



MgtE Homolog Ficl 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 Mg²⁺ transporter E (MgtE) family of passive Mg²⁺/Co²⁺ 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 Fe²⁺ and Mn²⁺. Here we characterize SO_3966, which encodes an MgtE homolog in *Shewanella oneidensis* that we name Ficl (ferrous iron and cobalt importer) 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 Fe²⁺ or Co²⁺ concentrations but exhibits wild-type Mg²⁺ transport and retention phenotypes. Conversely, deletion of *feoB*, which encodes an energy-dependent Fe²⁺ importer, causes a growth defect under conditions of low Fe²⁺ concentrations but not high Fe²⁺ concentrations. We propose that Ficl represents a secondary, less energy-dependent mechanism for iron uptake by *S. oneidensis* under high Fe²⁺ 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 Mg²⁺ and Co²⁺ transporters. This work broadens that designation with the discovery of an MgtE homolog in *S. oneidensis* that imports Fe²⁺ but not Mg²⁺. 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 (Fe³⁺) (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	Ficl	<i>Providencia stuartii</i> MgtE	<i>Thermus thermophilus</i> HB8 MgtE	<i>Pseudomonas aeruginosa</i> PAO1 MgtE
SO_1565	23					
Ficl	24	36				
<i>Providencia stuartii</i> MgtE (16)	31	24	25			
<i>Thermus thermophilus</i> HB8 MgtE (17)	33	25	29	33		
<i>Pseudomonas aeruginosa</i> PA14 MgtE (45)	27	35	40	28	29	
<i>Aeromonas piscicola</i> AH-3 MgtE (46)	29	25	25	55	35	27

(Fe²⁺), however, it may become soluble or be reincorporated into mixed-valence minerals, depending on environmental conditions (8, 9). Therefore, as Fe³⁺ 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), Mg²⁺ 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 Mg²⁺ concentration through reversibly bound Mg²⁺ ions (17, 18). MgtE adopts a closed-gate formation under high Mg²⁺ concentrations, when the Mg²⁺ ions are bound to the cytoplasmic domain; under low Mg²⁺ concentrations, bound Mg²⁺ 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 Fe²⁺ levels become too high, the inner membrane exporter FeoE removes excess Fe²⁺ from the cytoplasm (22); when iron levels are low, the trans-periplasmic TonB-dependent Fe³⁺ import system and the inner-membrane Fe²⁺ importer FeoB drive the uptake of iron into the cytoplasm using energy-dependent mechanisms (23–26). In this work, we describe a third Fe²⁺ transport protein in *S. oneidensis*, which imports Fe²⁺ at higher concentrations; we name this protein Ficl.

RESULTS

Deactivation of one of three *mgtE* homologs in the *S. oneidensis* genome affects fitness at high Fe²⁺ concentrations. Three genes in the *S. oneidensis* genome have been annotated as encoding MgtE-family Mg²⁺/Co²⁺ 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

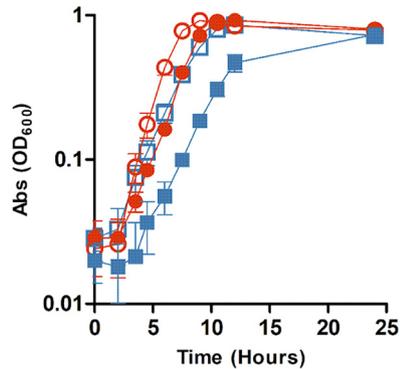


FIG 1 Anaerobic growth of wild-type and Δfcl strains with excess FeCl_2 . The rate of growth in anoxic tryptone medium with 20 mM lactate, 40 mM fumarate, and 2 mM FeCl_2 was measured for wild-type *S. oneidensis* with an empty pBBR1MCS-2 vector (open squares), wild-type *S. oneidensis* with pBBR1MCS-2::fcl (filled blue squares), *S. oneidensis* Δfcl with an empty pBBR1MCS-2 vector (open circles), and *S. oneidensis* Δfcl with pBBR1MCS-2::fcl (filled red circles). Results represent means for three biological replicates \pm 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 Ficl (*ferrous iron and cobalt importer*), and describe its role in mediating cytoplasmic Fe^{2+} and Co^{2+} concentrations.

