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ORIGINAL ARTICLE

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Characterization of the ZAT1p zinc transporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomes

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Abstract The ZAT1p zinc transporter from *Arabidopsis thaliana* (L.) Heynh. is a member of the cation diffusion facilitator (CDF) protein family. When heterologously expressed in *Escherichia coli*, ZAT1p bound zinc in a metal blot. Binding of zinc occurred mainly to the hydrophilic amino acid region from H_{182} to H_{232}. A ZAT1p/ZAT1p$^{*}\Delta(M_1–I_{25})$ protein mixture was purified and reconstituted into proteoliposomes. Uptake of zinc into the proteoliposomes did not require a proton gradient across the liposomal membrane. ZAT1p did not transport cobalt, and transported cadmium at only 1% of the zinc transport rate. ZAT1p functioned as an uptake system for $^{65}Zn^{2+}$ in two strains of the Gram-negative bacterium *Ralstonia metallidurans*, which were different in their content of zinc-efflux systems. The *ZAT1* gene did not rescue increased zinc sensitivity of a Δ*ZRC1* single-mutant strain or of a Δ*ZRC1* Δ*COT1* double-mutant strain of *Saccharomyces cerevisiae*, but *ZAT1* complemented this phenotype in a Δ*SpZRC1* mutant strain of *Schizosaccharomyces pombe*.

Keywords CDF-family · Heavy metal · *Ralstonia* · *Saccharomyces* · *Schizosaccharomyces* · Zinc-transporter

Abbreviations CDF: cation diffusion facilitator · DW: dry weight · SMM: supplemented mineral salts medium

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Introduction

Metal tolerance and homeostasis in plants is reached by controlled transport processes in connection with sequestration by specific metal-binding components like phytochelatins, metallo-chaperones and metallothioneins (Clemens 2001). Cation diffusion facilitators (CDF, T. C. 2.A.4.1.1; Saier 2000) are a family of metal-transport proteins that have been found in a variety of organisms (Nies and Brown 1998; Nies and Silver 1995; Paulsen and Saier1997). The first examples were the CzcD (Nies 1992) protein from the Gram-negative bacterium *Ralstonia metallidurans* strain CH34 (previously *Alcaligenes eutrophus*; Brim et al. 1999; Mergeay2000; Goris et al. 2001) and ZRC1p from the yeast *Saccharomyces cerevisiae* (Kamizomo et al. 1989). Both proteins are transporters also involved in regulation of metal ion homeostasis.

Ralstonia metallidurans is a model organism with which to study the impact of transport systems on heavy-metal homeostasis. At high concentrations (>100 μM Zn^{2+} in the growth medium), zinc homeostasis in this bacterium is maintained by the *czc* (cobalt-zinc-cadmium) metal-resistance determinant, which encodes two cation-efflux systems. A high-level resistance is mediated by the CzcCBA protein complex which seems to transport the cations across both membranes of the Gram-negative bacterium from the cytoplasm to the outside (Nies et al. 1987, 1989; Nies and Silver 1989; Nies 1995; Rensing et al. 1997; Goldberg et al. 1999). The driving force is the proton gradient Z·ΔpH, which is part of the proton motive force Δp. A low-level resistance, only visible in the absence of *czcCBA*, is mediated by CzcD, which is additionally also involved in the metal-dependent regulation of *czcCBA* expression (Nies 1992; van der Lelie et al. 1997; Anton et al. 1999; Große et al. 1999). ZRC1p was identified as a factor involved in zinc and cadmium resistance (Kamizomo et al. 1989) and independently as a factor required for regulation of glutathione synthesis (Inoue et al. 1993). In *S. cerevisiae*,

COT1p, a second CDF protein, is involved in cobalt transport (Conklin et al. 1992), and both yeast CDFs are able to complement each other at least partially (Conklin et al. 1994). The *S. cerevisiae* genome contains three additional CDF proteins (Paulsen et al. 1998); two are involved in iron transport (Li and Kaplan 1997), the third one also affects cellular zinc distribution (Li and Kaplan 2001). Mammals have at least four CDF proteins that are zinc transporters. ZnT-1seems to be a protein bound to the plasma membrane and may pump zinc out of the cell (Palmiter and Findley 1995). ZnT-2 may protect cells from zinc toxicity by facilitating zinc transport into an endosomal/lysosomal compartment (Palmiter et al. 1996a), while ZnT-3 facilitates the accumulation of zinc in synaptic vesicles (Palmiter et al. 1996b). Finally, ZnT-4 (Huang and Gitschier 1997) is responsible for the inherited zinc deficiency in the lethal-milk mouse.

For several years, the only clue for the existence of CDF-coding genes in plants was the 123PEP sequence, which was found in a screening for pathogen-induced genes (Leister et al. 1996). Recently, the *ZAT1* gene from plants was found in the course of the *Arabidopsis thaliana* sequencing project (gi|3510254; GenBank accession number AC005310), and fortuitously in an unrelated project (van der Zaal et al. 1999). The ZAT1p protein is expressed in all organs of the plant, and overexpression of *ZAT1* can lead to enhanced zinc resistance and accumulation in roots (van der Zaal et al. 1999).

This paper studies the function of ZAT1p after purification and reconstitution into proteoliposomes and after heterologous expression in the model organisms *R. metallidurans*, *S. cerevisiae* and *Schizosaccharomyces pombe*. It is the first reconstitution study with a purified protein of the CDF protein family and also the first investigation of the substrate-specificity of a CDF protein, which sheds some light on zinc homeostasis in plant cells.

