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A Ferrous Iron Exporter Mediates Iron Resistance in *Shewanella oneidensis* MR-1

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Shewanella oneidensis **strain MR-1 is a dissimilatory metal-reducing bacterium frequently found in aquatic sediments. In the absence of oxygen, *S. oneidensis* can respire extracellular, insoluble oxidized metals, such as iron (hydr)oxides, making it intimately involved in environmental metal and nutrient cycling. The reduction of ferric iron (Fe^{3+}) results in the production of ferrous iron (Fe^{2+}) ions, which remain soluble under certain conditions and are toxic to cells at higher concentrations. We have identified an inner membrane protein in *S. oneidensis*, encoded by the gene SO_4475 and here called FeoE, which is important for survival during anaerobic iron respiration. FeoE, a member of the cation diffusion facilitator (CDF) protein family, functions to export excess Fe^{2+} from the MR-1 cytoplasm. Mutants lacking *feoE* exhibit an increased sensitivity to Fe^{2+}. The export function of FeoE is specific for Fe^{2+}, as an *feoE* mutant is equally sensitive to other metal ions known to be substrates of other CDF proteins (Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}). The substrate specificity of FeoE differs from that of FieF, the *Escherichia coli* homolog of FeoE, which has been reported to be a Cd^{2+}/Zn^{2+} or Fe^{2+}/Zn^{2+} exporter. A complemented *feoE* mutant has an increased growth rate in the presence of excess Fe^{2+} compared to that of the Δ*feoE* mutant complemented with *fieF*. It is possible that FeoE has evolved to become an efficient and specific Fe^{2+} exporter in response to the high levels of iron often present in the types of environmental niches in which *Shewanella* species can be found.**

Shewanella oneidensis strain MR-1 is a versatile, facultatively anaerobic bacterium that lives in aquatic environments and is capable of respiring numerous organic and inorganic compounds in the absence of oxygen. The respiratory diversity of *S. oneidensis* has widespread effects on biogeochemical cycling (1) and has therefore been a focus for applications in biotechnology and bioremediation (2). Terminal electron acceptors that *S. oneidensis* can use, aside from oxygen, include dimethyl sulfoxide (DMSO), trimethylamine *N*-oxide, fumarate, nitrate, and sulfite (3–6), as well as oxidized metals, such as iron and manganese (hydr)oxides (3, 7), which are abundant in the types of sediments (8) in which *Shewanella* spp. are often found (1). The molecular mechanisms that allow dissimilatory metal-reducing bacteria to survive under iron-rich conditions, however, are not fully understood.

Respiration of ferric iron (Fe^{3+}) results in the production of ferrous iron (Fe^{2+}), which can remain as aqueous Fe^{2+} ions or become incorporated into solid-phase minerals (9, 10), depending on the environmental conditions. As iron respiration by *S. oneidensis* continues, the local concentration of aqueous Fe^{2+} may increase, and Fe^{2+} ions can be taken up by cells through transition metal ion uptake systems, primarily the iron transport complex FeoAB (11). At higher concentrations, however, Fe^{2+} is toxic to cells. Aerobically, Fe^{2+} toxicity is thought to be caused by oxidative damage from hydroxyl radicals produced through the Fenton reaction (12), but the cause of damage under anaerobic conditions is not well understood. Several possible causes of anaerobic Fe^{2+} toxicity have been proposed, such as the production of reactive nitrogen species (13) or inhibition of the F_oF_1 ATPase (14). Regardless of the basis for toxicity, microorganisms have evolved means of minimizing the cellular damage caused by high concentrations of Fe^{2+} and other metal ions.

One of the well-characterized mechanisms that microorganisms use to prevent metal toxicity is efflux via membrane transporters. Metal efflux proteins are widespread in all three domains of life and comprise multiple protein families and superfamilies. For example, the major facilitator family includes the tetracycline-metal ion transporter TetL in *Bacillus subtilis* (15) and the iron citrate exporter IceT in *Salmonella enterica* serovar Typhimurium (16). P-type ATPases, which couple the uptake or efflux of cations to ATP hydrolysis, include the cadmium exporter CadA in *Staphylococcus aureus* and *B. subtilis* (17, 18) and the copper transporter CopA in *Escherichia coli* (19). To date, however, no proteins mediating Fe^{2+} resistance have been described in *S. oneidensis*.