Loss of SO_3966 (*ficl*) enhances resistance to Fe^{2+} . To confirm the results of the transposon screen, an in-frame deletion of *ficl* was made in *S. oneidensis*. *S. oneidensis* Δfcl and wild-type *S. oneidensis* containing either an empty vector (pBBR1MCS-2) or a complementation vector (pBBR1MCS-2::fcl) were grown anaerobically in tryptone medium supplemented with lactate and fumarate, with or without 2 mM FeCl_2 . The Δfcl 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 Δfcl 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 Δfcl mutant with *ficl* enhanced sensitivity to Fe^{2+} (Fig. 1); the wild-type strain with the empty vector had the same growth phenotype as the complemented Δfcl strain (Fig. 1). These phenotypes are consistent with Ficl playing a role in Fe^{2+} uptake, where a strain lacking Ficl (the Δfcl strain with an empty pBBR1MCS-2 vector) is most resistant to Fe^{2+} , while a strain with high levels of Ficl (the wild type with pBBR1MCS-2::fcl) displays higher sensitivity to Fe^{2+} (Fig. 1).

Deletion of *ficl* decreases cellular iron retention. To determine whether the decreased Fe^{2+} sensitivity of the Δfcl mutant was due to altered Fe^{2+} uptake, iron retention assays were performed. The Δfcl and wild-type strains with the empty vector or a *ficl* 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 Δfcl 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 Δfcl strain with an empty vector retained approximately half the concentration of iron ($P < 0.0001$) that was retained by the wild-type or Δfcl strain carrying the complementation vector (Table 2). Given the annotation of Ficl 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 Δfcl 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::fcl	225.5	15.1
Δfcl strain + empty pBBR1MCS-2	99.9	4.0
Δfcl strain + pBBR1MCS-2::fcl	200.9	16.32
Co		
Wild type	55.4	2.1
Δfcl strain	26.0	1.4
Mg		
Wild type	204.2	10.4
Δfcl strain	204.6	13.2

Ficl imports Co²⁺ but not Mg²⁺. MgtE proteins have been described in other bacterial species as importers of Mg²⁺ and Co²⁺ (15, 16). To determine whether Ficl imports either of these metals in addition to Fe²⁺, *S. oneidensis* Δfcl and wild-type *S. oneidensis* cultures were grown anaerobically with or without concentrations of Mg²⁺ or Co²⁺ that impair growth (0.7 mM CoCl₂ or 230 mM MgCl₂). No difference in sensitivity to Mg²⁺ was seen between the wild-type and Δfcl strains (doubling times, 2.02 ± 0.14 and 2.03 ± 0.21 h, respectively) (Fig. 2A); however, the Δfcl strain displayed a higher growth rate than the wild type when grown with excess Co²⁺ (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 Δfcl strain in excess Co²⁺ was due to a decrease in Co²⁺ uptake, cobalt retention assays were performed on cultures grown aerobically in tryptone medium supplemented with 0.7 mM CoCl₂. The Δfcl strain retained >50% less cobalt ($P < 0.0001$) than the wild type (Table 2). There was no difference in magnesium retention when *S. oneidensis* Δfcl and wild-type *S. oneidensis* were grown aerobically in tryptone medium supplemented with 230 mM MgCl₂ (Table 2).

***S. oneidensis* Δfcl has enhanced survival when grown in the presence of [Co(III)-EDTA]⁻.** Since *fcl* deletion conferred a growth rate higher than that of the wild type in the presence of a high concentration of Co²⁺, we hypothesized that *fcl* deletion would confer a benefit under conditions where Co³⁺ is respired. Wild-type and Δfcl strains were grown anaerobically in *Shewanella* basal medium (SBM) with 20 mM lactate, 40 mM fumarate, and 5 mM [Co(III)-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