Materials and methods

Microbial strains, growth conditions and plasmids

Tris-buffered mineral salts medium containing 2 g/l sodium gluconate was used to cultivate *Ralstonia metallidurans* strains AE128(pMOL30), DN182(pMOL30-14, Δ*czcD*) and AE104 (plasmid-free, metal-sensitive) (Mergeay et al. 1985; Anton et al. 1999). *Saccharomyces cerevisiae* strain INVSC2 (*ura3-52 his3-Δ200*, mating type α; Invitrogen, Groningen, The Netherlands) was cultivated in supplemented mineral salts medium (SMM: 6.7 g yeast nitrogen base without amino acids and 20 g glucose per liter). *Schizosaccharomyces pombe* strain FY261 (h^+ *ade6-M216 leu1-32 ura4-Δ18 can1-1*; Susan Forsburg, Salk Institute, La Jolla, USA) was cultivated in Edinburgh minimal medium (EMM2; Nurse 1975).

For construction of plasmid pDNA308, the *ZAT1* gene (gi|3510254; AC005310) was PCR-amplified from total DNA isolated from *Arabidopsis thaliana* (L.) Heynh.. The primers used were 5′-AAAGGATTC**AGGAG**TTTCTATGGAGTCTTCAAGTCCCCA-3′ (bold: ribosome-binding site of *czcD*) and 5′-AAAGGATCCTTAGCGCTC GATTTGTATCGT-3′. The PCR fragment was double-digested with *Eco*RI and *Bam*HI and cloned under control of the *lac* promoter into the broad-host-range plasmid pVDZ′2 (Deretic et al. 1987). Expression from the *lac* promoter is constitutive in *R. metallidurans* (Nies et al. 1989).

For expression in *S. cerevisiae*, the *ZRC1* gene was PCR-amplified from genomic DNA of *S. cerevisiae* INVSC2 as a 1.3-kb PCR fragment (primer CCGGATCCGGACTCTTGATGTATCTCCGG and GGCTGCAGGGAACAAGCAGGCGCCCG, restriction sites underlined) and cloned into the yeast shuttle vector pYES2 (Amp^r *ura3*) under control of the *gal* promoter. Similarly, the *ZAT1* gene was cloned from the pASK expression vector (see below) into pYES2.

Constructions of mutant strains

Mutant strains of *S. cerevisiae* strain INVSC2 were constructed with gene-disruption cassettes (Güldener et al. 1996). The primers used for Δ*COT1* were 5′-ATGAAACTCGGAAGCAAACAGGTAAAAATTATATCCTTGTCAGCTGAAGCTTCGTACGC-3′ and 5′-AATGATCCTCTAAGCAATGAGCTGTGTTGCAGTTGGCAGCATAGGCCACTAGTGGATCTG-3′. The primers used for Δ*ZRC1* were 5′-ATGATCACCGGTAAAGAATTGAGAATCATCTCTCTTTTGACAGCTGAAGCTTCGTACGC-3′and 5′-GCAGGCAATTGGAAGTATTGCAGTTTACAGCGTCATCTACAGCATAGGCCACTAGTGGATCTG-3′. Briefly, the target gene was exchanged for a kanamycin-resistance gene (*kan*), which was deleted again in a second step by Cre-mediated double-site-specific recombination at two *loxP*-sequences flanking the *kan* gene.

Purification of ZAT1p

The *ZAT1* gene was PCR-amplified from total DNA isolated from *A. thaliana* using the primers 5′-AAAACCGCGGTGAGTCTTCAAGTCCCCACCAT-3′ and 5′-AAAACTGCAGGCGCTCGATTTGTATCGTGAC-3′ (restriction endonuclease sites for *Sac*II or *Pst*I underlined), and cloned into pASK-IBA3 (Amp^r; IBA, Göttingen, Germany) using the appropriate restriction enzymes. Correct cloning was verified by DNA sequence analysis. The resulting plasmid pECD698 was transferred into *Escherichia coli* strain BL21 (pUBS520;Stratagene Europe, Amsterdam, The Netherlands). Plasmid pUBS520 (Brinkmann 1990) is compatible with pECD698, and contains the *argU* gene for $tRNA_{Arg}$[AGA/AGG] and a kanamycin-resistance determinant for selection. The respective codons are rarely used in *E. coli*, but ZAT1p contains nine of them. Therefore, cells without plasmid pUSB520 did not express detectable amounts of ZAT1p (data not shown).

For construction of *ZAT1*Δ(H_{182}–H_{232}), the 5′ part was PCR-amplified from plasmid pECD698 with the primer pair AAAACCGCGGTGAGTCTTCAAGTCCCCACCAT and AAAGGTACCCCCTAGCAGAACAGCCATTATGAT, the 3′ part with the primer pair AAAACTGCAGG CGCTCGATTTGTATCGTGAC and AAAGGTACCGGGGATGTTACTGAGCAATTGTTG. The two resulting PCR fragments were digested with *Sac*II and *Kpn*I or *Kpn*I and *Pst*I, respectively, cloned into pGEM-T Easy (Promega, Madison, Wis., USA) in one step, verified by DNA sequence analysis and finally cloned again into pASK-IBA3 (Amp^r; IBA, Göttingen, Germany).

A pre-culture of the respective *E.coli* expression strain was diluted 1,000-fold with fresh LB-medium (Sambrook et al. 1989) containing 125 µg/ml ampicillin and 50 µg/ml kanamycin, and incubated with shaking (200 rpm) at 30 °C. When an optical density (at 600 nm) of 1.0 was reached, expression of *ZAT1* and *argU* was induced with 100 µg/l anhydrotetracycline and 300 µM isopropyl-thiogalactoside (IPTG). Incubation was continued with shaking at 30 °C for 3 h and the cells were harvested by centrifugation. The cells were suspended in buffer W [100 mM Tris-HCl (pH 8.0), 1 mM EDTA], additionally containing 10 g/l DNAse I and 1 mM phenylmethylsulfonyl fluoride. After treatment with a French press (three times, 1,200 psi), the cell debris was removed (1 h, 5,000 rpm, 4 °C; Hettich, Tuttlingen, Germany), the membranes

were sedimented (100,000 *g*, 90 min, 4 °C) and suspended in 4 ml of buffer W. The membrane protein concentration was adjusted to 5 g/l with buffer W, and 5 g *n*-dodecyl *β*-D-maltoside/g protein and 3.5 g/l phosphatidylcholine were added. The resulting suspension was stirred on ice for 1 h, and the membrane debris was removed by ultracentrifugation as before.

