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The Metal Permease ZupT from *Escherichia coli* Is a Transporter with a Broad Substrate Spectrum

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The *Escherichia coli zupT* (formerly *ygiE)* gene encodes a cytoplasmic membrane protein (ZupT) related to members of the eukaryotic ZIP family of divalent metal ion transporters. Previously, ZupT was shown to be responsible for uptake of zinc. In this study, we show that ZupT is a divalent metal cation transporter of broad substrate specificity. An *E. coli* strain with a disruption in all known iron uptake systems could grow in the presence of chelators only if *zupT* was expressed. Heterologous expression of *Arabidopsis thaliana* ZIP1 could also alleviate iron deficiency in this *E. coli* strain, as could expression of indigenous *mntH* or *feoABC*. Transport studies with intact cells showed that ZupT facilitates uptake of $^{55}Fe^{2+}$ similarly to uptake of MntH or Feo. Other divalent cations were also taken up by ZupT, as shown using $^{57}Co^{2+}$. Expression of *zupT* rendered *E. coli* cells hypersensitive to Co^{2+} and sensitive to Mn^{2+}. ZupT did not appear to be metal regulated: expression of a Φ(*zupT-lacZ*) operon fusion indicated that *zupT* is expressed constitutively at a low level.

The ZIP family derived its name from the first identified members, ZRT, IRT-like protein (15). These proteins were initially identified as iron or zinc transporters in eukaryotes, but some members were subsequently shown to also transport other metals, such as manganese or cadmium. However, the specificity and affinity for different metals change with each ZIP transporter (10, 15). ZupT is the first characterized bacterial member of the ZIP family and was shown to be responsible for zinc uptake in *Escherichia coli* (13).

The transport mechanism for members of the ZIP family is still unknown. All of the functionally characterized ZIP proteins are predicted to have similar membrane topologies, with eight transmembrane domains and the amino- and carboxy-terminal ends of the protein located on the outside surface of the plasma membrane (12). *Arabidopsis* ZIP proteins range from 326 to 425 amino acids in length, the difference being largely due to the extension of a variable region probably located in the cytoplasm between transmembrane domains III and IV. In most cases, this variable region contains a potential metal-binding domain rich in His residues. For example, in ZIP1, this motif is HAGHVHIHTHASHGHTH. Although the function of this motif is unknown, its conservation in many of the ZIP proteins suggests it may have a role in metal transport or regulation (15).

In this report, we investigated the role and metal specificity of ZupT. Studies examining factors determining metal specificity of an individual transporter are often complicated by redundant transport systems. In *E. coli*, ferrous iron (Fe^{2+}) is taken up with high affinity by the gene products of the *feo* locus (21). Ferrous iron may also be transported into the cytoplasm by the manganese permease MntH (24). The magnesium transporter CorA was also reported to be capable of ferrous iron uptake (4, 16), but recent work has demonstrated that Fe^{2+} is not transported by CorA and that Fe^{2+} does not significantly inhibit Mg^{2+} transport via CorA (27). Ferric iron (Fe^{3+}) is taken up by the gene products of the *fec* locus as a complex with the chelator citrate (17) or by several other receptors for iron chelates (siderophores) in the outer membrane.

Therefore, *E. coli* strains deficient in all relevant iron and manganese uptake systems were created. We found that ZupT can transport iron and cobalt in addition to zinc and possibly manganese. The *zupT* gene was not induced by the presence or absence of metals and appears to be constitutively expressed.

MATERIALS AND METHODS

Bacterial strains and growth media. Strains used are listed in Table 1. *E. coli* was grown in Luria-Bertani medium or Tris-buffered mineral salts medium (25) containing 2 g of glycerol and 3 g of Casamino Acids per liter. Antibiotics (chloramphenicol [15 to 20 μg/ml], kanamycin [25 μg/ml], ampicillin [100 μg/ml], and tetracycline [12.5 μg/ml]) and metals were added where appropriate.

Gene disruptions and deletions. Genes were disrupted by the insertion of Kan^r or Cam^r cassettes by employing a protocol developed in the laboratory of B. Wanner, based on the λ Red-recombinase system, as described previously (7). Multiple deletions were constructed by elimination of the respective resistance cassette and subsequent phage P1 transduction.

Deletion of *mntH* or *zupT*, respectively, in strain GR460 (Δ*feoABC*::*cat* Δ*entC)* led to strains GR489 (Δ*mntH*::*cat* Δ*feoABC* Δ*entC*) and GR507 (Δ*zupT*::*cat* Δ*feoABC* Δ*entC*). A quadruple deletion mutant, GR499 (Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*) was constructed from strain GR489. To delete all known iron uptake systems from *E. coli* necessary for growth in minimal medium, an additional deletion in the ferric-citrate uptake determinant, Δ*fecABCDE*, was introduced into the strains mentioned above, resulting in strains GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)*, GR537 (Δ*fecABCDE*::*kan* Δ*mntH*::*cat* Δ*entC* Δ*feoABC*), GR538 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*entC* Δ*feoABC*), and GR539 (Δ*fecABCDE*::*kan* Δ*entC*::*cat* Δ*feoABC*).

