A Single Amino Acid Change in the Yeast Vacular Metal Transporters Zrc1 and Cot1 Alters Their Substrate Specificity

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Iron is an essential nutrient but in excess may damage cells by generating reactive oxygen species due to Fenton reaction or by substituting for other transition metals in essential proteins. The budding yeast Saccharomyces cerevisiae detoxifies cytosolic iron by storage in the vacuole. Deletion of CCC1, which encodes the vacuolar iron importer, results in high iron sensitivity due to increased cytosolic iron. We selected mutants that permitted Δacc1 cells to grow under high iron conditions by UV mutagenesis. We identified a mutation (N44I) in the vacuolar zinc transporter ZRC1 that changed the substrate specificity of the transporter from zinc to iron. COT1, a vacuolar zinc and cobalt transporter, is a homologue of ZRC1 and both are members of the cation diffusion facilitator family. Mutation of the homologous amino acid (N45I) in COT1 results in an increased ability to transport iron and decreased ability to transport cobalt. These mutations are within the second hydrophobic domain of the transporters and show the essential nature of this domain in the specificity of metal transport.

The yeast vacuole plays an important role in transition metal storage and detoxification. In conditions of metal scarcity, metals stored in the vacuole can be mobilized by specific transporters and utilized for metabolic purposes. Conversely, export of metals from cytosol to vacuole is thought to prevent metal toxicity. Yeast mutants that are unable to store iron in the vacuole, either due to a lack of vacuolar structures (1), an inability to acidify vacuoles due to mutation in the V-ATPase (2, 3), or deletion of vacuolar metal transporters (4–6) show sensitivity to high concentrations of metals.

Specific transporters mediate the transport of different transition metals from cytosol to vacuole. Among the best studied of the vacuolar transition metal transporters are the zinc transporters Zrc1 and Cot1 (4, 5, 7–9). These homologous proteins, which are involved in the transport of zinc and cobalt, are members of the cation diffusion facilitator (CDF) family (for review see Ref. 10). Although CDF members show differences in size, cellular localization, and substrate metals transported, they share some common features. The majority of CDF family members have six putative transmembrane domains (TMD) and a highly conserved amino acid sequence extending from TMD II to III, which is a signature motif for the family. Based on the alignment of multiple sequences, highly conserved charged residues in TMD II and V are implicated in metal binding and transport. This finding is supported by structural studies on an Escherichia coli CDF family member Fief (also known as YiiP), a putative Zn2+ and Fe2+ transporter (11, 12).

Transport of iron from cytosol to vacuole is mediated in yeast by Ccc1 (6) and in plants by the CCC1 homologue VIT1 (13). These proteins define a unique family that appears to be restricted to fungi and plants. Little is known of the mechanism of transport, although it is clear that CCC1 is regulated by iron at transcriptional and post-transcriptional levels (14, 15). Deletion of CCC1 results in poor growth in high iron medium indicating that increased cytosolic iron may be toxic (6). The mechanism(s) leading to high iron toxicity is unknown. High iron toxicity occurs in cells deleted for CCC1 even in the absence of respiratory capacity (e.g. ρ0 cells) or anaerobically (16). These results call into question the assumption that high iron toxicity is due to the generation of iron-mediated reactive oxygen radicals. In an effort to define the mechanism of iron toxicity we initiated a genetic screen in which we identified mutant strains of Δacc1 that grew on high iron. Herein, we identify a missense mutation in the vacuolar zinc transporter Zrc1, which completely changes the substrate specificity of this transporter from Zn2+ to Fe2+. We show that a similar amino acid change in the homologous Cot1, a Co2+ and Zn2+ transporter, also results in a change in metal specificity.