The solubilized membrane proteins were directly applied onto a strep-tactin sepharose column (bed volume 1 ml; IBA, Göttingen, Germany) equilibrated with buffer W additionally containing 1 g/l *n*-dodecyl *β*-D-maltoside. The column was washed with 6 ml buffer W. The ZAT1p protein was eluted with 4 ml buffer E [100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 g/l *n*-dodecyl *β*-D-maltoside, 2.5 mM desthiobiotin], and 0.5-ml fractions were collected.

Metal blot

As published (Klimpel et al. 1994), 20 µg of the total membrane proteins of *E. coli* cells expressing ZAT1p or ZAT1pΔ(H_{182}–H_{232}), or 20 µg of purified ZAT1p protein, was separated on an SDS polyacrylamide gel and transferred onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Eschwege, Germany) by semi-dry blotting (Biometra, Göttingen, Germany) using a constant electric current of 1 mA/cm^2 gel area. The efficiency of transfer was controlled by Coomassie staining of the gel after blotting. The PVDF membrane was washed twice with 10 mM Tris-HCl/5 mM EDTA (pH 7.5) and five times with distilled water. The membrane was incubated for 3 h at 30 °C in 50 ml of buffer (100 µM $CaCl_2$, 10 mM Tris-HCl, pH 7.3) containing 0.1 MBq $^{65}ZnCl_2$ (185 GBq/g). The membrane was washed three times with the same buffer without $^{65}Zn^{2+}$ and exposed to an X-ray film for 3 days. As a control, both proteins were also visualised with an anti-strep-tag antibody and the strep-tag detection kit (IBA, Göttingen, Germany), following the manufacturer's instructions.

Reconstitution of ZAT1p

Soybean L-*α*-phosphatidylcholine (type II-S, 17% PC) was suspended in buffer R [20 mM Tris-HCl, pH 7.0, 2 mM dithiothreitol (DTT)] to yield a lipid concentration of 50 g/l. The suspension was ultrasonicated (60 W) on an ice/NaCl mixture for 30 s followed by cooling for 2 min until the solution was translucent (Reidlinger and Müller 1994). For detergent-destabilized reconstitution (Racker 1979), the liposomes were diluted to 10 g/l of lipid in buffer R, and Chaps [3-(3-cholamidopropyl)-dimethylammonio-1-propane sulphonate] was added to a final concentration of 11 mM. 2 g ZAT1p/l (protein:lipid = 1:50) was added and the suspension was dialysed for 72 h against buffer R. The proteoliposomes were sedimented by ultracentrifugation (100,000 *g*, 15 min 4 °C), suspended in 0.1 vol. buffer R, frozen in liquid nitrogen and stored in 20-µl aliquots at –80 °C until needed. Negative controls were liposomes treated in the same way but without ZAT1p protein added. Proteoliposomes and liposomes were loaded with 0.5 M NH_4Cl as published by Goldberg et al. (1999).

Quenching of acridine orange fluorescence

As published (Rosen 1986; Nies 1995), 5 µl of proteoliposomes was diluted into 2 ml buffer C [10 mM Tris-HCl (pH 8.0), 0.5 M choline chloride, 5 mM $MgCl_2$] containing 2 µM acridine orange (3,6 bis-dimethylaminoacridine). Acridine orange fluorescence was measured using an excitation wavelength of 493 nm and an emission wavelength of 530 nm (SFM25 spectrofluorometer; Kontron, Zürich, Switzerland) in stirred cuvettes at 24 °C.

Uptake experiments

Cation-uptake experiments using the filtration method were performed as described previously (Rosen 1986; Nies 1995; Goldberg et al. 1999) with some modifications. All experiments were repeated at least once with similar results. The NH_4Cl-containing proteoliposomes were diluted into Tris-choline buffer [0.5 M choline chloride, 10 mM Tris (pH 9.0), 5 mM $MgCl_2$] to a final volume of 30 µl. After 1 min, cation uptake was started by the addition of the radioactive cations $^{65}Zn^{2+}$, $^{57}Co^{2+}$ or $^{109}Cd^{2+}$ (Amersham), and the reaction mixture was incubated at 25 °C. Samples (5 µl) were filtered through membrane filters (pore size 0.45 µm; Schleicher and Schuell, Dassel, Germany) and rinsed twice with 0.5 ml of washing buffer [0.2 M choline chloride, 10 mM Tris (pH 7.0), 10 mM EDTA, 10 mM Mg^{2+}]. The radioactivity that remained on the membrane filter was determined with a scintillation counter (LS6500; Beckman, München, Germany). Control liposomes without ZAT1p were prepared using the same amounts of phospholipids, but no ZAT1p. To calculate a "mol zinc/mol ZAT1p" value with these negative controls, the amount of zinc accumulated by control liposomes was divided by the ZAT1p content of the ZAT1p-containing proteoliposomes in the parallel experiment, as published by Goldberg et al. (1999). All experiments were repeated at least once with similar results.