Construction of a *zupT-lacZ* operon fusion. Expression of *zupT* was analyzed using a transcriptional fusion with *lacZ* as a reporter gene. To construct the chromosomal Φ(*zupT-lacZ*) transcriptional fusion in strain *E. coli* SF1 [W3110 Φ(*zupT-lacZ*) Δ*lacZYA*::*kan*], the 400 bp upstream and downstream of the *zupT*

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype	Source or reference
E. coli strains		
W3110	Wild type	
GG161	W3110 Δ*lacZYA*::*kan*	11
SF1	W3110 Φ(*zupT-lacZ*) Δ*lacZYA*::*kan*	This study
SF4	W3110 Φ(*zupT-lacZ*) Δ*znuABC*::*cat* Δ*lacZYA*::*kan*	This study
SF14	W3110 Φ(*zupT-lacZ*) Δ*mntH* Δ*feoABC* Δ*entC* Δ*lacZYA*::*kan*	This study
GR417	W3110 Δ*entC*::*cat*	This study
GR460	W3110 Δ*feoABC*::*cat* Δ*entC*	This study
GR489	W3110 Δ*mntH*::*cat* Δ*feoABC* Δ*entC*	This study
GR499	W3110 Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*	This study
GR507	W3110 Δ*zupT*::*cat* Δ*feoABC* Δ*entC*	This study
GR532	W3110 Δ*zupT*::*cat* Δ*mntH*	This study
GR536	W3110 Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC*	This study
GR537	W3110 Δ*fecABCDE*::*kan* Δ*mntH*::*cat* Δ*entC* Δ*feoABC*	This study
GR538	W3110 Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*entC* Δ*feoABC*	This study
GR539	W3110 Δ*fecABCDE*::*kan* Δ*entC*::*cat* Δ*feoABC*	This study
ECA281	Δ*zupT*::*cat* Δ*corA*	This study
Plasmids		
pACYC184		New England Biolabs
pZUPT-low	*zupT* with appr. 300-bp upstream region EcoRI in pACYC184[a]	This study
pASK-IBA3		IBA GmbH, Göttingen, Germany
pZUPT	*zupT* from *E. coli* in pASK-IBA3	13
pZITB	*zitB* from *E. coli* in pASK-IBA3	11
pMntH	*mntH* from *E. coli* in pASK-IBA7	This study
pFEO	*feoABC* from *E. coli* in pASK-IBA7	This study
pYIIP	*yiiP* (*fieF*) from *E. coli* in pASK-IBA3	11
pCZCD	*czcD* from *Ralstonia metallidurans* CH34 in pASK-IBA5	2
pZIP1	ZIP1 from *Arabidopsis thaliana* in pACYC184	Natasha Grotz

[a] appr., approximately.

stop codon were separately amplified by PCR from chromosomal DNA of *E. coli* W3110. These fragments were digested with BamHI, and both fragments were joined and cloned into vector plasmid pGEM T-Easy (Promega, Madison, Wis.) in one step. As confirmed by sequencing, this led to a plasmid harboring an 800-bp *zupT* fragment with a BamHI site and an XbaI site located directly downstream of the stop codon of *zupT*, mutating the sequence CAT**TAA**TGGG ACAGC (the TAA stop codon of *zupT* is in boldface) to CAT**TAA**GGATCCG GGTCTAGAGGCCATTCACATCATCACCATTAATGGGACAGC (underlining indicates restriction sites for BamHI and XbaI). A promoterless *lacZ* gene was inserted into the BamHI/XbaI sites of this plasmid, and the fragment containing *zupT-lacZ* was cloned as a NotI fragment into plasmid pKO3 (23). Finally, the pKO3 hybrid plasmid with Φ(*zupT-lacZ*) was used in a double-recombination event to insert the *lacZ* gene downstream of *zupT* on the chromosome of *E. coli* GG161 (W3110 Δ*lacZYA*::*kan*) as described previously (11), resulting in strain SF1. The correct insertion and orientation of *lacZ* in *E. coli* strain SF1 were verified by PCR.

Cloning of *zupT*, *mntH*, and *feo*. The open reading frame of *zupT* with its upstream region was PCR amplified from chromosomal DNA of *E. coli* strain W3110 and cloned into plasmid pGEM T-Easy (Promega). Inserts were sequenced and subcloned into the EcoRI site of low-copy-number vector pACYC184. The *mntH* or *feoABC* gene was PCR amplified and cloned into expression vector pASK-IBA7 (IBA GmbH, Göttingen, Germany).

CAS agar plates. *E. coli* strains were grown overnight in Luria-Bertani medium with shaking at 37°C, diluted 1:500 into Tris-buffered minimal medium (25) supplemented with 2 ml of glycerol and 3 g of Casamino Acids per liter. Cultures were grown overnight and spread on Chrome Azurol S (CAS) agar plates. CAS agar plates were prepared as described previously (34).

Metal uptake. Uptake experiments were performed by filtration. Stationary-phase cultures were diluted to 30 Klett units in minimal medium. The cells were then grown to an optical density of 60 Klett units, and gene expression was initiated with the addition of 200 μg of anhydrotetracycline (AHT) per liter. After growth for 35 min, cells were washed with Tris-buffered mineral salt medium without Casamino Acids and iron or with 10 mM Tris-HCl, pH 7.0. Metal uptake was started by addition of a mixture of ascorbate (final concentration, 1 mM) and $FeSO_4$, labeled with $^{55}FeCl_3$, (final iron concentration, 5 μM), or $CoCl_2$ labeled with $^{57}CoCl_2$, (final cobalt concentration, 5 μM). The cells were incubated with shaking, and 0.4- or 0.5-ml aliquots were filtered through nitrocellulose membranes (0.45 μm) at various times and immediately washed with 6 ml of 0.1 mM LiCl (for iron) or buffer (10 mM Tris-HCl [pH 7.0], 10 mM $MgCl_2$) (for cobalt). The membranes were dried, and radioactivity was measured using a liquid scintillation counter (LS6500; Beckman, München, Germany). The dry weight (d.w.) was determined from the optical density using a calibration curve. $^{55}FeCl_3$ and $^{57}CoCl_2$ were from Perkin-Elmer (Boston, Mass.).

