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# Contributions of Five Secondary Metal Uptake Systems to Metal Homeostasis of *Cupriavidus metallidurans* CH34▽†

Andreas Kirsten,[1] Martin Herzberg,[1] Alexander Voigt,[1] Javier Seravalli,[2] Gregor Grass,[3] Judith Scherer,[1] and Dietrich H. Nies[1]*

*Molecular Microbiology, Institute for Biology/Microbiology, Martin Luther University, Halle-Wittenberg, Germany*[1]*; Department of Biochemistry, University of Nebraska—Lincoln, Lincoln, Nebraska*[2]*; and Bundeswehr Institute of Microbiology, Munich, Germany*[3]

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***Cupriavidus metallidurans* is adapted to high concentrations of transition metal cations and is a model system for studying metal homeostasis in difficult environments. The elemental composition of *C. metallidurans* cells cultivated under various conditions was determined, revealing the ability of the bacterium to shield homeostasis of one essential metal from the toxic action of another. The contribution of metal uptake systems to this ability was studied. *C. metallidurans* contains three CorA members of the metal inorganic transport (MIT) protein family of putative magnesium uptake systems, ZupT of the ZRT/IRT protein, or ZIP, family, and PitA, which imports metal phosphate complexes. Expression of the genes for all these transporters was regulated by zinc availability, as shown by reporter gene fusions. While expression of *zupT* was upregulated under conditions of zinc starvation, expression of the other genes was downregulated at high zinc concentrations. Only $corA_1$ expression was influenced by magnesium starvation. Deletion mutants were constructed to characterize the contribution of each system to transition metal import. This identified ZupT as the main zinc uptake system under conditions of low zinc availability, $CorA_1$ as the main secondary magnesium uptake system, and $CorA_2$ and $CorA_3$ as backup systems for metal cation import. PitA may function as a cation-phosphate uptake system, the main supplier of divalent metal cations and phosphate in phosphate-rich environments. Thus, metal homeostasis in *C. metallidurans* is achieved by highly redundant metal uptake systems, which have only minimal cation selectivity and are in combination with efflux systems that "worry later" about surplus cations.**

Sophisticated cellular biochemistry needs metals as cofactors. About 40% of all enzymes have them, ranking from Mg (16%) > Zn (9%) > Fe (8%) > Mn (6%) > Ca (2%) > Co and Cu (1%) down to K, Na, Ni, V, Mo, W, and only one example of Cd (59). It is an interesting question how the correct metal is allocated to the right protein, a challenge especially for the divalent metal cations $Mg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$. These metals compete with each other for the metal binding sites in enzymes (16). Additionally, $Fe^{2+/3+}$ and $Cu^{+/2+}$ promote dangerous reactive oxygen species in Fenton and Fenton-like reactions, as described by Haber and Weiss (13).

Part of the solution to this problem might be to keep the metal cation bouquet in any cellular compartment in a way that minimizes competition for metal binding sites and the Fenton reaction. This leads to the question of how the cellular metal cation bouquet can be maintained in environments that may contain a single metal in a concentration range from pM to mM. The betaproteobacterium *Cupriavidus metallidurans* strain CH34 is able to keep its metal homeostasis under a variety of such adverse conditions (19, 28, 30). The organism can be found in many mesophilic metal-contaminated environments around the globe, such as zinc deserts of Belgium (8). Key to this adaptive ability is an impressive array of metal efflux systems (highlighted for zinc in Table 1) (57).

Because the cytoplasmic concentration of each metal may be based on a kinetic flow equilibrium of uptake and efflux reactions (see Fig. S1 in the supplemental material), real understanding of multiple-metal homeostasis also requires knowledge of the uptake systems. However, little is known about the import of these cations into *C. metallidurans*. The divalent metal cations of zinc, cadmium, cobalt, nickel, and manganese are transported into cells of the plasmid-free *C. metallidurans* derivative AE104 by energy-dependent magnesium transport systems with $K_m$ values in the mid-μM range (41). Careful analysis of the uptake data (25) revealed the presence of at least two uptake systems for transition metal cations. A slow (low maximum rate of transport [$V_{max}$]) system functions mainly at low (≤100 μM) cation concentrations and a faster one at concentrations above this value. The slow uptake system displayed kinetic parameters similar to those of the previously published magnesium transport system(s). However, the molecular identities of all these uptake systems remain unknown, although the bacterium contains a unique repertoire of secondary transport systems that might be responsible for the observed transport processes (Table 1). Secondary transport systems are driven by a gradient across a biological membrane, such as the proton motive force, while primary systems form gradients by using other forms of chemical energy (e.g., ATP or PEP), redox differences, or light (49).

*C. metallidurans* does not contain a high-affinity $Zn^{2+}$ uptake system like the ABC (TC 3.A.1) uptake system ZnuABC from *Escherichia coli* (44) but an ortholog of ZupT (23) of the

* Corresponding author. Mailing address: Kurt-Mothes-Str. 3, 06120 Halle/Saale, Germany. Phone: 49/0-345-5526352. Fax: 49/0-345-5527010. E-mail: d.nies@mikrobiologie.uni-halle.de.

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TABLE 1. Inventory of possible and absent zinc transport systems in *C. metallidurans* strain CH34

| Name | Rmet no.[a] | Transporter family[b] | Possible function[c] | Reference(s) |
|---|---|---|---|---|
| Uptake systems | | | | |
| $CorA_1$ | Rmet_3052 | MIT, TC 1.A.35 | Importer for divalent metal cations; $Mg^{2+}$ regulation | |
| $CorA_2$ | Rmet_0036 | MIT, TC 1.A.35 | Importer for divalent metal cations; no $Mg^{2+}$ regulation | |
| $CorA_3$ | Rmet_3287 | MIT, TC 1.A.35 | Importer for divalent metal cations; no $Mg^{2+}$ regulation | |
| ZupT | Rmet_2621 | ZIP, TC 2.A.5 | Uptake of $Zn^{2+}$ and other cations under conditions of low zinc concentrations | |
| HoxN | Rmet_1533 | NiCoT, TC 2.A.52 | $Ni^{2+}$, $Co^{2+}$, synthesis of hydrogenases. | |
| PitA | Rmet_1973 | PiT, TC 2.A.20 | Phosphate-metal cation complexes: $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cd^{2+}$ | |
| MgtE | Absent | MgtE, TC 9.A.19 | Possible substrates $Mg^{2+}$, $Co^{2+}$ | |
| MntH | Absent | NRAMP, TC 2.A.55 | Possible substrates $Mn^{2+}$, $Fe^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Ni^{2+}$ | |
| ZnuABC | Absent | ABC, TC 3.A.1 | High-affinity uptake of zinc under conditions of zinc starvation | |
| MgtA | ? | P type, TC 3.A.3 | Primary uptake system for $Mg^{2+}$ and other divalent cations | |
| Efflux systems | | | | |
| CzcA | Rmet_5980 | RND, TC 2.A.6 | Together with CzcB and CzcC, outer membrane efflux of $Zn^{2+}$; other substrates $Cd^{2+}$ and $Co^{2+}$ | 10, 39, 51 |
| CzcP | Rmet_5970[d] | P-type, TC#3.A.3 | High $V_{max}$ efflux of loosely bound $Zn^{2+}$; other substrates $Cd^{2+}$ and $Co^{2+}$ | 51 |
| ZntA | Rmet_4594 | P type, TC 3.A.3 | Efflux of tightly bound $Zn^{2+}$; other substrate $Cd^{2+}$ | 24, 51 |
| PbrA | Rmet_5947 | P type, TC 3.A.3 | Efflux of tightly bound $Pb^{2+}$; may use $Zn^{2+}$ and $Cd^{2+}$ as substrates | 7, 51 |
| CadA | Rmet_2303[d] | P type, TC 3.A.3 | Efflux of tightly bound $Cd^{2+}$; may use $Zn^{2+}$ as substrate | 24, 51 |
| CzcD | Rmet_5979 | CDF, TC 2.A.4 | Efflux of loosely bound $Zn^{2+}$, other substrates $Cd^{2+}$ and $Co^{2+}$ | (2, 3, 36, 51) |
| DmeF | Rmet_0198 | CDF, TC 2.A.4 | Efflux of $Co^{2+}$; $Zn^{2+}$ not substrate in vivo | 32, 51 |
| FieF | Rmet_3406 | CDF, TC#2.A.4 | Efflux of $Fe^{2+}$; $Zn^{2+}$ not substrate in vivo | 32, 51 |
| Unclear | | | | |
| ZntB | Rmet_0549 | MIT, TC 1.A.35 | Efflux or uptake of $Zn^{2+}$? | |

[a] References 57 and 58; http://genome.ornl.gov/microbial/rmet/.
[b] http://www.tcdb.org/.
[c] Possible substrates were predicted according to the situations in *E. coli* (38) and other bacteria (35).
[d] Probably starts upstream of the annotated position (51).