EXPERIMENTAL PROCEDURES

Yeast Strains—The following yeast strains (W303 background) were used: DY150 (Matα ade2-1 his3-11 leu2-3,112 trp1-1 uRA3-52 can1-100(oc)) and DY1457 (Matα ade6 his3-11 leu2-3,112 trp1-1 uRA3-52 can1-100(oc)). Deletions of CCC1 and ZRC1 were generated by double fusion polymerase chain reaction using the HIS3 gene as a selectable marker (17). Primers for disruption of CCC1 were described (6). Primers for disruption of ZRC1 were Pri20–75 (5’-TCC CTT TGT ACC TTA GAC ACG-3’), Pri20–75 (5’-GTC GTG ACT GGG AAA ACC coupled plasma-optical emission spectrometer; TMD, transmembrane domain; YPD, yeast extract peptone dextrose medium.
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CTG GCG ATC GCT GCC ATG ATC GTG GAA-3'), Pri20–80 (TCC TGT GTG AAA TTG TTA TCC GCT GCT GAT CAG ATT CAA AGA GAG-3'), and Pri20–81 (GCA GTT TAC AGC GTC ATC TAC-3'). The CO17 gene was disrupted using IRA3 as described (4). Strains with a FET3-lacZ reporter integrated at the HO locus were constructed as described (18). Wild type BY4743 (Mat a/α his3Δ1/αhis3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 met15Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0), Δcot1::KanMX and Δpmr1::KanMX strains in the BY4743 background were obtained from Research Genetics.

Growth Media—Yeast strains were grown inYPD medium (1% yeast extract, 2% peptone, 2% dextrose) or in CM medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, 1% yeast extract, 2% peptone, 2% dextrose) or in CM medium containing 5 mM ferrous ammonium sulfate. Cells were grown for 22 h. The 8–10-kb genomic fraction was ligated to pRS416-

Isolation of Mutants—DY150 Δcct1 or DY1457 Δcct1 cells were plated on CM agar plates at a density of 500 cells/plate and exposed to UV light to give a 50% survival. Mutagenized cells were grown for 2 days and then replicated to CM plates containing 5 mM ferrous ammonium sulfate. Cells were grown for another 3 days. Colonies able to grow on high iron were selected for further study.

Identification of ZRC1(N44I)—The mutated gene in dominant mutant R1 was identified by constructing a library from the R1 genomic DNA using standard protocols (19). Briefly, Sau3A partially digested genomic DNA was fractionated on a 10–40% sucrose gradient, and centrifuged at 25,000 × g for 22 h. The 8–10-kb genomic fraction was ligated to pRS416-zero, a centromeric plasmid, digested with BamHI and dephosphorylated with calf intestinal phosphatase (New England Biolabs). Ligation products were transformed into Electromax™ DH10b™ E. coli (Invitrogen) (20). Plasmids were extracted from transformed bacteria and pooled. The pooled library was transformed into Δcct1 cells and plasmids that conferred resistance to high iron (3 mx) were recovered. The genomic fragments in these plasmids were identified by sequencing.

Plasmids Construction and Site-directed Mutagenesis—ZRC1(N44I) was subcloned from the genomic library using KanMX and BglII and inserted into pRS416 (a yeast centromeric vector), which had been digested with BglII and KpnI. Wild type ZRC1 with its own promoter and 3' end was generated using PCR from genomic DNA using pri54 (5'-GAT ATG AAA GTA GTT GCA TT-3') and pri60 (5'-TGT GTA CAG GGA ACA AG-3'). The PCR fragment was digested with BglII and KpnI and inserted into pRS416 digested with BglII and KpnI. To introduce the N44I mutation into wild type ZRC1, pri64 (5'-GGG CTG TAT GCG TCA TTC ATG TCA CAT GTT GAT GTA ATT CAT CTC TCT TTG AGG-3') and pri65 (5'-GCC ACT AAA AGA GAG ATG ATA TCA ATC AAC ATG TGA AAT GAA TCG GCA ATC AAG GAC-3') were used. To introduce the D45A mutation into wild type ZRC1, pri102 (5'-CAC ATG TTG AAT GCT ATC ATC TCT TTA GTG GCA C-3') and pri103 (5'-GTG CCA TTA AAA GAG AGA TGA TAG CAT TCA ACA TCA GAT G-3') were used. COT1/Yep352 was obtained from Dr. Douglas S. Conklin (4). To introduce N451 into wild type COT1, pri66 (5'-CGG GGA CTC ATT CCA TAT GCT AAT CGA TAT AAT TTC TCT TGT GG-3') and pri67 (5'-CCA GAA AAA TTA TAT CGA TTA GCA TAT GGA ATG AGT CCG CG-3') were used. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions. ZRC1 and ZRC1(N44I) were digested with KpnI and XbaI and inserted into a yeast episomal vector pTF62 (LEU2 marker). To generate His₅-tagged versions of CCCI, ZRC1, and ZRC1(N44I) under the control of the galactose inducible promoter (GALI) the following primers were used: CCCI, pri78 (5'-CGC GGA TCA TTC ATT GCA TGA CTA AAG-3') and pri79 (5'-CCG GAA TTT ACC CAG TAA ATT AAC AAA GAA-3'), for ZRC1 and ZRC1(N44I), pri80 (5'-GGG CGA AGA TCT ATG ATC ACC GGT AAA GAA TTG-3'), DNA was amplified by PCR to generate each open reading frame without the stop codon. The amplified products were inserted into BamHI- and EcoRI-digested pYES2/C (Invitrogen).

