Desulfovibrio DA2_CueO is a novel multicopper oxidase with cuprous, ferrous and phenol oxidase activity

Stefano Mancini,† Ranjeet Kumar,‡ Veena Mishra‡ and Marc Solioz†,‡,*

Abstract

Desulfovibrio sp. A2 is a novel Gram-negative sulfate-reducing bacterium that was isolated from sediments of the Norilsk mining/smelting area in Russia. The organism possesses a monocistronic operon encoding a 71 kDa periplasmic multicopper oxidase, which we call DA2_CueO. Histidine-tagged DA2_CueO expressed from a plasmid in Escherichia coli and purified by Ni–NTA affinity chromatography oxidizes Cu⁺ and Fe²⁺, and exhibits phenol oxidase activity with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), 2,3-dihydroxybenzoic acid and 2,6-dimethoxyphenol as substrates, using O₂ as the oxidant. When expressed in an E. coli cueO knock-out strain, DA2_CueO exhibits phenol oxidase activity in vivo and enhances the copper tolerance of the strain. These findings indicate that the DA2_CueO gene of Desulfovibrio sp. A2 encodes a multicopper oxidase with a role in metal ion resistance. The enzyme displays some novel structural features, which are discussed.

INTRODUCTION

Copper ions are an essential cofactor in many enzymes, such as Cu,Zn-superoxide dismutases, which are required for defence against oxidative stress, cytochrome aa₃-type terminal oxidases, which reduce molecular oxygen, or plastocyanins and azurins, which act as electron carriers, to name just a few [1–3]. It is currently believed that copper-loading of cuproenzymes in bacteria takes place in the periplasmic space and that there is no requirement for cytoplasmic copper, except in photosynthetic organisms [4]. While this concept is not yet fully proven, it is clear that excess cytoplasmic copper is toxic to bacteria. However, how copper enters the cytoplasm is still not resolved for most bacteria.

With copper being a redox-active metal, its intracellular toxicity has generally been ascribed to Fenton and Haber–Weiss reactions, which result in the production of hydroxyl radicals and reactive oxygen species (ROS) [5]. ROS can cause lipid peroxidation, and protein and DNA damage. Copper ions induce the soxRS regulatory system of Escherichia coli under aerobic conditions, indicating the generation of ROS. However, hypersensitivity to copper is only observed in mutants that are deficient in superoxide dismutases or repair enzymes for oxidative DNA damage, suggesting that wild-type E. coli can deal with copper-induced oxidative stress [6]. Indeed, the idea of copper-induced oxidative stress by in vivo Fenton chemistry has recently been challenged by several lines of research. First, the demonstration that the intracellular free copper concentration in E. coli may be in the zeptomolar (10⁻²¹ M) range makes Fenton chemistry unlikely [7]. Secondly, a large body of recent evidence suggests that in vivo copper toxicity is primarily due to the displacement of iron from iron–sulfur clusters, which leads to the inactivation of essential enzymes in glucose metabolism and branched-chain amino acid synthesis [8–13]. The degradation of iron–sulfur clusters leads to the release of Fe²⁺, which can cause further toxicity. In line with these copper toxicity concepts, Park et al. showed that intracellular hydroxyl radical levels are not significantly changed by the addition of Cu(II) to E. coli [14]. Rather, Cu(II) is reduced to Cu(I), which is considerably more toxic to cells than Cu(II) due to its higher thiophilicity and thus higher avidity for sulfhydryl residues of proteins, and the higher...
permeability of the cytoplasmic membrane for Cu(I) compared to Cu(II) [3, 15].

A key element in detoxification of the cytoplasm from copper is the copper export ATPases. In E. coli, the CopA copper-transporting ATPase serves this function under both aerobic and anaerobic conditions [16]. CopA expression is regulated by CueR, a transcriptional regulator induced by Cu(I) [17]. Closely related CopA-type ATPases are found in every bacterial genome sequence, including that of Desulfovibrio sp. A2 [18], but the transcriptional regulators of these enzymes are diverse and have probably evolved by convergent evolution [19]. The third apparently universal component of cytoplasmic copper detoxification is CpxZ-type copper chaperones, which are named after the founding member of Enterococcus hirae [2]. Such proteins from several bacteria have been structurally and functionally characterized. They bind cytoplasmic Cu(I) and deliver it to copper-dependent regulators and copper-export systems [20]. In Gram-negative organisms, a multi-subunit complex can serve to expel periplasmic copper. In E. coli, the respective CusCFBA complex has been studied in great detail. This efflux system belongs to the resistance–nodulation–cell division (RND) protein family and comprises a central RND proton–substrate antiporter, a membrane-fusion protein and an outer-membrane factor. The mechanism of substrate binding and subsequent efflux has yet to be elucidated [21].

