growth, harvesting of cells and preparation of cell-free extracts. *C. necator* H16 (DSM 428; Pohlmann *et al.*, 2006) was provided by B. Bowien (Georg-August-Universität, Göttingen, Germany). *D. acidovorans* NAT (DSM 17854; Mayer *et al.*, 2006) and *D. acidovorans* SPH-1 (DSM 14801; Schleheck *et al.*, 2004) were isolated in this laboratory.

Cultures of the three strains were grown aerobically at 30 °C in a phosphate-buffered mineral salts medium, pH 7.2 (Thurnheer *et al.*, 1986) with 20 mM taurine or acetate as the sole added source of carbon. Precultures (3 ml) were grown in 30 ml screw-cap tubes in a roller. Cultures for enzyme assays (50 ml in 300 ml Erlenmeyer flasks) or for protein purification (1 l in 5 l Erlenmeyer flasks) were grown on a shaker and harvested at OD₅₈₀ ~0.8 by centrifugation (20 000 g, 20 min, 4 °C). Cells were washed with the starting buffer of the first purification step [50 mM Tris/HCl buffer, pH 9.7 (containing 5 mM MgCl₂) or 50 mM Tris/HCl, pH 9.0] and stored frozen. Cell-free extracts free of nucleic acids (DNase-treated) were generated through disruption by three to four passages through a French pressure cell set at 140 MPa and centrifugation (Junker *et al.*, 1994).

Enzyme purification. The membrane/particulate fraction was removed from the crude extract by ultracentrifugation (170 000 g,

Fig. 1. Diagrammatic representation of the location and mechanism of the sulfite dehydrogenase (SorA and SorB) [EC 1.8.2.1] in *C. necator* H16. In contrast to the periplasmic, heterodimeric SorAB in *S. novella* (Kappler, 2007), the enzyme in *C. necator* has two components, the periplasmic SorA, which was purified, and the apparently membrane-bound SorB.

30 min, 4 °C) and the supernatant fluid was designated the soluble fraction.

Soluble fractions of *C. necator* H16 were brought to 1.7 M ammonium sulfate, the precipitate was discarded (30 000 g, 5 min, 4 °C), and the supernatant fluid subjected to hydrophobic interaction chromatography (HIC) on a Phenyl Superose HR 10/10 column (Pharmacia): the flow rate was 1 ml min⁻¹. A linear decreasing gradient of ammonium sulfate in 20 mM Tris/HCl buffer, pH 9.0, was applied, and SorA eluted at 0 mM ammonium sulfate. Active fractions were rebuffed on PD10 columns (Sephadex G-25, Pharmacia) with 50 mM MES, pH 5.5, and loaded on to a cation-exchange column (Mono S, HR 5/5, Pharmacia) at 1 ml min⁻¹. A linear increasing gradient to 0.5 M sodium sulfate was applied and SorA eluted at about 0.4 M sodium sulfate. Active fractions were pooled, concentrated using Vivaspin concentrators (10 kDa cut-off; Sartorius), transferred to 50 mM Tris/HCl, pH 9.7 and loaded on to an anion-exchange chromatography column (Mono Q, HR 10/10, Pharmacia) at a flow rate of 2 ml min⁻¹. A linear gradient of sodium sulfate (0.075–0.2 M over 45 min) eluted SorA at about 0.1 M salt. Concentrated active fractions were subjected to gel filtration (Superose 12 HR 10/30, Pharmacia) in 50 mM Tris/HCl, pH 7.5 with 150 mM sodium sulfate at a flow rate of 0.4 ml min⁻¹.

Soluble fraction of *D. acidovorans* SPH-1 in 50 mM Tris/HCl, pH 9.7 was loaded on to an anion-exchange chromatography column (Mono Q, HR 10/10, Pharmacia) at a flow rate of 1.0 ml min⁻¹. A linear gradient of sodium sulfate (0.075–0.2 M over 45 min) was applied. SorA did not bind to the column and eluted immediately, but other proteins did bind. Concentrated active fractions were brought to 1.7 M ammonium sulfate, and subjected to HIC on Phenyl Superose HR 10/10 columns (Pharmacia). A linear decreasing gradient of ammonium sulfate in 50 mM Tris/HCl buffer, pH 9.0, was applied, and SorA eluted at 0 mM ammonium sulfate. Gel filtration was used as a third purification step (Superose 12 HR 10/30, Pharmacia) in 50 mM Tris/HCl, pH 7.5 with 150 mM sodium sulfate at a flow rate of 0.4 ml min⁻¹. Other combinations of columns were also tested, but did not improve the purification factor in combination with a high recovery.