ZupT overexpression and purification. ZupT was purified by using Strep-TagII technology (IBA GmbH, Göttingen, Germany). The *zupT* gene was expressed from plasmid pZUPT in *E. coli* strain BL21 cells (Stratagene Europe, Amsterdam, The Netherlands). Cells were cultivated overnight at 37°C in Luria-Bertani broth, diluted 1:50 into 2 liters of fresh medium, and cultivated with shaking at 30°C up to an optical density at 600 nm of 1.0. Expression of *zupT* was induced by addition of 200 μg of anhydrotetracycline/liter, and incubation continued for 3 h. Cells were harvested by centrifugation (7,650 × *g*, 4°C, 15 min), suspended in 20 ml of buffer W (100 mM Tris-HCl [pH 8.0]), and broken twice via French press (SLM Aminco, Urbana, Ill., at 138 kPa) in the presence of protease inhibitor cocktail (Sigma-Aldrich, Deisenhofen, Germany) and DNaseI (10 g/liter). Debris was removed by centrifugation (23,400 × *g*, 15 min, 4°C), and the membrane fraction was isolated by ultracentrifugation (100,000 × *g*, 2 h, 4°C). The membrane pellet was suspended in buffer W to a final protein concentration of 10 g/liter. ZupT was solubilized with 1% (wt/vol) *n*-lauroyl sarcosine for 45 min on ice with stirring, and residual membrane fragments were removed by ultracentrifugation (100,000 × *g*, 30 min, 4°C). The resulting solubilized protein fraction was applied to a Strep-Tactin-Sepharose affinity chromatography column (bed volume, 2 ml), which was washed subsequently with 30 and 20 ml of buffer W containing 0.1% (wt/vol) *n*-lauroyl sarcosine with or without 1 M NaCl. Finally, ZupT was eluted with 100 mM Tris-HCl buffer (pH 8.0) containing 2.5 mM desthiobiotin and 0.05% (wt/vol) *n*-lauroyl sarcosine. Total ZupT protein yield was approximately 75 μg/liter of culture.

Immunoblotting. ZupT protein samples were separated on sodium dodecyl sulfate-polyacrylamide gels, blotted (SemiDry-Blot; Biometra, Göttingen, Germany) onto a polyvinylidene difluoride membrane, and incubated with a Strep-Tactin horseradish peroxidase conjugate. Blots were developed with a chromogenic substrate as described previously (22).

TABLE 2. Growth of several *E. coli* strains on Chrome Azurol S agar plates

Strain	Genotype	Characteristics
GR499	Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*	No growth
GR507	Δ*zupT*::*cat* Δ*feoABC* Δ*entC*	Growth after 2 to 3 days, no halos
GR489	Δ*mntH*::*cat* Δ*feoABC* Δ*entC*	Growth after 2 to 3 days, no halos
GR460	Δ*feoABC*::*cat* Δ*entC*	Growth overnight, no halos
GR417	Δ*entC*::*cat*	Growth overnight, no halos
GR532	Δ*zupT*::*cat* Δ*mntH*	Growth overnight, halos
W3110	Wild type	Growth overnight, halos

Miscellaneous. Standard molecular genetic techniques were used (33). Chromosomal DNA of *E. coli* strain W3110 was isolated by using Genomic-Tips (QIAGEN). PCR was performed with *Pwo* or *Taq* DNA polymerase (Roche, Fermentas). DNA sequencing was performed at the DNA Sequencing Service facility of the University of Arizona. The β-galactosidase activity in permeabilized cells was determined as published previously (11, 26).

RESULTS

ZupT is involved in iron uptake in *E. coli*. There are multiple pathways of iron uptake in *E. coli*. In an effort to elucidate the role of ZupT in iron transport in *E. coli*, all known systems required for iron uptake in defined minimal medium were deleted. Deletion of *entC*, coding for isochorismate synthase 2, results in an inability to synthesize enterobactin, the indigenous catechol siderophore of *E. coli* (29). Deletion of *feo* reduces the ability of high-affinity ferrous iron uptake (21). The manganese permease MntH was also deleted because it can transport ferrous iron at high iron concentrations (24).

Single deletions of *feoABC*, *mntH*, or *zupT* in *E. coli* strain W3110 did not result in an iron-dependent phenotype with growth in mineral salt medium (data not shown). Deletion of *entC* rendered *E. coli* unable to produce enterobactin, and growth was slightly impaired under iron-depleted conditions (data not shown). A double deletion of *feoABC* and *entC* in strain GR460 did not exhibit a phenotype towards iron depletion significantly different from that of strain GR417 (Δ*entC*::*cat*) (data not shown).

When multiple-deletion strains were streaked on CAS agar plates, triple-deletion strains, GR507 (Δ*zupT*::*cat* Δ*feoABC* Δ*entC*) or GR489 (Δ*mntH*::*cat* Δ*feoABC* Δ*entC*), grew after 2 to 3 days, while the quadruple-deletion strain GR499 (Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*) did not (Table 2). Cells lacking only enterobactin grew overnight, as did cells that expressed the siderophore but lacked both MntH and ZupT. The presence or absence of the high-affinity ferrous uptake system Feo in strains GR460 and GR417 had no effect on growth under this condition. These data suggested that for growth under conditions of iron deficiency on CAS agar plates, either production of enterobactin (EntC dependent) or any one of the other iron uptake systems allowed growth.