ZIP protein family (TC 1.A.35). Reminiscent of the situation with a multitude of paralogous efflux systems (57), *C. metallidurans* contains 4 members of the CorA or metal inorganic transport (MIT) (TC 1.A.35) protein family. The three $CorA_1$ to $CorA_3$ proteins exhibit a conserved "GMNFXXMPEL" sequence motif, while only the first "GMN" and the proline residue are conserved in the remaining MIT protein, ZntB. While CorA from *Salmonella* is not involved in zinc import (54), ZntB could be a zinc efflux protein (62). The structure of ZntB is different from that of CorA, and ZntB contains three zinc-binding sites, two of them probably required for zinc transport (60).

An NRAMP (TC 2.A.55) protein similar to *E. coli* MntH, which imports $Mn^{2+}$ but also $Fe^{2+}$ and the toxic $Cd^{2+}$, is missing in *C. metallidurans* (Table 1). Since a SitABC-type uptake system for manganese/iron and a housekeeping manganese-dependent superoxide dismutase are also absent, strain CH34 may have little use for manganese. Alternatively, zinc and other metals could be imported by PitA, which may transport phosphate-metal cation complexes at high transition metal concentrations, as in *E. coli* (6). Thus, PitA could be responsible for the observed fast uptake of metals at high ($>100$ μM) concentration (25). Finally, HoxN could be a slow nickel uptake system required to provide the metal for hydrogenase biosynthesis (9). However, the HoxN from *C. metallidurans* is not an ortholog of the HoxN from *Cupriavidus eutrophus* because it belongs to a different subfamily of NiCoT proteins (57).

To understand the contribution of uptake systems to the adjustment of the cellular metal bouquet in *C. metallidurans*, first, the metal bouquet itself was quantified, and how *C. metallidurans* maintains it under various conditions was investigated. Interestingly, the bacterium is able to shield homeostasis of all other metals when one metal is present at high concentrations, and it is able to handle high concentrations of many metals in parallel. The question was how this was achieved. The efflux systems of *C. metallidurans* each may be assigned to a central substrate cation (51), e.g., DmeF to cobalt, CadA to cadmium, ZntA to zinc, PbrA to lead, FieF to iron, and CnrT to nickel (see Fig. S1 in the supplemental material). How do the uptake systems fit into this picture? Do a specific uptake system and a specific efflux system, both controlled by their central substrate, line up to form a "shunt" (see Fig. S1 in the supplemental material), such as ZnuABC and ZntA in *E. coli* (43), or do the uptake systems import more or less what they can get and leave it to the efflux systems to "worry later" about any surplus cation?

To keep our approach manageable, here, we ignored all primary uptake systems, the putative nickel importer HoxN, and the possible efflux system ZntB. Moreover, to circumvent interference with the metal resistance determinants on the native plasmids pMOL28 and pMOL30 of *C. metallidurans*,

mutants in metal uptake systems were constructed in the megaplasmid-free strain AE104, which was also used in the first physiological characterization of metal import (41). The studied systems ZupT, PitA, and $CorA_1$, $CorA_2$, and $CorA_3$ were all controlled by the environmental zinc concentration (and magnesium in the case of $CorA_1$), and functions in metal homeostasis could be assigned to these importers.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains used for experiments were *E. coli* strain W3110 (5), *C. metallidurans* strain CH34(pMOL28, pMOL30) wild type, and derivatives of the megaplasmid-free strain AE104 (29). Tris-buffered mineral salts medium (29) containing 2 g sodium gluconate/liter (TMM) was used to cultivate these strains aerobically with shaking at 30°C. Analytical-grade salts of heavy metal chlorides were used to prepare 1 M stock solutions, which were sterilized by filtration. Solid Tris-buffered media contained 20 g agar/liter (Difco). If not otherwise indicated, TMM contained trace element solution SL6 (45), which added the following final concentrations to the TMM: 35 nM $ZnCl_2$, 15 nM $MnCl_2$, 84 nM $CoCl_2$, 5.9 nM $CuCl_2$, 8.4 nM $NiCl_2$, 12 nM $Na_2MoO_4$, and 485 nM $H_3BO_3$.

**Genetic techniques.** Standard molecular genetic techniques were used (34, 50). For conjugative gene transfer, overnight cultures of the donor strain *E. coli* S17/1 (53) and of the *C. metallidurans* recipient strains grown at 30°C in Tris-buffered medium were mixed (1:1) and plated onto nutrient broth agar. After 2 days, the bacteria were suspended in TMM, diluted, and plated onto selective media as previously described (34).

All primer pairs used are listed in Table S1 in the supplemental material. Plasmid pECD1003 was used to construct deletion mutants. It is a derivate of plasmid pCM184 (27). These plasmids harbor a kanamycin resistance cassette flanked by *loxP* recognition sites. Plasmid pECD1003 additionally carries alterations of 5 bp at each *loxP* site. Using these mutant *lox* sequences, multiple gene deletions within the same genome are possible without interference by secondary recombination events (1, 55).

**Deletion mutants.** Fragments of 300 bp upstream and downstream of the target gene were amplified by PCR, cloned into the vector pGEM-T Easy (Promega), sequenced, and further cloned into the plasmid pECD889 or pECD1003. The resulting plasmids were used in a double-crossover recombination in *C. metallidurans* strains to replace the respective target gene by the kanamycin resistance cassette, which was subsequently also deleted by transient introduction of the *cre* expression plasmid pCM157 (27). Cre recombinase is a site-specific recombinase from the phage P1 that catalyzes the *in vivo* excision of the kanamycin resistance cassette at the *loxP* recognition sites. The correct deletions of the respective transporter genes were verified by Southern DNA-DNA hybridization. For construction of multiple-deletion strains, these steps were repeated. The resulting mutants carried a small open reading frame instead of the wild-type gene to prevent polar effects.

**Gene insertions.** Although the $corA_1$ gene could be interrupted by a single-crossover experiment as described above, recombinant strains containing a $corA_1$ deletion as a consequence of a double-crossover event could never be isolated at this stage. Therefore, the $corA_1$ gene had to be inactivated by insertional mutagenesis. Its central part was amplified by PCR from total DNA of strain AE104, cloned as a PstI/XbaI fragment into the plasmid pGEM-T easy (Promega), verified by DNA sequence analysis, and cloned into plasmid pECD794 (pLO2-*lacZ*) (26). For reporter operon fusions, *lacZ* was inserted downstream of several targets. This was done without interrupting any open reading frame downstream of the target genes to prevent polar effects. The 300- to 400-bp 3′ ends of the respective target genes were amplified by PCR from total DNA of strain AE104, and the resulting fragments were cloned into plasmid pECD794 (pLO2-*lacZ*). The respective operon fusion cassettes were inserted into the open reading frame of the target gene by single-crossover recombination.

**Induction experiments.** *C. metallidurans* cells with a *lacZ* reporter gene fusion were cultivated in TMM with shaking at 30°C. At a cell density of 60 to 70 Klett units, heavy metal salts were added to various final concentrations and the cells were incubated with shaking for an additional 3 h or 16 h. The specific β-galactosidase activity was determined in permeabilized cells as published previously, with 1 U defined as the activity forming 1 nmol of *o*-nitrophenol per min at 30°C (36).

**Transport assays.** $^{65}Zn^{2+}$ uptake experiments using the filtration method were performed as described previously (42) with some modifications. Cation uptake was started by the addition of the radioactive $^{65}Zn^{2+}$ (NEN; 80 GBq/g). Samples (300 μl) were filtered through membrane filters (pore size, 0.45 μm; Schleicher and Schuell) and rinsed with 10 ml 10 mM Tris-HCl (pH 7.0) buffer containing 10 mM $MgCl_2$. The radioactivity remaining on the membrane filter was determined with a scintillation counter (LS6500; Beckman).

