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## Interplay between seven secondary metal uptake systems is required for full metal resistance of *Cupriavidus metallidurans*†

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The beta-proteobacterium *Cupriavidus metallidurans* is able to grow in metal-contaminated environments due to having sophisticated metal efflux systems. Here, the contribution of all seven known secondary metal uptake systems (ZupT, PitA, CorA<sub>1</sub>, CorA<sub>2</sub>, CorA<sub>3</sub>, ZntB, HoxN) to metal resistance is characterized. In a strategic deletion approach, all ten double deletion mutants, a variety of triple and quadruple mutants, and from the  $\Delta 4$  mutant ( $\Delta zupT \Delta corA_1 \Delta corA_2 \Delta corA_3$ ) the mutants  $\Delta 5$  ( $=\Delta 4 \Delta pitA$ ),  $\Delta 6$  ( $=\Delta 4 \Delta pitA \Delta zntB$ ), and finally  $\Delta 7$  ( $\Delta zupT \Delta corA_1 \Delta corA_2 \Delta corA_3 \Delta pitA \Delta zntB \Delta hoxN$ ) were constructed. Metal resistance, metal content, and regulation of expression of these genes were characterized in these mutants. The  $\Delta zupT$  single deletion strain exhibited an extended lag phase in Tris-buffered liquid mineral salts medium (TMM) compared to its parent strain AE104, indicating a decreased fitness level. Further deletions up to  $\Delta 6$  did not influence growth in TMM without added metals but fitness of the  $\Delta 7$  strain dropped to a lower level compared to  $\Delta 6$ ,  $\Delta 5$  and  $\Delta zupT$ . The cells of the  $\Delta 7$  multiple deletion strain still contained all essential metals, demonstrating that additional metal import systems must exist in *C. metallidurans*. PitA was an important contributor of metal:phosphate complexes to *C. metallidurans*. Up to  $\Delta 5$  no evidence was found for increased expression of the transporter genes to recruit substitutes for the deleted importers. Only the *hoxN-lacZ* reporter gene fusion displayed a changed expression pattern in the  $\Delta 6$  strain, indicating recruitment of HoxN. Metal resistance of the deletion strains decreased along the deletion series although all strains still contained metal efflux systems: up to the  $\Delta 6$  mutant the overall fitness was kept at the  $\Delta zupT$  mutant strain level at the cost of a diminished competence to handle  $\mu\text{M}$  concentrations of transition metals. Together, these data demonstrated an important contribution of the seven secondary metal import systems to metal homeostasis in this bacterium.

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## Introduction

The beta-proteobacterium *Cupriavidus metallidurans* owes its outstanding ability to survive in environments with high concentrations of transition metals to the possession of a variety of sophisticated heavy metal-resistance determinants.<sup>1–4</sup> Central to these are metal efflux systems that have been characterized in detail in the last decades.<sup>5–7</sup> Members of the P<sub>IB</sub>-type ATPase (TC#3.A.3), CDF (TC#2.A.4) or other transporter families {TC, transporter classification<sup>8–10</sup>} remove surplus transition metal cations from the cytoplasm to the periplasm. Transenvelope protein complexes containing RND proteins (TC#2.A.6), such as CzcCBA or CnrCBA as major component, are responsible *in vivo* for further export from the periplasm to the external medium.<sup>1,5,7,11</sup> Substrates of these efflux systems are metal cations that have been

imported by metal uptake systems, which are thus interaction partners of the exporters.

In contrast to the efflux systems, contribution of the metal uptake systems to metal resistance in *C. metallidurans* has not been characterized in detail. The genome of *C. metallidurans* predicts at least seven secondary uptake systems for transition metals: (i) the ZIP protein ZupT (TC#2.A.5); (ii) the metal:phosphate importer PitA (TC#2.A.20); (iii) three members of the MIT family CorA<sub>1</sub> through CorA<sub>3</sub> (TC#1.A.35); (iv) the additional MIT protein ZntB that might be an uptake or efflux system; (v) and the NiCoT protein HoxN (TC#2.A.52) that might import additional Ni<sup>2+</sup> cations into the cell for hydrogenase synthesis.<sup>1,11,12</sup> *C. metallidurans*<sup>13</sup> does not possess orthologs of the ABC-type (TC#3.A.1) import systems ZnuABC and NikABC from *E. coli* for zinc or nickel import, respectively,<sup>14–16</sup> so that the secondary transport systems should theoretically have an important role in the uptake of transition metal cations.

Using single gene deletion mutants and a  $\Delta zupT \Delta pitA$  double mutant, it could be demonstrated that neither of the

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five systems ZupT, PitA, CorA<sub>1</sub>, CorA<sub>2</sub> or CorA<sub>3</sub> alone was essential for the import of zinc or other transition metal ions.<sup>13</sup> Moreover, none of these systems pairs up with an efflux system to form a regulated “shunt” of a metal through the cell. Such a shunt might considerably have maintained the cytoplasmic concentration of a metal in homeostasis, by either increased export or increased import of the metal if its concentration would be too high or too low, respectively. Instead, these five import systems possibly form a battery of redundant uptake systems with low and overlapping substrate specificities, which supplies metals to the cell as they become available, relying on the subsequent efflux system to cope with any surplus. In this way, the exact composition of the cytoplasmic metal content and mélange composition is maintained in a way that minimizes negative interference between the individual metals.<sup>13</sup>

Expression of *zupT*, *corA<sub>1</sub>-corA<sub>3</sub>* and *pitA* is down-regulated by zinc excess. Only expression of *zupT* is up-regulated by zinc starvation via the Fur-type zinc uptake regulator Zur (previously FurC).<sup>17</sup> Expression of *corA<sub>1</sub>* is influenced by magnesium starvation.<sup>13</sup> The *pitA* gene is up-regulated with increasing phosphate concentrations up to 5 mM phosphate, which complexes zinc ions and lowers its availability. At higher phosphate concentrations, *pitA* is down-regulated again but remains on a high expression level in a  $\Delta zupT$  mutant,<sup>13</sup> indicating a central function of zinc in control of expression of *pitA*; Zur is not involved in this process.<sup>17</sup>

The ZupT importer of *Escherichia coli* has a broad substrate specificity.<sup>18–20</sup> Deletion of its ortholog in *C. metallidurans* does not impair net import of Zn(II) or any other metal<sup>13</sup> at least not at the first glance. A more detailed analysis, however, reveals a pleiotropic effect of the  $\Delta zupT$  deletion: (i) the mutant cell is no longer able to import Zn(II) at low zinc concentrations,<sup>13</sup> e.g. in the presence of the metal chelator ethylene-diamine-tetra-acetic acid (EDTA); (ii) the overall zinc content of the mutant cell cultivated in minerals salts medium amounts to about 20 000 zinc per cell while the parent contains 70 000 zinc per cell;<sup>21</sup> (iii) zinc cannot be efficiently allocated to the zinc-dependent beta-prime subunit RpoC of the RNA polymerase, leading to aggregation of RpoC into inclusion bodies;<sup>21</sup> (iv) the central CzcA subunit of the RND-driven transenvelope complex CzcCBA is either not translated or is rapidly degraded;<sup>21</sup> (v) surprisingly zinc is not efficiently allocated to the periplasmic Cu-Zn super-oxide dismutase SodC;<sup>21,22</sup> and (vi) a part of a genomic island that is silenced as a response to metal stress in the parent strain AE104 is un-silenced again in the  $\Delta zupT$  mutant.<sup>23</sup> So, the effect of the single  $\Delta zupT$  gene deletion revealed interesting phenomena upon close inspection. We ask here what more can be learned if all seven currently known secondary metal uptake systems are removed from the *C. metallidurans* cell.

## Results

### Deletion of seven genes for metal uptake systems decreases fitness of the resulting $\Delta 7$ mutant strain

All seven recognizable genes for secondary metal uptake systems (*zupT*, *pitA*, *corA<sub>1</sub>*, *corA<sub>2</sub>*, *corA<sub>3</sub>*, *zntB*, *hoxN*) were deleted from the

chromosome of the plasmid-free *C. metallidurans* strain AE104. This plasmid-free derivative was used as parent instead of *C. metallidurans* CH34 wild type to avoid interference through the plasmid-encoded metal efflux systems, and because the *czc*-containing plasmid pMOL30 was unstable in a  $\Delta zupT$  mutant.<sup>21</sup> Starting with the five genes *zupT*, *pitA*, *corA<sub>1</sub>*, *corA<sub>2</sub>* and *corA<sub>3</sub>*, all ten combinations of double mutants and four of the five possible quadruple mutants but only two triple mutants were constructed. During construction of the mutants, it was only once possible to obtain a “clean” (antibiotic marker-free deletion)  $\Delta corA_1$  deletion, in this case from the  $\Delta zupT$   $\Delta corA_2$   $\Delta corA_3$  triple deletion strain leading to the  $\Delta zupT$   $\Delta corA_1$   $\Delta corA_2$   $\Delta corA_3$  quadruple mutant, designated  $\Delta 4$ . In all other cases, the *corA<sub>1</sub>* gene had to be interrupted by insertion of a kanamycin resistance cassette. The “clean” quadruple deletion strain  $\Delta 4$  was the parent of  $\Delta 5$  (= $\Delta 4$  *ApitA*),  $\Delta 6$  (= $\Delta 4$  *ApitA* *ΔzntB*) and finally  $\Delta 7$  (= $\Delta 4$  *ApitA* *ΔzntB* *hoxN* =  $\Delta zupT$  *ApitA*  $\Delta corA_1$   $\Delta corA_2$   $\Delta corA_3$  *ΔzntB* *hoxN*).