A transposon screen identified mutations in gene locus SO_4475 resulting in a strong growth defect during ferric citrate respiration but not during respiration of fumarate or DMSO (E. D. Brutinel and J. A. Gralnick, unpublished data). SO_4475 is predicted to encode a metal ion exporter in the cation diffusion facilitator (CDF) family, a group of inner membrane proteins that utilize proton motive force (PMF) to export a range of divalent metal cations (20, 21). The closest homolog of SO_4475 described in the literature, at an amino acid sequence similarity of 60.9% and identity of 47.7%, is the *E. coli* protein FieF (YiiP). FieF from *E. coli* has been reported to export Zn^{2+}/Cd^{2+} by some researchers (22, 23) and Fe^{2+}/Zn^{2+} by others (24); the protein encoded by

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TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Description[a]	Reference or source
Strains		
JG274	*S. oneidensis* MR-1, wild type	3
JG2989	JG274 Δ*feoE*	This work
JG168	JG274 with empty pBBR1MCS-2, Km^r	27
JG2993	JG2989 with empty pBBR1MCS-2, Km^r	This work
JG2780	JG274 with pBBR1MCS-2::*feoE*, Km^r	This work
JG2994	JG2989 with pBBR1MCS-2::*feoE*, Km^r	This work
JG2997	JG2989 with pBBR1MCS-2::*fieF*, Km^r	This work
MG1655	*E. coli* K-12, wild type	Arkady Khodursky, University of Minnesota
JG3304	MG1655 Δ*fieF*	This work
JG3306	JG3304 with empty pBBR1MCS-2, Km^r	This work
JG3307	JG3304 with pBBR1MCS-2::*fieF*, Km^r	This work
JG3308	JG3304 with pBBR1MCS-2::*feoE*, Km^r	This work
UQ950	*E. coli* DH5α λ(*pir*) cloning host; F$^-$ Δ(*argF-lac*)*169* ϕ80d*lacZ58*ΔM15 *glnV44*(AS) *rfbD1 gyrA96* (Nal^r) *recA1 endA1 spoT1 thi-1 hsdR17 deoR* λ*pir*$^+$	26
WM3064	*E. coli* conjugation strain; *thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)*567* Δ*dapA1341*::[*erm pir*(wt)]	26
Plasmids		
pSMV3	Deletion vector, Km^r *sacB*	31
pBBR1MCS-2	Broad-host-range cloning vector, Km^r	30
pBBR1MCS-2::*feoE*	SO_4475 (*feoE*), 48 bp upstream, 51 bp downstream, Km^r	This work
pBBR1MCS-2::*fieF*	b3915 (*fieF*), 76 bp upstream, 26 bp downstream, Km^r	This work

[a] AS, amber (UAG) suppressor; wt, wild type.

SO_4475 was described as exporting Zn^{2+}/Cd^{2+} (25), although Fe^{2+} transport was not evaluated in that study. Here we characterize SO_4475, which we name *feoE* (for *fe*rr*o*us iron *e*xport), and show physiological evidence demonstrating that the protein encoded by *feoE* exports excess Fe^{2+} from *S. oneidensis* and is important for survival under iron-reducing conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. oneidensis* strain MR-1 was originally isolated from Lake Oneida in New York State (3). The *E. coli* strains used for cloning (UQ950) and mating (WM3064) have been previously described (26). *E. coli* K-12 strain MG1655 was used for FieF analysis. The strains used for cloning, derivative strains of MR-1 and MG1655, and the plasmids used in this study are found in Table 1. Liquid overnight Luria-Bertani (LB) cultures supplemented with 50 μg/ml kanamycin, when appropriate, were inoculated with colonies isolated from freshly streaked −80°C stocks. All cultures were grown at 30°C (*S. oneidensis*) or 37°C (*E. coli*); liquid cultures were shaken at 250 rpm. Unless otherwise noted, all experiments using liquid and solid media were performed with LB; where indicated, *Shewanella* basal medium (SBM) (27) supplemented with 0.05% (wt/vol) Casamino Acids, 5 ml/liter vitamin solution (28), and 5 ml/liter mineral solution (29) was used as a defined minimal medium. Anaerobic cultures were flushed with nitrogen gas and supplemented with 20 mM sodium lactate and an electron acceptor, as indicated below. Results are reported as the means from three biological replicates ± 1 standard deviation. Data were statistically analyzed using analysis of variance.

Plasmid and mutant construction. The primers used to construct the plasmids are listed in Table 2. In-frame deletion of *feoE* from the MR-1 genome and *fieF* from the MG1655 genome was performed as previously described (26). Briefly, fragments 1 kb upstream and downstream of *feoE* (SO_4475) with flanking SacI and BamHI sites were fused via a SpeI restriction site and ligated into the suicide vector pSMV3, which has kanamycin resistance and *sacB* cassettes. Fragments 1 kb upstream and downstream of *fieF* (b3915) with flanking SpeI and BamHI restriction sites were fused via a SacII site and ligated into pSMV3. To make the *feoE*

