Genetic Redundancy in Iron and Manganese Transport in the Metabolically Versatile Bacterium *Rhodopseudomonas palustris* TIE-1

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ABSTRACT The purple nonsulfur bacterium *Rhodopseudomonas palustris* TIE-1 can produce useful biochemicals such as bioplastics and biobutanol. Production of such biochemicals requires intracellular electron availability, which is governed by the availability and the transport of essential metals such as iron (Fe). Because of the distinct chemical properties of ferrous [Fe(II)] and ferric iron [Fe(III)], different systems are required for their transport and storage in bacteria. Although Fe(III) transport systems are well characterized, we know much less about Fe(II) transport systems except for the FeoAB system. Iron transporters can also import manganese (Mn). We studied Fe and Mn transport by five putative Fe transporters in TIE-1 under metal-replete, metal-depleted, oxic, and anoxic conditions. We observed that by overexpressing *feoAB*, *efeU*, and *nramp1AB*, the intracellular concentrations of Fe and Mn can be enhanced in TIE-1 under oxic and anoxic conditions, respectively. The deletion of a single gene/operon does not attenuate Fe or Mn uptake in TIE-1 regardless of the growth conditions used. This indicates that genetically dissimilar yet functionally redundant Fe transporters in TIE-1 can complement each other. Relative gene expression analysis shows that *feoAB* and *efeU* are expressed during Fe and Mn depletion under both oxic and anoxic conditions. The promoters of these transporter genes contain a combination of Fur and Fnr boxes, suggesting that their expression is regulated by both Fe and oxygen availability. The findings from this study will help us modulate intracellular Fe and Mn concentrations, ultimately improving TIE-1's ability to produce desirable biomolecules.

IMPORTANCE *Rhodopseudomonas palustris* TIE-1 is a metabolically versatile bacterium that can use various electron donors, including Fe(II) and poised electrodes, for photoautotrophic growth. TIE-1 can produce useful biomolecules, such as biofuels and bioplastics, under various growth conditions. Production of such reduced biomolecules is controlled by intracellular electron availability, which, in turn, is mediated by various iron-containing proteins in the cell. Several putative Fe transporters exist in TIE-1's genome. Some of these transporters can also transport Mn, part of several important cellular enzymes. Therefore, understanding the ability to transport and respond to various levels of Fe and Mn under different conditions is important to improve TIE-1's ability to produce useful biomolecules. Our data suggest that by overexpressing Fe transporter genes via plasmid-based expression, we can increase the import of Fe and Mn in TIE-1. Future work will leverage these data to improve TIE-1 as an attractive microbial chassis and future biotechnological workhorse.

KEYWORDS *Rhodopseudomonas palustris* TIE-1, biofuels and bioplastics, bioproduction, *efeU*, *feoAB*, iron transporters, microbial chassis

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Iron (Fe) and manganese (Mn) are essential nutrients for biological processes (1–3). Because of the unique redox properties of Fe, it plays an important role as a cofactor in enzymes involved in a variety of cellular processes (4). Similarly, Mn plays an essential role in lipid, protein, and carbohydrate metabolism (5). It also serves as a cofactor of Mn-dependent superoxide dismutase and can contribute to the catalytic detoxification of reactive oxygen species (ROS) (5–7). However, when in excess, these metals are toxic to cells. An evaluation of Fe toxicity revealed that the presence of 1 mM Fe(III) and 0.5 mM Fe(II) can significantly affect the growth of *Escherichia coli* (8). Such elevated concentrations can perturb intracellular redox conditions and produce reactive hydroxyl radicals (1). Likewise, excess Mn can inactivate enzymes that use other divalent ions. Mn is known to replace Fe from cellular proteins (6, 7). Therefore, the function of many Fe-containing proteins (e.g., cytochromes, dehydrogenases, and iron-sulfur proteins), which are involved in diverse cellular processes, may be affected due to replacement of Fe by Mn when a very high level of Mn is present (9). The interplay of the need and the toxicity of these metals forces organisms to carefully maintain metal homeostasis. This ensures that metal availability is in accordance with their physiological needs (1). This homeostasis is largely maintained by regulating specific metal transport systems across all biological systems (4, 10–17).

Depending on oxygen availability in the environment, iron exists in two forms: ferric [Fe(III)] and ferrous [Fe(II)]. In oxic environments, most bacteria acquire insoluble Fe(III) by synthesizing high-affinity Fe(III) siderophores via a process that involves multi-enzyme pathways (1, 18–20). In contrast, Fe(II) uptake is much simpler and involves direct transportation of Fe(II) into the cytoplasm through distinct Fe(II) transporters (21–23). Likewise, depending on oxygen availability, manganese is also found in two most stable forms: Mn(II) and Mn(IV). In many cases, Fe(II) transporters can also transport Mn(II) across the cytoplasmic membrane (24, 25). Although Fe(III) transport systems are well understood, we know much less about Fe(II) transport systems (except for the FeoAB system) and their ability to transport Mn(II) in bacteria, which is the focus of this study.

In this investigation, we studied Fe and Mn transport in a metabolically versatile anoxygenic phototroph *Rhodospseudomonas palustris* TIE-1. Depending on the availability of different energy and carbon sources, TIE-1 can perform multiple modes of metabolisms, such as photoautotrophy, photoheterotrophy, chemoautotrophy, and chemoheterotrophy (26–28). Similar to the other strains of *Rhodospseudomonas* that have been used to produce value-added compounds (29–34), TIE-1 can also produce compounds, such as bioplastics and biofuels, under various growth conditions (35; W. Bai, T. O. Ranaivoarisoa, R. Singh, K. Rengasamy, and A. Bose, submitted for publication). Electron transfer during the synthesis of these compounds requires Fe-containing proteins, such as cytochromes. The activity of such proteins depends on the bioavailability and the transport of Fe (28, 35, 36).

We studied the role of five genes (*feoAB*, *efeU*, *nramp1AB*, *nramp3AB*, and *sitABC*) that encode Fe(II) transporters in TIE-1 to import Fe and Mn under metal-replete and -depleted conditions with respect to oxygen availability. Using heterologous complementation, mutant analysis, and gene expression analysis, we showed that by manipulating TIE-1's Fe transport systems, we can enhance its ability to acquire Fe and Mn. Identifying such key transporters might be beneficial for developing TIE-1 as a bioproduction chassis.

