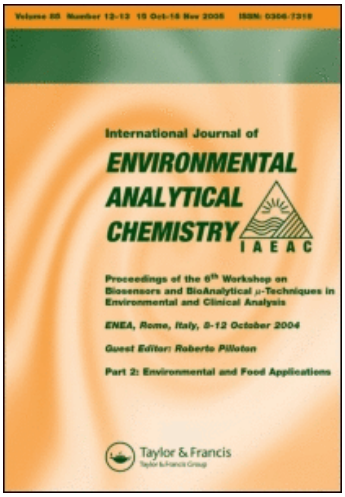

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Metal transport ATPase genes from Cupriavidus metallidurans CH34: a transcriptomic approach

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Metal transport ATPase genes from *Cupriavidus metallidurans* CH34: a transcriptomic approach

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Cupriavidus metallidurans strain CH34 is a multiple metal resistant β-proteobacterium isolated from the sediments of a zinc metallurgical plant. This strain possesses a large diversity of heavy-metal-resistance genes that are located mostly on two large plasmids, pMOL28 and pMOL30. The metal efflux ATPases constitute the major mechanism by which metal ions are removed and pumped out from the cytoplasmic pool of thiol groups. The genome of *C. metallidurans* CH34 contains eight such P1-ATPases. This high number of ATPases, compared with the P1-ATPase content in other micro-organisms, is a typical feature of the adaptation of *C. metallidurans* CH34 to metal-rich biotopes. We performed a phylogenetic analysis, and used quantitative PCR to study the metal-specific induction of the ATPases genes and, for some of them, their neighbouring genes. One of the ATPases, a homologue of the *E. coli zntA* gene, was characterized in detail by analysing the phenotypes of *zntA* mutants in different genetic backgrounds. In addition to the already-reported sensitivity to Zn(II), Cd(II), and Pb(II), *zntA* mutants also displayed a higher sensitivity to Tl(I) and Bi(III), indicating that the ZntA protein might play a role in the tolerance of very large cations.

Keywords: Ralstonia; *Cupriavidus metallidurans*; P1-type ATPase; Heavy-metal resistance; ZntA; Thallium; Bismuth

1. Introduction

Cupriavidus metallidurans CH34 [1] (formerly *Ralstonia* and more recently *Wautersia*) [2, 3] was isolated from the sediments of a Belgian zinc metallurgical plant [4]. Closely related strains were found in a variety of industrial biotopes linked to the metallurgical industry [5, 6]. This β-proteobacterium is a facultative hydrogenotroph that possesses two large plasmids carrying many genetic determinants for metal resistance. Altogether, at least 150 genes conferring resistance to Ag(I), As(III), As(V), Bi(III), Cd(II), Co(II), Cr(VI), Cu(I), Cu(II), Hg(II), Mn(II), Ni(II), Pb(II), Tl(I), and Zn(II) were identified

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(although some putatively) on the four replicons: one chromosome (3.9 Mb), one megaplasmid (2.6 Mb), and two large plasmids: pMOL30 (234 kb), and pMOL28 (171 kb) [7] (GenBank accession numbers NC_006525 and NC_006466, respectively). These plasmids contain at least 70 genes that are thought to be involved in heavy-metal resistance, augmenting the already-substantial tolerance of heavy metals governed by the chromosomal genes in this bacterium. Heavy-metal-resistance determinants include genes that encode reductases, membrane transporters, cation diffusion facilitators (CDFs), and tri-component cation efflux proton antiporter systems of the Resistance Nodulation Division–Heavy Metal Efflux family (RND-HMEs) [8]. The CDF and RND efflux systems remove free ions from the cytoplasm and the periplasm [7, 9]. The most recalcitrant fraction of toxic metals is linked to the cytoplasmic pool of thiols, and its removal is energy-intensive. This is where P-type ATPases, key enzymes of the cellular metabolism, come into play. These P-type ATPases are named according to their conserved DKTG motif involved in the phosphorylation of aspartate and are ancient, universal proteins found in all organisms [10, 11]. Thanks to their ability to hydrolyse ATP, these enzymes are able to generate a trans-membrane ionic gradient. Among the P1-ATPases are the P1-B subgroup—also known as the HM-ATPases (for heavy metal)—which have been implicated in the regulation of the cytoplasmic concentration of various heavy metals [12]. This sub-family clearly differs from the P1-A ATPases, which are mainly involved in K^+ transport. The family P1-B constitutes a large group of proteins [7] with two major branches. The first branch contains proteins related to *Staphylococcus aureus* CadA [13]. The second branch includes ATPases involved mainly in trafficking of copper, but also of silver.