In addition to the aforementioned copper defence mechanisms, a number of periplasmic functions contribute to copper detoxification. Among these are periplasmic copper oxidases, usually designated CueO, that can participate in copper tolerance [23, 24]. Among other things, they can convert Cu(I) to the less toxic Cu(II) ion [3]. For E. coli CueO, which we call Eco_CueO here, it has been shown that it can also curb copper toxicity by oxidizing catechols, which in their oxidized form avidly bind Cu(II) [25].

We recently sequenced the genome of a novel Gram-negative sulfate-reducing bacterium, Desulfovibrio sp. A2 [18]. Desulfovibrio bacteria are key players in microbial sulfate reduction in many environments. Sulfate reduction contributes to the precipitation of metal sulfides and, thereby, the immobilization of toxic metals [26]. In culture, Desulfovibrio sp. A2 exhibits unusually high copper tolerance, being able to grow at 40 mM copper [27]. The genome of Desulfovibrio sp. A2 encodes a putative periplasmic multicopper oxidase, which could contribute to the unusual copper resistance of this strain. In this work, the in vivo and in vitro function of this enzyme, DA2_CueO, was characterized. The enzyme was found to be a multicopper oxidase that is able to oxidize Cu(I), Fe(II) and a number of phenolic compounds. These activities of DA2_CueO could contribute to the copper resistance of Desulfovibrio sp. A2.

**METHODS**

**Strains and culture conditions**

Desulfovibrio sp. A2 was isolated and grown as previously described [28]. E. coli ΔcueO strain JW0119 and E. coli wild-type strain JW2922 were obtained from the Keio Collection, Japan. All E. coli strains were grown aerobically in Luria–Bertani (LB) media at 37°C, unless indicated otherwise.

**Cloning of DA2_cueO**

DNA was isolated from Desulfovibrio sp. A2 by alkaline lysis as described [29]. The DA2_cueO gene (locus tag DA2_0547 on contig 12 of GenBank bioproject PRJNA61255) was cloned by PCR amplification of genomic DNA with Pfu DNA polymerase and primers sm147 (5′-GACAGGATCCGTCGTCTCGATCCACCC) and sm148 (5′-CCATGTTCGCCCATCGACAGC). The resulting PCR product was cloned into PCR Blunt II TOPO (Invitrogen, CA, USA), yielding pDT01. The DA2_cueO gene was excised from this vector with BamHI and EcoRI, and subcloned into pProExHTa, digested with the same enzymes. The resulting construct, pDA01, encodes DA2_CueO with an N-terminal His-tag that can be cleaved with recombinant tobacco etch virus (rTEV) protease. In the cleaved protein, the original N-terminal methionine is replaced by GAMDP. The absence of mutations in the cloned DA2_cueO gene was verified by commercial DNA sequencing.