RESULTS

TIE-1 possesses six putative ferrous iron transporter genes. We identified six putative Fe(II) transporters in TIE-1 using whole-genome homology searches for previously characterized Fe(II) transport systems from *E. coli* and *R. palustris* CGA009 (Fig. 1). The first is the Fe(II)-specific FeoAB system. Unlike *E. coli*, TIE-1 contains only FeoA and FeoB and not FeoC. However, the Feo system lacking FeoC is fully functional for Fe(II) uptake in several bacteria (37). The second Fe(II)-specific transport system is EfeU, which is a member of the oxidase-dependent iron transporters (OFET) and is a homolog of the iron permease Ftr1p from yeast (38). It is a part of the *efeUOB* tricistronic operon,

FIG 1 Genomic organization of *R. palustris* TIE-1 genes that are likely involved in Fe(II) transport. Six candidates' loci were identified (A to F). The genes of interest are shown in black with their names bolded. Other genes are shown in gray with their locus tags, searchable at <https://img.jgi.doe.gov/cgi-bin/w/main.cgi>.

which is expressed in response to Fe availability in *E. coli* (24). The third system is SitABC (SitD is absent), which has been reported to have a much greater affinity for Mn than Fe (39). Fe- and Mn-dependent regulation of *sitABC* operon has been previously described for *Staphylococcus epidermidis* (40) and *Staphylococcus aureus* (41), respectively. The fourth transport system belongs to the natural resistance-associated macrophage protein (NRAMP) family. We found three *nramp* gene/operons: *nramp1AB*, *nramp2*, and *nramp3AB*, which are also known to be important for Fe and Mn uptake and homeostasis in other organisms (42, 43). Nramp1A belongs to the DNA binding transcriptional regulator LysR family, whereas Nramp3A is a hypothetical protein (Rpal_3713) that is predicted only for the genomes of TIE-1 and the related strain CGA009 (Fig. 1E). Nramp1B, Nramp2, and Nramp3B belong to a Mn transporter protein family, MntH from *E. coli* (42, 43). Because we were unable to delete *nramp2* from TIE-1, we excluded *nramp2* from our studies.

Putative ferrous iron transport systems from TIE-1 can rescue iron transport deficiencies in a heterologous host. To assess whether the putative Fe(II) transporters support iron uptake, we performed two heterologous-complementation experiments with *E. coli* as an initial test. For the first complementation experiment, we used *E. coli* H1771, a mutant defective for the Feo system and siderophore biosynthesis with a chromosomal *fhuF-lacZ* (encoding β -galactosidase) reporter system. This reporter system has been previously used in similar studies (44–46). The *fhuF* promoter of the reporter system is responsive to Fe, where repression of β -galactosidase activity represents adequate intracellular Fe concentration while an increase in β -galactosidase activity indicates Fe starvation (47, 48).

The identified Fe transporter genes/operons from TIE-1 were cloned into the low-copy-number vector pWKS30 (47) and were driven by the constitutive promoter P_{aphII} (49). We have also recently reported gene expression using the P_{aphII} promoter in TIE-1: we produced PioA in TIE-1 in a *pioA* deletion TIE-1 background using this promoter and confirmed protein expression by Western blotting (36). To further confirm the efficiency of the P_{aphII} promoter, we built a plasmid in which *mCherry* was driven by P_{aphII} . TIE-1 carrying this plasmid showed a 5,000-fold-higher fluorescence signal (normalized to optical density at 600 nm [OD₆₀₀]) than wild-type (WT) TIE-1 (see Fig. S1 in the supplemental material). This promoter has been also used previously in a closely related organism, *Rhodobacter capsulatus* (50).

Plasmids carrying the transporter genes were then transformed into *E. coli* H1771, and β -galactosidase (reporter) activity was assayed under four different conditions, namely, (i) Fe(III) replete [50 μ M Fe(III)-citrate], (ii) Fe(III) depleted [50 μ M Fe(III)-citrate with 100 μ M 2,2'-dipyridyl (DiP)], (iii) Fe(II) replete [50 μ M ascorbate-reduced Fe(III)-citrate], and (iv) Fe(II) depleted [50 μ M ascorbate-reduced Fe(III)-citrate with 100 μ M DiP]. The membrane-permeant chelator DiP was used to control the bioavailability of Fe(II) and Fe(III). DiP is a Fe(II)-specific bidentate organic ligand that acts by directly depleting intracellular stores of ferrous iron (51). The inherent *fhuF-lacZ* reporter system of *E. coli* H1771 carrying an empty vector expressed *lacZ* to a full extent due to a low intracellular Fe concentration (Fig. 2). *E. coli* H1771 complemented with K-12 *feoABC* through a plasmid served as a positive control and showed more than an 80% decrease in *lacZ* expression as determined by β -galactosidase activity compared to the vector control under all conditions (Fig. 2). Regardless of different Fe(III) concentrations and transporter genes from TIE-1, none of the complemented *E. coli* H1771 strains showed significant changes in β -galactosidase activity (Fig. 2). In contrast, all the complemented strains under Fe(II)-replete conditions showed a lower β -galactosidase activity (Fig. 2C) than both the vector control and the activities under Fe(II)-depleted conditions (Fig. 2D). The most pronounced difference in the β -galactosidase activity compared to the empty vector was observed for the *efeU*-complemented strain under Fe(II)-replete conditions (62% lower than the vector control) (Fig. 2C).

To further confirm the role of the putative Fe(II) transporters in cell growth, we employed a second heterologous-complementation experiment using *E. coli* GR536. *E. coli* GR536 is a mutant lacking all five known Fe transport systems ($\Delta fecABCDE::kan$ $\Delta zupT::cat$, $\Delta mnth$, $\Delta feoABC$, and $\Delta entC$), and therefore, it is unable to grow under Fe-depleted conditions (52). We expressed the Fe(II) transporter genes from TIE-1 in this heterologous host and examined if their expression can rescue the growth defect of *E. coli* GR536 under Fe-depleted conditions (Fig. 3A to E). Indeed, complementation of *E. coli* GR536 with *efeU*, *nramp1AB*, *nramp3AB*, and *sitABC* from TIE-1 restored its growth to various degrees after ~ 60 h (Fig. 3B to E). In contrast, although the growth defect was rescued by complementation with *feoABC* from *E. coli* K-12 (a positive control) within 24 h (Fig. 3F), the expression of the FeoAB system from TIE-1 could not rescue the growth defect (Fig. 3A). This result suggests that all three genes of the Feo system, including the FeoC gene, are important for Fe transport in *E. coli*.

Putative ferrous iron transporters from TIE-1 do not transport zinc, cadmium, or copper. Based on previous studies, the FeoAB and EfeU transporters are predicted to be specific for Fe uptake, while the Nramp and Sit systems are known to also transport other divalent cations (39, 42, 53, 54). Therefore, we further investigated the metal specificity of these putative Fe transporters in TIE-1. Although DiP is a strong intracellular Fe(II) chelator, it also binds to other divalent metals, such as copper [Cu(II)], cadmium [Cd(II)], and zinc [Zn(II)] (55). Because of this broader metal binding capacity of DiP, we further used this chelator in studying the metal specificity of these transport systems. Heterologous complementation was performed in two different strains: (i) *E. coli* GG48, which lacks key Zn transport genes, and (ii) *E. coli* $\Delta copA$ $\Delta cueO$ $\Delta cusCFBA::cat$, which lacks the key Cu transport genes (56). Complementation of these *E. coli* hosts with a functional Zn/Cu/Cd transport system is detrimental and is reflected by a lower growth rate when these metals are provided in the growth medium (56). We then