Genome mining of *C. metallidurans* strain CH34 showed the occurrence of up to 11 putative genes encoding ATPases (figure 1), among which we identified eight putative metal-transporting ATPases, also called CPx-ATPases according to a conserved CP*x* motif involved in metal binding (*x* stands for a cysteine, histidine, or serine residue). This high number of ATPases present in *C. metallidurans* compared with the average of three P1-ATPases in other micro-organisms [7] shows how well *C. metallidurans* is adapted to metal-rich biotopes. Using protein multi-alignment and phylogenetic analysis, as was similarly described for *Archaea* species [14] or *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [15, 16], we show in this work that these eight CPx-ATPases present on the genome of *C. metallidurans* CH34 all belong to the group of P_{1B}-ATPases [12, 17] (figure 1). These P_{1B}-type ATPases are often associated with mobile genetic elements: three are plasmid-bourne (pMOL30), PbrA, CzcP, and CopF, and two are harboured by the RMGI-2(C) genomic island [18], namely CadA and $FixI_1$, a cation transport ATPase similar to a FixI-like protein found in *Bradyrhizobium* sp. Two from the eight *C. metallidurans* P1-type ATPases, CadA and ZntA, are known to be involved in cadmium and zinc resistance, respectively [19]. The interplay between both P-ATPases has also been shown: a double mutant of *cadA* and *zntA* led to a sixfold decrease in zinc resistance and a 350-fold decrease in cadmium resistance, whereas a single mutant in the *C. metallidurans* plasmid-free derivative strain had only a moderate effect on zinc and cadmium resistance [19]. In this work, we have investigated the expression of those eight P_{1B}-ATPases by transcriptomic analysis (quantitative RT-PCR) by determining their genetic response after exposing bacterial cultures of *C. metallidurans* CH34 to different levels of various heavy metals, and the effect of a *zntA* mutant on zinc, cadmium, copper, lead, thallium, and bismuth resistance.