**Purification of DA2_CueO**

DA2_CueO was overexpressed from plasmid pDA01 in E. coli ΔcueO strain JW0119. Cells were grown aerobically at 37°C in 300 ml of LB medium containing 50 μg ml⁻¹ of ampicillin and 1 mM of CuSO₄ to an OD at 600 nm of 0.8. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for an additional 16 h at 20°C. The cells were harvested at 7000×g for 10 min and the resultant pellet was stored at −70°C until further use. His-tagged DA2_CueO was purified by resuspending pelleted cells in 2 ml per g of wet weight in lysis buffer [50 mM Na-4-(2-hydroxyethyl)–1-piperazinemethanesulfonic acid (HEPES), pH 7.5] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), freshly added. Two passages through a French press at 30 MPa. Cell debris was removed by centrifugation at 15 000×g for 30 min. The supernatant obtained was applied to a Ni-NTA column equilibrated with lysis buffer. The column was washed with five column volumes of lysis buffer, and this was followed by the elution of DA2_CueO with lysis buffer containing 50 mM imidazole. Eluted fractions were analysed by 10% sodiumdodecyl sulfate (SDS) polyacrylamide gels [30]. Size-exclusion chromatography was performed on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare Life Sciences) in 20 mM Tris–SO₄, pH 7.4, 150 mM NaCl. DA2_CueO-containing fractions were pooled and stored frozen at −20°C. The His-tag of DA2_CueO was removed by cleavage with 1/10 the amount of rTEV protease (purified inhouse) overnight at room temperature in the same buffer, followed by passage through a Ni-NTA column to remove...
uncleaved His-DA2_CueO and the His-tagged rTEV protease. The resulting 678 amino acid protein had the N-terminal M (the one-letter amino acid code will be used throughout) replaced by GAMDP and had a predicted molecular weight of 71.2 kDa. It exhibited a purity of ~90%, as determined by SDS polyacrylamide gel electrophoresis (Fig. S3, available with the online Supplementary Material). The protein concentration was determined by Bradford’s method, using bovine serum albumin as a standard [31].

Enzyme assays
All enzyme assays were conducted in a total volume of 1 ml at 30 °C, using purified DA2_CueO, preactivated by incubation with 50 µM CuSO₄. Enzyme reactions were started by the addition 20 µg of purified DA2_CueO. Phenol oxidase activity was determined spectrophotometrically in 50 mM Na-MOPS, pH 7, 50 µM CuSO₄, using the following substrates: 2,3-dihydroxybenzoic acid (DHB; λₘₐₓ=374 nm, ε=29.7 mM⁻¹ cm⁻¹ [32]) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; λₘₐₓ=436 nm, ε=39.3 mM⁻¹ cm⁻¹ [24]).

Cuprous oxidase activity was measured in 50 mM Na-MOPS, pH 7, 50 µM CuSO₄, using the previously described Cu(I)-bicinchoninic acid complex as the substrate [33]. [Cu(I)(BCA)₂]⁺ was prepared by mixing 160 µM BCA with 80 mM CuSO₄, followed by reduction with sodium dithionite, which resulted in the formation of the purple complex (λₘₐₓ=562 nm, ε=7.9 mM⁻¹ cm⁻¹, βₐ=10⁻⁶ M⁻¹ cm⁻² [33]). This complex was stable for several days in air.

Ferrous oxidase activity was measured anaerobically in 50 mM Na-acetate, pH 5.1, 50 µM CuSO₄. FeSO₄ was dissolved anaerobically just before use and added to the reaction at the concentrations indicated in the figures. Iron oxidation was followed in a UV2600 spectrophotometer (Shimadzu) at 304 nm at 30 °C, and an extinction coefficient of 1.9 mM⁻¹ cm⁻¹ for ferric iron was used for calculation [34]. In all of the assays, background controls without enzymes were included.

Spectroscopy
Spectra were recorded at 20 °C in 50 mM Na-acetate buffer, pH 5.2, using a Varian Cary 4000 UV-vis spectrophotometer (Agilent Technologies).

Disc inhibition zone assays
Wild-type E. coli (JW2902), the Δ cueO deletion strain (JW0119) and the Δ cueO strain containing plasmid pDA01 expressing DA2_CueO were grown aerobically in LB media at 37 °C to the stationary phase. To generate even bacterial lawns, 100 µl of culture was mixed with 2.5 ml of 0.7 % molten agar at 40 °C and poured onto LB plates. Filter disks containing 30 µl of 10 mM CuSO₄ were deposited on the plates and after 24 h of incubation at 37 °C, the plates were photographed with a digital camera and the inhibition zones were assessed.