TABLE 2 Primers used for mutant construction and complementation in this work

Primer	Sequence	Restriction site
4475USF	GTACGGATCCGCAGAGCGCGTAACACTTC	BamHI
4475USR	GTACACTAGTCCATTGTATATCAGCTTGGCG	SpeI
4475DSF	GTACACTAGTGCGACACTGAATCGATTATTCAAC	SpeI
4475DSR	GTACGAGCTCGCTCAGTCACAGCGGCATTAACAC	SacI
4475CompF	GTACGGATCCCGCCAAGCTGATATACAATGG	BamHI
4475CompR	GTACACTAGTGGCATAACCACTCCTTTGATTG	SpeI
ECfieFUSF	GATCACTAGTCGATACCATTTTTCTTCGGC	SpeI
ECfieFUSR	GATCCCGCGGCATAAATACTCCCGCTATCAAC	SacII
ECfieFDSF	GATCCCGCGGCGGTCTATGCTTTCATAATCAG	SacII
ECfieFDSR	GATCGGATCCCATACGGGAAGCCAGAATAC	BamHI
ECfiefF	GTACGGATCCCAATTTGCCTGCTGCTTAATGC	BamHI
ECfiefR	GTACACTAGTGCGGGTCTGGCTCTCTTTTATAC	SpeI

complementation vector, *feoE* was cloned from the *S. oneidensis* MR-1 genome with flanking BamHI and SpeI restriction sites and inserted into the pBBR1MCS-2 multiple-cloning site (30). To make the *fieF* complementation vector, *fieF* (b3915) was cloned from the *E. coli* MG1655 genome with flanking BamHI and SpeI restriction sites and inserted into the multiple-cloning site of pBBR1MCS-2.

Growth curves. Overnight cultures of each strain were pelleted, washed once, and resuspended in fresh LB or SBM. For aerobic and anaerobic Fe^{3+} cultures, SBM was supplemented with 20 mM sodium lactate and 80 mM ferric citrate. The growth of anaerobic Fe^{3+} cultures was measured by periodically plating serial 1:10 dilutions of each culture onto LB plates and performing colony counts after 1 day of incubation. The growth of aerobic Fe^{3+} cultures was measured by taking the optical density at 600 nm (OD_{600}). For cultures with divalent metals, LB was supplemented with 0.45 mM $CdCl_2$; 0.8 mM $CoCl_2$; 2.2 mM $CuCl_2$; 20 mM sodium lactate, 40 mM sodium fumarate, and 1.0 mM, 2.5 mM, 3.5 mM, 5.0 mM, or 7.0 mM $FeCl_2$; 20 mM sodium lactate, 40 mM sodium fumarate, and 8.0 mM $MnCl_2$; 1.0 mM $NiCl_2$; or 1.0 mM $ZnCl_2$. Cultures with $FeCl_2$ and $MnCl_2$ were grown anaerobically to prevent the oxidation of Fe^{2+} to Fe^{3+} or Mn^{2+} to Mn^{4+}. Growth was periodically measured by taking the OD_{600} using a spectrophotometer.

Iron citrate reduction assay. Fe^{3+} respiration was measured using ferrozine assays as previously described (31). Briefly, overnight cultures of each strain were pelleted, washed once, resuspended in fresh SBM, and adjusted to an OD_{600} of 1.00. Thirty microliters of this suspension was inoculated into 270 μl SBM with 20 mM sodium lactate, 5 mM ferric citrate, 5 ml/liter vitamin solution, and 5 ml/liter mineral solution in anaerobic 96-well plates. Fe^{2+} production was monitored over time by determining the ferrozine absorbance at 542 nm (32).

Iron retention assay. Overnight cultures of each strain were pelleted, washed once, and resuspended in fresh LB. Suspensions were inoculated into 5-ml anaerobic cultures of LB with 20 mM sodium lactate and 40 mM sodium fumarate at an OD_{600} of 0.05. Cultures were incubated at 30°C until the growth reached an OD_{600} of approximately 0.50. $FeCl_2$ was spiked into each culture at a concentration of 2.5 mM, and the cultures were incubated at 30°C for 1 h. Each culture was pelleted, washed once, and resuspended in 5 ml fresh SBM. Cell suspensions were assayed for iron concentration via inductively coupled plasma mass spectrometry (ICP-MS) analysis by the Analytical Geochemistry Lab in the Department of Earth Sciences at the University of Minnesota. Iron concentrations were normalized to the final OD_{600} before harvesting.

RESULTS

The Δ*feoE* mutant has decreased survival with ferric citrate as an electron acceptor. A transposon screen indicated that inactivation of *feoE* (SO_4475) in *S. oneidensis* caused a growth defect during anaerobic ferric citrate respiration but not during respiration of fumarate or DMSO (Brutinel and Gralnick, unpublished). These results indicate that the protein product of *feoE* is important for growth during Fe^{3+} respiration, rather than anaerobic growth in general. To confirm the results of the transposon screen, an in-frame deletion of *feoE* was made in *S. oneidensis*. No significant differences in the growth rate between the Δ*feoE* mutant and the wild type were found in anaerobic cultures supplemented with 20 mM lactate and 40 mM fumarate (doubling times, 1.94 ± 0.23 and 1.91 ± 0.22 h, respectively) or 20 mM lactate and 40 mM DMSO (doubling times, 1.48 ± 0.16 and 1.45 ± 0.15 h, respectively). To evaluate the importance of *feoE* during respiration of Fe^{3+}, growth was monitored in anaerobic cultures supplemented with 20 mM lactate and 80 mM ferric citrate. Deletion of *feoE* resulted in impaired growth over time during iron respiration compared to that of the wild type (Fig. 1). Complementation of the Δ*feoE* mutant and the wild type with pBBR1MCS-2::*feoE* enhanced the log-phase growth rate of both strains over that of the wild type with the empty vector (doubling times, 1.57 ± 0.48, 1.48 ± 0.66, and 4.71 ± 1.20 h, respectively). The complemented strains also displayed steeper die-off than either strain MR-1 or the Δ*feoE* mutant with the empty vector (Fig. 1). A similar growth impairment of the Δ*feoE* mutant was observed in anaerobic SBM cultures supplemented with 20 mM lactate, 40 mM fumarate, and 1 mM ferric citrate (see Fig. S1 in the supplemental material).