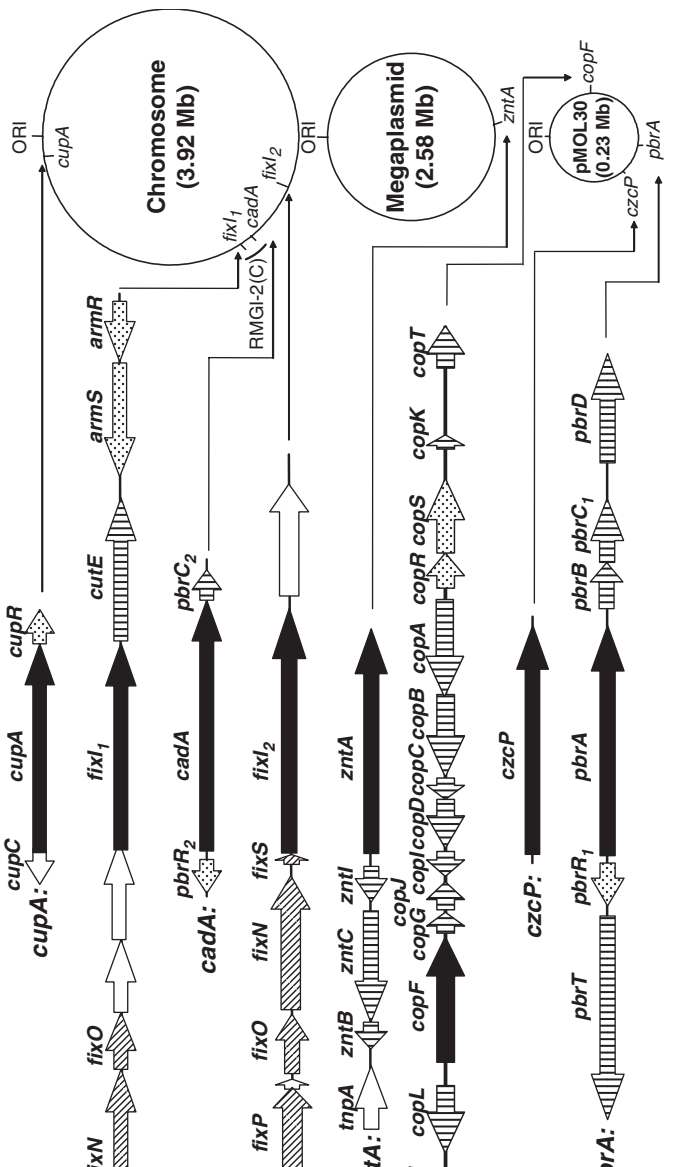

Figure 1. Eight P1-type ATPase gene clusters in *C. metallidurans* CH34. This figure shows the localization of the eight P1-Type ATPases within the *C. metallidurans* CH34 genome (four on the chomosome, one on the megaplasmid, and three on the plasmid pMOL30). The P1-type ATPases are grouped into three separate classes according to their metal selectivity. Class I contains four P1-type ATPases involved in Zn/Cd/Pb transport: CzcP and PbrA, both encoded by plasmid pMOL30, CadA encoded by the genomic island RMGI-2(C), and ZntA. Class II contains two P1-type ATPases involved in Cu/Ag transport: CupA, CopF located on plasmid pMOL30. In the last group, class III contains P1-type ATPases with still unknown specificity and includes two FixI ATPases, one carried by the genomic island RMGI-2(C). This representation shows (for ease of reading) the pMOL30 plasmid on a different scale compared with the two other replicons. The *cop* cluster that includes ATPase *copF* is also represented on a different scale to the other clusters.

2. Experimental

2.1 *Measurement of ATPase gene induction by real-time quantitative PCR (RT-qPCR)*

Liquid cultures were grown at 30°C with Tris mineral liquid medium shaken at 120 rpm on an orbital shaker until the OD at 660 nm reached 0.3 (early log phase). Ten millilitres of cultures were then transferred simultaneously into Falcon tubes and challenged with 0.8 mM Cd(II), 0.4 mM Pb(II), 0.8 mM Cu(II), 1 mM Zn(II), or 0.1 mM Tl(I). Cultures were incubated at 30°C for 15 min and 3 h, respectively. Total RNA was extracted from control and induced cultures of *C. metallidurans* using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Extracted RNAs were stored at −80°C until use. Reverse transcription of target mRNA was performed using the Taqman Reverse Transcription reagent (Roche), as specified by the manufacturer, in the AB Applied Microsystem (2700). Quantitative RT-PCR was conducted on the resulting cDNA using specific primer sets designed for each ATPase encoding gene. Using the PrimerExpress software (Applied Biosystems), the following primer sets were designed: *pbrA* (pMOL30): forward CAAAGGCGGCGTCTATCTG, reverse CCCCACACCACGAAATCG; *cadA* (RMGI-2(C)): forward AGAAGGCGCTTGCT TCCATT, reverse AGACTTTCCTCGGTCGGACAGT; *zntA* (Chr) forward AGA CGCTGATCCGCAACAAGAT, reverse AGTGAGTCGAGCGTGTGGTGAA; *czcP* (pMOL30) forward CGCCATTGAAGTGGAAGTGA, reverse GCTGGC TCGGTGACTTTTG; *cupA* (Chr) forward AACCTGTTCTGGGCCTTCAT, reverse GACCACGCTGACACTTGAAA; *copF* (pMOL30) forward GGTATTCGTGC TCGCAGTCATT, reverse CCACAATCAGTGCGGTAACCTT; $FixI_1$ (RMGI-2(C)) forward TTTGCCTCTGTTGCTGTCCTT, reverse CAGCAGAAAAGTGATCAA CATGGT; $FixI_2$ (Chr) forward TGTCGCCACCCTCTTGATC, reverse GCAAGGA CAGCTCACAACGA. Amplification of *rrnB* encoding 16S rRNA (presumed non-inducible by heavy-metal challenges) was used for normalization purposes.

