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Point mutations change specificity and kinetics of metal uptake by ZupT from *Escherichia coli*

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Abstract The ZIP (ZRT-, IRT-like Protein) protein ZupT from *Escherichia coli* is a transporter with a broad substrate range. Phenotypic and transport analysis showed that ZupT, in addition to Zn(II), Fe(II) and Co(II) uptake, is also involved in transport of Mn(II) and Cd(II). Competition experiments with other substrate cations suggested that ZupT has a slight preference for Zn(II) and kinetic parameters for Zn(II) in comparison to Co(II) and Mn(II) transport support this observation. Metal uptake into cells by ZupT was optimum at near neutral pH and inhibited by ionophores. Bicarbonate or other ions did not influence metal-uptake via ZupT. Amino acid residues of ZupT contributing to substrate specificity were identified by site directed mutagenesis. ZupT with a H89A exchange lost Co(II) and Fe(II) transport activity, while the S117V mutant no longer transported Mn(II). ZupT with E152D was impaired in overall metal uptake but completely lost its ability to transport the substrates Zn(II) and Mn(II). These experimental findings expand our knowledge on the substrate specificity of ZupT and provide further insight into the function of ZupT as a bacterial member of the vastly distributed and important ZIP family.

Keywords *Escherichia coli* · Metal · Transport · Site directed mutagenesis · ZIP protein

Abbreviations

DIP	2,2′-Dipyridyl
TPEN	*N*,*N*,*N*′,*N*′-Tetrakis-(2-pyridylmethyl)-ethylenediamine
EDTA	Ethylenediaminetetraacetic acid
FCCP	Carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
ZIP	ZRT1, IRT1-like Protein
TMH	Transmembrane helices
d.w.	Dry weight
MES	2-(*N*-morpholino)ethanesulfonic acid

Introduction

ZIP (ZRT-, IRT-like Protein) permeases play an important role for metal acquisition in plants and are required for metal trafficking in animals including humans (Rogers et al. 2000; Gaither and Eide 2000). Earlier

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work (Rogers et al. 2000) identified a variety of amino acid residues in IRT1 from *Arabidopsis thaliana* that are important for transport function. Residues in or near transmembrane helices (TMH) IV and V were critical for efficient metal-uptake (Rogers et al. 2000). A similar study with human hZIP1 (SLC39A1), indicated that histidines located within TMH and in connecting loop domains were necessary for Zn(II) transport (Milon et al. 2006). A variety of mechanisms were suggested to explain ZIP-mediated metal ion transport but experimental data to confirm these hypotheses are insufficient. Bicarbonate stimulated hZIP2 and a Zn(II)/bicarbonate co-transport was suggested (Gaither and Eide 2000). Bicarbonate probably also drives metal-uptake in other ZIP-transporters, such as ZIP8 (Slc39A8) and ZIP14 (Slc39A14) (He et al. 2006; Girijashanker et al. 2008).

The role of ZIP transporters in bacteria including their contribution for overall metal-homeostasis and their mechanism of action is not well understood. Only a few bacterial ZIP transporters have been studied and most data are derived from genome sequences or transcriptomics (Hudek et al. 2009). Moreover, most of what we know about protein function stems from *Escherichia coli* and its ZIP transporter ZupT. ZupT was the first bacterial ZIP identified and it contributes to Zn(II) uptake in this organism (Grass et al. 2002). However, the high-affinity Zn(II) uptake system Znu (Patzer and Hantke 1998) usually masks the action of ZupT. Recently, a role in pathogen fitness for ZupT, together with ZnuABC, during urinary tract infection was found in mice (Sabri et al. 2009). In addition to Zn(II), ZupT transports other metal cations into the cytoplasm including Fe(II), Co(II) and probably Mn(II) (Grass et al. 2005a). It is not known if a similar broad substrate spectrum is also typical for other bacterial ZIP transporters.

So far, there is no information on how substrates are recognized by ZupT and on the kinetics of transport. In the present report we employed *E. coli* deletion strains lacking all known transport systems for a given transition metal (Zn, Fe, Mn, Co or Cd) to expand our knowledge on the substrate spectrum of ZupT and for determining the transport parameters in vivo. We also altered charged and polar amino acid residues within ZupT predicted to be involved in substrate recognition or transport and then assessed the function and substrate spectrum of the mutants. Some of the mutant proteins lost the ability to transport certain metal substrates while transport of other metal substrates was unaffected.

Materials and methods

Bacterial strains, growth media

Strains used in this work for *zupT* expression were derivatives of *E. coli* strain K12 (W3110). Strain ECA580 (Δ*zupT* Δ*mntH* Δ*zntA* Δ*yodA*::*cat*) that lacks all known cadmium-uptake (MntH, ZupT), -binding (YodA) and major efflux (ZntA) systems was utilized for characterization of Cd(II) as a ZupT substrate. Strain ECA349 (Δ*corA* Δ*rcnA* Δ*zupT*::*cat*) deleted in genes for Co(II)-uptake (CorA, ZupT) and -efflux (RcnA) transporters was employed for Co(II) utilization studies. Strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) and GR354 (Δ*znuABC* Δ*zupT*::*cat*) for Zn(II). The strains lack all Zn(II)-uptake (ZnuABC, ZupT) and/or -efflux (ZntA, ZntB, ZitB) systems. Strains GR536 (Δ*entC* Δ*fecA-E*::*kan* Δ*feoABC* Δ*mntH* Δ*zupT*::*cat*) and its derivative ECA458 (Δ*entC* Δ*fecA-E* Δ*feoABC* Δ*mntH* Δ*zupT*) were used for Fe(II)- or Mn(II)-uptake, respectively. In these strains all iron and manganese uptake systems relevant for growth in minimal medium are missing. A complete list of all strains and plasmids is provided in supplemental table 1 (Grass 2009). *E. coli* was grown in Luria–Bertani (LB) medium or in Tris-buffered mineral salts medium (Mergeay et al. 1985) containing 2 ml glycerol and 3 g deferrated casamino acids per liter (where indicated) with or without supplemented iron and trace elements. For dose–response experiments, cultures of *E. coli* were grown overnight in LB at 37°C, diluted 1:400 in Tris-buffered mineral salts medium and grown overnight. Cultures were inoculated 1:400 in fresh Tris-buffered mineral salts medium (or in Tris-buffered mineral salts medium without iron and deferrated CAS) and after 2 h early log-cultures were diluted 1:400 in fresh medium with additives. Growth was monitored as OD_{600} after 16 or 24 h and the dry weight was determined from a calibration curve. $CdCl_2$, $CoCl_2$, $MnCl_2$, 2,2′-dipyridyl (DIP), *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), ethylenediaminetetraacetic acid (EDTA) or antibiotics [chloramphenicol (15 μg/ml), kanamycin (25 μg/ml), ampicillin (100 μg/ml)] were added where

appropriate. Glassware was acid treated prior to use in order to minimize variations in metal content.