The fitness of the mutant strains  $\Delta zupT$ ,  $\Delta 5$ ,  $\Delta 6$  and  $\Delta 7$  was compared to that of the parent strain AE104 in Tris-buffered mineral salts medium (TMM) without added metals. As already reported,<sup>22</sup> the  $\Delta zupT$  strain showed an extended lag phase compared to strain AE104 (Fig. 1A and Fig. S1A, ESI†). The lag phase of the  $\Delta 7$  ( $\Delta zupT$  *ApitA*  $\Delta corA_1$   $\Delta corA_2$   $\Delta corA_3$  *ΔzntB* *hoxN*) mutant strain was even more extended, while growth curves of the  $\Delta 5$  ( $\Delta zupT$  *ApitA*  $\Delta corA_1$   $\Delta corA_2$   $\Delta corA_3$ ) and  $\Delta 6$  ( $\Delta zupT$  *ApitA*  $\Delta corA_1$   $\Delta corA_2$  *ΔzntB*) mutants were not different from that of the  $\Delta zupT$  strain. While removal of the

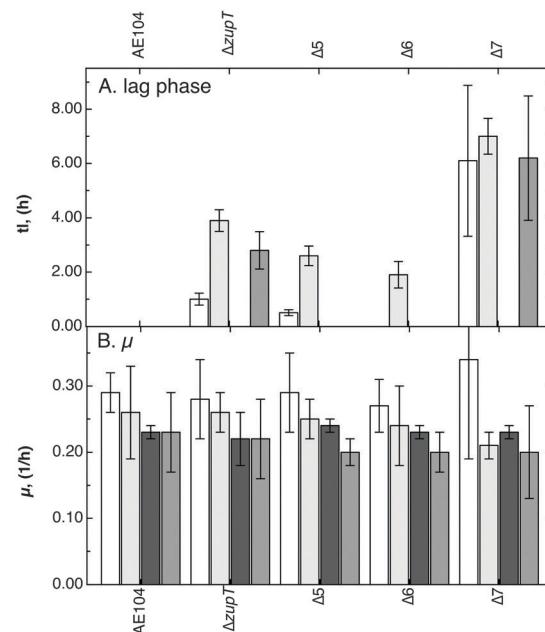


Fig. 1 Growth impairment of the mutant strains. The strains AE104,  $\Delta zupT$ ,  $\Delta 5$ ,  $\Delta 6$  or  $\Delta 7$  were cultivated in TMM at 30 °C without further additions (white bars) or in the presence of 10  $\mu$ M Co(II) (light grey bars), 10  $\mu$ M Zn(II) (dark grey bars), or 2.5  $\mu$ M Cd(II) (medium grey bars) and growth was measured as turbidity at 600 nm. The duration of the lag phase (panel A) and the growth rates (panel B) were calculated. Growth curves in Fig. S1 (ESI†), data for the lag phase and growth rate in a table below Fig. S1 (ESI†).  $n \geq 3$ , deviation bars indicated.



important zinc importer ZupT decreased fitness of *C. metallidurans* to a first level, the cells were able to compensate for deletion of an additional 5 genes leading to the  $\Delta 6$  strain but not that of the 6th gene leading to the  $\Delta 7$  strain. This decreased fitness to a second level, highlights the importance of the battery of seven metal importers for metal homeostasis of *C. metallidurans*.

### Metal content of the deletion strains

The cells of the  $\Delta 7$  multiple deletion strain still contained all essential metals, clearly indicating that additional metal import systems must exist in *C. metallidurans* (Fig. 2 and Table S1, ESI $\dagger$ ). These are likely to be primary importers or members of currently unknown uptake systems.

As published,<sup>21</sup> deletion of *zupT* resulted in a decreased zinc content visible in all  $\Delta zupT$ -containing multiple deletion strains up to  $\Delta 7$  (Fig. 2). Except in these  $\Delta zupT$  mutants, *C. metallidurans* cells were always able to compensate for the loss of a metal uptake system by adopting other systems, which altered the cellular metal content to some degree. First, also as published,<sup>13</sup> deletion of *pitA* led to a 3-fold increase in magnesium (and phosphorus) content in the  $\Delta zupT \Delta pitA$  double deletion strain of AE104. An increased magnesium content was also a feature of all investigated  $\Delta pitA$ -containing multiple deletion strains up to  $\Delta 7$  (Fig. 2). Second, deletion of *pitA* in  $\Delta 4$  leading to  $\Delta 5$  additionally increased the zinc, but decreased the iron, copper and nickel content. This indicated some re-arrangement of metal import of  $\Delta 5$  compared to  $\Delta 4$  as a result of the *pitA* deletion. Importers of magnesium and zinc were increasingly used in the  $\Delta 5$  strain while the decreased iron, copper and nickel content did not stimulate additional import of these metals, *e.g.* by up-regulation of iron import pathways *via* FurA or FurB.<sup>17</sup> Third, while deletion of *zntB* from  $\Delta 5$  (leading to  $\Delta 6$ ) had no significant effect on the metal content (Fig. 2), deletion of *hoxN* in  $\Delta 6$  (leading to  $\Delta 7$ ) increased the magnesium content, restored the iron and copper content to the higher and the zinc content to the lower level of the  $\Delta 4$  strain.

The cellular cobalt content decreased when *pitA* was deleted from the parent strain AE104. Together with the lowered iron, copper and nickel content of the  $\Delta 5$  strain ( $=\Delta 4 \Delta pitA$ ) compared to  $\Delta 4$ , this indicated that PitA may be an important contributor of iron, copper, nickel and cobalt in the form of the metal:phosphate complexes to *C. metallidurans*.

### Metal resistance of the deletion strains

The deletion strains up to  $\Delta 7$  were still able to import all essential transition metals, probably due to use of other uptake pathways (Fig. 2 and Table S1, ESI $\dagger$ ). All deletion mutants of the parent strain AE104 still contained the genes for the metal-exporting  $P_{IB2}$ -type ATPases ZntA and CadA, for three copper-exporting  $P_{IB1}$ -type ATPases such as CupA, and the two CDF proteins DmeF and FieF, which together should maintain a minimum zinc, cadmium, copper, cobalt and nickel resistance.<sup>7</sup> If efficient physiological function of these efflux systems requires a balanced import of metals by the seven secondary importers, successive removal of importers should lead to increased

imbalance in the import of transition metals, eventually to incompetence of the efflux systems, and ultimately to loss of metal resistance. To test this hypothesis, metal resistance of the deletion strains was analyzed in growth curves, liquid culture and on solid agar medium.

On solid TMM there was no significant effect in any of the studied multiple deletion strains on zinc, cadmium or copper resistance (Table S2, ESI $\dagger$ ). Nickel resistance of all  $\Delta corA_1$ -containing multiple mutant strains was enhanced. In addition, the  $\Delta zupT \Delta pitA \Delta corA_2 \Delta corA_3$  mutant, which still possessed a functional *corA1* gene, was also more resistant to nickel than the parent strain AE104, while its direct parent, the triple mutant  $\Delta zupT \Delta pitA \Delta corA_3$ , was not (Table S2, ESI $\dagger$ ). Together with the low nickel content of the  $\Delta 4$  and  $\Delta 5$  mutant strains cultivated in non-amended TMM (Fig. 2), this indicated that CorA<sub>1</sub> was involved in nickel uptake but needed the CorA<sub>2</sub> protein for full function. Due to the fact that CorA-like proteins are pentamers<sup>24</sup> and TMM-grown *C. metallidurans* cells contain  $117 \pm 8$  copies of CorA<sub>1</sub> (Rmet\_3052) per cell,  $38 \pm 19$  copies of CorA<sub>2</sub> (Rmet\_0036) and  $37 \pm 5$  copies of CorA<sub>3</sub> (Rmet\_3287), yielding a  $3.2 \pm 0.2 : 1.0 \pm 0.5 : 1 \pm 0.1$  ratio of CorA<sub>1</sub>:CorA<sub>2</sub>:CorA<sub>3</sub>,<sup>22</sup> this could mean that the CorAs of *C. metallidurans* form heteromultimers, as has been described for other metal transporters.<sup>25</sup>

The determination of the MIC values to cobalt in all  $\Delta zupT \Delta corA$  and  $\Delta pitA \Delta corA$ -containing double mutants were hampered by high deviations of the individual measurements. Cobalt resistance of two double mutants,  $\Delta corA_1 \Delta corA_2$  and  $\Delta corA_2 \Delta corA_3$  was very low, while that of the *corA<sub>123</sub>* triple mutant was higher than that of the parent strain AE104. Resistance of the three single  $\Delta corA$  deletion strains was similar to the AE104 level (Table S2, ESI $\dagger$ ). This could mean that a putative heteropentameric complex composed of two CorA species is able to function if PitA and ZupT are additionally present, whereas the presence of CorA<sub>3</sub> or CorA<sub>1</sub> alone results in a strongly decreased cobalt resistance. The three CorAs of *C. metallidurans* could be assigned to the import of nickel and cobalt, maybe in the form of a heteromultimeric complex CorA<sub>123</sub>, which interacts with ZupT and PitA as main metal supply routes in *C. metallidurans*.

Analysis of metal resistance of the mutants up to  $\Delta 5$  on solid growth medium did not yield further insights and the study was continued using liquid TMM together with low concentrations of Zn(II), Co(II) or Cd(II) (Fig. 1 and Fig. S1, ESI $\dagger$ ). The extended lag phase of the  $\Delta zupT$  mutant compared to the parent AE104 increased when  $10 \mu\text{M}$  Co(II) or  $2.5 \mu\text{M}$  Cd(II) was added while addition of  $10 \mu\text{M}$  Zn(II) ameliorated the effect of the  $\Delta zupT$  deletion. Growth of the  $\Delta 5$  and the  $\Delta 6$  mutant strains was similar to that of the  $\Delta zupT$  strain or was even better in the case of added cadmium. As in the case of non-amended medium (Fig. 1), deletion of *pitA*, *corA<sub>123</sub>* and *zntB* in addition to  $\Delta zupT$  did not impair fitness of the *C. metallidurans* strains further because the cells were able to compensate for the loss of these importers. However, in  $\Delta 7$  resistance to cobalt and cadmium was significantly decreased (Fig. 1 and Fig. S1, ESI $\dagger$ ), while zinc resistance was not so strongly affected. Addition of  $10 \mu\text{M}$  Zn(II) partially restored fitness of the multiple deletion strains up to  $\Delta 7$ .