In liquid mineral salt medium with an added chelator, 2,2′-dipyridyl (DIP), both triple-deletion strains, GR489 and GR507, were slightly less iron dependent than their parental double-deletion strain, GR460 (Δ*feoABC::cat* Δ*entC*) (Table 3). The quadruple-deletion mutant GR499 (Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*) was much more iron dependent in minimal medium with DIP than the triple-deletion mutants GR489 and GR507 (Table 3). However, all strains grew equally well on Luria-Bertani agar (data not shown). This suggested that ZupT could mediate iron uptake. Growth of both triple- and quadruple-deletion mutants was restored to the level of growth of the double-deletion mutant GR460 (Δ*feoABC*::*cat* Δ*entC*) when iron or manganese was added at a concentration equimolar to that of DIP (Table 3).

In dose-response experiments with iron deficiency induced by DIP, the quintuple-deletion strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC*) was affected most (Table 3). Growth of the quintuple-deletion mutant GR536 was only slightly restored by addition of iron, suggesting that all important iron uptake systems were deleted in this strain, and thus, the cells were no longer able to take up sufficient iron even when iron was replete. Interestingly, growth of this mutant could be restored when manganese was added to the

TABLE 3. Effect of different metals on iron depletion of *E. coli* strains harboring multiple gene deletions[a]

Strain	Genotype	Systems still functional	Growth yield in the presence of DIP and[b]:				
			No metal	$MgCl_2$	$ZnCl_2$	$MnCl_2$	$FeCl_3$
GR499	Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*	*Fec*	0.070 ± 0.01	0.09 ± 0.04	0.14 ± 0.02	0.64 ± 0.02	0.66 ± 0.08
GR507	Δ*zupT*::*cat* Δ*feoABC* Δ*entC*	*Fec, MntH*	0.39 ± 0.01	0.31 ± 0.01	0.51 ± 0.09	0.69 ± 0.03	0.65 ± 0.01
GR489	Δ*mntH*::*cat* Δ*feoABC* Δ*entC*	*Fec, ZupT*	0.45 ± 0.03	0.42 ± 0.01	0.42 ± 0.01	0.64 ± 0.01	0.69 ± 0.08
GR460	Δ*feoABC*::*cat* Δ*entC*	*Fec, ZupT, MntH*	0.29 ± 0.02	0.29 ± 0.02	0.35 ± 0.04	0.52 ± 0.02	0.65 ± 0.09
GR536	Δ*fecA-E*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*	None	0.09 ± 0.01	0.08 ± 0.02	0.17 ± 0.02	0.71 ± 0.01	0.22 ± 0.03
GR538	Δ*fecA-E*::*kan* Δ*zupT*::*cat* Δ*feoABC* Δ*entC*	*MntH*	0.52 ± 0	0.51 ± 0.01	0.51 ± 0.04	0.71 ± 0.01	0.81 ± 0
GR537	Δ*fecA-E*::*kan* Δ*mntH*::*cat* Δ*feoABC* Δ*entC*	*ZupT*	0.40 ± 0.2	0.42 ± 0.02	0.64 ± 0.01	0.76 ± 0.01	0.79 ± 0.02
GR539	Δ*fecA-E*::*kan* Δ*feoABC*::*cat* Δ*entC*	*ZupT, MntH*	0.50 ± 0.02	0.51 ± 0.05	0.68 ± 0.04	0.75 ± 0.02	0.83 ± 0.01

[a] Overnight cultures grown in Luria-Bertani broth were diluted 1:500 in minimal medium and grown overnight. Cells were diluted 1:500 in fresh minimal medium, and after 2 h, cells were diluted 1:500 into fresh medium with the indicated additives. The dry weight was determined after 16 h of incubation at 37°C with shaking. Experiments were performed independently in duplicate, and the average with deviation was calculated.

[b] All at a final concentration of 50 μM.