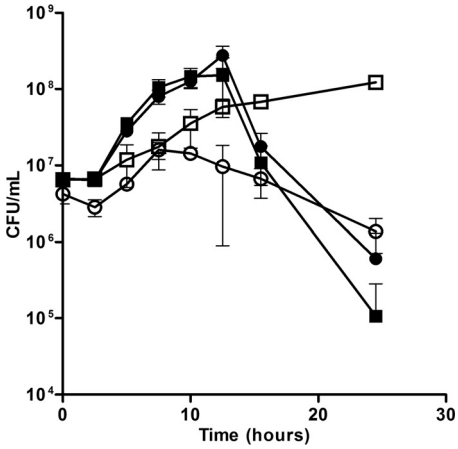

FIG 1 Anaerobic growth of the wild-type and Δ*feoE* strains on ferric citrate. The rate of growth in SBM with 20 mM lactate and 80 mM ferric citrate over time was measured for the Δ*feoE* mutant with empty pBBR1MCS-2 (○), MR-1 with empty pBBR1MCS-2 (□), the Δ*feoE* mutant with pBBR1MCS-2::*feoE* (●), and MR-1 with pBBR1MCS-2::*feoE* (■). Growth was determined by counting the numbers of CFU per milliliter of culture medium.

To rule out the possibility that the growth defect seen for the Δ*feoE* mutant during Fe^{3+} respiration was due to an increased sensitivity to citrate or soluble Fe^{3+}, the aerobic growth of cultures supplemented with 20 mM lactate and 80 mM ferric citrate was evaluated. The growth rate of the Δ*feoE* mutant showed no difference from that of the wild type during aerobic respiration in the presence of ferric citrate (doubling times, 1.16 ± 0.04 and 1.15 ± 0.04 h, respectively, during log phase).

Deletion of *feoE* does not impair ferric citrate respiration. To determine whether the impaired growth of the Δ*feoE* mutant during anaerobic Fe^{3+} respiration was due to a defect in the strain's ability to use Fe^{3+} as an electron acceptor, ferrozine assays were performed to measure the production of Fe^{2+} from the respiration of ferric citrate. No statistically significant difference ($P > 0.05$) in the initial rate of Fe^{2+} production was observed between the Δ*feoE* mutant and MR-1 whether they were complemented with pBBR1MCS-2::*feoE* or with the empty vector (Fig. 2). Complementation of the Δ*feoE* mutant and the wild type with pBBR1MCS-2::*feoE* led to the production of final concentrations of Fe^{2+} slightly higher than those produced by the strains complemented with the empty vector ($P < 0.01$; Fig. 2).

Δ*feoE* mutants exhibit greater sensitivity to Fe^{2+}. Because the Δ*feoE* mutant was not defective in reducing Fe^{3+}, we hypothesized that the growth defect displayed by the Δ*feoE* mutant during Fe^{3+} respiration was due to increased sensitivity to Fe^{2+}, the by-product of Fe^{3+} respiration. To determine if the Δ*feoE* mutant is more sensitive to Fe^{2+} than the wild type, cultures of the Δ*feoE* mutant and the wild type with pBBR1MCS-2 and pBBR1MCS-2::*feoE* were grown anaerobically in LB with 20 mM lactate, 40 mM fumarate, and 1 mM $FeCl_2$. The Δ*feoE* mutant displayed a greater

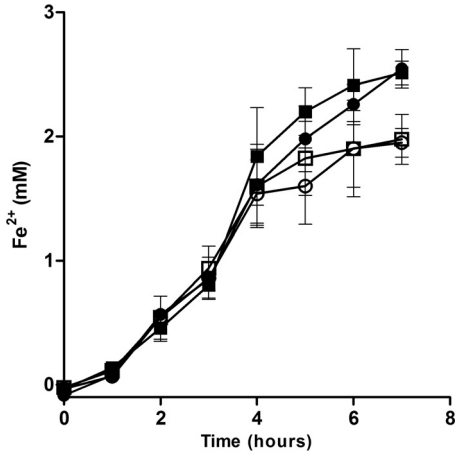

FIG 2 Ferric citrate reduction by the wild-type and the Δ*feoE* mutant strains. The rate of ferric citrate reduction was measured for the Δ*feoE* mutant with empty pBBR1MCS-2 (○), MR-1 with empty pBBR1MCS-2 (□), the Δ*feoE* mutant with pBBR1MCS-2::*feoE* (●), and MR-1 with pBBR1MCS-2::*feoE* (■).