Analytical methods. Sulfite was determined as the fuchsin adduct (Denger *et al.*, 2001); sulfate was measured as turbidity of a suspension of insoluble BaSO₄ (Sörbo, 1987). Sulfite and sulfate were also quantified by ion chromatography (Laue *et al.*, 1996). Protein in extracts was assayed by protein–dye binding (Bradford, 1976). Denatured proteins were separated on 13 % SDS-PAGE gels and stained with Coomassie Brilliant Blue R250 (Laemmli, 1970); on occasion, the presence of a haem group was tested for with bovine cytochrome *c* as the positive control (Francis & Becker, 1984). The N-terminal sequence of a blotted protein was determined after Edman degradation and HPLC separation under contract at TopLab (Martinsried, Germany).

Enzyme assay. Sulfite dehydrogenase [EC 1.8.2.1] was assayed photometrically with 1 mM potassium ferricyanide as the electron acceptor in 50 mM Tris/HCl, pH 8.0 (Reichenbecher *et al.*, 1999). Due to substrate inhibition and non-specific reaction with ferricyanide the concentration of sulfite was lowered to 0.4 mM. The protein concentration in the assay was in the range 0.5–50 µg ml⁻¹. The eukaryotic cytochromes *c* (equine and bovine) that were tested as electron acceptors gave only about 1 % [0.3 mkat (kg protein)⁻¹] of the specific activities obtained with ferricyanide. Activity of 1 kat represents the oxidation of 1 mol sulfite s⁻¹, for which 2 mol of ferricyanide ($\epsilon_{420}=0.9 \text{ mM}^{-1} \text{ cm}^{-1}$) or cytochrome *c* ($\epsilon_{550}=21.0 \text{ mM}^{-1} \text{ cm}^{-1}$) had to be reduced. The presence of the APS pathway was tested for by the addition of up to 1 mM AMP to the standard assay.

Cell fractionation. Whole cells were separated into periplasmic and cytoplasmic fractions by generating spheroplasts essentially as described elsewhere (Witholt *et al.*, 1976). A fresh culture was harvested by centrifugation at the end of the exponential growth phase and suspended to a density of about 10 mg protein ml⁻¹ in 0.2 M Tris/HCl, pH 8.0, with 0.5 mM EDTA and 0.5 M sucrose. Egg-white lysozyme was added to 300 µg ml⁻¹ and the suspension was incubated at room temperature for about 60 min: the process was followed microscopically and was considered complete when all rods disappeared and only spherical objects were visible. The spheroplasts were stabilized by the addition of MgCl₂ (to 20 mM) and removed by centrifugation (6000 g, 5 min, room temperature) to yield a periplasmic fraction (the supernatant fluid), which was removed, and the spheroplasts (the pellet). The spheroplasts were ruptured by the addition of water, which yielded the cytoplasmic enzymes in solution.

Molecular methods. The reverse primers 16S-533R (Weisburg *et al.*, 1991), H16xscR (5'-gggtgtagaagccacctgggtct-3') and H16sorBR (5'-acgtatgcggccgaatggcagat-3') were used for reverse transcription reactions. Subsequent PCRs were done as described previously (Innis *et al.*, 1990). For PCR, the reverse and forward primers H16sorAF (5'-ttcgaaggccatgatcggttctc-3') and H16sorAR (5'-tcaat-gacgttgccggcgatag-3') were used. Absence of DNA after RNA isolation was confirmed by PCR using the primers H16xscF (5'-accgacatcgg-caacatcaactc-3') and H16xscR. Positive controls for RNA integrity after isolation were done using the 16S rRNA-specific primers 16S-27F and 16S-533R (Weisburg *et al.*, 1991). RT-PCR products were visualized on 1.5 % agarose gels.