FIG 2 *E. coli* H1771 complementation by selected iron transport genes resulted in less iron stress when grown in Fe(II) supplied LB medium but had no effect when grown in Fe(III)-supplied LB medium. The Miller β -galactosidase assay was used to determine the level of iron stress. Four conditions were used: Fe(III) replete [50 μ M Fe(III)-citrate] (A), Fe(III) depleted [50 μ M Fe(III)-citrate with 100 μ M 2,2'-dipyridyl (DiP)] (B), Fe(II) replete [50 μ M ascorbate-reduced Fe(III)-citrate] (C), and Fe(II) depleted [50 μ M ascorbate-reduced Fe(III)-citrate with 100 μ M DiP] (D). Cells at the exponential phase were used for all assays. Values are represented as means $\pm$ standard errors from six independent biological measurements. The number on each bar represents the percent reduction (normalized to empty vector control) of Miller β -galactosidase activity. The x axis shows the genes used for complementation. TIE-1, *Rhodopseudomonas palustris* TIE-1; *Ec*, *E. coli* K-12.

determined the ability of the putative Fe(II) transport systems from TIE-1 to transport Zn(II), Cd(II), and Cu(II) by monitoring the growth rate of the heterologous *E. coli* host complemented with plasmids expressing TIE-1's putative Fe(II) transporters compared to the control strain (Table 1; $P \leq 0.05$). These data suggest that the TIE-1 Fe(II) transporters had either no effect or a positive effect on the growth rate of *E. coli*. The increase in growth rate could have accounted for the ability of these transporters to bring in extracellular Fe into the cell. The only case where we observed a negative effect on growth rate was under the Cd/Cu-supplemented conditions for *E. coli* Δ *copA* Δ *cueO* Δ *cusCFBA::cat* complemented with the *E. coli* *feoABC* system. Overall, these data suggest that the TIE-1's Fe transport systems do not import enough Zn/Cu/Cd into these *E. coli*

FIG 3 Complementation with selected Fe(II) transport genes aid growth of *E. coli* GR536 mutant (which lacks all known iron transport genes) during severe iron deprivation (in Tris-mineral salts medium at pH 7.0 with no added iron). *E. coli* GR536 was complemented by *Rhodopseudomonas palustris* TIE-1 *feoAB* (A), TIE-1 *efeU* (B), TIE-1 *nramp1AB* (C), TIE-1 *nramp3AB* (D), and TIE-1 *sitABC* (E) and *E. coli* K-12 *feoABC* (positive control) (F). Two representative growth conditions are shown: without and with 150 μ M DiP. Empty vector was used as a control for both conditions. Growth was measured as OD₆₀₀. Values represent the means $\pm$ standard deviations from biological triplicates.

mutant strains to observe a severe growth defect. The result implies that these TIE-1 Fe transport systems likely have a low affinity for Zn/Cu/Cd.

Overexpression of ferrous iron transporter genes increases intracellular iron and manganese in TIE-1 under oxic and anoxic conditions. After confirming the role of the putative Fe(II) transporters in Fe transport and growth of the heterologous host *E. coli*, we sought to investigate their role in Fe and Mn accumulation in the native organism TIE-1. We constructed markerless deletions of these genes in TIE-1 as described in Materials and Methods (27). We were unable to obtain some of the mutants despite screening >100 colonies. These include *feoA*, *feoB*, *nramp1A*, *nramp2*, and *sitA* mutants. We tested the ability of the five obtained mutants (Δ *feoAB*, Δ *efeU*, Δ *nramp1AB*, Δ *nramp3AB*, and Δ *sitABC* mutants), their complemented strains (each complemented by corresponding genes), and the WT (positive control) to intracellularly accumulate Fe and Mn under anoxic, oxic, metal-replete, and metal-depleted condi-

TABLE 1 Metal specificity tests using *E. coli* GG48 for Zn and *E. coli* $\Delta copA \Delta cueO \Delta cusCFBA::cat$ for Cd and Cu transformed with plasmids with genes encoding Fe transporters from *R. palustris* TIE-1 or *E. coli* K-12^a

Gene/operon	Zn	Cd	Cu
<i>R. palustris</i> TIE-1			
<i>feoAB</i>	0	+	0
<i>efeU</i>	0	0	0
<i>nramp1AB</i>	+	0	0
<i>nramp3AB</i>	0	+	0
<i>sitABC</i>	0	+	0
<i>E. coli</i> K-12			
<i>feoABC</i>	+	—	—

^aA "0" indicates no significant difference in growth from the empty vector control ($P > 0.05$), "+" a significantly higher growth rate for the experimental strain versus that of the control, and "—" a significantly lower growth rate versus that of the control ($P \leq 0.05$).

tions. The complemented transporter genes were expressed using the same plasmid, from the same *P_{aphII}* promoter and ribosome binding site as described above. Therefore, we assume that the expression level for each gene is similar. We quantified intracellularly accumulated Fe and Mn using inductively coupled plasma mass spectrometry (ICP-MS). The results are expressed as the ratio of metal accumulated by a strain normalized to the metal accumulated by the wild-type control strain. We did not observe any metal precipitation during our experiments.

TIE-1 is an ideal candidate to study Fe and Mn uptake due to its ability to grow under both oxic and anoxic conditions, which allowed us to study Fe(II), Fe(III), Mn(II), and Mn(IV) transport systems using the same organism. Although the ideal conditions to study the transport of these metal species is to use the same growth medium, we had to grow TIE-1 aerobically on yeast-peptone (YP) medium and anaerobically on defined freshwater (FW) medium as described in Materials and Methods. This is because TIE-1 does not grow robustly in YP medium under anoxic conditions or in FW medium under oxic conditions. Such different growth conditions have recently been used in a Fe transport study in *Shewanella oneidensis* (57). To further control the bioavailability of Fe and Mn, two different chelators, EDTA and DiP, were tested for all conditions. Being membrane-permeant (58), DiP has the potential to diffuse into cells and alter the intracellular metal distribution (59). It has stability constants of 17.5 and 16.3 with Fe(II) and Fe(III), respectively, and 6 with Mn(II) (60, 61). In contrast, EDTA is a large molecule that is membrane impermeant (58). In addition, the presence of two nitrogen atoms and four carboxyl groups in EDTA's structure makes it a strong metal chelator for iron [stability constants, 25.10 with Fe(III) and 14.33 with Fe(II)] and manganese [stability constants, 24.80 with Mn(III) and 14.04 with Mn(II)] (59). Due to its inability to enter cells, it can only bind to extracellular Fe or Mn via metal-ligand interaction, eventually limiting their transports (58). Our results indicate that the complemented strains accumulate amounts of Fe comparable to those accumulated by the WT and the mutant strains under anoxic growth conditions. When Fe was depleted by adding EDTA or DiP under anoxic conditions, growth was suppressed (OD_{660} , ~ 0.1 [Fig. S2]) and Fe acquisition was abolished (Fig. 4A to E). Two exceptions were with the $\Delta nramp3AB$ and $\Delta sitABC$ mutants, for which growth was observed with EDTA and/or DiP in the mutants but not in the WT or the complemented mutants (Fig. S2). Because we could not obtain a measurable metal concentration from the WT, with which we normalized the other values, we could not report the Fe concentration from the $\Delta nramp3AB$ and $\Delta sitABC$ mutants during growth with EDTA and/or DiP.