RESULTS
Cloning of the DA2_cueO gene of Desulfovibrio sp. A2
In an effort to unravel the unusually high copper resistance of Desulfovibrio sp. A2, we identified a monocistronic gene, DA2_cueO, predicted to code for a multicopper oxidase (MCO). Upstream of the gene, there is a ribosome binding site of consensus GGAGG, spaced seven nucleotides from an ATG start codon. The predicted translation product of the DA2_cueO gene exhibits 34 % similarity to E. coli CueO, the best characterized member of this rather diverse protein family, but also shared extensive sequence similarity with other MCOs (Fig. S1). However, native DA2_CueO has a predicted molecular weight of 70.8 kDa and is thus considerably larger than the 56.7 kDa Eco_CueO. The larger size of DA2_CueO compared to Eco_CueO is due to an additional 40 amino acids at the N-terminus and four major insertions of 11 to 32 amino acids along the protein chain. The crystal structure of many CueO-like enzymes has been determined and the amino acids coordinating the four copper centers have been identified [35]. As shown in Fig. S1, the T1, T2 and T3 copper-binding sites are conserved DA2_CueO, suggesting that this enzyme has similar catalytic activities to other CueO-like enzymes.

DA2_CueO possesses an N-terminal twin-arginine motif, suggesting a periplasmic location (Fig. S2a). This is further supported by the prediction of the cellular location of DA2_CueO with the programs TatT, SignalP, PSORT and PolyPhobius (Fig. S2B). Since Desulfovibrio sp. A2 only grows under extreme conditions, it proved to be impossible to assess the cellular location of DA2_CueO directly in vivo. A number of methods to release DA2_CueO expressed in a Δ cueO E. coli strain were tested, such as osmotic shock [36], detergent treatment [37] and the formation of protoplast by lysozyme treatment [38]. However, none of these methods released detectable DA2_CueO activity from the cells, but they also failed to release Eco_CueO from E. coli wild-type cells. Nevertheless, the phenotypic observations described below suggest that DA2_CueO has a periplasmic location in E. coli.

In vivo effects of DA2_CueO expression in E. coli
MCOs can oxidize a range of phenolic compounds. It has for example been shown that Eco_CueO oxidizes DHB, a precursor of the bacterial siderophore enterobactin [25]. To test whether DA2_CueO is functional in E. coli, cultures were grown in the presence of 50 µM CuSO₄ and 200 µM DHB. Following overnight growth, there is intense colour development from wild-type E. coli, while no colour develops with a Δ cueO mutant (Fig. 1a). The Δ cueO mutant complemented with the DA2_CueO-expressing plasmid also leads to DHB oxidation and colour development. Induction of DA2_CueO expression with 10 µM IPTG does not further enhance colour development and induction with 100 nM IPTG even leads to partial inhibition of growth.
Using a filter disc diffusion assay, the sensitivity of *E. coli* wild-type cells to copper was compared to that of an *E. coli* ΔcueO mutant and an *E. coli* ΔcueO mutant complemented with plasmid pDA01, which expresses DA2_CueO. Fig. 1(b) shows that the wild-type strain is more resistant to copper than the cueO knock-out strain, while the knock-out strain complemented with a plasmid expressing DA2_CueO exhibits the same copper sensitivity as the wild-type. DA2_CueO also exhibits phenol oxidase activity when expressed in *E. coli*. Thus, DA2_CueO appears to be functional in enhancing copper resistance in *E. coli*, as has previously been observed for Eco_CueO [24]. Induction with IPTG is not required for this phenotype to be apparent, which is similar to what has been observed for DHB oxidation. These findings show that DA2_CueO is active in *E. coli*.

**In vitro properties of DA2_CueO**

MCOs feature four copper atoms, designated type 1 (T1), type 2 (T2) and type 3 (T3), which exhibit specific spectroscopic properties. The T1 copper site produces an absorption peak at ~600 nm and confers a blue colour that is typical of MCOs. The T2 and T3 copper atoms that make up the trinuclear center are associated with an absorption peak at around 330 nm [39]. The copper binding sites T1, T2 and T3 identified in Eco_CueO are also present in DA2_CueO. However, DA2_CueO as isolated by Ni–NTA affinity chromatography did not exhibit any enzymatic activity and lacked the blue colour typical of MCOs, even though cultures for DA2_CueO expression were supplemented with 1 mM CuSO₄. Loss of copper and the need to reconstitute copper sites with exogenously added copper has previously been reported for Eco_CueO and other MCOs [40].