Gene deletion and plasmid construction

Genes from *E. coli* strain W3110 were deleted by the consecutive insertion of Cam^R or Kan^R cassettes using the λ Red-recombinase system of plasmid pKD46 (Datsenko and Wanner 2000) and removal of resistance cassettes by FLP-recombinase encoded by plasmid pCP20 (Datsenko and Wanner 2000). Primers for deletion of *yodA* are listed in supplemental table 2 (Grass 2009). Gene deletions were also combined by generalized transduction with bacteriophage P1*vir* as described previously (Grass et al. 2005a). Plasmids with mutant *zupT* genes were generated by the Quick-Change method (Stratagene, Heidelberg, Germany) using the mutagenesis primers listed in supplemental table 2 (Grass 2009) and plasmids pECD1046 (pASK-IBA3::*zupT*) (Grass et al. 2002), pECD754 (pACYC184::*zupT*) (Grass et al. 2005a) and pECD1061 (pACYC177::*zupT*) as templates. For plasmid pECD1061 the open reading frame of *zupT* including a 300 bp upstream region was PCR amplified from chromosomal DNA of *E. coli* strain W3110 and cloned into the *Sca*I site of low copy-number vector pACYC177 (New England Biolabs GmbH, Frankfurt, Germany). Plasmids expressing *zupT* mutants that resulted in ambiguous results in metal uptake and growth experiments were constructed de novo from wild-type plasmid templates and retested. All plasmids were checked by restriction and DNA sequence analysis.

^{55}Fe, ^{54}Mn, ^{109}Cd, ^{65}Zn or ^{57}Co uptake

Iron uptake experiments were performed with *E. coli* strain GR536 (Δ*entC* Δ*fecABCDE*::*kan* Δ*feoABC* Δ*mntH* Δ*zupT*::*cat*). An isogenic strain (but lacking the antibiotics resistance markers) was used for manganese uptake experiments. For radioactive Cd-uptake experiments strain ECA580 (*mntH zupT zntA yodA*::*cat*) was employed. Strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) was used for zinc-uptake experiments and strain ECA349 (Δ*corA* Δ*rcnA* Δ*zupT*::*cat*) for cobalt-uptake assays. Uptake experiments were performed by the filtration method (Grass et al. 2005b). For this, stationary phase cultures grown in Tris-buffered mineral salts medium were diluted to 30 Klett units (64 μg/ml dry weight) into minimal medium. Cultures were then grown to an optical density of 60 Klett units (128 μg/ml dry weight), and gene expression was initiated by adding 200 μg of anhydrotetracycline (AHT) per liter. After growth for 1 h, cells were washed with Tris-buffered mineral salts medium without casamino acids and without iron for iron uptake experiments; in Tris-buffered mineral salts medium for Zn-uptake experiments, in 10 mM Tris–HCl buffer pH 7.0 for Co- and Cd-uptake experiments and with Tris-buffered mineral salts medium without casamino acids, iron, phosphate and trace elements for manganese uptake experiments. After washing, optical densities of cell suspensions were determined and diluted to an optical density (OD_{600}) of 0.4 (118 μg/ml dry weight). Iron uptake was started by adding a mixture of ascorbate (final concentration, 1 mM) and $FeSO_4$ (final iron concentration, 5 μM), labeled with $^{55}FeCl_3$. Other uptake mixes were $ZnCl_2$ (final concentration 1 μM), labeled with $^{65}ZnCl_2$, $CdCl_2$ (final concentration 1 μM), labeled with $^{109}CdCl_2$; $CoCl_2$ (final cobalt concentration, 2.5 μM) labeled with $^{57}CoCl_2$; and $MnCl_2$ (final concentration 1.4 μM) labeled with $^{54}MnCl_2$. The cells were incubated with shaking, and at various times 0.3 or 0.4 ml aliquots were withdrawn and filtered through nitrocellulose membranes (0.45 μm) and immediately washed with 12 ml of 0.1 mM LiCl (for iron) or wash buffer (10 mM Tris–HCl pH 7.0, 10 mM $MgCl_2$). The membranes were dried, and radioactivity was measured using a liquid scintillation counter (LS6500; Beckman, Munich, Germany). $^{55}FeCl_3$, $^{57}CoCl_2$, $^{109}CdCl_2$, $^{65}ZnCl_2$ and $^{54}MnCl_2$ were purchased from Perkin-Elmer (Boston, MA).

Competition studies and kinetic determinations were performed in Tris-buffered mineral salts medium without casamino acids, iron, phosphate and trace elements to avoid interference with other metals. Competition studies were started by adding 1 μM final concentration of isotope-labeled $ZnCl_2$ (control) or 1 μM isotope-labeled $ZnCl_2$ plus the indicated concentrations of "cold" $ZnCl_2$, $CdCl_2$, $CuCl_2$, $CoCl_2$, $NiCl_2$, $MnCl_2$ or $FeSO_4$. After 3 min, 0.3 ml samples were withdrawn, filtered and membranes washed with buffer (Tris–HCl pH 7.0, 10 mM $MgCl_2$) as described above.

Investigations of pH dependency of ZupT-mediated Zn-uptake were performed in MES/Tris-buffered

mineral salts medium without casamino acids, iron, phosphate or trace elements at pH 6.0, 6.5 and 7.2 and metal uptake quantified as described above.

Miscellaneous

Standard molecular genetic techniques were used (Sambrook et al. 1989). PCR was performed in the presence of *Tgo* or *Taq* DNA polymerase (Roche, Mannheim, Germany). DNA sequencing and N-terminal sequencing of the ZupT protein were performed by commercial services (Microsynth, Lindau, Germany and Toplab GmbH, Martinsried, Germany, respectively). StrepTactin-Sepharose, StrepTactin horse radish peroxidase (HRP) conjugate for western blot and anhydrotetracycline (AHT) were from IBA GmbH (Göttingen, Germany). Ionophores FCCP, CCCP, chelators and metal salts were from Sigma-Aldrich (Munich, Germany).

