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Essential role of the *czc* determinant for cadmium, cobalt and zinc resistance in *Gluconacetobacter diazotrophicus* PAl 5

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Summary. The mechanisms of cadmium, cobalt and zinc resistance were characterized in the plant-growth-promoting bacterium *Gluconacetobacter diazotrophicus* PAl 5. The resistance level of the wild-type strain was evaluated through the establishment of minimum inhibitory concentrations (MIC) of the soluble compounds $CdCl_2 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$ and $ZnCl_2$. *Gluconacetobacter diazotrophicus* PAl 5 was resistant to high concentrations of Cd, Co and Zn, with MICs of 1.2, 20 and 20 mM, respectively. Screening of an insertion library from transposon EZ-Tn5<R6Kyori/KAN-2> in the presence of ZnO revealed that the mutant GDP30H3 was unable to grow in the presence of the compound. This mutant was also highly sensitive to $CdCl_2 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$ and $ZnCl_2$. Molecular characterization established that the mutation affected the *czcA* gene, which encodes a protein involved in metal efflux. *In silico* analysis showed that *czcA* is a component of the *czcCBARS* operon together with four other genes. This work provides evidence of the high tolerance of *G. diazotrophicus* PAl 5 to heavy metals and that *czc* is a determinant for metal resistance in this bacterium. [**Int Microbiol** 2012; 15(2):69-78]

Keywords: *Gluconacetobacter diazotrophicus* PAl 5 · *czc* determinant · cadmium · cobalt · zinc · metal resistance

Introduction

Metals are natural components of the environment. Some metals, such as zinc and copper, serve as essential nutrients for living organisms and play a role in gene expression, biomolecular activity and structural DNA stabilization [4,23]. Other metals, such as mercury and lead, do not have a known biological function and are toxic to living organisms even at trace amounts [42]. In microorganisms, an excess of certain metals may affect growth, morphology and metabolism [13]. Toxicity occurs through the displacement of essential ions present in biologically active molecules, deleterious interactions with ligands or the formation of non-specific complex compounds in the cell wall [36]. This results in alterations in the conformational structure of macromolecules and interferes with oxidative phosphorylation and the cellular osmotic balance [4].

Throughout evolution, microorganisms have developed several mechanisms to attenuate the toxicity from an excess of metals [43]. Active metal efflux, the synthesis of ligand

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compounds, the accumulation and complexation of metals inside the cell and the reduction of metals to less toxic forms have been highlighted [12,36]. The primary intracellular process for regulating an excess of metals is based on transporting the metals through the cytoplasmic membrane [34]. Under normal conditions, essential and non-essential metals are transported by nonspecific entry systems. However, when metal ions are in excess, specific ion efflux protein complexes may be synthesized to aid in the elimination of non-essential metals [34].

CzcA is one of the primary proteins in cadmium, cobalt and zinc resistance in several microorganisms, including the tolerant bacterium *Cupriavidus metallidurans* CH34 [22,31,37,57]. CzcA has been characterized also in other species associated with metal resistance, such as *Caulobacter crescentus* CB15N [18], *Pseudomonas putida* CD2 [19] and *Sinorhizobium meliloti* 1021 [45]. Together with other genes, *czcA* forms the *czc* determinant, which encodes a multi-protein complex associated with a high level resistance to cadmium, cobalt and zinc in bacteria [37].

Gluconacetobacter diazotrophicus PAl 5 is an endophytic bacterium that promotes plant growth [48] and was first isolated in sugarcane [6]. Later, this bacterium was detected in several other hosts, such as Cameroon grass, finger millet, coffee, sweet potato, tea, banana, pineapple, carrot, radish, beetroot and wetland rice [48]. Nitrogen fixation, phytohormone production, activity against phytopathogens, and the solubilization of mineral nutrients are several of the characteristics of plant growth promoted by *G. diazotrophicus* PAl 5 [48]. Moreover, this bacterium is resistant to antibiotics [33], which suggests the existence of multi-resistance mechanisms against drugs and/or the expression of efflux pumps [41].

The *G. diazotrophicus* PAl 5 genome has been sequenced, allowing further functional genomic studies [3]. The large number of transport systems described is consistent with several characteristics related to the endophytic lifestyle of the bacterium [3]. The availability of this information has facilitated characterization of the relevant metabolic pathways of this bacterium [21].

In the present study, the tolerance levels for cadmium, cobalt and zinc were evaluated in *G. diazotrophicus* PAl 5. The minimum inhibitory concentration of each metal that affected the development of the wild-type strain was measured in solid medium. Molecular characterization of a defective mutant sensitive to these metals enabled the identification of a gene crucial for cadmium, cobalt and zinc resistance in *G. diazotrophicus* PAl 5.

Materials and methods

Bacterial strains, media and growth conditions. The *G. diazotrophicus* PAl 5 wild-type strain used in the present work was kindly provided by Dr. Fábio L. Olivares, from the Culture Collection of the State University of North Fluminense Darcy Ribeiro (Campos dos Goytacazes, RJ, Brazil). *Gluconacetobacter diazotrophicus* PAl 5 and the GDP30H3 mutant [21] were grown in DYGS medium [21,44] and modified LGI (g/l composition: glucose 10, K_2HPO_4 0.2, KH_2PO_4 0.6, $MgS0_4{\cdot}7H_2O$ 0.2, $[NH_4]_2SO_4$ 1.32, $CaCl_2{\cdot}2\ H_2O$ 0.02, $NaMoO_4{\cdot}2\ H_2O$ 0.002 and $FeCl_3{\cdot}6\ H_2O$ 0.018) at 30 °C [6]. *Escherichia coli* TransforMax EC100D (*pir* $^-$) (Epicentre, Madison, WI, USA) was used for cloning studies following the manufacturer's instructions. The cells were grown in LB medium-Miller at 37 °C [21,30]. Kanamycin (50 μg/ml) was added when necessary [21].

Minimum inhibitory concentration. In brief, 100 ml of wild-type strain was grown to exponential phase in 1000-ml Erlenmeyer flasks capped with baffles and containing LGI medium. Drops (10 μl) of the culture (10^9 cells/ml) were plated onto LGI solid medium containing the varying concentrations of metal salts ($CdCl_2{\cdot}H_2O$, $CoCl_2{\cdot}6H_2O$ and $ZnCl_2$). For each metal, the lowest concentration that inhibited visible growth within 6 days was determined. The assay was repeated at least three times.