Supplementation of 50 μ M Fe(II) did not increase Fe accumulation by these strains under anoxic conditions (Fig. 4A to E; Table S1a to e). In contrast, compared to the WT and their respective mutants, Fe(II) supplementation to the basal YP (yeast-peptone supplemented with 1 mM succinate) medium under oxic conditions significantly increased intracellular Fe concentration (up to ~ 2 - to ~ 7 -fold higher) by the comple-

FIG 4 Fe acquisition by *Rhodopseudomonas palustris* TIE-1 mutants, complemented strains, and WT under Fe-replete, Fe-depleted, oxic, and anoxic conditions using inductively coupled plasma mass spectrometry (ICP-MS). The iron content of the cell is normalized to the Fe acquisition by the WT. (A to E) Fe acquisition under anoxic conditions. (F to J) Fe acquisition under oxic conditions. Values represent the means $\pm$ standard deviations (Continued on next page)

mented strains of the $\Delta feoAB$, $\Delta efeU$, $\Delta nramp1AB$, and $\Delta sitABC$ mutants (Fig. 4F, G, H, and J; $P < 0.05$; Table S1). Similar to the anoxic growth conditions, the addition of EDTA suppressed cell growth ($OD_{660} < 0.1$) as well as Fe transport by all the strains (Fig. 4F to J). However, Fe(II) supplementation restored Fe transport and rescued their growth except for the complemented strains. Because gentamicin was added to maintain plasmid (pBBRIMCS-5) (62) expressing the individual genes from TIE-1 in the complementation strains, we hypothesize that the lack of growth is likely because of a combined effect of gentamicin and EDTA. A combination of EDTA and gentamicin has been previously reported to inhibit the growth of *Pseudomonas aeruginosa* (63). The growth inhibition in the presence of EDTA was consistently observed in all the complemented strains under oxic conditions despite the addition of Fe (Fig. S2k). In contrast, the same effect did not hold true under anoxic conditions with FW medium. Even the complemented strains accumulated amounts of Fe very similar to those accumulated by the mutants and WT strains with their subsequent growth (Fig. 4A to E; Fig. S2). The combined toxic effects of EDTA and gentamicin might have been reversed by the presence of other cations in the FW medium including magnesium and calcium. The addition of magnesium, calcium, and iron appears to block cell death and detachment of *P. aeruginosa* biofilms (63). This could suggest that the addition of other divalent metals increases the binding competition for EDTA and frees more Fe for cell growth. However, future work will be required to determine the specific role of medium composition in this process. In contrast, combination of DiP and gentamicin under oxic conditions did not seem to have any toxic effects on the complemented strains and their subsequent metal accumulation (Fig. 4F to J).

To investigate if the putative Fe(II) transporters also play a role in Mn transport, we quantified the total amounts of Mn accumulated by the different strains of TIE-1. Under anoxic growth conditions with a supply of Mn and EDTA, the complemented strains of the $\Delta feoAB$ and $\Delta efeU$ mutants accumulated noticeably more Mn (~ 3.6 - and ~ 1.8 -fold, respectively) than the WTs and their mutants (Fig. 5A and B; $P < 0.05$; Table S1k and l). However, when the strains were cultured in the basal FW medium supplemented with Mn(II), only the $\Delta efeU$ complemented strain accumulated a significant amount of Mn compared to those accumulated by the WT and its mutant (Fig. 5B; Table S1l). Similar to the Fe studies, the addition of EDTA and DiP to the basal medium abolished cell growth and Mn accumulation by all the strains under anoxic conditions, with the two exceptions noted above (Fig. 5A to E; Fig. S2).

Under oxic growth conditions, only the $\Delta efeU$ complemented strain accumulated a larger amount of Mn than the WT as well as the mutant (~ 3 -fold) when Mn(II) was added to the basal YP medium (Fig. 5G; $P < 0.005$; Table S1q). Mn accumulation by the rest of the TIE-1 strains was similar under oxic conditions (Fig. 5F to J; Table S1p to t). The combined toxic effects of EDTA and gentamicin under oxic conditions were also observed in this case. None of the complemented strains accumulated Mn when the medium was supplemented with Mn(II) and EDTA (Fig. 5F to J; Fig. S2f to j), including the complemented strains of the $\Delta nramp1AB$, $\Delta nramp3AB$, and $\Delta sitABC$ mutants, which showed modest growth ($OD_{660} \sim 0.1$) (Fig. S2h to j). The amount of Mn accumulated by these three strains was below our detection limit. Under Mn-depleted conditions using EDTA, we observed neither growth ($OD_{660} < 0.1$) nor Mn accumulation by these strains (Fig. 5F to J; Fig. S2). Overall, the Mn accumulation results show that the Fe transporter *efeU* also participates in Mn transport in TIE-1 (Fig. 5G).

Gene expression of ferrous iron transporters varies in response to iron and manganese availability and oxygen tension. Because Fe transporter genes are regulated primarily by the availability of Fe and oxygen in the environment (64, 65), we analyzed the gene expression profiles of these transport systems in wild-type TIE-1 in

FIG 4 Legend (Continued)

from biological triplicates. FWS, freshwater media supplemented with 1 mM succinate; YPS, yeast-peptone supplemented with 1 mM succinate; NG, no growth. *, conditions under which an OD_{660} of > 0.1 was observed only for the mutant and not the WT or the complemented strain (see Fig. S2).

FIG 5 Mn acquisition by *R. palustris* TIE-1 mutants, complemented strains, and WT under Mn-replete, Mn-depleted, oxic, and anoxic conditions. The Mn content of the cells was normalized to the Mn acquisition by WT. (A to E) Mn acquisition under the anoxic conditions. (F to J) Mn acquisition under oxic conditions. Values represent the means $\pm$ standard deviations from biological triplicates. *, conditions under which an OD_{660} of >0.1 was observed only for the mutant and not WT or the complemented strain (see Fig. S2).

response to Fe, Mn, and oxygen availability. We used reverse transcription-quantitative PCR (RT-qPCR) with *recA* and *clpX* as reference genes as performed previously (28). Data reported here are with respect to *recA* for consistency with previous studies (28, 35, 36, 64, 65). The trends obtained were similar to those with *clpX* as a reference gene. Under conditions where no growth was observed ($OD_{660} < 0.1$), we collected large volumes of cells to extract sufficient RNA for RT-qPCR.

Fe depletion by the addition of EDTA elevated the expression of *feoB* (up to 20-fold), *efeU* (2.5-fold), *nramp3B* (5-fold), and *sitA* (5.6-fold) under anoxic conditions (Fig. 6A to E; Table S2a to e). This overexpression was reversed when the medium was supplemented with 50 μ M Fe(II). Similarly, Fe depletion by DiP increased the expression of *feoB* ~8-fold. However, this upregulation was not reversed by Fe(II) supplementation (Fig. 6A; $P < 0.05$; Table S2a). Among these genes, *nramp1B* appears to be less responsive to Fe bioavailability, as the addition of chelators or Fe did not show a significant effect on its expression (Fig. 6C; Table S2c).

Under oxic growth conditions, the addition of both Fe(II) and EDTA increased the expression of the *feoB* (~8-fold), *efeU* (~3-fold), and *sitA* (~3-fold) genes (Fig. 6F, G, and J; $P < 0.05$; Table S2f, g, and j). Overall, relative gene expression obtained in the presence of EDTA was higher than the expression observed in the presence of DiP (Fig. 6F to J). *nramp3B* was minimally expressed (Fig. 6I).