**ICP-MS analysis.** To determine the metal content, cells were cultivated in TMM to the end of the exponential phase of growth, and 1.5 ml cell suspension was centrifuged for 5 min at 4,500 × *g*. The supernatant was discarded, and the residual liquid was carefully removed with a sterile cotton swab. Additional washing of the cells did not improve the reproducibility of the method. The pellet was suspended in 20 μl concentrated 70% (wt/vol) $HNO_3$ (trace metal grade; Mallinckrodt) and mineralized at 70°C for 3 h. Samples were diluted to a final concentration of 5% (wt/vol) nitric acid. Gallium [as $Ga(NO_3)_3$] was added as an internal standard at a final concentration of 50 ppb. Elemental analysis was performed via inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent ICP-MS 7500cx instrument operating with a collision cell and flow rates of 3.5 ml $min^{-1}$ of $H_2$ and 1.5 ml $min^{-1}$ of He, with an Ar carrier flow rate of 0.95 liter $min^{-1}$ and an Ar makeup flow rate of 0.15 liter $min^{-1}$. Data acquisition for each sample was accumulated in triplicate for 100 milliseconds. An external calibration curve was recorded with gallium in 5% (wt/vol) nitric acid. Samples were loaded onto 96-well plates prior to analysis, and an autosampler (Elemental Scientific Inc.) was used to inject samples via a 6-port valve at a flow of 70 μl $min^{-1}$. The results were transformed from ppm, ppb, or ppt via molar units into atoms per sample and divided by the number of cells per sample, which had been determined before as CFU.

## RESULTS

**Elemental composition of *C. metallidurans*.** To understand the adjustment of the metal bouquet in *C. metallidurans*, the elemental composition of *C. metallidurans* CH34 wild-type cells was determined and compared to that of the megaplasmid-free strain AE104 and that of *E. coli* W3110 as a "reference bacterium" (Table 2). Cells of strain CH34 contained the divalent metal cations in the order Mg ≫ Ca > Fe ≫ Zn > Cu > Ni ≫ Co ≫ Mn > Cd, indicating that iron and zinc are the most important transition metals (Table 2). Cadmium may have originated from contamination of the water or chemicals used. *E. coli* contained half as much iron but twice as much calcium and copper as *C. metallidurans* CH34. The biggest difference between the bacteria, however, was a nearly 12-fold-higher content of manganese in *E. coli* than in strain CH34, matching the absence of an NRAMP manganese uptake system and manganese-dependent superoxide dismutase (Table 1) in CH34.

Megaplasmid-free *C. metallidurans* strain AE104 (29) accumulated half as much nickel and copper but twice as much cobalt as strain CH34; the latter value, however, had a large deviation (Table 2). Deletion of the genes for the P-type ATPases ZntA and CadA from the chromosome of strain AE104 did not change most metal contents under these conditions. Only the cobalt content decreased to the CH34 wild-type level, and the cadmium content was slightly elevated (Table 2). Thus, deletion of the two efflux P-type ATPases ZntA and CadA or of the metal resistance plasmids pMOL28 and pMOL30 did not disturb metal homeostasis at ambient metal concentrations.

**Accumulation of metals under conditions of metal stress.** In the second step, the consequences of increasing concentrations of a metal for the cellular contents of the same metal and of other metals was examined in the three strains CH34, AE104, and *E. coli* W3110. Cell-bound metal increased when a particular metal was added to the growth medium (see Table S2 in the supplemental material). Nonamended TMM contained these metals in nM concentrations from trace element solution

TABLE 2. Elemental compositions of *C. metallidurans* and mutant strains in comparison to that of *E. coli* strain W3110

| Strain or mutant | Composition (%)[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mg | P | Ca | Fe | Co | Ni | Cu | Zn | Mn | Cd |
| *E. coli* | | | | | | | | | | |
| W3110 | 93 ± 3 | 70 ± 2 | **234 ± 38** | 54 ± 19 | 113 ± 47 | 90 ± 88 | **279 ± 167** | 125 ± 34 | **1,174 ± 159** | 87 ± 83 |
| *C. metallidurans* | | | | | | | | | | |
| AE104 | 90 ± 4 | 92 ± 1 | 96 ± 11 | 80 ± 9 | **189 ± 116** | 48 ± 9 | 59 ± 23 | 95 ± 6 | 83 ± 26 | 84 ± 34 |
| Δ*zntA* Δ*cadA* | 94 ± 6 | 94 ± 3 | 96 ± 1 | 90 ± 2 | 91 ± 1 | 38 ± 2 | 46 ± 4 | 104 ± 1 | 79 ± 7 | **150 ± 69** |
| Δ*zupT* | 104 ± 6 | 103 ± 7 | 131 ± 35 | 118 ± 16 | **362 ± 211** | 116 ± 40 | 92 ± 47 | 104 ± 5 | 122 ± 57 | 100 ± 35 |
| Δ*zupT* Δ*pitA* | **305 ± 24** | **179 ± 8** | 109 ± 5 | 90 ± 5 | **264 ± 127** | 87 ± 10 | 49 ± 10 | 82 ± 9 | 111 ± 16 | 85 ± 66 |

[a] The elemental composition of *C. metallidurans* strain CH34, cultivated in Tris-buffered mineral salts medium, was determined by ICP-MS and calculated as atoms per cell as follows: Mg, $13.7 \times 10^6 \pm 0.8 \times 10^6$; P, $110 \times 10^6 \pm 3 \times 10^6$; Ca, $875 \times 10^3 \pm 165 \times 10^3$; Fe, $537 \times 10^3 \pm 103 \times 10^3$; Co, $3.97 \times 10^3 \pm 1.31 \times 10^3$; Ni, $10.8 \times 10^3 \pm 3.8 \times 10^3$; Cu, $60.7 \times 10^3 \pm 22.1 \times 10^3$; Zn, $90.9 \times 10^3 \pm 9.5 \times 10^3$; Mn, $993 \times 10^0 \pm 123 \times 10^0$; and Cd, $221 \times 10^0 \pm 254 \times 10^0$. Other elements were as follows: B, $27 \pm 14 \times 10^3$; Na, $96.2 \times 10^6 \pm 45.3 \times 10^6$; S, $38.8 \times 10^6 \pm 13.8 \times 10^6$; K, $33.5 \times 10^6 \pm 8.0 \times 10^6$; Se, $13 \times 10^6 \pm 5 \times 10^6$; and Mo, $3.45 \pm 0 \times 10^3$. Assuming a total cellular volume of 0.57 fl (11), these values would calculate to the following quotas (43) in decreasing order: 320 mM P, 280 mM Na, 110 mM S, 98 mM K, 40 mM Mg, 26 mM Ca, 16 mM Fe, 260 μM Zn, 180 μM Cu, 80 μM B, 31 μM Ni, 12 μM Co, 10 μM Mo, 2.9 μM Mn, 640 nM Cd, and 39 nM Se. The contents of the other *C. metallidurans* strains and that of *E. coli* strain W3110 are given as percentages of the *C. metallidurans* strain CH34 value. Interesting values are in boldface.

SL6 (45). Despite the more than 1,000-fold-higher concentrations, the total content of the added metal of the cells increased less than 20-fold under some bacterial strain/metal combinations: up to 100 μM $Zn^{2+}$ for *E. coli* and strain AE104 and up to 100 μM $Cu^{2+}$ or 30 μM $Ni^{2+}$ for all three strains. Under most conditions, the cellular metal content was a linear function of the outside concentration (see Table S2 in the supplemental material, shaded areas), and the strains did not differ much in the total amount of metal bound to the cells. This indicated that binding of metals by the cells may have masked a possible adjustment of the periplasmic or cytoplasmic metal concentration by transport mechanisms.

Under three conditions (see Table S2 in the supplemental material, boxed areas), the cellular metal content increased more than the outside concentration: in *E. coli* and when $Co^{2+}$ was ≥30 μM or $Cd^{2+}$ was ≥10 μM. In strain CH34, the zinc content also increased strongly (see Table S2 in the supplemental material). Interestingly, increasing the content of one metal 1,000- or even 10,000-fold above the concentration in the TMM growth medium did not disturb the homeostasis of all the other metals (see Table S2 in the supplemental material). Most exceptions were observed when the cells approached the limits of their metal tolerances. In the case of CH34 and zinc, some coprecipitation with the zinc-binding process may have occurred. Otherwise, none of the three strains showed any disturbance of their ability to control homeostasis of one metal in the presence of another. To test the limits of the metal homeostasis system of strain CH34, the five cations $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ were provided in a 1:1:1:1:1 ratio, and the metal content of CH34 cells was compared to that of the same metal under conditions when only a single metal was added (see Table S3 in the supplemental material). Up to 30 μM, the total content of each metal was about the same as if only a single metal had been added to the culture, and no disturbance of the homeostasis of another metal was measured. At 100 μM, however, all metal contents increased, and at 300 μM, growth stopped.

