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PHYSIOLOGY

MgtE Homolog FicI Acts as a Secondary Ferrous Iron Importer in *Shewanella oneidensis* Strain MR-1

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ABSTRACT The transport of metals into and out of cells is necessary for the maintenance of appropriate intracellular concentrations. Metals are needed for incorporation into metalloproteins but become toxic at higher concentrations. Many metal transport proteins have been discovered in bacteria, including the Mg^{2+} transporter E (MgtE) family of passive Mg^{2+}/Co^{2+} cation-selective channels. Low sequence identity exists between members of the MgtE family, indicating that substrate specificity may differ among MgtE transporters. Under anoxic conditions, dissimilatory metal-reducing bacteria, such as *Shewanella* and *Geobacter* species, are exposed to high levels of soluble metals, including Fe^{2+} and Mn^{2+}. Here we characterize SO_3966, which encodes an MgtE homolog in *Shewanella oneidensis* that we name FicI (*f*errous *i*ron and *c*obalt *i*mporter) based on its role in maintaining metal homeostasis. A SO_3966 deletion mutant exhibits enhanced growth over that of the wild type under conditions with high Fe^{2+} or Co^{2+} concentrations but exhibits wild-type Mg^{2+} transport and retention phenotypes. Conversely, deletion of *feoB*, which encodes an energy-dependent Fe^{2+} importer, causes a growth defect under conditions of low Fe^{2+} concentrations but not high Fe^{2+} concentrations. We propose that FicI represents a secondary, less energy-dependent mechanism for iron uptake by *S. oneidensis* under high Fe^{2+} concentrations.

IMPORTANCE *Shewanella oneidensis* MR-1 is a target of microbial engineering for potential uses in biotechnology and the bioremediation of heavy-metal-contaminated environments. A full understanding of the ways in which *S. oneidensis* interacts with metals, including the means by which it transports metal ions, is important for optimal genetic engineering of this and other organisms for biotechnology purposes such as biosorption. The MgtE family of metal importers has been described previously as Mg^{2+} and Co^{2+} transporters. This work broadens that designation with the discovery of an MgtE homolog in *S. oneidensis* that imports Fe^{2+} but not Mg^{2+}. The research presented here also expands our knowledge of the means by which microorganisms have adapted to take up essential nutrients such as iron under various conditions.

KEYWORDS *Shewanella oneidensis*, iron acquisition, iron transport

The dissimilatory metal-reducing bacterium *Shewanella oneidensis* strain MR-1, commonly found in the oxic-anoxic transition zones of aquatic sediments (1), can impact the geochemical cycling of metals. Known for their highly versatile respiratory capacity, most *Shewanella* species are able to use numerous compounds as terminal electron acceptors, including oxygen, fumarate, nitrate, and sulfite (1–3), as well as metals such as iron(III), manganese(IV), chromium(IV), arsenate(VI), uranium(IV), and cobalt(III) (1, 4–7). The oxidation or reduction of a metal can influence its physical properties, including solubility. Iron, for example, is commonly found in soils and sediments as an insoluble ferric (Fe^{3+}) (hydr)oxide; upon reduction to ferrous iron

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TABLE 1 Amino acid identities between MgtE homologs

MgtE homolog (reference)	Amino acid identity (%) with: SO_1145	SO_1565	FicI	*Providencia stuartii* MgtE	*Thermus thermophilus* HB8 MgtE	*Pseudomonas aeruginosa* PAO1 MgtE
SO_1565	23					
FicI	24	36				
Providencia stuartii MgtE (16)	31	24	25			
Thermus thermophilus HB8 MgtE (17)	33	25	29	33		
Pseudomonas aeruginosa PA14 MgtE (45)	27	35	40	28	29	
Aeromonas piscicola AH-3 MgtE (46)	29	25	25	55	35	27

(Fe^{2+}), however, it may become soluble or be reincorporated into mixed-valence minerals, depending on environmental conditions (8, 9). Therefore, as Fe^{3+} respiration by dissimilatory metal-reducing bacteria proceeds, minerals can dissolve, releasing into the surrounding medium bioavailable metals, toxins, and nutrients that had previously been adsorbed to the minerals. When the sediment is again exposed to oxygen, the iron becomes oxidized and re-forms into insoluble minerals. Due to the cyclic nature of the availability of electron acceptors and soluble metals in their environments, *S. oneidensis* and other bacteria living under metal-rich conditions have likely evolved means of adapting rapidly to changes in extracellular metal concentrations.

Microorganisms require metals to facilitate structural stability and enzymatic activity in numerous proteins, including cytochromes, peptidases, and oxidoreductases (10). The major mechanism by which bacteria maintain appropriate intracellular metal concentrations is the activity of transport proteins. Numerous metal transport proteins have been described across all three domains of life, including members of the cation diffusion facilitator (11, 12), metal ion transporter (13, 14), Mg^{2+} transporter E (MgtE) (15–18), and P-type ATPase (19–21) protein families. MgtE transporters appear to act as channels in which the cytoplasmic domain senses the Mg^{2+} concentration through reversibly bound Mg^{2+} ions (17, 18). MgtE adopts a closed-gate formation under high Mg^{2+} concentrations, when the Mg^{2+} ions are bound to the cytoplasmic domain; under low Mg^{2+} concentrations, bound Mg^{2+} ions are released, and the protein becomes an open channel (17, 18). Thus far, no energetic mechanism necessary for the opening or closing of the MgtE channel has been discovered.