Metal Analysis—For whole cell metal analysis, 20 OD cells (about 2 × 10⁸ cells) at log phase were collected, washed three times with 50 mM Tris-Cl (pH 6.5), 10 mM EDTA and once with deionized water. Vacuoles were prepared using Ficoll gradients as described previously (6). Samples were digested in 200 μl of nitric acid at 80 °C for 1 h, then diluted to 1 ml with deionized water. Metals were analyzed in a Perkin-Elmer Inductively Coupled Plasma-Optical Emission Spectrometer (ICP) and calculated using a standard curve generated from mixed metal standards.

Western Blot, β-Galactosidase Activity, and Protein Concentration Assay—Cells were disrupted with glass beads in the presence of protease inhibitors (1.0 mM phenylmethylsulfonyl fluoride, 1.0 μM pepstatin A, and 1.0 μM leupeptin) (Sigma). Samples (20 μg) were run on a 12% SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-His₅ tag (1:2000 Abcam) or mouse anti-Vma1 antibody (1:4000, Molecular Probes), followed by peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10,000, Jackson Immuno-Research). Membranes were developed using chemiluminescence reagents (PerkinElmer Life Sciences). The reporter constructs FET3-lacZ and CCC1-lacZ were described previously (15, 18). β-Galactosidase activity was performed in 96-well plates using ortho-nitrophenyl-β-galactoside as a substrate. The generation of ortho-nitrophenol was monitored at 420 nm and the data are presented as nanomole/min/mg of protein (18). Protein concentration was determined by the bichinchoninic acid method (Pierce) using bovine serum albumin as standard.

RESULTS

Identification of Yeast Mutants Resistant to High Iron—Deletion of CCC1 results in sensitivity to high iron (6, 15). We took advantage of this phenotype to identify UV-induced mutants of Δcct1 cells that were capable of growth on high iron medium.
We screened 12,400 colonies and identified 15 mutants that would survive on high iron medium. The mutants were backcrossed to the parental strain (H9004 ccc1) to determine whether they were single gene defects and whether the mutants were dominant or recessive. Seven of the mutants were single gene recessive mutations that fell into two complementation groups. Eight of the mutants were dominant. The mutants were characterized as having different sensitivity to high iron, as shown by liquid (Fig. 1A) and plate growth assays (Fig. 1B).

There are several potential mechanisms that might lead to high iron-resistant growth: decreased iron uptake, increased iron export, increased iron storage, or increased antioxidant defenses. To determine whether the mutant strains had decreased iron acquisition, cells were grown in CM medium overnight and cellular iron levels were measured by ICP. Mutants W2 and W7 accumulate less iron than control H9004 ccc1 cells. R1 and W17 accumulated more iron than the parental H9004 strain (Fig. 2A). If resistance to high iron was due to increased iron export from cells, then the mutant strains might not grow on low iron. This situation was observed for a mutant PDR1 in which high iron resistance was correlated with low iron sensitivity (21). The Δccc1 mutant strains were able to...
grow on iron-limited medium, which requires a functional iron transport system (Fig. 2B). These results suggest that the resistance to high iron cannot be explained only by decreased iron acquisition or increased iron export. If iron toxicity was due to increased oxidative damage resulting from Fenton chemistry, then increased antioxidant defenses might reduce such damage. To test this possibility we examined the ability of mutant cells to resist the effect of the oxidants H2O2 and paraquat. One mutant (W17) showed a slight increase in paraquat resistance, but in general the mutants were as sensitive to oxidants as the parental strains (Fig. 2C).