How may *C. metallidurans* handle high concentrations of multiple metals in parallel? Is it by a "shunt" of an uptake system for a single metal lined up with an efflux system for the same metal, with both controlled by the metal (see Fig. S1 in the supplemental material)? Or do the uptake systems take up what they can get and leave it to the efflux systems to "worry later" about any surplus metals?

**Regulation of the expression of the genes for putative secondary metal uptake systems.** To discriminate between these possibilities, *lacZ* reporter gene fusions of the genes $corA_1$, $corA_2$, $corA_3$, *pitA*, and *zupT* were constructed as operon fusions, leaving the gene of the importer intact, and regulation of these fusions by transition metals was studied. Expression of all five genes was downregulated with increasing zinc concentrations in the order *zupT-lacZ* (4.8-fold) > *pitA-lacZ* (3.6-fold) > $corA_3$-lacZ (3.3-fold) > $corA_2$-lacZ (3.0-fold) > $corA_1$-lacZ (1.5-fold) (Fig. 1 and 2). The four other cations ($Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Cd^{2+}$) had no effect on the expression of these genes, with the exception of $corA_1$, which was slightly (1.4-fold) downregulated by either $Cu^{2+}$ or $Cd^{2+}$ (data not shown). This indicated that zinc availability was a major control element of transition metal homeostasis in *C. metallidurans*.

**$CorA_1$ production is downregulated by magnesium.** The $corA_1$ gene, encoding the MIT transporter with the highest similarity to CorA from *E. coli* (data not shown), was the only gene that was upregulated with decreasing magnesium concentrations (Fig. 3A). Expression of the other four genes was not at all influenced by the $Mg^{2+}$ concentrations when the trace element solution SL6 (containing 35 nM $ZnCl_2$ in addition to other transition metal salts) was added to the TMM (shown in Fig. 3 for $corA_2$-lacZ and $corA_3$-lacZ; other data not shown). In the absence of SL6, the magnesium concentration affected the expression of these genes less than 2-fold (data not shown). Regulation of $corA_1$-lacZ was not influenced by SL6 (data not shown). This indicated that $CorA_1$ may be the main secondary uptake system for $Mg^{2+}$ in *C. metallidurans*.

**PitA production is upregulated by phosphate.** On the other hand, all transport systems except $corA_1$ were upregulated at high phosphate concentrations, although the effect of increasing phosphate concentrations on $corA_2$-lacZ or $corA_3$-lacZ activity was below 2-fold (Fig. 2A [for *zupT-lacZ*] and data not shown). Since phosphate may complex with substrate cations, such as $Zn^{2+}$ [solubility constant of $Zn_3(PO_4)_2$ complex, $9 \times$

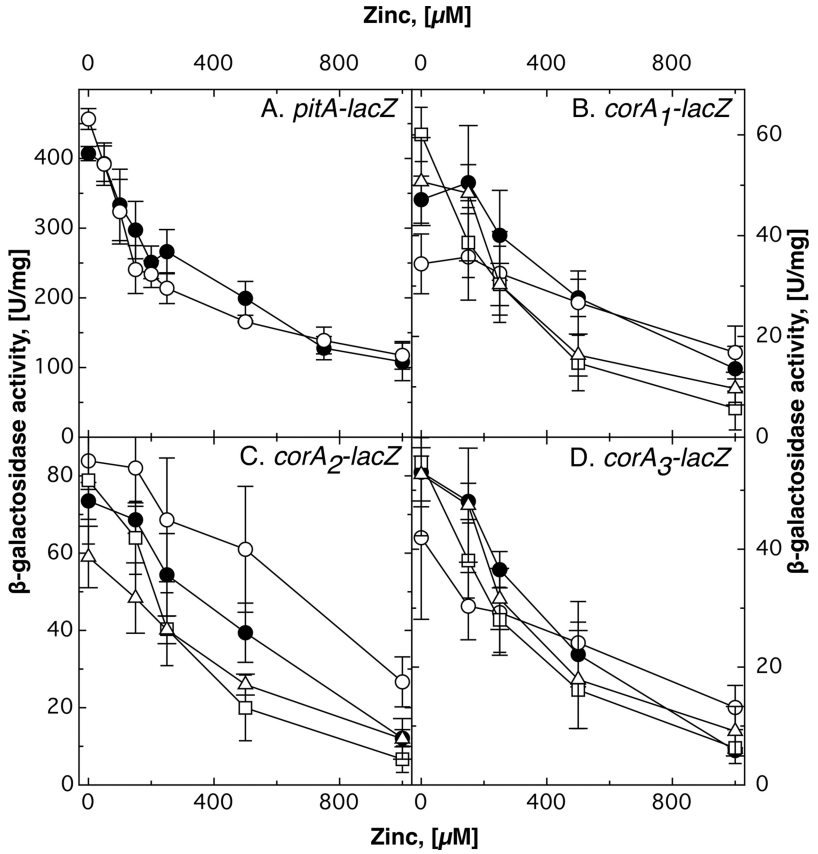


FIG. 1. Regulation of expression of *lacZ* reporter gene fusions by zinc in mutant strains. Cells of *C. metallidurans* AE104 (●) mutant strains were incubated in TMM containing various concentrations of $Zn^{2+}$. These strains carried *lacZ* fusions of *pitA* (A), $corA_1$ (B), $corA_2$ (C), or $corA_3$ (D). The mutant strains were a Δ*zupT* deletion strain (○) (A to D) and a Δ*pitA* (□) and a Δ*pitA* Δ*zupT* (△) deletion strain (B, C, and D). At least three independent determinations were made. The error bars indicate standard deviations.

$10^{-33}$ (61)], this upregulation may also have been due to complexation of zinc by phosphate, which decreased zinc availability to the cells.

The phosphate uptake system PitA was about 5-fold upregulated at a phosphate concentration of 5 mM. The expression level declined again at higher phosphate concentrations (Fig. 3B). This was unusual, since expression of the gene of an uptake system should be downregulated by increasing substrate concentrations, indicating that the focus of the cellular action of PitA may be more the cation uptake than that of phosphate.

**Only *zupT* expression was upregulated by transition metal starvation.** Expression of *zupT-lacZ* was strongly upregulated, not only by EDTA and phosphate, but also by DPTA (diethylenetriaminepentaacetate) and TPEN [*N*,*N*,*N*′,*N*-tetrakis(2-pyridylmethyl) ethylenediamine] (Fig. 2). In contrast, none of the genes $corA_{1i}$, $corA_2$, $corA_3$, and *pitA* was influenced by the EDTA concentration (data not shown). This indicates that *zupT* was the only gene for a secondary uptake system studied that was upregulated by starvation for divalent metal cations.

The influence of EDTA and TPEN on *zupT-lacZ* expression was more closely examined. Upregulation of *zupT-lacZ* expression by TPEN (Fig. 2C) was retarded in the presence of zinc, cobalt, and manganese (the effects were in the order $Zn^{2+} > Co^{2+} > Mn^{2+}$ [Fig. 2C]), while the effect of $Fe^{3+}$ was negligible. A concentration of 50 μM EDTA yielded 2.4- ± 0.5-fold upregulation. Addition of equimolar amounts of metal cations decreased this upregulation (Fig. 2D). Again, $Zn^{2+}$ had the strongest effect; $Ni^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Cu^{2+}$, and $Mn^{2+}$ had some effect (shown for $Ni^{2+}$, $Co^{2+}$, and $Mn^{2+}$ in Fig. 2D; $Cd^{2+}$ not shown but similar to $Co^{2+}$; $Cu^{2+}$ not shown but similar to $Mn^{2+}$); and $Fe^{3+}$, $Ca^{2+}$, and $Mg^{2+}$ had no effect (data not shown).

Taken together, the environmental zinc concentrations, but not those of other metals, regulated the expression of all five genes. Additionally, zinc starvation regulates the expression of *zupT*. Moreover, $corA_1$ was upregulated by magnesium starvation. Thus, no evidence for "shunts" was obtained.