Supplementation of purified DA2_CueO with CuSO₄ activates the enzyme and renders it blue. Maximal enzyme activities and spectral features are obtained at 50 µM CuSO₄ in the medium, irrespective of the protein concentration. Fig. 2(a) shows the stimulation of ferrous oxidase activity by exogenously added copper, which peaks at 50 µM added CuSO₄. Fig. 2(b) displays the spectral features of copper-reconstituted DA2_CueO, with a shoulder at 330–340 nm and a peak at 600 nm, which are typical for T2/T3 and T1 copper sites, respectively [33]. ε₆₀₀ for *E. coli* CueO is 5 mM⁻¹ cm⁻¹. DA2_CueO exhibits a calculated ε₆₀₀ of 2 mM⁻¹ cm⁻¹. This could indicate that the copper centers of DA2_CueO have a different architecture from those of Eco_CueO, or, more likely, that the copper sites of DA2_CueO are not fully reconstituted by the addition of copper.

Copper-reconstituted DA2_CueO oxidizes ABTS, a substrate commonly employed to determine MCO activity (Fig. 3). DA2_CueO catalyzes ABTS oxidation in 50 mM Na-MOPS, pH 7, 50 µM CuSO₄, with an apparent kₗ for 200 min⁻¹ and a Kₘ of 22±2 µM (Table 1). No significant ABTS oxidase activity was observed in Na-acetate buffer of pH 5.1. The affinity of DA2_CueO for ABTS was about 10-fold lower and the kₗ about 100-fold lower than the respective values for Eco_CueO reported by Kim et al. [41]. However, considerably lower kₗ values have also been reported for Eco_CueO [24, 40]. DHB, another substrate frequently used to test MCOs activity, was oxidized by DA2_CueO under the same conditions with a kₗ of 2.3±0.4 min⁻¹, compared to the value of 200 min⁻¹ reported for Eco_CueO [41]. No detailed kinetic analysis to determine substrate affinity was performed for DHB.

The cuprous and ferrous oxidase activities of DA2_CueO were also analysed, since Cu(I) and Fe(II) may be the natural substrates of the enzyme. Cu(I) oxidase activity was measured with a preformed [Cu(I)(BCA)₃]₂⁻ complex as the substrate in 50 mM Na-MOPS, pH 7, 50 µM CuSO₄ [33]. This reaction exhibited a biphasic behaviour, with a maximal kₗ of 7.7±1.2 min⁻¹ exhibited at 5 µM [Cu(I)(BCA)₃]⁻ (Fig. 4). At higher substrate concentrations, the enzyme was inhibited, conceivably by the unnatural [Cu(I)(BCA)₃]⁻ substrate. No activity was observed with this substrate in Na-acetate buffer of pH 5.1.

Fe²⁺ oxidation was measured in Na-acetate buffer of pH 5.1, 50 µM CuSO₄; spontaneous iron oxidation was too rapid at pH 7. The ferrous oxidase activity of DA2_CueO exhibited
the following kinetic parameters: $K_m=112\pm22\ \text{µM}$, $k_{cat}=182\pm13\ \text{min}^{-1}$ (Fig. 5 and Table 1). These values are very similar to those reported for Eco_CueO, namely $K_m=129\pm15\ \text{µM}$, $k_{cat}=215\pm9\ \text{min}^{-1}$ [42].

In summary, we have described a novel MCO, DA2_CueO from the recently sequenced Gram-negative sulfate-reducing bacterium Desulfovibrio sp. A2 [18]. This organism is difficult to grow and is not readily amenable to biochemical experimentation. However, DA2_CueO appears to be functional in E. coli: it can contribute to copper resistance and can catalyze DHB oxidation. Purified DA2_CueO exhibits typical MCO activities, namely DHB, ABTS, cuprous and ferrous oxidase. The relatively large molecular weight of DA2_CueO raises interesting questions regarding possible additional activities of this enzyme.

DISCUSSION

Most CueO-like enzymes have a periplasmic location and contain a twin-arginine motif, which is typical for proteins exported by the Tat pathway for the secretion of folded proteins into the periplasmic space [43]. Notable exceptions are CotA of Bacillus subtilis, which is a spore-coat protein with a presumed function in melanin synthesis for UV protection [44], and BmcO of Brucella melitensis, which does function as a multicopper oxidase, but is devoid of the N-terminal twin-arginine motif [45]. Eco_CueO was shown to be transported across the cytoplasmic membrane by the Tat pathway as an apo-protein. Notably, cofactor assembly was not required for Tat transport [46]. Periplasmic CueO was only activated by the addition of copper ions, either in vitro by the addition of CuSO$_4$ or in vivo under copper stress conditions. This is in line with our finding that periplasmic DA2_CueO requires activation by copper.