We tested the possibility that the mutants were able to store or sequester cytosolic iron by measuring the expression of an iron-responsive reporter. Transcription of CCC1 or a CCC1-lacZ reporter construct is increased by iron through activation of the transcription factor Yap5 (15). Wild type, Δccc1, and Δccc1 mutants were transformed with a CCC1-lacZ construct and the effect of iron on induction of β-galactosidase activity was determined. Compared with wild type cells, Δccc1 cells showed a higher expression of CCC1-lacZ, indicating the presence of increased cytosolic iron. This induction of CCC1-lacZ was repressed when the CCC1 gene was introduced back to Δccc1 cells (Fig. 2D). Mutants W2 and W17 showed similar amounts of CCC1-lacZ activity compared with the parental Δccc1 strain. R1 and W7, however, showed lower levels of β-galactosidase compared with the parental strains. The R1 mutant was the most resistant to growth inhibition by high iron (cf. Fig. 1A). Mutant R1 had a slight increase in cellular iron compared with Δccc1 but less cytosolic iron as indicated by the CCC1-lacZ reporter assay. This implies that the resistance to high iron growth might be due to its ability to sequester cytosolic iron. Taken together these results showed that different mechanisms may contribute to high iron resistance in mutant strains.

The High Iron Resistance of Mutant R1 Is Caused by a Mutation in ZRC1—To identify the gene responsible for the high iron resistance of R1 a low copy genomic library was generated from R1 cells. The library was transformed into the Δccc1 cells and transformants able to grow on high iron were identified. Plasmids conferring resistance to high iron were isolated and transformants able to grow on high iron were identified. Plasmids containing overlapping segments of chromosome XIII were isolated. ZRC1, a gene that encodes a vacuolar zinc transporter (5, 22), was identified as a common gene on all rescued plasmids. Subcloning of the insert showed that the R1-specific ZRC1 plasmid conferred iron resistance when transformed into Δccc1 cells (data not shown). Sequence analysis of R1-ZRC1 revealed a mutation at position 131 of the coding sequence that altered an adenine to thymidine, which resulted in the substitution of isoleucine for asparagine at amino acid position 44 (referred to henceforth as ZRC1(N44I)). To confirm that this single amino acid change was responsible for high iron resistance, the asparagine at position 44 of the wild type Zrc1 was changed to an isoleucine by site-directed mutagenesis. When transformed into Δccc1 cells, ZRC1(N44I) was able to confer high iron resistance to Δccc1 cells (Fig. 3A).

Zrc1 was identified initially as a high copy suppressor of zincc-sensitive growth (5) and deletion of ZRC1 increases zinc sensitivity (7). Cells with a deletion in ZRC1 showed zincc-sensitive growth that was suppressed by expression of plasmid containing ZRC1 but not by a plasmid containing ZRC1(N44I) (Fig. 3B). This result demonstrates that Zrc1(N44I) has lost its intrinsic zinc transport activity, and may explain why the R1 mutant is sensitive to high zinc concentration (data not shown).

It is possible that a loss of function of ZRC1 protects Δccc1 from high iron toxicity by altering zinc homeostasis and modulating the entire yeast transcriptome. To test this we generated a mutant ZRC1 based on the sequence alignment of Zrc1 with the E. coli FieF, another member of the cation diffusion facilitator family (23). The crystal structure of FieF suggests that Asp99 is involved in binding the zinc substrate. The homologous residue in Zrc1 is Asp45 and site-specific mutagenesis of ZRC1(D45A) resulted in an expressed protein that was unable to protect Δzrc1 cells from high zinc toxicity (Fig. 3B). This construct was unable to permit Δccc1 cells to grow on high iron medium (Fig. 3A). Furthermore, deletion of ZRC1 in Δccc1 cells did not protect Δccc1 from high concentrations of iron (Fig. 3C). Together, these data suggest that loss of zinc transport activity is not sufficient to protect Δccc1 from high iron toxicity and also explains why mutant R1 shows dominant characteristics.

