Mtr extracellular electron-transfer pathways in Fe(III)-reducing or Fe(II)-oxidizing bacteria: a genomic perspective

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Abstract

Originally discovered in the dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1 (MR-1), key components of the Mtr (i.e. metal-reducing) pathway exist in all strains of metal-reducing *Shewanella* characterized. The protein components identified to date for the Mtr pathway of MR-1 include four multihaem *c*-Cyts (*c*-type cytochromes), CymA, MtrA, MtrC and OmcA, and a porin-like outer membrane protein MtrB. They are strategically positioned along the width of the MR-1 cell envelope to mediate electron transfer from the quinone/quinol pool in the inner membrane to Fe(III)-containing minerals external to the bacterial cells. A survey of microbial genomes has identified homologues of the Mtr pathway in other dissimilatory Fe(III)-reducing bacteria, including *Aeromonas hydrophila*, *Ferrimonas balearica* and *Rhodoferax ferrireducens*, and in the Fe(II)-oxidizing bacteria *Dechloromonas aromatica* RCB, *Gallionella capsiferriformans* ES-2 and *Sideroxydans lithotrophicus* ES-1. The apparent widespread distribution of Mtr pathways in both Fe(III)-reducing and Fe(II)-oxidizing bacteria suggests a bidirectional electron transfer role, and emphasizes the importance of this type of extracellular electron-transfer pathway in microbial redox transformation of iron. The organizational and electron-transfer characteristics of the Mtr pathways may be shared by other pathways used by micro-organisms for exchanging electrons with their extracellular environments.

Introduction

In the absence of O_2 and other electron acceptors, the Gram-negative bacterium *Shewanella oneidensis* MR-1 (MR-1) can use solid-phase minerals, such as Fe(III)- or Mn(III/IV)-containing minerals, as the terminal electron acceptors for anaerobic respiration (i.e. dissimilatory metal reduction) [1,2]. Because of their insolubility at circumneutral pH, mineral-associated Fe(III) or Mn(III/IV) cannot cross the bacterial outer membrane to the periplasm and the inner membrane where bacterial terminal reductases for other electron acceptors, such as O_2, nitrate and sulfate, are usually located. MR-1 and related strains of metal-reducing *Shewanella* have thus evolved a pathway (i.e. metal-reducing or Mtr pathway) for transferring electrons from the inner membrane through the periplasm and across the outer membrane to the minerals external to the bacterial cells. The protein components identified to date for the Mtr pathway of MR-1include four multihaem *c*-Cyts (*c*-type cytochromes), CymA, MtrA, MtrC and OmcA, and a trans-outer membrane and porin-like protein MtrB [3–12]. The tetrahaem *c*-Cyt CymA is an inner-membrane quinol dehydrogenase that oxidizes quinol in the inner membrane and transfers the released electrons to MtrA either directly or indirectly via other periplasmic proteins [13–15]. MtrA is a decahaem *c*-Cyt that is thought to be inserted into MtrB. Together, MtrAB mediate electron transfer across the bacterial outer membrane to MtrC and OmcA [16,17]. Located on the bacterial surface, MtrC and OmcA are two outer-membrane decahaem *c*-Cyts that reduce Fe(III)-containing minerals directly and indirectly via extracellular electron shuttles, such as flavins secreted by MR-1 cells [11,18–25] (Figure 1). The Mtr pathway of MR-1 is the best characterized pathway used by micro-organisms for extracellular electron-transfer reactions, and characterization of the Mtr pathway has made significant contributions to a molecular-level understanding of microbial extracellular electron transfer (for reviews, see [26–31]). It should be noted that electron-transfer reactions mediated by the Mtr pathway of MR-1 can be bidirectional as electron transfer from an extracellular electrodes to the quinone/quinol pool in the inner membrane has been demonstrated [32]. Thus characterization of the Mtr pathway has not only advanced our understanding of the molecular mechanisms used by micro-organisms for exchanging electrons with their extracellular environments, but also paved the way for biotechnological applications of the Mtr pathway in electrobiosynthesis of valuable materials, chemicals or fuels.