Ralstonia metallidurans cells were incubated in 10 mM Tris-HCl (pH 7.0) buffer containing 2 g/l sodium gluconate. The 300-µl samples were washed twice with 10 vol. 10 mM Tris-HCl (pH 7.0) buffer containing 10 mM $MgCl_2$. *S. cerevisiae* cells were incubated in MES buffer (10 mM MES, 20 g/l glucose, pH 6.1) and the 350-µl samples were washed three times with SSW buffer (1 mM NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 20 mM Na_3-citrate, 1 mM EDTA, pH 4.2). With both organisms, the dry weight was determined via the optical density and an equilibration curve.

Results

Zinc binds to a hydrophilic domain of ZAT1p

The ZAT1p protein was heterologously expressed in *E. coli* as a strep-tag fusion protein (predicted size 44.839 kDa) and 20 µg of membrane protein from these cells was separated by polyacrylamide gel electrophoresis. A metal blot experiment with $^{65}Zn^{2+}$ gave two signals at molecular masses of about 42 kDa and about 83 kDa (Fig. 1A, lane 1). A Western blot control gave two signals at sizes of about 42 kDa and 44 kDa (Fig. 1B), but no signal at 83 kDa. To obtain a negative control, the amino acid sequence 182-HDHGHSH GHGHGHGHDHHNHSHGVTVTTHHHHHDHEH-GHSHGHGEDKHHAH-232 was deleted from the *ZAT1* gene leading to *ZAT1*Δ(H_{182}–H_{232}). The mutant protein [ZAT1p′Δ(H_{182}–H_{232}), predicted size 39.105 kDa] was also expressed in *E. coli* as a strep-tag fusion protein. The presence of the ZAT1p′Δ(H_{182}–H_{232}) protein could be detected by anti-strep-tag antibodies in the cytoplasmic membrane at sizes of about 32 kDa and 39 kDa (Fig. 1C), but it did not bind $^{65}Zn^{2+}$ in the metal blot experiment (Fig. 1A, lane 3). A 80-kDa zinc-binding activity was not observed with the ZAT1p′Δ(H_{182}–H_{232}) protein (Fig. 1A, lane 3). Taken together, ZAT1p bound zinc to the His-rich part from H_{182} to H_{232}.

Reconstitution of ZAT into proteoliposomes

In contrast to ZAT1p′Δ(H_{182}–H_{232}), the ZAT1p strep-tag fusion protein could be purified to homogeneity

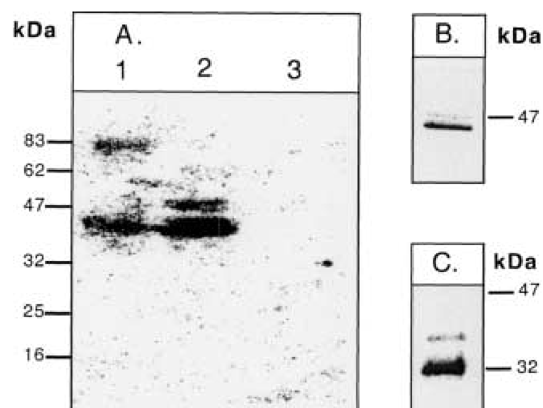


Fig. 1A–C ZAT1p from *Arabidopsis thaliana* binds zinc. **A** The proteins ZAT1p (*lanes 1, 2*) and ZAT1pΔ(H_{182}–H_{232}) (*lane 3*) were heterologously expressed as strep-tag fusion proteins in *Escherichia coli*. The membrane fractions were isolated and ZAT1p was purified after solubilisation with *n*-dodecyl *β*-D-maltoside. Either 20 µg of total membrane protein [*lane 1* ZAT1p, *lane 3* ZAT1pΔ(H_{182}–H_{232})] or of purified ZAT1p protein (*lane 2*) was separated by 12.5% SDS PAGE, transferred onto a PVDF membrane and incubated with $^{65}Zn^{2+}$ (0.1 MBq) for 3 h at 23 °C. Unbound zinc was removed in three washing steps and autoradiography was performed. The *arrow* indicates a possible signal from ZAT1pΔ(H_{182}-H_{232}). **B, C** Western controls with the membrane fractions containing ZAT1p (**B**) or ZAT1pΔ(H_{182}–H_{232}) (**C**). A strep-tag-specific antibody was used for labelling

(data not shown). Purified ZAT1p gave two signals in the zinc blot experiment at 42 kDa and 44 kDa (Fig. 1A, lane 2). N-terminal sequence analysis of these two bands (data not shown) demonstrated that the 44-kDa protein was the native ZAT1p strep-tag protein, and the 42-kDa protein was a ZAT1p protein carrying a deletion of the first 25 amino acid residues [ZAT1p$^{*}\Delta$(M_1–I_{25}), predicted size 42.041 kDa]. Thus, the ZAT1p protein preparation used was a ZAT1p/ZAT1p$^{*}\Delta$(M_1–I_{25}) mixture.

This ZAT1p protein preparation was reconstituted into proteoliposomes. In the presence of various zinc concentrations, ZAT1p-containing proteoliposomes accumulated substantially more $^{65}Zn^{2+}$ than control liposomes (shown for 1 mM Zn^{2+} in Fig. 2A; 100 µM and 10 mM Zn^{2+} not shown). In contrast, cobalt was not transported by ZAT1p, and cadmium was transported at only 1% of the transport activity of a similar concentration of zinc (Fig. 2B, C). The velocity of zinc transport by ZAT1p into proteoliposomes did not show substrate saturation behaviour at concentrations up to 10 mM (data not shown). Higher concentrations denatured the liposomes and were not tested.