Quantitative RT-PCR was performed with Syber Green (Syber Green PCR Master mix; Applied Biosystems), while fluorescence was continuously monitored by the Sequence Detection System 5700 (Applied Biosystems). Data were analysed by normalization, first according to the amount of 16S rDNA amplicon produced as an internal control, and second according to the negative controls (growth without addition of heavy metals).

2.2 *Construction of zntA insertional mutants of the wild-type* C. metallidurans *strain CH34 and its plasmid free derivative AE104*

An internal gene fragment was amplified from the *zntA* gene using a specifically designed primer set. Amplification was carried out using a MJ-PTC-100 thermocycler (MJ Research), and each 100 µL reaction mixture contained 1× amplification buffer and 1 U Taq DNA polymerase (Promega). A hot start of 3 min at 94°C was followed by 35 cycles of denaturation (45 s at 94°C), annealing (30 s at 55°C), extension (2 min at 72°C), and a final extension (6 min at 72°C). To check for successful amplification, electrophoretic analysis of 5 µL of amplicons was carried out on horizontal ethidium bromide stained 1% agarose gels (BDH) in TBE buffer (40 mM Tris-HCl, 4 mM sodium acetate, 1 mM EDTA, pH 7.9) using the Biorad DNA submarine system.

Internal ATPase PCR fragments were further cloned into the 2.1 Topo Cloning Vector (Invitrogen) via A/T complementation according to the manufacturer's instructions, resulting in the construction of a *lacZ::zntA*Δ plasmid that was electroporated into competent DH5α *E. coli* strains as previously specified [11]. White transformant colonies were selected on LB agar supplemented with X-gal, IPTG, and 100 μg mL^{-1} of ampicillin. Plasmids were extracted from the transformants using the Qiagen mini plasmid extraction kit (Qiagen), and subsequently used as a template for sequence determination. Sequencing reactions (total volume 10 μL) contained approximately 300 ng of DNA template (4 μL), 1.5 μL of ABI PRISM BigDye Terminator v1.1 Cycle Sequencing RR-100 Mix, 1.2 μL of 5× sequencing buffer and 2.4 pmol of primers T7 or M13. The sequencing programme involved an initial denaturation step (94°C for 60 s) followed by 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reactions were precipitated with 40 μL of 76% ethanol for 30 min, washed with 70% ethanol, and re-suspended in 10 μL of 0.3 mM EDTA buffer. Electrophoresis, data collection, and fragment analysis were performed using an ABI PRISM 3700 Genetic Analyser. Sequences were checked by alignment with the *C. metallidurans* CH34 genome sequence data available at the JGI website http://genome.jgi-psf.org/draft_microbes/ralme/ralme.home.html. Subsequently, internal ATPase fragments were sub-cloned into pPW78 [derived from the pALTER-1 mutagenesis vector] (Promega) as *Bam*HI to *Xba*I fragments [20]. The pPW78 vector was customized by the insertion of a *mob* RK2 origin of replication, resulting in a mutagenic mobilisable vector. The vector also carried a *lacZ* gene allowing for traditional blue/white screening of the insert. The obtained construction was electroporated into *E. coli* S17.1. This strain contains an RP4 derivative integrated into the chromosome and is able to efficiently mobilize Mob$^+$ plasmids and vectors to a variety of recipients without transferring RP4. White transformant colonies were then selected on LB agar supplemented with 10 μg mL^{-1} of tetracycline, Xgal and IPTG. Plasmids were extracted from the transformants using the Qiagen mini plasmid extraction kit (Qiagen) and subsequently digested with *Xba*I–*Bam*HI to check for the correct construction. Transfer of the construct (via the S17.1 derivative) from the donor into *C. metallidurans* (recipient) was performed by conjugation. Recombinant *C. metallidurans* clones with the integrated pPW78::*zntA*Δ resulted in the disruption of the counterpart *zntA* gene and were selected on Tris minimal gluconate medium supplemented with 20 μg mL^{-1} of tetracycline.