S. oneidensis requires a large iron supply for the production of heme groups, the redox-active cofactors in the many *c*-type cytochromes that allow *S. oneidensis* to respire numerous electron acceptors anaerobically. Bacteria have multiple mechanisms for the maintenance of intracellular iron concentrations. In *S. oneidensis* growing anaerobically, when Fe^{2+} levels become too high, the inner membrane exporter FeoE removes excess Fe^{2+} from the cytoplasm (22); when iron levels are low, the trans-periplasmic TonB-dependent Fe^{3+} import system and the inner-membrane Fe^{2+} importer FeoB drive the uptake of iron into the cytoplasm using energy-dependent mechanisms (23–26). In this work, we describe a third Fe^{2+} transport protein in *S. oneidensis*, which imports Fe^{2+} at higher concentrations; we name this protein FicI.

RESULTS

Deactivation of one of three *mgtE* homologs in the *S. oneidensis* genome affects fitness at high Fe^{2+} concentrations. Three genes in the *S. oneidensis* genome have been annotated as encoding MgtE-family Mg^{2+}/Co^{2+} transporters: SO_1145, SO_1565, and SO_3966 (27, 28). Homologs of these three MgtE proteins are well conserved among many *Shewanella* species (of 36 *Shewanella* genomes, 16 encode a homolog of SO_1145, 21 encode a homolog of SO_1565, and 26 encode a homolog of SO_3966). However, the amino acid sequences encoded by each *S. oneidensis mgtE* gene share little sequence identity with each other and with MgtE proteins identified in other species (Table 1). While the mechanism of metal transport is likely to be similar for the *S. oneidensis* homologs and other described MgtE family members, substrate

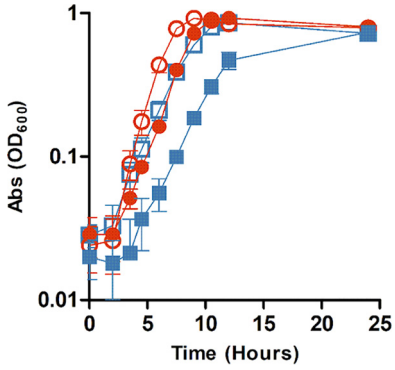

FIG 1 Anaerobic growth of wild-type and Δ*ficI* strains with excess $FeCl_2$. The rate of growth in anoxic tryptone medium with 20 mM lactate, 40 mM fumarate, and 2 mM $FeCl_2$ was measured for wild-type *S. oneidensis* with an empty pBBR1MCS-2 vector (open squares), wild-type *S. oneidensis* with pBBR1MCS-2::*ficI* (filled blue squares), *S. oneidensis* Δ*ficI* with an empty pBBR1MCS-2 vector (open circles), and *S. oneidensis* Δ*ficI* with pBBR1MCS-2::*ficI* (filled red circles). Results represent means for three biological replicates ± 1 standard deviation. Abs, absorbance.

specificity is more difficult to predict (29, 30). A transposon sequencing (Tn-Seq) screen indicated that while inactivation of SO_3966 had no effect on the growth rate in the presence of low Fe^{2+} concentrations, the mutation conferred a fitness benefit under high Fe^{2+} concentrations; however, no significant fitness effect was seen for either SO_1145 or SO_1565 under either condition (49). Here we characterize the function and specificity of SO_3966, which we name FicI (*f*errous *i*ron and *c*obalt *i*mporter), and describe its role in mediating cytoplasmic Fe^{2+} and Co^{2+} concentrations.

Loss of SO_3966 (*ficI*) enhances resistance to Fe^{2+}. To confirm the results of the transposon screen, an in-frame deletion of *ficI* was made in *S. oneidensis*. *S. oneidensis* Δ*ficI* and wild-type *S. oneidensis* containing either an empty vector (pBBR1MCS-2) or a complementation vector (pBBR1MCS-2::*ficI*) were grown anaerobically in tryptone medium supplemented with lactate and fumarate, with or without 2 mM $FeCl_2$. The Δ*ficI* strain displayed no difference in phenotype from the wild type while growing on tryptone medium without metal supplementation (doubling times, 0.90 ± 0.08 and 0.88 ± 0.08 h, respectively). However, when $FeCl_2$ was added to the medium, the Δ*ficI* strain with the empty vector displayed enhanced growth over that of the wild type with the empty vector (Fig. 1). Complementation of either the wild type or the Δ*ficI* mutant with *ficI* enhanced sensitivity to Fe^{2+} (Fig. 1); the wild-type strain with the empty vector had the same growth phenotype as the complemented Δ*ficI* strain (Fig. 1). These phenotypes are consistent with FicI playing a role in Fe^{2+} uptake, where a strain lacking FicI (the Δ*ficI* strain with an empty pBBR1MCS-2 vector) is most resistant to Fe^{2+}, while a strain with high levels of FicI (the wild type with pBBR1MCS-2::*ficI*) displays higher sensitivity to Fe^{2+} (Fig. 1).

