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Reconstructing electron transfer components from an Fe(II) oxidizing bacterium

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Abstract

Neutrophilic Fe(II) oxidizing bacteria play an important role in biogeochemical processes and have also received attention for multiple technological applications. These micro-organisms are thought to couple their metabolism with extracellular electron transfer (EET) while oxidizing Fe(II) as electron donor outside the cell. *Sideroxydans lithotrophicus* ES-1 is a freshwater chemolithoautotrophic Fe(II) oxidizing bacterium that is challenging to culture and not yet genetically tractable. Analysis of the *S. lithotrophicus* ES-1 genome predicts multiple EET pathways, which are proposed to be involved in Fe(II) oxidation, but not yet validated. Here we expressed components of two of the proposed EET pathways, including the Mto and Slit_0867–0870 PCC3 pathways**,** from *S. lithotrophicus* ES-1 into *Aeromonas hydrophila*, an established model EET organism. We demonstrate that combinations of putative inner membrane and periplasmic components from the Mto and Slit_0867–0870 PCC3 pathways partially complemented EET activity in *Aeromonas* mutants lacking native components. Our results provide evidence for electron transfer functionality and interactions of inner membrane and periplasmic components from the Mto and Slit_0867–0870 PCC3 pathways. Based on these findings, we suggest that EET in *S. lithotrophicus* ES-1 could be more complicated than previously considered and raises questions regarding directionality of these electron transfer pathways.

INTRODUCTION

Fe(III) reducing and Fe(II) oxidizing bacteria are known to perform extracellular electron transfer (EET) to either reduce acceptors or oxidize donors outside the cell [1–5]. These bacteria are known to play an important role in multiple biogeochemical cycles and are of interest for a range of biotechnological applications [6]. EET pathways in Fe(III) reducing bacteria have been well characterized through *in vivo* genetic studies [1–4, 7]. However, only recently has a genetic system been reported in a neutrophilic Fe(II) oxidizing bacterium, *Mariprofundus ferrooxydans* PV-1 [8]. Our knowledge of biochemical and electron transfer pathways involved in growth and survival of neutrophilic Fe(II) oxidizing bacteria remain limited to bioinformatic, heterologous expression and *in vitro* studies [9–11]. The Mtr pathway from *Shewanella oneidensis* MR-1 is the best understood EET system [1] and variations have been reported in *Aeromonas hydrophila* and *Vibrio natriegens* [2, 3]. The Mtr system consists of a porin-periplasmic *c*-type cytochrome complex (PCC) formed by MtrB and MtrA, respectively, along with the extracellular *c*-type cytochrome MtrC [12]. Based on the PCC model of the Mtr pathway, bioinformatic studies have predicted novel EET pathways that are presumed to be involved in Fe(II) oxidation in *Sideroxydans lithotrophicus* ES-1, a freshwater neutrophilic Fe(II) oxidizing chemolithoautotrophic bacterium [9, 13]. EET pathways in *S. lithotrophicus* ES-1 were based on co-localization of genes predicted to encode porins and periplasmic *c*-type cytochromes, sometimes with extracellular *c*-type cytochromes [9]. Genomic analysis identified three different PCC type EET pathways in *S. lithotrophicus* ES-1 [9]. The first pathway, designated as the Mto pathway, is encoded by four genes, *Slit_2495–2498* (Fig. 1a) and includes MtoAB, which are homologues of MtrAB from *S. oneidensis* and other iron reducing bacteria [9, 10]. *S. lithotrophicus* does not encode either FccA or CctA, which are known to transfer electrons to MtrA during Fe(III) reduction in *S. oneidensis* [14]. However, *mtoD* from this cluster is predicted to encode a mono-haem periplasmic *c*-type cytochrome that is proposed to accept electrons from MtoA during Fe(II) oxidation

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Abbreviations: DAP, diaminopimelic acid; EET, extracellular electron transfer; LB, lysogeny broth; MWMM, modified Wolfe's mineral medium; PCC, porin-periplasmic c-type cytochrome complex; SBM, Shewanella basal medium.
One supplementary figure and one supplementary table are available with the online version of this article.