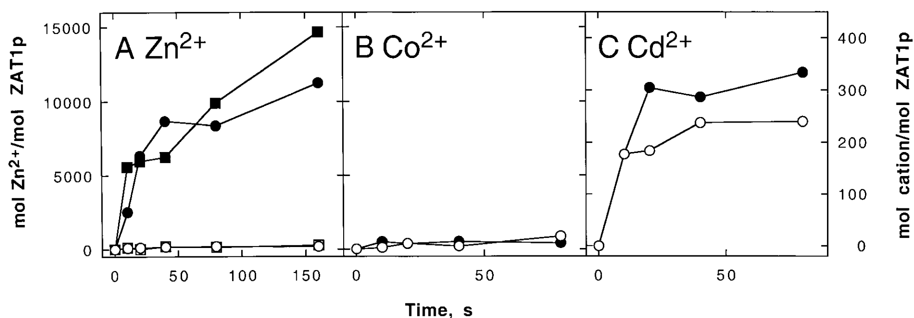
ZAT1p-mediated zinc transport was also determined in NH_4Cl-loaded proteoliposomes. Again, ZAT1p-containing proteoliposomes accumulated more $^{65}Zn^{2+}$ than the liposomes (shown for 1 mM Zn^{2+} in Fig. 2A; 10 mM Zn^{2+} not shown). There was no difference, however, between metal accumulation of proteoliposomes with or without a proton gradient. Formation of the proton gradient with NH_4Cl-loaded liposomes and proteoliposomes was demonstrated with the acridine orange quenching assay; the amount of liposomes used quenched the fluorescence signal down to 40% of the value before addition of the liposomes (data not shown). Thus, transport of zinc by ZAT1p required only the zinc concentration gradient across a membrane as an energy source for transport.

Expression of ZAT1p in *R. metallidurans*

Since the *ZAT1* gene did not contain introns, it was again amplified with PCR from total DNA isolated from *A. thaliana*. The upstream primer used for amplification attached the ribosome-binding site of *czcD* to *ZAT1* to prevent translational artefacts. The resulting gene was cloned, verified by DNA sequence analysis, and subcloned into the broad-host-range expression plasmid pVDZ'2 (Deretic et al. 1987) leading to plasmid pDNA308. Expression of *ZAT1* in the metal-sensitive *R. metallidurans* strain AE104 (Mergeay et al. 1985) did not change resistance to Zn^{2+} in liquid culture (data not shown).

When the uptake of metal cations by AE104 (pVDZ'2) was compared with that by AE104(pDNA308) (Fig. 3), it was found that expression of *ZAT1* led to increased accumulation of Zn^{2+} at concentrations between 100 µM and 1 mM. The initial velocities in cells

Fig. 2A–C Metal ion uptake by ZAT1p in proteoliposomes. The purified *A. thaliana* ZAT1p protein was reconstituted into detergent-destabilized liposomes (*black circles*) and the uptake 1 mM of $^{65}Zn^{2+}$(**A**), $^{57}Co^{2+}$ (**B**) or $^{109}Cd^{2+}$ (**C**) was determined. Control liposomes (*open circles*) were always treated in the same way as proteoliposomes and served as a negative control. Additionally, liposomes (*open squares*) and proteoliposomes (*black squares*) were also loaded with 1 mM $NHCl_4$ to generate a proton gradient prior to the addition of zinc



containing ZAT1p were about 3-fold higher than the velocities of control cells (Fig. 3B). The initial uptake velocities were linear functions of the zinc concentrations used, with slopes of 4.75 ± 0.12 μmol min^{-1} mM^{-1} $(g\ DW)^{-1}$ without ZAT1p and 13.95 ± 0.08 μmol min^{-1} mM^{-1} $(g\ DW)^{-1}$ with ZAT1p. The linear functions indicated that the uptake by ZAT1p was not saturated at 1 mM, which was in agreement with the data obtained with reconstituted proteoliposomes. Thus, ZAT1p catalyzed zinc uptake into *R. metallidurans*. The levels of cell-bound zinc reached after 1 h were 1.5- to 2-fold higher in cells containing ZAT1p than in control cells (shown for 100 μM Zn^{2+} in Fig. 3A, other data not shown). In contrast to zinc, however, no increased or decreased metal content could be observed when *R. metallidurans* cells containing ZAT1p were incubated with 100 μM or 1 mM Co^{2+} or 100 μM or 500 μM Cd^{2+} (data not shown).

The effect of ZAT1p on zinc transport was additionally demonstrated in a different physiological background in *R. metallidurans* strains containing the zinc-inducible CzcCBA high-resistance zinc-efflux system. Strain DN182 carries an *in frame* Δ*czcD* deletion on a modified derivative of plasmid pMOL30, pMOL30-14 (Anton et al. 1999). This mutation leads to a 7-fold higher expression of the CzcCBA efflux pump in cells not induced with zinc. This effect is only visible at high (≥100 μM) zinc concentrations: when non-induced cells of strain DN182 and its wild type AE128 were incubated in the presence of 1 mM Zn^{2+}, the non-induced wild-type cells accumulated about 200 μmol Zn^{2+}/g DW within 1 h while the mutant strain with 7 times more efflux system took up about 10% of this amount (data not shown).