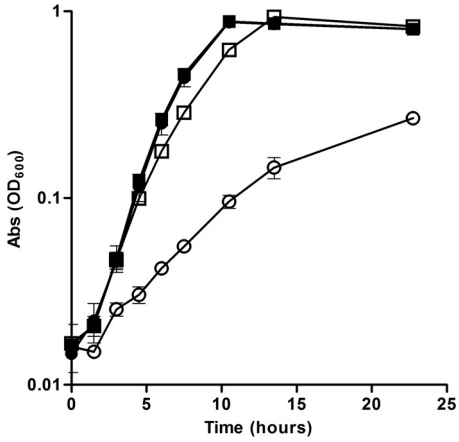

FIG 3 Growth of the wild-type and Δ*feoE* strains in the presence of excess Fe^{2+}. The rate of growth in anaerobic LB with 20 mM lactate, 40 mM fumarate, and 1 mM $FeCl_2$ was measured for the Δ*feoE* mutant with empty pBBR1MCS-2 (○), MR-1 with empty pBBR1MCS-2 (□), the Δ*feoE* mutant with pBBR1MCS-2::*feoE* (●), and MR-1 with pBBR1MCS-2::*feoE* (■). Abs, absorbance.

sensitivity to Fe^{2+} than the parent strain, as indicated by a lower growth rate (Fig. 3). Complementation of the mutant with pBBR1MCS-2::*feoE* restored the rate of growth in the presence of Fe^{2+} to that of the wild type (Fig. 3). The average doubling times during log phase were 3.69 ± 0.14 h for the Δ*feoE* mutant with the empty vector, 2.35 ± 0.16 h for the Δ*feoE* mutant with pBBR1MCS-2::*feoE*, 2.36 ± 0.12 h for the wild type with the empty vector, and 2.41 ± 0.16 h for the wild type with pBBR1MCS-2::*feoE*.

Loss of *feoE* increases Fe^{2+} retention. The protein product of *feoE* has been annotated as belonging to the CDF protein family, the members of which confer increased resistance to a variety of divalent metal ions via active export of the ions from a cell's cytoplasm (21). In order to determine whether the increased sensitivity to Fe^{2+} seen in the Δ*feoE* mutant was due to an impaired ability to export Fe^{2+}, iron retention assays were performed. The wild type and the Δ*feoE* mutant carrying the empty vector and the pBBR1MCS-2::*feoE* vector were grown anaerobically with 20 mM lactate and 40 mM fumarate into log phase (OD_{600}, approximately 0.5) and then spiked with 2.5 mM $FeCl_2$. The concentration of 2.5 mM $FeCl_2$ was chosen because the growth of both the Δ*feoE* mutant and the wild type is decreased but not arrested at this concentration. After incubation with Fe^{2+} for 1 h, cells in each culture were harvested and analyzed by ICP-MS for total iron content. Δ*feoE* mutant cells with the empty pBBR1MCS-2 vector retained a significantly larger amount of iron ($P < 0.0001$) than the Δ*feoE* mutant with pBBR1MCS-2::*feoE*, the wild type with the empty vector, or the wild type with pBBR1MCS-2::*feoE* (218.0 ± 9.1, 148.9 ± 6.3, 141.3 ± 8.0, and 137.3 ± 7.8 ng · ml^{-1} · $OD_{600}{}^{-1}$ iron, respectively). Similar results were observed in experiments performed using SBM in place of LB (data not shown).

The export function of FeoE is specific for Fe^{2+}. To determine the export specificity of FeoE, the sensitivity of the wild type and the Δ*feoE* mutant to various divalent metals was tested. Cultures of the Δ*feoE* mutant and the wild type were grown aerobically in LB with excess $CdCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$, or $ZnCl_2$ and anaerobically in LB with 20 mM lactate, 40 mM fumarate, and excess $MnCl_2$. No difference in sensitivity to any of these divalent metals was observed between the Δ*feoE* mutant and the wild type (Fig. 4). Similarly, no difference in sensitivity to any of these metals was seen in zone-of-inhibition assays performed anaerobically on tryptone medium plates using Noble agar as the solidifying agent (see Table S1 in the supplemental material).

FeoE confers greater resistance to Fe^{2+} than the *E. coli* homolog FieF. An earlier study indicated that *E. coli* FieF, the closest characterized homolog of FeoE, may export Fe^{2+} (25). In order to determine how the function of FeoE compares to that of FieF, a cross-complementation study was performed in which the Δ*feoE* mutant was transformed with pBBR1MCS-2::*fieF*. Anaerobic cultures of the Δ*feoE* mutant with the empty vector, pBBR1MCS-2::*feoE*, or pBBR1MCS-2::*fieF* were grown in LB with 20 mM lactate, 40 mM fumarate, and 1.0 mM, 2.5 mM, 3.5 mM, or 5.0 mM $FeCl_2$. The growth rates of the Δ*feoE* mutant complemented with pBBR1MCS-2::*fieF* were similar to those of the Δ*feoE* mutant complemented with pBBR1MCS-2::*feoE* at 1.0 and 2.5 mM $FeCl_2$, but at higher Fe^{2+} concentrations, the growth of the *fieF*-complemented mutant was considerably diminished compared to that of the mutant complemented with *feoE* (Fig. 5). Similarly, *E. coli* Δ*fieF* grown anaerobically in LB with 20 mM lactate, 40 mM fumarate, and 7 mM $FeCl_2$ showed significantly impaired growth when complemented with pBBR1MCS-2::*fieF* compared with that when complemented with pBBR1MCS-2::*feoE* ($P < 0.001$; see Fig. S2 in the supplemental material).