Zrc1(N44I) Increased Vacuolar Iron—Because Zrc1 localizes to the vacuolar membrane (9), it is possible that Zrc1(N44I) transports iron into the vacuole, which would decrease cytosolic iron. We tested this possibility by assaying the expression of a FET3-lacZ reporter. This reporter construct is regulated by the low iron sensing transcription factor Aft1 (24). Overexpres-
iron was measured in vacuoles isolated from Δccc1 cells transformed with empty vector or plasmids expressing ZRC1 or ZRC1(N44I). Overexpression of wild type ZRC1 showed a slight increase in vacuolar iron relative to vector-transformed cells (Fig. 4C). Overexpression of ZRC1(N44I), however, resulted in a 3–4-fold increase in vacuolar iron. Together, these results show that ZRC1(N44I) is a gain of function allele that can confer iron-resistant growth by exporting iron from cytosol to vacuole.

These data show that Zrc1(N44I) can transport iron but it is hard to compare the intrinsic iron transport activity of Zrc1(N44I) with that of Ccc1, as expression of each transporter is regulated differently. CCC1 transcription and mRNA stability are increased by iron, whereas ZRC1 (and by implication ZRC1(N44I)) shows increased transcription due to low zinc (7). To determine the intrinsic iron transport activity of Ccc1, Zrc1, and Zrc1(N44I), each was cloned into a GAL1-regulated vector. We placed a His6 epitope at the carboxyl terminus of each gene, which permitted us to determine protein levels. GAL1 regulated CCC1-HIS6 and ZRC1(N44I)-HIS6 were able to suppress the high iron growth defect of Δccc1 cells, whereas as a GAL1 regulated ZRC1-HIS6 was not (Fig. 5A). Under similar growth conditions, Zrc1-HIS6 and Zrc1(N44I)-HIS6 were expressed to higher levels than Ccc1-HIS6 (Fig. 5B). Measurement of iron transport activity based on FET3-lacZ expression showed that Zrc1-HIS6 had no measurable iron transport activity, whereas both Zrc1(N44I)-HIS6 or Ccc1-HIS6 induced expression of FET3-lacZ (Fig. 5C). Based on the expression levels of the proteins Zrc1(N44I)-HIS6 has ~10–15% of the iron transport activity of Ccc1-HIS6. Δccc1 cells transformed with empty vector or galactose-regulated constructs were grown in galactose medium with 100 μM FeSO4. Vacuoles were isolated and iron content determined. Overexpression of CCC1-HIS6 and ZRC1(N44I)-HIS6, but not ZRC1-HIS6, increased vacuolar iron compared with vector-transformed control cells (Fig. 5D). There was more iron in Ccc1-HIS6 vacuoles than in Zrc1(N44I)-HIS6 vacuoles, again indicating that Ccc1 was more efficient in transporting iron into vacuoles.

It might be possible that iron is not transported into the vacuole but rather the iron is tightly bound to the cytosolic facing surface of Zrc1(N44I). The difference in vacuolar iron between Δccc1 cells overexpressing ZRC1(N44I) and control cells (empty vector) was about 10–12 nmol/mg of vacuolar protein. The crystal structure of the E. coli CDF family member FieF suggests that it has 4 Zn binding sites (11). Assuming one molecule of Zrc1(N44I) binds 4 atoms of iron, then based on the molecular mass of Zrc1 (48,344 daltons), would require ~0.12–0.15 mg of Zrc1 protein/mg of vacuole protein to bind the measured iron. This means that Zrc1(N44I) would have to constitute 12–15% of vacuolar proteins. To test this prediction we grew Δccc1 cells, transformed with either a control vector or GAL1-regulated ZRC1(N44I) in galactose medium overnight and then in galactose medium containing 100 μM FeSO4 for 4 h. Cells were harvested, vacuolar iron determined, and vacuolar proteins applied to SDS-PAGE and the gel analyzed by Western blot and silver stain. Vacuoles isolated from control cells only showed a slight increase in iron over the time course of the experiment, probably due to endocytosis of iron from the cul-

sion of CCC1, by transporting iron into vacuoles, leads to lower cytosolic iron inducing the expression of a FET3-lacZ reporter (6) (Fig. 4A). Expression of ZRC1(N44I) was also able to induce FET3-lacZ activity compared with cells transformed with either a vector or wild type ZRC1. Conversely, expression of ZRC1(N44I) but not wild type ZRC1 was able to reduce the activity of a CCC1-lacZ reporter construct (Fig. 4B). These results confirm that expression of ZRC1(N44I) lowers cytosolic iron. To prove that Zrc1(N44I) transports iron into vacuoles, cells transformed with empty vector, ZRC1, ZRC1(N44I), or CCC1 under their own promoters. Cells were grown to log phase and β-galactosidase activity determined. B, WT cells with empty vector, Δccc1 cells with empty vector, ZRC1, or ZRC1(N44I) were transformed with a CCC1-lacZ reporter construct. Cells were grown in CM medium to log phase and β-galactosidase activity determined. C, Δccc1 cells transformed with a high copy empty vector, ZRC1, or ZRC1(N44I) under the ZRC1 promoter were grown in CM medium with 50 μM FeSO4 overnight. Vacuoles were isolated and iron content was determined by ICP. All data were normalized for protein concentrations and error bars represent S.D. from three experiments.

