

Characterisation of MtoD from *Sideroxydans lithotrophicus*: a cytochrome c electron shuttle used in lithoautotrophic growth.

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Abstract

The autotrophic *Sideroxydans lithotrophicus* ES-1 can grow by coupling the oxidation of ferrous iron to the reduction of oxygen. Soluble ferrous iron is oxidised at the surface of the cell by an MtoAB porin-cytochrome complex that functions as an electron conduit through the outer membrane. Electrons are then transported to the cytoplasmic membrane where they are used to generate proton motive force (for ATP synthesis) and NADH for autotrophic processes such as carbon fixation.

As part of the *mtoAB* gene cluster, *S. lithotrophicus* also contains the gene *mtoD* that is proposed to encode a cytochrome c protein. We isolated *mtoD* from a *Shewanella oneidensis* expression system where the *mtoD* gene was expressed on a pBAD plasmid vector. Biochemical, biophysical and crystallographic characterisation of the purified MtoD revealed it as an 11 kDa monomeric protein containing a single heme. Sequence and structural alignment indicated that MtoD belonged to the class-1 cytochrome c family and had a similar fold to ferricytochrome c552 family, however the MtoD heme is bis-histidine coordinated and is substantially more exposed than the hemes of other family members. The reduction potential of the MtoD heme at pH 7 was +155 mV vs. Standard Hydrogen Electrode, which is approximately 100 mV lower than that of mitochondrial cytochromes *c*. Consideration of the properties of MtoD in the context of the potential respiratory partners identified from the genome suggests that MtoD could associate to multiple electron transfer partners as the primary periplasmic electron shuttle.

1. Introduction

The potential for bacteria to utilise iron as an energy source has been widely recognised in recent years (Bird et al., 2011; Konhauser et al., 2011). Several families of bacteria live at the microoxic/ferrous iron interface where they can survive by coupling the oxidation of ferrous iron to the reduction of oxygen (Hedrich et al., 2011). These bacteria are typically autotrophic and generate sufficient energy from this reaction to fix carbon dioxide and dinitrogen. Bacteria that are capable of this chemolithoautotrophic growth include acidophilic bacteria involved in acid mine drainage such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Rawlings et al., 1999; Ferguson and Ingledew, 2008; Mishra and Rhee, 2014); the marine stalk-forming *Gallionella ferruginea* and the freshwater *Gallionella capsiferriformans* ES-2 and *Sideroxydans lithotrophicus* ES-1 (Hallbeck et al., 1993; Emerson and Moyer, 1997). The genomes of these organisms have been sequenced revealing a range of putative metabolic pathways (Emerson et al., 2013) and analysis of these pathways poses a range of interesting questions: how do the bacteria extract the electrons from the ferrous iron, and how those electrons are ultimately coupled to the generation of NADH and a proton motive force (PMF).

The best studied of the iron oxidising bacteria is the acidophilic *A. ferrooxidans*, where an outer membrane monoheme Cyc2 collects electrons from the oxidation of Fe(II) to Fe(III) and transfers them to a periplasmic rusticyanin which then transfers the electrons to two potential shuttles, the diheme cytochromes Cyc1 and CycA1. These diheme cytochromes then transfer the electrons down divergent routes, either to a cytochrome bc₁ complex where electrons enter the quinol pool to ultimately generate NADH, or an aa₃ oxidase where oxygen is reduced to water together with the transport of protons across the membrane (Bonney and Holmes, 2012; Roger et al., 2012).

The neutrophilic *S. lithotrophicus* ES-1 does not contain any genes with significant homology for *cyc2*, but instead contains the genes *mtoA* (*Slit_2497*) and *mtoB* (*Slit_2496*) (Liu et al., 2012). These are homologues of *S. oneidensis* *mtrA* and *mtrB*, which encode a decaheme cytochrome MtrA and transmembrane porin MtrB. These two proteins form a porin-cytochrome complex in the outer membrane of *S. oneidensis* that allows for efficient electron transport through the outer membrane via a chain of hemes (Hartshorne et al., 2009; White et al., 2013). It has been proposed that MtoA and MtoB fulfil a similar function in *S. lithotrophicus* ES-1, in forming an electron conduit that allows electrons to be collected from the oxidation of ferrous iron at the surface of the cell and transported through to the periplasm (Liu et al., 2012).