DISCUSSION

Efflux proteins, responsible for the maintenance of intracellular concentrations of various small molecules, are found among all domains of life. The actions of cation exporters allow cells to maintain subtoxic intracellular levels of heavy metals, most frequently, Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Zn^{2+} (33). Here we have characterized FeoE, a member of the CDF family found in *S. oneidensis* MR-1 and encoded by the gene locus SO_4475 which specifically exports Fe^{2+} and is important for survival during Fe^{3+} respiration.

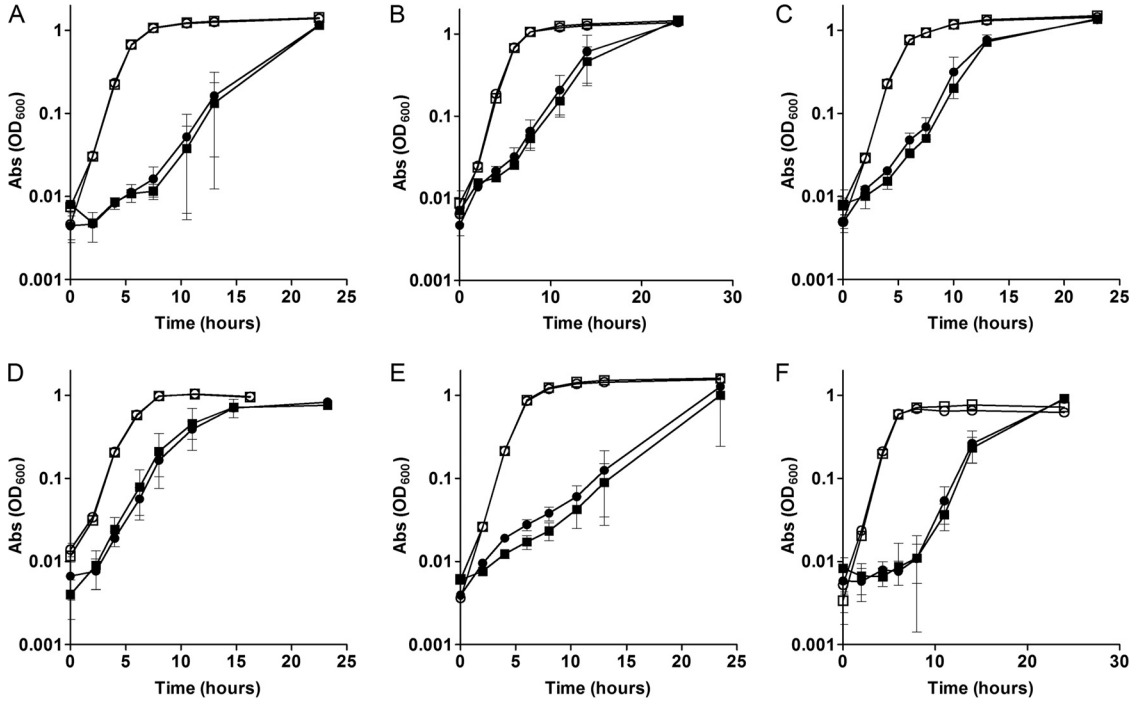

FIG 4 Growth of the wild type and the Δ*feoE* mutant in the presence of divalent metals. Growth was measured over time in LB with 0 mM or 0.45 mM $CdCl_2$ (A); 0 mM or 0.8 mM $CoCl_2$ (B); 0 mM or 2.2 mM $CuCl_2$ (C); 20 mM sodium lactate, 40 mM fumarate, and 0 mM or 8 mM $MnCl_2$ (D); 0 mM or 1.0 mM $NiCl_2$ (E); or 0 mM or 1.0 mM $ZnCl_2$ (F). □, MR-1 with no metal added; ○, the Δ*feoE* mutant with no metal added; ■, MR-1 with metal added; ●, the Δ*feoE* mutant with metal added.

FeoE is well conserved throughout the shewanellae, with closely related homologs being found in the other metal-reducing *Shewanella* spp., *Shewanella* sp. strains ANA-3, MR-4, and MR-7 and *Shewanella putrefaciens* CN-32 (92.0 to 94.3% identity), suggesting that Fe^{2+} efflux is an important strategy for these iron-respiring organisms. Interestingly, distantly related FeoE homologs can also be found in the metal-reducing *Deltaproteobacteria* species *Geobacter metallireducens* and *Geobacter sulfurreducens*, but the sequence similarity between these proteins and FeoE (23.1 to 25.6% identity) is too low to draw conclusions about function without additional experimentation.