FIGURE 4. ZRC1(N44I) alters cytosolic iron by transporting iron into the vacuole. A, wild type (WT) cells with integrated FET3-lacZ were transformed with empty vector, ZRC1, ZRC1(N44I), or CCC1 under their own promoters. Cells were grown to log phase and β-galactosidase activity determined. B, WT cells with empty vector, Δccc1 cells with empty vector, ZRC1, or ZRC1(N44I) were transformed with a CCC1-lacZ reporter construct. Cells were grown in CM medium to log phase and β-galactosidase activity determined. C, Δccc1 cells transformed with a high copy empty vector, ZRC1, or ZRC1(N44I) under the ZRC1 promoter were grown in CM medium with 50 μM FeSO4 overnight. Vacuoles were isolated and iron content was determined by ICP. All data were normalized for protein concentrations and error bars represent S.D. from three experiments.
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**FIGURE 5. Iron transport efficiency of Zrc1(N44I).** A, wild type (WT) cells transformed with empty vector (pYES2 GAL1 promoter), Δccc1 cells transformed with pYES2, ZRC1, ZRC1(N44I), or CCC1 tagged with His6 at the carboxyl terminus were grown in CM with 2% raffinose at 30 °C overnight. Cells were washed and spotted onto CM-Ura medium containing 2% galactose with or without 3 or 5 mM ferrous ammonium sulfate. Plates were incubated at 30 °C for 3 days. B, Δccc1 cells as in A were grown in CM medium with 2% raffinose and then incubated in CM medium with 2% galactose for 12 h. Cells were harvested, disrupted by glass beads, and protein levels measured by Western blot using a rabbit anti-His6 antibody or a mouse anti-Vma1 antibody followed by peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. The vacuolar protein Vma1 was used as a loading control. C, Δccc1 cells with an integrated FET3-lacZ at the HO locus were transformed with empty vector pYES2, ZRC1, ZRC1(N44I), or CCC1 as in A. Cells were grown in CM medium with 2% raffinose and inoculated into CM with 2% galactose for 12 h. Cells were harvested and β-galactosidase activity determined. The data are normalized for protein concentrations and error bars represent S.D. from three experiments. D, cells as in A were grown in CM-Ura medium containing 2% galactose with 100 μM FeSO4 and 1 mM ascorbate overnight. Vacuoles were isolated and iron content was determined by ICP. All data were normalized for protein concentrations and error bars represent S.D. from three experiments.

**FIGURE 6. Measurement of vacuolar iron content and Zrc1(N44I) protein abundance.** A, Δccc1 cells were transformed with a control vector (pYES2) or a ZRC1(N44I)-HIS6 containing plasmid. Cells were grown in galactose CM overnight and then incubated in the same medium containing 100 μM FeSO4. Vacuoles were isolated from control and ZRC1(N44I) expressing cells at time 0 and 4 h after incubation in iron-rich medium. The iron content of isolated vacuoles was determined by ICP and the data normalized to protein content. B, isolated vacuoles were solubilized and analyzed by SDS-PAGE and Western blot using a rabbit anti-His6 antibody or a mouse anti-carboxypeptidase Y antibody followed by peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. C, the same samples as in B were also analyzed by silver staining. The arrow represents the predicted mass of Zrc1(N44I)-HIS6.

Vacuoles isolated from Zrc1(N44I) showed a much greater increase in vacuolar iron (Fig. 6A). Western blot showed that during this time course, the Zrc1(N44I)-HIS6 protein was expressed and remained constant (Fig. 6B). Silver staining of vacuolar proteins from both control and Zrc1(N44I)-HIS6 expressing cells had a similar protein distribution in which the band corresponding to Zrc1(N44I)-HIS6 was not notable (Fig. 6C), suggesting that the protein abundance would be insufficient to bind vacuolar iron.