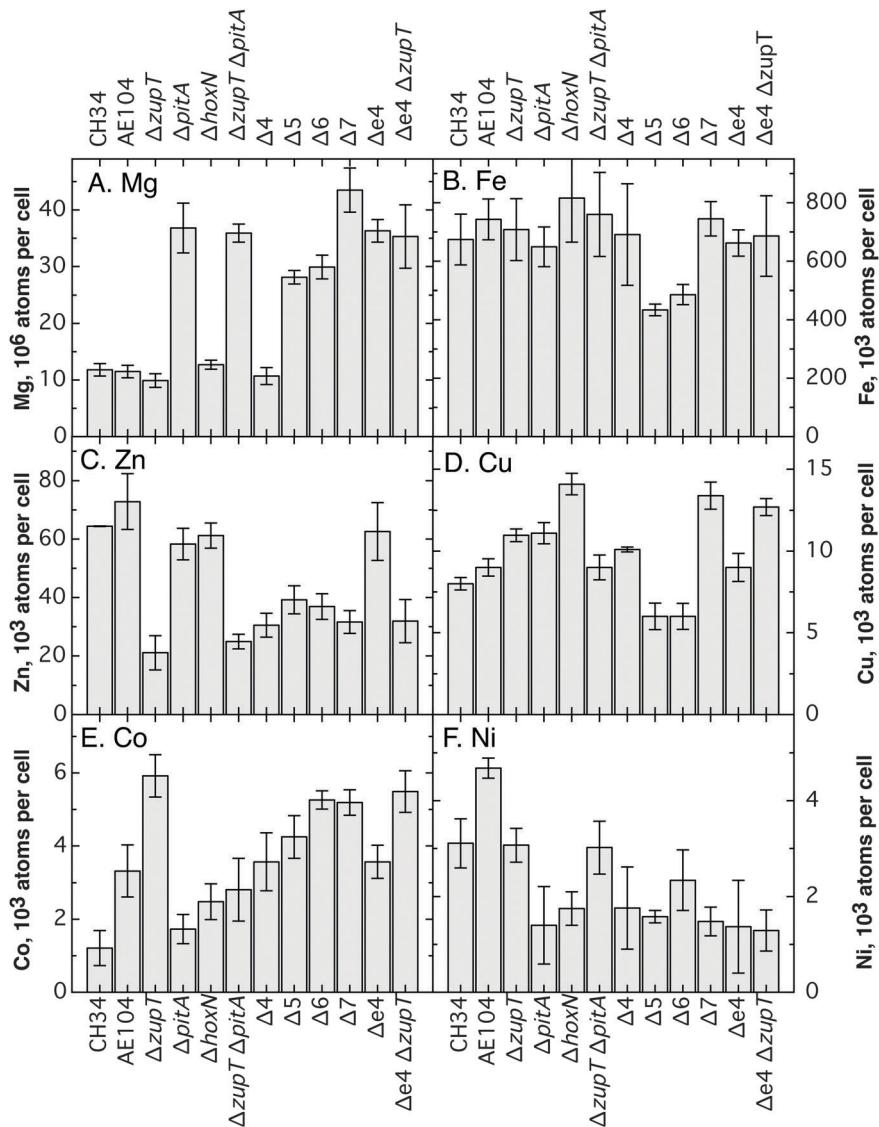


Fig. 2 Metal content of *C. metallidurans* strains. As determined by ICP-MS. The data are also shown in Table S1 (ESI†).

The mutant cells were cultivated in the presence of higher concentrations of transition metals in liquid TMM in dose-response experiments and the  $IC_{50}$  values were calculated (Fig. 3 and Table S3, ESI†). These experiments (up to 20 h) revealed more effectively the differences in metal resistance between the mutant strains than the long-term (up to 3 days) MIC determinations. Zinc, cobalt and cadmium resistance of the  $\Delta zupT$  strain was decreased compared to the parent strain, and that of the  $\Delta 7$  strain was decreased compared to  $\Delta zupT$  (Fig. 3).

During the course of the deletion analysis it was observed that zinc resistance decreased in series from the parent strain AE104 to the  $\Delta zupT$  and to the  $\Delta pitA$  mutants (Fig. 3A). In the case of the  $\Delta zupT \Delta pitA$  mutant resistance remained at the  $\Delta zupT$  level and increased to the level of the parent strain again in  $\Delta 4$  ( $\Delta zupT \Delta corA_{123}$ ). Decreased zinc resistance in the  $\Delta pitA$  mutant strain was mediated partially by ZupT and decreased zinc resistance in the  $\Delta zupT$  strain by CorA<sub>123</sub>. Zinc resistance

of the  $\Delta 5$  strain ( $\Delta zupT \Delta pitA \Delta corA_{123}$ ) was similar to that of the  $\Delta pitA$  strain demonstrating that the interplay between these uptake systems, which substituted for each other, and the efflux systems was required for resistance to upper  $\mu\text{M}$  Zn(II) concentrations. Deletion of  $zntB$  in  $\Delta 5$  generating  $\Delta 6$  increased zinc resistance again. ZntB was partially responsible for the low zinc resistance level of the  $\Delta 5$  strain, although this deletion did not change the zinc content of TMM-grown cells (Fig. 2C). Zinc resistance finally decreased to the lowest level in the  $\Delta 7$  strain following deletion of  $hoxN$ , probably due to use of additional unknown zinc uptake systems that were not in balance with the efflux systems. This demonstrated that all seven systems were involved in zinc homeostasis, either as probable zinc importers (CorA<sub>123</sub> in  $\Delta zupT$ , ZntB in  $\Delta 5$ ) or by interfering with activation of other uptake routes.

A second series of deletion mutants in the order  $\Delta hoxN \rightarrow \Delta zupT \rightarrow \Delta zntB \rightarrow \Delta pitA$  also demonstrated this order of decreasing zinc resistance (Fig. 3A). Zinc resistance decreased

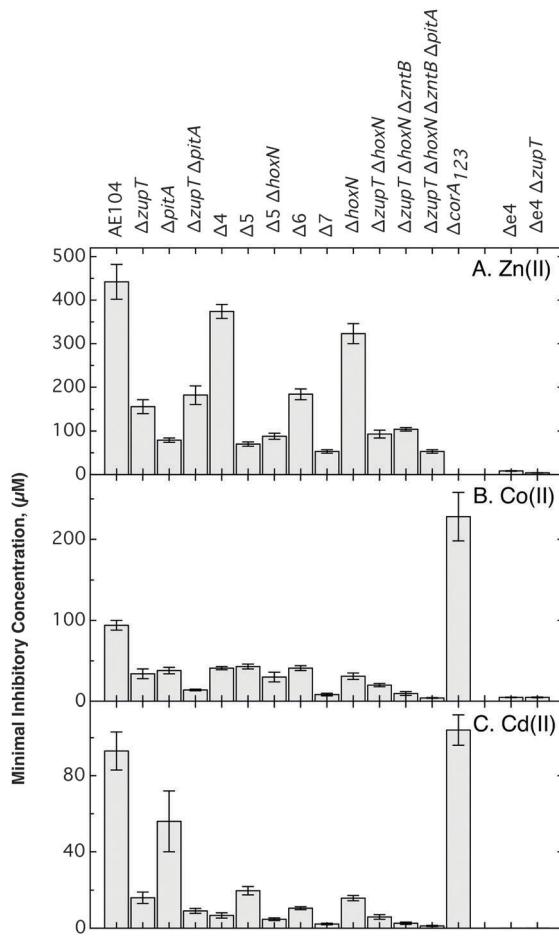


Fig. 3 Metal resistance of deletion strains in liquid culture. Dose response experiments were performed ( $n > 3$  per conditions) in the presence of Zn(II) (panel A), Co(II) (panel B) and Cd(II) (panel C), and the IC<sub>50</sub> values calculated. The data are also shown in Table S3 (ESI<sup>†</sup>) for a better documentation of the differences in cobalt and cadmium resistance of the mutant strains.

in this order except when *zntB* was deleted in the  $\Delta zupT \Delta hoxN$  double mutant. The presence of ZntB was not required when PitA and CorA<sub>123</sub> were still present, and it was recruited only in the Δ5 strain. These results also defined a ranking order of the zinc importers: first, PitA because *ApitA* had the strongest effect on zinc resistance and zinc resistance of Δ4 was close to that of the parent strain AE104; second, ZupT because of the required supply of zinc to zinc-dependent proteins; third, CorA<sub>123</sub> which was partially responsible for zinc sensitivity of the  $\Delta zupT$  strain; fourth, ZntB as metal importer in Δ5; and finally HoxN.

Additionally, cobalt and cadmium resistance decreased with each step along the second deletion series. With one exception, resistance to cadmium also decreased along the first deletion series (Fig. 3B, C and Fig. S3 (ESI<sup>†</sup>), upper part): it increased about three-fold when *pitA* was deleted from the Δ4 strain leading to Δ5 so that PitA was responsible for cadmium imbalance in the Δ4 strain, indicating that PitA also imported cadmium:phosphate.

Cobalt resistance was especially low in the  $\Delta zupT \Delta pitA$  double mutant and increased again when the *corAs* were deleted.

Similar to what was observed with the experiments on solid TMM, the three CorAs seemed to be important cobalt uptake systems. In agreement with this, cobalt resistance of the ΔcorA<sub>123</sub> triple mutant was twice as high in liquid culture as that of the parent strain AE104 (Fig. 3B).

