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CzcD Is a Heavy Metal Ion Transporter Involved in Regulation of Heavy Metal Resistance in *Ralstonia* sp. Strain CH34

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The Czc system of *Ralstonia* sp. strain CH34 mediates resistance to cobalt, zinc, and cadmium through ion efflux catalyzed by the CzcCB$_2$A cation-proton antiporter. The CzcD protein is involved in the regulation of the Czc system. It is a membrane-bound protein with at least four transmembrane α-helices and is a member of a subfamily of the cation diffusion facilitator (CDF) protein family, which occurs in all three domains of life. The deletion of *czcD* in a *Ralstonia* sp. led to partially constitutive expression of the Czc system due to an increased transcription of the structural *czcCBA* genes, both in the absence and presence of inducers. The *czcD* deletion could be fully complemented in *trans* by CzcD and two other CDF proteins from *Saccharomyces cerevisiae*, ZRC1p and COT1p. All three proteins mediated a small but significant resistance to cobalt, zinc, and cadmium in *Ralstonia*, and this resistance was based on a reduced accumulation of the cations. Thus, CzcD appeared to repress the Czc system by an export of the inducing cations.

CzcD from *Ralstonia* sp. strain CH34 (formerly *Alcaligenes eutrophus* [1, 13]) and ZRC1p from *Saccharomyces cerevisiae* (6) were the first two published members of the cation diffusion facilitator (CDF) protein family (17, 23). The members of this family are all predicted to be membrane-bound proteins, mostly with six assumed transmembrane-spanning α-helices. The CDF proteins seem to interact with the divalent cations of zinc, cadmium, and cobalt. The transport of divalent heavy metal cations has been shown for COT1p from *S. cerevisiae* (2), for some mammalian ZnT proteins (21, 22), and for a CDF protein from *Staphylococcus aureus* (28).

On the other hand, CDF proteins were found to be involved in regulatory processes (5, 13): ZRC1p seems to regulate glutathione biosynthesis, and CzcD is involved in the regulation of a zinc, cobalt, and cadmium efflux system, the Czc system, which mediates resistance to these heavy metal cations in *Ralstonia* sp. strain CH34. The Czc system probably transports the toxic heavy metals across both membranes of the gram-negative bacterium (24). This transenvelope efflux is mediated by the CzcCB$_2$A protein complex, a proton-cation antiporter (12). The *czcCBA* genes (16) are located on one of the two megaplasmids of strain CH34, plasmid pMOL30, and are flanked by genes encoding regulators, *czcNI* upstream and *czcDRS* downstream of *czcCBA* (13, 27). Transcription probably starts at four promoters, *czcNp*, *czcIp*, *czcCp*, and *czcDp*, and leads to a variety of transcripts in the *czcNICBA* region and to a tricistronic *czcDRS* message (4). In the current model of Czc system regulation (4), CzcN and CzcI may regulate the activity of a hypothetical extracellular function sigma factor while the two-component regulatory system made up of CzcR (response regulator) and CzcS (sensor histidine kinase) regulates the expression of CzcN.

CzcD is not essential for Czc system regulation but is needed to regulate the expression of a *czcC*::*lacZ* fusion when a constitutively expressed CzcCB$_2$A efflux complex diminishes the cytoplasmic inducer concentration (13). In this publication, the function of CzcD in the Czc regulatory network is defined.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Tris-buffered mineral salts medium containing 2 g of sodium gluconate/liter was used to cultivate *Ralstonia* strains, all derivatives of the wild-type strain CH34(pMOL28, pMOL30) (9). Strain AE128(pMOL30) harbors megaplasmid pMOL30 with the *czc* determinant only, and strain AE104 is a plasmid-free, metal-sensitive derivative of strain CH34 (9). Strain DN175(pMOL30-9) has an insertion of a *lacZ* gene in the *czcCBA* operon between *czcC* and *czcB* (4). The *czcD* gene was cloned into the broad-host-range plasmid pVDZ′2 (3) under the control of the *lac* promoter which is constitutively expressed by *Ralstonia* (16), leading to plasmid pDNA176. Additionally, the *S. cerevisiae* genes *ZRC1* and *COT1* were PCR amplified and cloned into the same vector plasmid, leading to plasmids pDNA178 and pDNA177, respectively. Analytical-grade salts of $CdCl_2 \cdot H_2O$, $ZnCl_2$, and $CoCl_2 \cdot 6H_2O$ were used to prepare 1 M stock solutions, which were sterilized by filtration. Solid Tris-buffered media contained 20 g of agar/liter. Uptake experiments were performed by the filtration technique as described previously (19), but 10 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$ was used to wash the cells on the filters. $^{109}CdCl_2$ (1 Ci/g) and $^{65}ZnCl_2$ (1.84 Ci/g) were from NEN (Brussels, Belgium), and $^{57}CoCl_2$ (4,000 Ci/g) was from Amersham (Braunschweig, Germany).