Software for sequence analyses and accession numbers. Sequence analyses of the genome (accession no. NC_008314) of *C. necator* H16 and of the draft genome of *D. acidovorans* SPH-1 (accession no. NZ_AAVD000000000) were done using the BLAST algorithm on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). Sequence data were manipulated with different subroutines from the LASERGENE program package (DNASTAR). Transmembrane regions were predicted using the program TMHMM, while leader peptides were predicted by SignalP (Bendtsen *et al.*, 2004), both at the Center for Biological Sequence Analysis (CBS; <http://www.cbs.dtu.dk/services/>).

RESULTS

Sulfite dehydrogenase, not sulfite oxidase, is expressed

Growth of *C. necator* H16 in e.g. taurine-salts medium was concomitant with substrate utilization and the sulfonate moiety was recovered quantitatively as sulfate; no intermediate sulfite was detected and the specific degradation rate in taurine-grown cells was calculated from the specific growth rate and the molar growth yield to be about 5.8 mkat (kg protein)⁻¹. Putative sulfite dehydrogenase activity was found to be inducible in cultures of strain H16 and specific activities of 20–35 mkat (kg protein)⁻¹ were observed with routine colorimetric assays of crude extracts from cells grown in taurine-, isethionate- or sulfoacetate-salts medium, which confirmed earlier data (Weinitschke *et al.*, 2007). The specific activity of sulfite dehydrogenase in crude extracts was thus sufficient to support the growth rate. Crude extract from these taurine-grown cells depleted sulfite from stirred reaction mixtures containing only

dioxygen as the terminal electron acceptor. When the particulate fraction was removed by ultracentrifugation, the soluble fraction showed negligible ability to cause sulfite to disappear in the presence of dioxygen (and the separated enzyme was inactive). When this supernatant fluid was then supplied with ferricyanide as the electron acceptor, sulfite was depleted at high specific activity (see above). The enzyme under examination obviously did not interact directly with dioxygen and was considered to be a sulfite dehydrogenase. The natural catalytic electron acceptor (with electron transport) was obviously in the particulate fraction.

The addition of AMP to the standard assay did not stimulate the reaction. The APS pathway was thus unlikely to be present.

Separation of the sulfite dehydrogenase from *D. acidovorans* SPH-1

Work was initiated with taurine-grown cells of *D. acidovorans* NAT, which inducibly expresses the 'atypical' sulfite dehydrogenase (Mayer *et al.*, 2006) reported in *D. acidovorans* P53 (Reichenbecher *et al.*, 1999), but was transferred to the metabolically identical *D. acidovorans* SPH-1, whose draft genome sequence had become available. The poor binding of this sulfite dehydrogenase to anion-exchangers (Reichenbecher *et al.*, 1999) was confirmed, and exploited to remove all proteins that bound to the column. Results from different variants of the separation steps led to the conclusion that a protein of about 40 kDa (SDS-PAGE), which could be purified about 270-fold in the presence of other major protein bands, probably represented the sulfite dehydrogenase. The N-terminal amino-acid sequence was determined to be AQAQAQA. The peptide represented either positions 38–44 or 40–46 of Daci_0055 (417 aa), which had been annotated 'sulfite oxidase'. The predicted cleavage site was amino acid 39, so we tentatively assume the prediction to be correct. The molecular mass of the mature protein was deduced to be 39.8 kDa with a pI of 8.7, in good agreement with the SDS-PAGE data and the failure to interact with the anion-exchange column at pH 8.5. We considered that the enzyme was a sulfite oxidoreductase (see below) and used the abbreviation SorA, encoded by *sorA*. After a gap of

85 nt, the downstream gene, which we tentatively named *sorB*, encodes a hypothetical cytochrome *c*.

The separated SorA was chromatographed on a calibrated gel-filtration column. The retention time of SorA corresponded to a molecular mass of about 67 kDa, the value reported for *D. acidovorans* P53 (Reichenbecher *et al.*, 1999), so, knowing that the method is prone to significant errors (le Maire *et al.*, 1996), we tentatively presume native SorA to be a homodimer.