**Effect of *zupT* deletion on metal homeostasis.** Deletion of *zupT* did not change the metal resistance of *C. metallidurans* strain AE104 significantly (see Table S4 in the supplemental material). After cultivation in Tris-buffered mineral salts medium, the metal content of Δ*zupT* cells was similar to that of strain AE104 with the exception of increased cobalt content (Table 2). ZupT was therefore not the only uptake system for any metal. However, Δ*zupT* led to a strong decrease of EDTA resistance on solid growth medium from a MIC of 1.5 mM to about 300 μM (Fig. 4). Addition of zinc more than that of cobalt abolished EDTA sensitivity (0.5 mol metal/mol EDTA;

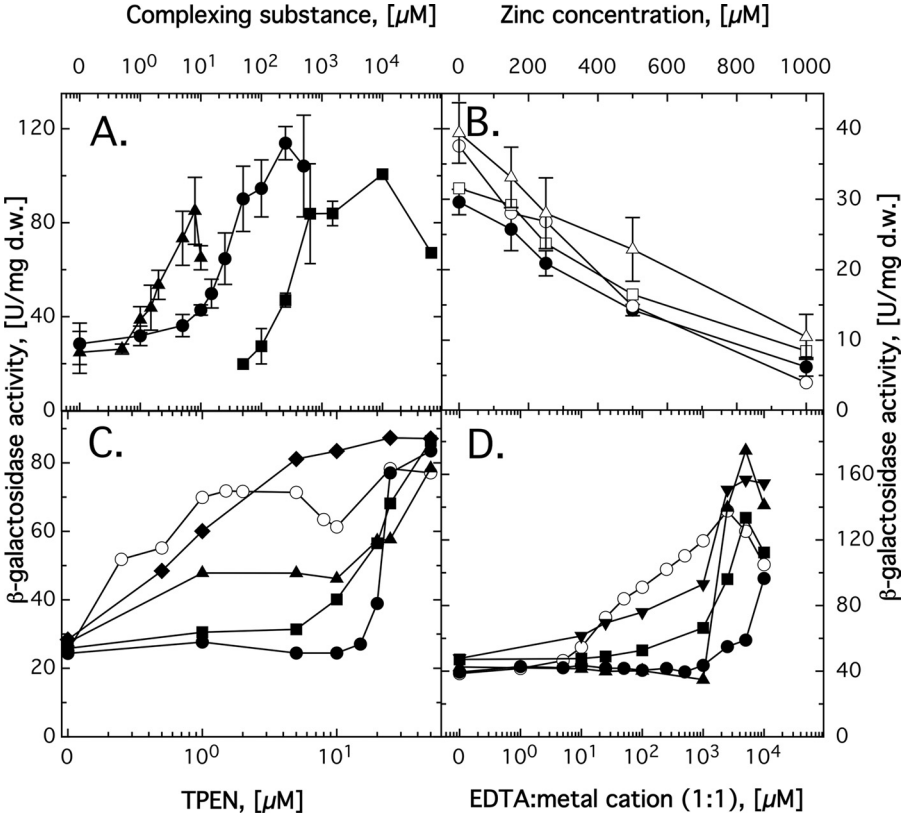


FIG. 2. Regulation of expression of a *zupT-lacZ* reporter gene fusion. Cells of *C. metallidurans* AE104 carrying a *zupT-lacZ* fusion were incubated in TMM containing various concentrations of the chelating substance DPTA (▲), EDTA (●), or phosphate (■) (A); zinc in the AE104 parent (●) or the Δ*pitA* (○), Δ$corA_2$ (□), or Δ$corA_3$ (△) mutant (B); increasing concentrations of the chelating substance TPEN in the presence of 20 μM $Fe^{3+}$ (♦), $Mn^{2+}$ (▲), $Co^{2+}$ (■), or $Zn^{2+}$ (●) or without added metal cations (○) (C); or increasing concentrations of EDTA-metal complexes in a 1:1 ratio, i.e., no metal (○), $Zn^{2+}$ (●), $Co^{2+}$ (■), $Mn^{2+}$ (▲), or $Ni^{2+}$ (▼) (D). Incubation was continued for 16 h (phosphate) or 3 h (all others), and the β-galactosidase activity was determined. At least three independent determinations were made. Error bars indicating standard deviations are shown in panels A and B, but not in panels C and D to avoid cluttering. d.w., dry mass. Note that only panel B has a linear *x* axis.

see Table S5 in the supplemental material). This indicated that ZupT was required to obtain zinc and possibly also cobalt under conditions of low cation availability.

The effect of EDTA on the metal content of *C. metallidurans* was further analyzed using ICP-MS (Fig. 5). With increasing EDTA concentrations, the zinc contents of *C. metallidurans* strain CH34 wild type and the plasmid-free strain AE104 decreased gradually. *E. coli* W3110, which was used for comparison and possesses a ZnuABC and a ZupT system, contained a much higher zinc content at high EDTA concentrations than *C. metallidurans*. In contrast, the zinc content of the Δ*zupT* strain decreased sharply at low EDTA concentrations (Fig. 5A). The Ni and Co contents in all four strains decreased sharply at low EDTA concentrations, while those of the monovalent cations Na, K, Mn, Ca, and Fe did not change (data not shown). Thus, *C. metallidurans* CH34 and AE104 are able to obtain zinc under conditions of low availability, and ZupT is required for this feature.

Uptake of zinc was more closely examined using $^{65}Zn^{2+}$ (Fig. 6 and Table 3). Comparing the $^{65}Zn^{2+}$ uptake of strain AE104 parent cells under various conditions reproduced a 10-fold-increased zinc uptake under conditions of magnesium starvation (41) and about 50% increase of zinc uptake when the cells were cultivated in the absence of trace element solution SL6. Deletion of *zupT* did not impair the ability of the cells to take up $^{65}Zn^{2+}$ under conditions of sufficient zinc in the environment. Under magnesium starvation, uptake of zinc by the Δ*zupT* mutant cells was similar to that of the control cells (Fig. 6A and B). Probably, other systems were upregulated under these conditions, imported zinc efficiently, and led to a concomitant increase in the cellular cobalt content (Table 2).

With sufficient magnesium and in cells cultivated without trace element solution SL6, zinc import of the Δ*zupT* mutants was 2-fold decreased compared to the parent strain, AE104, but 3-fold increased when the cells were cultivated in the presence of SL6 (Fig. 6C and D). Zinc import by Δ*zupT* cells without SL6 was very similar to that of AE104 parent cells with SL6, indicating that ZupT may have been responsible for 50% of the zinc import in the parent cells cultivated in the presence of high magnesium concentrations but without SL6, in agreement with the observed upregulation of *zupT* under conditions of zinc starvation (Fig. 2). The 3-fold increase in zinc import in the Δ*zupT* mutant at 1 mM magnesium plus SL6 might be