Genetic techniques. Standard molecular genetic techniques were used (10, 25). For conjugal gene transfer, overnight cultures of donor strain *Escherichia coli* S17/1 (26) and of the *Ralstonia* recipient strains grown at 30°C in complex medium were mixed (1:1) and plated onto nutrient broth agar. After overnight growth, the bacteria were suspended in saline (9 g of NaCl/liter), diluted, and plated onto selective media as previously described (11). The total RNA of the *Ralstonia* organisms was isolated as described previously (20). PCGENE (IntelliGenetics, Mountain View, Calif.) was used as the standard computer program for the analysis of DNA sequences.

Reporter gene fusions. Fusion vectors pECD499 (*lacZ* fusions) and pECD500 (*phoA* fusions) (24) and *E. coli* CC118 were used (8). The specific activities of alkaline phosphatase (8) and β-galactosidase (13) were determined in triplicate as published previously (15). From each mean value, the negative control value (vector control without insert) was subtracted. The result was divided by the highest specific activity, which was 2.12 U/g (dry weight) for PhoA (fusion I-115) and 37.3 U/mg (dry weight) (control of a nonfused *lacZ* gene without leader) for LacZ, leading to the relative activities for each fusion point.

Construction of knockout mutations in *czc* genes (4). To prevent polar effects mediated by the deletion of the *czcD* gene from megaplasmids pMOL30 containing *czcNICBADRS* (9) and pMOL30-9 harboring Φ(*czcNIC-lacZ-czcBADRS*) (4), *czcD* was exchanged for a small open reading frame encoding a polypeptide of 20 amino acids (aa). The first nine aa coded by this small open reading frame were identical with the nine amino-terminal amino acids of CzcD, and the last 9 aa were identical with the last carboxy-terminal amino acids of CzcD. Residues 10 and 11 were E and L, respectively, and were coded by the hexanucleotide recognition sequence CAATTG of the restriction endonuclease *Mun*I. Thus, the 500 bp upstream of *czcD* were amplified by PCR, and this fragment ended with the 27-bp sequence coding for the first 9 aa of the respective

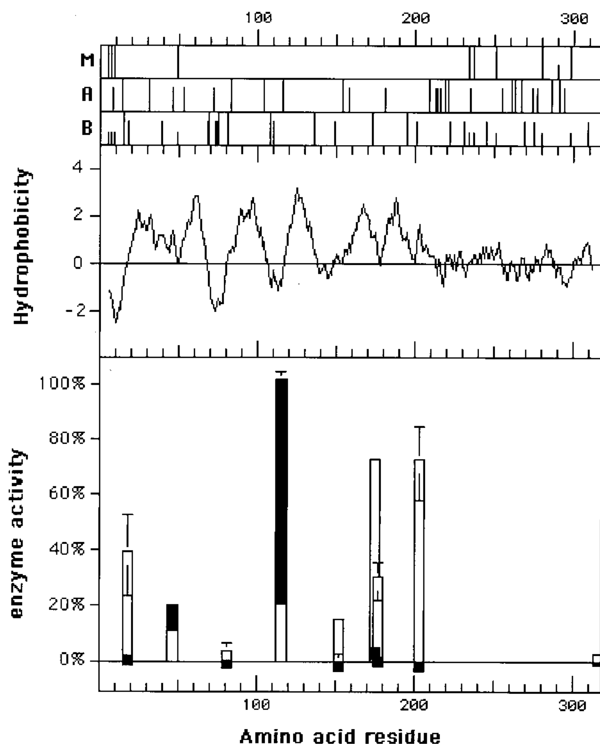

FIG. 1. Topological structure of CzcD. Parts of the *czcD* gene of *Ralstonia* were fused with the *lacZ* or *phoA* topological reporter gene. The *czcD* parts were from the 5′ end of the gene up to the points indicated by the positions of the bars. The relative activities of the reporter enzymes are indicated by overlapping white (LacZ) and black bars (PhoA), with error bars representing standard deviations. Above the hydrophobicity plot are the positions of putative metal-binding amino acid residues (in panel M, residues H [full-size bars] and C [half bars]), acidic amino acid residues (in panel A, residues D [two-thirds bars] and E [full-size bars]), and basic amino acid residues (in panel B, residues H [half bars], R [full-size bars], and K [two-thirds bars]).