Results

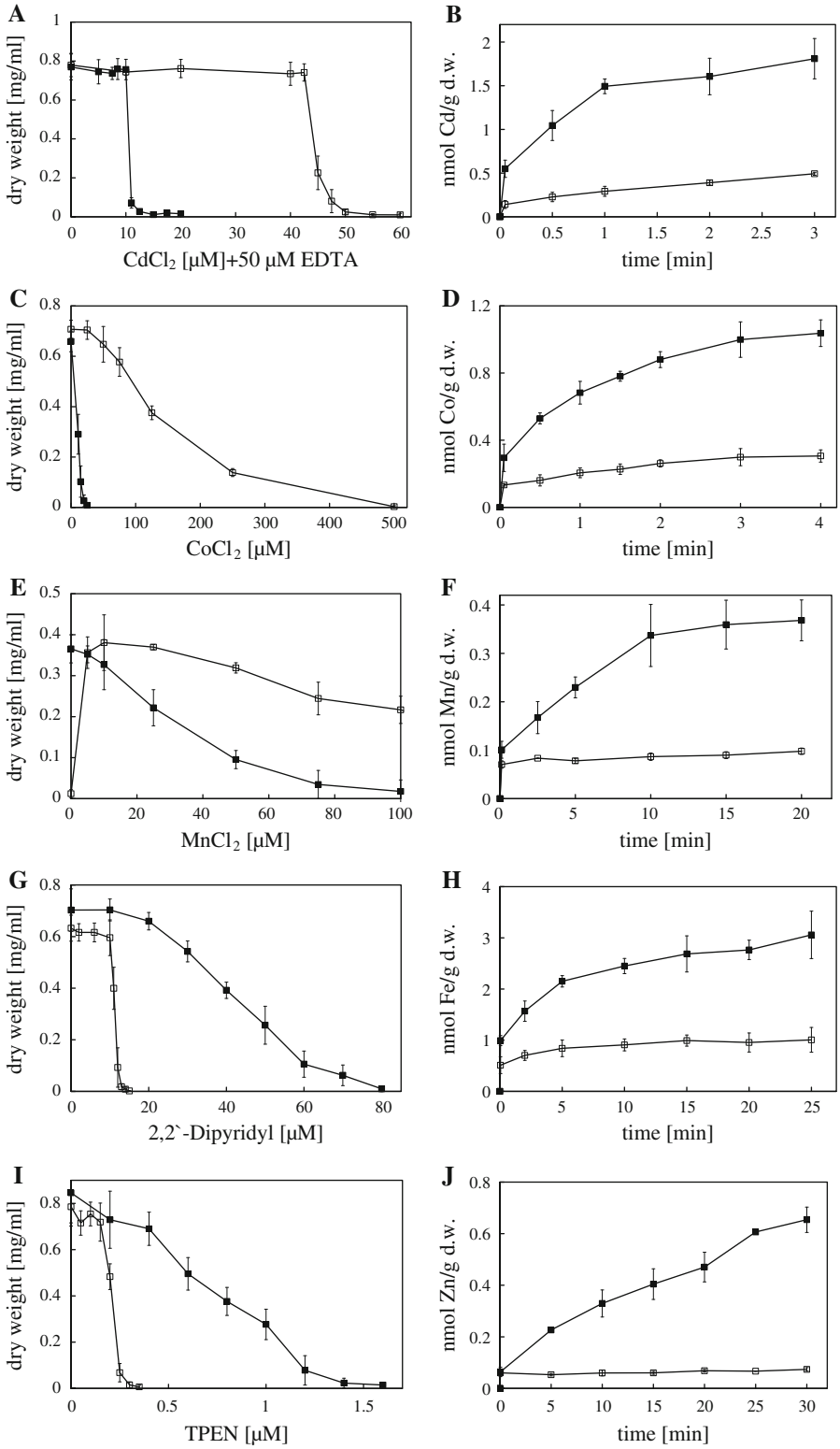
ZupT transports cadmium and manganese in addition to zinc, cobalt and iron

Previously, we showed that ZupT is involved in Zn(II), Co(II) and Fe(II) uptake (Grass et al. 2005a). Additional strains, ECA458 (Δ*entC* Δ*fecA-E* Δ*feoABC* Δ*mntH* Δ*zupT*) and ECA580 (Δ*zupT* Δ*mntH* Δ*zntA* Δ*yodA*::*cat*) were constructed to investigate if ZupT also transports Mn(II) and Cd(II), respectively. Strain ECA349 (Δ*corA* Δ*rcnA* Δ*zupT*::*cat*) was used for Co(II)-uptake and growth studies. Strain GR536 (Δ*entC* Δ*fecA-E*::*kan* Δ*feoABC* Δ*mntH* Δ*zupT*::*cat*) (Grass et al. 2005a) was utilized for Fe(II) studies. Similarly, strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) (Grass et al. 2005a) and strain GR354 (Δ*znuABC* Δ*zupT*::*cat*) (Grass et al. 2002) were used for Zn(II)-uptake and growth under Zn(II)-deprivation.

Strains ECA580 (Δ*zupT* Δ*mntH* Δ*zntA* Δ*yodA*::*cat*) and ECA349 (Δ*corA* Δ*rcnA* Δ*zupT*::*cat*) were constructed in a manner that Cd(II)- or Co(II)-transport would result in metal overload inside the cell resulting in toxicity. In these strains the respective efflux systems (ZntA or RcnA) are missing. If ZupT also transported Cd(II), this would result in a growth defect in the presence of this cation, similar to what can be observed in the presence of Co(II). Strains expressing *zupT* were more sensitive to Cd(II) or Co(II) than the plasmid-only controls (Fig. 1a, c). The IC50 for the ZupT expressing strain was about 10.5 μM for Cd(II) and 8 μM for Co(II) whereas the IC50 for the controls were about 44 μM Cd(II) and 175 μM Co(II), respectively. Uptake experiment also demonstrated Cd(II) and Co(II) transport by ZupT. Cells expressing *zupT* exhibited time-dependent Cd(II) and Co(II) uptake at a rate higher than that of the plasmid-only control (Fig. 1b, d).

When grown for 16 h strain ECA458 (Δ*entC* Δ*fecA-E* Δ*feoABC* Δ*mntH* Δ*zupT*) pACYC184 had not yet entered logarithmic growth phase (Fig. 1e). After this growth period the same strain expressing *zupT* has already reached a cell density of about 0.37 μg/ml (dry weight). Because this strain is also challenged in its ability to take up iron, this growth difference is probably owed to iron acquisition via ZupT. Remarkably, increasing concentrations of Mn(II) in the growth media led to increasingly inhibited growth of the *zupT* expressing strain. In contrast, growth of the strain lacking ZupT was improved by low Mn(II) concentrations reaching a maximum at 10 μM Mn(II). At higher concentrations Mn(II) became inhibitory, similar to the *zupT* expressing strain. This inhibition was probably facilitated by the Mn(II) transport activity of ZupT (Fig. 1f) suggesting that Mn(II) at higher cellular concentrations is becoming toxic in iron-deprived cells.

Fig. 1 ZupT-mediated growth and metal uptake in *E. coli* strains. Dose response growth experiments for *E. coli* strains harboring plasmids pZUPT-low (*filled square*) or pACYC184/pACYC177 (*open square*) (**a**, **c**, **e**, **g**, **i**) or metal uptake for strains with plasmids pZUPT (*filled square*) or pASK-IBA3 (*open square*) (**b**, **d**, **f**, **h**, **j**), from at least triplicate experiments with standard deviations (*error bars*) are shown. Cultures were strain ECA580 (Δ*zupT* Δ*mntH* Δ*zntA* Δ*yodA*::*cat*) grown with 50 μM EDTA and increasing concentrations of $CdCl_2$ (**a**) or assayed for ^{109}Cd uptake (**b**), ECA349 (Δ*corA* Δ*rcnA* Δ*zupT*::*cat*) grown with increasing concentrations of $CoCl_2$ (**c**) or assayed for ^{57}Co uptake (**d**), ECA458 (Δ*entC* Δ*fecA-E* Δ*feoABC* Δ*mntH* Δ*zupT*) grown with increasing concentrations of $MnCl_2$ (**e**) or assayed for ^{54}Mn uptake (**f**), GR536 (Δ*entC* Δ*fecA-E*::*kan* Δ*feoABC* Δ*mntH* Δ*zupT*::*cat*) grown with increasing concentrations of the iron chelator 2,2′-dipyridyl (**g**) or assayed for ^{55}Fe uptake (**h**) and GR354 (Δ*znuABC* Δ*zupT*::*cat*) grown with increasing concentrations of zinc chelator TPEN (**i**) or GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) assayed for ^{65}Zn uptake (**j**)


A
dry weight [mg/ml]
CdCl2 [μM]+50 μM EDTA
B
nmol Cd/g d.w.
time [min]
C
dry weight [mg/ml]
CoCl2 [μM]
D
nmol Co/g d.w.
time [min]
E
dry weight [mg/ml]
MnCl2 [μM]
F
nmol Mn/g d.w.
time [min]
G
dry weight [mg/ml]
2,2`-Dipyridyl [μM]
H
nmol Fe/g d.w.
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I
dry weight [mg/ml]
TPEN [μM]
J
nmol Zn/g d.w.
time [min]

ZupT also mediated uptake of Zn(II) and Fe(II) in strains deleted in all known zinc- or iron-uptake transporters and improved growth when cells where challenged with chelators TPEN or DIP, respectively (Fig. 1g–j). Thus, the substrate spectrum of ZupT can be expanded from Zn(II), Fe(II) and Co(II) to Mn(II), and the toxic metal Cd(II).