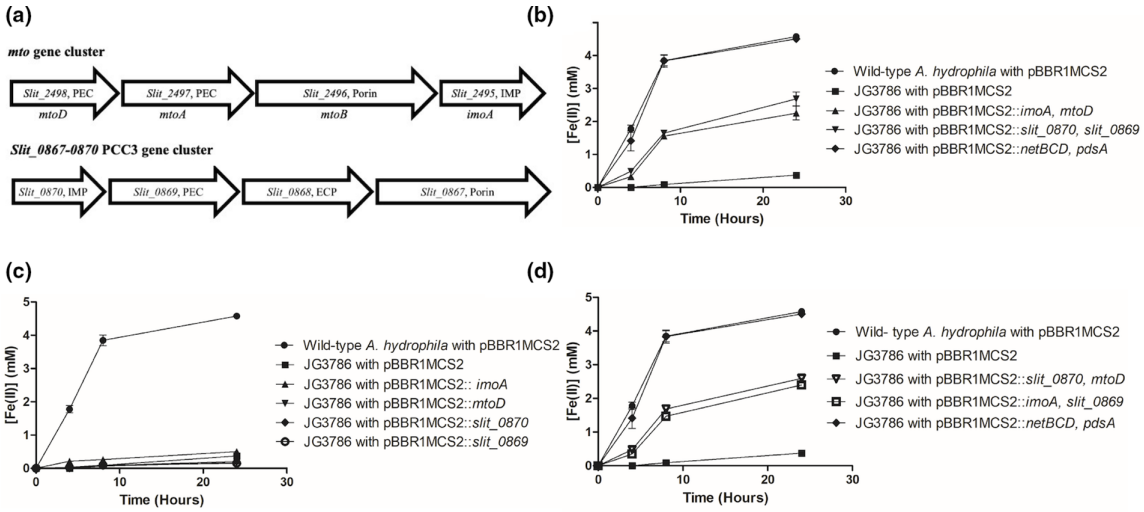

Fig. 1. Testing the functionality and interactions of the Mto and Slit_0867–0870 PCC3 pathways components. (a) Arrangement, putative cellular location and locus tags of the genes in the *mto* and *Slit_0867–0870* PCC3 clusters. PEC, putative periplasmic electron carrier; IMP, putative inner membrane protein; ECP, putative extracellular *c*-type cytochrome. Note genes are not to scale. (b) Resting cell Fe(III) citrate reduction by *ΔnetBCD ΔpdsA Aeromonas* (JG3786) containing inner membrane and periplasmic components from the Mto and Slit_0867–0870 PCC3 pathways, (c) individual inner membrane and periplasmic components from the Mto and Slit_0867–0870 PCC3 pathways respectively and (d) cross combinations of inner membrane and periplasmic components from the Mto and Slit_0867–0870 PCC3 pathways, respectively. Error bars represent standard deviations from the mean of two independent experiments performed in duplicates.

[9, 10, 15]. Once reduced, MtoD is thought to transfer electrons to Slit_2495, a putative cytoplasmic membrane protein encoded by the fourth gene in the *mto* cluster [9, 10] and recently renamed ImoA [16]. The remaining two pathways in *S. lithotrophicus* are paralogous gene clusters *Slit_0867–0870* (Fig. 1a) and *Slit_1446–1449* that are both classified as a third type of PCC configuration (PCC3), predicted to encode an inner membrane protein, a periplasmic multiheme *c*-type cytochrome, an outer membrane porin and an extracellular *c*-type multiheme cytochrome [9]. Interestingly, the presumed inner membrane components of these PCC3 pathways do not contain clear motifs for electron transferring cofactors [9]. There is no direct evidence establishing the role of either the Mto or PCC3 pathways in EET, and the directionality of electron flow remains ambiguous. We focused this work on the *mto* gene cluster (~4.6 kb) given its similarity to known Fe(III) reduction pathways and the *Slit_0867–0870* PCC3 cluster due to its smaller size (~6.5 kb) compared to the *Slit_1446–1449* gene cluster (~8.3 kb).