We also tested the relative gene expressions under different degrees of Mn availability. Under anoxic Mn-depleted conditions (with EDTA), all five genes, namely, *feoB*, *efeU*, *nramp1B*, *nramp3B*, and *sitA*, were upregulated (up to ~2.7-fold) (Fig. 7A to E; Table S2k to o). However, the expression was downregulated when excess Mn(II) was added. This pattern remained consistent even when the chelator was changed from EDTA to DiP, except for the *feoB* gene (Fig. 7A). The supply of Mn(II) upregulated *feoB*, similar to the trend observed with Fe(II). Unlike the highly variable gene expression observed under anoxic conditions, gene expression under oxic conditions did not vary significantly from that under the control conditions (Fig. 7F to J; Table S2p to t). We observed that expression of the previously known Fe-specific transporter systems, such as Feo and EfeU, can be regulated by Mn bioavailability in TIE-1.

Promoter predictions. Because Fe transporter genes are regulated primarily by the availability of Fe and oxygen in the environment, next we analyzed the promoter regions of these Fe transporters to identify the binding sites for Fe and oxygen-responsive regulators. We found a putative Fur-like binding site (Table S3). Fur is known to be a transcriptional regulator of Fe transporter genes in *E. coli* and many other bacteria (66, 67). We also identified a putative binding site for Fnr family proteins in the *feoAB* promoter along with a Fur/Irr binding site (Fig. S3) (68). Fnr is a transcriptional activator under anoxic conditions (69) that turns on the transcription of the *feo* operon in the absence of oxygen (22). The *efeU* promoter has a putative Fnr box (Fig. S4) but only a weak Fur box (68). Similar binding sites have been identified in the *Nramp1AB* promoter sequence (Fig. S5). We found a weak Fur box and an Fnr box in the *nramp3B* upstream region that encompasses the small open reading frame (ORF) *nramp3A*. We were unable to detect *nramp3A* mRNA under any condition tested, which suggests that this ORF is likely not transcribed. The *sitABC* promoter also contains an Fnr box along with a Fur box (Fig. S7) (68).

DISCUSSION

This study provided insight into the role of five putative Fe(II) transporter genes (Fig. 1) to transport Fe and Mn in TIE-1 under differing degrees of Fe, Mn, and oxygen availability. The important role of these transporters in Fe uptake and cellular growth was determined first by heterologous complementation of *E. coli* H1771 (lacking the Feo system and siderophore biosynthesis genes) and *E. coli* GR536 (lacking all Fe transporter systems), respectively (Fig. 2D and Fig. 3). Total Fe and Mn quantification in the wild-type, single-gene/operon deletion mutants, and complemented strains of *R. palustris* TIE-1 showed that these transporter genes are important for Fe and Mn transport in TIE-1. Under anoxic conditions, all TIE-1 mutant strains accumulated similar

FIG 6 Determination of relative mRNA abundance of Fe-transporting genes in TIE-1 using RT-qPCR under Fe-replete, Fe-depleted, oxic, and anoxic conditions. (A to E) Relative gene expression under anoxic conditions. (F to J) Relative gene expression under oxic conditions. Values represent the means $\pm$ standard deviations from biological triplicates.

FIG 7 Determination of relative abundances of Fe-transporting genes in TIE-1 using RT-qPCR under Mn-replete, Mn-depleted, oxic, and anoxic conditions. (A to E) Relative gene expression under anoxic conditions. (F to J) Relative gene expression under oxic conditions. Values represent the means $\pm$ standard deviations from biological triplicates.

amounts of Fe, suggesting that the deletion of no single transporter gene affects iron homeostasis in TIE-1. However, some TIE-1 strains under oxic conditions, particularly the complemented strains of the *feoAB*, *efeU*, *nramp1AB*, and *sitABC* mutants, accumulated a larger amount of Fe than did the WT and mutants (Fig. 4F to J). Under oxic conditions, Fe likely exists as Fe(III). Because overexpressing Fe(II) transporters increases total Fe content under oxic conditions, it is possible that assimilatory ferric reductases may allow reduction of Fe(III) to Fe(II). Fe(II) in the periplasm is subsequently transported by these Fe(II) transporters (present in the inner membrane). This would lead to an increase in intracellular Fe concentration when these transporters are overexpressed under a strong constitutive promoter under oxic conditions. Ferric reductases are known to be involved in Fe acquisition under oxic conditions in several microbes (70). We also found that expressing the putative Fe(II) transporters, including the previously described Fe-specific transporters such as *feoAB* and *efeU*, can increase intracellular Mn in TIE-1 under both anoxic and oxic conditions (Fig. 5). Under oxic conditions, Mn likely exists as Mn(IV). Similar to Fe(III) uptake, Mn reductases likely mediate Mn(IV) reduction in the periplasm to Mn(II) (71), which can be then transported via Fe transporters.

Our metal accumulation data are also supported by the relative gene expression of these transporters under anoxic and oxic conditions with respect to metal availability. Production of metal transporters is often induced as the metal concentrations are lowered (72, 73). Because the presence of metal chelators reduces metal availability, we observed the upregulation of putative Fe transporters under anoxic metal-depleted conditions (Fig. 6A to E; Fig. 7A to E). A similar observation has been made previously for *Salmonella enterica*, in which the FeoB protein was overexpressed when levels of both oxygen and Fe were low (74). However, a supply of Fe(II) significantly downregulated the expression. This trend appears more obvious in the case of *feoB* with iron. Upstream regulatory elements such as ferric iron regulator (*fur*) have been widely found to regulate the expression of *feo* in the presence or absence of iron (22, 75). When iron is limited by chelators, Fur releases repression, upregulating the expression of iron transport genes (22, 75). Fur protein has been also indicated to directly or indirectly mediate manganese-dependent regulation of gene expression (76). Likewise, fumarate and nitrate reductase transcriptional regulator (Fnr), which is a transcriptional activator of anaerobic respiratory genes (69), is known to activate transcription of the Feo system under anoxic conditions (48) that can increase the expression level of Feo up to 3-fold (22). Indeed, the genome of TIE-1 appears to have at least 11 identifiable Fnr family proteins (Table S3) that are involved in oxygen sensing (50).

Curiously, we observed opposite gene expression trends under oxic conditions. The expression of *feoB* and *efeU* in the basal YP medium was lower with EDTA than the expression obtained under the control conditions (Fig. 6 and 7). We also observed growth inhibition of WT strains with EDTA (Fig. S2f to j). EDTA has been reported to kill both planktonic cells and biofilms of *P. aeruginosa* (63). We speculate that EDTA may have prevented TIE-1's growth (Fig. S2f to j) and subsequent gene expression of *feoB* and *efeU*. This could be also true in the case of growth with Mn (Fig. 7F to J). However, the addition of Fe or Mn may reduce the negative effect of EDTA via its interaction with added metals. This is also reflected by their increased growth when Fe or Mn was added to the YP medium (Fig. S2f to j). Therefore, growth and metal limitation in the presence of Fe and EDTA may have contributed to the increased relative gene expression of *feoB* and *efeU* under oxic conditions. However, under anoxic conditions, the presence of other divalent cations, such as Ca and Mg in FW medium (stability constants of 10.6 and 8.7, respectively) (77), may increase the binding competition for EDTA, freeing more Fe for cells. Future work, however, will be required to confirm this hypothesis.