In the MR-1 genome, the genes that encode MtrABC and OmcA are located in the same region, which also includes *mtrD* (an *mtrA* homologue), *mtrE* (an *mtrB* homologue) and *mtrF* (an *mtrC* homologue) whose function is currently unknown [32a]. They are clustered in sequential order of *mtrD-mtrE-mtrF-omcA-mtrC-mtrA-mtrB* (Figure 2). Further

Key words: *c*-type cytochrome, dissimilatory Fe(III) reduction, Fe(II) oxidation, genomics, Mtr extracellular electron-transfer pathway, *Shewanella oneidensis* MR-1.

Abbreviations used: *c*-Cyt, *c*-type cytochrome.

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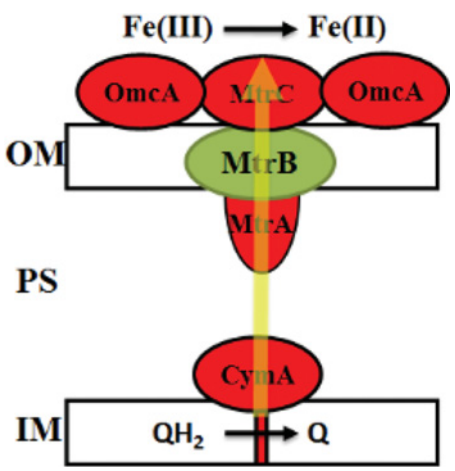
Figure 1 | The proposed Mtr extracellular electron-transfer pathway of the dissimilatory Fe(III)-reducing bacterium *S. oneidensis* MR-1

The protein components identified to date for the Mtr pathway include CymA, MtrA, MtrB, MtrC and OmcA. CymA is a tetrahaem *c*-Cyt that belongs to the NapC/NrfH family of quinol dehydrogenases. Through its N-terminal region, CymA is anchored in the inner membrane (IM) where it oxidizes quinol (QH_2) to quinone (Q) in the IM and transfers the released electrons to MtrA in the outer membrane (OM) either directly or indirectly via other periplasmic proteins. MtrA is a decahaem *c*-Cyt that is thought to be embedded in MtrB, a trans-outer membrane and porin-like protein. Together, MtrAB facilitate electron transfer across the outer membrane to the MtrC and OmcA on the bacterial surface. Both MtrC and OmcA are the outer membrane decahaem *c*-Cyts that transfer electrons to Fe(III)-containing minerals directly and indirectly via electron shuttles, such as flavins. *c*-Cyts are labelled in red, and the direction of electron transfer is indicated by a yellow arrow. PS, periplasmic space.



analysis of the genomes of an additional 19 metal-reducing *Shewanella* strains reveals that (i) *mtrC-mtrA-mtrB* genes are well conserved among all analysed genomes, (ii) *omcA* is sometimes replaced by *undA* or *undA1*, the genes predicted to encode 11-haem *c*-Cyts, and (iii) numbers of the genes found in the *mtr* clusters of the *Shewanella* strains analysed vary from four, such as *omcA-mtrC-mtrA-mtrB* in *Shewanella frigidimarina*, to nine, such as *mtrD-mtrE-mtrF-omcA-undA-omcA-mtrC-mtrA-mtrB* in *Shewanella halifaxensis* [26]. In addition to reduction of Fe(III)- or Mn(III/IV)-containing minerals, MtrAB homologues are also involved in the extracellular reduction of DMSO by MR-1 and extracellular Fe(II) oxidation by *Rhodopseudomonas palustris* TIE-1 [33,34]. Thus MtrAB of MR-1 are believed to be the prototype of a model system for electron transfer across the bacterial outer membrane [16]. Previous surveys indicated that MtrAB homologues were widespread in the Gram-negative bacteria [16,35]. However, it remains unclear as to what extent that the Mtr extracellular electron-transfer pathway is employed by other Fe(III)-reducing or Fe(II)-oxidizing bacteria. To this end, we searched the microbial genomes available on 31 January 2012 for Mtr homologues using a method that was described previously [35–37].

Mtr homologues of Fe(III)-reducing bacteria

As shown in Table 1, *mtrCAB* homologues are found in the genomes of dissimilatory Fe(III)-reducing bacteria *Aeromonas hydrophila*, *Ferrimonas balearica* and *Rhodoferax ferrireducens* [38–40]. Like those in metal-reducing *Shewanella* strains, the *mtrCAB* homologues in these organisms are clustered in the same sequential order of *mtrC-mtrA-mtrB* (Figure 2), indicating that they may have co-evolved in these bacteria. The observation of co-evolution of *mtrCAB* in these Fe(III)-reducing bacteria is consistent with the fact that, in MR-1, MtrCAB form a stable protein complex [16,41]. Identification of MtrCAB homologues in *A. hydrophila* is also consistent with previous observations that Fe(III) reduction by *A. hydrophila* is mediated by *c*-Cyts [39]. In contrast with the situation in *Shewanella*, no *omcA*, *undA* or *undA1*homologue is found in *A. hydrophila*. Absence of additional outer membrane *c*-Cyt in *A. hydrophila* indicates that OmcA and probably UndA and UndA1 are not necessary for microbial reduction of Fe(III).