Zn(II)-uptake by ZupT is efficient and can be competitively inhibited

So far, no kinetic data is available for any bacterial ZIP transporter including ZupT. ^{65}Zn(II) accumulation in cells of strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) with pZUPT (pECD1046) demonstrated that zinc uptake is a saturable process and time- and concentration-dependent. When assayed over a range of zinc concentrations (0.125–1.75 μM), the in vivo transport activity of ZupT showed Michaelis–Menten kinetics with an apparent K_m value of 0.71 ± 0.14 μM zinc and a V_{max} of 1.87 ± 0.03 nmol × g (d.w.)$^{-1}$ × min^{-1}. Affinities for Co(II) or Mn(II) were slightly lower with K_m values of 0.91 ± 0.09 μM Co(II) or 1.16 ± 0.29 μM Mn(II). Similarly, V_{max} for Co(II) of 0.49 ± 0.03 nmol × g (d.w.)$^{-1}$ × min^{-1} or Mn(II) of 0.85 ± 0.09 nmol × g (d.w.)$^{-1}$ × min^{-1} did not reach the velocity of that for Zn(II).

Metal competition experiments were conducted to corroborate the zinc preference of ZupT. Is ZupT-mediated Zn(II) transport inhibited by other metals cations and, if so, by what concentrations? The *zupT* gene was expressed in strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) pZUPT (pECD1046) and for zinc uptake experiments 1 μM ^{65}Zn(II) was mixed with $CoCl_2$, $CdCl_2$, $CuCl_2$, $MnCl_2$, $NiCl_2$, $FeSO_4$ or "cold" $ZnCl_2$ as a control. At 5 μM competing metal concentration Cd(II) inhibited Zn(II) uptake as much as when the available Zn(II) concentration was raised by 5 μM (Fig. 2). Thus, Cd(II) was an efficient competitor for ZupT-mediated uptake. In fact, addition of as low as 1 μM Cd(II) led to a significant reduction in Zn(II) uptake (data not shown). Similar to Cd(II), Cu(II), however at 20 μM, also strongly inhibited Zn(II) transport. This is a 20-fold excess of Cu(II) over the Zn(II) concentration. A concentration of 50 μM was required for an inhibitory effect of Co(II), Mn(II) or Ni(II) on Zn(II) uptake indicating that Zn(II) is

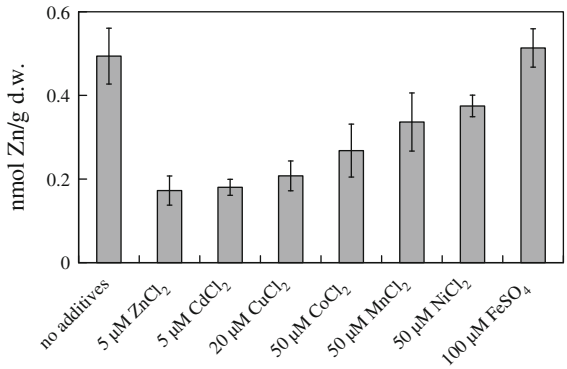

Fig. 2 Inhibition of ZupT-mediated Zn(II) transport by other divalent metal cations. Cultures of *E. coli* strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) harboring plasmid pZUPT were centrifuged and cells resuspended in Tris-buffered mineral salts medium (without iron, casamino acids, phosphate and trace elements) and adjusted to an optical density OD_{600} of 0.4. Uptake experiments were initiated by adding 1 μM $^{65}ZnCl_2$ (final concentration) alone or with the indicated concentrations of competing metals and zinc uptake measured after 3 min. Shown are averages of three independent measurements with standard deviations (*error bars*)

favored by ZupT over these substrates. It should be noted that these results only appear to be in contrast to the kinetic data because strain GR362 was not used for manganese- or cobalt-uptake kinetics; it harbors all Mn(II) and Co(II) homeostatic factors except ZupT. This strain lacks, however, all zinc transporters and is thus, ideally suited for competition analysis.

Finally, Fe(II) failed to compete with Zn(II) even at 100 μM notwithstanding Fe being a substrate of ZupT (Fig. 2). Overall these results emphasize that ZupT is a broad-substrate permease and that the non-physiological metal Cd(II) can efficiently inhibited Zn(II)-uptake competitively.

The proton motive force but not bicarbonate or other ions might be involved in ZupT-mediated metal-uptake

Currently it is not known how ZupT-mediated metal transport is energized. Moreover, the identity of the driving force for most of the ZIP proteins is controversial. There is no ATP-binding domain in ZupT or other ZIPs ruling out primary transport by ATP-hydrolysis but secondary transport by a metal symport mechanism has been previously suggested (Zhao and Eide 1996). If the proton motive force drives substrate-uptake through ZupT, the addition of