Initial experiments indicated that deletion of SO_4475 (*feoE*) resulted in decreased cell density over time during growth with ferric citrate as a terminal electron acceptor (Fig. 1; see also Fig. S1 in the supplemental material) but not during respiration of DMSO or fumarate. Complementation of both the wild type and the Δ*feoE* mutant with a plasmid carrying *feoE* conferred an increased rate of growth to each strain during ferric citrate respiration (Fig. 1), indicating that having multiple copies of the gene allows cells to minimize the inhibitory effects of increased Fe^{2+}. Unexpectedly, both *feoE*-complemented strains displayed a rapid die-off after reaching stationary phase (Fig. 1). One possible explanation for this phenomenon is that production of excess FeoE is energetically taxing to cells. However, we think that a more likely explanation is that the initial expansion to a high cell density causes the *feoE*-complemented strains to run out of a limiting nutrient earlier than the strains complemented with an empty vector, given that the strains grow much faster with *feoE* constitu-

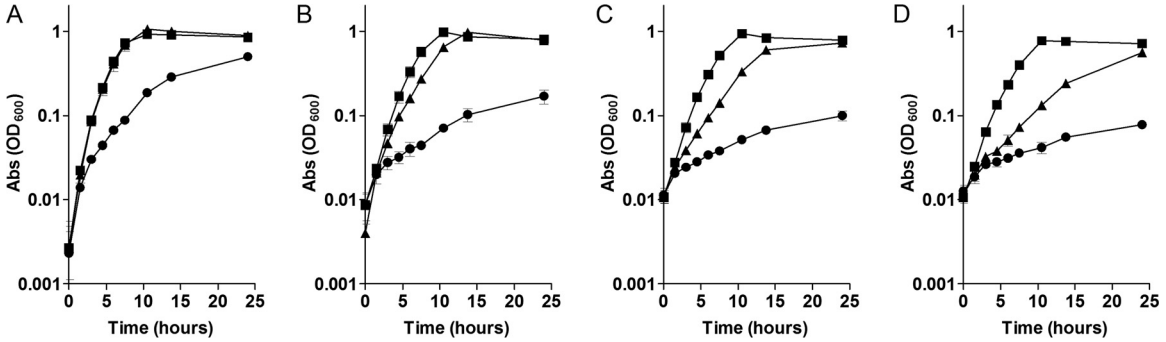

FIG 5 Growth of the Δ*feoE* mutant complemented with *feoE* or *fieF* from *E. coli* in the presence of excess Fe^{2+}. Growth in anaerobic LB with 20 mM lactate, 40 mM fumarate, and 1.0 mM (A), 2.5 mM (B), 3.5 mM (C), or 5.0 mM (D) $FeCl_2$ was measured for the Δ*feoE* mutant with empty pBBR1MCS-2 (●), pBBR1MCS-2::*feoE* (■), or pBBR1MCS-2::*fieF* (▲).

tively expressed from a multicopy vector. Alternatively, the increased amounts of FeoE in cells could result in the nonspecific efflux of a trace metal(s) required for growth.

To confirm that the decrease in cell density seen for the Δ*feoE* mutant during growth on ferric citrate (Fig. 1) was not due to a respiratory defect, production of Fe^{2+} from ferric citrate respiration by resting cells was measured. Both the Δ*feoE* mutant with the empty vector and the Δ*feoE* mutant with pBBR1MCS-2::*feoE* produced Fe^{2+} at the same rate as the corresponding wild-type strains (Fig. 2). Interestingly, the *feoE*-complemented wild-type and Δ*feoE* strains produced slightly but statistically significant ($P < 0.01$) increased amounts of Fe^{2+} near the end of the assay (Fig. 2). It appears that, similar to the results seen in the ferric citrate growth curve (Fig. 1), production of more copies of the FeoE transporter allows *feoE*-complemented strains to minimize inhibition by Fe^{2+} and thus increase metabolic processes.

To determine whether the decline in cell density seen for the Δ*feoE* mutant during ferric citrate respiration (Fig. 1) was due to an increased susceptibility to Fe^{2+} toxicity, growth curves were performed with 1 mM $FeCl_2$. The *feoE* mutant with the empty vector displayed a notably lower rate of growth than the Δ*feoE* mutant with pBBR1MCS-2::*feoE*, the wild type with an empty vector, or the wild type with pBBR1MCS-2::*feoE*, indicating that deletion of *feoE* caused greater sensitivity to Fe^{2+} (Fig. 3). Commensurate with the findings of the ferric citrate respiration assays (Fig. 1 and 2), both the wild type and the Δ*feoE* mutant complemented with *feoE* displayed significant ($P < 0.0001$) increases in log-phase growth rate over that of the wild type with the empty vector in the presence of excess Fe^{2+} (Fig. 3), again suggesting that the enhanced activity of FeoE facilitates greater resistance to Fe^{2+}.