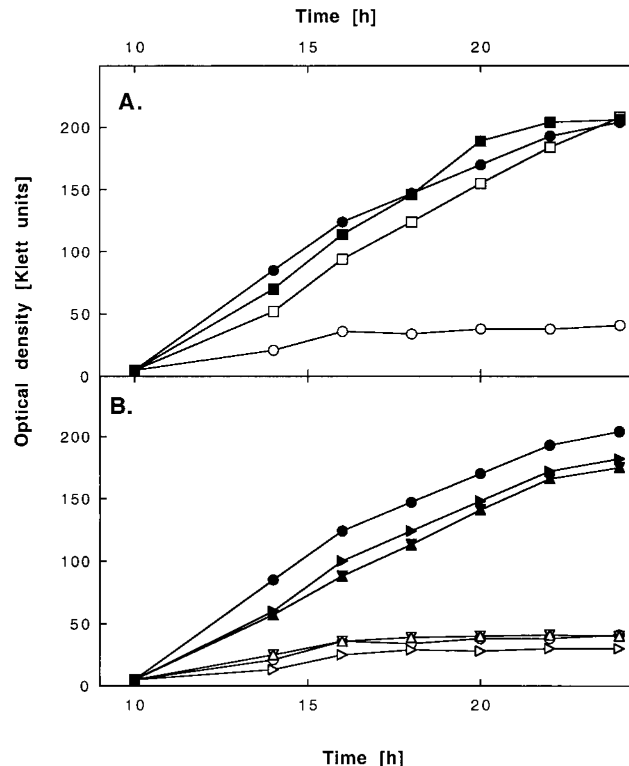

FIG. 2. Effect of Δ*czcD* mutation on growth in presence of zinc. Cells of strain AE128(pMOL30) (● and ○), its Δ*czcD* mutant strain DN182(pMOL30-14) (■ and □), strain DN182 complemented in *trans* with pDNA176 containing *czcD* (▲ and △), pDNA178 containing *ZRC1* (▼ and ▽), and pDNA177 containing *COT1* (▶ and ▷) were cultivated for 48 h at 30°C in Tris-buffered mineral salts medium containing 2 g of sodium gluconate/liter as the carbon source. The cells were diluted in fresh medium containing 300 μM Zn^{2+} as the inducer (closed symbols) or no inducer (open symbols). Incubation was continued for 10 h, and then the cells were diluted to a cell density of up to 10 Klett units in fresh medium containing 2.5 mM Zn^{2+}.

gene product, followed by a *Mun*I hexanucleotide. Secondly, the 500 bp downstream of *czcD* were amplified by PCR, and this fragment started with the *Mun*I recognition sequence and the last 27 bp of the respective gene. Both fragments were fused by *Mun*I restriction and ligation, cloned, verified by DNA sequencing, and finally cloned into pLO2 (7). The resulting plasmid was used for mutating *Ralstonia* organisms as described previously (4), leading to plasmid pMOL30-14 in strain DN182(pMOL30-14) (*czcNICBA* Δ*czcD czcRS*) and plasmid pMOL30-15 in strain DN183(pMOL30-15) [Φ(*czcNIC-lacZ-czcBA*) Δ*czcD czcRS*]. The mutant genotypes were verified by PCR and DNA sequencing.