Overall, our results show that TIE-1 tightly regulates and maintains Fe and Mn homeostasis. The deletion of any single gene or operon does not affect the metal uptake capacity and viability of TIE-1. This suggests that the existence of compensatory systems for metal uptake in TIE-1 is crucial for its metabolic adaptability under different levels of Fe/Mn and oxygen.

R. palustris TIE-1 has homologs for both Fur and two Irr proteins that mediate

iron-dependent regulation of gene expression (Table S3) (68). The genome of TIE-1 appears to have at least 11 identifiable Fnr family proteins (Table S3) that are involved in oxygen sensing (78). One of these homologs is the FixK protein, which has a Fe-S cluster, and its oxygen sensing response is linked to the two-component regulation system FixLJ (64).

We have recently shown that TIE-1 can produce bioplastics and biofuels such as *n*-butanol under various growth conditions (35; Bai et al., submitted). Other strains of *Rhodospseudomonas* have also been used to produce value-added compounds (29–34). Production of such biomolecules is controlled by intracellular electron availability, which, in turn, is modulated by the action of various Fe-containing proteins. Mn is part of many enzymes in central metabolism. By controlling Fe and Mn intake via synthetic biology, we can modulate bioproduction by *R. palustris* TIE-1. This will be investigated in future studies in which overexpression of the two Fe transporters *feoAB* and *efeU* (involved in Fe and Mn transport that we have identified here) will be performed with bioplastic and biofuel production strains to see if product titers can be increased.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains were obtained from the sources indicated in Table 2. They were grown in lysogeny broth (LB) at pH 7.0 at 37°C with shaking at 225 rpm. *Rhodospseudomonas palustris* TIE-1 was obtained from Dianne K. Newman, Division of Biology and Biological Engineering and the Division of Geological and Planetary Sciences, California Institute of Technology. Prior to photoferrotrophy [Fe(II) as an electron donor], TIE-1 cells were grown chemoheterotrophically in medium with 0.3% yeast extract and 0.3% peptone (YP), with 1 mM sodium succinate and 10 mM MOPS [3-*N*-(morpholino)propanesulfonic acid] at pH 7.0 in the dark at 30°C with shaking at 250 rpm (64). Time course cell growth was monitored using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific, USA). To adapt the cells to photoautotrophic growth using H₂ as the sole electron donor, chemoheterotrophically grown TIE-1 cells were transferred into anoxic (purged with 34.5 kPa N₂-CO₂ [80%-20%]) bicarbonate-buffered freshwater (FW) (79) medium with ammonium chloride (NH₄Cl; 5.61 mM) as a nitrogen source and H₂ as an electron donor. The cells were incubated at 30°C under a 60-W incandescent light source placed at a distance of 12.5 cm. For photoautotrophic growth with Fe(II) as an electron donor, photoautotrophically grown TIE-1 cells were inoculated into anoxic FW medium prepared under a flow of 34.5 kPa N₂-CO₂ (80%-20%) and dispensed into presterilized serum bottles/Balch tubes purged with 34.5 kPa N₂-CO₂ (80%-20%). The containers were then sealed using sterile butyl rubber stoppers with aluminum crimps and stored at room temperature for at least a day before supplementation with anoxic sterile stocks of FeCl₂ to a final concentration of 5 mM. For anoxic growth, all sample manipulations were performed inside an anaerobic chamber (Coy; MI). For growth on solid medium, LB or YP medium was solidified with 1.5% agar and supplemented with antibiotics as described previously (64).

DNA isolation and plasmid and strain construction. Genomic DNA of TIE-1 was isolated using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) and used as a template for PCRs. All nucleic acids isolated in this study were quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). A QIAprep spin miniprep kit (Qiagen, Valencia, CA) was used for plasmid DNA isolation from *E. coli*. All primers used in this study were obtained from Integrated DNA Technologies, Coralville, IA, and their sequences are available upon request. The identity of all DNA constructs was confirmed via DNA sequencing at Genewiz Inc., South Plainfield, NJ. *E. coli* strains were transformed by electroporation using an *E. coli* Gene Pulser (Bio-Rad, Hercules, CA), as recommended by the supplier. Plasmids were mobilized from *E. coli* S17-1/λ pir into TIE-1 by conjugation on YP agar. All plasmids constructed are indicated in Table S4. Mutant construction was performed as described previously for TIE-1 (28, 36, 64, 65). Briefly, 1-kb DNA fragments upstream and downstream of the gene of interest were PCR amplified using TIE-1 genomic DNA as the template and cloned into pJQ200KS, a suicide vector. The constructs were mobilized into TIE-1 via conjugation with *E. coli* S17-1. Homologous recombination was allowed to occur on selective YP medium containing 400 μg/ml of gentamicin. Vector loss was selected using media containing 10% sucrose. Mutants lacking the target genes were screened using PCR. The mutants created are listed in Table 2. The primers used for verification are provided in Table 3. All the mutants and plasmids are maintained by the lab as frozen stocks and will be distributed upon request.

***E. coli* β-galactosidase assays.** *E. coli* H1771 derivatives were grown in LB medium at pH 7.0 at 37°C with shaking at 225 rpm. Four different growth conditions were tested for each strain: (i) Fe(III) replete [50 μM Fe(III)-citrate], (ii) Fe(III) depleted [50 μM Fe(III)-citrate with 100 μM DiP], (iii) Fe(II)-replete [50 μM ascorbate-reduced Fe(III)-citrate], and (iv) Fe(II)-depleted [50 μM ascorbate-reduced Fe(III)-citrate with 100 μM DiP]. Ascorbate-reduced Fe(III)-citrate was prepared as previously described (32). The *E. coli* strains were grown with 100 μg/ml of ampicillin to maintain the pWKS30 plasmid derivatives. Overnight cultures of these strains were inoculated into the respective media with a dilution factor of 1:100. The cultures were grown at 37°C with shaking at 225 rpm for 5 h. These cells were then assayed for β-galactosidase activity as previously described using the Miller assay (80). β-Galactosidase activity, expressed in Miller units, was calculated using the following formula: Miller units = (OD₄₂₀ × 1,000)/