Zinc solubilization assay. The wild-type strain and defective mutant were grown to reach exponential phase in DYGS medium. Drops (10 μl) of the culture (10^9 cells/ml) were inoculated on a DYGS agar plate control and on a DYGS agar plate supplemented with 0.1 % (w/v) ZnO at 0.12 %. Petri plates were incubated for 2 days [21,49]. Colony growth was observed and compared. This experiment was retested at least three times.

Determination of metal sensitivity in the defective mutant. The wild-type strain and defective mutant were grown to reach exponential phase in LGI medium. Drops (10 μl) of the culture (10^9 cells/ml) were plated onto LGI medium containing 0.3 and 0.5 mM $CdCl_2{\cdot}H_2O$, 2.0 and 8.0 mM $CoCl_2{\cdot}6H_2O$, 2.0 and 8.0 mM $ZnCl_2$. The inhibitory concentrations were defined based on the MIC values determined for the wild-type strain at low and medium concentrations. The metal compounds were supplied in concentrations allowing viable growth of the wild-type strain. Higher concentrations were not measured because the mutant had a "loss-of-function" phenotype. Therewith, growth was monitored after 6 days. This experiment was repeated at least three times.

DNA isolation and Southern hybridization for mutant. Genomic DNA from bacteria was isolated using Plant DNAzol Reagent (Invitrogen, Grand Island, NY, USA), following the manufacturer's protocol. The genomic DNA was digested with *Eco*RI (New England Biolabs, Ipswich, MA, USA), separated by electrophoresis on an 0.8 % (w/v) agarose gel and then transferred onto a nylon membrane (Hybond-N+, Amersham, GE Healthcare, Little Chalfont, UK). The entire transposon (2001 bp) was amplified using primer MEint (5′-CTG TCT CTT ATA CAC ATC T-3′) from the selected mutant. The PCRs (20 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of primer MEint, 1 U of Taq DNA polymerase (Fermentas, Burlington, ON, CA) and 10 ng of DNA template. Reactions were carried out in a thermocycler (MasterCycler Gradient-Eppendorf) with an initial denaturation at 95 °C for

5 min followed by 40 cycles of 95 °C for 60 s, 55 °C for 60 s, 72 °C for 90 s and a final extension at 72 °C for 30 min. The amplified fragment was used as a hybridization probe. Probe labeling and hybridization were performed according to the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare, Little Chalfont, UK). The membrane was then washed and exposed to X-ray [47].

Identification of the insertion site of transposon Tn5. The genomic regions flanking the transposon insertion point were rescued by self-ligation of total DNA digested with EcoRI and electroporation in *E. coli* TransforMax EC100D (*pir*$^-$) competent cells following the instructions of the EZ-Tn5<R6Kyori/KAN-2>Tnp Transposome kit (Epicentre, Madison, WI, USA). After electroporation, the cells were plated onto LB agar plates containing kanamycin. Colonies were selected and their plasmids were then purified.

The nucleotide sequences were determined by using an ABI 3130 automatic sequencer (Applied Biosystems) and the Big Dye Terminator kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The transposon-specific primers KAN-2-FP-1 and KAN-2-RP-1, available in the EZ-n5<R6Kyori/KAN-2>Tnp Transposome Kit , were used. Approximately 900 nucleotides were sequenced in each flanking region. BLAST searches were performed at the National Center for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/BLAST]. There are two genome sequences of *G. diazotrophicus* PAl 5 available in the NCBI database (RefSeq: NC_010125 and NC_011365). These sequences were generated by two distinct groups and contain a considerable number of differences. In this study, data were checked in both genomes, and showed similar results for the sequences studied.

Genomic organization of the *czc* determinant. The sequences of genes comprising the *czc* determinant in *Gluconacetobacter diazotrophicus* PAl 5 (RefSeq: NC_010125) and related bacterial species were obtained through the NCBI database. The *czc* locus of *Gluconacetobacter xylinus* NBRC 3288 - pGXY010 (RefSeq: NC_016037), which belongs to the same genus as *G. diazotrophicus* PAl 5, and of *Herbaspirillum seropedicae* SmR1 (RefSeq: NC_014323), another sugarcane endophyte, were included in the sequence analysis. *Pseudomonas aeruginosa* PAO1 (RefSeq: NC_002516), *Pseudomonas putida* KT2440 (RefSeq: NC_002947) and *Cupriavidus metallidurans* CH34 - pMOL30 (RefSeq: NC_007971) were also evaluated because their *czc* determinants have already been characterized [5,16,17]. The BioCyc [http://biocyc.org/] [7] and Microbes Online [10] databases [http://www.microbesonline.org/] were used for an *in silico* prediction of the operon organization.

Phylogenetic analysis of the CzcA protein. The protein sequence of CzcA from *G. diazotrophicus* PAl 5, similar sequences from nitrogen-fixing bacteria, and proteins of the resistance, nodulation and cell division (RND) superfamily [53] were used for phylogenetic analysis. These proteins were aligned using ClustalW (default settings) [http://align.genome.jp/] [54] and Needle algorithm [htpp://www.ebi.ac.uk]. The HAE3 protein from *Methanoregula boonei* 6A8 was used as outgroup (Uniprot accession number: A7I766). Optimal substitution models for phylogenetic analysis were selected using the software ProtTest [9].

The alignment result served as input for MEGA software version 5.0 [52] to generate a phylogenetic tree using the maximum likelihood (ML) method. The parameters used were the model of substitution WAG+F with the invariant site (I) and the gamma-distributed site, 1000 bootstraps and four substitution rate categories. The results were visualized using MEGA software version 5.0 [52].

Predictions for the structural and transmembrane domains (TMH) of CzcA proteins were also obtained and evaluated. This analysis was performed using simple modular architecture research tool –(SMART) [http://smart.embl-heidelberg.de/] [28,50] and TMHMM Server v.2.0, [http://www.cbs.dtu.dk/services/TMHMM/] [26], respectively.