Additional genes that may be involved in electron-transfer reactions are also clustered with the *mtrCAB* homologues in *F. balearica* and *R. ferrireducens* (Figure 2). In *F. balearica*, these genes include the homologues of *cymA* (locus tag Fbal_1365), *mtrDEF* (Fbal_1364 to 1362), *mtrG* (Fbal_1358, a gene predicted to encode a decahaem outer-membrane *c*-Cyt) [26], *omcA* (Fbal_1359), *undA* (Fbal_1361) and a gene (*orfA*/Fbal_1360) whose function is unknown (Table 1). They are organized in a sequential order of *cymA-mtrD-mtrE-mtrF-undA-orfA-omcA-mtrG-mtrC-mtrA-mtrB*, in which the direction of predicted transcription of *cymA* is opposite to that of rest of the genes (Figure 2). It should be noted that *F. balearica* possesses a third copy of *mtrAB* homologues that is part of the DMSO reductase gene cluster (Fbal_2475–Fbal_2478).

In *R. ferrireducens*, the genes predicted to encode a nona-haem inner-membrane *c*-Cyt (MtrH/Rfer_4073), two CymA homologues (CymA1/Rfer_4075 and CymA2/Rfer_4076), two outer-membrane decahaem *c*-Cyts (MtrI/Rfer_4079 and MtrJ/Rfer_4080), two proteins without any known function (OrfB/Rfer_4074 and OrfC/Rfer_4078) and three monohaem *c*-Cyts (MtrK1/Rfer_4077, MtrK2/Rfer_4084 and MtrK3/Rfer_4085) are found to be adjacent to the *mtrCAB* homologues. They are organized in a sequential order of *mtrK3-mtrK2-mtrC-mtrA-mtrB-mtrJ-mtrI-orfC-mtrK1-cymA2-cymA1-orfB-mtrH* (Table 1 and Figure 2).

In the metal-reducing *Shewanella*, *cymA* is not located in the *mtr* gene cluster. But, in *F. balearica* and *R. ferrireducens*, *cymA* homologues are part of the *mtr* clusters. In fact, two *cymA* genes exist in the *mtr* cluster of *R. ferrireducens*. Furthermore, *mtrH* of *R. ferrireducens* is predicted to encode a unique *c*-Cyt whose N-terminal half (1–330 amino acids) contains nine CX_2H (i.e. haem-binding) motifs and whose C-terminal half (331–662 amino acids) is a homologue of the cytochrome *b* subunit of bacterial formate dehydrogenase. As an inner-membrane protein, the cytochrome *b* subunit

Table 1 | **Mtr homologues found in Fe(III)-reducing or Fe(II)-oxidizing bacteria**