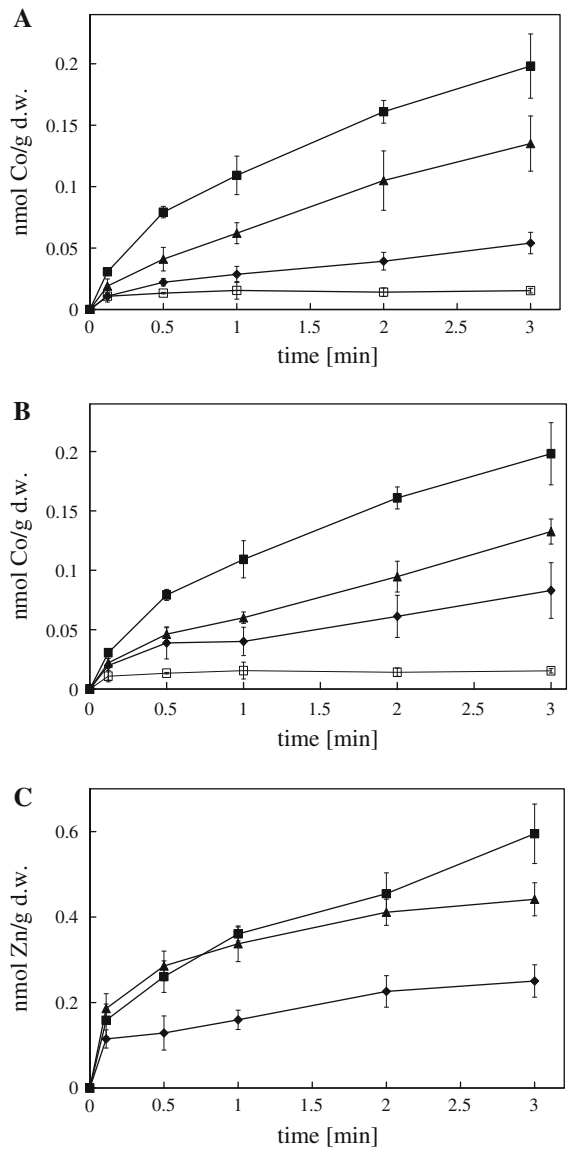

Fig. 3 Metal uptake by cells expressing *zupT* in the presence of protonophores FCCP or CCCP and under different pH values. Cultures of *E. coli* strain ECA349 (Δ*corA* Δ*rcna* Δ*zupT*::*cat*) (**a**, **b**) or strain GR362 (Δ*znuABC* Δ*zupT* Δ*zntB* Δ*zitB*::*cat* Δ*zntA*::kan_{Dis}) (**c**) harboring pZUPT (*closed symbols*) or vector only (*open symbols*) were resuspended in Tris-buffered mineral salts medium (without iron, casamino acids, phosphate and trace elements), adjusted to an optical density OD_{600} of 0.4 and incubated for 5 min without (*filled square*) or with 10 μM FCCP (*filled triangle*), 25 μM FCCP (*filled diamond*) (**a**) or without (*filled square*) or with 10 μM CCCP (*filled triangle*), 25 μM CCCP (*filled diamond*) (**b**) or cells of *E. coli* strain GR362 harboring pZUPT were resuspended in buffered media with different pH values, pH 6.0 (*filled diamond*), pH 6.5 (*filled triangle*) and pH 7.2 (*filled square*) (**c**). Uptake was initiated by adding 1 μM $^{57}CoCl_2$ (**a**, **b**) or 1 μM $^{65}ZnCl_2$ (**c**). Cultures without protonophores served as positive controls and cells lacking *zupT* as negative controls (**a**, **b**). Shown are averages of at least three independent measurements with standard deviations (*error bars*)

ionophores should diminish metal-transport in a concentration-dependent manner. Indeed, when the ionophores FCCP or CCCP were added to transport assays, increasing concentrations of these ionophores resulted in a decrease of metal-uptake in cells expressing *zupT*. At 25 μM FCCP or CCCP more than half of the activity of ZupT was inhibited (data shown for Co(II) in Fig. 3a, b; data for Mn(II) and Zn(II) not shown). Solvents in which the ionophores were solubilized did not negatively affect the assay at the concentrations used (data not shown). To rule out that cells were killed by FCCP or CCCP, treated and untreated cultures were plated after 5, 10 and 20 min on solid media and the survivors counted as colony forming units (CFU). No significant differences in CFU numbers were observed (data not shown).

We also performed uptake assays in media buffered at different pH values. If metal-transport is energized by the proton gradient across the cytoplasmic membrane, then a low pH, e.g. a higher outside proton concentration should improve ZupT transport activity. However, the transport-rate at pH 7.2 was higher than that at pH 6.0 or 6.5 arguing against a proton/metal symport mechanism (data shown for Zn(II) in Fig. 3c; data for Mn(II) and Co(II) not shown). Higher pH values could not be tested because the metal cations precipitated under these conditions as evidenced by a decrease in total isotope counts in the liquid of the uptake-assay. Other potential co-substrates for symport were also tested. Addition of NaCl, $CaCl_2$, $MgCl_2$ or KCl did not change the metal-uptake rate of ZupT (data not shown). Thus, even though ionophores inhibited ZupT-mediated metal-uptake no clear contribution of the proton-motive force could be demonstrated for ZupT-mediated transport.

Zinc transport by the human or murine ZIP-permeases was shown to be stimulated by the addition of bicarbonate (Gaither and Eide 2000; Girijashanker et al. 2008). To investigate if metal-ion uptake by ZupT is likewise improved by bicarbonate, freshly prepared bicarbonate solution (0.5 or 1 mM) was added to cultures expressing *zupT* and the transport activity was compared to a control lacking HCO_3^-. At these concentrations bicarbonate did not

alter the pH of the transport buffer and thus the transport-rate. In Cd(II)-, Co(II)- or Zn(II)-uptake-assays, there was no increase in metal-transport observed when bicarbonate was present (data not shown) suggesting metal-uptake via ZupT is not metal/bicarbonate symport.

Changes in amino acid residues within ZupT result in altered substrate specificity

IRT1 from *A. thaliana* is the only ZIP proteins for which site specific mutant analysis is available (Rogers et al. 2000). As a consequence not much is known about how substrate specificity is accomplished by ZIP transporters. We used the amino acid residues changes characterized in the *At*IRT1 mutants and in silico analysis of conserved amino acid residues from a variety of different ZIP proteins as a road map for site-directed mutagenesis analysis of ZupT. Because ZupT is a much more hydrophophic protein than most eukaryotic ZIPs, with small loop domains between transmembrane-spanning helical (TMH) segments, a variety of conserved amino acid residues in eukaryotic ZIP proteins are absent from ZupT. Some of these also comprise residues found to be involved in substrate discrimination in IRT1 (Rogers et al. 2000). Therefore, IRT1 and ZupT cannot be directly compared and as a consequence negatively charged or polar amino acid residues and histidines of ZupT were selected for site directed mutagenesis.

Figure 4 shows which amino acid residues of ZupT were changed. They mostly include residues within TMH putatively involved in substrate translocation across the cytoplasmic membrane (M62, S117, H119, E123, H148 and E152) and negatively charged residues and histidine residues within loop domain II and III possibly implicated in substrate recognition and binding (D82, H87 and H89). In *E. coli* the *zupT* gene is naturally expressed on a low and constitutive level and high-level expression of *zupT* easily becomes toxic to cells (Grass et al. 2005a). This property of ZupT also made it difficult to overproduce the protein for purification (Taudte and Grass, unpublished observation). For growth experiments under metal deprivation (and for Cd or Co toxicity tests), wild-type and mutated *zupT* genes were expressed in mutant strains at low levels from plasmid pACYC184. This ensured that ZupT did not become toxic to the cells but this also precluded detection of ZupT and its variants and thus, confirmation that the variant proteins are synthesized. In contrast ZupT variants encoded by the overproduction vector pASK-IBA3 can be detected by immunoblotting using the C-terminally attached Strep-tag epitope (Fig. 5) and were used for uptake-assays. However, prolonged growth of such strains is retarded probably because of the adverse effects of ZupT overproduction (data not shown).

Cells harboring ZupT variants M62A D82A, H87R and H89R were indistinguishable from cells with wild-type ZupT (Table 1) both during growth under low metal conditions and in uptake assays. The mutant proteins were produced at similar levels as the wild-type ZupT when overproduced and could be detected in purified cytoplasmic membrane fractions (Fig. 5). This suggested that M62 D82, H87 and H89 were not necessary for full ZupT activity.

However, when H87 or H89 were changed to alanines the proteins exhibited altered properties. This drastic change of the histidine residue rendered H87A unstable when this mutant *zupT* gene was highly expressed (Fig. 5). Under long-term low-level expression the protein still fulfilled a wild-type level function for growth with any of the tested metal cations (Table 1) but the protein was inactive in uptake assays. In contrast, H89A was stable in cells but strains with H89A lost all Co(II) and most of Fe(II) transport activity, whereas Zn(II), Mn(II) and Cd(II) uptake was almost unaffected. Thus, while the H89R mutant was unremarkable, an alanine residue at the same location resulted in a ZupT protein that lost the ability to transport two of its substrates, Co(II) and Fe(II) and growth of the H89A mutant in the presence of Co(II) or Fe(II) was similar to the vector only control (Table 1).