METHODS

Bacterial cultivation

Sideroxydans lithotrophicus ES-1 was obtained from the National Center for Marine Algae and Microbiota culture collection (https://ncma.bigelow.org) and was grown on modified Wolfe's mineral medium (MWMM) [13], buffered to pH 6.5 with MES buffer. The culture was grown in 60 ml serum bottles containing 20 ml of MWMM medium with N_2:CO_2 (80:20) headspace and sealed with butyl rubber stoppers. Sealed serum bottles containing the medium were autoclaved and added with 0.1 ml of filtered ferrous chloride solution (100 mM), and 0.5 ml of filtered air to introduce oxygen as electron acceptor. Overall, 0.1 ml of filtered ferrous chloride solution (100 mM), and 0.5 ml of filtered air were added at every 48 h to the serum bottles. *Shewanella* and *Aeromonas* strains were grown in lysogeny broth (LB) medium. Then, 50 µM or 100 µM kanamycin was added to the medium when required.

Strain construction

Strains used in this study are listed in Table 1. Plasmid and primers used in this study are listed in Table S1. Genomic DNA of *S. lithotrophicus* ES-1 was extracted using Qiagen DNeasy PowerSoil kit. Specific gene clusters and genes were amplified using genomic DNA of *S. lithotrophicus* ES-1 as the template. Amplicons were restriction digested, ligated and cloned into pBBR1MCS2 expression vectors [17], which were transformed into chemically competent *E. coli* UQ950 cells [18]. Purified plasmids from *E. coli* UQ950 cells were transformed into *E. coli* WM3064 [18] cells followed by selection on LB medium plates containing 50 µM kanamycin and 360 µM diaminopimelic acid (DAP). *E. coli* WM3064 cells containing the plasmids were used to conjugate the plasmids into *Shewanella* and *Aeromonas* mutants. *Shewanella* and *Aeromonas* cells containing the plasmids were selected on LB plates containing 50 µM and 100 µM kanamycin, respectively, without DAP.

Table 1. Strains used in this study

Bacterial strains (strain no.)	Source
Aeromonas hydrophila with pBBR1MCS2 (JG3522)	2
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2 (JG3790)	2
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2::*mtoABD, imoA* (JG4452)	This study
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2::*Slit_0867–0870* (JG4453)	This study
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2::*imoA, mtoD* (JG4454)	This study
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2::*Slit_0870, Slit_0869* (JG4455)	This study
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2::*imoA, Slit_0869* (JG4456)	This study
ΔnetBCD ΔpdsA A. hydrophila with pBBR1MCS2::*Slit_0870, mtoD* (JG4457)	This study
Δ*netBCD* Δ*pdsA A. hydrophila* with pBBR1MCS2::*imoA* (JG4458)	This study
Δ*netBCD* Δ*pdsA A. hydrophila* with pBBR1MCS2::*mtoD* (JG4459)	This study
Δ*netBCD* Δ*pdsA A. hydrophila* with pBBR1MCS2::*Slit_0870* (JG4460)	This study
Δ*netBCD* Δ*pdsA A. hydrophila* with pBBR1MCS2::*Slit_0869* (JG4461)	This study
Δ*mtrA A. hydrophila* with pBBR1MCS2 (JG3634)	2
Δ*mtrA A. hydrophila* with pBBR1MCS2::*mtoABD, imoA* (JG4462)	This study
Δ*mtrA A. hydrophila* with pBBR1MCS2::*Slit_0867–0870* (JG4463)	This study
Escherichia coli UQ950	24
E. coli WM3064	24

Fe(III) citrate reduction assay

Shewanella and *Aeromonas* cells were freshly struck from −80°C glycerol stocks to LB plates containing 50 and 100 µM kanamycin, respectively. Oxic LB liquid medium containing appropriate kanamycin concentration were inoculated with single colonies and incubated in a shaker at 30°C. The cells were washed with *Shewanella* basal medium (SBM) [2] and resuspended in the same medium to obtain a cell density of 10^9 cells ml^{-1}. Altogether, 30 µl of the resuspended cells were added to 270 µl of the SBM medium containing 20 mM sodium lactate and 5 mM of ferric citrate on a 96-well plate. The 96-well plate was placed inside an anaerobic chamber, which was made anaerobic by flushing with oxygen free argon, and incubated at 30°C. Samples were collected periodically to quantify Fe(II), produced as a result of Fe(III) reduction, using the ferrozine assay [19].