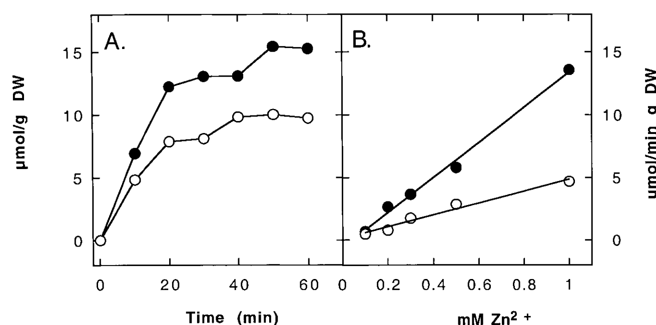


Fig. 3A, B Expression of ZAT1p in *Ralstonia metallidurans* strain AE104. The *ZAT1* gene was cloned under control of the *czcD* ribosome-binding site and the *lac* promoter (which is constitutively expressed in *R. metallidurans*) in the broad-host-range plasmid pVDZ′2 (Deretic et al. 1987) and conjugated into the plasmid-free *R. metallidurans* strain AE104. The cells were cultivated in Tris-buffered mineral salts medium containing 2 g sodium gluconate/l and harvested in the late exponential phase of growth. The cells were suspended into Tris-HCl buffer (10 mM, pH 7.0), and $^{65}ZnCl_2$ was added to 100 μM (**A**) or higher concentrations. Incubation was continued with shaking at 30 °C, samples were removed and the amount of cell-associated metal cation was determined by the filtration method. The initial velocity of zinc uptake was determined and plotted against the zinc concentration used in the uptake experiment (**B**). A Lineweaver-Burke-plot was not used in **B** since the observed uptake velocity was at least the result of two uptake systems in strain AE104(pVDZ′2) (data not shown), plus ZAT1p in AE104(pDNA308). *Black circles* Plasmid pDNA308, *open circles* pVDZ′2 vector control

At 100 μM Zn^{2+}, however, the cytoplasmic zinc concentration was well below the activity threshold of the CzcCBA pump, which is based on the sigmoidal substrate-saturation behaviour of this efflux system (Nies 1995; Goldberg et al. 1999). Therefore, strain DN182 and its wild type AE128 did not differ at 100 μM Zn^{2+} (Fig. 4) and the apparent cytoplasmic concentration was in equilibrium at 2 μmol/g DW (=0.7 mM apparent cytoplasmic concentration). Expression of ZAT1p in this equilibrium situation substantially increased the cellular zinc content: the cells rapidly accumulated 5 μmol/g DW and the cellular zinc content increased in the following hour up to 7 μmol/g DW. Thus, ZAT1p functions as a zinc-uptake system in *R. metallidurans* under two completely different physiological situations.

ZAT1p did not complement a Δ*ZRC1* Δ*COT1* double mutation in *S. cerevisiae*

To study ZAT1p in a eukaryotic cell, the two CDF-coding genes *ZRC1* and *COT1* were deleted from the genome of a haploid strain of *S. cerevisiae*. The resulting single-deletion strains showed a decreased resistance to Co^{2+}, Zn^{2+} and Cd^{2+} (Table 1, Fig. 5A), with Δ*COT* influencing primarily cobalt resistance and Δ*ZRC1* zinc resistance. Moreover, a Δ*COT1* Δ*ZRC1* double-deletion mutant was also constructed (Table 1). The Δ*COT1* strain accumulated only about 20% of the $^{65}Zn^{2+}$ amount compared to the wild-type cell (Fig. 6A). The double-mutant strain and the Δ*ZRC1* strain accumulated intermediary levels of $^{65}Zn^{2+}$.

Zinc resistance of the Δ*ZRC1* mutant strain could be complemented *in trans* with a *ZRC1* gene back to the wild-type level. Complementation of the double-mutant

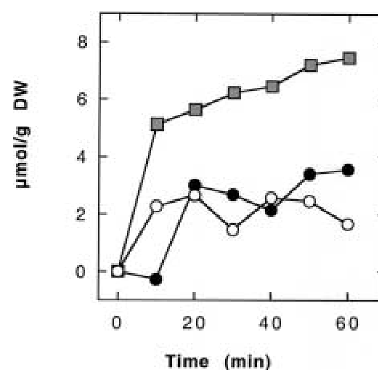


Fig. 4 Uptake of zinc by derivatives of *R. metallidurans* AE128(pMOL30). The uptake of $^{65}Zn^{2+}$ by cells of AE128 (pMOL30) (*black circles*), its *czcD* mutant strain DN182(pMOL30-14) (*open circles*) and of DN182(pMOL30-14, pDNA308) (*grey squares*) was determined by the filtration method at 100 μM of $^{65}Zn^{2+}$

Table 1 Mutants of *Saccharomyces cerevisiae* with deletions in CDF-encoding genes. The cells were cultivated for 5 days at 30 °C on solid SMM medium containing increasing concentrations of the heavy-metal chlorides. Formation of single colonies was judged as growth. The experiment was done twice with identical results

Strain	Genotype	Heavy-metal chloride (mM)		
		Co^{2+}	Zn^{2+}	Cd^{2+}
INVSC2	WT	2.0	12	0.2
YK40	Δ*COT1*	0.8	12	0.1
YK41	Δ*ZRC1*	1.2	7.5	0.15
YK44	Δ*COT1* Δ*ZRC1*	0.8	1.0	0.075

strain with a *ZRC1* gene led to the zinc resistance level of the Δ*ZRC1* single-mutant strain (Fig. 5B). However, neither the Δ*ZRC1* nor the double-mutant strain gained any zinc resistance when complemented with the *ZAT1* gene (Fig. 5C). In contrast, zinc resistance of the Δ*ZRC1* strain was even diminished when the *ZAT1* gene was expressed. When accumulation of $^{65}Zn^{2+}$ was compared in the double-mutant strains complemented with *ZRC1* or *ZAT1*, expression of both proteins led to a decreased level of zinc accumulation (Fig. 6B), but increased zinc accumulation in the short term (up to about 10 min).