Both *mtoA* and *mtoB* are located in a gene cluster in the *S. lithotrophicus* ES-1 genome that also contains two other *c*-type cytochromes; *Slit_2498* that encodes MtoD, a small mono-heme cytochrome and *Slit_2495*, which encodes CymA_{ES-1}, a tetraheme quinol oxidoreductase. As part of the same cluster it is possible that all four of these genes are co-transcribed and expressed as part of an operon. This would provide a porin-cytochrome complex (MtoAB), a soluble periplasmic cytochrome (MtoD) and a quinol oxidoreductase in the cytoplasmic membrane (CymA_{ES-1}) (Emerson et al., 2013).

S. lithotrophicus ES-1 also contains the genes necessary to express two distinct oxygenases, a cytochrome bb₃ oxidase, (cbb₃) and a cytochrome bd₁ oxidase (cbd₁). These have been characterised in other bacteria and shown to have low K_M values for

oxygen: typically cbd_1 operates in the micromolar range while cbb_3 operates in the sub-micromolar range. cbb_3 couples O_2 reduction to proton pumping and receives electrons from a cytochrome c , while cbd_1 has no proton pumping mechanism and receives electrons directly from the quinol pool (Pitcher and Watmough, 2004).

The bioenergetic and biochemical mechanism of precisely how iron oxidising bacteria are able to couple Fe oxidation to O_2 reduction is still unclear. How do electrons from the MtoAB complex enter the cytochrome oxidase? It was previously suggested that CymA_{ES-1} was the redox partner, however, this would result in the loss of protonmotive force. In order to better understand the possible roles of the periplasmic redox partners in *S. lithotrophicus* ES-1 the MtoD cytochrome was expressed in a recombinant form, purified and characterised using a range of biochemical, spectroscopic and crystallographic techniques.

2. Materials and Methods

2.1. Expression and isolation of Strep-II tagged MtoD

The 354 bp sequence encoding *mtoD* was synthesised and cloned into a puc57 vector by GENScript. *mtoD* was amplified from pUC57 using the following primers: *mtoD_1_F*: 5'-ATG ACT CGT CAA GCT TAT TCC TCA ATG TTG and *mtoD_1_R*: 5'-GAG CGA AAG GAT CCA GTC CAC CAG. A second pair of primers were used to make modifications to *mtoD* including the addition of a 5' CACC overhang, making the *mtoD* insert compatible with the desired pBAD202 D-TOPO cloning kit, followed by a ribosome binding sequence and a 3' 6xCAC repeat coding for a C-terminal polyhistidine tag. *mtoD_2_F*: 5'-CAC CTA AGA AGG AGA TAT ACA TCC CAT GAC TCG TCA AGC TTA TTC. *mtoD_R_6xHis*: 5'-CTA GTG GTG GTG GTG GTG GTG GAG CGA AAG GAT C. A pBAD directional TOPO[®] expression kit was used clone *mtoD*-His into a pBAD202 expression vector. One Shot TOP10 *E. coli* cells were transformed with pBAD202_*mtoD*-His using methods described in the pBAD D-TOPO user guide. pBAD202_*mtoD*-His was conjugated from TOP10 to *Shewanella oneidensis* MR-1 using *E. coli* helper strain DH5 α pRK2013. Kanamycin and carbenicillin were used to select for successfully conjugated MR-1. DNA sequencing performed by Eurofins MWG operon using primers *mtoD_2_F* and *mtoD_R_6xHis* confirmed successful conjugation of pBAD202_*mtoD*-His into *S. oneidensis* MR-1. For the production of Strep II-tagged MtoD pBAD202_*mtoD*-His was isolated from *S. oneidensis* MR-1 using a miniprep kit and the whole plasmid was amplified using the primers *mtoD_SII_F*: 5'-AAT TCG AGA AGT AGA AGG GCG AGC TCA AGC TTG AAG GTA and *mtoD_SII_R*: 5'-GTG GAT GAG ACC AGA GCG AAA GGA TCC AGT CCA CAG G. The pBAD202_*mtoD*-His template was removed by DpnI digestion followed by PCR clean up. T4 polynucleotide kinase was used to phosphorylate the linear product and a blunt end ligation was performed using DNA ligase to circularise the pBAD202_*mtoD*-SII product. One Shot TOP10 *E. coli* were transformed with pBAD202_*mtoD*-SII and conjugation was used to produce recombinant *S. oneidensis* MR-1 as before.