Competitive RT-PCR (5). DNase-treated total RNA was isolated from cells of *Ralstonia* sp. strains AE128(pMOL30) (9) and DN182(pMOL30-14) (Δ*czcD*) either without induction or after induction for 10 min with 300 μM Zn^{2+}. One microgram of this total RNA was reverse transcribed with 100 U of Superscript II RT (Gibco BRL, Karlsruhe, Germany) and 50 pmol of random primer in a total volume of 20 μl. To determine the amount of *czcCBA* mRNA-specific cDNA for each strain and induction condition, different amounts of an internal DNA standard were added to 0.5 μl of the resulting cDNA solution, and the mixture was amplified with 100 μM concentrations of deoxynucleoside triphosphates, 10 pmol of primers (3′ antisense primer B, ATGCCACCGATTACCA CCGTTGCGA, positions 7144 to 7120 [gbX98451] in *czcA* [positions 4092 to 7283], and 5′ sense primer A, ATTGGTTCATTCGTGCCCG, positions 6615 to 6633 in *czcA*] and 1 U of *Taq* polymerase (Qiagen, Hilden, Germany) in a total volume of 50 μl by the following PCR program: 2.30 min at 94°C, 1 min at 60°C, and 1 min at 72°C as the initial cycle, and a further 28 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with final extension for 5 min at 72°C. A 10-μl amount of each PCR product was analyzed on a 1.5% agarose gel stained with ethidium bromide. The relative amounts of *czcCBA* cDNA (529 bp) and internal DNA standard (237 bp) products were quantified after densitometric analysis with ScanPack 2.0 software (Biometra, Göttingen, Germany). For each lane, which represents cDNA with one of the internal DNA standards, the resulting spot intensities were first normalized for the lengths of the cDNA (529 and 237 bp, respectively, for *czcCBA* cDNA and the internal standard), and then the normalized *czcCBA* cDNA intensity was divided by the normalized density of the internal standard. For all lanes, the logarithm of this quotient was plotted against the logarithm of the amount of the internal standard used in the respective competition experiment. A linear regression was calculated for these points, and this line intercepts the *x* axis exactly at the point where the amount of the internal standard is identical with the amount of the *czcCBA*-specific cDNA in the reverse transcription (RT) probe. This value was used to calculate the *czcCBA*-specific cDNA per microgram of total RNA used.

Control assays were as follows: a complete assay without template, a complete assay with RNA template but without RT, and a complete assay with total RNA isolated from the plasmid-free *Ralstonia* strain AE104. All controls were negative.

For the construction of a specific internal standard, a 529-bp part of *czcA* was amplified with primers A and B. This fragment was purified and used as the template in a PCR experiment with a loop-out primer (ATTGGTTCATTCGT GCCCG**GGCGGTGCTCAATGGTCTG**) and primer B. The loop-out primer was

TABLE 1. Induction of *czcCBA*::*lacZ* in a Δ*czcD* mutant strain[a]

Inducer[b]	β-Galactosidase activity (mean ± SD) in bacterial strain	
	DN175(pMOL30-9)	DN183(pMOL30-15) (Δ*czcD*)
None	120 ± 34	207 ± 36
10 μM Zn^{2+}	256 ± 7	461 ± 42
300 μM Co^{2+}	206 ± 15	293 ± 12
100 μM Cd^{2+}	192 ± 32	341 ± 43

[a] Means for two independent experiments for the determinations of the β-galactosidase activity are given in units per milligram (dry weight).

[b] The cells were induced for 2 h with the respective heavy-metal cation.

TABLE 2. Concentration of *czcCBA* mRNA-originating cDNA depends on the presence of CzcD[a]

Bacterial strain	Genotype	cDNA concn (ng/g of RNA) in:		Fold induction[c]
		Uninduced cells	Induced cells[b]	
AE128(pMOL30)[d]	Wild type	6, 16	762, 846	74
DN182(pMOL30-14)	Δ*czcD*	106, 268	4,950, 10,150	42

[a] Two independent determinations are given.
[b] The cells were induced for 10 min with 300 μM Zn^{2+} before the RNA was isolated.
[c] Determined as the concentration in induced cells/concentration in uninduced cells.
[d] Data from reference 4.

identical in the first 19 nucleotides (underlined) to primer A, and the remaining 19 nucleotides (bold) were the base pairs in positions 6926 to 6944 (gbX98451) of *czcA*, which are located between the positions of primers A and B. Thus, a 237-bp PCR product which could be clearly differentiated from the 529-bp *czcCBA* cDNA product was amplified, but it had the same ends as the 529-bp fragment. The 237-bp PCR product was isolated, cleaned with QIAquick, quantified (GeneQuant; Pharmacia, Uppsala, Sweden), diluted, and used for competitive RT-PCR.

RESULTS

Topological analysis of CzcD. The structure of the CzcD protein from *Ralstonia* was investigated by using translational *lacZ* and *phoA* fusions. Starting with the 5′ end, variously sized parts of the respective *czcD* gene were amplified and cloned into the fusion vectors pECD499 and pECD500 (24).