Deletion of *ficI* decreases cellular iron retention. To determine whether the decreased Fe^{2+} sensitivity of the Δ*ficI* mutant was due to altered Fe^{2+} uptake, iron retention assays were performed. The Δ*ficI* and wild-type strains with the empty vector or a *ficI* complementation vector were grown anaerobically in tryptone medium with 20 mM lactate and 40 mM fumarate to log phase (optical density at 600 nm [OD_{600}], ~0.5) and were then supplemented with 2.5 mM $FeCl_2$. Cultures were harvested after 1 h of further incubation and were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for total iron content. The average amount of iron retained by the Δ*ficI* strain with an empty vector was 91% that of the median amount retained by the wild type with an empty vector ($P < 0.0001$) (Table 2). The wild-type or Δ*ficI* strain with an empty vector retained approximately half the concentration of iron ($P < 0.0001$) that was retained by the wild-type or Δ*ficI* strain carrying the complementation vector (Table 2). Given the annotation of FicI as a homolog of MgtE, we tested other divalent metals known to be transported by this class of channels.

TABLE 2 Metal retention by wild-type and Δ*ficI* strains

Strain	Avg ng · OD_{600}^{-1} · ml^{-1} of indicated element	SD
	Fe	
Wild type + empty pBBR1MCS-2	109.1	12.0
Wild type + pBBR1MCS-2::*ficI*	225.5	15.1
Δ*ficI* strain + empty pBBR1MCS-2	99.9	4.0
Δ*ficI* strain + pBBR1MCS-2::*ficI*	200.9	16.32
	Co	
Wild type	55.4	2.1
Δ*ficI* strain	26.0	1.4
	Mg	
Wild type	204.2	10.4
Δ*ficI* strain	204.6	13.2

FicI imports Co^{2+} but not Mg^{2+}. MgtE proteins have been described in other bacterial species as importers of Mg^{2+} and Co^{2+} (15, 16). To determine whether FicI imports either of these metals in addition to Fe^{2+}, *S. oneidensis* Δ*ficI* and wild-type *S. oneidensis* cultures were grown anaerobically with or without concentrations of Mg^{2+} or Co^{2+} that impair growth (0.7 mM $CoCl_2$ or 230 mM $MgCl_2$). No difference in sensitivity to Mg^{2+} was seen between the wild-type and Δ*ficI* strains (doubling times, 2.02 ± 0.14 and 2.03 ± 0.21 h, respectively) (Fig. 2A); however, the Δ*ficI* strain displayed a higher growth rate than the wild type when grown with excess Co^{2+} (doubling times, 1.37 ± 0.13 and 1.72 ± 0.28 h, respectively) (Fig. 2B). To determine whether the increased growth rate seen for the Δ*ficI* strain in excess Co^{2+} was due to a decrease in Co^{2+} uptake, cobalt retention assays were performed on cultures grown aerobically in tryptone medium supplemented with 0.7 mM $CoCl_2$. The Δ*ficI* strain retained >50% less cobalt ($P < 0.0001$) than the wild type (Table 2). There was no difference in magnesium retention when *S. oneidensis* Δ*ficI* and wild-type *S. oneidensis* were grown aerobically in tryptone medium supplemented with 230 mM $MgCl_2$ (Table 2).

***S. oneidensis* Δ*ficI* has enhanced survival when grown in the presence of $[Co(III)\text{-}EDTA]^-$.** Since *ficI* deletion conferred a growth rate higher than that of the wild type in the presence of a high concentration of Co^{2+}, we hypothesized that *ficI* deletion would confer a benefit under conditions where Co^{3+} is respired. Wild-type and Δ*ficI* strains were grown anaerobically in *Shewanella* basal medium (SBM) with 20 mM lactate, 40 mM fumarate, and 5 mM $[Co(III)\text{-}EDTA]^-$. Fumarate was added to enhance the growth of the culture. No difference in growth rate was seen for the two strains; however, after entry into stationary phase, the wild type displayed a significant survival

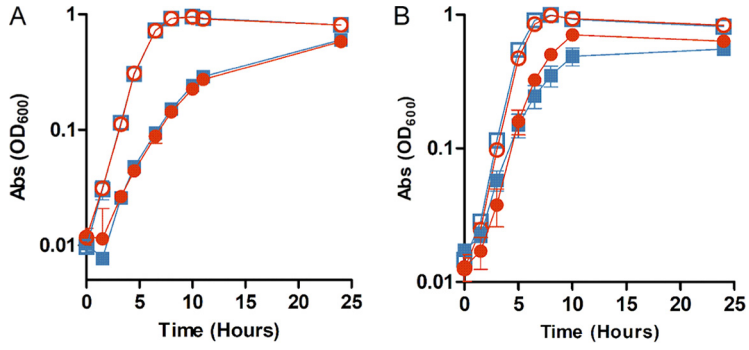

FIG 2 Growth of wild-type *S. oneidensis* and *S. oneidensis* Δ*ficI* with excess $MgCl_2$ or $CoCl_2$. The rate of growth was measured for wild-type *S. oneidensis* and *S. oneidensis* Δ*ficI* in anoxic tryptone medium with 20 mM lactate, 40 mM fumarate, and either $MgCl_2$ at 230 mM (A) or $CoCl_2$ at 0.7 mM (B). Symbols: open squares, wild type with no metal added; open circles, Δ*ficI* strain with no metal added; filled blue squares, wild type with metal added; filled red circles, Δ*ficI* strain with metal added. Results represent means for three biological replicates ± 1 standard deviation.