S117V and E152D were derivatives that specifically lost their ability to transport one or two metal cations but retained most of their activity for the others. Cells harboring S117V were no longer able to transport Mn(II) and lost some activity towards Co(II). Growth of these mutant cells was undistinguishable from the negative control. Cells with the E152D exchange lost Zn(II) and Mn(II) uptake activity and showed negative control-like growth with these cations. However, E152D also lost considerable activity towards the other substrates. A related mutant, with E152A that lacked the

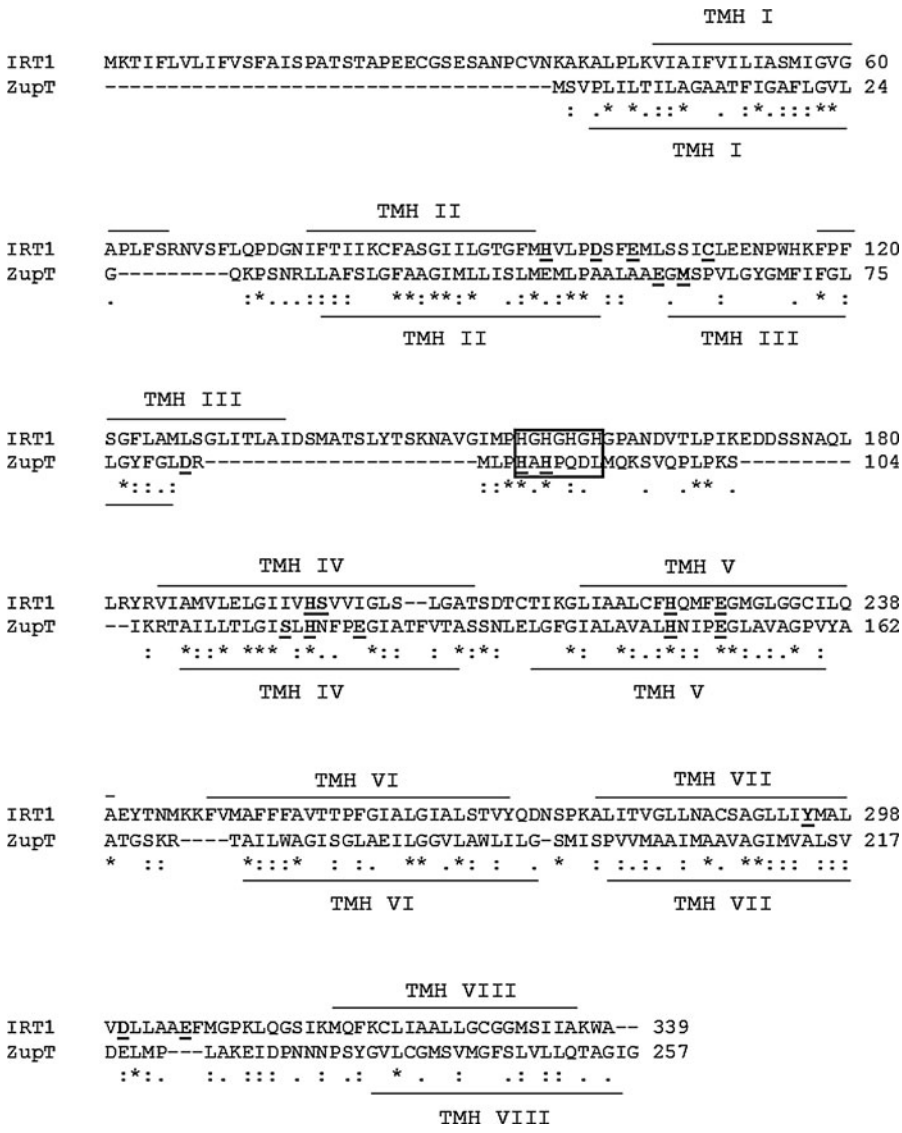

Fig. 4 Alignment of ZupT from *E. coli* and IRT1 from *A. thaliana*. Localizations of putative transmembrane helices (roman numbers I–VIII) were derived from in silico analysis (Sonnhammer et al. 1998; Tusnady and Simon 2001). The amino acid residues that were changed by site-directed mutagenesis from ZupT or IRT1 (Rogers et al. 2000) are shown in *bold*. A *box* indicates the variable, histidine-rich region between helices III and IV

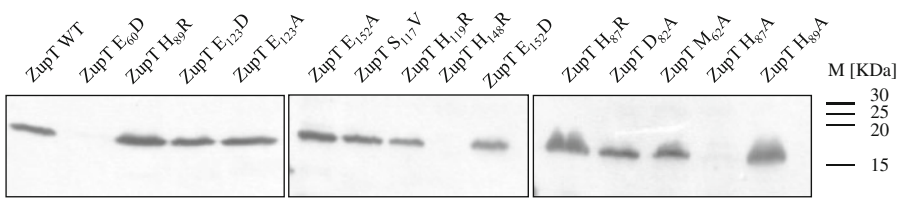

Fig. 5 Detection of ZupT mutant proteins in cytoplasmic membrane fractions. Cultures of *E. coli* strain ECA580 (Δ*zupT* Δ*mntH* Δ*zntA* Δ*yodA*::*cat*) with different mutant derivatives of plasmid pZUPT were incubated with anhydrotetracycline for 1 h grown to allow for *zupT* gene expression and membrane fractions were isolated. Membrane fractions (50 µg) were separated on SDS PAGE, immunoblotted and ZupT proteins were visualized via colorimetric detection of the StrepTagII epitope. Sizes of marker *PageRuler*™ *Unstained Protein Ladder*, MBI Fermentas, (St. Leon-Rot, Germany) are indicated on the *right*

Table 1 Growth and metal-uptake by strains expressing *zupT* mutant genes in comparison to wild-type *zupT*

Mutant[a]	Addition of									
	Co		Fe		Zn		Mn		Cd	
	Growth	Uptake	Growth	Uptake	Growth	Uptake	Growth	Uptake	Growth	Uptake
$E_{60}D$	++−−	−	++++	−	++++	−	++++	−	+−−−	+−−−
$M_{62}A$	+++++	+++++	++++	++++	++++	++++	+++++	++++	+++++	+++++
$D_{82}A$	+++++	+++++	++++	++++	++++	++++	+++++	++++	+++++	+++++
$H_{87}R$	+++++	+++++	++++	++++	++++	++−−	+++++	++++	++++	++++
$H_{87}A$	+++++	−	++++	−	++++	−	+++++	−	++++	−
$H_{89}R$	+++++	+++++	++++	++++	++++	++++	+++++	++++	++++	++++
$H_{89}A$	−	−	−	+−−−	++−−	++++	++−−	++++	++++	++++
$S_{117}V$	++−−	++−−	++++	++++	++++	++++	−	−	++++	++++
$H_{119}R$	−	−	−	−	++−−	+−−−	−	−	−	+++−
$E_{123}D$	−	−	−	−	−	−	−	−	−	++−−
$E_{123}A$	−	−	−	−	++−−	++−−	−	−	−	+−−−
$H_{148}R$	−	−	−	−	++−−	−	−	−	−	−
$E_{152}D$	++−−	++−−	++−−	++−−	−	−	−	−	++−−	+−−−
$E_{152}A$	+−−−	−	+−−−	−	++++	+−−−	−	−	++++	+++−

All *zupT* derivative genes were expressed in different *E. coli* strains lacking metal ion uptake systems to determine whether they are able to complement relative to a wild-type allele

++++, like wild-type; +++, similar to wild-type; ++−−, intermediate; +−−−, almost all activity lost; −, activity lost

[a] Amino acid exchanges and positions within the ZupT peptides are indicated

negative charge at this position was predominantly inactive for all substrates except Zn(II) and Cd(II) (Table 1).