TABLE 2 Strains used and constructed in this study

Strain	Description ^a	Reference or source
<i>E. coli</i> strains		
H1771	MC4100 <i>aroB feoB7 fhuF::Δplac Mu</i>	48
GR536	<i>ΔfecABCDE::kan ΔzupT::cat ΔmntH ΔfeoABC ΔentC</i>	52
GG48	<i>zntA::kan ΔzitB::cam</i>	56
<i>ΔcopA ΔcueO ΔcusCFBA::cat</i> mutant	<i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	Kindly provided by Gregor Grass
S17-1	<i>Tp^r Sm^r recA thi pro hsdR^{M+} RP4-2 Tc::Mu Km::Tn7</i>	85
AB445	pWKS30 in <i>E. coli</i> H1771	This study
AB450	pAB446 in <i>E. coli</i> H1771	This study
AB451	pAB447 in <i>E. coli</i> H1771	This study
AB452	pAB448 in <i>E. coli</i> H1771	This study
AB453	pAB449 in <i>E. coli</i> H1771	This study
AB455	pAB454 in <i>E. coli</i> H1771	This study
AB457	pAB456 in <i>E. coli</i> H1771	This study
AB463	pAB462 in <i>E. coli</i> H1771	This study
AB464	pAB446 in <i>E. coli</i> GR536	This study
AB465	pAB447 in <i>E. coli</i> GR536	This study
AB466	pAB448 in <i>E. coli</i> GR536	This study
AB467	pAB449 in <i>E. coli</i> GR536	This study
AB468	pAB454 in <i>E. coli</i> GR536	This study
AB469	pAB456 in <i>E. coli</i> GR536	This study
AB470	pAB462 in <i>E. coli</i> GR536	This study
AB471	pWKS30 in <i>E. coli</i> GR536	This study
AB472	pAB434 in <i>E. coli</i> GR536	This study
AB486	pWKS30 in <i>E. coli</i> GG48	This study
AB487	pAB434 in <i>E. coli</i> GG48	This study
AB488	pAB446 in <i>E. coli</i> GG48	This study
AB489	pAB447 in <i>E. coli</i> GG48	This study
AB490	pAB448 in <i>E. coli</i> GG48	This study
AB491	pAB449 in <i>E. coli</i> GG48	This study
AB492	pAB454 in <i>E. coli</i> GG48	This study
AB493	pAB456 in <i>E. coli</i> GG48	This study
AB494	pAB462 in <i>E. coli</i> GG48	This study
AB588	pAB413 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB589	pAB434 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB590	pAB446 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB591	pAB447 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB592	pAB448 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB593	pAB449 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB594	pAB454 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB595	pAB456 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
AB596	pAB462 in <i>E. coli</i> <i>ΔcopA ΔcueO ΔcusCFBA::cat</i>	This study
<i>R. palustris</i> TIE-1 strains		
DKN379	WT	26
AB41	<i>ΔfeoAB</i>	This study
AB63	Complemented <i>ΔfeoAB</i> strain	This study
AB42	<i>ΔefeU</i>	This study
AB64	Complemented <i>ΔefeU</i> strain	This study
AB68	<i>Δnramp1B</i>	This study
AB43	<i>Δnramp1AB</i>	This study
AB65	Complemented <i>Δnramp1AB</i> strain	This study
AB77	<i>Δnramp3A</i>	This study
AB70	<i>Δnramp3B</i>	This study
AB44	<i>Δnramp3AB</i>	This study
AB66	Complemented <i>Δnramp3AB</i> strain	This study
AB72	<i>ΔsitB</i>	This study
AB74	<i>ΔsitC</i>	This study
AB45	<i>ΔsitABC</i>	This study
AB67	Complemented <i>ΔsitABC</i> strain	This study

^aTp^r, trimethoprim resistance; Sm^r, streptomycin resistance.

(OD₆₀₀ × V × t), where V is volume of culture processed for each sample and t is time elapsed from start to end of the reaction.

E. coli growth curves. *E. coli* GR536 derivatives were maintained in LB medium with pH 7.0 with 25 μg/ml of chloramphenicol and 50 μg/ml of kanamycin. The strains carrying pWKS30 derivatives had 100 μg/ml of ampicillin in addition to chloramphenicol and kanamycin. Overnight cultures of these

TABLE 3 Primers used to construct and check mutants constructed in this study

Primer	Sequence
Mutant construction	
Δ feoBupforApal	ACTAGTGGGCCCCGACTCCGCGTCATGCCCCGGCCTCGTG
Δ feoBuprevSmaI	ACTAGTCCCGGGCGCGCAATCGCCAAATCAAGAAGCTGCAATCTG
Δ feoAdnforEcoRV	ACTAGTGATATCTGACGCGCAGATTGCAGCTTCTTGATTG
Δ feoAdnrevSpeI	GGATCCACTAGTGAACGCGCGCGCCATGTAGC
Δ efeUupforXhoI	ACTAGTCTCGAGCGCGGCTTCGACCATCAAGCAC
Δ efeUuprevBamHI	ACTAGTGGATCCCTGCGCCAACCTCGCTCGCCCAAC
Δ efeUdnforBamHI	ACTAGTGGATCCTGAGCCGAACCTGCAGCG
Δ efeUdnrevNotI	ACTAGTGCGGCCGCCACGCGTTCCACGGAATCAAGC
Δ nramp1upforPstI	ACTAGTCTGCAGGAATGACGACGAGAAGAATC
Δ nramp1uprevBamHI	ACTAGTGGATCCCGAAAGCGTCTCTGATGCCTAG
Δ nramp1dnforBamHI	ACTAGTGGATCCTAGCCTCGCGCAGTCATTCCG
Δ nramp1dnrevNotI	ACTAGTGCGGCCGCTGTTACGCTGCGCGAACT
Δ nramp3upforPstI	ACTAGTCTGCAGGCCTATTACGGCGACAGCTAC
Δ nramp3uprevBamHI	ACTAGTGGATCCACGGTGAGATCAGGCGGCTAGC
Δ nramp3dnforBamHI	ACTAGTGGATCCTGATGCATCCGGCGGTTGCTG
Δ nramp3dnrevSpeI	GGCGGCCACTAGTTGAACCTGGTGACGCGGTC
Δ sitABCupforSall	ACTAGTGTGACTGACCGACGCCAGACCACGCTG
Δ sitABCuprevBamHI	ACTAGTGGATCCGGTCACCTCATTGCCACCAACAC
Δ sitABCdnforBamHI	ACTAGTGGATCCTGACGACGCGCGGTCCTCCGCTG
Δ sitABCdnrevNotI	ACTAGTGCGGCCGCGTCTGCACCAATTGCTGCAGC
Mutant check	
Δ feoAupckfor	TTTGTGCCAAATCAAACCA
Δ feoAdnckrev	TGAACAGGGAGGTCTTACCG
Δ feoBupckfor	GAAGTGCTGCACGAGGGTAT
Δ feoBdnckrev	AACTCATCACGTTGGTCGTG
Δ nramp1upckfor	GACCTGCTCGGTGTGATCC
Δ nramp1dnckrev	GGACTCTGCCCCAACTAATT
Δ nramp3upckfor	CGAAATCAGCATCAGACGTG
Δ nramp3dnckrev	ATCTGCGATCTCGACGATGT
Δ sitAupckfor	CCTCCCTCTCAATCCATT
Δ sitAdnckrev	CATGAATTCGAATCGGTGA
Δ sitBupckfor	CCGACAACCAGGTGGAAG
Δ sitBdnckrev	GGACGCGGTATAGACGTGAA
Δ sitCupckfor	CCAGCCATCATCCTGGTC
Δ sitCdnckrev	GGTCATCAGGCAGTTACTCCA

strains were grown and inoculated into Tris-mineral salts medium with 3 g/liter of Casamino Acids and 0.2% sodium gluconate (50 mM Tris-Cl [pH 7.0], 80 mM NaCl, 20 mM KCl, 20 mM NH_4Cl , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 μM iron ammonium citrate, 3 mM $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 50 μM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) with $0.1 \times \text{SL6}$ trace elements solution. Increasing concentrations of 2,2'-dipyridyl (DiP) were added to the medium, with the highest concentration at 150 μM . Similar to *E. coli* GR536 derivatives, *E. coli* GG48 derivatives were also maintained in LB medium with pH 7.0 with 25 $\mu\text{g}/\text{ml}$ of chloramphenicol and 50 $\mu\text{g}/\text{ml}$ of kanamycin. The zinc, cadmium, and copper toxicity tests were performed in LB medium at pH 7.0 with increasing concentrations of added ZnCl_2 from 0 to 400 μM , CdCl_2 from 0 to 100 μM , and CuCl_2 from 0 to 400 μM , respectively. The cultures were grown at 37°C with shaking at 225 rpm. Samples were withdrawn periodically for OD_{600} measurements. *E. coli* Δ copA Δ cueO Δ cusCFBA::cat derivatives were maintained in pH 7.0 LB medium with 25 $\mu\text{g}/\text{ml}$ of chloramphenicol. The strains carrying pWKS30 derivatives had 100 $\mu\text{g}/\text{ml}$ of ampicillin in addition to chloramphenicol.