2.3 *Phenotypic analysis of* zntA *mutants on zinc, cadmium, thallium, and bismuth*

C. metallidurans strains were streaked on 284 Tris minimal medium plates that were supplemented with increased concentrations of heavy metals. Similar studies were performed in liquid media. A more accurate definition of the phenotype was carried out by determining viable counts on plates [21] supplemented with zinc (sulphate) in the concentration range from 0.05 to 12.6 mM, cadmium (chloride) in the concentration range from 0.05 to 6.4 mM, thallium (nitrate) in the concentration range lying between 0.05 and 1.6 mM, lead (acetate) in the concentration lying between 0.05 and 6.4 mM, copper (sulphate) in the concentration range lying between 0.05 and 6.4 mM, and bismuth (ammonium citrate) in the concentration range from 0.0125 to 0.8 mM. The determination of viable counts was carried out by plating 10–20 μL droplets

of appropriate dilutions on the same plate and incubation at 30°C for 5–9 days. The minimal inhibiting concentration (MIC) was then determinated for each strain.

2.4 *Phylogenetic analysis*

Phylogenetic trees were built using the Phylip package. Proteins similar to the individual ATPases selected were identified by searching the NCBI protein database using BLASTP. Multiple alignments in Phylip format were performed using the ClustalW software at http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl. The alignments were analysed with 'protdist' (Phylip package) that uses protein sequences to compute a distance matrix. The distance for each pair of species provides an estimate of the total branch length between the two species and was used in the distance matrix program NEIGHBOR. The 'protdist' output was then analysed with the 'NEIGHBOR' program (Phylip package), which implements the Neighbour-Joining method. NEIGHBOR constructs a tree by successive clustering of lineages, setting branch lengths as the lineages join. A bootstrap analysis was performed using both 'protpars' (to generate 100 random trees) and 'consense' (to compute a consensus tree). The resulting tree was displayed using 'treeview' in an unrooted format. NCBI accession numbers are given in parentheses.

3. Results

3.1 *Function of* C. metallidurans *P-ATPases and organization of the corresponding gene clusters/operons*

P-type ATPase structure predictions show that the eight *C. metallidurans* CH34 ATPases share the classic features of canonical heavy-metal ATPases [8, 10, 12, 22]. They possess one or more heavy metal binding sites on the N-terminal extremity [7], a conserved intramembranous CP*x* motif (*x* = Cys, His, or Ser), a conserved dipeptide His–Pro (HP) in the second cytoplasmic domain, and a unique number and topology of the membrane-spanning domains [23]. Next to the polar amino terminus, the polypeptide chain was predicted to cross the membrane fourfold before the first cytoplasmic domain (except for the chromosomal ZntA and the pMOL30-borne CzcP). At the carboxy-terminal end, following the second cytoplasmic domain, only two putative membrane helices were observed. Through this study, we distinguish three classes of P-type ATPases in *C. metallidurans* according to their metal specificities [7].

