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# Mind the gap: diversity and reactivity relationships among multiheme cytochromes of the MtrA/DmsE family

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**Abstract**:

***Shewanella oneidensis* MR-1 has the ability to use many external, terminal electron acceptors during anaerobic respiration, such as dimethyl sulfoxide (DMSO). The pathway that facilitates this electron transfer includes the decaheme cytochrome DmsE, a paralog of the MtrA family of decaheme cytochromes. Although both DmsE and MtrA are decaheme cytochromes implicated in the long-range electron transfer across a ~300 Å wide periplasmic "gap", MtrA has been shown to be only 105 Å in maximal length. Here, DmsE is further characterized via protein film voltammetry, revealing that the electrochemistry of the DmsE heme cofactors display macroscopic potentials lower than those of MtrA by 100 mV. It is possible this tuning of the redox potential of DmsE is required to shuttle electrons to the outer-membrane proteins specific to DMSO reduction. Other decaheme cytochromes found in *S. oneidensis*, such as the outer-membrane proteins MtrC, MtrF and OmcA, have been shown to have similar electrochemical properties to MtrA, yet possess a different evolutionary relationship.**

**Key Words**:
Electron Transfer, Cytochrome, anaerobic respiration, heme

## Introduction: A periplasmic "gap"

*Shewanella oneidensis* MR-1 is a facultative anaerobe that is being studied intensely due to its ability to utilize a diverse set of terminal electron acceptors [1, 2], such as iron and manganese oxides, chromate, uranium, nitrate/nitrite and dimethyl sulfoxide (DMSO) [3-9]. This versatility stems from the various internal electron transfer pathways that are able to shuttle electrons from the inner membrane through the periplasm and, as needed, across the outer membrane where the terminal electron acceptor is located (Figure 1). It is believed these pathways share a common mechanism to acquire electrons: the quinol pool and the tetraheme cytochrome, CymA [10-12]. From CymA, the pathways branch as there are numerous proteins that can serve as its redox partner [13-17], including members of the MtrA family, a class of decaheme *c*-type cytochromes. MtrA in particular has been shown to receive electrons from CymA [15, 17], and subsequently pass electrons to an outer-membrane decaheme cytochrome (MtrC), when docked in a MtrCAB complex [18], where MtrB is an outer-membrane porin protein. Additionally, MtrC has been demonstrated to interact with yet another outer-membrane decaheme cytochrome, OmcA [19, 20].

Recently, measurements of the size of the periplasmic space of *Shewanella oneidensis* MR-1 using microscopy have shown that the distance needed to be traversed by trans-periplasmic electron transfer events is approximately 240 Å [21], whereas the outer-membrane is typically estimated to have a thickness of 40 to 70 Å as well [22]. While prior models suggested that MtrA might be able to span the periplasmic space [1, 23, 24], recently we have shown that small angle x-ray scattering (SAXS) of purified MtrA indicates that at most, MtrA has 2:1 aspect ratio with the maximal length dimension of 105 Å [25]. The SAXS analysis, in excellent agreement with supporting studies using analytical ultracentrifugation, shows that spanning a range of high nM to μM concentration regimes, MtrA is a monomeric protein consistent with dimensions of 25-50 x 100 Å, meaning that it is insufficiently long to span both the periplasmic and outer-membrane compartments [25]. How extra-cellular electron transfer is achieved is still an open question, even when considering just the periplasmic compartment. As the majority of studies of *Shewanella* electron transfer pathways have examined MtrA only with a single report of another MtrA ortholog in the literature (MtoA, from metal oxidizing *Sideroxydans lithotrophicus* [26]), here we review the current understanding of the biochemical and biophysical properties of the MtrA family, highlighting, DmsE, which is associated with outer-membrane DMSO reduction, in order to identify the commonalities associated with extra-cellular electron transfer chemistry.