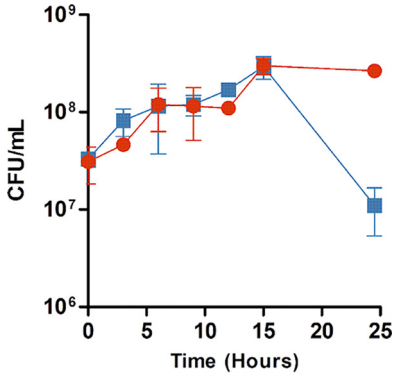

FIG 3 Anaerobic growth of wild-type *S. oneidensis* and *S. oneidensis* Δ*ficI* on $[Co(III)\text{-}EDTA]^-$. The rate of growth in anoxic SBM with 20 mM lactate, 40 mM fumarate, and 5 mM $[Co(III)\text{-}EDTA]^-$ was measured for wild-type *S. oneidensis* (filled blue squares) and *S. oneidensis* Δ*ficI* (filled red circles). Growth was determined by counting CFU per milliliter of culture medium. Results represent means for three biological replicates ± 1 standard deviation.

defect relative to the Δ*ficI* strain (Fig. 3). Dissipation of the red color associated with $[Co(III)\text{-}EDTA]^-$ (31) indicated that the bacteria were respiring the cobalt.

Characterization of MgtE homologs in *S. oneidensis*. To determine the substrate specificities of the other two MgtE homologs encoded in the *S. oneidensis* genome, SO_1155 and SO_1565, growth curves with an excess of Co^{2+}, Mg^{2+}, or Fe^{2+} were performed. Cultures of wild-type *S. oneidensis* or the ΔSO_1155, ΔSO_1565, or Δ*ficI* strain were grown anaerobically in tryptone medium with or without 2.5 mM $FeCl_2$ or 0.63 mM $CoCl_2$. Only the Δ*ficI* strain displayed a growth benefit in the presence of either excess Fe^{2+} or Co^{2+} (Fig. 4) (doubling times in excess Fe^{2+} were 1.32 ± 0.16 h for the wild type, 1.26 ± 0.18 h for the ΔSO_1145 strain, 1.29 ± 0.54 h for the ΔSO_1565 strain, and 1.03 ± 0.10 h for the Δ*ficI* strain). While deletion of the two annotated *mgtE* homologs did not exhibit phenotypes like those displayed by the Δ*ficI* mutant, we wanted to test if overexpression of these genes would result in similar phenotypes. Cultures of the wild type carrying an empty pBBR1MCS-2 vector, pBBR1MCS-2::SO_1145, pBBR1MCS-2::SO_1565, or pBBR1MCS-2::*ficI* were grown either aerobically in tryptone medium with or without 0.65 mM $CoCl_2$ or 300 mM $MgCl_2$ or anaerobically in tryptone medium supplemented with 20 mM lactate and 40 mM fumarate with or without 2.5 mM $FeCl_2$. No difference in the growth rate, as would be expected if these proteins were able to facilitate Mg^{2+} transport, was observed between any of the four

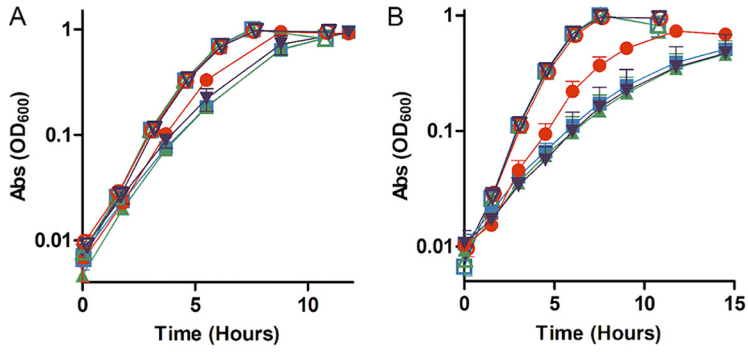

FIG 4 Anaerobic growth of the wild-type, ΔSO_1145, ΔSO_1565, and Δ*ficI* strains in excess Fe^{2+} or Co^{2+}. The rate of growth in anoxic LB with 20 mM lactate, 40 mM fumarate, and 2.5 mM $FeCl_2$ (A) or 0.63 mM $CoCl_2$ (B) was measured for wild-type *S. oneidensis* (squares) and for the Δ*ficI* (circles), ΔSO_1565 (inverted triangles), and ΔSO_1145 (triangles) strains. Open symbols, no metal added; filled symbols, excess metal. Data points for wild-type *S. oneidensis*, *S. oneidensis* ΔSO_1565, and *S. oneidensis* ΔSO_1145 overlap under each condition, and the data points for *S. oneidensis* Δ*ficI* overlap those for the other three strains when no metal is added. Results represent means for three biological replicates ± 1 standard deviation.