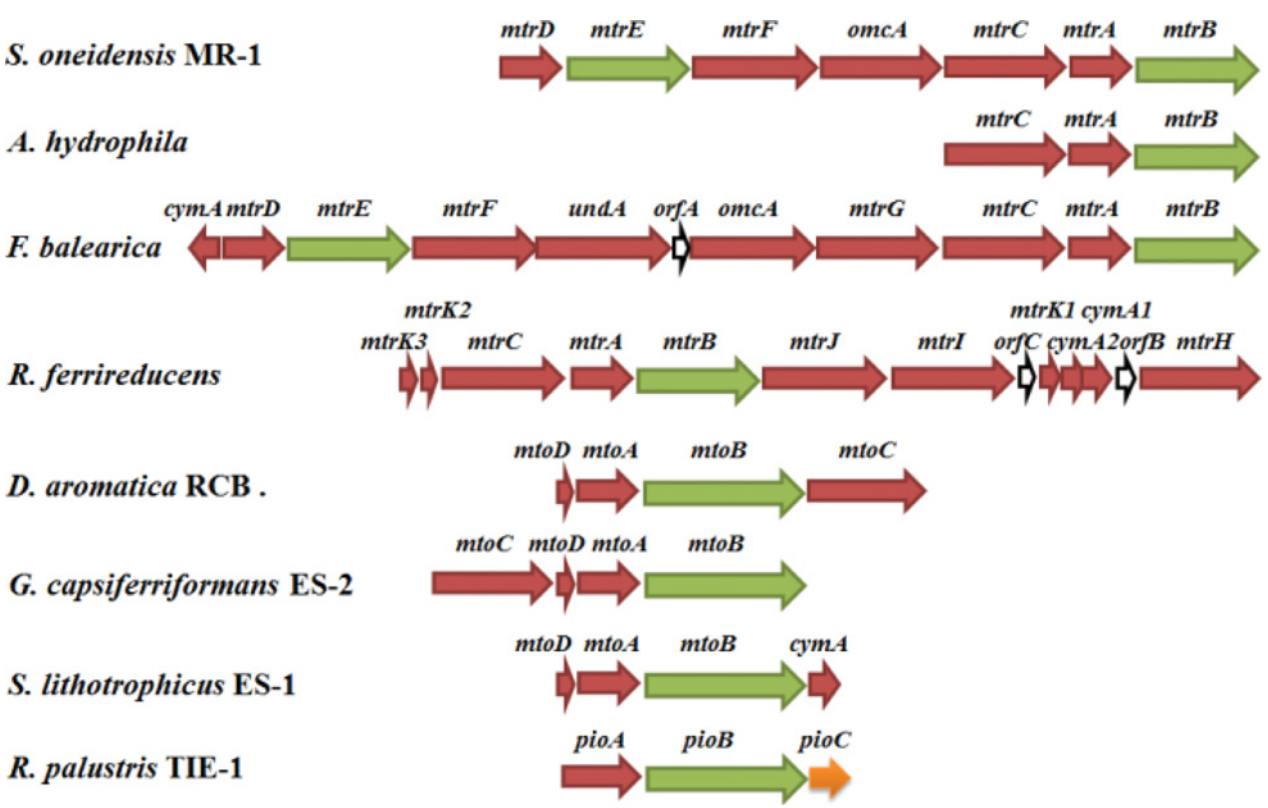
The 'others' column includes the genes predicted to encode (i) outer-membrane multihaem *c*-Cyts (Fbal_1358, Fbal_1359, Fbal_1361, Rfer_4079 and Rfer_4080); (ii) inner-membrane multihaem *c*-Cyts, such as homologues of CymA (Fbal_1365, Rfer_4075, Rfer_4076 and Slit_2495) and MtrH/MtoC (Rfer_4073, Daro_1404 and Galf_2006); (iii) monohaem *c*-Cyts MtrK/MtoD (Rfer_4077, Rfer_4084, Rfer_4085, Daro_1401, Galf_2005 and Slit_2498); and (iv) proteins whose functions are unknown (Fbal_1360, Rfer_4074 and Rfer_4078).

Name	MtrA	MtrB	MtrC	Others
Fe(III) reducers				
A. hydrophila	AHA_2765*	AHA_2766	AHA_2764	
F. balearica	Fbal_1356; Fbal_1364	Fbal_1355; Fbal_1363	Fbal_1357; Fbal_1362	Fbal_1358; Fbal_1359; Fbal_1360; Fbal_1361; Fbal_1365
R. ferrireducens	Rfer_4082	Rfer_4081	Rfer_4083	Rfer_4073; Rfer_4074; Rfer_4075; Rfer_4076; Rfer_4077; Rfer_4078; Rfer_4079; Rfer_4080; Rfer_4084; Rfer_4085
Fe(II) oxidizers				
D. aromatica RCB	Daro_1402	Daro_1403		Daro_1401; Daro_1404
G. capsiferriformans ES-2	Galf_2004	Galf_2003		Galf_2005; Galf_2006
S. lithotrophicus ES-1	Slit_2497	Slit_2496		Slit_2495; Slit_2498

*Locus tag.