Finally, a group of mutations rendered ZupT largely incapable of transport of most substrates and these cells exhibited growth similar to the vector-only control. These mutations were H119R, E123D, E123A, H148R. H119R and E123A mediated some residual Zn(II) and Cd(II) transport activity with no transport of Co(II), Fe(II) or Mn(II). Growth with Zn(II) of the H119R and E123A mutants was intermediate between wild-type ZupT and the negative control. Mutants with E123D or H148R only retained intermediate Cd(II)-uptake or Zn(II)-sensitivity, respectively, whereas growth and uptake-activity for all other cations was lost. One mutation leading to E60D yielded an unstable protein that could not be detected in membrane fractions (Fig. 5) but low level expression still conferred a wild-type like phenotype for all substrates except Cd(II) and Co(II).

Overall, mutant analysis indicated that transport function of ZupT becomes compromised when conserved negatively charged amino acid and histidine residues located within TMH IV and V were altered (Fig. 4). Manipulations within this part of ZupT caused almost complete loss of function to the mutant protein or when function was retained, Mn(II)-transport was always lost. Similarly, Co(II)- and Fe(II)-transport activity also declined in a variety of mutants, mostly when residues within TMHs were targeted, and the substrates to remain last were Zn(II) and Cd(II) (Table 1). It is also noteworthy that the only mutation which specifically abolished Co(II)- and almost all Fe(II)-transport, H89A, is located within a putative cytoplasmic loop domain of ZupT suggesting a potential regulatory function of this protein domain.

Discussion

ZIP proteins as a group of metal-only transporters have attracted little attention in prokaryotes but are studied frequently in eukaryotic systems including humans. We show here and previously (Grass et al.

2005a) that ZupT is a transporter of broad substrate specificity. As such ZupT is at the cross roads of transition metal homeostasis in *E. coli*. The metal cation substrates transported by ZupT are very similar to those of eukaryotic ZIP proteins. Zn(II) and Cd(II) but also Cu(II) and Mn(II) were identified as substrates for the hZIP2 protein through competition experiments. Fe(II) was not tested as a substrate of hZIP2. However, Fe(II) inhibited Zn(II) uptake indicating Fe(II) being a substrate of hZIP2 (Gaither and Eide 2000). Fe(II), Zn(II), Mn(II) or Cd(II) are transported by Arabidopsis IRT1 making it a broad substrate range transporter (Korshunova et al. 1999). ZIP14 and ZIP8 are further examples of eukaryotic ZIPs transporting a variety of substrates (He et al. 2006; Liu et al. 2008). Murine ZIP8 transports Mn(II), Zn(II) and toxic Cd(II). Similarly, Zn(II), Mn(II), Cu(II) and Cd(II) were found to be substrates of murine ZIP14 (Girijashanker et al. 2008). Involvement of ZupT in copper-uptake into *E. coli* cells is physiologically probably not relevant because there is no cytoplasmic protein known that requires a copper co-factor in *E. coli*.

Here, we compare a bacterial ZIP transporter to family members from eukaryotes. Thus far, transport kinetics exclusively of eukaryotic ZIP proteins have been characterized. The transport kinetics of ZIP8 for Cd(II) and Mn(II) were determined by employing retroviral infection of fetal mouse fibroblast cultures with ZIP8 cDNA. Apparent K_m values were determined as 2.2 and 0.69 μM as well as V_{max} values of 73.8 and 92.1 pmol × min^{-1} × mg $protein^{-1}$, respectively (He et al. 2006). This is relatively close to what we have determined for ZupT and its substrate manganese [K_m 1.16 ± 0.29 μM Mn(II); V_{max} 850 ± 90 pmol × g $d.w.^{-1}$ × min^{-1}]. Metal competition studies indicated that ZIP8-mediated Cd(II)-transport was inhibited by other metal cations especially by Mn(II), Hg(II) and Pb(II) but not by Fe(II) or Ni(II). Recently, Cd(II) transport into Xenopus oocytes through ZIP8 was shown to be inhibited efficiently by Zn(II) but not by Mn(II) (Liu et al. 2008). The apparent affinity of ZupT for Zn(II) [0.71 ± 0.14 μM Zn(II))] in *E. coli* was in the same order of magnitude to what was determined for ZIP8 and Zn(II) [K_m = 0.26 μM Zn(II)] and similar to what was measured for IRT1 expressed in yeast [K_m = 2.8 μM Zn(II)] (Korshunova et al. 1999).

Zn(II)-transport by ZupT was not inhibited but slightly increased by Fe(II) (data not shown) even though Fe(II) is a substrate of ZupT (Grass et al. 2005a). Remarkably, in IRT1 the substrate Fe(II) but not Fe(III) also led to an increased Zn(II)-uptake rate (Korshunova et al. 1999). Similarly, Zn(II) transport by ZIP8 and ZIP14 was also not inhibited but rather increased in the presence of the alternative substrate Fe(II) (Girijashanker et al. 2008). The authors suggested co-transport of both metals cations or that Fe(II) might allosterically regulate Zn(II) transport. Co-transport, however, cannot explain increased Zn(II) transport by IRT1 (Eide et al. 1996) or by ZupT in the presence of Fe(II). Transport by ZIP proteins ZIP8, ZIP14, IRT1 and ZupT follows typical Michaelis–Menten kinetics (Girijashanker et al. 2008; Eide et al. 1996). If allosteric regulation by Fe(II) of ZupT-mediated Zn(II)-transport was involved, this would mean that in an inverse experiment, Fe(II) transport would be modulated by Zn(II). This, however, was not observed: a 50-fold surplus of Zn(II) over Fe(II) did not diminish Fe(II)-uptake by ZupT (data not shown).

The proton motive force seemed to be involved in the metal-uptake process mediated by ZupT because the protonophores FCCP and CCCP inhibited transport. Uptake experiments were also performed at different pH values to differentiate if ΔpH or $\Delta\psi$ energized ZupT-mediated uptake. If metal-transport occurred in symport with protons, as demonstrated for e.g. MntH (Courville et al. 2008), maximum transport should be measured at low pH. However, both for ZupT and for other ZIP transporters, the opposite was observed. ZupT worked best at physiological pH. There are a variety of possible explanations for this. The affinity of ZupT to its substrates might increase with increasing pH or transport efficiency is higher under these conditions. For MntH it was shown in vivo that the affinity for Cd(II) increased with decreasing pH but also that the reaction velocity increased with increasing pH (Courville et al. 2008). A similar process would explain increased Zn(II)-transport by ZupT at increasing pH. Further, depending on the pH, the protonation-state of the amino acid residues within the ZupT polypeptide might change and modulate ZupT activity. Possibly, the observed higher activity of ZupT at pH 7.2 (Fig. 3c) resulted from an indirect effect of the

different outside pH values on amino acid residues within the ZupT polypeptide involved in metal translocation or regulation.