Iron and manganese uptake assays. Iron and manganese uptake assays were performed as previously described (48), with some modifications. For anoxic growth conditions, TIE-1 strains were grown in FW medium at pH 7.0 with 1 mM sodium succinate at 30°C under a 60-W incandescent light source placed at a distance of 12.5 cm from the cultures. Time course OD_{600} was measured by withdrawing the samples under anoxic conditions. Late-exponential-phase cultures were then aliquoted into presterilized anoxic Balch tubes, followed by headspace flushing with 34.5 kPa $\text{N}_2\text{-CO}_2$ (80%-20%). The tubes were placed on ice until use. For oxic growth conditions, TIE-1 was grown in YP medium with 3 g/liter of yeast extract and 3 g/liter of peptone supplemented with 1 mM succinate, with shaking at 250 rpm for 120 h.

To test iron and manganese accumulation by single-gene mutants and complemented and WT strains and their responses to metal-replete and -depleted conditions with respect to oxygen availability, the following experiments were included: (i) normal level of Fe(II) or Mn(II) (basal media only), (ii) basal media with 100 μM DiP or EDTA, (iii) basal media with 50 μM Fe(II) or Mn(II), and (iv) basal media with 100 μM DiP or EDTA and 50 μM Fe(II) or Mn(II), under oxic and anoxic conditions. Fe(II), Mn(II), EDTA, and DiP were added from a 50 mM stock solution at the beginning of the growth.

Iron and manganese analysis by ICP-MS analysis. A 1-ml sample was taken from the cultures incubated with iron or manganese and centrifuged at $21,000 \times g$ for 5 min. Samples were washed with

TABLE 4 RT-qPCR primers used in this study

Primer name	Sequence	Reference or source
recAfor	ATCGGCCAGATCAAGGAAC	64
recArev	GAATTCGACCTGCTTGAACG	64
clpXfor	GGAGATCTGCAAGGTTCTCG	64
clpXrev	CCGCTTGTAGTGATTGTGGA	64
feoAfor	CCCATCACAAACGGCCATAG	This study
feoArev	ACCCAGCTCGATCAGTC	This study
feoBfor	TCGATTCTCAGCCGTTCAAC	This study
feoBrev	GTAGTTCGCCACCTTCTGAC	This study
efeUfor	TGACCAGCCGAGAAATCTATG	This study
efeUrev	TGATGGTTTCCTGTCGCAG	This study
nramp1Afor	CTTTCAGATCGTGCTGTTT	This study
nramp1Arev	AATCGGAGACACCGACTTTG	This study
nramp1Bfor	CGCCCTATCTGTTCTTCTGG	This study
nramp1Brev	GGTGTCTTGATCAGCGGTTT	This study
nramp2for	CGATCACTGCCACCGATCT	This study
nramp2rev	GCGGAATCCGAACAACA	This study
nramp3Afor	AACTTCAAAACCGCTTGGTG	This study
nramp3Arev	GTTGTCGTTGGCGTGGAC	This study
nramp3Bfor	ATTCGGCACATACACCCAG	This study
nramp3Brev	CCATCTCCTGATTGACGTACAG	This study
sitAfor	TGGCACGCTGTATTCCG	This study
sitArev	TGCCGGACCATATCAATGTAAG	This study
sitBfor	AAGGGCACCAACATCGATC	This study
sitBrev	TCGACGCATTCAATACCAG	This study
sitCfor	ACCTTTAACGATGTCACCCTC	This study
sitCrev	TTCAGGATACCAACGATGCC	This study

1 ml of Tris-EDTA (TE) buffer and 1 ml of ultrapure water. Washed pellets were then added with 50 μ l of high-purity HNO_3 trace element (Fisher) and digested with a CEM MARS 6 microwave at 180°C for 20 min. Digested samples were then diluted 2-fold with ultrapure water and filtered with a 0.22- μ m polyether-sulfone (PES) filter. Filtered samples were analyzed using a Perkin Elmer Elan DRC II ICP-MS. Iron was analyzed as a 55.845 isotope and manganese as a 54.94 isotope. Yttrium was used as an internal standard. Standard curves were built from ICP-MS-grade iron and manganese (Inorganic Venture, VA) using the following concentrations: 0, 1, 10, 50, 100, and 200 ppb. Standards were also used as abiotic controls. Obtained Fe and Mn concentrations were normalized to the OD of the sample and further normalized to the concentration obtained from the WT TIE-1.

Gene expression analysis. Gene expression analysis was performed using RT-qPCR. We quantified the mRNA abundances of the putative Fe(II) transporter genes in TIE-1 under the following conditions: (i) basal medium only (Fe, ~ 4 nM; Mn, ~ 0.25 nM), (ii) basal medium with 100 μ M DiP or EDTA, (iii) basal medium with 50 μ M Fe(II) or Mn(II), and (iv) basal medium with 100 μ M DiP or EDTA and 50 μ M Fe(II) or Mn(II), under oxic and anoxic conditions. The data were normalized to the gene expression obtained from the basal medium only. The comparative threshold cycle (C_T) method was used as described previously (64) to assess the expression of the relevant genes. Primer efficiencies were determined using the manufacturer's protocol (Applied Biosystems Inc.; user bulletin number 2). *clpX* and *recA* were used as the two internal standards; they have been previously used and validated as internal standards (64). The primers used for the assays are indicated in Table 4. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used for reverse transcription. iTaq FAST SYBR green Supermix with ROX (Bio-Rad) and the Stratagene Mx3005P qPCR system (Agilent, Santa Clara, CA) were used for all quantitative assays.

Bioinformatics tools. For identifying iron transporters in TIE-1, delta-blast (81), FASTA (82; <http://www.ebi.ac.uk/Tools/sss/fasta/>), and the IMG ortholog neighborhood search (83; <http://img.jgi.doe.gov/cgi-bin/w/main.cgi>) were used. Transmembrane predictions were made using the TMHMM server v. 2.0 at <http://www.cbs.dtu.dk/services/TMHMM/>. Promoter predictions were made using Virtual Footprint v. 3.0 at <http://www.prodoric.de/vfp/> (84).

Statistical analysis. All statistical analyses (two-tailed Student's *t* test) were performed with Python. A *P* value of <0.05 was considered significant. All the experiments were carried out using biological triplicates.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.05 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

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