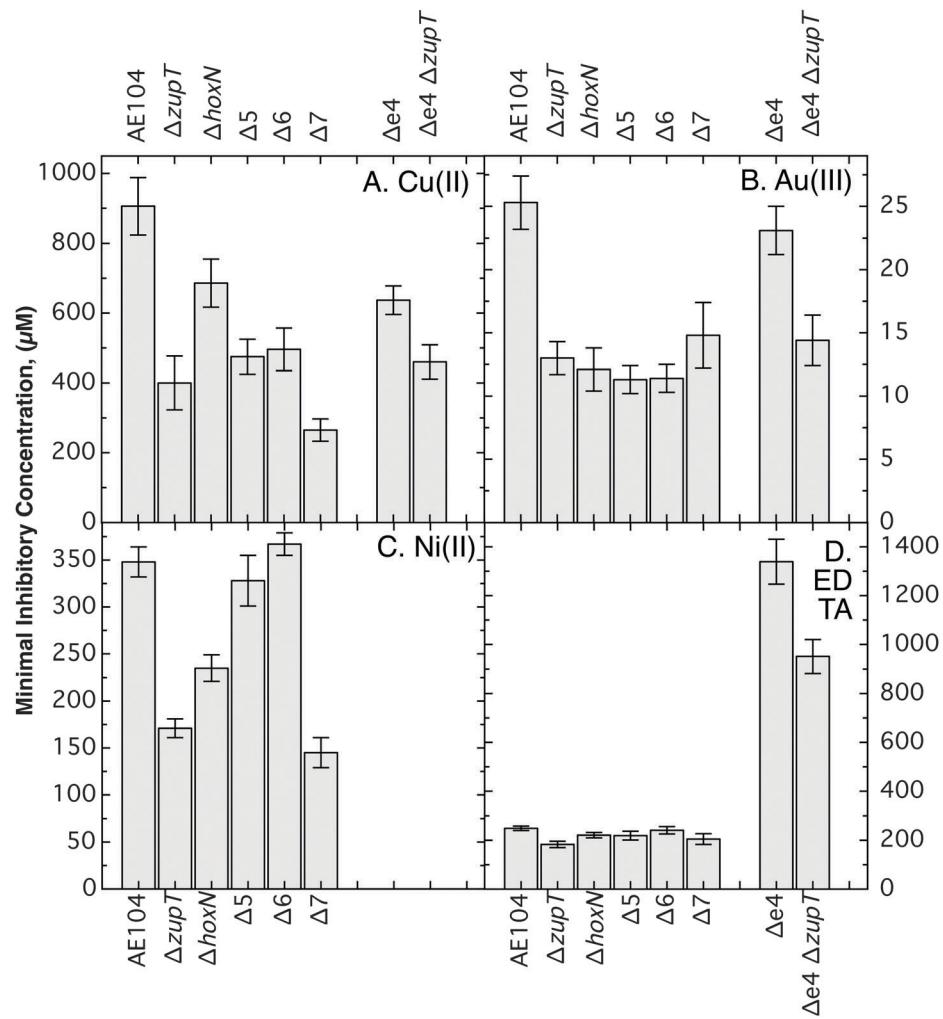
Since the cobalt resistance level of most mutant strains was so surprisingly low, resistance of the uptake mutants was also compared to that of the quadruple efflux deletion mutant Δe4 (=ΔzntA ΔcadA ΔdmeF ΔfieF) and to that of the Δe4 ΔzupT mutant (Fig. 3). The IC<sub>50</sub> of Δe4 for zinc was only 8 μM, much lower than that of the Δ7 mutant which had an IC<sub>50</sub> of 53 μM. Deletion of *zupT* always decreased zinc resistance down to 1/2 or 1/3, in the AE104 background from 440 μM to 156 μM, in the ΔhoxN background from 323 μM to 93 μM, and in the Δe4 background from 8.3 to 3.9 μM. This occurred despite the fact that the cellular zinc content decreased (Fig. 2) and addition of zinc ameliorated the retarded growth of the  $\Delta zupT$  mutant strain (Fig. 1). Because the CorAs were responsible for the decreased zinc resistance in the  $\Delta zupT$  single mutant (Fig. 3), the cells were able to partially compensate for the loss of ZupT by use of the CorAs, which led to imbalanced zinc import that could not be compensated by the metal efflux systems.

Cobalt resistance of the Δe4, Δe4 ΔzupT and of the  $\Delta zupT \Delta hoxN \Delta zntB \Delta pitA$  quadruple mutant was similar with an IC<sub>50</sub> of 4 to 5 μM Co(II). It doubled when the *corAs* were deleted in the quadruple mutant, leading to Δ7 (Fig. 3 and Table S3, ESI<sup>†</sup>). Again, balanced import was required to allow efficient export. Cadmium resistance of the Δe4 mutant was 60-times lower than that of the most sensitive  $\Delta zupT \Delta hoxN \Delta zntB \Delta pitA$  quadruple mutant showing the greater importance of the efflux systems compared to the uptake systems in resistance to this “toxic-only” metal cation. A balanced metal import and export was important for the “essential-but-also-toxic” metals but efflux was the main contributor to resistance to the “toxic-only” cadmium cation.

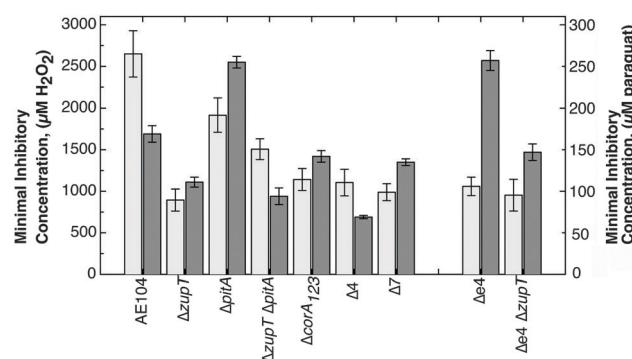
The Δ7 strain also displayed decreased resistance to copper, gold and nickel compared to strain AE104 (Fig. 4). While copper resistance of the  $\Delta zupT$ , Δ5 and Δ6 strains was on the same level in-between Δ7 and AE104, nickel resistance increased from  $\Delta zupT$  to Δ5 and Δ6, and decreased again from Δ6 to Δ7 as a result of the deletion of the nickel importer HoxN. Gold resistance of all mutant strains was similar and lower than that of the parent strain AE104 (Fig. 4). Resistance to the metal-complexing component EDTA decreased due to the  $\Delta zupT$  deletion in the AE104 parent strain and in the Δe4 efflux mutant. Here, in the absence of efflux systems, EDTA resistance was 5-times higher than in AE104 (Fig. 4).

When oxidative stress resistance of the mutant strains was examined as a marker of general stress tolerance (Fig. 5), Δ7 was as resistant to H<sub>2</sub>O<sub>2</sub> and paraquat as the  $\Delta zupT$  mutant. The ΔpitA and Δe4 strains demonstrated a higher paraquat, but not H<sub>2</sub>O<sub>2</sub>, resistance compared to the parent strain, which was decreased again by additional deletion of *zupT*.<sup>22</sup> This demonstrates further the importance of ZupT for oxidative stress resistance.

These data clearly demonstrate that the interaction between metal uptake and efflux systems, and not only the activity of the



**Fig. 4** Metal resistance of deletion strains in liquid culture. Dose response experiments were performed ( $n > 3$  per conditions) in the presence of Cu(II) (panel A), Au(III) (panel B), Ni(II) (panel C) and EDTA (panel D), and the  $IC_{50}$  values calculated. Bold-faced values indicate significant deviations from metal resistance of the direct parent strain. The data are also shown in Table S4 (ESI†) for a better visibility of the differences between the strains.



**Fig. 5** Oxidative stress resistance of deletion strains in liquid culture. Dose-response experiments were performed ( $n > 3$  per conditions) with  $H_2O_2$  (light grey bars) and paraquat (dark grey bars), and the  $IC_{50}$  values calculated. The data are also shown in Table S5 (ESI†).

uptake systems, is responsible for a balanced supply of “essential-but-also-toxic” metals to *C. metallidurans*. Deletion of *zupT* resulted in decreased fitness of the mutant and use of other metal import

systems, which subsequently resulted in imbalanced metal import and decreased overall metal resistance. PitA was involved in homeostasis of all metals tested, while the three CorAs were required for cobalt and nickel homeostasis but they were also responsible for the decreased zinc sensitivity of the  $\Delta zupT$  mutant. Up to the  $\Delta 5$  and  $\Delta 6$  mutant, the fitness level of the mutant strains could be kept at that of the  $\Delta zupT$  mutant but at the cost of increasing problems in handling higher ( $\mu M$ ) concentrations of metals. This was first accomplished by PitA and the CorAs complementing loss of ZupT, subsequently the action of ZntB that was responsible for the low zinc resistance of the  $\Delta 5$  strain, and finally of HoxN. When all seven systems were inactivated, overall fitness and metal resistance reached a low level, despite the presence of the metal efflux systems.

#### Regulation of expression of the genes for the secondary uptake systems

As published,<sup>13</sup> expression of a *zupT-lacZ* reporter gene fusion was up-regulated under zinc starvation conditions, that of *corA1-lacZ* was down-regulated by increasing magnesium concentrations

and that of *pitA-lacZ* by increasing phosphate concentrations. During the strategic construction of the deletion series, the *lacZ* gene was inserted downstream of all seven transporter genes mentioned in this study, always leaving the open reading frame of the transporter gene intact. These fusions were constructed in strain AE104 and several mutant strains, and regulation of expression of the reporter gene by divalent transition metal cations and EDTA was measured. With three exceptions, this did not yield any insights or differences in the regulatory patterns between the strains (data not shown). Exception 1 was the already known global down-regulation of most genes by increasing zinc concentrations, as published.<sup>13</sup>

Exception 2 concerned expression of *zntB-lacZ*, which was introduced into AE104,  $\Delta$ *zupT*,  $\Delta$ 5 (Fig. 6) and  $\Delta$ *pitA* (data not shown). In all these mutants, addition of zinc chloride up to 1 mM Zn(II) decreased expression of the reporter construct by half. Addition of cadmium at 0.5 mM Cd(II) decreased *zntB-lacZ* expression down to 25% of the value without added metals. No difference was visible in *zntB-lacZ* expression between the AE104,  $\Delta$ *zupT* and  $\Delta$ 5 derivatives (Fig. 6). EDTA, Co(II), or Mn(II) had no effect but Cu(II) also resulted in a down-regulation of *zntB-lacZ* expression (data not shown). Decreased expression at the high metal cation concentrations could also result from metal toxicity in addition to the metal-dependent changes. ZntB was regulated as would be expected for an uptake system, and was down-regulated when high concentrations of thiol-binding metals such as cadmium and copper were challenging the cells. Regulation was independent of the presence of *ZupT*, *PitA* or *CorA<sub>123</sub>*. Although ZntB was involved in the low zinc resistance level of the  $\Delta$ 5 strain, its gene was not up-regulated, nor in the double, triple or quadruple mutants tested. As the altered cellular metal content and metal resistance indicated that ZntB, the CorAs and PitA were subsequently used in the respective mutant strains, activation may have been on various levels, *e.g.* the translational level, by flux control or other post-transcriptional mechanisms in addition to the already published changes in *zupT* and *pitA* expression.<sup>13,17</sup> This indicated a cross-talk between PitA, *ZupT*, *CorA<sub>123</sub>* and ZntB, which organized metal import into *C. metallidurans* cells and was flexible enough to compensate for loss of some of these proteins, albeit not at higher metal concentrations.