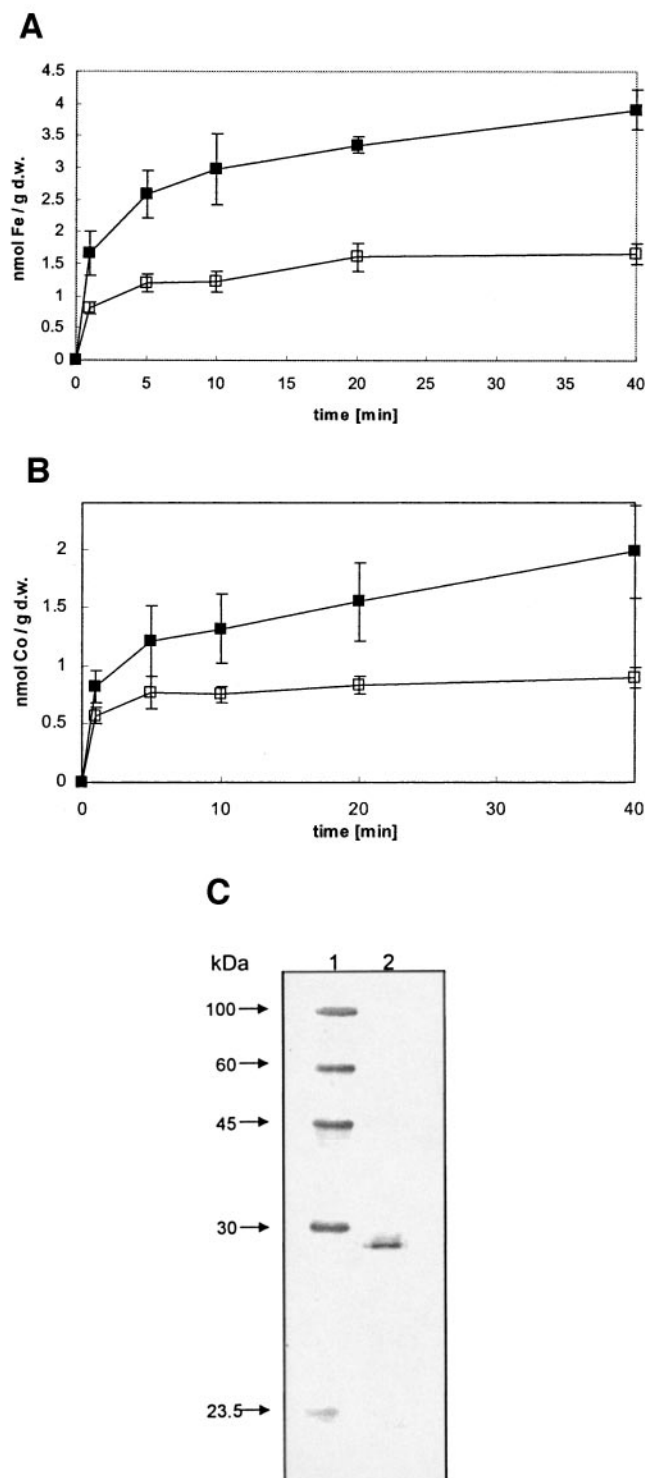

FIG. 1. Metal uptake by *E. coli* expressing *zupT*. Overnight cultures grown in Luria-Bertani broth were diluted 1:500 in Tris-buffered mineral salt medium, grown overnight, inoculated at 30 Klett units into fresh medium at 37°C, and grown to 60 Klett units. Expression of *zupT* was induced with of 200 ng of AHT/ml for 35 min. Uptake was started by addition of (A) a reaction mix of ^{55}Fe (1 μCi), $FeSO_4$ (final concentration, 5 μM), and 1 mM ascorbate or (B) $^{57}CoCl_2$ (1 μCi of ^{57}Co) (final concentration, 5 μM). At defined time points, cellular metal accumulation was determined by the filtration method. Shown are (A) *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* pZupT (■) or pASK-IBA3 (□) and (B) *E. coli* strain ECA281 (Δ*zupT*::*cat* Δ*corA)* pZupT (■) or pASK-IBA3 (□). Averages with standard deviations for three independent experiments are shown. (C) Western blot of Strep-TagII-labeled ZupT protein expressed from plasmid pZUPT in *E. coli*: lane 1, Strep-Tag protein ladder (IBA GmbH, Göttingen, Germany); lane 2, ZupT (0.6 μg of ZupT protein). d.w., dry weight.

growth medium in equimolar concentrations with the chelator DIP.

If ZupT takes up iron, expression of *zupT* in *E. coli* should lead to enhanced growth of an iron uptake-deficient mutant. In the *E. coli* quadruple-deletion strain GR499 (Δ*zupT*::*cat* Δ*mntH* Δ*feoABC* Δ*entC*), expression of *zupT* in *trans* from the low-copy-number plasmid pACYC184 (pZUPT-low) resulted in growth in complex medium containing DIP (data not shown), as opposed to the control strain that harbored only the vector plasmid.

ZupT mediates uptake of ^{55}Fe and ^{57}Co. *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* was transformed with plasmid pZUPT in order to measure uptake of ^{55}Fe by ZupT, and expression of *zupT* was induced with AHT. Cells containing pZUPT showed a significant increase in $^{55}Fe^{2+}$ uptake compared to the *E. coli* strain GR536 pASK-IBA3 vector control (Fig. 1A). This suggested that ZupT is responsible for iron uptake under these conditions. Since the experiments were performed in the presence of excess ascorbate, and thus, the iron would be present mainly in the ferrous state, the transported species is probably ferrous iron.

Likewise, the presence of pZupT in *E. coli* strain ECA281 (Δ*zupT*::*cat* Δ*corA*), which also contains a deletion in the gene of the cobalt transporter CorA, resulted in increased cobalt accumulation, which was not observed for the vector-only control (Fig. 1B). These results indicated that ZupT is able to transport iron and cobalt in addition to zinc.

Iron transport by different transporters of *E. coli*. To compare the ability of ZupT to transport iron with that of other *E. coli* iron transporters, time course experiments were performed under iron depletion conditions. The transporter gene *zupT*, *mntH*, or *feoABC*, respectively, was expressed from the inducible *tet* promoter of high-copy-number plasmid pASK-IBA3 in *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* with increasing concentrations of EDTA. Additionally, *zupT* was expressed from low-copy-number plasmid pACYC184 (pZUPT-low) under its native promoter. Figure 2A shows that MntH and Feo enabled strain GR536 to grow in the presence of EDTA, with Feo being a little more efficient than MntH. The presence of plasmid pZUPT-low resulted in the best growth in the presence of EDTA of all strains tested. However, *zupT* expressed from pZUPT exhibited a phenotype very similar to that of the negative control. This suggested that the expression level of *zupT* has to be low.