Results

***Gluconacetobacter diazotrophicus* PAl 5 and cadmium, cobalt and zinc resistance.** The resistance of this bacterium to metals when grown in solid medium containing $CdCl_2 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$ and $ZnCl_2$ was evaluated; the results are shown in Fig. 1A. *Gluconacetobacter diazotrophicus* PAl 5 was resistant to high levels of Cd, Co and Zn, with maximum tolerated concentrations of 1.0, 18 and 16 mM, respectively. The MICs for Cd, Co and Zn were 1.2, 20 and 20 mM, respectively. *Gluconacetobacter diazotrophicus* PAl 5 was found to be susceptible to theses metals in the following order: Co = Zn < Cd.

Metal-sensitive mutant. Figure 1B shows that the mutant GDP30H3 did not grow in the presence of ZnO. As this phenotype was due to the presence of zinc in the culture medium, the sensitivity of GDP30H3 to soluble cadmium, cobalt and zinc was evaluated. Figure 1C shows that, in the absence of metals, GDP30H3 had a growth rate similar to the wild-type strain. However, the mutant had high sensitivity to the presence of cadmium, cobalt and zinc. GDP30H3 did not grow when high concentrations of these metals were added, and growth was negatively affected even when they were supplied at low concentrations. These data indicate the crucial role of the affected gene in the resistance of the bacterium to the aforementioned heavy metals.

Characterization of the Tn5 insertion in the GDP30H3 mutant. The number of transposon insertions in the chromosome of the mutant was measured by Southern hybridization, performed using *Eco*RI-digested genomic DNA and the transposon sequence as a probe. Figure 1D shows the presence of one only band for the mutant. As predicted by in silico analysis, the size of the hybridized fragment corresponded to the distance between the EcoR1 restriction sites (4601 bp) observed in the genome sequence plus the inserted transposon sequence (2001 bp), totaling 6002 bp.

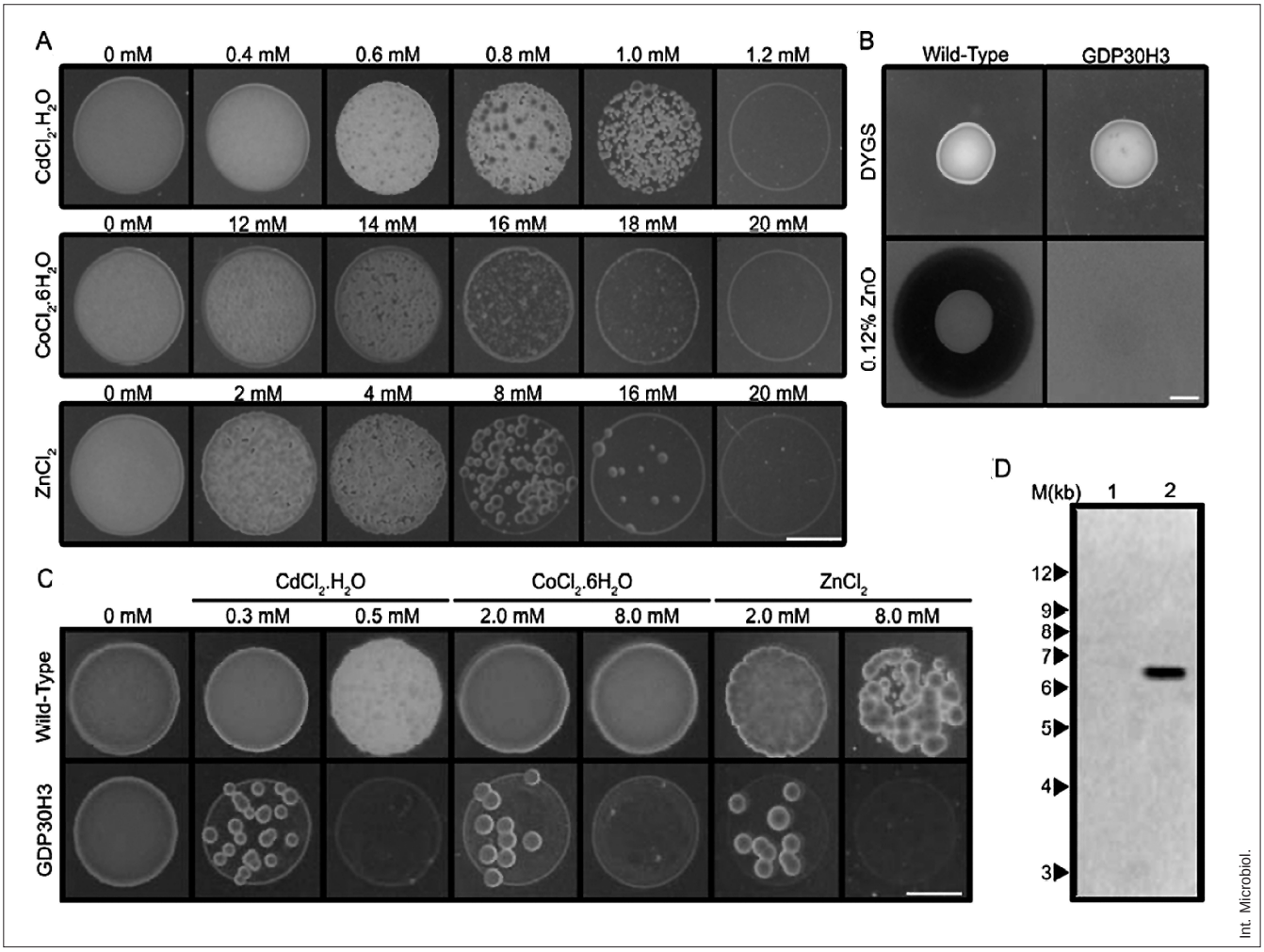

Fig. 1. (**A**) Sensitivity of *Gluconacetobacter diazotrophicus* PAl 5 to cadmium, cobalt and zinc on LGI media. The first concentrations are those that first affected bacterial growth, i.e., lower concentrations did not reduce the colony density. (**B**) *Gluconacetobacter diazotrophicus* PAl 5 mutant sensitive to zinc in ZnO solubilization assay in DYGS medium. (**C**) Comparison of sensitivities to cadmium, cobalt and zinc of the *G. diazotrophicus* PAl 5 wild-type strain and the mutant strain on LGI medium. All experiments were repeated at least three times. The white bar represents 1 cm. (**D**) Southern hybridization analysis of the *G. diazotrophicus* PAl 5 wild-type strain and the transformant. Lane 1: wild-type strain. Lane 2: GDP30H3. The molecular ladder is 1 kb Plus DNA Ladder (Invitrogen).