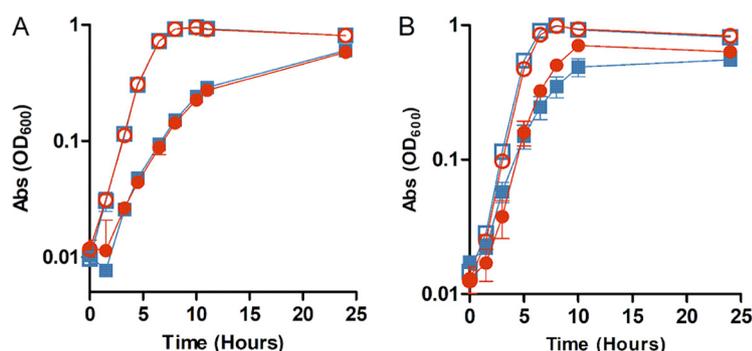


FIG 2 Growth of wild-type *S. oneidensis* and *S. oneidensis* Δfcl with excess MgCl₂ or CoCl₂. The rate of growth was measured for wild-type *S. oneidensis* and *S. oneidensis* Δfcl in anoxic tryptone medium with 20 mM lactate, 40 mM fumarate, and either MgCl₂ at 230 mM (A) or CoCl₂ at 0.7 mM (B). Symbols: open squares, wild type with no metal added; open circles, Δfcl strain with no metal added; filled blue squares, wild type with metal added; filled red circles, Δfcl strain with metal added. Results represent means for three biological replicates ± 1 standard deviation.

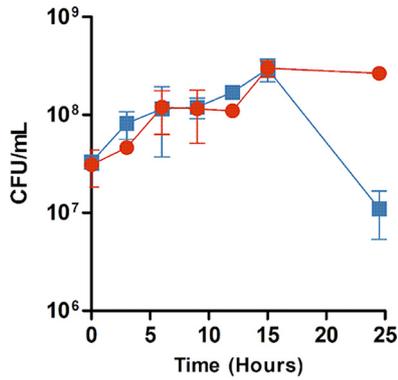


FIG 3 Anaerobic growth of wild-type *S. oneidensis* and *S. oneidensis* Δfcl on $[\text{Co(III)-EDTA}]^-$. The rate of growth in anoxic SBM with 20 mM lactate, 40 mM fumarate, and 5 mM $[\text{Co(III)-EDTA}]^-$ was measured for wild-type *S. oneidensis* (filled blue squares) and *S. oneidensis* Δfcl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

defect relative to the Δfcl strain (Fig. 3). Dissipation of the red color associated with $[\text{Co(III)-EDTA}]^-$ (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 Δfcl strain were grown anaerobically in tryptone medium with or without 2.5 mM FeCl_2 or 0.63 mM CoCl_2 . Only the Δfcl 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 Δfcl strain). While deletion of the two annotated *mgtE* homologs did not exhibit phenotypes like those displayed by the Δfcl 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::*fcl* 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

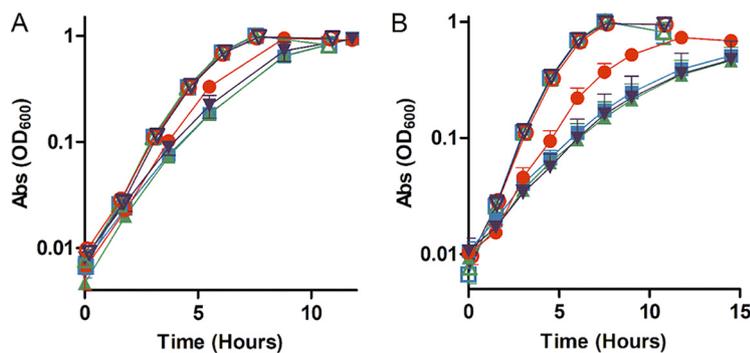


FIG 4 Anaerobic growth of the wild-type, ΔSO_1145 , ΔSO_1565 , and Δfcl strains in excess Fe^{2+} or Co^{2+} . The rate of growth in anoxic LB with 20 mM lactate, 40 mM fumarate, and 2.5 mM FeCl_2 (A) or 0.63 mM CoCl_2 (B) was measured for wild-type *S. oneidensis* (squares) and for the Δfcl (circles), 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* Δfcl overlap those for the other three strains when no metal is added. Results represent means for three biological replicates \pm 1 standard deviation.