Iron uptake experiments with *E. coli* strain GR538, expressing only *zupT*, *mntH*, or *feoABC*, were performed (Fig. 2B). While the presence of the vector control did not lead to increased iron accumulation, the expression of any iron transporter resulted in increased uptake of iron. Under the condi-

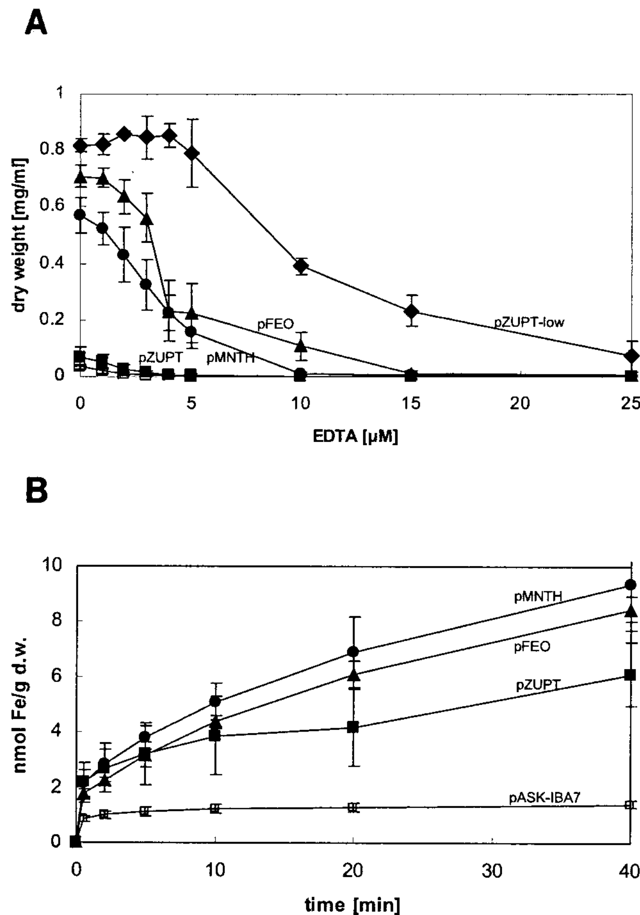

FIG. 2. Growth and iron uptake in *E. coli* expressing different iron transporters. Dose response experiments (panel A) or iron uptake (panel B) for *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* harboring plasmids pASK-IBA7 (□), pMNTH (●), pFEO (▲), pZUPT (■), or pZUPT-low (♦) from at least triplicate experiments with standard deviations are shown. Overnight cultures of *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* grown in Luria-Bertani broth were diluted 1:500 in Tris-buffered mineral salt medium and grown overnight. (A) Cells were diluted 1:500 in fresh medium, and after 2 h of growth at 37°C, cells were diluted 1:500 in fresh medium with iron or different concentrations of EDTA. Cell growth was monitored as the optical density at 600 nm after 16 h of incubation at 37°C with shaking, and the final growth yield is given, or (B) 30 Klett units of cultures were inoculated into fresh medium at 37°C and grown to 60 Klett units. Expression of genes under control of the *tet* promoter from plasmid pASK-IBA7 was induced with of 200 ng of AHT/ml for 35 min. Uptake was started by addition of a reaction mix of ^{55}Fe (1 μCi), $FeSO_4$ (final concentration, 5 μM), and 1 mM ascorbate.

tions tested, MntH was the most effective, followed by Feo and ZupT. In contrast to the dose-response curves (Fig. 2A), expression of pZUPT-low did not lead to significantly increased iron accumulation compared to results with the vector control (data not shown). A possible explanation is that increased and unregulated iron accumulation caused by high-level expression of *zupT* resulted in disadvantageous iron overload of the cells and thus diminished growth.

Expression of *zupT* from a plasmid renders *E. coli* W3110 more sensitive to cobalt and manganese. In plants, members of