Figure 2 | **Genetic organization of the *mtr* clusters identified in the dissimilatory Fe(III)-reducing bacteria *A. hydrophila*, *F. balearica* and *R. ferrireducens* and the *mto* clusters from the Fe(II)-oxidizing bacteria *D. aromatica* RCB, *G. capsiferriformans* ES-2 and *S. lithotrophicus* ES-1**

Shown are the relative positions of genes identified within the complete nucleotide sequence of these bacteria. The genes are labelled by arrows whose sizes and orientations indicate their relative lengths and directions in which they are presumed to be transcribed. For comparison, the *mtr* cluster of *S. oneidensis* MR-1 and the *pio* cluster of *R. palustris* TIE-1 are included. The genes predicted to encode *c*-type cytochromes are labelled in red, whereas those predicted to encode β-barrel outer membrane proteins are labelled in green.



receives electrons from the [FeS] electron-transfer subunit of formate dehydrogenase located in the periplasm and then uses the electrons received to reduce quinone to quinol in the inner membrane [28]. Given that CymA oxidizes the quinol in the inner membrane and relays the released electrons to the periplasmic proteins during Fe(III) reduction by MR-1, it is reasonable to speculate that, like CymA, MtrH may participate in Fe(III) reduction by functioning as an inner-membrane quinol dehydrogenase that oxidizes quinol and transfers the released electrons to the periplasmic proteins via its N-terminal half. To the best of our knowledge, this is the first finding that three genes predicted to encode quinol dehydrogenases exist in the same cluster as *mtr* genes. In MR-1, CymA is the entry point for electrons into the Mtr pathway [31]. The presence of *cymA1*, *cymA2* and *mtrH* in the *mtr* cluster of *R. ferrireducens* suggests the important roles of these *c*-Cyts in mediating extracellular electron-transfer reactions during Fe(III) reduction by this organism. *MtrK*, predicted to encode a monohaem *c*-Cyt, is associated with the *mtr* cluster of *R. ferrireducens* and is not observed in any other Fe(III)-reducing bacteria characterized to date. Because it is predicted to be localized to the periplasm, MtrK may transfer electrons from CymA and MtrH that are associated with the inner membrane across the periplasm to MtrA during Fe(III) reduction in *R. ferrireducens*. Notably, homologues of *mtrH* as well as *mtrK* are also found to be associated with the *mtrAB* homologues found in the Fe(II)-oxidizing bacteria (see the next section).

During this search, we also found an *mtrCAB* cluster in the genomes of the soil bacterium *Candidatus* Solibacter usitatus Ellin6076 and the marine bacteria *Vibrio* sp. Ex25, *Vibrio parahaemolyticus*, *Vibrio vulnificus* CMCP6, *V. vulnificus* MO6-24/O and *V. vulnificus* YJ016. Although none of these bacteria has been shown to be capable of reducing Fe(III) or of other forms of extracellular electron transfer and most of the identified *Vibrio* strains are opportunistic human pathogens, they all inhabit environments where Fe(III) minerals can be abundant and where O_2 may be limited [42–44]. Identification of MtrCAB homologues in these bacteria suggests that they may be able to respire on Fe(III) in the absence of other electron acceptors or that MtrCAB have other, as yet undiscovered, functions. Anaerobic respiration on Fe(III) minerals could provide an alternative means of energy conservation in these bacteria during periodic anoxia. A previous survey also indicated the existence of MtrAB homologues in a *Geobacter* strain [16]. Analyses of all sequenced *Geobacter* genomes reveal that only *Geobacter* sp. M21 contains *mtrAB* homologues (GM21_0397 and GM21_0398), but no *mtrC* homologue is found to be next to the *mtrAB* homologues identified, which suggests that these *mtrAB* homologues may not be involved in metal reduction. The lack of apparent Mtr homologues in the most metal-reducing *Geobacter* strains is consistent with previous findings that the electron-transfer pathways used by metal-reducing *Geobacter* strains for extracellular reduction of Fe(III)-containing minerals have evolved independently from the Mtr pathways [29,30].