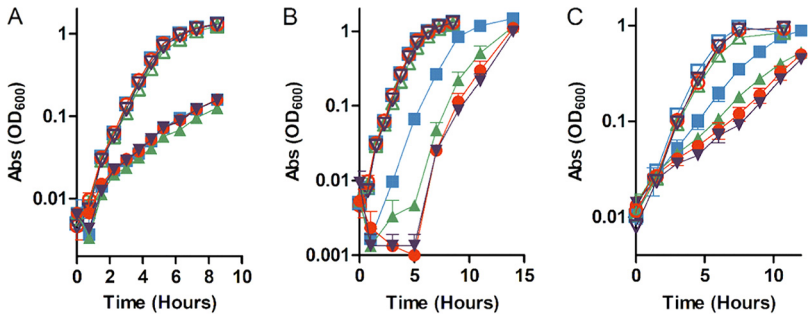

FIG 5 Growth of wild-type strains overexpressing *mgtE* homologs. The rate of growth was measured in oxic tryptone medium with or without 300 mM $MgCl_2$ (A), oxic tryptone medium with or without 0.65 mM $CoCl_2$ (B), or anoxic tryptone medium with 20 mM lactate, 40 mM fumarate, and 2.5 mM $FeCl_2$ (C). The wild-type strain carried either an empty pBBR1MCS-2 vector (squares), pBBR1MCS-2::*ficI* (circles), pBBR1MCS-2::SO_1145 (triangles), or pBBR1MCS-2::SO_1565 (inverted triangles). Open symbols, no metal added; filled symbols, excess metal. Data points for all four strains overlap under conditions with no metal added or with added $MgCl_2$. Results represent means for three biological replicates ± 1 standard deviation.

strains under excess Mg^{2+} conditions (Fig. 5A). Each strain of the wild type overexpressing an *mgtE* homolog displayed a lower growth rate under high Fe^{2+} or Co^{2+} conditions than the wild type with an empty vector (Fig. 5B and C) (doubling times at high Fe^{2+} concentrations, 1.67 ± 0.27 h for the wild type with an empty vector, 2.16 ± 0.31 h for the wild type overexpressing SO_1145, 2.82 ± 0.67 h for the wild type overexpressing SO_1565, and 3.18 ± 0.55 h for the wild type overexpressing *ficI*).

***ficI* and *feoB* mutants differ in Fe^{2+} requirements and sensitivity.** The iron retention phenotypes of strains either lacking *ficI* or with enhanced expression of *ficI* are consistent with a role for this putative transporter in Fe^{2+} import. The *S. oneidensis* genome also encodes the energy-dependent FeoAB import system, which has been described as the primary Fe^{2+} importer in multiple bacterial species (25, 26). To determine the conditions under which FeoB and FicI import Fe^{2+}, cultures of wild-type *S. oneidensis* and the Δ*feoB* and Δ*ficI* strains were grown anaerobically in tryptone medium supplemented with 20 mM lactate and 40 mM fumarate, with or without 2 mM $FeCl_2$. Under conditions without added Fe^{2+}, the Δ*ficI* and wild-type strains displayed the same growth phenotype, whereas the Δ*feoB* strain displayed a significantly lower growth rate (Fig. 6A). Under high Fe^{2+} conditions, the Δ*feoB* strain displayed the same growth rate as the wild type, while the Δ*ficI* strain had a higher growth rate than the other two strains (Fig. 6B).

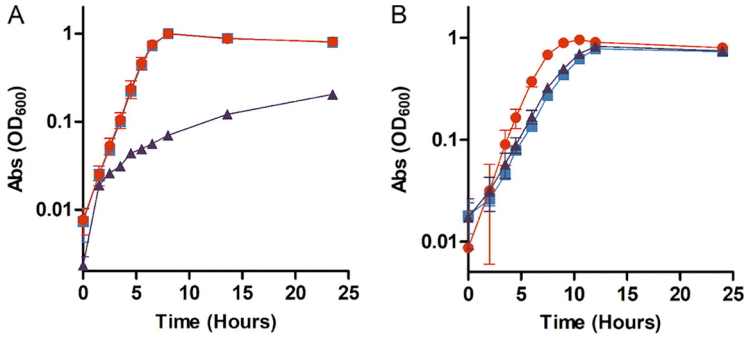

FIG 6 Growth of wild-type, Δ*ficI*, and Δ*feoB* strains. The rate of growth in anoxic tryptone medium with 20 mM lactate and 40 mM fumarate, either without $FeCl_2$ (A) or with 2 mM $FeCl_2$ (B), was measured for wild-type *S. oneidensis* (filled blue squares), *S. oneidensis* Δ*ficI* (filled red circles), and *S. oneidensis* Δ*feoB* (filled purple triangles). Data points overlap for wild-type *S. oneidensis* and *S. oneidensis* Δ*ficI* in panel A and for wild-type *S. oneidensis* and *S. oneidensis* Δ*feoB* in panel B. Results represent means for three biological replicates ± 1 standard deviation.