The gene interrupted by the transposon insertion in the mutant was identified by sequencing the flanking regions of the transposon insertion. According to the NCBI database, the gene ID is 5789610. Figure 2A shows the genomic region of the transposon insertion. GDP30H3 was altered at gene *czcA* (Locus tag: GDI_1513), which encodes a cobalt-zinc-cadmium resistance protein. This protein has been characterized in several species of bacteria, where it was shown to be involved in the efflux of cations (cobalt, zinc and cadmium).

The *czc* determinant and metal resistance in *Gluconacetobacter diazotrophicus* PAl 5. Analysis of the location of *czcA* revealed an overlap of the gene's coding region ends with the adjacent genes, *czcR* and *czcB* (Locus tag: GDI_1512 and GDI_1514, respectively). An evaluation of the subsequent genes showed that *czcS* and *czcC* (Locus tag: GDI_1511 and GDI_1515, respectively) also had overlapping regions (Fig. 2B). An *in silico* prediction using the BioCyc and Microbes databases suggested the organization of these genes in an operon. This set of genes is known as the *czc* determinant, which is widely found in bacteria that have high resistance levels for metals [27,37]. Two other genes involved in the efflux of heavy metals, *copA* (Locus tag: GDI_1508) and *CopB* (Locus tag: GDI_1509), are located near this genomic region. They encode the copper resistance proteins A and B, respectively.