**Zrc1(N44I) Transports Manganese—**Many transporters that transport iron also transport Mn2+ (25, 26). Deletion of CCC1 results in sensitivity to high Mn2+ (27), although with the concentrations and strain used here the sensitivity was slight at best. Expression of ZRC1(N44I) suppressed Mn2+ toxicity in Δccc1 cells to a greater extent than ZRC1 (Fig. 7A). Pmr1 is a Golgi membrane P-type ATPase involved in transporting Ca2+ and Mn2+ into the Golgi (28). Deletion of PMR1 leads to accumulation of Mn2+ in the cytosol, increasing the sensitivity of cells to high concentrations of Mn2+. Indeed, CCC1 was identified as a high copy suppressor of Δpmr1 Mn2+ toxicity phenotype, indicating it also transports Mn2+ into vacuoles (27).
Overexpression of CCC1 or ZRC1(N44I), but not wild type ZRC1, protected Δpmr1 cells from Mn\(^{2+}\) toxicity (Fig. 7B). These results indicate that Zrc(N44I) also has the ability to transport Mn\(^{2+}\). To examine whether ZRC1(N44I) can transport copper into vacuoles, we studied the effect of overexpression of ZRC1(N44I) in copper-sensitive cells. ACE1 encodes a transcription factor that regulates the expression of metallothioneins, small intracellular proteins that can bind and detoxify copper (29). Deletion of ACE1 renders cells copper sensitive but overexpression of ZRC1 or ZRC1(N44I) did not suppress or enhance the copper sensitivity of Δace1 cells (Fig. 7C).

Cot1(N45I) Shows Increased Iron Transport and Decreased Cobalt Transport—The N45I mutation in Zrc1 is in the second hydrophobic domain of the sequence of this domain is highly conserved in homologous CDF proteins involved in Zn\(^{2+}\) transport, which are found throughout the biological kingdoms (Fig. 8). This sequence is highly conserved in the Zrc1 homologue Cot1, which shows 78% amino acids similarity to Zrc1. Cot1 was initially identified as a gene that conferred cobalt resistance when overexpressed (4). Cot1 has Zn\(^{2+}\) transport activity, as overexpression of COT1 confers zinc resistance and deletion of COT1 increases zinc sensitivity in Δcct1 cells (30). The corresponding asparagine at position 45 of Cot1 was changed to isoleucine by site-directed mutagenesis to examine the effect on the substrate specificity of Cot1. Wild type COT1 or COT1(N45I) were transformed into Δcct1 cells and growth on high iron medium was examined. High copy expression of COT1 in Δcct1 cells was able to confer high iron resistance (Fig. 9A). Expression of COT1(N45I) increased the resistance of Δcct1 cells to high iron. Overexpressed COT1(N45I) was able to induce FET3-lacZ reporter expression (Fig. 9B) and reduce expression of the CCC1-lacZ reporter (Fig. 9C) indicating an alteration of iron homeostasis. The N45I mutation, however, decreased the ability of COT1 to confer cobalt resistance on Δcct1 cells (Fig. 9D).

Overexpression of COT1 can partially suppress the high iron growth deficit of Δcct1 cells (Fig. 9A), suggesting that Cot1 may be a low affinity iron transporter. In CM medium, however, expression of COT1 has little effect on Fet3-lacZ activity. COT1 is a target of the transcription factors Aft1 and Aft2 (31) and shows a modest induction under low iron conditions. Low iron conditions prevent accumulation of the vacuolar iron transporter Ccc1, as CCC1 transcription is activated under high iron conditions (15) and CCC1 mRNA is destabilized under low iron conditions by the Aft1-regulated gene CTH2 (14). We considered the possibility that expression of a low affinity vacuolar iron transporter might protect cells against iron shock. Compelling evidence shows that expression of Zrc1 by low zinc conditions protects cells against sudden increases in cytosolic zinc, termed zinc shock (7). We tested the possibility that Cot1 might play an analogous role and protect cells from “iron shock.” Cells grown in low iron were transferred to high iron medium and growth assayed. Wild type cells showed no obvious growth defect when incubated in high iron (Fig. 10). In contrast, Δcct1 cells showed a severe growth deficiency. Deletion of COT1 by itself did not affect growth in high iron nor did it exacerbate the growth defect of Δcct1 deletion. These results do not support a role for Cot1 in either iron shock conditions or in protecting cells from high iron.