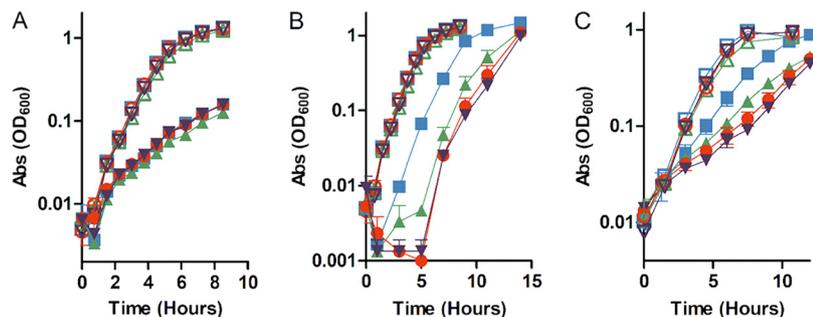


FIG 5 Growth of wild-type strains overexpressing *mgtE* homologs. The rate of growth was measured in oxic tryptone medium with or without 300 mM MgCl_2 (A), oxic tryptone medium with or without 0.65 mM CoCl_2 (B), or anoxic tryptone medium with 20 mM lactate, 40 mM fumarate, and 2.5 mM FeCl_2 (C). The wild-type strain carried either an empty pBBR1MCS-2 vector (squares), pBBR1MCS-2::*fcl* (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 \pm 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 *fcl*).

***fcl* and *feoB* mutants differ in Fe^{2+} requirements and sensitivity.** The iron retention phenotypes of strains either lacking *fcl* or with enhanced expression of *fcl* 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 Ficl import Fe^{2+} , cultures of wild-type *S. oneidensis* and the ΔfeoB and Δfcl 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 Δfcl and wild-type strains displayed the same growth phenotype, whereas the ΔfeoB strain displayed a significantly lower growth rate (Fig. 6A). Under high Fe^{2+} conditions, the ΔfeoB strain displayed the same growth rate as the wild type, while the Δfcl strain had a higher growth rate than the other two strains (Fig. 6B).

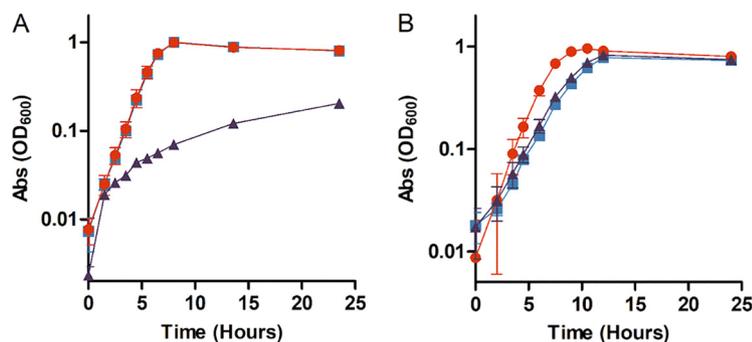


FIG 6 Growth of wild-type, Δfcl , and ΔfeoB strains. The rate of growth in anoxic tryptone medium with 20 mM lactate and 40 mM fumarate, either without FeCl_2 (A) or with 2 mM FeCl_2 (B), was measured for wild-type *S. oneidensis* (filled blue squares), *S. oneidensis* Δfcl (filled red circles), and *S. oneidensis* ΔfeoB (filled purple triangles). Data points overlap for wild-type *S. oneidensis* and *S. oneidensis* Δfcl in panel A and for wild-type *S. oneidensis* and *S. oneidensis* ΔfeoB in panel B. Results represent means for three biological replicates \pm 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 Fe²⁺ and Co²⁺ importer in *S. oneidensis* that belongs to the MgtE protein family, members of which have until now been described as inner-membrane Mg²⁺ and Co²⁺ importers (15, 16).