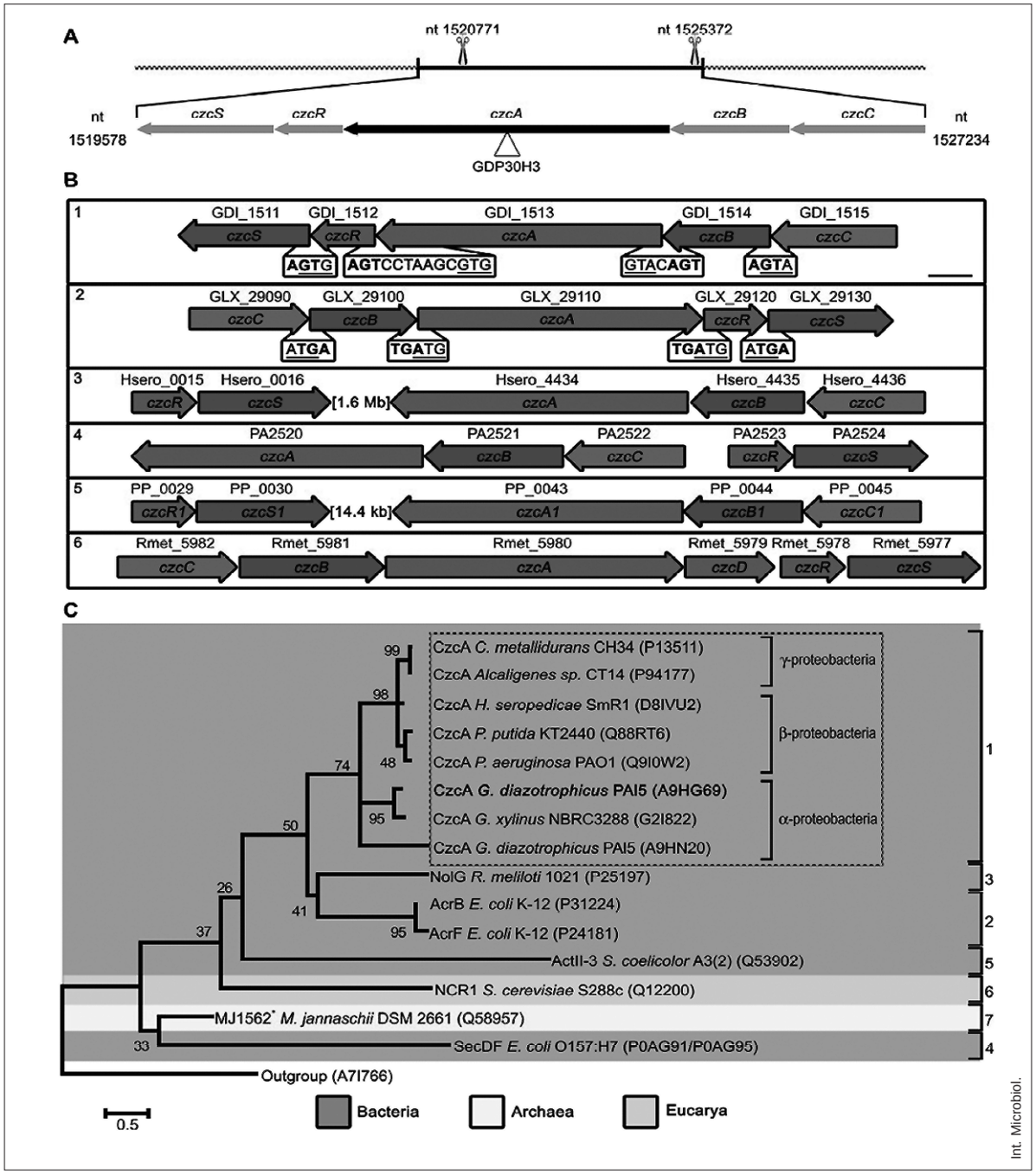

Fig. 2. (**A**) Genomic organization of genes flanking Tn5 insertions in *Gluconacetobacter diazotrophicus* PAl 5 according to the NCBI database. Gene cluster flanking *czc*A. The arrows indicate the orientation of the ORF. *Black arrows*: genes disrupted in this study, *gray arrows*: genes not disrupted, *scissors*: EcoRI restriction site, *white triangles*: transposon insertion sites, *nt*: nucleotide. (**B**) Comparison of the genomic organization of the *czc* determinant in *G. diazotrophicus* PAl 5 and related species following the RefSeq NCBI database: (1) *G. diazotrophicus* PAl 5 (NC_010125), (2) *G. xylinus* NBRC 3288 (NC_016037), (3) *H. seropedicae* SmR1 (NC_014323), (4) *P. aeruginosa* PAO1 (NC_002516), (5) *P. putida* KT2440 (NC_002947), and (6) *C. metallidurans* CH34 (NC_007971). Underlined letters in the boxes, start codon; and bold letters, termination codon. The arrows indicate the orientation of the ORF. The codes above the arrows are the locus tag of the sequences. Black bar: 500 bp. (**C**) Multiple alignment and phylogenetic relationship of the amino acid sequences of members of the RND superfamily. Families of the RND superfamily: (1) the heavy metal efflux (HME), (2) the hydrophobe/amphiphile efflux-1 (HAE1), (3) the nodulation factor exporter (NFE), (4) the SecDF protein-secretion accessory protein (SecDF), (5) the hydrophobe/amphiphile efflux-2 (HAE2), (6) the eukaryotic sterol homeostasis (ESH), and (7) the hydrophobe/amphiphile efflux-3 (HAE3). HME family is indicated in the box. CzcA from *G. diazotrophicus* PAl 5 is in bold. The numbers at the nodes indicate bootstrap support. Branch length is proportional to evolutionary distance (scale bar). Asterisk indicates a putative membrane protein MJ1562. Codes next to the proteins are the accession number of their sequences. All of the amino acids sequence analyses were performed using the UniProt database.

Table 1. Sequence comparison of the *Gluconacetobacter diazotrophicus* PAl 5 CzcA protein with homologous CzcA proteins

Organism	Similarity (%)	Identity (%)	Length[a]	TMH[b]	Number[c]
Gluconacetobacter diazotrophicus PAl 5	100	100	1031	12	A9HG69
G. diazotrophicus PAl 5[d]	52.3	34.3	1033	12	A9HN20
G. xylinus NBRC 3288	82.9	73.2	1024	12	G2I822
Cupriavidus metallidurans CH34	58.9	39.9	1063	11	P13511
Alcaligenes sp. CT14	59.1	40.0	1063	11	P94177
Herbaspirillum seropedicae SmR1	58.2	39.2	1073	10	D8IVU2
Pseudomonas aeruginosa PAO1	59.4	39.7	1051	10	Q9I0W2
P. putida KT2440	59.4	40.4	1053	10	Q88RT6

[a]Number of amino acids.
[b]Number of transmembrane domains (TMH).
[c]Accession number from UniProt.
[d]Protein corresponding to the copy of the *czcA* gene (Locus tag: GDI_2438).

In addition, a second copy of the *czcA* gene was identified in another region of the *G. diazotrophicus* PAl 5 chromosome (Locus tag: GDI_2438). However, the other genetic components of the czcCBARS determinant were not observed in the vicinity. The genes adjacent to this region encode an efflux pump outer membrane protein (Locus tag: GDI_2437) and a HlyD family secretion protein (Locus tag: GDI_2439). This region also included transposase (Locus tag: and GDI_2423 GDI_2424) and integrase genes (Locus tag: and GDI_2426 GDI_2443). A low percentage of sequence similarity (52.3 %) and identity (34.3 %) was determined between the two *czcA* copies, as shown in Table 1. No *czc* gene was found in the sequences of the three plasmids of this bacterium deposited in the NCBI database (pGDIPAl5I - RefSeq: NC_010124, pGDIPal5II - RefSeq: NC_010123 and pGDIA01 - RefSeq: NC_011367).

High similarity with the genomic organization of plasmid pGXY010 from *G. xylinus* NBRC 3288 was observed in a comparison of the organization of the *czc* genes of *G. diazotrophicus* PAl 5 and related homologs sequences (Fig. 2B). A second *czc* determinant was also found in the chromosome of *G. xylinus* NBRC 3288. Comparative analysis with the genome of *Herbaspirillum seropedicae* SmR1, another sugarcane endophyte [2], revealed the presence of two distinct coding regions of the *czc* determinant in the chromosome of this bacterium. These were also observed in *Pseudomonas aeruginosa* CMG103 and *P. putida* KT2440 (Fig. 2B) [5,17]. The genomic organization of *Cupriavidus metallidurans* CH34, a proteobacterium widely characterized with respect to its metal resistance, presents two *czc* determinants. Some non-functional *czc* genes are located on chromosome 2 (*czcICBA-ubiG-czcSRL*), but the majority of the genes is located on its plasmid, pMOL30 (*czcMNICBADRSEJ*), which is fully functional [22,31, 56,57]. Figure 2B shows the genes located on plasmid pMOL30, which is organized into two operons, *czcCBA* and *czcDRS* [16].

Figure 2C shows the phylogeny of representative members of the seven families that form the RND superfamily [53], which consists of permeases that act as efflux pumps to transport various compounds. CzcA belongs to this RND superfamily.

Table 1 provides a comparison of CzcA from *G. diazotrophicus* PAl 5 with other homologs of CzcA proteins that have been described from other Proteobacteria, including *nccA* (*C. metallidurans* A31), *czrA* (*P. aeruginosa* CMG103) and *cusA* (*E. coli* W3110 and *Myxococcus xanthus* DZF1) [11,17,32], all of which are part of triple-gene (CBA) loci encoding chemiosmotic antiporter complexes. The highest protein identity and similarity occurred with *G. xylinus* NBRC 3288 (73.2 % and 82.9 %, respectively). The sizes of the CzcA proteins in these species ranged from 1024 to 1073 amino acids. Functional prediction based on the amino acid sequence of the *czcA* gene using SMART and TMHMM revealed the presence of 10–12 transmembrane regions in CzcA, which is characteristic of this type of transporter. All of the analyzed proteins had an ADAM cysteine-rich (ACR) domain, covering most of the total length.

Discussion

Gluconacetobacter diazotrophicus PAl 5 is a plant-growth-promoting bacterium capable of solubilizing nutrients such as zinc. It is therefore likely to express genes that confer metal resistance. In the present study, transposon mutagenesis was used to analyze the metal-resistant determinant of PA15, resulting in the identification of a chemiosmotic antiporter.