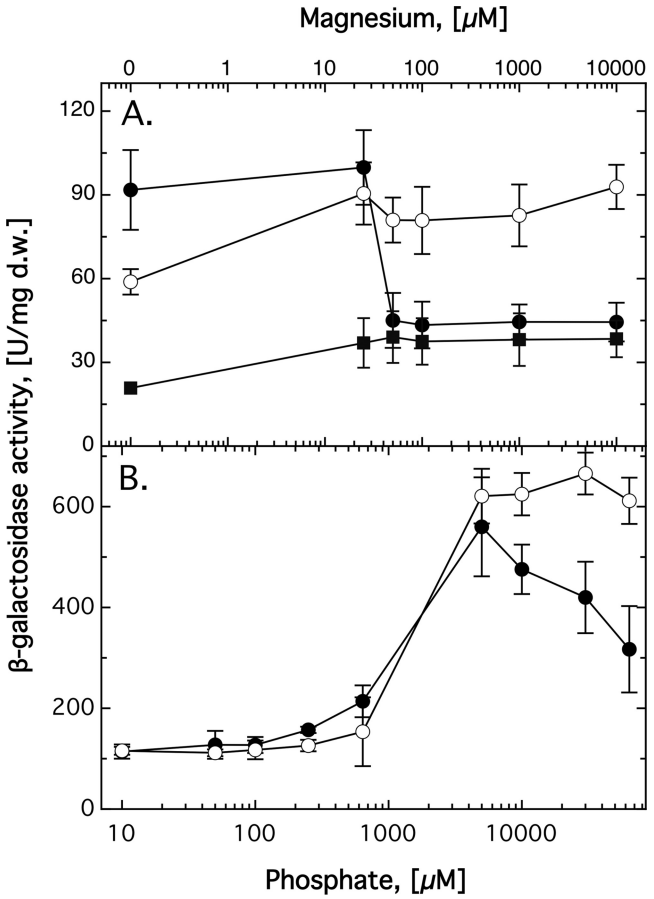


FIG. 3. Regulation of expression of *pitA* and the *corA* proteins. (A) Influence of increasing $Mg^{2+}$ concentrations on the expression of a $corA_1$-lacZ (●), a $corA_2$-lacZ (○), and a $corA_3$-lacZ (■) fusion. (B) Influence of increasing phosphate concentrations on the expression of a *pitA-lacZ* fusion (with *pitA* intact) in a parental background (●) and in a Δ*zupT* deletion strain (○). Precultures were washed twice and used to inoculate the main cultures, which were incubated for 16 h with shaking at 30°C in TMM containing SL6. Following incubation, β-galactosidase activity was determined. Note the log scale of the *x* axis in panel B. Five to seven independent determinations were made. The error bars indicate standard deviations. d.w., dry mass.

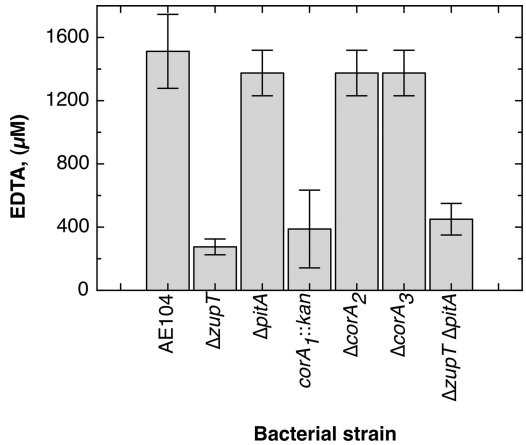


FIG. 4. MICs of various *C. metallidurans* mutant strains. The strains were grown for 48 h in TMM, diluted 1:100, and streaked on TMM agar plates with increasing EDTA concentrations. Growth was monitored after 5 days at 30°C. The results were confirmed by at least three independent experiments. The error bars indicate standard deviations.

explained by upregulation of other metal importers in the Δ*zupT* strain under these conditions.

Regulation of expression of *pitA-lacZ*, $corA_1$-lacZ, $corA_2$-lacZ, and $corA_3$-lacZ by the external zinc concentration did not change in the Δ*zupT* strain compared to the parent control (Fig. 1). However, the pattern of the phosphate-dependent regulation of *pitA-lacZ* was different in the Δ*zupT* and the parent backgrounds (Fig. 3B): in both strains, a maximum expression level of 600 U/mg was reached at 5 mM phosphate. This level decreased 2-fold in the parent background at higher phosphate concentrations but remained at a high level in the Δ*zupT* mutant. Thus, PitA may have been able to substitute for ZupT with respect to zinc uptake at high phosphate concentrations and in the presence of SL6.

**Effect of the *pitA* deletion on metal homeostasis.** Deletion of *pitA* did not change *zupT-lacZ* expression (Fig. 2B) or resistance to metal cations, to EDTA (Fig. 4; see Table S4 in the supplemental material), or to metal-EDTA complexes (see Table S5 in the supplemental material). The Δ*pitA* deletion strain accumulated more $^{65}Zn^{2+}$ than the parental strain, AE104, when cultivated in sufficient magnesium (with or without SL6) but contained 25% less zinc when the cells were starved for magnesium and trace elements (Fig. 6 and Table 3). Thus, import of cation-phosphate complexes by PitA might be an important metal source; however, other zinc transfer systems were able to substitute for a missing PitA system. Regulation of expression of the three *corA-lacZ* fusions by zinc did not change in the Δ*pitA* mutant strain (Fig. 1).

**Deletion of *zupT* and *pitA*.** The metal resistance of the Δ*zupT* Δ*pitA* double mutant was similar to that of the single mutants or the AE104 parent with the exception of decreased cobalt resistance (see Table S4 in the supplemental material). The resistance to EDTA of the double mutant was similar to that of the Δ*zupT* single mutant, whereas that of the Δ*pitA* single mutant was on the parental level. Further deletion of *pitA* did not change the EDTA sensitivity of the Δ*zupT* mutant. Addition of zinc (0.5 mol zinc per mol EDTA) completely abolished the EDTA sensitivity of the double mutant, similar to that of the Δ*zupT* single mutant. While cobalt (0.5 mol/mol EDTA) could also counteract the EDTA sensitivity of the Δ*zupT* single mutant, this was not observed for the double mutant (see Table S5 in the supplemental material). Cells of the double mutant showed 3-fold-increased magnesium and 2-fold-increased phosphate contents (Table 2 and Fig. 5B) compared to the parent strain, AE104, plus an increased cobalt content similar to that of Δ*zupT* cells. Deletion of *pitA* from the Δ*zupT* mutant obviously led to magnesium and phosphate starvation, perhaps leading to upregulation of additional metal and phosphate importers. Thus, PitA seemed to be an important magnesium-phosphate (in general, metal cation-phosphate) uptake system.

The zinc content, however, in cells of the Δ*pitA* Δ*zupT* double mutant was similar to that of the AE104 parent (Table 2). Similar to the Δ*zupT* single mutant, double-mutant cells were unable to obtain zinc from EDTA complexes (Fig. 5A). Uptake of zinc was on the same level as for the Δ*pitA* single-

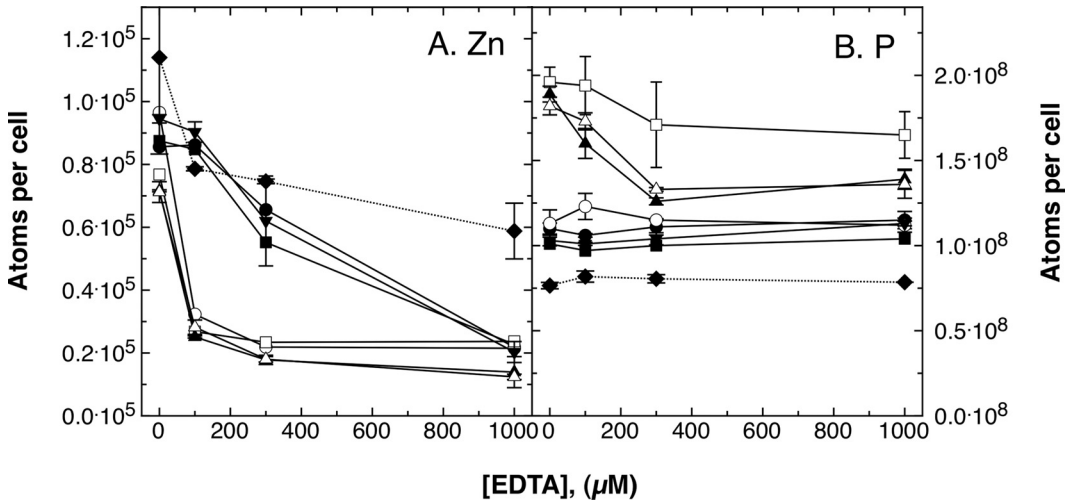


FIG. 5. Zinc and phosphate contents of *C. metallidurans* and *E. coli* cells. The contents of zinc and phosphate atoms per cell were determined by ICP-MS in *C. metallidurans* CH34 wild type (●), megaplasmid-free AE104 (■), and the AE104 Δ*zupT* (○) and Δ*zupT* Δ*pitA* (□) mutant strains, as well as *E. coli* strain W3110 (♦). The cells were cultivated for 20 h in TMM with shaking at 30°C. Three independent determinations were made. The error bars indicate standard deviations.

mutant strain (Fig. 6 and Table 3). Thus, if zinc was not sequestered, e.g., by EDTA, the double mutant still contained enough importers for zinc and the other metal cations. Since the zinc-dependent downregulation of the three *corA-lacZ* reporter fusions was not changed in the double mutant compared to the parental background (Fig. 1), this indicates the presence of unknown uptake systems.