Bicarbonate did not influence metal transport in cells expressing *zupT*. This is in contrast to results from eukaryotic ZIP proteins. Previously, a link between bicarbonate and metal cation transport for the human ZIP protein hZIP2 was described and the authors ruled out the proton motive force to drive metal transport (Gaither and Eide 2000). Nevertheless, Zn(II) transport by hZIP2 was pH-dependent and high pH values resulted in increased zinc-uptake. Because Zn(II)-transport was stimulated by bicarbonate this pH-dependence was thought to correlate with dependence of bicarbonate concentration on pH. Here we also demonstrated pH-dependence of ZupT-transport (Fig. 3c). However, at pH 8.0 Zn(II) probably precipitated during the assay as evidenced by decreasing apparent total counts of the radioactive isotope in samples withdrawn and analyzed. Disregarding this decrease for calculations of isotope-uptake would give the false impression of increased metal transport at high pH.

Metal uptake by ZupT was independent of K^+-, Na^+- or Mg^+-ions arguing against these cations mediating substrate co-transport. For mammalian ZIP8 it was shown that Cd(II)-transport was energy-dependent but independent of K^+-, Na^+- or Cl^--ions (He et al. 2006). Further, pH-dependence was noted for Cd(II)-transport and transport was maximum at pH 7.5 (He et al. 2006). This result argues against protons as driving force for transport only if precipitation artifacts are not considered. Finally, Cd(II)-transport by ZIP8 was stimulated by bicarbonate indicative for HCO_3^-/metal symport (He et al. 2006). Similar results were reported for ZIP14-mediated Cd(II)-transport. This transporter was energy-dependent, was not dependent on K^+-, Na^+- or Cl^--gradients, had optimum transport-activity at pH 7.5 and was stimulated by bicarbonate (Girijashanker et al. 2008).

Overall, results obtained here for ZupT indicate that the proton-motive force might contribute to metal uptake by this bacterial ZIP-transporter but alkali metal cations or alkaline earth metal cations are not. Also, ZupT seems to differ from eukaryotic ZIP proteins by its lack of stimulation by bicarbonate. In an alternative model, metal-uptake by ZupT might be driven simply by the concentration gradient of labile transition metal cations likely to exist across the plasma membrane (Outten and O'Halloran 2001). Thus, changing the pH gradient across the membrane by two orders of magnitude is probably insignificant when the concentration gradient of Zn(II) across the cytoplasmic membrane is at least 14 orders of magnitude (Outten and O'Halloran 2001) and this pH change would probably only increase the availability of metal cations in the extracellular space.

In silico analysis suggested that most ZIP transporters including ZupT (Fig. 4) have a topology comprising eight TMH and an expanded loop domain between TMH III and IV (Rogers et al. 2000; Milon et al. 2006). Analysis of amino acid sequences of different ZIPs indicate few conserved residues between eukaryotic and prokaryotic ZIP proteins, which are predominantly located in TMH VI and V. So far, only a single study (Rogers et al. 2000) explored the potential contribution of these residues for transport activity and substrate specificity. The first identified ZIP transporter IRT1 from Arabidopsis (Eide et al. 1996) has, like ZupT, a broad substrate specificity and thus, IRT1 was used as a model for characterization of a eukaryotic ZIP (Rogers et al. 2000).

We used the IRT1 model as a starting point for a similar functional study of ZupT. Some of the amino acid residues found to be involved in substrate specificity in IRT1, D100 and E103, located in the soluble loop domain between TMH II and III, (Rogers et al. 2000) have no counterparts in ZupT. There is only one glutamate (E60) in ZupT in this region. Replacement of this residue only led to loss of Cd(II) [and to a lesser degree also Co(II)] transport without affecting the other substrates. Remarkably, while the ZupT E60D mutant behaved like wild-type ZupT in growth experiments, activity was abrogated in uptake-studies (Table 1). This might indicate that while still functional, this mutant protein was impaired in transport velocity even though the negative charge of this glutamate residue was retained by the mutation to aspartate. However, this variant ZupT protein was hardly detectable by immuno-blotting suggesting it is synthesized in low quantity.

Four amino acid residues in IRT1 (H197, S198, H224, E228) were found to be essential for transport activity (Rogers et al. 2000). Similar residues can be found in the shorter ZupT peptide at similar positions

but not in conserved order (S117, H119, H148, E152). However, in ZupT only mutations of H119 and H148 from this group of residues rendered the protein largely inactive. Conversely, alterations of S117, and E152 had very interesting phenotypes. The S117V mutant lost specifically its Mn(II)-transport activity (accompanied by a small reduction in Co(II)-uptake as well) and cells with E152D or E152A were no longer able to take up Zn(II) and Cd(II) or Zn(II), respectively.

The loop domain between TMH III and IV is a variable region (Guerinot 2000) and a feature shared by many ZIP transporters. Length and degree of conservation of amino acid residues within this region vary greatly within the ZIP family. As such, the role of this region is not well understood. It was suspected that the variable region is involved in metal-transport and constitutes a metal-binding site (Eide et al. 1996). ZupT possesses two histidine residues within this variable loop domain. Mutation of either one to arginine did not alter substrate specificity or transport-activity of ZupT. However, cells with H87A exhibited wild-type substrate spectrum for growth with any substrate but transport was impaired for all metals. Probably, low protein levels of this variant protein caused this reduced activity (Table 1; Fig. 5). In contrast, H89A specifically lost Co(II) and Fe(II) as substrates but retained most of its transport activity for the other metals. Another study demonstrated the necessity of two histidine residues in this cytoplasmic loop domain located between transmembrane domains III and IV of the human hZIP1 for Zn(II) transport (Milon et al. 2006). The authors suggested that these histidine residues together with an aspartate residue located within the same variable region contribute to coordination of Zn(II) during uptake. It was also speculated that the cytoplasmic loop domain was important for tertiary structure of the transporter protein and would provide histidines in close proximity to TMH IV. Finally, the loop domain of hZIP1was thought to be a "reentrant loop" (Grunewald et al. 2002) that might be inserted into the membrane from the inside without completely traversing the membrane. In this way the loop domain might contribute to both binding and transport of the substrates (Milon et al. 2006). Our results for the histidine exchange mutants in this loop domain of ZupT are not consistent with this model. Arginine residues at either of these positions 87 and 89 resulted in wild-type like proteins indicating a structural role for these positions. Alanine residues at these sites cannot contribute to metal cation ligation, yet cells with these ZupT variants still functioned in metal homeostasis. However, in case of H89A activity specifically towards Co(II) and Fe(II) was lost. For ZupT this result suggests a regulatory function of this histidine residue influencing substrate specificity rather than a direct contribution to metal transport.

Overall, in this study we have extended our knowledge on the substrate spectrum of ZupT, demonstrated that the proton motive force might be involved in driving ZupT-mediated metal-uptake and generated a variety of interesting variant proteins of ZupT (H89A, S117V or E152D) unable to transport some substrate metals. These mutants should be useful to gain further insight into how substrate specificity is achieved once structural data is available for ZupT or related transporters.

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