MCOs are encoded in the genomes of Eukarya, Bacteria and Archaea. These enzymes are unique in that they contain at least four Cu prosthetic groups organized into three copper sites. These have been classified as type 1 (T1), type 2 (T2) and binuclear type 3 (T3), based on their spectral properties [47]. Extensive studies of Eco_CueO have revealed that the T1 site is involved in substrate oxidation, while the T2 and T3 sites make up the trinuclear copper cluster that catalyzes the four-electron reduction of dioxygen to two H$_2$O [48]. Eco_CueO contains a 45-amino acid ‘methionine-rich’ domain (residues 355–402, 14 Met; 5 His; underlined in Fig. S1) that is absent in DA2_CueO. This domain contains an extra copper binding site (Cu5) that blocks solvent access to the T1 site. Occupancy of the Cu5 site by copper is required for full oxidase activity of Eco_CueO [35]. Two additional high-occupancy copper-binding sites (Cu6 and Cu7), located near the Cu5 center, have also recently been discovered and have been shown to be implicated in oxidase activity [49]. Cu5, Cu6 and Cu7 have been shown to play related but functionally distinct roles in Eco_CueO oxidase activity. This raises the question of how the function of DA2_CueO is affected by the absence of a methionine-rich domain.

Fig. 2. Copper stimulation and absorption spectrum of purified DA2_CueO. (a) DA2_CueO was preincubated for 1 h at 30 °C at varying CuSO$_4$ concentrations in 50 mM Na-MOPS, pH 7, followed by measurement of the ferrous oxidase activity, as described in the Methods section. The error bars indicate the standard deviations of three independent experiments. (b) The spectrum of 5 µM DA2_CueO, purified by size-exclusion chromatography, was recorded in 50 mM Na-acetate, pH 5.2, at 20 °C in the presence of 50 µM CuSO$_4$.

Fig. 3. ABTS oxidase activity. Lineweaver-Burk plot of enzyme activity measured spectrophotometrically in 50 mM Na-MOPS, pH 7, 50 µM CuSO$_4$, as described in the Methods section; the kinetic parameters were determined by linear regression ($R^2=0.98$). The resultant values are given in Table 1. The error bars indicate the standard deviations of three independent experiments.
In Eco_CueO, Cu5 serves as an electron-transfer mediator between the solvent-accessible Cu6 and Cu7 sites and the buried T1 site. Cu6 and Cu7 are the dominant substrate docking-oxidation sites on the Eco_CueO surface [35, 48, 49]. However, the methionine-rich sequence present in Eco_CueO is absent in many CueO-like proteins, including DA2_CueO. This may explain why DA2_CueO displays approximately 100-fold lower $k_{\text{cat,ABTS}}$ values for ABTS, DHB and Cu(I) oxidation (see Table 1). Further, in Eco_CueO, high phenol and metal oxidase activity require all Cu(II) atoms to be present in all the sites to serve as electron-transfer mediators between the substrates and the buried T1 copper center. Due to the lability of the Cu5, Cu6 and Cu7 centers, and the limited copper availability under standard growth conditions, phenol oxidase activity is unlikely to be the predominant activity of Eco_CueO. However, if excess copper is present in the ambient environment, the labile Cu5, Cu6, and Cu7 sites may be occupied and Eco_CueO could serve in copper detoxification via phenol oxidation and copper binding to the oxidation products [24].