Separation and purification of sulfite dehydrogenase from *C. necator* H16

The work with *D. acidovorans* did not yield a pure protein, so when it became clear that *C. necator* H16 encoded a taurine degradative pathway, grew faster than strain SPH-1 with taurine and expressed a similar sulfite dehydrogenase (Weinitschke *et al.*, 2007; see also Pohlmann *et al.*, 2006), work on sulfite dehydrogenase was transferred to strain H16. When fractions from the cation exchanger were brought to pH 9.7 and loaded on to an anion-exchange column, the sulfite dehydrogenase interacted with the column and was eluted from the column during the salt gradient. This allowed a purification in a relatively low volume (Table 1) and finally allowed the protein to be purified, though in low yield (Table 1, Fig. 2). The N-terminal amino-acid sequence (ARISDGV) was compared with the proteins deduced to be encoded by the genome of *C. necator* H16, which yielded a unique peptide at locus H16_B0860. The peptide represented positions 42–48 of the derived protein (420 aa), which had been annotated 'sulfite oxidase'. The molecular mass of the mature protein was deduced to be 40.7 kDa: this was in good agreement with the experimental data (~40 kDa, Fig. 2). We termed the sulfite dehydrogenase SorA, encoded by *sorA*. The downstream gene, after a gap of 9 nt, encodes a hypothetical cytochrome *c*, which we termed *sorB* (see below).

Some preparations of sulfite dehydrogenases contain a haem group (Toghrol & Southerland, 1983). The positive control (cytochrome *c*), some membrane proteins and contaminant proteins in some proteins from strains H16 and SPH-1 examined on SDS-PAGE gels reacted with the haem stain; the SorA proteins did not.

Table 1. Purification of sulfite dehydrogenase from *C. necator* H16

Purification step	Total protein (mg)	Total activity (nkat)	Recovery (%)	Specific activity (mkat kg ⁻¹)	Purification (-fold)
Crude extract	240	5160	100	21.5	1
Soluble fraction	170	5124	99	30.1	1
HIC	11.1	1403	27	127	6
Cation exchanger	1.9	1068	21	578	27
Anion exchanger	0.3	463	9	1838	85
Gel filtration	0.01	61	1	6850	319

Fig. 2. Separation by SDS-PAGE of proteins in different fractions from the purification of SorA from *C. necator* H16. Lanes 1, crude extract; 2, soluble fraction; 3, hydrophobic interaction column; 4, cation exchange column; 5, anion exchange column; 6, gel filtration column (different experiment); 7, molecular mass markers.

Transcription of *sorAB* in *C. necator* H16

The *sorA* gene was shown by RT-PCR to be inducibly transcribed, which confirms the enzyme data. When H16sorBR cDNA was used with the primer set for *sorAB*, an amplicon was obtained (not shown). The *sorAB* genes were obviously cotranscribed.

Some properties of SorA from *C. necator* H16

Denatured SorA had an observed molecular mass of 40 kDa (Fig. 2): the protein eluted from the gel filtration column in the range 49–55 kDa. We suspect that the mature, native protein could be monomeric. The enzyme was colourless. Initial steps in the purification contained in the SorA-fraction a protein(s) with the spectral properties of a *c*-type cytochrome, but the separated cytochrome *c* did not oxidize SorA (and was thus not SorB), though it was obviously active, because it did enable taurine dehydrogenase to deaminate taurine (cf. Weinitschke *et al.*, 2005, 2007).

The presence of a leader peptide, which was subject to cleavage from each separated SorA, allowed the hypothesis that the enzyme is periplasmic, as found in *S. novella* (Kappler *et al.*, 2000). Taurine-grown cells of *C. necator* H16 were fractionated. The periplasmic fraction contained SorA at about 105 mkat (kg protein)^{−1}, fivefold higher than in crude extract (Table 1), whereas the specific activity in the cytoplasmic fraction was 10 mkat (kg protein)^{−1}, some 30 % of that in crude extract. This was taken as direct evidence for a periplasmic SorA.