ZAT1p complemented an *S. pombe* Δ*ZRC1* knock-out strain

In order to study ZAT1p in another eukaryotic cell better suited for ZAT1p expression, the protein was expressed with a C-terminal HA-tag in an *S. pombe* strain carrying a disruption of the *SpZRC1* gene (GenBank accession number Z98559). This gene is one of three CDF genes in the fission-yeast genome and has the highest similarity to ZAT1p/CzcD/COT1p and ZRC1p. The Δ*SpZRC1* deletion strain is extremely sensitive to Zn^{2+} and Co^{2+} (data not shown). Expression of *ZAT1* resulted in complementation of the Zn^{2+} sensitivity (Fig. 7). The degree of complementation correlated with the amount of ZAT1p protein. In contrast, the similarly pronounced inhibition of ΔSpZRC growth in the presence of Co^{2+} was not rescued by ZAT1p (data not shown).

Discussion

Zinc binds to the hydrophilic, histidine-rich amino acid stretch in the middle of the ZAT1p protein. ZAT1p may

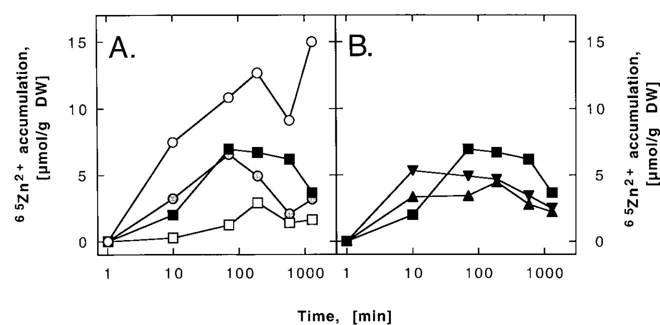
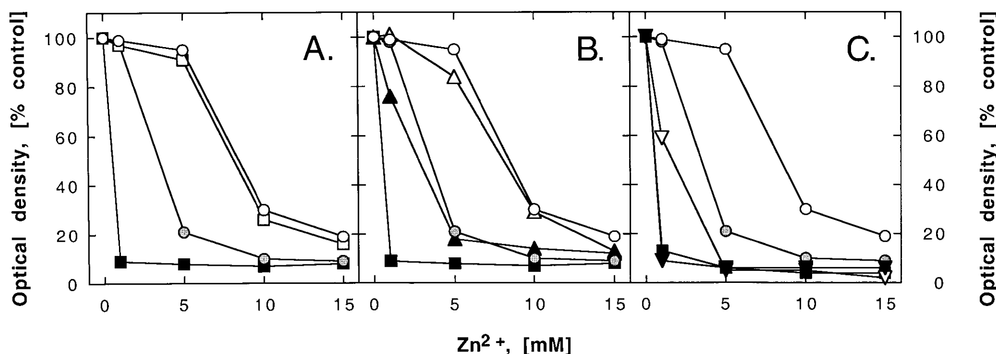


Fig. 6A, B Zinc uptake by *S. cerevisiae* strains with different sets of CDF proteins. Uptake of 100 µM $^{65}Zn^{2+}$ by various yeast strains was determined by the filtration method. **A** Wild type INVSC2 (*open circles*), YK40 Δ*COT1* (*grey squares*), YK41 Δ*ZRC1* (*grey circles*), and YK44 Δ*COT1* Δ*ZRC1* (*black squares*). **B** Complementation of the double-mutant strain YK44 (*squares*) with the genes *ZAT1* (*inverted triangles*) or *ZRC1* (*triangles*) *in trans*

Fig. 5A–C Zinc resistance in *Saccharomyces cerevisiae* strains with different sets of CDF proteins. The yeast strains were cultivated in SMM medium containing various concentrations of $ZnCl_2$ for 24 h with shaking at 30 °C. The optical density at 600 nm was determined and divided by the value for the wild-type strain INVSC2 (*open circles*) cultivated without added zinc. **A** YK40 Δ*COT1* (*grey squares*), YK41 Δ*ZRC1* (*grey circles*), and YK44 Δ*COT1* Δ*ZRC1* (*black squares*). **B** Complementation of the Δ*ZRC1* strain YK41 (*grey circles*) with the *ZRC1* gene *in trans* (*open triangles*) and the double-mutant strain YK44 Δ*COT1* Δ*ZRC1* (*black squares*) with *ZRC1* (*black triangles*). **C** Complementation of the Δ*ZRC1* strain YK41 (*grey circles*) with the *ZAT1* gene *in trans* (*inverted open trangles*) and of the double-mutant strain YK44 Δ*COT1* Δ*ZRC1* (*black squares*) with *ZAT1* (*inverted black triangles*)



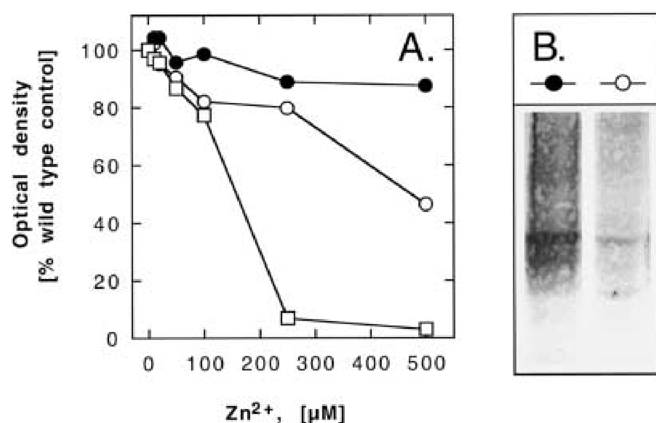


Fig. 7A, B Expression of *ZAT1* in *Schizosaccharomyces pombe*. **A** Cells of *S. pombe* wild type, its Δ*SpZRC1* deletion strain (*squares*) and of the Δ*SpZRC1* deletion strain complemented *in trans* with the *ZAT1* gene at a high (*black circles*) or a low (*open circles*) expression level were cultivated in EMM2 medium containing various concentrations of $ZnCl_2$. After 24 h of cultivation at 30 °C with shaking, the optical density was determined at 600 nm and divided by the optical density of wild-type cells incubated without added zinc. **B** Control Western blots indicating the expression levels of ZAT1p in the cells. For this purpose, antibodies against the HA-tags of both proteins were used for visualisation

also form dimers when present in membranes, as indicated by the 83-kDa signal in Fig. 1A (lane 1), but the evidence obtained in the metal-blot experiment was not sufficient to prove this fact. However, formation of dimers or trimers of CDF proteins within a cytoplasmic membrane has also been demonstrated with CzcD (Andreas Anton, Martin Luther University, Halle-Wittenberg, Germany, personal communication).