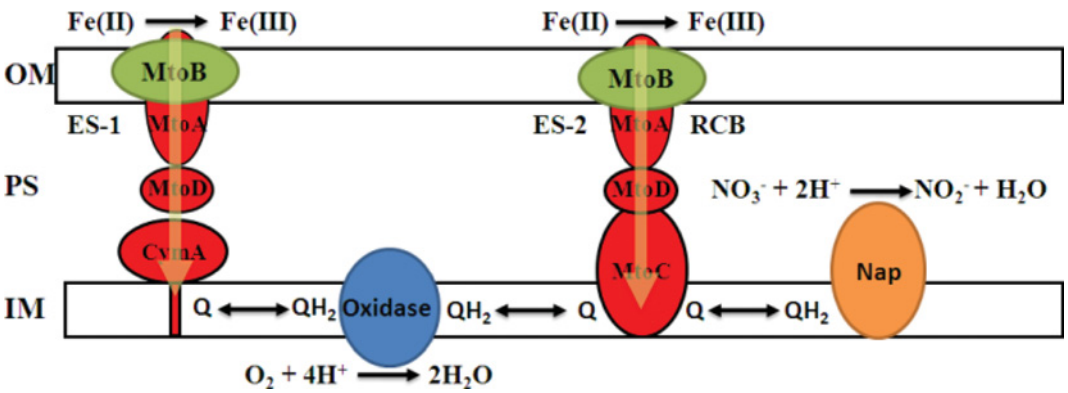
Mto proteins, the Mtr homologues identified from Fe(II)-oxidizing bacteria

mtrAB homologues have been identified in the Fe(II)-oxidizing bacteria *Dechloromonas aromatica* RCB (RCB), *Gallionella capsiferriformans* ES-2 (ES-2) and *Sideroxydans lithotrophicus* ES-1 (ES-1) [35,45–47]. Additional genes predicted to encode *c*-Cyts are also found to be adjacent to the *mtrAB* homologues identified, which include homologues of *cymA* (Slit_2495 in ES-1), *mtrH* (Daro_1404 in RCB and Galf_2006 in ES-2) and *mtrK* (Daro_1401 in RCB, Galf_2005 in ES-2 and Slit_2498 in ES-1). To distinguish them from those found in the Fe(III)-reducing bacteria, the MtrA, MtrB, MtrH and MtrK homologues identified from the Fe(II)-oxidizing bacteria are named MtoA, MtoB, MtoC and MtoD respectively [35] (Table 1 and Figure 2). In the Fe(II)-oxidizing bacterium *R. palustris* TIE-1, *pioAB* (i.e. *mtrAB* homologues) are clustered with *pioC* that encodes a high-redox-potential [FeS] protein [34]. However, no apparent *pioC* homologue is found in ES-1, ES-2 or RCB [35]. Similar to *mtrA* genes of the Fe(III)-reducing bacteria, all identified *mtoA* genes are smaller than *pioA*, whereas all *mtoB* genes identified from our study and *pioB* are similar in size and are bigger than *mtrB* genes of the Fe(III)-reducing bacteria [35] (Figure 2).

In the Fe(III)-reducing bacteria investigated, *mtrC* is always clustered with *mtrAB* (Figure 2). However, no *mtrC* homologue or other genes encoding for outer-membrane multihaem *c*-Cyts is found in the Fe(II)-oxidizing bacteria surveyed in our study. The presence of MtrC-like outer-membrane multihaem *c*-Cyts in the Fe(III)-reducing bacteria and its absence from the Fe(II)-oxidizing bacteria suggest a distinct role for this type of outer-membrane multihaem *c*-Cyts in extracellular reduction of Fe(III)-containing minerals. In the Fe(II)-oxidizing bacteria identified in our study, *mtoD*, a gene predicted to encode a monohaem *c*-Cyt, is at the position where *mtrC* is in the *mtr* clusters of the Fe(III)-reducing bacteria surveyed (Figure 2). Similar to its homologue MtrK of the Fe(III)-reducing bacteria, MtoD may also transfer electrons across the periplasm. As discussed above, both CymA and MtoC (an MtrH homologue) are inner-membrane *c*-Cyts that mediate electron transfer between the quinone/quinol pool in the inner membrane and redox proteins in the periplasm. Like MtrAB, MtoAB are believed to transfer electrons across the bacterial outer membrane. Consistent with this suggestion, the cloned *mtoA* of ES-1 partially complements an MR-1 mutant without MtrA with regard to ferrihydrite [a poorly crystalline Fe(III) oxide] reduction, suggesting that MtoA of ES-1 can be inserted into MR-1 MtrB and transfer electrons across the outer membrane to MR-1 MtrC during ferrihydrite reduction [35]. Furthermore, purified ES-1 MtoA is a decahaem *c*-Cyt that oxidizes Fe(II). Although it rapidly reaches equilibrium, Fe(II) oxidation by ES-1 MtoA involves all of its ten haems. Involvement of all ten haems during Fe(II) oxidation implies the ability of ES-1 MtoA to transfer electrons from extracellular Fe(II) across the outer membrane and into the