Deletion of *ficl* increases resistance to high Fe²⁺ concentrations over that of the wild type, while *ficl* overexpression confers increased Fe²⁺ sensitivity (Fig. 1). These results are not due to an inherently higher growth rate for the $\Delta ficl$ strain (Fig. 6A). Strains overexpressing *ficl* retain approximately twice as much iron as strains with an empty vector (Table 2). Taking these phenotypes together, uptake of Fe²⁺ and sensitivity to Fe²⁺ appear to increase concomitantly with copies of *ficl*, supporting the hypothesis that *ficl* encodes a Fe²⁺ importer.

Since MgtE proteins have previously been described as Mg²⁺ and/or Co²⁺ importers, we wanted to determine whether Ficl imports either of these metals in addition to Fe²⁺. The $\Delta ficl$ strain had the same phenotype as the wild type when grown in excess Mg²⁺ but had a growth benefit over the wild type when grown in excess Co²⁺ (Fig. 2). Metal retention assays showed that while $\Delta ficl$ 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 *ficl* encodes a transporter that imports Fe²⁺ and Co²⁺ but not Mg²⁺. We also determined that, due to the uptake of Co²⁺ by Ficl, *ficl* deletion confers a survival benefit during the respiration of [Co(III)-EDTA]⁻ and consequent production of Co²⁺ (Fig. 3). The uptake of Fe²⁺ or Co²⁺ via Ficl could be a double-edged sword for *S. oneidensis*: Ficl 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 Ficl does not appear to transport Mg²⁺, 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 Fe²⁺ concentrations (49). Growth curves showed that deletion of SO_1145 or SO_1565 did not affect the growth rate in the presence of Fe²⁺ or Co²⁺ (Fig. 4), but, surprisingly, overexpression of all three *mgtE* homologs resulted in increased sensitivity to both Fe²⁺ and Co²⁺ (Fig. 5). The Mg²⁺ growth curves indicate that none of the MgtE homologs in *S. oneidensis* is an Mg²⁺ 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 Mg²⁺ 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 Mg²⁺ import than MgtA, MgtB, or MgtE in other species of *Gammaproteobacteria* (16, 35), suggesting that CorA is most likely to be the primary Mg²⁺ importer even in species encoding multiple Mg²⁺ transporters. Since no other Mg²⁺ import genes are annotated in the MR-1 genome, we postulate that CorA is likely to be the predominant Mg²⁺ importer in *S. oneidensis*.