In spite of the low rate of phenol and Cu(I) oxidation by DA2_CueO in vitro, the enzyme is able to participate in phenol oxidation and copper detoxification when expressed in *E. coli* (cf. Fig. 1). This might be due to copper-activation of the enzyme by an alternative mechanism. The alignment of CueO-like proteins reveals a high degree of conservation of the copper-binding sites T1, T2 and T3 that constitute the reaction center for the four-electron reduction of oxygen, but generally a low degree of conservation of the Cu5, Cu6 and Cu7 sites. Since the T1 site is most likely also buried in DA2_CueO, and the methionine-rich sequence of Eco_CueO is absent, DA2_CueO is expected to contain an alternative substrate-binding oxidation site to mediate electron transfer to the T1 site. DA2_CueO contains several sequence insertions that are not present in most other CueO-like enzymes, making up for its larger size. One of these insertions (residues 149–180) contains four histidines that may well constitute additional copper-binding sites with functions analogous to those of the Cu6 and Cu7 sites of Eco_CueO. Due to significant sequence diversity between DA2_CueO and the CueO-like enzymes of known structure, it was not possible to reliably model the DA2_CueO structure and compare it to known structures. Elucidation of the three-dimensional structure of DA2_CueO is thus of primary importance to gain mechanistic insights into this enzyme.

In contrast to the low rate of Cu(I) oxidation by DA2_CueO in vitro, Fe(II) oxidation exhibits a $k_{\text{cat}}$ value similar to that of Eco_CueO. This could suggest that the in vivo function of DA2_CueO does not primarily lie in copper detoxification, but rather in iron oxidation for iron acquisition. For

### Table 1. Enzymatic activity of DA2_CueO and Eco_CueO

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_{\text{m}}$(µM)</th>
<th>$k_{\text{cat}}$(min$^{-1}$)</th>
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<tr>
<td></td>
<td>DA2_CueO</td>
<td>Eco_CueO</td>
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<tr>
<td>ABTS</td>
<td>22±2</td>
<td>2.5 x 10$^3$</td>
</tr>
<tr>
<td>DHB</td>
<td>ND</td>
<td>70$^*$</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>112±22</td>
<td>129±15$^+$</td>
</tr>
<tr>
<td>Cu$^+$</td>
<td>ND</td>
<td>169±24$^+$</td>
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ND, not determined.
$^*$Values from [41]; lower copper-dependent $k_{\text{cat,ABTS}}$ values were reported in [40].
$^+$Values from [42]; similar values were reported in [49].

Fig. 4. Cuprous oxidase activity. Cuprous oxidase activity by DA2_CueO was determined spectrophotometrically in 50 mM NaMOPS, pH 7, 50 µM CuSO$_4$, using the [Cu(II)BCA]$^{3-}$ substrate as described in the Methods section. The error bars indicate the standard deviations of three independent experiments.

Fig. 5. Ferrous oxidase activity. Hanes–Woolf plot of ferrous oxidase activity by DA2_CueO measured spectrophotometrically in 50 mM NaMOPS, pH 7, 50 µM CuSO$_4$, as described in the Methods section; the kinetic parameters were determined by linear regression ($R^2=0.99$). The resultant values are given in Table 1. The error bars indicate the standard deviations of three independent experiments.
Pseudomonas aeruginosa, it has been shown that a CueO-like enzyme is required for Fe(II) oxidation to allow citrate-mediated iron uptake via the citrate-inducible FecA receptor and the FeoB ferrous iron transporter [50]. A similar mechanism could apply to Desulfovibrio A2, but the complex growth conditions for this organism preclude in vivo functional analysis in this regard.

Although sulfate-reducing bacteria are traditionally considered to be strict anaerobes, many oxygen-requiring enzymes have been described in these organisms over the years (see [51] and references therein). Desulfovibrio vulgaris was even shown to possess an active ccaaa3-type cytochrome c oxidase. Subunits I, II and III of this enzyme are conserved in Desulfovibrio sp. A2 with 83, 67 and 66 % sequence identity, respectively. As in other sulfate-reducing bacteria, oxygen seems to play a role in the metabolism of Desulfovibrio sp. A2, which is further supported by the presence of the DA2_CueO multicopper oxidase described here. Proteins similar to DA2_CueO are encoded by the genomes of many other Desulfovibrio species. Since oxygen-requiring enzymes appear to be widespread in 'strict anaerobes', the role of oxygen in these organisms requires reappraisal.

Overall, we have presented a novel CueO-like multicopper oxidase that exhibits functional similarities to the well-characterized E. coli CueO, but exhibits extensive sequence divergence. The enzyme thus represents a new member of the CueO family. The significantly larger size of this enzyme compared to Eco_CueO suggests that DA2_CueO might harbour additional functions that remain to be discovered.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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