Figure 3 | The proposed Mto extracellular electron-transfer pathways of the Fe(II)-oxidizing bacteria *D. aromatica* RCB, *G. capsiferriformans* ES-2 and *S. lithotrophicus* ES-1

Like its homologue MtrA in the Fe(III)-reducing bacteria, decahaem *c*-Cyt MtoA is inserted into the porin-like outer-membrane (OM) protein MtoB that is a homologue of MtrB. MtoA oxidizes Fe(II) directly on the bacterial surface and transfers the released electrons across the outer membrane to the periplasmic monohaem *c*-Cyt MtoD. MtoD relays the electrons through the periplasmic space (PS) to the tetrahaem *c*-Cyt CymA in *S. lithotrophicus* ES-1 (ES-1) or nonahaem *c*-Cyt MtoC in *G. capsiferriformans* ES-2 (ES-2) and *D. aromatica* RCB (RCB). Both CymA and MtoC are inner-membrane (IM) proteins that are proposed to reduce quinone (Q) to quinol (QH_2). QH_2 is oxidized either by oxidase in ES-1 and ES-2 or by periplasmic nitrate reductase (Nap) in RCB. *c*-Cyts are labelled in red, and the direction of electron transfer is indicated by yellow arrows. For simplicity, multisubunits of oxidase or NAP are drawn as a single circle.



periplasm, which is consistent with the complementation results [35]. Initial characterization results also show that recombinant MtoC of RCB is a membrane *c*-Cyt (L. Shi, unpublished work). All of these findings suggest that MtoABD and MtoC or CymA may have functions similar to the Mtr electron-transfer systems of Fe(III)-reducing bacteria and may be responsible for electron transfer from extracellular Fe(II) across the bacterial cell envelope to the quinone/quinol pool in the inner membrane. Hence the direction of electron-transfer reactions conducted by the Mto proteins is opposite to that of the Mtr pathways of the Fe(III)-reducing bacteria. In these proposed pathways, MtoA, which is presumably inserted in MtoB, is hypothesized to oxidize Fe(II) extracellularly and transfer the electrons acquired inward across the outer membrane to the periplasmic MtoD. The current conceptual model for this process has MtoD relaying the electrons to CymA in ES-1 or MtoC in ES-2 and RCB. CymA and MtoC then uses the electrons received to reduce quinone to quinol in the inner membrane. Quinol could then shuttle the electrons to the redox proteins in the inner membrane for reducing O_2 in ES-1 and ES-2 or for reducing nitrate in RCB (Figure 3).

Conclusions

A survey of recently sequenced microbial genomes shows that the homologues of the Mtr extracellular electron-transfer pathway of *S. oneidensis* MR-1 exist in the Fe(III)-reducing bacteria *A. hydrophila*, *F. balearica* and *R. ferrireducens* and the Fe(III)-oxidizing bacteria *D. aromatica* RCB, *G. capsiferriformans* ES-2 and *S. lithotrophicus* ES-1. Like those in *S. oneidensis* MR-1, the homologues identified from these bacteria are clustered together, indicating that their protein products probably also work together to facilitate electron transfer across the cell envelop. Widespread distribution of the Mtr pathways in the Fe(III)-reducing bacteria as well as the Fe(II)-oxidizing bacteria emphasizes the versatility of these proteins for exchanging electrons with carriers and acceptors external to the bacteria cells, including extracellular redox transformations of iron by the Gram-negative bacteria. These two different functional groups of bacteria employ a common set of proteins, which are strategically positioned along the width of bacterial cell envelope, such as MtrAB/MtoAB in the outer membrane, MtrK/MtoD in the periplasm, and CymA and MtrH/MtoC in the inner membrane, for mediating electron transfer between extracellular Fe(III) or Fe(II) and the quinone/quinol pool in the inner membrane. Thus the electron-transfer reactions mediated by the Mtr pathways are bidirectional. The relative locations between the electron source and sink dictate the direction of electron-transfer reactions. These characteristics observed in the Mtr pathways may also be shared by the other pathways used by microorganisms for exchanging electrons with their extracellular environments. Moreover, results from our study also suggest that sequence-based approaches (e.g. metagenomics and functional gene arrays) can be used to better assess the potentials of the electron-transfer processes mediated by the Mtr pathways in the environmental samples and to explore natural diversity of these pathways.

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