The third exception was revealed by the *hoxN-lacZ* expression pattern (Fig. 6B): the gene was not regulated by EDTA, nickel or cobalt in all tested strains (data not shown), and was not regulated by zinc in strain AE104 (Fig. 6B), staying at a low specific activity of  $9.4 \pm 2.7$  U mg<sup>-1</sup> dry mass. In  $\Delta$ *zupT* and  $\Delta$ 5, *hoxN-lacZ* was expressed on a low level but 2-fold up-regulated with increasing zinc concentrations up to 1 mM. Surprisingly, the operon fusion was strongly expressed in the  $\Delta$ 6 strain at  $81 \pm 5$  U mg<sup>-1</sup> and down-regulated 1.6-fold with increasing zinc concentrations (Fig. 6B). It is likely that HoxN was recruited for metal import but only in the  $\Delta$ 6 mutant when all other known secondary metal import systems were inactivated.

Although metal uptake systems were removed stepwise up to  $\Delta$ 5, the mutant cells remained at the fitness level of the  $\Delta$ *zupT* mutant by use of substitute importers, members or non-

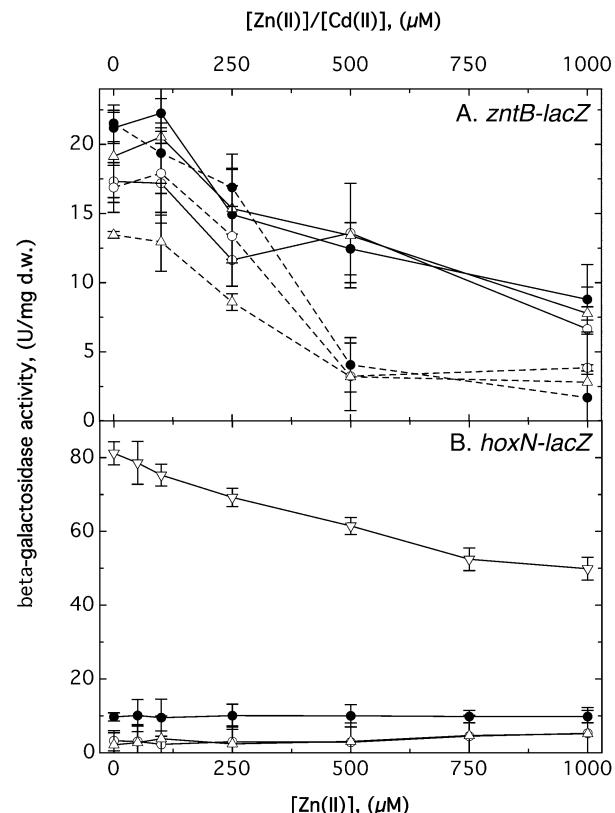


Fig. 6 Regulation of *zntB*- and *hoxN-lacZ* fusions in *C. metallidurans*. Exponentially growing cells of strain AE104 (closed circles, ●),  $\Delta$ *zupT* (open circles, ○),  $\Delta$ 5 (open triangles, Δ) or  $\Delta$ 6 (open inverted triangle, ▽, only in panel B), all carrying a *lacZ*-fusion with the full-length *zntB* (panel A) or *hoxN* (panel B) gene, were divided into parallel cultures and zinc chloride (solid lines) or cadmium chloride (dashed lines) was added. Incubation was continued with shaking at 30 °C and the specific activity of the  $\beta$ -galactosidase was determined after 3 h.

members of the group of seven studied transport systems, but metal resistance decreased along the series of deletion strains despite the presence of metal efflux systems. In  $\Delta$ 6, increased expression of *hoxN* was required for the strain to remain at the  $\Delta$ *zupT* fitness level. When *hoxN* was also gone, fitness and metal resistance decreased a second step to the level of the  $\Delta$ 7 mutant strain.

#### Intracellular zinc homeostasis

Deletion of *zupT* had two main effects: zinc could not be supplied efficiently to the zinc-dependent beta-prime subunit RpoC of the RNA polymerase, leading to accumulation of RpoC in inclusion bodies; and the central subunit CzcA of the CzcCBA transenvelope complex for cobalt, zinc, cadmium resistance could not be maintained in the cells although *czcA* was transcribed and constitutively expressed.<sup>21,22</sup> Both effects were used here as reporters for the ability of the mutant strains to maintain their intracellular zinc homeostasis.

Deletion of *zupT* always resulted in loss of the CzcA band, which was visible in the parent strain AE104 and the  $\Delta$ *pitA* single deletion strain (Fig. 7). This corresponded to a zinc content below 40 000 atoms per cell (Fig. 2) in all these strains.

The situation was more complicated concerning zinc allocation to RpoC. If this ability was disturbed, as in the  $\Delta zupT$  single mutant, RpoC protein was transferred to inclusion bodies and the RpoC band was very intense in the Coomassie-stained polyacrylamide gels after separation of crude extracts (Fig. 7A, lane 2). Surprisingly, and accompanied by a decrease of the cellular zinc content from 73 000 to 58 000 atoms per cell (Fig. 2), probably misfolded RpoC also accumulated in the  $\Delta pitA$  strain (Fig. 7B, lane 3). In contrast, extracts of the  $\Delta zupT$   $\Delta pitA$  double and the  $\Delta 4$  quadruple mutant did not display a strong RpoC band, indicating efficient zinc allocation. Starting with the  $\Delta 5$  mutant, crude extracts of all subsequent strains exhibited a strong RpoC band ( $\Delta 5$  and  $\Delta 6$  in lanes 6 and 7 of Fig. 7A,  $\Delta 7$  not shown). Allocation of zinc to RpoC was efficient in the parent strain AE104, hampered when ZupT or when PitA were removed, efficient again when only CorA<sub>123</sub> or only PitA was present, and no longer efficient in the subsequent higher order deletion strains. This could mean some negative interference between PitA and CorA<sub>123</sub> on the one hand and of ZupT and CorA<sub>123</sub> on the other, which prevented zinc allocation in the  $\Delta zupT$  and  $\Delta pitA$  mutant, respectively. On the other hand, PitA or CorA<sub>123</sub> alone were able to substitute for ZupT when zinc allocation to RpoC was concerned, but their activity did not allow the stable presence of CzcA.

In agreement with a high cellular zinc content and resistance to EDTA, the  $\Delta e4$  efflux mutant synthesized the CzcA protein and folded RpoC properly to prevent aggregation of this protein in inclusion bodies (Fig. 7, lanes 8). When *zupT* was deleted in  $\Delta e4$  and the zinc content dropped to 32 000 atoms per cell,

zinc allocation to RpoC was still efficient (Fig. 7A, lane 9), indicating that the negative interference of PitA and CorA<sub>123</sub> did not occur in the  $\Delta e4$  efflux mutant strain. The CzcA band was visible in Western blots of fresh cultures of the  $\Delta e4$   $\Delta zupT$  mutant strains (data not shown) but similar to the situation in strain AE104 and its  $\Delta zupT$  mutant, zinc resistance, as determined as IC<sub>50</sub> values, decreased after more than 5 transfers on strain collection plates (Fig. S3, ESI†) and CzcA was no longer present in the cells (Fig. 7B, lane 9). While other import pathways could complement for a missing ZupT importer when it comes to cytoplasmic zinc allocation, the long-term presence of CzcA strictly depended on the presence of ZupT if the cells were not kept on media with increased zinc content.

## Discussion

Heavy metal resistance can be defined as the ability to maintain metal homeostasis in the presence of high concentrations of metals or metal mixtures. In *C. metallidurans*, the plasmid-encoded RND-driven transenvelope systems such as CzcCBA and CnrCBA are responsible for the high resistance level to zinc and nickel, respectively.<sup>27–29</sup> It is highly likely that efflux by these transenvelope protein complexes pre-adjusts the periplasmic metal content for subsequent uptake into the cytoplasm, or they transport metal ions to the outside that were previously exported from the cytoplasm to the periplasm by efflux systems of the inner membrane,<sup>5,6</sup> such as P-type ATPases and CDF proteins. This demonstrates the importance of the metal transportome,<sup>30,31</sup> the totality of all metal export and import systems, to metal resistance and homeostasis in *C. metallidurans*. The combined actions of metal uptake and efflux systems might result in a kinetic flow-equilibrium of the transition metal concentration in both cellular compartments,<sup>32</sup> which adjusts individual concentrations and the composition of the overall mixture to prevent negative interference between “essential-but-also-toxic” metal cations such as Zn(II) and Ni(II).

*E. coli* does not possess CzcCBA- or CnrCBA-like efflux systems.<sup>33</sup> In this gut bacterium, a controlled shunt of zinc import and export systems seems to be important for zinc homeostasis. Import is by a variety of systems with broad substrate specificity such as ZupT,<sup>18</sup> complemented by the ABC transport system ZnuABC under zinc starvation conditions,<sup>14,15</sup> or alternatively by ZntA-dependent export when the metal is in surplus.<sup>34–36</sup> Expression of *znuABC* is under control of the Zur regulator of the Fur family of proteins,<sup>15,37</sup> that of *zntA* for the zinc-transporting P<sub>IB2</sub>-type ATPases regulated by the MerR member ZnrT.<sup>38</sup> While *zntA* expression is 50% up-regulated at about 1 fM “free” zinc in the cytoplasm, that of *znuABC* is 50% up-regulated at about 0.2 fM “free” zinc, and both systems are below 25% expression level at about 0.5 fM.<sup>39</sup> This equilibrium concentration of zinc in *E. coli* corresponds to a quota of 0.2 mM or 200 000 atoms per cell and a ratio of  $2.5 \times 10^{-12}$  between the zinc quota and “free” zinc. This can be explained by sequestration of Zn(II) in the cytoplasm by glutathione and zinc-binding proteins.<sup>22,40</sup> This means that the distribution of Zn(II) between zinc-binding sites