The susceptibility of *G. diazotrophicus* PAl 5 to cadmium, cobalt and zinc was first evaluated in an MIC assay. The results showed that this bacterium was highly tolerant of metals compared to other previously characterized organisms. Trajanovska et al. [55] evaluated bacteria isolated from environments contaminated with metal ions and identified *Corynebacterium* sp. AB18, *Arthrobacer* sp. E11 and *Cupriavidus metallidurans* CH34 among the isolated microorganisms [55]; the latter is a model organism used in metal resistance studies [22,31,37,57]. Overall, the MIC values determined for these organisms were lower than the values established in the present study for *G. diazotrophicus* PAl 5, especially those for cobalt and zinc [55] (Table 2). Similarly, studies performed with *Pseudomonas putida* 06909 [51] and *Pseudomonas aeruginosa* CMG466 [1] reported MIC values lower than those of *G. diazotrophicus* PAl 5, with the highest difference found for cobalt stress. Table 2 shows data from these bacteria relative to the values obtained in the present study for *G. diazotrophicus* PAl 5. However, these assays were performed in distinct culture media, and nutritional requirements differ among microorganisms. The bioavailability or toxicity of the metal ion may vary depending upon the chemical components of the culture medium [20].

After metal resistance was confirmed in *G. diazotrophicus* PAl 5, the molecular mechanism responsible for this property was studied. An insertion mutant (GDP30H3) highly susceptible to stress caused by soluble cadmium, cobalt and zinc was identified. Sequencing of the transposon insertion ends revealed that the *czcA* gene was altered in GDP30H3. This gene appears to be essential for resistance to all of the evaluated stress conditions because its disruption resulted in impaired bacterial growth even at the lowest metal concentrations. *CzcA* encodes a protein that belongs to the RND superfamily of transporters, which includes the heavy metal efflux family (HME-RND) belonging to the HME1 group [53]. The CzcA transporter is directly involved in the resistance to cobalt, zinc and cadmium, which explains why bacteria expressing the *czcA* gene are highly resistant to these metals [37].

CzcA is one of the proteins that belong to the *czc* determinant, which codes for CBA transporters, a group of trans-envelope pumps in gram-negative bacteria. These pumps are formed by three components that act as chemiosmotic antiporters [27]. The CzcCBA pump is composed of several proteins with defined functions. CzcC, an outer membrane factor (OMF), acts at the outer membrane and transports cations out of the cell [25]. CzcB, a membrane fusion protein, is distributed throughout the periplasmic space and is a metal transporter, preventing the release of free cations into the periplasm. CzcA binds to CzcB, which in turn is bound to CzcC, and removes these ions from the cytoplasm [8]. Thus, detoxification occurs through ion efflux driven by a proton motive force [15]. Two additional regulators of CzcR and CzcS gene expression form a two-component regulatory system made up of two proteins, the sensor (CzcS, a histidine

Table 2. MICs (mM) of metal ions for *Gluconacetobacter diazotrophicus* PAl 5 and related bacterial species

Organism	Cadmium	MIC	Cobalt	MIC	Zinc	MIC	Reference
Gluconacetobacter diazotrophicus PAl 5	$CdCl_2 \cdot H_2O$	1.2	$CoCl_2 \cdot 6\ H_2O$	20.0	$ZnCl_2$	20.0	This study
Cupriavidus metallidurans CH34	$CdCl_2$	0.2	$Co(NO_3)_2$	1.9	$Zn(NO_3)_2$	2.7	[55]
Corynebacterium sp. AB18[a]	$CdCl_2$	1.2	$Co(NO_3)_2$	2.7	$Zn(NO_3)_2$	2.5	[55]
Arthrobacter sp. E11[a]	$CdCl_2$	2.1	$Co(NO_3)_2$	2.5	$Zn(NO_3)_2$	3.1	[55]
Pseudomonas aeruginosa CMG466	$CdCl_2$	2.0	$CoCl_2$	0.5	$ZnCl_2$	1.5	[1]
P. putida 06909	$CdCl_2 \cdot 2.5\ H_2O$	1.7	$CoCl_2 \cdot 6\ H_2O$	0.3	$ZnSO_4 \cdot 7\ H_2O$	11.5	[51]

[a]Bacteria isolated from soil contaminated with metals.

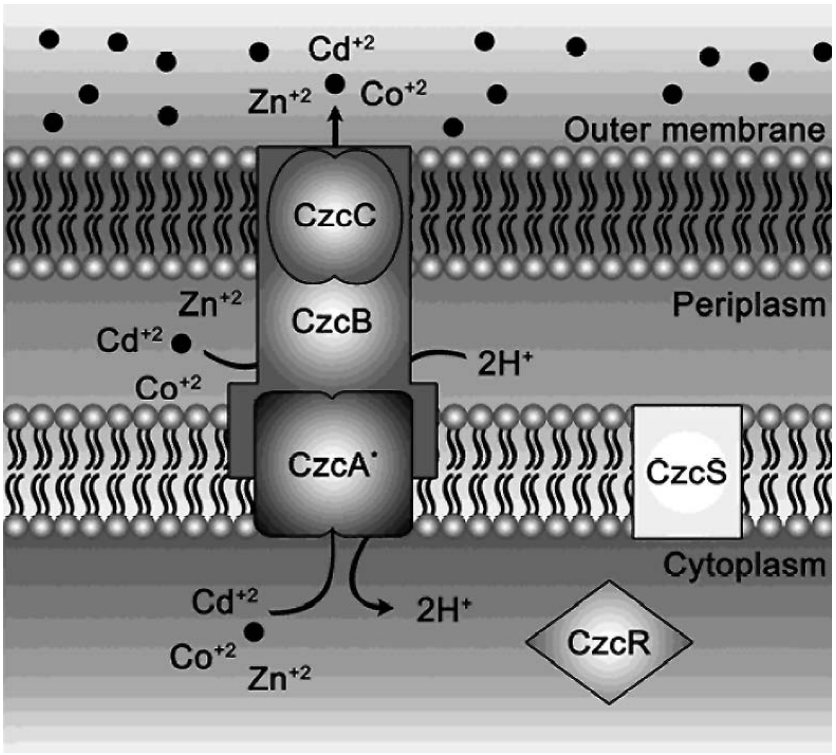

Fig. 3. Model for cadmium, cobalt and zinc resistance in *Gluconacetobacter diazotrophicus* PAl 5 as mediated by the CBA transporters, which act in the detoxification of toxic molecules. Asterisk indicates the mutated protein in this study. The black circles represent metals. Adapted from [37].

kinase) and the response regulator (CzcR), which regulates the expression of *czcCBA* through a phosphorylation cascade [35].