## Extra-cellular DMSO Reduction

The current model for extra-cellular DMSO reduction in *Shewanella* posits DmsE transfers electrons to the DmsAB protein complex via the integral outer-membrane beta-barrel protein, DmsF [9]. DmsAB is proposed to be similar to the DMSO reductase heterodimer of *E. coli*, with molybdopterin and iron-sulfur cluster cofactors [9, 27]. While on the surface, MtrA and DmsE may appear to be highly similar, *in vivo* studies have examined the modularity of the different electron transfer pathways available in *Shewanella*, to probe the interchangeability of the different components [28-30]. Although MtrA and DmsE are both periplasmic decaheme cytochromes with similar amino acid sequences, they need not behave identically. During reduction of ferric citrate, DmsE is able to partially function in the place of an MtrA/MtrD knockout, suggesting some modularity, however, the reverse is not true [28]. It has been shown MtrA is not able to recover DMSO reduction activity in DmsE knockout strains, suggesting these proteins do have unique reactivity. A caveat is that although DmsE is required for robust DMSO reduction activity, in its absence the CctA protein may aid in electron transfer across the periplasm, recovering some activity [28].

**Redox characteristics of the MtrA family**

As predicted by the appearance of CXXCH motifs in the primary amino acid sequence, recombinant DmsE prepared as described previously [15] is found to have ten *c*-type heme groups. The oxidized and reduced electronic absorbance spectra (Figure S1) and the as-isolated EPR (electron paramagnetic resonance) spectrum (Figure S2) are nearly identical to MtrA [14]. The CD spectra of DmsE and MtrA are also strongly similar (Figure S3). As described below, the electrochemical analysis of DmsE, however, differs from MtrA [31].

Purified recombinant DmsE was characterized electrochemically using protein film voltammetry (PFV). DmsE was deposited on a PGE electrode and non-turnover voltammograms were collected at a span of pH values. These reversible and symmetric non-turnover voltammograms show DmsE has a wide window of potential (Figure 2). Using the "potential window" metric describing the overall width of the electrochemical response [31], DmsE electrochemistry can be compared with other members of the DMSO reduction and DMR pathways (Table S1). The window of potential for DmsE spans 270 mV at pH 6 (Figure 2A). This width is in good agreement with other decaheme cytochromes (300 mV for MtrA and 275 mV for MtrC) [18, 23, 31]. DmsE has a window of potential between -90 mV and -360 mV vs SHE, which is shifted ~100 mV lower than MtrA (Figure 2B). There is overlap between the redox potential windows of DmsE and CymA (Figure 2C), indicating that it is feasible for electron transfer to occur between these cytochromes. The voltammograms of DmsE show there is little change in potential at neutral and high pH, however, there is a ~150 mV shift to higher potential at low pH (Figure S4). This behavior is not seen with MtrA [31]. MtrA has a potential window that is dependent upon pH at both high and low pH values. The electron transfer rate for DmsE is ~122 mV sec$^{-1}$ (Figure S5) which is equivalent to MtrA [18, 31]. In comparison, the reduction potential range for MtoA, the MtrA ortholog implicated in iron oxidation roughly 100 mV more positive than MtrA, with a potential window of approximately + 50 mV to -300 mV at pH 7.1 [26]. Thus, the overall shift in macroscopic redox potentials appears to be a major difference between members of the MtrA family.

This difference in thermodynamics may be important for the next electron transfer step in the respective pathways. While it has been established through visible assays of the MtrCAB complex, and electrochemically detected protein:protein interactions that MtrC can clearly receive electrons from MtrA [15, 16, 18], DmsAB from *S. oneidensis* has not been characterized at all to date. Yet, presuming similarity with DMSO reductase from *E. coli* (DmsABC heterotrimer) [9, 32], it is likely that *S. oneidensis* DmsE is responsible for passage of electrons into an FeS-cluster binding DmsB subunit, which in turn supplies electrons to DmsA, which houses the molybdopterin active site. Notably, *Ec*DmsB contains four [4Fe-4S] clusters with midpoint potentials ranging from -330 mV to -50 mV [33], and the sequence of *So*DmsB contains 16 conserved cysteines, which are predicted to be the ligands of the iron-sulfur clusters. Thus, we hypothesize that DmsE may possess a lower range of heme reduction potentials in order to facilitate electron transfer into the low-potential clusters of DmsB, and could equally well reduce the heme cofactors of MtrC, providing the partial recovery observed for substituting DmsE into the Δ*mtrA* mutant reported by Gralnick and co-workers [28]. In such a model, MtrA itself would not be able to serve as a DmsE substitute, as its potentials are too high to support efficient electron transfer into DmsAB.