High-level expression of ZAT1p in *E. coli* mainly led to the production of a ZAT1p*Δ (M_1–I_{25}) derivative. The codon of V_{26} of ZAT1p is a "GTG", with a "GAAGAGAGAA" sequence 5 bp upstream. This sequence may have served as translational start site in *E. coli*. Alternatively, the protein may have been degraded at the amino terminus during purification. Since the first predicted transmembrane region of the native ZAT1p starts after K_{57}, the overall structure and function of ZAT1p may not be influenced by deletion of the first 25 amino acid residues. Indeed, the ZAT1p*Δ(M_1–I_{25}) protein transported zinc when reconstituted into proteoliposomes. Transport did not require an energy source such as ATP or the proton motive force; thus, the zinc concentration gradient was sufficient to drive zinc transport by ZAT1p*Δ(M_1–I_{25}). It could be argued that the 25-amino-acid N-terminus of ZAT1p may be essential for energy coupling. To obtain additional data about ZAT1p function from a completely different direction, the impact of ZAT1p expression was studied in a bacterial system.

In the bacterial model organism *R. metallidurans*, ZAT1p was an uptake system. Because of the proton motive force, uptake of a cation into a bacterial cell is never a facilitated diffusion, but always at least a ΔΨ-driven uniport. Thus, *ZAT1* expression should have led to accumulation of Zn^{2+}, which has been observed. At an outside concentration of 100 µM, ZAT1p-containing *R. metallidurans* cells accumulated 15 µmol zinc/g DW (Fig. 3), which calculated (Li et al. 1994) to an apparent cytoplasmic concentration of about 5 mM and an accumulation factor inside/outside of 50-fold. This quotient is slightly higher than the theoretical value of 46-fold calculated for a 100 mV-driven uniport of a monovalent cation in thermodynamic equilibrium; therefore, zinc transport into *R. metallidurans* cells by ZAT1p fits to a ΔΨ-driven uniport of Zn^{2+}.

In proteoliposomes as well as in *R. metallidurans*, the rate of zinc transport by ZAT1p did not show substrate saturation in the concentration range where testing was possible. Thus, the K_m value for Zn^{2+} seems to be well above 10 mM. Zinc-transporting P-type ATPases have a K_m value in the micromolar range; however, these enzymes seem to transport Zn^{2+} cations bound to thiol groups, for instance to glutathione (Rensing et al. 1999). The K_{50} value for the zinc transporter CzcA, on the other hand, was 6.6 mM (Goldberg et al. 1999).

When other CDF proteins were expressed in the megaplasmid-free *R. metallidurans* strain AE104, ZRC1p and CzcD led to diminished accumulation of Zn^{2+}, Co^{2+} and Cd^{2+} at various concentrations (Anton et al. 1999). For instance, at 100 µM Zn^{2+}, when ZAT1p increased the zinc content of the cells from 10 µmol/g DW in the control to 15 µmol/g DW (Fig. 3), the other CDF proteins reduced the zinc content under identical conditions down to 3.5 µmol/g DW (Anton et al. 1999).

Deletion of *czcD* from the megaplasmid pMOL30 led to a strain that expressed the high-resistance pump CzcCBA at a 7-fold higher value than the wild-type strain (Anton et al. 1999). The difference between the two strains was visible at a medium concentration of 1 mM Zn^{2+} (data not shown), but not at 100 µM Zn^{2+} (Fig. 4), because the apparent cytoplasmic concentration of zinc remained below the K_{50} threshold value of the CzcCBA pump of 6.6 mM (=20 µmol/g DW; Li et al. 1994). Thus, even in the presence of high-activity efflux systems, ZAT1p functioned as an uptake system for zinc in *R. metallidurans*, while CzcD and ZRC1p acted as zinc-efflux systems. The COT1p protein was also an uptake system, but only at an outside concentration of 1 µM Zn^{2+} (Anton et al. 1999). Therefore, CDF proteins may transport metal cations in both directions. The direction taken depends on the protein, the cation and the concentration of the substrate.

The action of ZAT1p as an uptake system is in agreement with the data obtained with whole plants (van der Zaal et al. 1999). It also explains why ZAT1p was not able to complement the diminished zinc tolerance of an *S. cerevisiae* Δ*ZRC1* or a Δ*ZRC1* Δ*COT1* mutant strain. ZRC1p and COT1p were mainly efflux systems when expressed in *R. metallidurans* (Anton et al. 1999). Both proteins may export divalent cations, e.g. as cation/proton antiporters. ZRC1p transports Zn^{2+} into

the vacuole (Miyabe et al. 2001). Thus, detoxification of the cytoplasm of *S. cerevisiae* by ZRC1p and COT1p may be driven by the proton gradient across the vacuolar membrane of this yeast (Nishimura et al. 1998). In contrast, ZAT1p as a facilitator was not able to import sufficient amounts of Zn^{2+} into the *S. cerevisiae* vacuole to affect zinc resistance in the deletion strains.

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