**DISCUSSION**

Deletion of the vacuolar iron transporter Ccc1 results in growth inhibition on high iron medium. We initiated this study to identify genes that suppressed the high iron growth defect. Our study identified both recessive and dominant muta-
Metal Specificity of Vacular Transporters

Only one of the mutants showed notable resistance to oxidants, suggesting that modulation of iron-dependent oxygen toxicity is not a prominent mechanism for high iron resistance. This result confirms our previous observation that high iron toxicity does not necessarily result from increased Fenton chemistry, as high iron growth deficit occurs anaerobically.

We identified the molecular basis of one of the dominant iron-resistant mutants to be a single amino acid mutation in the vacular Zn\(^{2+}\) transporter Zrc1 (22, 30). Targeted site-specific mutagenesis is the most common approach to examining the substrate specificity of enzymes or transporters. In combination with either structural studies or genome wide informatics, targeted mutations have revealed much about the importance of specific domains or amino acids in determining substrate specificity. The role of amino acids in determining the substrate selectivity or transporters has been reported previously for such transporters, as the plant potassium transporter HKT1 (32), \(H^+\)-sucrose symporter AtSUC1 from *Arabidopsis thaliana* (33), plasma membrane \(H^+\)-ATPase PMA2 from *Nicotiana plumbaginifolia* (34), the plant *A. thaliana* ZIP family member metal transporter IRT1 (35), and yeast *Saccharomyces cerevisiae* Mn\(^{2+}\)/Ca\(^{2+}\) transporter Pmr1 (36). Amino acid substitutions generally abolish transporter activity or increase the transport activity of one of its substrates relative to another. Here we show that a single amino acid substitution in Zrc1 or Cot1 dramatically changed the substrate specificity. Genetic and biochemical studies have shown that Zrc1 is able to transport Zn\(^{2+}\), Ni\(^{2+}\), Cd\(^{2+}\), but not Fe\(^{2+}\) or Mn\(^{2+}\) (9). We confirmed this conclusion by measuring resistance to high iron growth conditions and by reporter constructs that assay cytosolic iron levels. Substitution of an asparagine at position 44 to isoleucine abolished the ability of Zrc1 to transport Zn\(^{2+}\) but conferred the ability to transport Fe\(^{2+}\) and Mn\(^{2+}\). A similar change in Cot1 also altered its substrate specificity to iron while reducing its ability to transport Co\(^{2+}\).

Zrc1 and Cot1 belong to the CDF family of transition metal transporters. Members of this family are found in all biological kingdoms and most usually transport metals out of the cytosol either into organelles or out of cells. CDF transporters usually have six transmembrane regions in which there is a high degree of sequence conservation in the charged residues of transmem-
brane domains II and V, which is thought to bind metals and form a transmembrane pore. The structure of the E. coli CDF member FieF, which transports iron, has been determined with Zn$^{2+}$ in the metal binding site (23). Structural and mutagenesis studies using E. coli FieF have focused on the importance of the Asp$^{45}$ and Asp$^{49}$ in transmembrane TMD II, and His$^{153}$ and Asp$^{157}$ in TMD V as residues that coordinate Zn$^{2+}$ in the crystal structure, and by implication iron in the native transporter. These charged residues are conserved in CDF family members although each family member shows different substrate specificities. The mutated asparagine is adjacent to the conserved aspartic acid in TMD II of Zrc1 and CotI. The substitution of a hydrophilic residue with a hydrophobic residue may alter the conformation of the metal binding site, changing the metal specificity of transport. Our study suggests that the microenvironment of the amino acids adjacent to the metal binding asparagine determines the substrate selectivity. The mutated ZRC1 was identified by a screen using random UV mutagenesis to select for cells showing high iron resistance. The approach of using a strong selection system in conjunction with mutagenesis of genes encoding transporters offers the possibility of facile identification of other residues critical for determining the substrate specificity of CDF transporters.

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REFERENCES

A Single Amino Acid Change in the Yeast Vacuolar Metal Transporters Zrc1 and Cot1 Alters Their Substrate Specificity

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