The amino terminus of CzcD is followed by four hydrophobic peaks (Fig. 1). Fusions between those peaks gave evidence for an alternation between periplasmic and cytoplasmic localizations of the fusion points (Fig. 1). Thus, the amino terminus of CzcD is followed by four transmembrane α-helices, I, II, III, and IV. The activity of the fusions plus the pattern of positively charged amino acid residues indicate a cytoplasmic localization of the N terminus and of the regions between helices II and III and downstream of helix IV. After these four spans (fusion position Y152), two more hydrophobic peaks occur. Although the fusions at position S203 after both peaks strongly indicate a cytoplasmic localization, two independent fusions between both peaks (T175 and W177) gave no evidence for a periplasmic localization (Fig. 1). LacZ and PhoA fusions at the carboxy terminus of CzcD had a very low specific activity. However, the absence of hydrophobic peaks downstream of S203 and the high LacZ activity of the S203 fusion could mean a cytoplasmic localization of the C terminus of CzcD.

Thus, both termini of CzcD are probably in the cytoplasm and the amino terminus is clearly followed by four transmembrane α-helices. The existence of the two subsequent transmembrane spans could not be proven; this may be the result of a limitation of the method used, or spans V and VI may be reversibly integrated into the membrane as part of the catalytic cycle or a regulatory event. However, CzcD should be an integral protein of the cytoplasmic membrane, with at least four and a maximum of six transmembrane α-helices.

A Δ*czcD* mutant is impaired in metal sensing. Mutants with in-frame Δ*czcD* deletions were constructed from *Ralstonia* strains AE128(pMOL30) (*czcNICBADRS*) (9) and DN175 (pMOL30-9) [Φ(*czcNIC-lacZ-czcBADRS*)] (4), leading to strains DN182(pMOL30-14) (*czcNICBA* Δ*czcD czcRS*) and DN183(pMOL30-15) [Φ(*czcNIC-lacZ-czcBA*) Δ*czcD czcRS*], respectively. As judged from the MICs, the resistances of the Δ*czcD* strains DN182 and DN183 to zinc were slightly lower than those of their isogenic $CzcD^+$ strains, AE128 and DN175, respectively. In the presence of 7 mM Zn^{2+}, both Δ*czcD* deletion strains produced only a few single colonies on solid medium while the wild-type strains displayed full growth (data not shown). There was no effect on the MICs of cobalt and cadmium and no significant difference in the induction of the β-galactosidase reporter gene at most metal cation concentrations when strains DN175 and DN183 were compared (data not shown). However, when the β-galactosidase activities of the Δ*czcD* strain and the wild-type strain were compared after induction by 300 μM Co^{2+}, 100 μM Cd^{2+}, or 10 μM Zn^{2+}, the Δ*czcD* strain reached a higher expression level after 2 h (Table

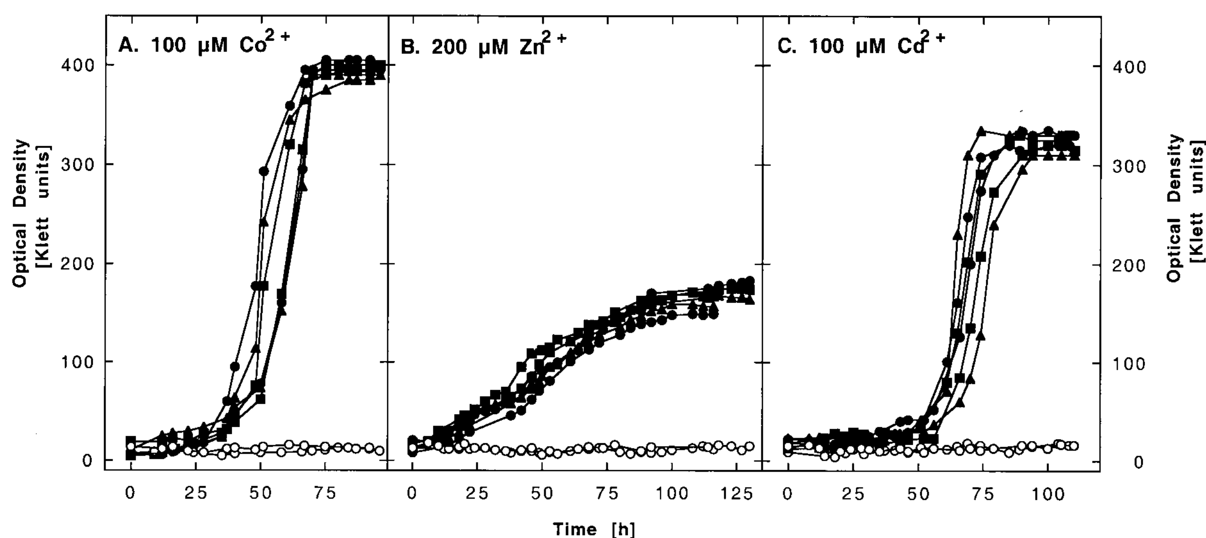

FIG. 3. CDF proteins mediate metal ion resistance. *Ralstonia* strain AE104 containing plasmids pVDZ′2 without an insert (○), pDNA176 with *czcD* (●), pDNA177 with *COT1* (▲), or pDNA178 with *ZRC1* (■) was cultivated in the presence of 100 μM Co^{2+} (A), 200 μM Zn^{2+} (B), or 100 μM Cd^{2+} (C), and the optical density over time was monitored. For each strain and metal ion, the results of two independent experiments are shown.