DISCUSSION

The import of metals, and the maintenance of intracellular metal homeostasis, is crucial for the growth and survival of all types of living cells. As such, transport proteins facilitating the import and export of biologically relevant metals have evolved in all three domains of life. Here we have described an Fe^{2+} and Co^{2+} importer in *S. oneidensis* that belongs to the MgtE protein family, members of which have until now been described as inner-membrane Mg^{2+} and Co^{2+} importers (15, 16).

Deletion of *ficI* increases resistance to high Fe^{2+} concentrations over that of the wild type, while *ficI* overexpression confers increased Fe^{2+} sensitivity (Fig. 1). These results are not due to an inherently higher growth rate for the Δ*ficI* strain (Fig. 6A). Strains overexpressing *ficI* retain approximately twice as much iron as strains with an empty vector (Table 2). Taking these phenotypes together, uptake of Fe^{2+} and sensitivity to Fe^{2+} appear to increase concomitantly with copies of *ficI*, supporting the hypothesis that *ficI* encodes a Fe^{2+} importer.

Since MgtE proteins have previously been described as Mg^{2+} and/or Co^{2+} importers, we wanted to determine whether FicI imports either of these metals in addition to Fe^{2+}. The Δ*ficI* strain had the same phenotype as the wild type when grown in excess Mg^{2+} but had a growth benefit over the wild type when grown in excess Co^{2+} (Fig. 2). Metal retention assays showed that while Δ*ficI* cells contained less cobalt than wild-type cells, there was no difference in magnesium content between the strains (Table 2). Together, these results indicate that *ficI* encodes a transporter that imports Fe^{2+} and Co^{2+} but not Mg^{2+}. We also determined that, due to the uptake of Co^{2+} by FicI, *ficI* deletion confers a survival benefit during the respiration of $[Co(III)\text{-}EDTA]^{-}$ and consequent production of Co^{2+} (Fig. 3). The uptake of Fe^{2+} or Co^{2+} via FicI could be a double-edged sword for *S. oneidensis*: FicI presents a lower-energy option for obtaining metals required for cellular functions, but excessive influx can cause toxicity, as seen when *S. oneidensis* is exposed to high concentrations of either metal (Fig. 1 and 2B).

Since FicI does not appear to transport Mg^{2+}, we wanted to determine the activities of the proteins encoded by SO_1145 and SO_1565, the other two genes in the *S. oneidensis* genome annotated as encoding MgtE homologs. In a transposon screen, inactivation of SO_1145 and SO_1565 had no significant fitness effect at either normal or high Fe^{2+} concentrations (49). Growth curves showed that deletion of SO_1145 or SO_1565 did not affect the growth rate in the presence of Fe^{2+} or Co^{2+} (Fig. 4), but, surprisingly, overexpression of all three *mgtE* homologs resulted in increased sensitivity to both Fe^{2+} and Co^{2+} (Fig. 5). The Mg^{2+} growth curves indicate that none of the MgtE homologs in *S. oneidensis* is an Mg^{2+} importer under the conditions we tested, underscoring the importance of determining a gene's physiological role with experimental data.

Aside from MgtE, three other families of Mg^{2+} importers have been discovered in bacteria: CorA, MgtA, and MgtB (32). CorA, like MgtE, is predicted to be an ion channel that imports cations along an electrochemical gradient (33), while MgtA and MgtB are P-type ATPases (20, 34). There are no genes annotated as *mgtA* or *mgtB* homologs in the *S. oneidensis* genome, but there is a predicted *corA* gene (SO_1941). CorA is more widespread in bacteria and has been shown to have a lower K_m for Mg^{2+} import than MgtA, MgtB, or MgtE in other species of *Gammaproteobacteria* (16, 35), suggesting that CorA is most likely to be the primary Mg^{2+} importer even in species encoding multiple Mg^{2+} transporters. Since no other Mg^{2+} import genes are annotated in the MR-1 genome, we postulate that CorA is likely to be the predominant Mg^{2+} importer in *S. oneidensis*.

Global expression profiles of *S. oneidensis* have reported no differential expression for SO_1145, SO_1565, or *ficI* under numerous conditions, including the respiration of various substrates (including iron and other metals) (36–38), metal stress (39, 40), and deletion of the iron response regulator Fur (41, 42). SO_1145 expression may increase approximately 2-fold upon exposure to an alkaline pH (43), the only condition shown to induce an expression change for any of these three genes. The discrepancy between

the phenotypes observed for transposon and deletion mutants (49) (Fig. 4) and overexpression strains (Fig. 5), along with the results from various expression profiles, indicates that SO_1145 and SO_1565 are likely expressed at low levels in wild-type cells, at least under the conditions tested thus far. Low expression of SO_1145 and SO_1565 could indicate that the proteins encoded by SO_1145 and SO_1565 are not normally produced under the conditions tested in our experiments, explaining why the knockout of each gene would not manifest in a detectable phenotype. Overexpression of each gene indicates that the function of each of the three MgtE transporters in *S. oneidensis* is the import of Fe^{2+} and Co^{2+}; however, the level of transport by SO_1145 and SO_1565 is likely to be low under physiologically relevant conditions, i.e., the natural environs of the bacterium. The evolutionary imperative for retaining three distinct MgtE-encoding genes in the *S. oneidensis* genome, and the roles of SO_1145 and SO_1565 in metal ion transport, remains unclear at this time, but it is likely related to the optimization of each protein for different environmental conditions.