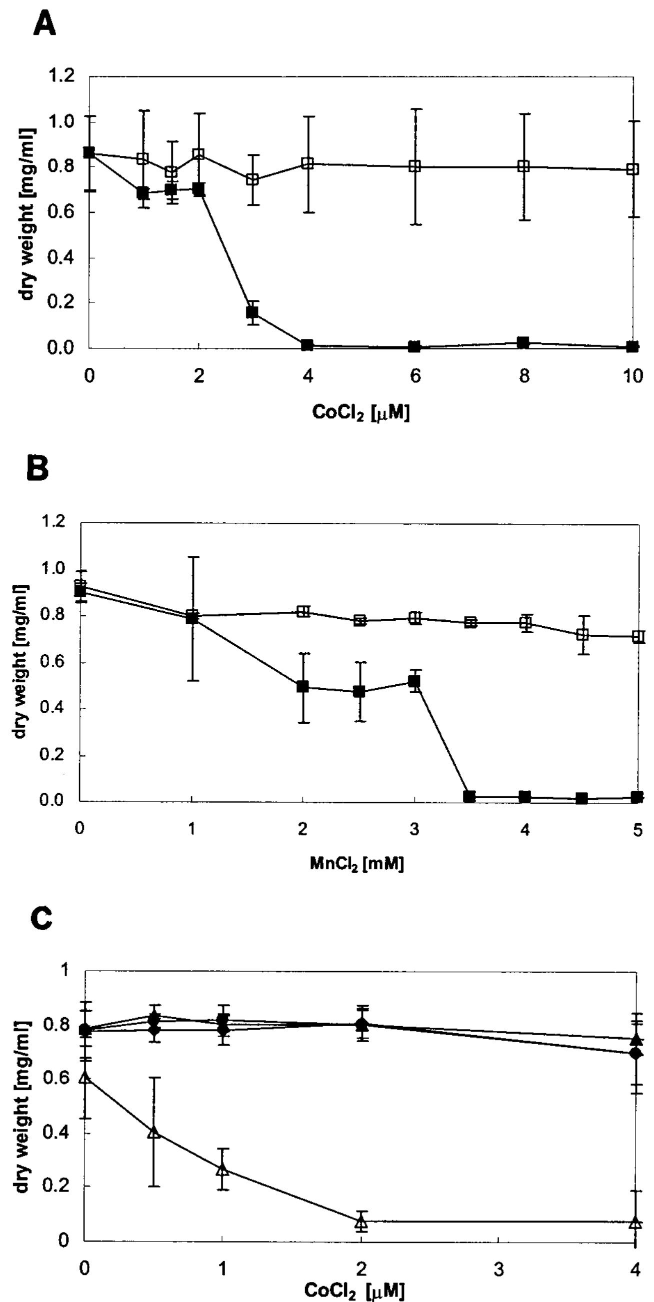

FIG. 3. Effect of cobalt or manganese on growth of *E. coli* W3110 expressing *zupT* and alleviation of cobalt toxicity by CDF transporters. Dose-response curves with different $CoCl_2$ concentrations are shown. Cultures were grown as described in the legend to Fig. 2 and challenged with the indicated concentrations of $CoCl_2$. Cell growth was monitored as the optical density at 600 nm after 16 h of incubation at 37°C with shaking, and the dry weight was determined. *E. coli* strains were (A, B) W3110 pZUPT-low (■) and *E. coli* W3110 pACYC184 (□) and (C) W3110 pZUPT-low pASK-IBA3 (△), pZUPT-low pZITB (▲), pZUPT-low pYIIP, (●), and pZUPT-low pCZCD (♦). Experiments were performed in duplicate (A) or triplicate (B), and the averages and standard deviations were calculated.

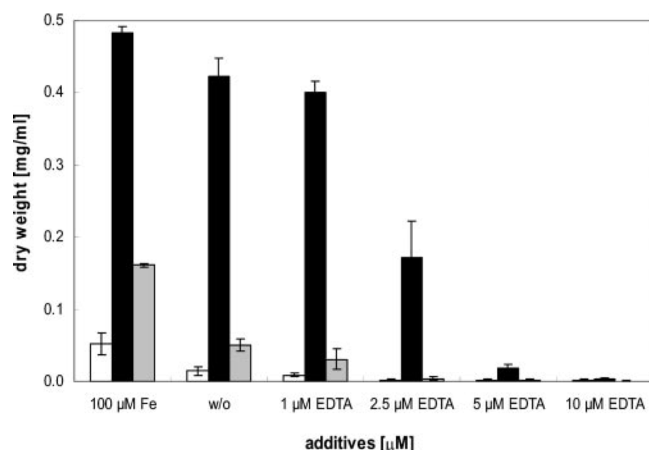

FIG. 4. Activity of *A. thaliana* ZIP1 in *E. coli*. Overnight cultures of *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* grown in Luria-Bertani broth were diluted 1:500 into Tris-buffered mineral salt medium and grown overnight. Cells were diluted 1:500 into fresh medium, and after 2 h of growth at 37°C, cells were diluted 1:500 into fresh medium with iron or different concentrations of EDTA. Cell growth was monitored as the optical density at 600 nm after 16 h of incubation at 37°C with shaking, and the dry weight was determined. *E. coli* strain GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* pACYC184 (white), pZUPT-low (black), and pZIP1 (grey) are shown. Experiments were performed in triplicate, and the averages with standard deviations were calculated.

the ZIP family are responsible for iron, zinc, cadmium, and manganese transport (15). In *E. coli*, expression of *zupT* from a medium-copy-number plasmid rendered cells hypersensitive to zinc and cadmium (13). To study ZupT function, *zupT* including its putative promoter region was expressed from the low-copy-number plasmid pACYC184 (pZUPT-low). pZUPT-low expressed in wild-type *E. coli* W3110 resulted in a moderately hypersensitive phenotype against zinc (data not shown). However, in medium without added metals, growth was identical to that of a plasmid-only control. Thus, this strain could be used as a tool for studying sensitivity to other divalent metal cations mediated by ZupT.

Studies of radioactive $^{57}Co^{2+}$ uptake showed that ZupT can mediate Co^{2+} accumulation, and this was corroborated by growth experiments. Figure 3A shows that *E. coli* strain W3110 was rendered Co^{2+} hypersensitive when *zupT* was expressed in *trans* from the low-copy-number plasmid pACYC184. In addition to ZupT, cobalt might be taken up nonspecifically by the magnesium uptake system CorA in *E. coli* but probably with a much lower affinity (30). There was a limited range of tolerance against this cation between 1 and 2 μM Co^{2+}. Expression of *zupT* also rendered cells more sensitive to manganese. However, the inhibitory concentration for Co^{2+} (Fig. 3A) was lower by a factor of 10^{-3} than that for Mn^{2+} (Fig. 3B). Thus, ZupT likely mediates manganese uptake but with poor affinity.

Cation diffusion facilitators (CDF) comprise a family of metal permeases whose members are found in all kingdoms of life (32). We reasoned that if pZUPT-low rendered *E. coli* hypersensitive to Co^{2+}, coexpression of a CDF permease that catalyzes efflux of Co^{2+} should result in alleviation of this hypersensitivity if Co^{2+} were a substrate of these CDF proteins. When *zitB*, *yiiP* (*fieF*), or *czcD* was expressed in strain *E. coli* W3110 pZUPT-low on pASK-IBA vectors, increasing concentrations of cobalt no longer led to growth inhibition in minimal medium, in contrast to results with strain W3110 pZUPT-low pASK-IBA3 (Fig. 3C). In bacteria, the first characterized member of this family, CzcD, was shown to confer resistance to Co^{2+}, Zn^{2+}, and Cd^{2+} (1, 28). Recently we showed that ZitB, one of the two intrinsic CDF proteins in *E. coli*, is responsible for Zn^{2+} resistance (11). The second CDF permease, YiiP (now named FieF), was recently shown to be involved in iron detoxification (12). Growth restoration clearly indicated that the three CDF transporters tested are capable of also transporting Co^{2+} across the cytoplasmic membrane and thereby probably counteract Co^{2+} uptake by ZupT.