CzcA has also been identified in other studies of metal resistance in various bacteria. In a screening of *P. putida* CD2 mutants susceptible to cadmium, five *czc* chemiosmotic antiporter operons were shown to be involved in the response to this divalent metal [19]. The expression of *czcA* has also been detected in other studies in which transcriptional whole-genome profiling in response to metals was carried out in *Sinorhizobium meliloti* 1021 and *Caulobacter crescentus* CB15N [18,45]. The induction of *czc* genes in the presence of copper has been demonstrated in *Myxococcus xanthus* [32]. The annotation of copA and CopB in the genome of *G. diazotrophicus* PAl 5, besides the *czc* determinant, therefore justifies further investigations regarding the copper resistance of this bacterium.

Five *czc* operons are present in *Comamonas testosteroni* S44. The expression of these genes in response to Zn^{2+} stress was analyzed, revealing that *czcA* genes are either Zn^{2+}-induced or downregulated by Zn^{2+} [58]. These results are consistent with those found for *C. metallidurans* CH34, in which one *czc* operon is induced by Zn^{2+}, another is expressed constitutively, and the third is repressed [38,57]. The presence of two different copies of the *czcA* gene in the genome *G. diazotrophicus* PAl 5 suggests their different roles in the response to heavy metals.

Transporters belonging to the RND family aare widespread especially among gram-negative bacteria. They form a tripartite structure, traversing both the outer and inner membrane to detoxify the cytoplasm [39,40]. They are also found in Archaea and Eukarya domains, indicating the protein's evolutionary importance [53] and suggesting that this metal resistance mechanism should have been present in the last universal common ancestor (LUCA). The widespread nature of this resistance mechanism indicates the biological relevance of maintaining this superfamily within the distinct evolutionary domains. In *G. diazotrophicus* PAl 5, CzcA function is essential for cell viability under high concentrations of cadmium, cobalt and zinc. However, few RND proteins have been characterized. Thus, additional information on members of this family is relevant and could be extended to homologues, considering the similarity of the remaining sequences [53].

Data obtained in the present study suggest that, in the presence of heavy metals (cobalt, cadmium and zinc), *G. diazotrophicus* PAl 5 reaches cellular homeostasis through the efflux of intracellular cation excess by means of transporters. If the detoxification system is damaged, cellular growth is impaired under these stressful conditions. The mechanism of metal efflux is mediated through CBA transporters, which act as antiporters formed by a protein complex. This complex bridges the cell membrane from the cytoplasm to the outside and acts in the efflux of toxic compounds [24,29]. Figure 3 illustrates the role of *czc* in the process of detoxification of cadmium, zinc and cobalt in *G. diazotrophicus* PAl 5. The layout of this model was based on the structure proposed for CBA complexes [35].

This study demonstrated that *G. diazotrophicus* PAl 5 is resistant to cadmium, cobalt and zinc cations and identified the CzcA protein as essential for metal resistance, which allowed for the *in silico* analysis of related proteins. When combined, these data reveal the crucial role of the *czc* operon in *G. diazotrophicus* PAl 5 and provide possibilities for further analyses on the importance of this mechanism in plant colonization by bacteria and in the tolerance of colonized plants to heavy metals present in soil.

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References

1. Ahmed N, Nawaz A, Badar U (2005) Screening of copper tolerant bacterial strains and their potential to remove copper from environment. Bull Environ Contam Toxicol 74:219-226
2. Baldani JI, Baldani VLD, Seldin L, Döbereiner J (1986) Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root associated nitrogen fixing bacterium. Int J Syst Bacteriol 36:86-93
3. Bertalan M, Albano R, Padua V, et al. (2010) Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* PAL 5. BMC Genomics 10:450
4. Bruins MR, Kapil S, Oehme FW (2000) Microbial resistance to metals in the environment. Ecotoxicol Environ Saf 45:198-207
5. Cánovas D, Cases I, Lorenzo V (2003) Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Environ Microbiol 5:1242-1256
6. Cavalcante VA, Döbereiner J (1988) A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant Soil 108:23-31
7. Caspi R, Altman T, Dale JM, et al. (2010) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res 38:D473-D479
8. Choudhury R, Srivastava S (2001) Zinc resistance mechanisms in bacteria. Curr Sci 81:768-775
9. Darriba D, Taboada GL, Doallo R, Posada D (2011) ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27: 1164-1165
10. Dehal PS, Joachimiak MP, Price MN, Bates JT, Baumohl JK, Chivian D, Friedland GD, Huang KH, Keller K, Novichkov PS, Dubchak IL, Alm EJ, Arkin AP (2010) MicrobesOnline: an integrated portal for comparative and functional genomics. Nucleic Acids Res 2010 38:D396-D400
11. Franke S, Grass G, Rensing C, Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol 185:3804-3812
12. Gadd GM (2004) Microbial influence on metal mobility and application for bioremediation. Geoderma 122:109-119
13. Giller KE, Witter E, Mcgrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol Biochem 30:1389-1414
14. Giongo A, Tyler HL, Zipperer UN, Triplett EW (2010) Two genome sequences of the same bacterial strain, *Gluconacetobacter diazotrophicus* PAl 5, suggest a new standard in genome sequence submission. Stand Genomic Sci 2:309-317
15. Goldberg M, Pribyl T, Juhnke S, Nies DH (1999) Energetics and topology of CzcA, a cation/proton antiporter of the RND protein family. J Biol Chem 274:26065-26070
16. Große C, Anton A, Hoffmann T, Franke S, Schleuder G, Nies DH (2004) Identification of a regulatory pathway that controls the heavy-metal resistance system Czc via promoter czcNp in *Ralstonia metallidurans*. Arch Microbiol 182:109-118
17. Hassan M, van der Lelie D, Springael D, Römling U, Ahmed N, Mergeay M (1999) Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. Gene 238:417-425
18. Hu P, Brodie EL, Suzuki Y, McAdams HH, Andersen GL (2005) Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. J Bacteriol 187:8437-8449
19. Hu N, Zhao B (2007) Key genes involved in heavy-metal resistance in *Pseudomonas putida* CD2. FEMS Microbiol Lett 267:17-22
20. Hughes MN, Poole RK (1991) Metal speciation and microbial growth-the hard (and soft) facts. J Gen Microbiol 137:725-734
21. Intorne AC, Oliveira MVV, Lima ML, Silva JF, Olivares FL, de Souza Filho GA (2009) Identification and characterization of *Gluconacetobacter diazotrophicus* mutants defective in the solubilization of phosphorus and zinc. Arch Microbiol 191:477-483.
22. Janssen PJ, Houdt RV, Moors H, et al. (2010) The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. PLoS ONE 5:e10433
23. Ji G, Silver S (1995) Bacterial resistance mechanism for heavy metals of environmental concern. J Ind Microbiol 14:61-75
24. Kim E, Nies DH, McEvoy MM, Rensing C (2011) Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis. J Bacteriol 193:2381-2387

25. Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature 405:914-919
26. Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305:567-580
27. Leedjärv A, Ivask A, Virta M (2008) Interplay of different transporters in the mediation of divalent heavy metal resistance in *Pseudomonas putida* KT2440. J Bacteriol 190:2680-2689
28. Letunic I, Doerks T, Bork P (2009) SMART 6: recent updates and new developments. Nucleic Acids Res 37:D229-D232
29. Long F, Su C, Lei H, Bolla JR, Do SV, Yu EW (2012) Structure and mechanism of the tripartite CusCBA heavy-metal efflux complex. Phil Trans R Soc B 367:1047-1058
30. Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA
31. Monchy S, Benotmane MA, Janssen P, Vallaeys T, Taghavi S, van der Lelie D, Mergeay M (2007) Plasmids pMOL28 and pMOL30 of *Cupriavidus metallidurans* are specialized in the maximal viable response to heavy metals. J Bacteriol 189:7417-7425
32. Moraleda-Muñoz A, Pérez J, Extremera AL, Muñoz-Dorado J (2010) Differential regulation of six heavy metal efflux systems in the response of *Myxococcus xanthus* to copper. Appl Environ Microbiol 76:6069-6076
33. Mowade S, Bhattacharyya P (2000) Resistance of P-solubilizing *Acetobacter diazotrophicus* to antibiotics. Curr Sci 79:1591-1594
34. Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol 14:189-199
35. Nies DH, Brown N (1998) Two-component systems in the regulation of heavy metal resistance. In: Silver S, Walden W (eds) Metal ions in gene regulation, Chapman and Hall, London, UK, pp 77-103
36. Nies DH (1999) Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51:730-750
37. Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313-339
38. Nies DH, Rehbein G, Hoffmann T, Baumann C, Grosse C (2006) Paralogs of genes encoding metal resistance proteins in *Cupriavidus metallidurans* strain CH34. J Mol Microbiol Biotechnol 11:82-93
39. Nikaido H (2011) Structure and mechanism of RND-type multidrug efflux pumps. Adv Enzymol Relat Areas Mol Biol 77:1-60
40. Nikaido H, Takatsuka Y (2009) Mechanisms of RND multidrug efflux pumps. Biochim Biophys Acta 1794:769-781
41. Pages D, Rose J, Conrod S, Cuine S, Carrier P, Heulin T, Achouak W (2008) Heavy metal tolerance in *Stenotrophomonas maltophilia.* PLoS ONE 3:e1539
42. Pan K, Wang W-X (2012) Trace metal contamination in estuarine and coastal environments in China. Sci Total Environ 421-422:3-16
43. Prapagdee B, Watcharamusik A (2009) Adaptive and cross-protective responses against cadmium and zinc toxicity in cadmium-resistant bacterium isolated from a zinc mine. Braz J Microbiol 40:838-845
44. Rodrigues Neto J, Malavolta Jr VA, Victor O (1986) Meio simples para isolamento e cultivo de *Xanthomonas campestris* pv. *citri* tipo B. Summa Phytopathol 12:16
45. Rossbach S, Mai DJ, Carter EL, Sauviac L, Capela D, Bruand C, Bruijn FJ (2008) Response of *Sinorhizobium meliloti* to elevated concentrations of cadmium and zinc. Appl Environ Microbiol, 74:4218-4221
46. Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, USA, pp 365-386
47. Sambrook J, Russel WD (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA
48. Saravanan VS, Madhaiyan M, Osborne J, Thangaraju M, Sa TM (2007) Ecological occurrence of *Gluconacetobacter diazotrophicus* and nitrogen-fixing *Acetobacteraceae* members: their possible role in plant growth promotion. Microb Ecol 1:1-11
49. Saravanan VS, Madhaiyan M, Thangaraju M (2007) Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacer diazotrophicus*. Chemosphere 66:1794-1798
50. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. Proc Natl Acad Sci USA 95:5857-5864
51. Seon-Woo L, Glickmann E, Cooksey DA (2001) Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium-transporting ATPase and a MerR Family Response Regulator. Appl Environ Microbiol 67:1437-1444
52. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739
53. Tseng T, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier MHJ (1999) The RND Permease Superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J Mol Microbiol Biotechnol 1:107-125
54. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680
55. Trajanovska S, Britz ML, Bhave M (1997) Detection of heavy metal ion resistance genes in Gram-positive and Gram-negative bacteria isolated from a lead-contaminated site. Biodegradation 8:113-124
56. van der Lelie D, Schwuchow T, Schwidetzky U, Wuertz S, Baeyens W, Megeay M, Nies DH (1997) Two-componente regulatory system involved in trasncriptional control of heavy-metal homeostasis in *Alcaligenes eutrophus*. Mol Microbiol 23:493-503
57. von Rozycki T, Nies DH (2009) *Cupriavidus metallidurans*: evolution of a metal-resistant bacterium. Antonie van Leeuwenhoek 96:115-139
58. Xiong J, Li D, Li H, He M, Miller SJ, Yu L, Rensing C, Wang G (2011) Genome analysis and characterization of zinc efflux systems of a highly zinc-resistant bacterium, *Comamonas testosteroni* S44. Res Microbiol 162:671-679