When strain AE104 and the Δ*zupT* single- and the Δ*zupT* Δ*pitA* double-mutant strains were cultivated under conditions of multiple-metal stress, strain AE104 maintained metal ion homeostasis at 10 μM (each) of the five metal cations but started to lose control and accumulate sodium at 30 μM. Growth stopped at 100 μM (see Table S6 in the supplemental material). The deletion strains did not even survive at 30 μM. The double mutant was less able to cope with multiple-cation stress at 10 μM each metal than the Δ*zupT* single mutant. This indicated that both systems were required for full metal ion resistance already in the plasmid-free strain AE104.

**Single *corA* gene deletions.** In agreement with the proposed function as the main secondary magnesium uptake system, magnesium resistance in the Δ$corA_1$ mutant strain increased from a MIC of 55 mM (strain AE104) to 70 mM (data not shown). Since $corA_1$ expression was upregulated when cells were cultivated in the presence of 10 μM $Mg^{2+}$ (Fig. 5A), $CorA_1$ could serve as a zinc importer under these conditions. However, uptake of $^{65}Zn^{2+}$ in Δ$corA_1$, Δ$corA_2$, or Δ$corA_3$ single-deletion strains was not compromised (Table 3).

Deletion of any *corA* gene did not change the metal resistance of strain AE104 or expression of *zupT-lacZ* significantly (Table 3 and Fig. 2). Deletion of $corA_1$, but not of the other two *corA* genes, led to decreased EDTA resistance, similar to deletion of *zupT* (Fig. 4; see Table S4 in the supplemental material). However, and in contrast to Δ*zupT*, any metal tested increased EDTA resistance when added at 0.5 mol/mol EDTA, with magnesium being less efficient than transition metal cations (see Table S5 in the supplemental material). Probably, each of the metals mobilizes sufficient zinc or other metals from the EDTA complexes to be taken up by ZupT or PitA. Thus, none of the three CorA systems was required as long as the other two CorA proteins, PitA and ZupT, were present in the cell.

## DISCUSSION

**Living with many metals.** *C. metallidurans* strain CH34 is a facultative chemolithoautotrophic bacterium that has evolved to become superbly adapted to environments containing high concentrations of transition metals (19, 57). When the metal contents of *C. metallidurans* strains CH34 and AE104 and of *E. coli* strain W3110 were compared, their metal contents increased linearly with the outside concentration, and there was not much difference between the sensitive strains AE104 and W3110 and the resistant strain CH34, indicating that metal resistance did not influence the cellular metal content. This suggested that binding of the metals to the surfaces of the bacteria contributed to the measured values. Nevertheless, zinc binding to cells of strain CH34 requires living cells and is therefore an active process (25). Since strain AE104 did not display similarly increased zinc-binding properties (see Table S2 in the supplemental material), increased zinc binding should also be a plasmid-mediated activity. The many periplasmic proteins that are encoded by parts of the *czc* operon may be involved in this process (40, 63).

The upper concentration limits of metal tolerance also became visible when cells were simultaneously treated with five toxic metals. Strain CH34 tolerated up to 100 μM each of the five metals and started to show growth inhibition at this concentration, strain AE104 did not grow at 100 μM but did at 30 μM, and the Δ*zupT* and Δ*zupT* Δ*pitA* strains did not grow at 30 μM. Both deletion strains continued to grow at 10 μM, but with enhanced accumulation of other metals in the case of the double mutant. Therefore, the functions of at least these two uptake systems were required for parallel handling of five toxic metals.

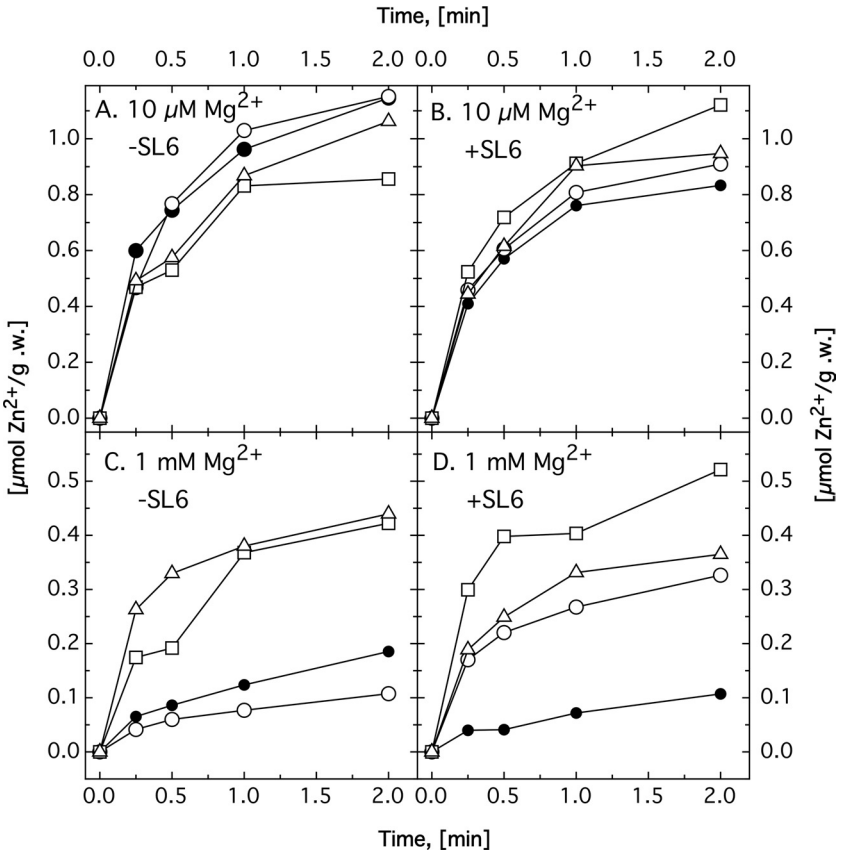


FIG. 6. Zinc uptake by *C. metallidurans* mutant strains. *C. metallidurans* strain AE104 (●) and its Δ*zupT* (○), Δ*pitA* (□), and Δ*zupT* Δ*pitA* (△) derivatives were cultivated in TMM with decreased magnesium content, 10 μM (A and B) or 1 mM (C and D) $Mg^{2+}$, either without (A and C) or with (B and D) the trace element solution SL6 in the medium. The cells were harvested by centrifugation, suspended in Tris buffer, and used in uptake experiments with $^{65}Zn^{2+}$. Mean values of at least three experiments are shown. Error bars were omitted to avoid cluttering.

Below this upper metal tolerance limit and under most conditions tested, a high concentration of one metal in the growth medium did not influence the cellular content of another. This might be accomplished by "metal homeostasis shunts," combinations of uptake and efflux systems assigned to each metal and regulated in their expression and activities by the metal (see Fig. S1, inset, in the supplemental material). Indeed, the previously identified efflux systems can be assigned to a single central metal cation as a substrate, so they could play their part should such a shunt exist (see Fig. S1 in the supplemental material). The three $P_{IB2}$-type ATPases (TC 3.A.3) ZntA, CadA, and PbrA remove $Zn^{2+}$, $Cd^{2+}$, and $Pb^{2+}$, respectively, from the cytoplasm (4, 7, 24, 49, 51, 56). The CDF proteins (TC 2.A.3) DmeF and FieF export $Co^{2+}/Ni^{2+}$ or $Fe^{2+}$, respectively (12, 32, 51), into the periplasm. From there, the RND-driven (TC 2.A.6) transenvelope efflux systems CzcCBA and CnrCBA transport $Co^{2+}/Zn^{2+}/Cd^{2+}$ or $Co^{2+}/Ni^{2+}$, respectively, from the periplasm to the outside (37). The $P_{IB4}$-type ATPase CzcP, the CDF protein CzcD, and the DMT protein CnrT (TC 2.A.7) enhance export of $Zn^{2+}$, $Co^{2+}/Zn^{2+}/Cd^{2+}$, or $Ni^{2+}$, respectively, from the cytoplasm to the periplasm to feed additional substrate cations into the RND-driven efflux systems (2, 3, 51). Up to four $P_{IB1}$-type ATPases, two RND-driven efflux systems, and a variety of other proteins maintain copper homeostasis (31) and may also be involved in silver and gold transformation (22, 47).