**Protein Family Relationships**

Previous analysis of the collection of sequences of multiheme cytochromes found in prokaryotes has revealed there are two distinct groups of decaheme proteins [34]. The first group contains the periplasmic proteins DmsE and MtrA (*Shewanella oneidensis*) while the second group contains the outer-membrane proteins OmcA and MtrF (*Shewanella oneidensis*). The pentaheme class of proteins related to NrfB has been proposed to be evolutionarily related to

MtrA/DmsE proteins [35], and it has been suggested that MtrA has evolved from a dimer (or gene duplication) of NrfB, though we have illustrated that the homology between NrfB and MtrA only holds for the N-terminal portion of MtrA itself (Figure S6). Protein similarity networks provides a tool for visually assessing questions of relatedness using criteria of either sequence or structural similarity, which can then be depicted with the freely available program Cytoscape [36]. The deployment of Cytoscape in this case allows us to pose the question, "How similar is DmsE to other decaheme cytochromes *c* and NrfB families?"

Using DmsE from *Shewanella oneidensis* (NP_717047.1) as the basis for queries using protein sequence-based Basic Local Alignment Search Tool (BLAST) searches [37], a list of protein sequences with an E-Value less than $1x10^{-7}$ and having more than five CXXCH motifs was generated. The resulting list contained 154 sequences (Table S2) used for generating and visualizing a protein sequence similarity network in Cytoscape, where each node represents a unique sequence and each edge represents a level of similarity that is equal to the E-Value cut-off.

Figure 3A shows the sequence-similarity relationships between the three families of multiheme cytochromes. As expected, NrfB and MtrA/DmsE cluster together and are more closely related to each other than the two decaheme protein families. In fact, the relationship of OmcA and DmsE is not much greater than functionally unrelated proteins [34]. By a cutoff E-value of $1x10^{-12}$ the OmcA family is completely separate, and NrfB is beginning to separate entirely from the MtrA/DmsE cluster. Figure 3B follows the MtrA/DmsE family, visualized by Cytoscape and labeled by heme content. Notably, the cluster contains a wider range of heme-containing protein than might be predicted, and is not limited to decaheme proteins from gamma proteobacteria (Figure S7). The decaheme cytochromes of this family, however, cluster tightly together and it takes an extremely strict cutoff value to separate DmsE (orange triangle) and MtrA (yellow hexagon). The list of proteins that cluster with each are found in Tables S3 and S4.

An observation that has been mentioned before [9] but highlighted here, is that not all species of *Shewanella* have a *dms* operon. *Shewanella loihica*, for example, has been shown to lack the ability to respire on DMSO [38] and does not have a DMSO reductase, and indeed, Cytoscape-based visualization of the similarity of MtrA family members shows that although *S. loihica* contains two decaheme proteins, both protein sequences cluster with MtrA and not with DmsE. In contrast, it has previously been shown that *Shewanella baltica* does not respire on DMSO [9], yet one subspecies (*S. baltica* OS195) does contain a gene product that clusters with DmsE. The reasoning behind some species containing the components to enable DSMO reduction while others are lacking is unknown. In this way, protein sequence similarity networks may be a useful tool for elucidating multiheme protein function: the *S. oneidensis* gene product SO_4360 (NP_719884.1) has been proposed to be a potential homolog of DmsE [9, 28]. This gene product is found in the DmsE family and clusters with the other decaheme proteinss initially, but at a cutoff value of $1x10^{-125}$, when DmsE and MtrA still cluster, it is no longer connected. Thus, we hypothesize that while SO_4360 is coded by a gene that exists within an operon bearing other *dms* genes, it does not cluster with DmsE, and therefore will likely have distinguishing biochemical/biophysical characteristics.