Recombinant *S. oneidensis* MR-1_*mtoD*-SII was cultured aerobically at 30°C in 20 L batches in LB media. Expression of tagged protein was induced at mid-exponential phase (OD₆₀₀: 0.5) by addition of L-arabinose to a working concentration of 2 mM. Cultures were incubated for a further 5 hours and harvested by centrifugation at 6,000

g for 15 minutes. Recombinant MR-1 cell pellets were re-suspended in 20 mM HEPES pH 7 buffer and 3 passes of French Press treatment at 1,000 psi (6.89 MPa) were used to lyse the cells. The soluble cell fraction was isolated by ultracentrifugation of the lysate at 205,000 g for 2 hours. The supernatant was retained for purification of tagged MtoD. MtoD was isolated using 5 mL Strep-Tactin affinity column (GE healthcare). The column was run with a 20 mM HEPES pH 7, 150 mM NaCl equilibration/wash buffer. Four cycles of loading and eluting were performed to isolate all of the expressed MtoD. SDS PAGE was used to analyse eluted fractions; MtoD fractions were dialysed with 20 mM HEPES pH 7, 150 mM NaCl overnight then concentrated in preparation for size exclusion chromatography. Gel filtration was performed using a Superdex S75 16/60 column. The column was equilibrated with 20 mM HEPES pH 7, 150 mM NaCl before loading MtoD and running at a flow rate of 0.5 mL.min⁻¹. Pure fractions of MtoD, determined by SDS PAGE analysis, were pooled, concentrated and dialysed with 20 mM HEPES pH 7, 100 mM NaCl overnight at 4°C.

2.2. Pyridine Hemochrome analysis of MtoD.

Oxidised spectra of horse heart cytochrome c and MtoD were prepared in 20 mM HEPES pH 7 buffer containing 2 mM CaCl₂ for cytochrome c, and 100 mM NaCl for MtoD. Each sample was fully oxidised with 10 µM K₃Fe(CN)₆ before measurement of UV/vis electronic absorption spectrum. The pyridine hemochrome method was used to quantify the concentration of heme in the purified MtoD sample and to determine the extinction coefficient of the Soret maxima of MtoD (Berry and Trumpower, 1987). Briefly cytochrome c and MtoD were mixed with pyridine and NaOH to give final concentrations of 3 mM and 75 mM respectively. These samples were divided into two Suba-sealed quartz cuvettes. Samples were oxidized using 10 µM K₃Fe(CN)₆ and reduced with 3 mM Na₂S₂O₄ to give the oxidized and reduced spectra of heme-pyridine. A Bio-Rad protein assay kit was used to determine the total protein concentration.

2.3. Sedimentation velocity.

410 µL of MtoD samples of 3.5 and 8.5 µM were measured into sample chambers of a two chamber cell assembly and 20 mM HEPES pH 7, 100 mM NaCl was measured into the reference chambers. A Beckman Optima XL-A analytical ultracentrifuge with an An50Ti rotor was used to optically measure sedimentation, monitoring absorbance at 406 nm with a rotational speed of 42,000 rpm. 300 radial scans were performed over a 20-hour period at a constant temperature of 20°C. Analyses of the sedimentation velocity data were performed using SEDFIT (Brown and Schuck, 2006). The C(s) distribution model was applied to the data and non-linear fitting was performed. Buffer density and viscosity parameters (1.0039 g.mL⁻¹ and 1.0264x10⁻² ρ respectively) in addition to predicted \bar{v} for MtoD (0.7282 mL.g⁻¹), were included in the C(s) model to solve Lamm equations.