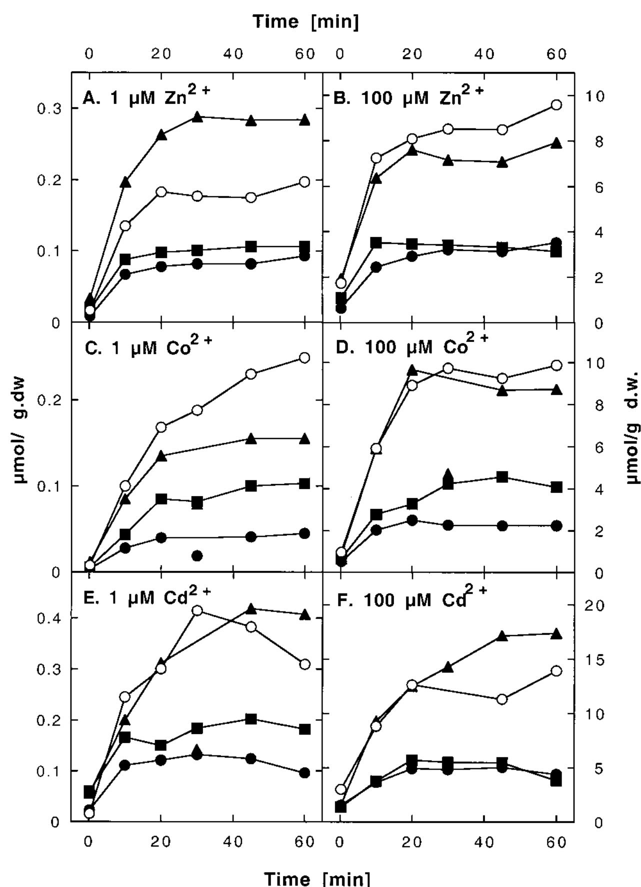

FIG. 4. Accumulation of heavy metal ions by *Ralstonia* strains expressing various CDF genes. *Ralstonia* strain AE104 containing plasmids pVDZ′2 without an insert (○), pDNA176 with *czcD* (●), pDNA177 with *COT1* (▲), or pDNA178 with *ZRC1* (■) was cultivated in Tris-buffered mineral salts medium containing 2 g of sodium gluconate/liter. The cells were harvested, washed, and suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 2 g of sodium gluconate/liter. Radioactive metal isotopes at 1 or 100 μM were added, and incubation was continued with shaking at 30°C. Samples of 200 μl were removed, filtered (pore diameter, 0.45 μm), and washed twice on the filter with 2 ml of 10 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$. Radioactivity was determined with a scintillation counter (Beckman, Munich, Germany), and the dry weight (dw) was determined from the optical density with a calibration curve.

1). In addition, the β-galactosidase activity in uninduced cells of the Δ*czcD* strain was twice as high as the activity in uninduced wild-type cells (Table 1).

When strains AE128(pMOL30) and DN182(pMOL30-14) (Δ*czcD*) were precultivated in the absence of Zn^{2+} or in 300 μM Zn^{2+} and then transferred to a liquid medium containing 2.5 mM Zn^{2+}, the Δ*czcD* deletion strain started to grow immediately in both cases but the wild-type strain grew only without a lag phase when it was preadapted in the presence of 300 μM Zn^{2+} (Fig. 2A). To examine this effect more closely, the concentration of *czcCBA* mRNA in both strains was judged by the amount of respective cDNA measured by competitive RT-PCR (Table 2). In both the deletion and wild-type strains, *czcCBA* was inducible, but the mRNA level in the deletion strain was 10-fold higher than that in the wild-type strain under noninduced and induced conditions (Table 2). Thus, CzcD represses *czc* induction either by inducer exclusion or by some kind of protein-protein interaction.