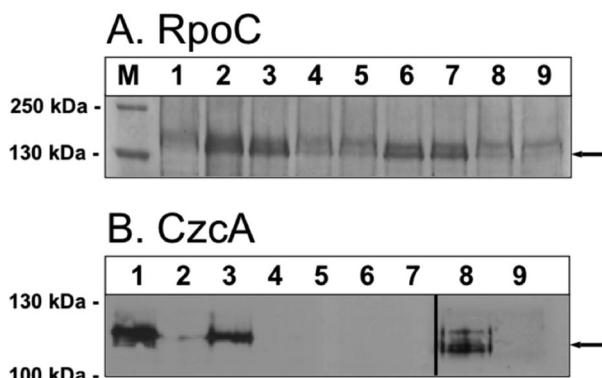


Fig. 7 Presence of RpoC and CzcA proteins in mutant strains of *C. metallidurans* AE104. Accumulation of the RpoC subunit of the RNA polymerase (panel A, arrow) was demonstrated in crude extracts in Coomassie-stained SDS gel.<sup>21</sup> Samples corresponding to 20  $\mu$ g cellular dry mass was used per lane. Only the part of gel between the 130 kDa and the 250 kDa marker band is shown; the complete gels serving as loading control are shown in Fig. S2 (ESI†). The plasmid pDNA130<sup>26</sup> that expressed the *czcCBAD* determinant constitutively in *C. metallidurans* cells had been transferred by conjugation into the mutant strains and their ability to produce the CzcA (panel B, arrow) central component of the CzcCBA efflux pump was determined in a Western blot using anti-CzcA antibodies, as published.<sup>21</sup> Marker (M); AE104 negative control (1);  $\Delta zupT$  positive control (2);  $\Delta pitA$  (3);  $\Delta zupT$   $\Delta pitA$  (4),  $\Delta 4$  (5);  $\Delta 5$  (6);  $\Delta 6$  (7);  $\Delta e4$  (8);  $\Delta e4$   $\Delta zupT$  (9). As indicated by the bar, the experiment shown in panel B lanes 8 and 9 was on a different gel than that in B lanes 1 to 7.



in ZntR, Zur and other proteins, *e.g.* those of the ribosome,<sup>41</sup> may contribute to maintenance of the zinc homeostasis by activating alternatively zinc import by ZunABC or export by ZntA, so that this shunt controls the level of zinc in the cytoplasm of *E. coli*.

*C. metallidurans* cells contain a minimum of 20 000 zinc per cell, with approximately 70 000 when cultivated in TMM without added zinc. When zinc is added to the medium there are 120 000 zinc per cell due to the action of the efflux systems, and the bacterium cannot grow when it harbors more than 250 000 zinc per cell.<sup>21</sup> This corresponds to a quota of 58  $\mu\text{M}$ , 204  $\mu\text{M}$ , 350  $\mu\text{M}$  and 728  $\mu\text{M}$ , respectively (assumed cell volume of 0.57 fL<sup>42</sup>), so that the zinc quota of *E. coli* and *C. metallidurans* are similar. Unexpectedly, no evidence for a shunt was found in *C. metallidurans*.<sup>13</sup> There is no ortholog in this bacterium for a ZnuABC uptake system but for a ZntA<sup>43</sup> efflux pump. While expression of *zupT* in *E. coli* was constitutive,<sup>18</sup> *zupT* in *C. metallidurans* was controlled by its Zur ortholog.<sup>13,17</sup> A main substrate and a role in basic resistance or high-level resistance could be assigned to all efflux systems in *C. metallidurans*,<sup>7</sup> however this could not be done with the studied secondary metal import systems.<sup>13</sup> This led to the hypothesis that a battery of redundant importers with low and overlapping substrate specificities was responsible for uptake of zinc and other transition metals in *C. metallidurans*.

Here, further evidence was found to support this hypothesis. It appears that *C. metallidurans* takes Zn(II), Ni(II) and Co(II) up as they become available. It relies on its efflux systems to remove surplus ions. Using the plasmid-encoded CzcCBA and CnrCBA systems it pre-adjusts the periplasmic metal composition, as well as concentration, prior to uptake across the inner membrane. The plasmid-free *C. metallidurans* derivative AE104<sup>27</sup> is in this aspect an anomaly because it is not able to perform this periplasmic pre-adjustment but is forced to import zinc, cobalt, and nickel "as they come along", while *E. coli* escapes this situation by using the ZnuABC/ZntA shunt for zinc, and maybe a similar shunt (NikABC/RcnA) for nickel.<sup>44,45</sup> This also explains why strain AE104 suffers from transition metal stress in the cytoplasm, resulting in silencing of genomic islands even in TMM without added metals.<sup>23</sup> However, it was not possible to construct similar deletion mutants in CH34 wild type because even deletion of *zupT* efficiently cures the *czc*-containing plasmid pMOL30,<sup>21</sup> providing evidence that the interplay of metal uptake and efflux systems is important in *C. metallidurans*.

When the genes for the seven secondary uptake systems were deleted, the cells were able to compensate for some of the consequences of this, revealing flexibility of the metal uptake transportome. However if any other were removed no compensation was observed, pointing to unique and important contributions of individual systems. Removal of all seven importers did not impair the overall metal content of the cells (Fig. 2) so that additional metal uptake systems must exist. Up to the quintuple deletion mutant ( $\Delta 5$ ) *lacZ* reporter gene fusions in various mutant strains did not show any up-regulation of the genes for the remaining uptake systems, nor was such a process evident on the transcriptome<sup>23</sup> or proteome<sup>22</sup> level of the  $\Delta zupT$

deletion strain, despite the obvious zinc starvation conditions this strain was experiencing.<sup>23</sup> Only in the sextuple deletion mutant,  $\Delta 6$ , was an altered expression pattern of the *hoxN* gene measured (Fig. 6B). This was due to recruitment of the predicted nickel importer HoxN for import and to compensate for the loss of the other systems. With the exception of HoxN, all the other known and unknown metal uptake systems were activated by means other than increased gene expression, such as flux control of, or protein–protein interaction with, already synthesized proteins. If this hypothesis is true, it would indicate the existence of a novel control mechanism of metal homeostasis in *C. metallidurans*.

Along the series of deletion mutants, the following observations were made: (i) deletion of *zupT* in the parent strain AE104 decreased fitness (Fig. 1); (ii) deletion of *corA* genes revealed an interplay of the CorA proteins (Table S2, ESI†); (iii) deletion of *pitA* increased the cellular magnesium content (Fig. 2) and influenced homeostasis of, and resistance to, most transition metals tested (Fig. 3). Nevertheless, deletion of these five genes did not decrease fitness (Fig. 1), only metal resistance (Fig. 3), indicating that use of other known (*e.g.* ZntB) or unknown importers compensated for the loss of the deleted genes. This also resulted in unbalanced metal import at  $\mu\text{M}$  metal concentrations that could no longer be compensated for by the efflux systems. Loss of *zntB* needed increased *hoxN* expression (Fig. 6B) to keep the fitness at the level of the  $\Delta zupT$  strain (Fig. 1) but this was no longer possible in the  $\Delta 7$  mutant (Fig. 1). Fitness and metal resistance dropped to a second, lower level (Fig. 1 and 3).

First, all these findings highlight the importance of ZupT among the other secondary metal uptake systems in *C. metallidurans*. ZupT was required in strain AE104 to allocate zinc to the client protein RpoC efficiently and this function could only partially be compensated for by an increased cellular zinc content in the  $\Delta zupT$  mutant.<sup>21</sup> Zinc imported by ZupT was required to allow a stable presence of the RND protein CzcA or the CzcCBA zinc efflux complex.<sup>21</sup> While ZupT was absolutely essential for the long-term presence of CzcA, PitA and the CorAs were able to mediate efficient allocation of zinc to RpoC, but only when the respective other importer was not present in the cell (Fig. 7). Moreover, this negative interference between PitA and the CorAs did not occur in the  $\Delta zupT$  mutant of the quadruple efflux mutant  $\Delta e4$ , which was able to fold RpoC efficiently but unable to harbor CzcA (Fig. 7). Replenishment of the zinc pool required for RpoC folding nevertheless was not sufficient to allow stable synthesis of CzcA.<sup>21</sup> PitA and the CorAs must somehow interact, *e.g.* on the hypothetical post-translational control level of metal homeostasis in *C. metallidurans*.

Second, the three CorAs are able to provide Zn(II) to RpoC in the  $\Delta zupT \Delta pitA$  mutant (Fig. 7). Moreover, CorA<sub>1</sub> and CorA<sub>2</sub> are involved in nickel import. CorA<sub>1</sub> and CorA<sub>3</sub> have something to do with cobalt toxicity although CorA<sub>123</sub> together functioned as cobalt importers. TMM-grown *C. metallidurans* cells contain  $117 \pm 8$  copies of CorA<sub>1</sub> (Rmet\_3052) per cell,  $38 \pm 19$  copies of CorA<sub>2</sub> (Rmet\_0036) and  $37 \pm 5$  copies of CorA<sub>3</sub> (Rmet\_3287), yielding a  $3.2 \pm 0.2 : 1.0 \pm 0.5 : 1 \pm 0.1$  ratio of CorA<sub>1</sub> : CorA<sub>2</sub> : CorA<sub>3</sub>.<sup>22</sup> Since CorA-like proteins are pentamers,<sup>24</sup> this could



mean that the CorA of *C. metallidurans* is actually a (CorA<sub>1</sub>)<sub>3</sub>-(CorA<sub>2</sub>)<sub>1</sub>(CorA<sub>3</sub>)<sub>1</sub> heteromultimer. CorA is the primary magnesium channel in *Salmonella*<sup>46</sup> and essential for virulence.<sup>47</sup> Metal uptake in the archaeum *Methanococcus jannaschii* is in the order Co(II) > Ni(II) > Mn(II) > Mg(II) > Ca(II).<sup>48</sup> Co(II) and Ni(II) – but not Ca(II) and Fe(II/III) transport – was also demonstrated for a homolog from the plant *Arabidopsis thaliana*.<sup>49</sup> In *C. metallidurans*, contribution of CorA<sub>123</sub> to cobalt, zinc, nickel, and cadmium import explains the metal-resistance phenotypes of the deletion mutants.