2.4. MtoD crystallisation and data collection.

A pure solution of MtoD was concentrated to 30 mg.mL⁻¹ and sparse matrix screening, using the sitting drop method, was performed to explore potential crystallisation conditions. Crystals formed at 16 °C in 0.6 µl drops at ratios of 1:1 or 2:1 mother liquor/protein. The mother liquor was 0.1 M KCN, 30% PEG 2000 MME. Crystals were cryo-protected by transferring to a solution of mother liquor containing 12 % glycerol before being vitrified by plunging into liquid nitrogen. Data were collected on MtoD crystals in a gaseous stream of nitrogen at 100 K on beamlines I03 at the

Diamond Light Source (UK). MtoD crystals were of space group P22121 with typical cell dimensions of $a=29.70$ $b=40.20$ $c=92.180$ Å. A SAD dataset was collected at a wavelength of 1.72 Å to a final resolution of 2.5 Å. Further datasets from single crystals were collected using an x-ray wavelength of 0.97 Å.

2.5. MtoD structure determination and refinement.

MtoD datasets were processed using XIA2 (Winter, 2010). The SAD dataset of MtoD was analysed using the autosol pipeline within the PHENIX software suite (Adams et al., 2010). The program HySS located 1 heavy atom site and the electron density maps calculated with PHASER/RESOLVE were sufficiently interpretable to manually place a single heme corresponding to a single MtoD molecule in the asymmetric unit. The model building program Phenix AutoBuild was used to build residues followed by alternating rounds of manual building and refinement using PHENIX (Adams et al., 2010) or REFMAC (Murshudov et al., 2011). The final model was refined to an Rcryst (Rfree) value of 19.4 (24.5) %. This model has no residues in the disallowed region of the Ramachandran plot. Coordinates have been deposited in the RCSB Protein Data bank under accession code 4XXL.

2.6. Electrochemistry.

Mediated redox spectropotentiometry of a solution of MtoD was performed using methods described previously (Bamford et al., 2002). Experiments were performed under continuous argon gas flow with a solution of 5.5 μ M MtoD in 20 mM HEPES pH 7, 100 mM NaCl containing 10 μ M each of the following mediators: 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD), phenazine methosulphate (PMS), phenazine ethosulphate (PES), 5-hydroxy-1,4-naphthoquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone, 2-methyl-1,4-naphthoquinone, 9,10-anthraquinone-2,6-disulphonic acid, anthraquinone-2-sulphonic acid and 1,1'-dibenzyl-4,4'-bipyridinium dichloride. The electrochemical potential was raised or lowered through the addition of aliquots of anaerobic solutions containing potassium ferricyanide or sodium dithionite respectively.

3. Results

3.1. Expression, purification and characterisation of *S. lithotrophicus* MtoD

MtoD was purified to homogeneity from *Shewanella oneidensis* as described in *Methods*. SDS-PAGE analysis revealed that MtoD ran as a single band with apparent molecular weight of 13 kDa, slightly larger than the predicted molecular weight of 11 kDa due to the c-terminal strep-II tag (Fig. 1A). Edman degradation (PNAC facility, Cambridge UK) revealed the N-terminal sequence began AVDVD, matching the cleavage site predicted by SignalP (Petersen et al., 2011) and pyridine hemochrome assays revealed that the sample of MtoD contained approximately stoichiometric ratio of 0.85 heme: protein, giving an ϵ_{410} coefficient of 105.2 $\text{mM}^{-1} \text{cm}^{-1}$.

Solutions of MtoD display electronic absorbance spectra consistent with the presence of a low-spin c-type heme (Fig. 1B). The characteristic Soret peak in the oxidized protein has a maximum at 406 nm, giving a 280/406 nm absorbance ratio of 0.19. The 406 nm maximum shifts to 416 nm on reduction of MtoD and α and β peaks appear with maxima at 520 and 549 nm respectively. Spectral features above 600 nm would be indicative of high-spin ferric heme or low-spin ferric heme with His/Met axial heme ligation as is typically observed in other class-1 cytochrome-c family members (Bertini

et al., 2006). However, MtoD displayed no detectable absorbance above 600 nm in agreement with the heme ligand set resolved by X-ray diffraction as described below. Mediated potentiometric titration of MtoD monitored by electronic absorbance spectroscopy defined the heme redox properties (Fig. 2A). Changes in the absorbance at 549 nm due to the ferric/ferrous heme interconversion were fully reversible with change of solution potential (Fig. 2B). The behavior was in excellent agreement with that predicted by the Nernst equation for a single redox center undergoing a one-electron redox transformation with a mid-point potential of 155 ± 10 mV vs SHE.