The deactivation of *feoB*, which encodes an Fe^{2+} importer (23, 25), confers a strong fitness defect at lower Fe^{2+} concentrations but has little effect on growth at high Fe^{2+} concentrations (Fig. 6) (49). Conversely, a *ficI* mutant has the same growth rate as the wild type at lower Fe^{2+} concentrations but is less sensitive to high Fe^{2+} concentrations (Fig. 6) (49). These results indicate that FeoB is likely the primary importer of Fe^{2+} when concentrations are low, and the transporter encoded by *ficI* is likely a secondary importer, active only when extracellular Fe^{2+} concentrations are high. That *S. oneidensis* has evolved to have at least two separate systems for Fe^{2+} import should not be surprising, considering that it inhabits redox transition zones of metal-rich sediments (1). The transitory respiration of solid Fe^{3+} minerals creates temporarily high local concentrations of soluble Fe^{2+} available for uptake. Additionally, *S. oneidensis* produces a wider range of multiheme cytochromes than, for example, *Escherichia coli* or *Salmonella enterica*, and it thus has a high requirement for iron when growing anaerobically. As such, it is likely that *S. oneidensis* has adapted to have two different systems for Fe^{2+} transport under different conditions: FeoB, which uses energy to take up Fe^{2+} (25, 26), and FicI, a member of the MgtE family of passive metal importers (18). In this way, *S. oneidensis* can maximize energy conservation by using FicI when local Fe^{2+} concentrations are high and energy production is low.

Conclusion. Metal-respiring bacteria have adapted to metal-rich environments and large fluctuations in local concentrations of soluble metals. Their mechanisms for the maintenance of metal homeostasis are extremely important for survival. Here we have presented a transport protein in *S. oneidensis* that imports Fe^{2+} and Co^{2+}, which was unexpected based on phenotypic descriptions of MgtE proteins in other bacterial species. Additionally, we have determined that none of the three MgtE homologs encoded by the *S. oneidensis* genome is an Mg^{2+} importer but that all three can import Fe^{2+} and Co^{2+} when overexpressed. Furthermore, while SO_1145 and SO_1565 may not import physiologically relevant concentrations of Fe^{2+}, we suggest that FicI is a secondary, less-energy-dependent Fe^{2+} importer active at high Fe^{2+} concentrations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are summarized in Table 3. *S. oneidensis* MR-1 was originally isolated from Lake Oneida in New York State, USA (1). *Escherichia coli* strains for cloning (UQ950) and transformation (WM3064) have been described previously (5). Liquid lysogeny broth (LB) cultures were supplemented with 50 μg/ml kanamycin when appropriate and were grown overnight using colonies from freshly streaked −80°C stocks. *E. coli* cultures were grown at 37°C and *S. oneidensis* at 30°C; all liquid cultures were shaken at 250 rpm. Experimental cultures were grown in *Shewanella* basal medium (SBM) supplemented with 5 ml/liter vitamins, 5 ml/liter trace minerals, and 0.05% Casamino Acids (31) or in tryptone medium (15 g tryptone, 5 g NaCl, and 0.1 g NaOH per liter). Tryptone medium was chosen because it exhibited more consistent results with metal augmentation than LB. Where indicated, cultures were made anoxic by flushing with nitrogen gas and were supplemented with 20 mM sodium lactate and 40 mM sodium fumarate. $[Co(III)\text{-}EDTA]^-$ was made as described previously (44). The results of all experiments are reported as means for three biological replicates ± 1 standard deviation. Statistical analysis was performed using analysis of variance (ANOVA).

TABLE 3 Strains and plasmids used in this work

Strain or plasmid	Description	Source or reference
Strains		
JG274	*S. oneidensis* MR-1; wild type	1
JG3275	JG274 Δ*ficI* (SO_3966)	This work
JG3574	JG274 Δ*feoB* (SO_1784)	This work
JG168	JG274 with empty pBBR1MCS-2	31
JG3376	JG3275 with empty pBBR1MCS-2	This work
JG3379	JG3275 with pBBR1MCS-2::*ficI*	This work
JG3373	JG274 with pBBR1MCS-2::SO_1145	This work
JG3374	JG274 with pBBR1MCS-2::SO_1565	This work
JG3375	JG274 with pBBR1MCS-2::*ficI*	This work
UQ950	*E. coli* DH5α λ(pir) cloning host; F⁻ Δ(*argF-lac*)*169* ϕ80d*lacZ58*(ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(NalR) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir*⁺	5
WM3064	*E. coli* conjugation strain; *thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)*567* Δ*dapA1341*::[*erm pir*(*wt*)]	5
Plasmids		
pSMV3	Deletion vector; Km^r *sacB*	47
pBBR1MCS-2	Broad-range cloning vector; Km^r	48
pBBR1MCS-2::SO_1145	SO_1145, 11 bp upstream, 54 bp downstream; Km^r	This work
pBBR1MCS-2::SO_1565	SO_1565, 18 bp upstream, 15 bp downstream; Km^r	This work
pBBR1MCS-2::*ficI*	*ficI*, 39 bp upstream, 24 bp downstream; Km^r	This work