Import activity of CorA from *T. maritima* is under flux control. The pentameric complex is locked in a transport-incompetent conformation by allosteric, sequential binding of cytoplasmic Mg(II) cations to the five protomers.<sup>50</sup> Because the amino acids required for transport and flux control by gating are all conserved within the MIT protein family, eukaryotic homologs included, this flux-controlled uptake of magnesium seems to be a general mechanism.<sup>51</sup> This may indicate that the individual protomers of the possible heteromultimeric CorA<sub>123</sub> importer in *C. metallidurans* could be under different flux control regimes, CorA<sub>1</sub> by Mg(II), CorA<sub>2</sub> by Ni(II) and CorA<sub>3</sub> by Co(II). These CorA substrates were identified already a long time ago in *E. coli* and *Aerobacter (=Enterobacter) aerogenes*,<sup>52</sup> nickel uptake by a magnesium uptake system in *Cupriavidus necator* (=*Ralstonia eutropha*, *Alcaligenes eutrophus*)<sup>53</sup> and *C. metallidurans*.<sup>52,54</sup> Moreover, expression of *corA*<sub>1</sub> is up-regulated 3-fold under conditions of magnesium starvation while that of *corA*<sub>2</sub> and of *corA*<sub>3</sub> is slightly down-regulated,<sup>13</sup> so that the complex may change from a homopentameric CorA<sub>1</sub> under magnesium flux control at low (10  $\mu$ M) magnesium concentrations to a heteropentameric CorA<sub>123</sub> under combined magnesium, nickel and cobalt flux control at magnesium concentrations above 100  $\mu$ M. Heteromultimers with different functions have also been described for other metal transporters<sup>25</sup> so that this hypothesis should be tested.

Third, PitA was an important metal uptake system. In *E. coli*, PitA also seems to be involved in zinc uptake<sup>55</sup> and some strains of this bacterium even contain a paralog of PitA, PitB.<sup>56</sup> Consequently, mutations in *pitA* cause zinc resistance.<sup>57</sup> PitA also imports phosphate complexes with magnesium and calcium, and Zn(II) competes with Mg(II) for import by PitA.<sup>57</sup> This would be a very energy-efficient import route for essential micro- and macroelements. If, however, all 110 million phosphate molecules needed by *C. metallidurans*<sup>13</sup> would be imported as metal:phosphate complexes by PitA, far too many metal cations would be imported into the cytoplasm, e.g. 76 million Mg(II) with 11 million actually needed. These surplus cations would have to be exported again.

As an alternative for energy-dependent efflux of surplus Mg(II) and Ca(II), metal:phosphate import by the low-affinity PitA transporter could be synchronized with phosphate import by the high-affinity ABC-type import system PstABC, which transports non-metallated H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and HPO<sub>4</sub><sup>2-</sup>.<sup>58,59</sup> *C. metallidurans* contains 1700 copies of the periplasmic-binding component PstS. Its genome possesses genes for two additional paralogs of this protein, one synthesized in 100 copies per cell.<sup>22</sup>

This argues for a strong participation of PstABC in phosphate import in TMM-grown cells, which may have the role to prevent any over-accumulation of metal cations. This scenario agrees with the fact that PitA is up-regulated with increasing phosphate concentrations<sup>13</sup> starting at 100  $\mu$ M phosphate and reaching maximum expression at 5 mM phosphate: at such a high phosphate concentration, use of the energy-efficient PitA system would be much more attractive than importing phosphate by the “expensive” PstACB pathway.

Last, there are also conflicting data concerning the 4th member of the MIT protein family in *C. metallidurans*, ZntB. In *Salmonella*, mutations in its *zntB* gene conferred decreased zinc and cadmium resistance and capacity for zinc efflux was demonstrated.<sup>60</sup> The structure of ZntB is that of a pentameric funnel similar to CorA.<sup>61</sup> In *C. metallidurans*, however, a *zntB-lacZ* fusion was down-regulated with increasing zinc and especially cadmium concentrations (Fig. 6). This regulatory pattern agrees more with that of an importer than with an exporter. The consequence of a *zntB* deletion, even from the genome of the  $\Delta 5$  mutant with *ZupT*, PitA and CorA<sub>123</sub> removed, was minor with the exception of a two-fold increase in zinc and cadmium resistance. This assigned to ZntB the role of a minor zinc and cadmium importer in *C. metallidurans*. Expression of a *hoxN-lacZ* fusion increased in the  $\Delta 6$  strain compared to  $\Delta 5$ , indicating that additional deletion of *zntB* resulted in activation of the last known secondary import system with some potential in zinc import. Consequently, the  $\Delta 6$  strain performed not much differently from the  $\Delta zupT$  single deletion strain, while the growth problems of the  $\Delta 7$  (= $\Delta 6$  *hoxB*) were considerable.

## Experimental

### Materials and methods

**Bacterial strains and growth conditions.** Strains used for experiments were *C. metallidurans* CH34 wild type, its plasmid-free derivative strain AE104,<sup>27</sup> and further derivatives of these strains Table 1. Tris-buffered mineral salts medium<sup>27</sup> containing 2 g L<sup>-1</sup> sodium gluconate (TMM) was used to cultivate these strains aerobically with shaking at 30 °C. Solid TMM contained 20 g L<sup>-1</sup> agar. Analytical grade salts of metal chlorides or sodium-tetrachloroaurate(III) (NaAuCl<sub>4</sub>) (Sigma-Aldrich, USA or Alfa Aesar, Germany) were used to prepare 0.1 or 1 M stock solutions.

**Genetic techniques.** Standard molecular genetic techniques were used.<sup>28,62</sup> For conjugal gene transfer, overnight cultures of donor strain *E. coli* S17/1<sup>63</sup> 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 d, the bacteria were suspended in TMM, diluted, and plated onto selective media as previously described.<sup>28</sup> All primer pairs used were listed in Table S6 (ESI†). Plasmid pECD1003<sup>7</sup> was used to construct deletion mutants. It is a derivative of plasmid pECD889<sup>64</sup> and therefore a derivative of plasmid pCM184.<sup>65</sup> These plasmids harbor a kanamycin-resistance cassette flanked by *loxP* recognition sites. Plasmid pECD1003 additionally carries an exchange of 5 bp at each *loxP*-site. Using these mutant *lox*

sequences, multiple gene deletions within the same genome are possible without secondary recombination events.<sup>66,67</sup>

**Deletion mutants.** Fragments of 300 bp upstream and downstream of the target gene were amplified by PCR and cloned into vector pGEM T-Easy (Promega, Madison, Wisc., USA),

further cloned into plasmid pECD1003, and sequenced. The resulting plasmids were used in a double-cross-over recombination in *C. metallidurans* strains to replace the respective target gene with the kanamycin resistance cassette, which was also subsequently deleted by transient introduction of *cre*

**Table 1** Bacterial strains and plasmids

| Name                             | Relevant markers, TCDB of the product <sup>70</sup>   | Ref.       |
|----------------------------------|---|------------|
| Bacterial strains                |   |            |
| <i>Cupriavidus metallidurans</i> |   |            |
| AE104                            | Plasmid-free  | 27         |
| DN515                            | $\Delta zupT$ , (TC2.A.5)   | 13         |
| DN527                            | $\Delta pitA$ , (TC2.A.20)  | 13         |
| DN675                            | $corA_1::kan$ , (TC1.A.35)  | 13         |
| DN528                            | $\Delta corA_2$ , (TC1.A.35)  | 13         |
| DN529                            | $\Delta corA_3$ , (TC1.A.35)  | 13         |
| DN785                            | $\Delta zntB$ , (TC1.A.35)  | 13         |
| DN786                            | $\Delta hoxN$ , (TC2.A.52)  | 13         |
| DN578, $\Delta e4$               | $\Delta zntA \Delta cad \Delta dmeF \Delta fieF$  | 7          |
| DN579, $\Delta e4z$              | $\Delta e4 \Delta zupT$   | 21         |
| DN532                            | $\Delta zupT \Delta pitA$   | 13         |
| DN674                            | $\Delta zupT corA_1::kan$   | This study |
| DN533                            | $\Delta zupT \Delta corA_2$   | This study |
| DN534                            | $\Delta zupT \Delta corA_3$   | This study |
| DN671                            | $\Delta pitA corA_1::kan$   | This study |
| DN530                            | $\Delta pitA \Delta corA_2$   | This study |
| DN531                            | $\Delta pitA \Delta corA_3$   | This study |
| DN666                            | $corA_1::kan \Delta corA_2$   | This study |
| DN667                            | $corA_1::kan \Delta corA_3$   | This study |
| DN787                            | $\Delta corA_2 \Delta corA_3$   | This study |
| DN788                            | $\Delta zupT \Delta hoxN$   | This study |
| DN535                            | $\Delta zupT \Delta pitA \Delta corA_3$   | This study |
| DN789                            | $corA_1::kan \Delta corA_2 \Delta corA_3$   | This study |
| DN790                            | $\Delta zupT \Delta hoxN \Delta zntB$   | This study |
| DN580, $\Delta 4$                | $\Delta zupT \Delta corA_1 \Delta corA_2 \Delta corA_3$                                     | This study |
| DN791                            | $\Delta zupT \Delta pitA corA_1::kan \Delta corA_2$   | This study |
| DN668                            | $\Delta zupT \Delta pitA corA_1::kan \Delta corA_3$   | This study |
| DN792                            | $\Delta zupT \Delta pitA \Delta corA_2 \Delta corA_3$                                       | This study |
| DN793                            | $\Delta zupT \Delta pitA \Delta hoxN \Delta zntB$   | This study |
| DN665, $\Delta 5$                | $\Delta zupT \Delta pitA \Delta corA_1 \Delta corA_2 \Delta corA_3$                         | This study |
| DN676, $\Delta 6$                | $\Delta zupT \Delta pitA \Delta corA_1 \Delta corA_2 \Delta corA_3 \Delta zntB$             | This study |
| DN794                            | $\Delta zupT \Delta pitA \Delta corA_1 \Delta corA_2 \Delta corA_3 \Delta hoxN$             | This study |
| DN681, $\Delta 7$                | $\Delta zupT \Delta pitA \Delta corA_1 \Delta corA_2 \Delta corA_3 \Delta zntB \Delta hoxN$ | This study |
| Conjugator strain                |   |            |
| <i>Escherichia coli</i>          |   |            |
| S17/1                            | Plasmids  | 63         |
| pECD794-1                        | <i>lacZ</i> , derivate of pLO2  | 7          |
| pCM157                           | Contains Cre recombinase  | 65         |
| pECD1003                         | Mutant <i>lox</i> sites, derivate of pECD889  | 7          |
| pVDZ2                            | Broad host range expression vector  | 71         |
| pDNA130                          | <i>pVDZ2::czcCBAD'</i>  | 26         |
| pECD977                          | pECD1003 construct for <i>zupT</i> deletion by <i>cre-lox</i> system                        | 13         |
| pECD981                          | pECD1003 construct for <i>pitA</i> deletion by <i>cre-lox</i> system                        | 13         |
| pECD982                          | pECD1003 construct for <i>corA<sub>1</sub></i> deletion by <i>cre-lox</i> system            | 13         |
| pECD983                          | pECD1003 construct for <i>corA<sub>2</sub></i> deletion by <i>cre-lox</i> system            | 13         |
| pECD984                          | pECD1003 construct for <i>corA<sub>3</sub></i> deletion by <i>cre-lox</i> system            | 13         |
| pECD1260                         | pECD1003 constr. for <i>zntB</i> deletion by <i>cre-lox</i> system                          | This study |
| pECD1144                         | pECD1003 constr. for <i>hoxN</i> del. by <i>cre-lox</i> system                              | This study |
| pECD1259                         | pECD794 constr. for <i>corA<sub>1</sub></i> disruption                                      | 13         |
| pECD985                          | pECD794 constr. for <i>zupT</i> <i>LacZ</i> reporter gene                                   | 13         |
| pECD986                          | pECD794 constr. for <i>pitA</i> <i>LacZ</i> reporter gene                                   | 13         |
| pECD988                          | pECD794 constr. for <i>corA<sub>1</sub></i> <i>LacZ</i> reporter gene                       | 13         |
| pECD989                          | pECD794 constr. for <i>corA<sub>2</sub></i> <i>LacZ</i> reporter gene                       | 13         |
| pECD990                          | pECD794 constr. for <i>corA<sub>3</sub></i> <i>LacZ</i> reporter gene                       | 13         |
| pECD1486                         | pECD794 constr. for <i>zntB</i> <i>LacZ</i> reporter gene                                   | This study |
| pECD1487                         | pECD794 constr. for <i>hoxN</i> <i>LacZ</i> reporter gene                                   | This study |
| pECD1490                         | pECD794 constr. for <i>rpoB</i> <i>LacZ</i> reporter gene                                   | This study |
| pECD1491                         | pECD794 constr. for <i>Enolase</i> <i>LacZ</i> reporter gene                                | This study |