The biophysical properties of MtoD in solution were examined using sedimentation velocity. MtoD samples at $3.5 \mu\text{M}$ and $8.5 \mu\text{M}$ were centrifuged as described in methods and the migration profile of MtoD at 406 nm was measured over 5 hours (Fig. 3A). The data was fitted using the software program SEDFIT which revealed a single species with a sedimentation coefficient of 1.55 S and a molecular mass of 11.7 kDa (Fig. 3B). Further analysis of the sedimentation data gave a f/f_0 coefficient of 1.24, indicating that MtoD behaved like a monomeric globular protein in solution.

The crystal structure of MtoD was solved to an initial resolution of 2.29 Å by single wavelength anomalous dispersion (SAD) using the anomalous signal caused by the single iron atom contained within the heme group. The initial model was used as a template for molecular replacement to obtain a final resolution of 1.47 Å. The overall statistics obtained for data collection and structure refinement are given in Supplementary Table 1. The crystal structure contained residues 28-119 of the processed amino acid sequence and a single c-type heme covalently attached to Cys₄₃ and Cys₄₆ via thioether bonds (Fig. 4A), consistent with the histidine ligands predicted from the sequence alignment. The iron atom of the heme group was coordinated by the imidazoles of the porin cofactor and His₄₇ and His₆₈ of the active site. The imidazole side chains of the two histidines coordinating the heme iron are arranged near-parallel relative to one another, at an angle of approximately 35° (Fig. 4B).

The sequence alignment between MtoD and the other structurally resolved cytochromes revealed that cytochrome c552 from *Nitrosomas europaea* had the highest amino acid sequence homology (Supplemental Fig. 1). When the structure of MtoD was compared with the other available structures of bacterial monoheme c-type cytochromes, the closest structural homologue was cytochrome c552 from *Hydrogenobacter thermophiles* (PDB id. 1YNR). Superposition of the main chain of this cytochrome with MtoD gave an average root-mean-square-displacement of 2.59 Å (Fig. 4B, Table 1). Despite the high level of secondary structure conservation there are notable structural differences between MtoD and the other known c-type cytochromes. One obvious difference is that the axial coordination of the MtoD heme is bis-Histidine, while other crystallised cytochromes have Histidine/Methionine coordination (Table 1). The class 1 cytochrome family typically contain a flexible loop that covers the front of the heme, however in MtoD this region is restrained by a hydrogen bonding network that causes the formation of a β -loop and prevents the peptide sidechains from interacting with the heme propionate groups (Fig. 4C). This causes an increase in the overall surface exposure of the MtoD heme, giving an overall exposed heme of 152 Å² compared to 30-61 Å² for other class 1 cytochromes. Typically the hemes of these cytochrome are exposed on one side, next to the thiolated cysteine residues, with the propionate edge being completely covered. To date the heme group of MtoD is significantly more exposed to solvent than the heme of any

other structurally resolved monoheme cytochrome, suggesting that the properties of the heme are likely to be extremely susceptible to changes within the local environment.

Table 1: Structural comparison of MtoD with similar monoheme c-type cytochromes. Six cytochromes were identified through sequence similarity to MtoD using BLAST. Structures were downloaded from the RCSB protein data bank. The root mean square displacement (RMSD) was measured using SUPERPOSE (Krissinel and Henrick, 2004). Accessible heme area was measured using a 1.4 Å probe using AREAIMOL (Shrake and Rupley, 1973))