The deployment of protein sequence similarity networks simultaneously allows for the delineation of known functionality, as well as suggests regions of sequence space that may correlate with novel chemistries. For example, considering MtrA paralogs implicated in iron oxidation (versus extra-cellular iron reduction), our analysis identified MtrA homologs including PioA (YP_001989850.1), which participates in phototrophic iron oxidation in *Rhodopseudomonas palustris* TIE-1 [39], and MtoA (YP_00352109.1) a decaheme cytochrome from *Sideroxydans lithotrophicus*, which is presumed to oxidize extracellular iron through MtoAB and CymA (MtrAB and CymA homologs) [26]. PioA and MtoA are both found initially clustered with MtrA and DmsE, yet both diverge from the iron-reducing paralogs at more stringent E-values. MtoA, which possesses a similar molecular weight as MtrA and similar

(though more positive) reduction potentials, diverges from MtrA/DmsE around an E-value of $1 \times 10^{-80}$. PioA, which includes an N-terminal extension of unknown function, diverges from the main MtrA/DmsE cluster at a similar E-value, yet forms its own small cluster with related proteins from other alphaproteobacteria at an E-value of $1 \times 10^{-125}$. Thus, we hypothesize that MtrA paralogs that participate in iron oxidation will similarly diverge, and that bioinformatics methods will be useful tools in the future identification of novel functionalities for MtrA-family members involved in extra-cellular electron transfer.

## Conclusions

While it is clear that periplasmic, decaheme electron transfer proteins such as MtrA and DmsE are vital components to the dissimilatory metal reduction and DMSO reduction pathways in *Shewanella oneidensis*, a truly molecular view of extra-cellular electron transfer remains to be elusive. While MtrA has been an object of study for over a decade, only recent efforts have demonstrated that MtrA itself cannot span the periplasmic space as a static molecular "wire", though certainly the disposition of its ten heme moieties is critical to successful long range electron transfer. As demonstrated by the measurements made by ourselves and others, there is clearly a gap in our understanding of what periplasmic events must occur in order to move electrons nearly 300 Å. We propose that the comparative study of MtrA orthologs, will elucidate the function and electron transfer properties of these proteins, the most "heme dense" proteins that have been identified to date [34]. We have shown that biochemically MtrA and DmsE share most properties (number of hemes, electronic absorption, EPR and electron transfer rate), yet they differ in terms of overall thermodynamics of electron transfer, consistent with the observation of Gralnick and co-workers that MtrA and DmsE are not completely interchangeable *in vivo*. Whether or not structural differences in these two proteins contribute to the differences in redox potential is unknown. Undoubtedly, through the systematic structural and biophysical interrogation of newly elucidated MtrA orthologs, coupled to additional biological and bioinformatic approaches, the gap of understanding may finally become closed.

## Figures

Figure 1   A model for DMSO reduction by DmsEFAB and iron reduction by MtrABC(DEF). The arrows indicate the flow of electrons.  DmsE and MtrA(D) are proposed to accept electrons from the menaquinone pool via CymA.  Heme groups in CymA, MtrACDF and DmsE are shown.

Figure 2  A cyclic voltammogram of DmsE on PGE, raw and baseline subtracted data, pH 6, 4°C (A).  Normalized voltammograms showing the difference in potential window of DmsE (black trace) and MtrA (dashed trace), pH 6, 4°C (B).  A bar-graph comparison of DmsE, MtrA and CymA (C).  Even though DmsE is lower in potential than MtrA, its potential window still overlaps with CymA.