expression plasmid pCM157.<sup>65</sup> 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. For gene disruption experiments, only the first step was performed.

**Minimal inhibitory concentration.** The MIC was determined in triplicate as the lowest concentration inhibiting bacterial growth on solid TMM. A pre-culture was incubated at 30 °C and shaken at 250 rpm up to early stationary phase, then diluted 1:20 in fresh medium and incubated for 24 h at 30 °C and 250 rpm. This 24 h culture was diluted 1:100 in fresh medium and used for streaking onto plates containing different concentration of the respective metal salts. The plates were incubated at 30 °C for 3 days and cell growth was monitored.

**Dose-response growth curves in 96-well plates.** These were also conducted in TMM. A pre-culture was incubated at 30 °C, 250 rpm up to early stationary phase, then diluted 1:20 in fresh medium and incubated for 24 h at 30 °C and 250 rpm. Overnight cultures were used to inoculate parallel cultures with increasing metal concentrations in 96-well plates (Greiner). Cells were cultivated for 20 h at 30 °C and 1300 rpm in a neoLab Shaker DTS-2 (neoLab, Heidelberg, Germany) and the optical density was determined at 500 nm or 600 nm as indicated in a TECAN infinite 200 PRO reader (TECAN, Männersdorf, Switzerland). To calculate the IC<sub>50</sub> value (metal concentration that led to turbidity reduction by half) and the corresponding *b*-value (measure of the slope of the sigmoidal dose-response curve), the data were adapted to the formula  $OD(c) = OD_0 / \{1 + \exp((c - IC50)/b)\}$ , which is a simplified version of a Hill-type equation as introduced by Pace and Scholtz (1997)<sup>68</sup> as published.<sup>69</sup> OD(*c*) is the turbidity at a given metal concentration; OD<sub>0</sub>, that at no added metal and *c* the metal concentration.

**β-Galactosidase assay and lacZ-reporter constructions.** *C. metallidurans* cells with a lacZ reporter gene fusion were cultivated as a pre-culture in TMM containing 1.5 mg L<sup>-1</sup> kanamycin at 30 °C, 250 rpm up to early stationary phase, then diluted into fresh medium with a cell density of 30 Klett units and incubated at 30 °C. At a cell density of 60 to 70 Klett units, metal salts were added in different concentrations and cells were incubated with shaking for a further 3 h. The specific beta-galactosidase activity was determined in permeabilized cells as previously published with 1 U defined as the activity forming 1 nmol of *o*-nitrophenol min<sup>-1</sup> at 30 °C.<sup>72</sup> The lacZ reporter gene was inserted within (disruption) or downstream of the gene after the stop-codon (*lacZ* fusion) of several target genes to construct reporter operon fusions. This was done by single cross-over recombination in *C. metallidurans* strains. A 300–400 bp PCR-product of the 3' end or in the middle region of the target genes was amplified from total DNA of strain AE104 and the resulting fragments were cloned into plasmid pECD794-1 (pLO2-lacZ).<sup>7</sup> All primer pairs used are listed in Table S6 (ESI<sup>†</sup>).

The respective operon fusion cassettes were inserted into the open reading frame of the target gene by conjugation and single cross-over recombination.

**SDS-PAGE and Western blot.** Cells were cultivated under various conditions and the cell density was determined from the turbidity with an equilibration curve. A sample representing a dry weight of 20 mg was mixed 1:1 with cracking buffer (final concentrations 125 mM Tris-HCl, pH 6.8, 20 g L<sup>-1</sup> SDS; 0.5% (vol/vol) β-mercaptoethanol, 0.01 g L<sup>-1</sup> bromophenol blue, 50% (vol/vol) glycerol), incubated for 30 min at 50 °C and loaded onto a SDS gel.<sup>73</sup> The gel was stained with Coomassie Brilliant Blue or blotted (Trans-Blot, BioRad) onto a nitrocellulose membrane, dried by air and then blocked in PBS (4 mM KH<sub>2</sub>PO<sub>4</sub>, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 115 mM NaCl), 5 mL Tween20 and 50 g skimmed milk per l with shaking at 23 °C for 16 h. The membrane was washed 3 times for 5 min with PBS-Tween (PBS, 1 mL Tween20 L<sup>-1</sup>) at 23 °C. The membrane was incubated for 1 h at 23 °C with shaking with the first antibody {polyclonal CzcA-antibody 1:15 000, diluted in PBS-Tween, antibody raised in rabbits after CzcA purification<sup>74</sup>}. The membrane was washed three times for 5 min with PBS-Tween and twice for 5 min with PBS, incubated with the second antibody {a monoclonal anti-rabbit-IgG conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:50 000 in PBS-Tween} for 1 h at 23 °C with shaking. Unbound antibody was washed off the membrane 3 times for 5 min with PBS-Tween and once for 5 min with PBS. For detection, the membrane was incubated with 5 mL solution 1 {10 mM TrisHCl (pH 8.0), 150 mM NaCl, 0.4 mM *p*-coumaric acid, 0.25 mM luminol} and 5 mL solution 2 {10 mM TrisHCl (pH 8.0), 150 mM NaCl, 5.4 M H<sub>2</sub>O<sub>2</sub>} for 1 min. The excess liquid was wiped off and the membrane was exposed for 5 s to 5–15 min to chemo-luminescence detection film (Roche).

**ICP-MS analysis.** To determine the metal content, cells were cultivated up to 100 Klett units in TMM and the different metal salts or EDTA was added. When the cells had reached the middle of the exponential phase of growth at 150 Klett units, 10 mL cell suspension were centrifuged for 30 min at 4500 × g. Cells were washed twice with 50 mM Tris-HCl buffer containing 10 mM EDTA (pH 7). The supernatant was discarded and the residual liquid carefully removed at each step. The pellet was suspended in concentrated 67% (wt/vol) HNO<sub>3</sub> (trace metal grade; Normatom/PROLABO) and mineralized at 70 °C for 2 h. Samples were diluted to a final concentration of 2% (wt/vol) nitric acid. Indium was added as internal standard at a final concentration of 10 ppb. Elemental analysis was performed *via* inductively coupled plasma mass spectrometry (ICP-MS) using ESI-sampler SC-2 (Elemental Scientific, Inc., Omaha) and an X-Series II ICP-MS instrument (Thermo Fisher Scientific, Bremen) operating with a collision cell and flow rates of 5 mL × min<sup>-1</sup> of He/H<sub>2</sub> (93%/7%), with an Ar carrier flow rate of 0.76 L × min<sup>-1</sup> and an Ar make-up flow rate at 15 L × min<sup>-1</sup>. An external calibration curve was recorded with ICP-multi-element standard solution XVI (Merck) in 2% (v/v) nitric acid. The sample was introduced *via* a peristaltic pump and analyzed for its metal content. For blank measurement and quality/quantity thresholds, calculations based on DIN32645 TMM were used. 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 colony-forming units.

## Conclusions

The seven secondary metal uptake systems characterized in this study appear to communicate to form the core of the metal transportome<sup>30,31</sup> of *C. metallidurans* AE104 by an interplay with the four metal efflux systems ZntA, CadA, DmeF and FieF. This is supported by a remarkable similarity of the metal content and cobalt resistance, a feature severely affected by deletion of metal import genes, of the  $\Delta 7$  metal uptake and the  $\Delta e4$  efflux mutant. Since  $\Delta 7$  is to some degree still able to maintain its cellular metal composition, more metal uptake systems must exist in *C. metallidurans* beyond the seven secondary importers. Up to  $\Delta 6$ , the plasticity of the metal uptake transportome revealed here seems not to involve increased gene expression for substitute importers but rather post-transcriptional events take over. Taken together with the disappearance of CzcA in *zupT* mutants, an important post-transcriptional level of control of the metal transportome seems to exist in *C. metallidurans*.

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