Name	Class	PDB id	Axial ligands	Accessible Heme area (Å ²)	RMSD (Å)
<i>S. lithotrophicus</i> MtoD	-	4XXL	His/His	152.5	-
<i>N. europea</i> c552	c552	1A56	His/Met	54.2	3.23
<i>P. aeruginosa</i> c551	c551	451C	His/Met	52.6	3.25
<i>H. thermolotulus</i> c552	c552	2D0S	His/Met	61.3	2.87
<i>H. thermophilus</i> c552	c552	1YNR	His/Met	57.4	2.59
<i>P. stutzeri</i> c551	c552	1COR	His/Met	55.2	3.08
<i>A. aeolicus</i> c555	c555	2ZZY	His/Met	37.5	3.54

4 Discussion.

Autotrophic iron oxidising bacteria face a significant bioenergetic challenge in generating both the reducing equivalents (NADH) and chemical energy (ATP) required for carbon fixation and other anabolic reactions essential for cell survival. The energy source for both NADH and ATP production is generated from the liberation of electrons obtained from the oxidation of iron at the cell surface. At pH 7.0 the redox potential of this iron couple $\text{Fe}(\text{OH})_3/\text{Fe}^{2+}$ is -236 mV vs. SHE, which is low enough to allow electron transfer across the outer membrane through the MtoAB complex (Widdel et al., 1993; Liu et al., 2012). The electrons obtained from iron oxidation are required both for PMF-coupled oxygen reduction, and the reduction of NADH at the cytoplasmic membrane. At an initial potential around -236 mV the energy associated with these electrons would be sufficient to catalyse the reduction of oxygen (+816 mV), but they would not be able to reduce NAD^+ to NADH (-320 mV). As a consequence electrons destined for NADH formation will have to be pumped ‘uphill’ through different carriers before reducing NADH. The energy source for this uphill electron transfer is most likely to be obtained from the proton gradient. In *A. ferrooxidans* two periplasmic diheme cytochromes Cyc1 and CycA1 take electrons to either the cytochrome oxidase or the cytochrome bc_1 complex, which then runs in reverse to reduce ubiquinone. These cytochromes are located in two separate operons; the *rus* operon contains *cyc1* as well as *cyc2*, *rusA* and the genes for a cytochrome c oxidase, while the *petl* operon contains *cycA1* and the genes for a cytochrome bc_1 complex (Roger et al., 2012). In contrast the *mto* gene cluster of *S. lithotrophicus* contains the gene for only one monoheme cytochrome: *mtoD*, while the gene clusters that contain the genes of cytochrome bc_1 and cytochrome bb_3 do not contain any soluble monoheme cytochromes.

The MtoD cytochrome has a number of unusual features. Comparative analysis of the MtoD amino acid sequence with the sequences of other structurally defined class-1 cytochromes revealed that MtoD is unusual in having bis-His coordination instead of His/Met, and that the sequence around the distal ligand is not proline rich as observed for other cytochromes (Table 1 and Supplemental Fig. 1). The measured redox potential of +155 mV for the MtoD heme is high for a bis-His coordinated heme and the heme group is significantly more solvent exposed than in monoheme cytochromes. It is possible that these unusual features of the MtoD heme group allow it to function as an electron donor to cytochrome bb_3 , cytochrome bc_1 and/or CymA_{ES-1} (Fig. 5).

Previous studies on the potentials of the heme groups of MtoA indicate that they span a mid-point potential range between +30 to -350 mV (Liu et al., 2012), which is lower than the potential of MtoD and indicates that electron transfer from MtoA to MtoD would be thermodynamically favourable. This supports the hypothesis that MtoD could be the electron transfer shuttle between the outer membrane MtoAB complex and the cytochrome bb_3 oxidase on the cytoplasmic membrane, allowing the efficient generation of a PMF.

A membrane bound NADH dehydrogenase is proposed to reduce NAD^+ to NADH using electrons provided by the quinol pool and energy generated by the PMF (Emerson et al., 2013). Either the cytochrome bc_1 complex or CymA_{ES-1} could generate quinol, using electrons supplied by MtoD (Fig. 5). At +155 mV, the potential of MtoD is only slightly higher than the midpoint potential of ubiquinol (+100 mV vs. SHE) and consequently electron transfer from MtoD to cytochrome bc_1 could occur in order to drive NADH production. The generation of reduced ubiquinol from cytochrome bc_1 has been observed in other iron oxidising bacteria, notably *A. ferroxydans* (Elbehti et al., 2000). In this system, the two half reactions of the cytochrome bc_1 complex run in reverse, causing the net transport of 2 protons across the cytoplasmic membrane for every ubiquinone reduced.