The ZIP1 transporter from *Arabidopsis thaliana* functions as an iron uptake system in *E. coli*. Growth of the quintuple deletion mutant GR536 (Δ*fecABCDE*::*kan* Δ*zupT*::*cat* Δ*mntH* Δ*entC* Δ*feoABC)* was severely affected by iron limitation (Table 3). This strain appeared ideally suited for heterologous expression of genes coding for iron uptake transporters and to study their function without interference by other systems. ZIP1 was chosen because it is an important iron uptake system in roots of higher plants (14). *ZIP1* from *A. thaliana* was functionally expressed in *E. coli* strain GR536. Growth of the mutant strain could be slightly restored by ZIP1 in iron-depleted medium (Fig. 4). Expression of *zupT* in *trans* in this strain resulted in a much higher level of tolerance to EDTA than expression of *ZIP1* (Fig. 4). This difference could be due to a much higher level of expression of *zupT*, since we were able to obtain a strong signal for ZupT but not for ZIP1 in a Western blot. Nevertheless, *E. coli* strain GR536 could be successfully employed for functional expression of a eukaryotic ZIP transporter in bacteria.

The gene encoding the divalent metal permease ZupT is expressed constitutively. In plants, expression of the ZIP transporters ZIP1 and ZIP3 or IRT1 are induced in roots in response to zinc or iron deficiency (5, 8, 14). To elucidate regulation of the gene for the sole ZIP transporter ZupT from *E. coli*, its expression was investigated. Analysis in silico of the coding region of *zupT* suggested that the gene was expressed as a monocistronic transcript. No potential regulatory gene could be identified immediately up- or downstream of its open reading frame. Regulator-binding sites for known metal-responsive transcription factors also could not be detected. To study transcriptional regulation of *zupT*, a *zupT-lacZ* operon fusion was constructed in *E. coli*. Thereby, the *zupT* open reading frame was not altered, resulting in a functional ZupT protein.

E. coli strain SF1 Φ(*zupT-lacZ*) was challenged with the metal chelators EDTA or DIP to induce metal depletion. The addition of chelators did not lead to a significant change in Φ(*zupT-lacZ*) expression. While unchallenged cells exhibited 50.3 ± 4.2 Miller units of β-galactosidase activity, the presence of DIP resulted in 46.9 ± 3.9 Miller units, and the presence of EDTA resulted in 55.4 ± 1.0 Miller units. Moreover, addition of metals (Zn^{2+}, Co^{2+}, Mn^{2+}, or Fe^{2+}) to strain SF1 Φ(*zupT-lacZ*) did not lead to altered expression of Φ(*zupT-lacZ*) (activities ranging from 42.2 ± 5.4 to 50.2 ± 5.2 Miller units). This indicated that in a wild-type background, expression of *zupT* was constitutive and the level of expression was rather low.

E. coli harbors several divalent cation uptake mechanisms, and we have shown that ZupT functions as a zinc uptake permease (13). To examine *zupT* expression without the inter-

ference of other metal uptake systems, the Φ(*zupT-lacZ*) fusion was constructed in *E. coli* strain SF4 [Φ(*zupT-lacZ*) Δ*znuABC*::*cat* Δ*lacZYA*::*kan*], lacking the high-affinity zinc uptake system (31). The reporter Φ(*zupT-lacZ*) was also introduced into *E. coli* strain GR489 (Δ*mntH*::*cat* Δ*feoABC* Δ*entC*), lacking ferrous iron and manganese uptake systems and unable to synthesize the siderophore enterobactin, leading to *E. coli* strain SF14 [Φ(*zupT-lacZ*) Δ*mntH* Δ*feoABC* Δ*entC* Δ*lacZYA*::*kan*]. Addition of chelators or metals did not significantly alter Φ(*zupT-lacZ*) expression in those reporter strains (data not shown) compared to results with the reporter in the wild-type strain. This suggested that expression of *zupT* in *E. coli* might always be constitutive at a low level, even when other high-affinity uptake systems were deleted.

DISCUSSION

In this report, we establish that ZupT is a broad-range metal ion transporter in *E. coli.* ZupT appears to be constitutively expressed at a low level and able to take up the divalent cations Zn^{2+}, Fe^{2+}, Co^{2+}, and possibly Mn^{2+}. These results are in agreement with the broad substrate specificity of some members of the ZIP family, such as IRT1 (15). ZIP proteins have not previously been shown to transport Co^{2+}. The transport mechanism for the ZIP family is at present unknown. The transporters of the ZIP family could work as a channel, since the cations would move along their concentration gradient and membrane potential. A symport is also possible; hZIP2 is reported to cotransport zinc and bicarbonate (9).

E. coli strain GR536, which is devoid of all iron uptake systems relevant for growth in mineral salt medium, was used for studies of single iron transport systems and could be highly useful in future studies of the physiological and biochemical parameters of diverse transporters. Interestingly, many of our mutants were deficient not only in iron uptake but also in manganese uptake. Addition of manganese to *E. coli* strain GR536 fully restored growth. A similar phenomenon was also observed with metal uptake mutants of *Salmonella enterica* serovar Typhimurium and *Streptococcus pyogenes* (3, 19). Thus, some organisms apparently can live without iron but then have an absolute requirement for manganese (18). For *E. coli*, excess cytoplasmic levels of iron can lead to growth inhibition, but under physiological conditions this is countered by at least two iron efflux pumps (12). Iron appears to exert its toxic effect by production of superoxide in *Streptococcus pneumoniae* (20). In addition, manganese appears to counter the effect of iron toxicity, as recently observed with *S. pneumoniae* and *Deinococcus radiodurans* (20, 6). In *E. coli* the iron and manganese interplay needs to be further elucidated.

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