Global expression profiles of *S. oneidensis* have reported no differential expression for SO_1145, SO_1565, or *ficl* 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 knock-out 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 Fe²⁺ and Co²⁺; 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 Fe²⁺ importer (23, 25), confers a strong fitness defect at lower Fe²⁺ concentrations but has little effect on growth at high Fe²⁺ concentrations (Fig. 6) (49). Conversely, a *ficl* mutant has the same growth rate as the wild type at lower Fe²⁺ concentrations but is less sensitive to high Fe²⁺ concentrations (Fig. 6) (49). These results indicate that FeoB is likely the primary importer of Fe²⁺ when concentrations are low, and the transporter encoded by *ficl* is likely a secondary importer, active only when extracellular Fe²⁺ concentrations are high. That *S. oneidensis* has evolved to have at least two separate systems for Fe²⁺ import should not be surprising, considering that it inhabits redox transition zones of metal-rich sediments (1). The transitory respiration of solid Fe³⁺ minerals creates temporarily high local concentrations of soluble Fe²⁺ 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 Fe²⁺ transport under different conditions: FeoB, which uses energy to take up Fe²⁺ (25, 26), and Ficl, a member of the MgtE family of passive metal importers (18). In this way, *S. oneidensis* can maximize energy conservation by using Ficl when local Fe²⁺ 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 Fe²⁺ and Co²⁺, 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 Mg²⁺ importer but that all three can import Fe²⁺ and Co²⁺ when overexpressed. Furthermore, while SO_1145 and SO_1565 may not import physiologically relevant concentrations of Fe²⁺, we suggest that Ficl is a secondary, less-energy-dependent Fe²⁺ importer active at high Fe²⁺ 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)-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	<i>S. oneidensis</i> MR-1; wild type	1
JG3275	JG274 Δ <i>ficI</i> (SO_3966)	This work
JG3574	JG274 Δ <i>feoB</i> (SO_1784)	This work
JG168	JG274 with empty pBBR1MCS-2	31
JG3376	JG3275 with empty pBBR1MCS-2	This work
JG3379	JG3275 with pBBR1MCS-2:: <i>ficI</i>	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:: <i>ficI</i>	This work
UQ950	<i>E. coli</i> DH5 α λ (pir) cloning host; F ⁻ Δ (<i>argF-lac</i>)169 ϕ 80 <i>dlacZ58</i> (Δ M15) <i>glnV44</i> (AS) <i>rfdD1</i> <i>gyrA96</i> (NalR) <i>recA1</i> <i>endA1</i> <i>spoT1</i> <i>thi-1</i> <i>hsdR17</i> <i>deoR</i> λ pir ⁺	5
WM3064	<i>E. coli</i> conjugation strain; <i>thrB1004</i> <i>pro</i> <i>thi</i> <i>rpsL</i> <i>hsdS</i> <i>lacZ</i> Δ M15 RP4-1360 Δ (<i>araBAD</i>)567 Δ <i>dapA1341</i> ::[<i>erm</i> <i>pir</i> (wt)]	5
Plasmids		
pSMV3	Deletion vector; Km ^r <i>sacB</i>	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:: <i>ficI</i>	<i>ficI</i> , 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 *Apal* (*ficI*) or *SpeI* and *BamHI* (*feoB*) restriction sites were fused via internal *Scal* (*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. Co²⁺, Mg²⁺, and Fe²⁺ growth curves were performed in tryptone medium supplemented with either 0.63, 0.65, or 0.70 mM CoCl₂, 230 or 300 mM MgCl₂, or 2.0 or 2.5 mM FeCl₂, 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. Co³⁺ 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 (OD₆₀₀). 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 Co²⁺ and Mg²⁺

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

Primer	Sequence	Restriction site
FeoBUSF	GTACACTAGTGTATGATTACCCAGCGGG	<i>SpeI</i>
FeoBUSR	GTACGCTAGCGTGACGCAATGAACTGCTTAG	<i>NheI</i>
FeoBDSF	GTACGCTAGCCGGATTACTGAGTAAACCC	<i>NheI</i>
FeoBDSR	GTACGGATCCCTCATATTGACGAGTACGATTTGG	<i>BamHI</i>
3966USF	GTACGAGCTCCCATTAACCTCGAAGGCAAGC	<i>SacI</i>
3966USR	GTACAGTACTCATGTTTCCTCCAGGGTG	<i>Scal</i>
3966DSF	GTACAGTACTGCGACCTTGATTTAATGCACTAG	<i>Scal</i>
3966DSR	GTACGGGCCCCATTGATGGCGGGTATGG	<i>Apal</i>
1145CompF	GTACGGTACCCGAGAATGAACTATGAACATGAAC	<i>KpnI</i>
1145CompR	GTACGAGCTCGCTCGTACCTTTTACGCAGC	<i>SacI</i>
1565CompF	GTACCTCGAGCAGCAGAAGGGCGTTTAG	<i>XhoI</i>
1565CompR	GTA CTCTAGACGCTTAATATCAAACCTAAAGG	<i>XbaI</i>
3966CompF	GTA CTCTGAGCAACCTATGCTCCACCGC	<i>XhoI</i>
3966CompR	GTA CTCTAGAGCTTTAGCAAGGCTTGGG	<i>XbaI</i>

experiments) or anoxic tryptone medium supplemented with 20 mM sodium lactate and 40 mM sodium fumarate (for Fe²⁺ experiments) to an OD₆₀₀ of 0.05. Cultures were incubated at 30°C until growth reached an OD₆₀₀ of ~0.5. CoCl₂, MgCl₂, or FeCl₂ 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 OD₆₀₀ before pelleting.

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