Fe(II) oxidation assay

Fe(II) oxidation assays were performed in 25 ml Balch tubes containing 5 ml of the SBM medium. Balch tubes were sparged with high-purity argon gas to remove oxygen and sealed with butyl rubber stoppers. After autoclaving, sealed Balch tubes were added with 200 µl of filtered ferrous chloride solution (100 mM) to obtain final Fe(II) concentration of 2 mM. 0.5 ml of filtered air was added to introduce oxygen as electron acceptor. Cells were prepared as explained for the Fe(III) citrate reduction assays and added to the Balch tubes to obtain a final cell density of approximately 2×10^8 cells ml^{-1}. Sodium azide (50 µM) was used as controls to measure abiotic rate of Fe(II) oxidation. Samples were collected periodically to quantify Fe(II) using the ferrozine assay [19].

RESULTS AND DISCUSSION

The initial goals of our work were to test the electron transfer functionality, component interaction, and directionality of the Mto and Slit_0867–0870 PCC3 pathways through heterologous expression in chassis strains of *S. oneidensis* and *A. hydrophila* missing native EET components. Our preliminary experiments showed that heterologous expression of these pathways did not confer the ability to oxidize Fe(II) in either *Shewanella* or *Aeromonas* backgrounds (data not shown). However, the expression of either the Mto or Slit_0867–0870 PCC3 pathway partially complemented Fe(III) citrate reduction activity in an *A. hydrophila* mutant lacking native inner membrane and the periplasmic EET components (*ΔnetBCD ΔpdsA,* Fig. S1, available in the online version of this article). Neither the Mto pathway, nor the Slit_0867–0870 PCC3 pathway complemented Fe(III) citrate reduction in a Δ*mtrA A. hydrophila* background, suggesting the native MtrCAB proteins were required for Fe(III) citrate reduction (data not shown). None of the *S. oneidensis* strains containing *Slit_0867–0870* showed Fe(III) citrate reduction activity (data not shown), highlighting the utility of heterologous expression and functional testing of novel EET

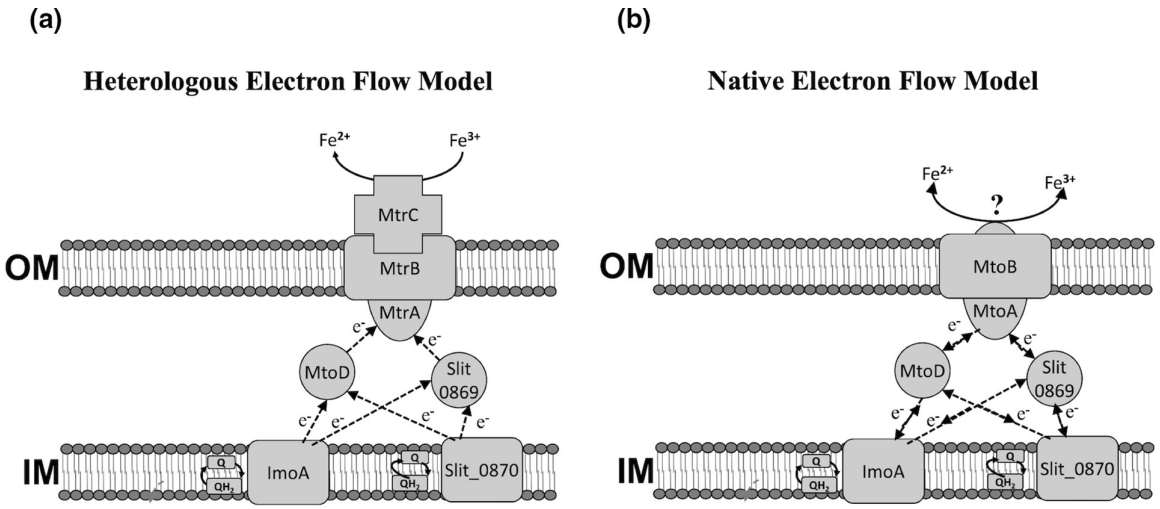

Fig. 2. Putative electron flow models of *S. lithotrophicus* components in *A. hydrophila* (a) and in *S. lithotrophicus* ES-1 (b). OM, outer membrane; IM, inner membrane.

components in multiple chassis strains. Based on these results, we hypothesized that expression of the cytoplasmic membrane and periplasmic EET components encoded in the Mto and Slit_0867–0870 PCC3 from *S. lithotrophicus* would restore Fe(III) citrate reduction in *A. hydrophila* mutant backgrounds.