To verify that the increased Fe^{2+} sensitivity of the Δ*feoE* mutant was due to higher cellular concentrations of iron, analysis of the iron concentration in the wild type and the Δ*feoE* mutant strains was performed. Δ*feoE* mutant cells with the empty vector retained considerably more iron than the Δ*feoE* mutant with pBBR1MCS-2::*feoE*, the wild type with the empty vector, or the wild type with pBBR1MCS-2::*feoE*. Greater iron retention by the Δ*feoE* mutant indicates that the increased sensitivity of the Δ*feoE* mutant to Fe^{2+} (Fig. 3) is due to the inability of the mutant to export excess Fe^{2+} from the cytoplasm through FeoE.

Previous studies have characterized *E. coli* FieF as being an Fe^{2+}, Cd^{2+}, and/or Zn^{2+} exporter (23, 24). FieF and FeoE have a 47.7% amino acid sequence identity, which is below the typical threshold for substrate specificity prediction (34, 35). In order to determine whether FeoE is responsible for the export of any other divalent metals known to be substrates of cation diffusion facilitators, growth curves in LB with or without an excess of Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+} were performed for the wild-type and Δ*feoE* mutant *S. oneidensis* strains. No difference in sensitivity to any of these metals was seen between the two strains (Fig. 4; see Table S1 in the supplemental material), indicating that FeoE is specific for the export of Fe^{2+}.

In order to compare the functionality of *S. oneidensis* FeoE to that of *E. coli* FieF, the growth rates of the respective mutants and complemented strains of each species were compared. The mutant strains complemented with pBBR1MCS-2::*feoE* and pBBR1MCS-2::*fieF* had similar growth rates at an Fe^{2+} concentration of 1.0 or 2.5 mM (Fig. 5), but a difference in the ability of each strain to resist Fe^{2+} toxicity emerged at higher concentrations (Fig. 5; see also Fig. S2 in the supplemental material). The strains carrying *fieF* had lower growth rates in the presence of higher Fe^{2+} concentrations, suggesting that FeoE activity results in more effective Fe^{2+} export. The vector and expression strategies used for *fieF* and *feoE* complementation were identical; therefore, expression levels should not influence the activity differences observed *in vivo*.

To determine a potential explanation for the difference in transport specificity and efficiency between FeoE and FieF, we compared the protein sequences of each. Structural studies of FieF have determined that metal binding sites A and B are responsible for Zn^{2+} transport, while binding site C is important for the structural integrity of the homodimer (25, 36). Despite an amino acid sequence identity between FeoE and FieF of 47.7%, the key metal-coordinating residues in the three metal binding sites, as well as those responsible for salt bridge formation (36), were conserved among all 47 *E. coli* and 29 *Shewanella* strains investigated (see Table S2 in the supplemental material), aside from one metal-binding residue at site 285 in *E. coli* M605. However, one difference between FieF and FeoE was found in a residue at the cytoplasmic end of transmembrane helix 2 (TM2). In all *E. coli* strains investigated, this residue is a glutamine at position 65, which forms a hydrogen bond with a zinc-coordinating histidine at position 75 in binding site B (36). The genomes of nearly all *Shewanella* spp. investigated encode a valine in place of a glutamine at this position. *Shewanella denitrificans* and *Shewanella amazonensis* encode an alanine and a threonine at this position, respectively. The replacement of glutamine, a polar residue, with a hydrophobic one, such as valine, could alter the conformation of binding site B, thereby changing the metal coordination geometry. Additionally, two residues important for dimerization in *E. coli*, an aspartic acid at position 69 and a serine at position 70, also located at the base of TM2 (36), are poorly conserved among the shewanellae. Any of these three substitutions could also affect the orientation of TM2 and therefore change the coordination geometry of metal binding site A. Alternatively, one or more of these substitutions could influence the hinge architecture at the base of TM2, affecting the conformational change that occurs to facilitate cation exchange (25). Currently there is no high-resolution structural information available for FeoE. Further investigation would be needed to determine if these residues affect transport specificity and efficiency.

The difference in Fe^{2+} transport efficiency between FieF and FeoE should not be surprising, considering the environmental conditions in which their respective species are found: the primary environmental niche of *E. coli* is the mammalian and avian intestinal tract (37, 38), where microorganisms must frequently scavenge for adequate iron rather than mitigate the toxic effects of high iron concentrations (39). Meanwhile, *Shewanella* spp. thrive in the oxic/anoxic transition zones of sediments, often rich in iron and manganese cycling between their oxidized and reduced states (1, 40), causing continual shifting between aerobic and anaerobic respiratory strategies in the cells. Retaining a low concentration of intracellular iron would become especially important upon a return to oxygen respiration, in order to minimize the production of damaging reactive oxygen species. *S. oneidensis* has therefore likely evolved a greater Fe^{2+} export efficiency by FeoE, affording it better survival in iron-rich, redox-active environments.

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