Figure 3  Cytoscape analysis of MtrA (burgundy), OmcA (blue) and NrfB (yellow) families of multiheme cytochromes is shown in (A).  MtrA and NrfB families are more closely related and still cluster together at an E-value of $1x10^{-12}$.  Cytoscape analysis of the MtrA family of proteins is shown in (B).  Each node is labeled by the number of CXXCH motifs contained in each protein.  Several proteins of interest are highlighted: DmsE (orange triangle), MtrA (yellow hexagon), MtrD (green circle), PioA (black diamond), and MtoA (blue square).
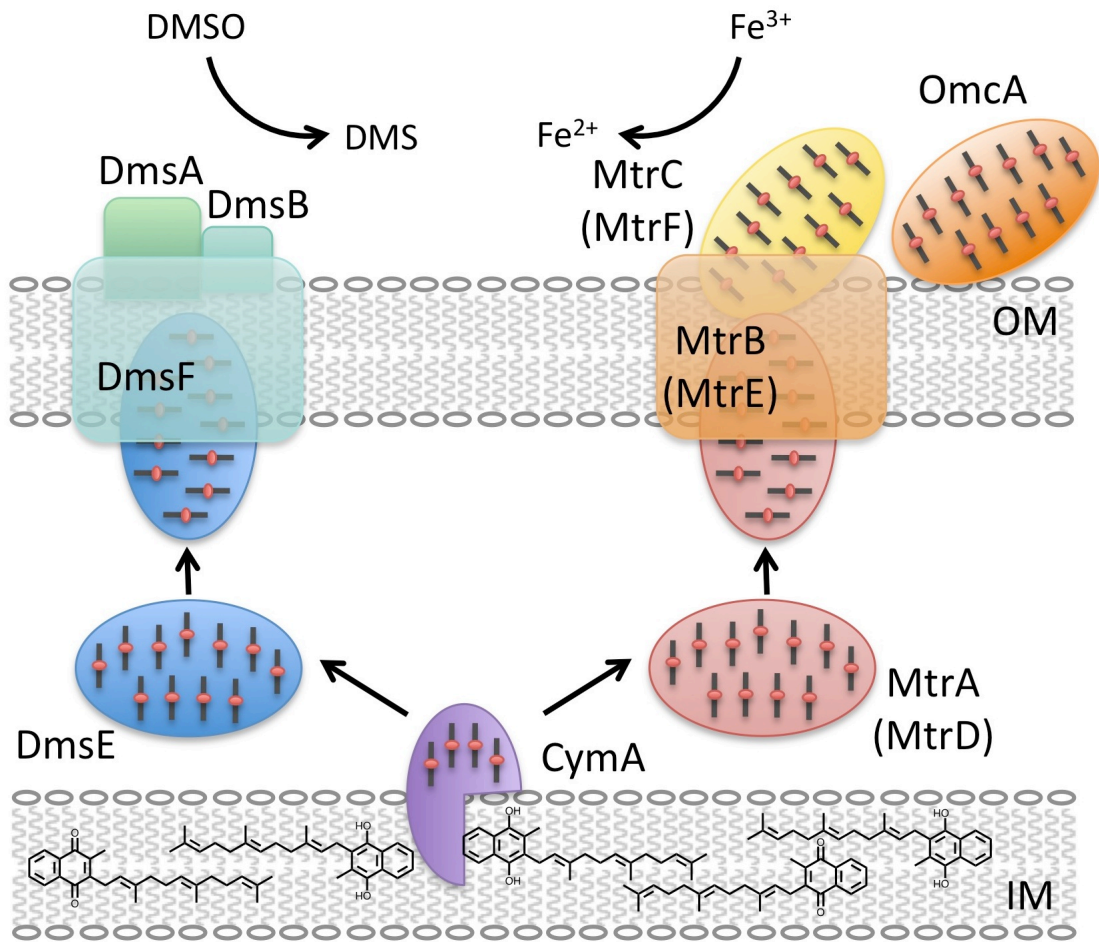
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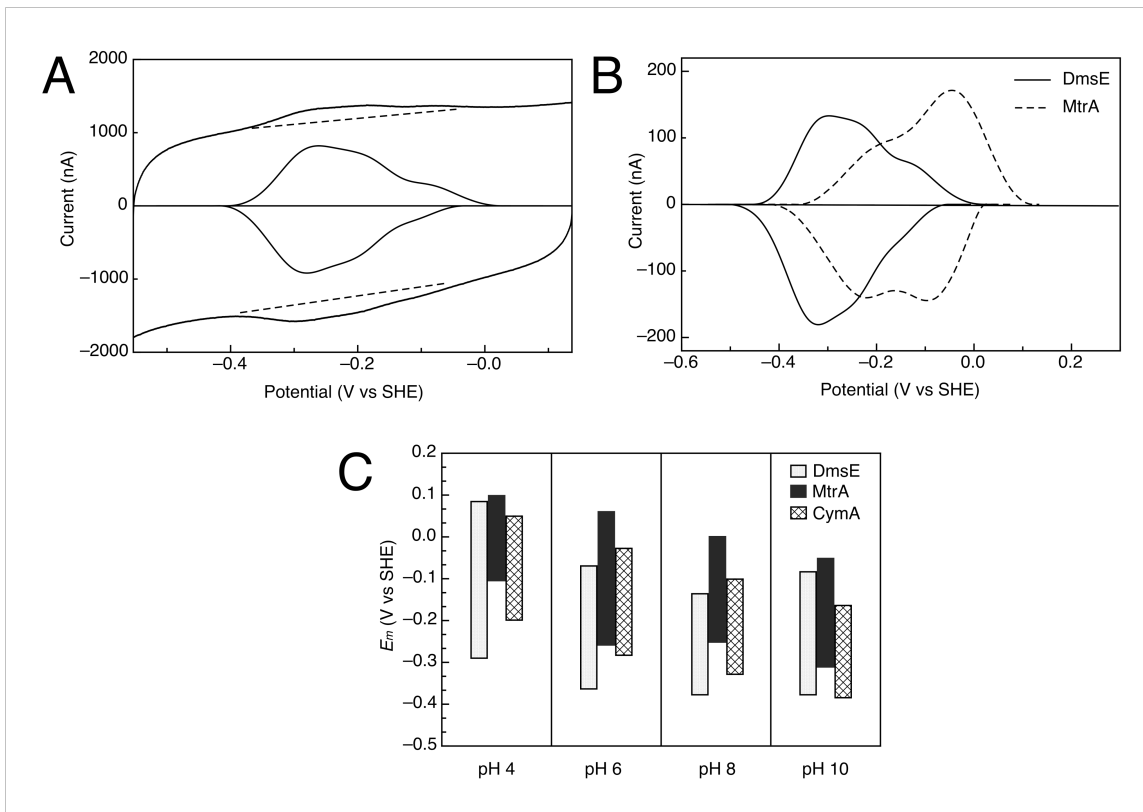
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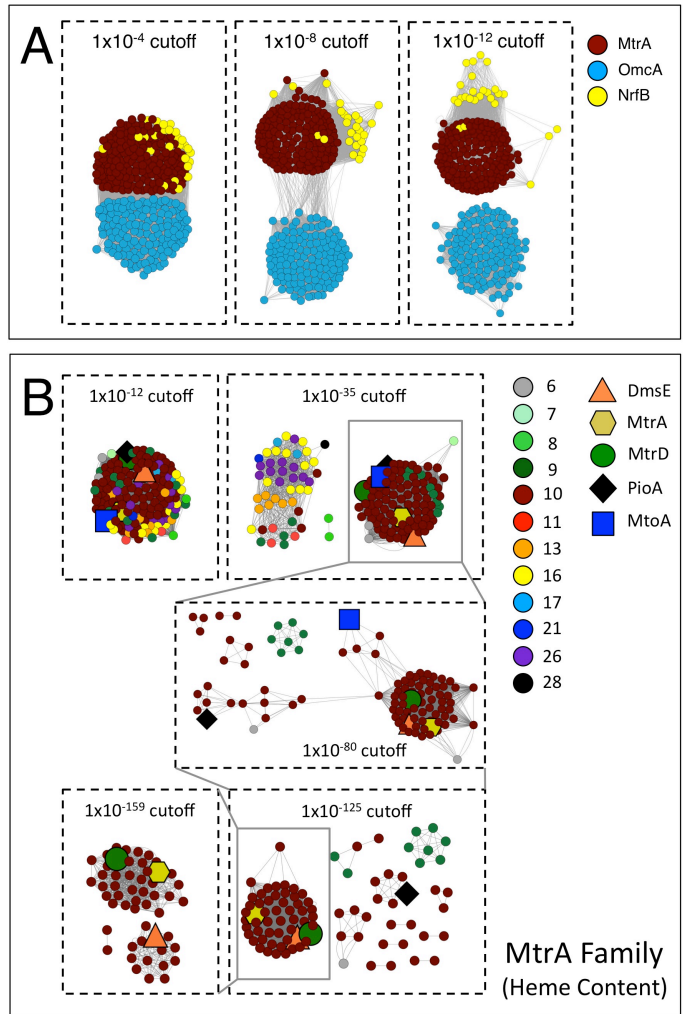
**Figure 1**

**Figure 2**

**Figure 3**