By comparing the mRNA data and the growth curves, it was found that a 15-fold overexpression of *czcCBA* in the Δ*czcD* deletion strain was sufficient for the cells to start growing immediately in the presence of 2.5 mM Zn^{2+}. However, differences in the β-galactosidase activity were visible only in the presence of 10 μM Zn^{2+}. This difference cannot be explained at the moment.

CzcD mediates a low-level metal ion resistance, probably by efflux. To determine if CzcD transports cations, the *czcD* gene was cloned into plasmid pVDZ′2 (3), leading to plasmid pDNA176. This plasmid was transferred to plasmid-free *Ralstonia* strain AE104. At most metal ion concentrations, no difference in metal ion resistance between AE104(pDNA176) and the negative control strain AE104(pVDZ′2) (data not shown) was observed. However, with 100 μM cobalt, 100 μM cadmium, or 200 μM zinc, the control strain AE104(pVDZ′2) was not able to grow, in contrast to AE104(pDNA176) (Fig. 3). Thus, the expression of CzcD mediates a small degree of metal ion resistance which is below the resistance level obtained when the protein CzcA is expressed alone (24). With the protection of CzcA, it took the cells 20 h to grow up to 300 Klett units in the presence of 200 μM Zn^{2+} (24); with CzcD, it took 125 h for the cells to reach 200 Klett units (Fig. 3). In both cases, the negative control strain did not grow.

Resistance mediated by a membrane-integrated histidine-rich protein may be based on the binding of the metal by the histidine residues and/or metal ion efflux. Binding should increase the amount of metal ions in resistant cells compared to that in sensitive ones, while efflux should decrease the amount of cell-bound metal ions. When levels of cell-bound metal ions in cells with and without overexpressed CzcD were compared, resistant cells contained only 20 to 50% of the cobalt, zinc, or cadmium that the respective control cells contained (Fig. 4). There was no difference in the initial velocity of zinc or cadmium entry between cells of both strains (Fig. 5A and C). The uptake of cobalt in the first minutes was slow, with strong fluctuations between different experiments (Fig. 5B). Thus, CzcD mediates a reduced accumulation of Zn^{2+}, Co^{2+}, and Cd^{2+}. Since the initial velocities of metal cation uptake with and without CzcD were identical, this reduced accumulation is probably based on the efflux of metal cations, at least in the cases of zinc and cadmium. Since CzcD is located in the cytoplasmic membrane, indicated by the fusion data, this efflux is across the cytoplasmic membrane.

ZRC1p and COT1p mediate a low-level metal ion resistance. The genes of the two CDF proteins from yeast were cloned into plasmid pVDZ′2, leading to plasmids pDNA178 (containing *ZRC1*) and pDNA177 (containing *COT1*). Both plasmids mediated a degree of metal ion resistance comparable to that mediated by pDNA176 containing *czcD* (Fig. 3).

ZRC1p expression led to effects similar to CzcD expression concerning zinc and cadmium (Fig. 4 and 5), but ZRC1p was not as efficient as CzcD concerning the reduced accumulation of cobalt. If CzcD constitutes an efflux system for zinc, cobalt, and cadmium, then ZRC1p should function in the same manner. Although COT1p provided the same degree of metal resistance as ZRC1p and CzcD, the effect obtained by COT1p expression varied with the metal ion and its concentration (Fig. 4). With 100 μM Zn^{2+} or 1 μM Co^{2+}, COT1p mediated a reduced accumulation, and it is probably an efflux system. In contrast, with 1 μM Zn^{2+}, COT1p-containing cells accumulated more zinc than control cells, so COT1p should enhance the uptake and/or binding of zinc (Fig. 4A). Moreover, the uptake of 100 μM Zn^{2+} was faster in COT1p-containing cells than in CzcD- or ZRC1p-containing cells or control cells (Fig. 5A). Thus, the yeast CDF proteins work similarly to CzcD; however, the transport of cations across the cytoplasmic mem-