A simple rate diagram of the sulfite dehydrogenase reaction of strain H16 (Fig. 3) indicated a value for K_m^{app} of 50–100 μM for sulfite and substrate inhibition. This is in the

Fig. 3. Specific activity of SorA from *C. necator* H16 as a function of the substrate (sulfite) concentration. Partially purified enzyme was used.

same order of magnitude as reported for SorA from *S. novella* (30 μM ; Kappler *et al.*, 2000). The corresponding value for the artificial electron acceptor ferricyanide was 0.9 mM.

SorA was stable for at least 3 months at 4 °C or at −20 °C in the pH range 5.5–10.5 in MES, phosphate, Tris or CAPS buffer, as appropriate.

sorAB-like genes in genome projects involving sulfonate dissimilation

There are a large number of orthologues of SorA (from *S. novella*) in the NCBI database, and they are found in different subgroups (Kappler, 2007; see also Introduction), so we limited our collection to SorA (and SorB)-candidates in presumably desulfonative organisms (51 candidate organisms, 48 aerobes) with at least partially sequenced genomes (see Supplementary Table S1, available with the online version of this paper; excerpts are given in Table 2). The CDD classification of SorA (from *S. novella*) is a conserved domain (cd02114) covering most of the protein and representing the binding site for the Moco. Only three gene products in desulfonative organisms in Supplementary Table S1 contain this domain, *C. necator* H16_B0860 (above), *C. necator* JMP134 (Reut_A3183) and *Herminiimonas arsenicoxydans* (HEAR2349) (another member of the Burkholderiales). *C. necator* H16 contains three other genes which potentially encode proteins with a binding site for a Moco, namely a *soxC* in a *sox* gene cluster, an isolated *soxC(D)* and a gene encoding a protein of unknown function (YedY; cd02107), none of which was detected as a sulfite dehydrogenase in this work.

The SorA from *D. acidovorans*, Daci_0055, has a different binding site for the Moco, to judge from the CDD prediction (cd02110; Table 2). If the activity of this SorA is representative, some 11 other organisms could also catalyse the sulfite dehydrogenase reaction with this type of enzyme (Supplementary Table S1).

Table 2. Desulfonative organisms with sequenced genomes and the candidate loci to encode enzymes that bind the Moco

The domain to which the organism belongs is given in square brackets [A, archaea; B, bacteria] and the substrate in parentheses (i, isethionate; s, sulfolactate; t, taurine); a question mark (?) indicates a substrate deduced from the presence of genes we identified on the genome of the organism. The identifier of the genome is given to save space in the subsequent columns. Candidates are distributed according to the conserved domain (cd) attributed by NCBI. As there is only one purified sulfite dehydrogenase (SorA), the nomenclature in CDD is speculative, and derived from work in multicellular eukaryotes. Usually an organism has only one candidate per cd, but in some cases there are paralogues, which are listed alpha-numerically. The list of organisms includes the two used in this paper (indicated in bold type), with the separated enzymes (indicated in bold type) and a strictly anaerobic bacterium, which contains no candidate sulfite dehydrogenase, and is known to reduce sulfite to sulfide

Organism [domain] (sulfonate utilized)	Locus identifier	Locus for gene products with the named CDD conserved domain for the Moco							
		cd02114 <i>sorA</i>	cd02113 <i>soxC</i> in Sox cluster	cd02113 <i>soxC</i> alone	cd02110	cd02109	cd02108	cd02107 YedY	cd00321
<i>Burkholderia multivorans</i> ATCC 17616 [B] (t?)	BmulDRAFT_	—	—	—	—	—	—	_1256	—
<i>Burkholderia phytofirmans</i> PsJN [B] (t?)	BphytDRAFT_	—	—	—	—	—	_5604	—	—
<i>Burkholderia xenovorans</i> LB400 [B] (t)	Bxe_	—	—	A2166, C0151	—	—	A2177, B1752	A0349	—
<i>Chromohalobacter salexigens</i> DSM 3043 [B] (s?)	Csa_	—	—	—	—	—	—	—	—
<i>Cupriavidus necator</i> H16 [B] (t)	H16_	B0860	A3572	B2222	—	—	—	A3445	—
<i>Delftia acidovorans</i> SPH-1 [B] (t)	Daci_	—	—	—	_0055	—	_3134	_5586	—
<i>Desulfitobacterium hafniense</i> DCB-2 [B] (i)	DhafDRAFT_	—	—	—	—	—	—	—	—
<i>Fulvimarina pelagi</i> HTCC2506 [B] (s?)	FP2506_	—	_12519	—	—	—	—	—	—
<i>Rhodobacter sphaeroides</i> 2.4.1 [B] (t)	RSP_	—	—	—	—	—	—	_1410	—
<i>Rhodococcus</i> sp. RHA1 [B] (t)	RHA1_	—	—	—	ro01563	—	—	—	ro04266
<i>Sulfolobus solfataricus</i> P2 [A] (s)	SSO	—	—	—	—	_3201	—	—	_2826
<i>Thermoplasma acidophilum</i> DSM 1728 [A] (s?)	Ta	—	—	—	—	_0136	—	—	—