Testing the Mto and Slit_0867–0870 PCC3 pathway components

Plasmids expressing both the inner membrane and periplasmic components of either the Mto or Slit_0867–0870 PCC3 pathway partially complemented Fe(III) citrate reduction in a strain of *A. hydrophila* (JG3786) missing its native cytoplasmic (*netBCD*) and periplasmic (*pdsA*) EET components (*ΔnetBCD ΔpdsA*) (Fig. 1b). Plasmids expressing single genes, either *mtoD*, *imoA, Slit_0869* or *Slit_0870,* did not complement Fe(III) citrate reduction in *ΔnetBCD ΔpdsA* (Fig. 1c). The Fe(III) citrate reduction phenotype of *ΔnetBCD ΔpdsA* strains containing both the inner membrane and periplasmic components of either the Mto or Slit_0867–0870 PCC3 pathways (Fig. 1b–d) provide evidence for the proposed electron transfer functionality of these components and their ability to interact to facilitate electron transfer from quinone pools to MtrCAB in *A. hydrophila*.

Inner membrane and periplasmic components of the Mto and Slit_0867–0870 PCC3 pathways are modular

The ability of both the Mto and Slit_0867–0870 PCC3 pathway components to transfer electrons to the native MtrA in *Aeromonas* (Fig. 1b) prompted experiments to test the possible modularity of periplasmic and inner membrane components from ES-1. A plasmid expressing the inner membrane component of the Mto pathway, *imoA* and periplasmic component of the Slit_0867–0870 PCC3 pathway, *Slit_0869*, partially restored Fe(III) citrate reduction activity in *ΔnetBCD ΔpdsA*, as did a construct with the reciprocal components (*Slit_0870* and *mtoD*) (Fig. 1d). The results presented in Fig. 1 suggest that the inner membrane and periplasmic components of the Mto and the Slit_0867–0870 PCC3 pathways are modular and can interact to transfer electrons to MtrA in *Aeromonas*, facilitating Fe(III) citrate reduction (Fig. 2a).

MtoA is a homologue of MtrA and PioA, which are known to be involved in Fe(III) reduction and Fe(II) oxidation, respectively [20, 21], and therefore we presume that MtoA is also involved in Fe(II)/Fe(III) redox transformation. However, the ability of the Mto and Slit_0867–0870 pathway components to perform outward EET in *A. hydrophila* raises questions regarding the directionality and possible reversibility of these pathways in *S. lithotrophicus* ES-1 (Fig. 2b). Based on the results presented in Fig. 1d, we speculate that the Mto and Slit_0867–0870 PCC3 pathways can interact in *S. lithotrophicus* ES-1 (Fig. 2b). The presence of a second PCC3 cluster encoded by *Slit_1446_1449* and seven additional paralogs of MtoD [16] encoded in the genome suggest the possibility of multiple interacting EET pathways in *S. lithotrophicus* ES-1. The *S. lithotrophicus* ES-1 genome also encodes other inner membrane proteins including a cytochrome bc_1 complex and an alternative complex III that are presumed to be involved in Fe(II) oxidation in obligate Fe(II) oxidizing bacteria [22]. The presence of multiple EET pathways, and modularity of certain components may provide growth/survival advantages to *S. lithotrophicus* ES-1 under dynamic redox conditions, analogous to how *Geobacter sulfurreducens* has evolved distinct EET pathways based on redox potential and utilizing different soluble and insoluble sources and/or sinks of electrons in the environment [4, 23]. Recent work quantifying expression of genes encoding candidate iron oxidation pathway genes from *S. lithotrophicus* found that paralogues of *cyc2,* encoding a protein recently shown to exhibit Fe(II) oxidation activity from *M. ferrooxydans* [24], were

expressed more highly than *mtoA* under iron-oxidizing and non-iron oxidizing conditions [25]. These results suggest that Cyc2, and not Mto, is the major pathway for Fe(II) oxidation in *S. lithotrophicus* under the conditions tested [25] and that more work should be done to investigate the role of Mto in EET. In conclusion, we established the electron transfer functionality, interactions and modularity of novel EET components from an Fe(II) oxidizing bacterium that is dependent on EET for its primary metabolism. These insights suggest that multiple cross-interacting extracellular electron transfer pathways could be functional in *S. lithotrophicus* ES-1.

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Conflicts of interest
The authors declare no conflicts of interest.

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