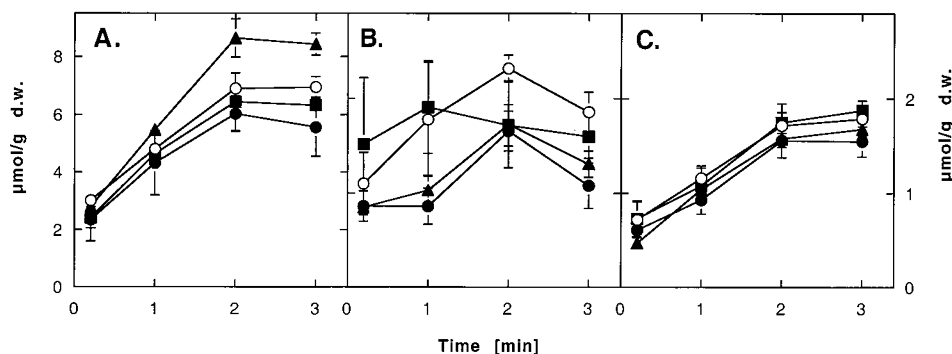

FIG. 5. Fast uptake of heavy metal ions by *Ralstonia* strains expressing various CDF genes. *Ralstonia* strain AE104 containing plasmids pVDZ′2 without an insert (○), pDNA176 with *czcD* (●), pDNA177 with *COT1* (▲), or pDNA178 with *ZRC1* (■) was cultivated in Tris-buffered mineral salts medium containing 2 g of sodium gluconate/liter. The cells were harvested, washed, and suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 2 g of sodium gluconate/liter. The radioactive metal isotopes $^{65}Zn^{2+}$ (A), $^{57}Co^{2+}$ (B), and $^{109}Cd^{2+}$ (C) at 100 μM were added, and metal uptake was measured as described for Fig. 4. d.w., dry weight.

brane may be in both directions, depending on the concentration.

Expression of ZRC1p and COT1p complements a *czcD* mutation. To analyze the complementation of the Δ*czcD* mutation by *czcD* in *trans*, plasmid pDNA176 was transferred into DN182. The resulting transconjugant strain showed a lag phase of growth in the presence of 2.5 mM Zn^{2+} when the cells were not adapted by precultivation in 300 μM Zn^{2+} (Fig. 2B). Similarly to pDNA176, the *ZRC1*- and *COT1*-containing plasmids pDNA178 and pDNA177 were transferred into DN182 (pMOL30-14) (Δ*czcD*). Both plasmids complemented the Δ*czcD* mutation as well as the *czcD*-containing plasmid (Fig. 2B). Thus, the two yeast CDF proteins were fully functional as participants of the Czc regulatory network.

DISCUSSION

In *Ralstonia* spp., CzcD and the two yeast CDF proteins ZRC1p and COT1p catalyze a reduced accumulation of heavy-metal ions, which is probably based on metal cation efflux. Since CzcD is located in the cytoplasmic membrane, this means there is an export of heavy-metal cations from the cytoplasm into the periplasm.

COT1p, which does not function well as an efflux system with 100 μM Co^{2+} in *Ralstonia*, nevertheless gives the same degree of resistance to 100 μM Co^{2+} as ZRC1p and CzcD do; therefore, the large histidine-rich domains of the protein might detoxify cobalt cations by binding them. The CDF proteins might function as a kind of heavy-metal buffer for the cell by performing the following: importing heavy metals when the magnesium transport system (18) is too slow to supply sufficient amounts of the trace elements cobalt or zinc (e.g., due to competitive inhibition by high magnesium concentrations), binding the heavy-metal cations, and exporting the heavy-metal cations when the cytoplasmic concentration becomes too high. Only when the capacity of this system is exhausted by too low or too high heavy-metal concentrations may additional energy sources have to be tapped to drive increased accumulation (e.g., by P-type or ATP-binding cassette uptake ATPases) or increased efflux (e.g., by P-type export ATPases or the Czc proton-cation antiporter).

CzcD not only protects the cell against toxic heavy metals, albeit at a lower level than $CzcCB_2A$ does, but also is involved in the regulation of expression of the $CzcCB_2A$ efflux system. Deletion of the *czcD* gene results in a higher *czcCBA* mRNA level in uninduced and induced cells, which is sufficient to produce enough $CzcCB_2A$ efflux complex for an initial protection against 2.5 mM Zn^{2+}. As shown by primer extension and RT-PCR, the *czcNICBA* region is transcribed from three promoters, *czcNp*, *czcIp*, and *czcCp* (4). The two-component regulatory system CzcRS regulates only *czcNp* (4). CzcS, the sensor protein, may sense only cytoplasmic cations (14). Thus, since no other Czc protein is located in the cytoplasm except for CzcR, the presence of heavy metals in the cytoplasm should lead to transcription initiation from *czcNp* with a signal chain via CzcS and CzcR. Because deletion of the genes *czcR* or *czcS* does not abolish Czc system induction (4), other metal-sensing components are involved in Czc system regulation, and these may be located in the periplasm (e.g., CzcI) or the cytoplasmic membrane (e.g., CzcN).

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