DISCUSSION

This project was initiated because an ‘atypical’ sulfite dehydrogenase (Reichenbecher *et al.*, 1999), which is widespread (Cook *et al.*, 2007), was amenable to analysis. The novelty was assumed to lie in the electron transport (see Introduction). In fact, the reaction can be assumed to involve both the established Moco, with its oxo-transfer, and a cytochrome *c* for electron transport (Fig. 1). The novelty is that the enzyme assay does not function well with one artificial electron acceptor (eukaryotic cytochrome *c*), but it is effective with an alternative artificial acceptor (ferricyanide). There are differences in detail, but not in principle, from the enzyme characterized by Kappler *et al.* (2000).

Monomeric, periplasmic SorA together with a membrane-bound SorB seem to represent sulfite dehydrogenase in *C. necator* H16, in contrast to the heterodimeric, periplasmic SorAB in *S. novella* (Kappler *et al.*, 2000). Neither Moco nor SorB has been explored in this context in *C. necator* H16, but the high level of purification required and the poor yield of SorA did not permit much more than

identification of the genes involved, far less analysis of the Moco. SorB is apparently membrane bound (Fig. 1). We presume that heterologous expression of SorA and SorB at high levels (or homologous overexpression), possibly with pre-fractionation of periplasmic proteins, is needed to allow comprehensive analyses of the enzyme and its Moco.

The SorA of *C. necator* with its conserved domain (cd02114) and SorB (precursor molecular mass 22.8 kDa; mature molecular mass 20.6 kDa) are orthologous to SorAB from *S. novella*, but this SorA is not representative of sulfite dehydrogenases in most organisms which need this enzyme activity to dissimilate organosulfonates (Supplementary Table S1). The SorA of *D. acidovorans* has a different conserved domain (cd02110) and interacts with a different SorB (precursor molecular mass 11.7 kDa; mature molecular mass 8.7 kDa). The conserved domain (most of the protein) in this group of undefined proteins presumably represents not only a binding site for the Moco, but also for the cytochrome *c* (SorB). Assuming that the SorA-orthologues (Supplementary Table S1) with domain cd02110 are, in fact, active sulfite dehydrogenases during dissimilation of organosulfonates, we have identified only

about 29% of the sulfite dehydrogenases in currently identified sulfite-dehydrogenase-requiring organisms.

Many organisms, including the bacteria examined in this paper (Table 2, Supplementary Table S1), have several candidate genes to encode hypothetical Moco-binding proteins, which could represent SorA (and a corresponding SorB). Organisms with domain cd02109, cd02108 or cd02107 as apparently the sole Moco-containing enzymes are *Thermoplasma acidophilum* DSM1728, *Burkholderia phytofirmans* PsJN and *Rhodobacter sphaeroides* 2.4.1, respectively (Table 2). We hypothesize that different classes of Moco-binding proteins might be sulfite dehydrogenases. Correspondingly, we believe that the nomenclature in CDD and genome annotation should be altered to eliminate the incompatibility of calling sulfite dehydrogenase [EC 1.8.2.1] a sulfite oxidase [EC 1.8.3.1].

The N-terminal amino acid sequence of the mature SorA from *C. necator* H16 is currently unique. In contrast, the N-terminal amino acid sequence of the mature SorA from *D. acidovorans* SPH-1 is found in about 500 proteins.

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