The role of CymA_{ES-1} is less clear. The homologous CymA cytochrome from *S. oneidensis* MR-1 was shown to be specific for menaquinone, with a midpoint potential of -70 mV vs. SHE (McMillan et al., 2012b;a). This suggests that the measured potential of MtoD would not allow thermodynamically favourable electron transfer to CymA_{ES-1}. However, the highly exposed heme surface of MtoD is likely to be sensitive to changes in the local environment, such as those caused by protein complex formation. This may allow lowering of the MtoD midpoint potential on association with CymA_{ES-1}, facilitating electron exchange and menaquinone reduction.

In summary, the structure and electrochemical properties of MtoD are consistent with its possible role as an electron shuttle between MtoAB on the outer membrane and a range of potential electron acceptors on the inner membrane. However, a full biochemical analysis will be required to verify the true redox partners and confirm the pathway of electron transfer through this iron oxidising bacterium.

Conflict of Interest Statement.

The Authors declare they have no conflict of interest.

Authors and contributors.

CB, ME, ML and LS carried out data acquisition and analysis. CB, ME, JB DR TC carried out data analysis and interpretation. CB, ME, ML, LS, JB, DR, TC drafted the manuscript. CB, ME, JB, DR, TC revised the manuscript. All authors approved the final manuscript.

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Figure 1: Isolation and preliminary characterisation of recombinant MtoD: (A) SDS-polyacrylamide gels of Coomassie stained MtoD. (B) UV-visible wavelength spectrum of fully oxidised (black) and fully reduced (red) MtoD. MtoD was prepared by addition of potassium ferricyanide to obtain a fully oxidised spectrum and addition of sodium dithionite to obtain a fully reduced spectrum. *Inset* the region between 500 and 700 shows the $\alpha\beta$ peaks associated with the c-type heme.

Figure 2. Redox characterisation of MtoD. (A) Electronic absorbance of MtoD equilibrated at potentials between 0.3 and -0.3 V during a mediated potentiometric titration. Spectra are coloured from red to blue corresponding to increasingly reduced protein. (B) Variation of absorbance at 549 nm (A_{549}) with solution potential (points) and best fit (line) to the Nernst equation for a one-electron transformation with a mid-point potential of +155 mV vs. SHE. Experiments performed in 20 mM HEPES pH 7, 100 mM NaCl at 21 °C with a mediator range as listed in the methods.

Figure 3: Solution based biophysical characterisation using sedimentation velocity. (A) Absorption at 406 nm was used to track the boundary migration of an 8.5 μ M sample of MtoD in 20 mM HEPES pH 7, 100 mM NaCl at 42,000 rpm. The

change in absorbance was fitted using SEDFIT with the residual fit shown below the data (B). The C(s) distribution of the fitted data indicates the presence of a single, non-interacting species with a sedimentation coefficient of 1.55 S and a corresponding molecular weight of 11.7 kDa, corrected for temperature and buffer parameters.

Figure 4: 1.8 Å Crystal structure of MtoD. (A) Top view of MtoD; (B) Side view of MtoD revealing the near-parallel histidine side chains co-ordinating the haem iron. (C) Superposition of MtoD over the secondary structure of cytochrome c552 from *Hydrogenbacter thermophiles*. (D) Close up of the loop region that exposes the MtoD heme. In cytochrome c552 the conserved tryptophan Trp54 covers much of the exposed heme.

Figure 5: Proposed dual role of MtoD in supporting both proton translocation and NADH production. Possible electron transfer pathways through MtoA and MtoD are indicated by dashed lines. UQ and UQH₂ refer to ubiquinone and ubiquinol respectively, while MK and MKH₂ refer to menaquinone and menaquinol. Q and QH₂ represent non-specific quinones and quinols. The known redox midpoint potentials for *S. lithotrophicus* ES-1 MtoD and MtoA are shown in parentheses.