Plasmid and mutant construction. The primers used to create deletion and expression plasmids are listed in Table 4. In-frame deletion of *ficI* and *feoB* from the *S. oneidensis* genome was performed as described previously (5). Briefly, 1-kb fragments upstream and downstream of each gene with flanking SacI and ApaI (*ficI*) or SpeI and BamHI (*feoB*) restriction sites were fused via internal ScaI (*ficI*) or NheI (*feoB*) restriction sites and were ligated into pSMV3, which has *sacB* and kanamycin resistance cassettes. SO_1145, SO_1565, and *ficI* multicopy expression plasmids were created by cloning each gene from the *S. oneidensis* genome and inserting it into the multiple cloning site of pBBR1MCS-2 via KpnI and SacI (SO_1145) or XhoI and XbaI (SO_1565 and *ficI*) restriction sites in the proper orientation to be constitutively active.

Growth curves. Overnight cultures of each strain were pelleted, washed once, and resuspended in 1 ml fresh tryptone medium or SBM. Co^{2+}, Mg^{2+}, and Fe^{2+} growth curves were performed in tryptone medium supplemented with either 0.63, 0.65, or 0.70 mM $CoCl_2$, 230 or 300 mM $MgCl_2$, or 2.0 or 2.5 mM $FeCl_2$, respectively. Metals were added at slightly different concentrations in different batches of media in order to impair growth enough to exhibit the effects of toxicity without abolishing growth entirely. Co^{3+} growth curves were performed in anoxic SBM supplemented with 20 mM sodium lactate, 40 mM sodium fumarate, and 5 mM [Co(III)-EDTA]$^-$. The growth of tryptone medium cultures was measured by taking the optical density at 600 nm (OD_{600}). The growth of [Co(III)-EDTA]$^-$ cultures was measured by periodically plating serial 1:10 dilutions of culture aliquots onto LB agar and counting colonies after 1 day of incubation.

Metal retention assays. Overnight cultures were pelleted, washed once, and resuspended in fresh tryptone medium. Suspensions were inoculated into oxic tryptone medium (for Co^{2+} and Mg^{2+}

TABLE 4 Primers used for mutant construction and complementation in this work

Primer	Sequence	Restriction site
FeoBUSF	GTACACTAGTGTTATGATTACCCAGCGGG	SpeI
FeoBUSR	GTACGCTAGCGTGACGCAATGAAACTGCTTAG	NheI
FeoBDSF	GTACGCTAGCCGGATTATTACTGAGTAAACCC	NheI
FeoBDSR	GTACGGATCCCTCATATTGACGAGTACGATTTGG	BamHI
3966USF	GTACGAGCTCCCATTAAACTCGAAGGCAAGC	SacI
3966USR	GTACAGTACTCATGTTTCCTCCAGGGTG	ScaI
3966DSF	GTACAGTACTGCGACCTTGTATTTAATGCACTAG	ScaI
3966DSR	GTACGGGCCCCATTGATGGCGGGTATGG	ApaI
1145CompF	GTACGGTACCGAGAATGAACTATGAACATGAAC	KpnI
1145CompR	GTACGAGCTCGCTCGTACCTTTTACGCAGC	SacI
1565CompF	GTACCTCGAGCAGCAGAAGGGCGTTTAG	XhoI
1565CompR	GTACTCTAGACGCTTAATATCAAACTTAAAGG	XbaI
3966CompF	GTACCTCGAGCAACCTATGCTCCACCGC	XhoI
3966CompR	GTACTCTAGAGCTTTAGCAAGGCTTGGG	XbaI

experiments) or anoxic tryptone medium supplemented with 20 mM sodium lactate and 40 mM sodium fumarate (for Fe^{2+} experiments) to an OD_{600} of 0.05. Cultures were incubated at 30°C until growth reached an OD_{600} of ~0.5. $CoCl_2$, $MgCl_2$, or $FeCl_2$ was added to cultures at a concentration of 0.7 mM, 230 mM, or 2.5 mM, respectively, and the cultures were incubated for a further 1 h. Cultures were pelleted, washed once in 0.9% NaCl, and resuspended in deionized water. Cell suspensions were analyzed for cobalt, magnesium, or iron concentrations using ICP-MS at the Analytical Geochemistry Lab in the Department of Earth Sciences at the University of Minnesota. Metal concentrations were normalized to the final OD_{600} before pelleting.

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