This arrangement assigns a basal export system plus a system for high metal concentrations to each individual metal cation: for $Zn^{2+}$, these are ZntA (basal) plus CzcP/CzcD/CzcCBA (high); for $Cd^{2+}$, CadA (basal) plus CzcD/CzcCBA (high); for

TABLE 3. Uptake of $^{65}Zn^{2+}$ by various *C. metallidurans* mutant strains

| Strain or mutant | Uptake of $^{65}Zn^{2+}$[a] | | | |
|---|---|---|---|---|
| | 10 μM $Mg^{2+}$ | | 1 mM $Mg^{2+}$ | |
| | Without SL6 | With SL6 | Without SL6 | With SL6 |
| AE104 | 1.15 ± 0.60 | 0.83 ± 0.07 | 0.19 ± 003 | 0.11 ± 0.01 |
| Δ*zupT* | 1.15 ± 0.18 | 0.91 ± 0.13 | 0.11 ± 0.04 | 0.33 ± 0.26 |
| Δ*pitA* | 0.86 ± 0.03 | 1.12 ± 0.02 | 0.42 ± 0.13 | 0.52 ± 0.25 |
| *corA*$_1$::*kan* | 1.10 ± 0.28 | 1.11 ± 0.18 | 0.22 ± 0.07 | 0.20 ± 0.06 |
| Δ*corA*$_2$ | 1.13 ± 0.03 | 1.00 ± 0.02 | 0.20 ± 0.06 | 0.22 ± 0.03 |
| Δ*corA*$_3$ | 1.45 ± 0.24 | 0.90 ± 0.10 | 0.18 ± 0.04 | 0.24 ± 0.07 |
| Δ*zupT* Δ*pitA* | 1.06 ± 0.34 | 0.95 ± 0.09 | 0.44 ± 0.34 | 0.37 ± 0.14 |

[a] As for Fig. 6, *C. metallidurans* strain AE104 and its mutant derivatives were cultivated in TMM with decreased magnesium content (10 μM) (Fig. 6A and B) or 1 mM $Mg^{2+}$ (Fig. 6C and D), either without (Fig. 6A and C) or with (Fig. 6B and D) the trace element solution SL6 in the medium. The cells were harvested by centrifugation, suspended in Tris buffer, and used in uptake experiments with $^{65}Zn^{2+}$. The values at a *t* of 2 min are given in μmol $^{65}Zn^{2+}$/g dry mass. At least 3 reproductions.

$Ni^{2+}$, DmeF (basal) plus CnrT/CnrCBA (high); and for $Co^{2+}$, DmeF (basal) plus CzcD/CzcCBA/CnrCBA (high). Moreover, backup systems are ready to compensate for failure of the main systems. ZntA, CadA, and PbrA may substitute for each other, and CzcP and many RND-driven system may have broad substrate specificities when on a higher expression level (cobalt for CzcP and zinc for CnrCBA) or are only expressed to phenotypic relevance after a mutation (40, 51, 57).

**There are no metal homeostasis shunts.** Now, do the uptake systems line up with the efflux systems to form a shunt? They do in *E. coli*, and ABC uptake systems are needed for this. Examples are ZnuACB for zinc (44), NikABC for nickel (33), and ModABCD for molybdate (46, 52). ZnuABC, ZntA, and their regulators Zur and ZntR form a shunt as described above, controlling the cytoplasmic zinc content in the bacterium (43). NickABC, RncA, NikR, and RcnR could be a second shunt (17, 21), albeit linked to iron and cobalt homeostasis.

However, there are no known ABC uptake systems for divalent metal cations in *C. metallidurans*, and none of the five systems ZupT, PitA, and $CorA_1$, $CorA_2$, and, $CorA_3$ could be assigned to a single metal so that a specific homeostasis shunt of an uptake and efflux system is formed. The only regulatory signals in control of the expression levels of the five systems were zinc starvation for *zupT*, magnesium starvation for $corA_1$, and the zinc concentration in general for all five systems. Other metals, such as $Co^{2+}$, $Ni^{2+}$, and $Cd^{2+}$, did not regulate the expression of any of the studied genes under the conditions tested. Nevertheless, these metals are bound to the *C. metallidurans* cell (Table 2) and were imported (41). High zinc concentrations downregulated expression of all five genes studied but did not decrease their expression level to zero. Thus, for most metal cations, no "shunt" seems to exist.

***C. metallidurans* seems not to use much manganese.** This leaves the "worry later" hypothesis: highly redundant uptake systems with low ion selectivity take up what they can get and leave it to the more specific efflux systems to remove anything in surplus.

However, not anything is being taken up. *C. metallidurans* does not contain a known manganese efflux system—no SitABC-type and no MntH manganese/cadmium importer of the NRAMP protein family—as *E. coli* does. Consequently, the manganese content of *E. coli* cells grown in TMM was 12-fold higher than that of strain CH34. When challenged with 30 μM cadmium, *E. coli* contained many more atoms of cadmium per cell than CH34 or AE104 and started to lose control of its metal homeostasis system (see Table S2 in the supplemental material), probably indicating cytoplasmic accumulation of the metal (14, 15). The main Mn-containing enzyme of *E. coli* is the superoxide dismutase SodA (38). The main superoxide dismutase in CH34, however, is an iron-containing SodB-type protein (48), while the only Mn-containing SOD-like protein, ChrC, has very low activity and is part of the chromate detoxification system (20). This indicated that CH34 has no MntH and with it the highly active housekeeping protein SodA, presumably to decrease the uptake of the nonspecific MntH substrate cadmium. Thus, absence of MntH in strain CH34 may be a prerequisite for its cadmium resistance, in addition to the possession of CadA, CzcD, and CzcCBA.

**Functions of the five uptake systems.** ZupT is likely the main uptake system for $Zn^{2+}$ under conditions of zinc starvation, such as in the presence of EDTA. On the other hand, ZupT is not the only system able to import zinc. When *C. metallidurans* strain AE104 was cultivated under conditions of magnesium starvation, deletion of *zupT* or *pitA* or double deletion of both genes did not change zinc uptake by the cells (Fig. 6). Obviously, magnesium starvation led to upregulation of a transport system that imported zinc. This "magnesium uptake system" was assigned to metal uptake in *C. metallidurans* previously (41). Since deletion of the genes for any of the transport systems tested did not hamper zinc import in cells grown under magnesium starvation (Table 3 and Fig. 6), PitA, ZntA, or the three CorA proteins are not this "magnesium uptake system."

As judged by the activity of the reporter gene fusion, *pitA* had the highest expression level among the five studied systems. Its deletion led to increased ability for zinc import into cells of strain AE104. While deletion of *zupT* doubled the amount of cell-bound cobalt, the additional deletion of *pitA* also increased that of phosphate and magnesium (Table 2). Increase in the phosphate content (Fig. 5) can be explained by upregulation of the phosphate-specific system PstABC, which is encoded on chromosome 1 by the putative operon Rmet_2179 to Rmet_2185 and includes the genes for an ABC transport system, the periplasmic phosphate-binding protein PhoS, and the regulatory factors PhoU, PhoB, and PhoR. The higher magnesium content may be the result of upregulation of $CorA_1$ or the unknown "magnesium uptake system." Thus, upregulation of PitA by high phosphate concentrations (Fig. 3B) indicated that PitA is a metal-phosphate uptake system. This has been observed before for *E. coli* (18). The affinity of metals for phosphate from the solubility product constants (http://www.ktf-split.hr/periodni/en/abc/kpt.html) indicates that most metal cations may be available as parts of phosphate complexes in TMM. Thus, PitA may be an important metal importer in bacteria. $CorA_1$ seemed to be a central magnesium uptake system, and $CorA_2$/$CorA_3$ seem to be backup systems.

Taking the data together, in *C. metallidurans*, adaptation to metal-rich environments seems to have led to a genetic outfit with uptake systems with rather broad substrate specificity that import metal phosphate complexes (PitA), magnesium and other metals ($CorA_1$), or zinc and other metals (ZupT), leaving it to the cell to "worry later" what to do with a nonoptimal cytoplasmic metal ion bouquet. Here, efflux systems control and regulate the content and bouquet composition of this metal pool (see Fig. S2 in the supplemental material). In addition, *C. metallidurans* contains a variety of RND-driven efflux pumps that may adjust the periplasmic concentrations of the transition metals, thus dealing with the substrates of the uptake systems. This action may lead to substrate competition between these uptake systems and RND-driven pumps for metals. In this model, the intrinsic function of ZupT would be to deal with this situation.

## ACKNOWLEDGMENTS

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