3.1.1 Class I of ATPases: Zn/Cd/Pb P-type ATPases. This class is composed of four ATPases that appear to be related to Cd(II), Zn(II), Pb(II) efflux (figure 1):

(1) ZntA, which [19] is part of a small cluster of genes, *zntBCIA*, that includes *zntCI*, a locus highly similar to *czcC–czcI*, and *zntB* which resembles a partial *czcB* (figure 1). The *zntA* gene is transcribed in the opposite direction to the putative transcription of *zntC* and is close to a transposase gene related to the Tn*3* family which does not match a regular IS-element. In the pMOL30 *czc* gene cluster [24–27], *czcI* encodes a protein that acts as an activator of

transcription in the presence of Zn(II) or Cd(II) [28]. We thus assume that ZntI could have a similar role (table 1).

(2) CadA, encoded by a gene located on the RMGI genomic island under the arrangement *pbrR*$_2$ *cadA pbrC*$_2$ [7, 18]. The protein PbrC$_2$ is 45% homologous to PbrC$_1$ encoded by pMOL30 on the *pbrTRABCD* cluster of genes. The gene *pbrC* is coding for the lipoprotein signal peptidase PbrC. The protein PbrR$_2$ is 52% homologue to PbrR$_1$ encoded by pMOL30. The *pbrR*$_2$ gene that encodes a merR-like regulatory protein is transcribed in the opposite direction to cadA (table 1) (figure 1) and is almost exclusively activated by Pb(II) [29].

(3) PbrA expressed by the pMOL30 *pbrA* gene of the locus *pbrTR*$_1$*ABC*$_1$*D* [11].

(4) CzcP shows less similarity with the other members of this group. The *czcP* gene is likely to be a 'lonely' gene that is located 4.6 kb downstream of the *czcNICBADRSE* locus (involved in resistance to Co(II), Zn(II), and Cd(II)) of pMOL30. An *ISRme3* is located between the ATPase *czcP* and the *czc* cluster. CzcP was so called because it seems to be regulated by *czcRS* (D. Nies, pers. comm.). This ATPase groups with the Zn/Cd ATPases from *R. capsulatus* and *C. crescentus*, and, to a lesser extent, with the Cd(II) ATPases from diverse *Firmicutes*.

3.1.2 Class II P-ATPases: Cu and Ag. This class is composed of two ATPases that appear to be related to Cu(I) and Ag(I) efflux:

(1) CupA is encoded by the gene *cupA* which lies on the chromosome in a small cluster including *cupR* and *cupC*, which encode a regulatory protein belonging to the MerR family of regulatory proteins and a copper chaperone, respectively (table 1) (figure 1). CupA is similar to other copper P-type ATPases present in pseudomonads (figure 2), but the real function of the chromosomal *cupA* ATPase is not yet known. However, it could have a detoxification function similar to that previously reported for the chromosomal *E. coli* CopA P-ATPase [30]. The CupA denomination is used in reference to the chromosomal *E. coli* CopA P-ATPase but also aims to avoid confusion with the CopA periplasmic proteins encoded by the *copSRABCD* gene clusters that are located on the pMOL30 plasmid as well as on the chromosome.

(2) The CopF encoding gene *copF* is phylogenetically linked to *silP*, which codes for a plasmid-borne *Salmonella typhimurium* P-ATPase involved in silver resistance and, to a lesser extent, to the *Enterococcus hirae copB* P-ATPase involved in copper export (figure 2). The corresponding *copF* gene mapped in a large cluster of 14 copper resistance genes: *copTKSRABCDIJGFLH* [31] (figure 1).

3.1.3 Class III: P-type ATPase with unknown substrates. The last class of P-type ATPases contains two ATPases that show similarities with FixI proteins of *Bradyrhizobium* sp. (figure 2): the FixI$_1$ (located on the genomic island RMGI-2(C) that is 100% identical to the PAGI-2(C) island of *P. aeruginosa* gene number C22) [18] and the FixI$_2$. The former, FixI$_1$, is part of a locus that includes an *arm*RS two-component regulatory system, four proteins that display similarities to cytochrome oxidases, and a CutE homologue [7, 18] whereas the latter, FixI$_2$, is bordered upstream

Table 1. Proposed nomenclature and function for the genes from the P1-type ATPases operons found in *C. metallidurans* CH34.[a]

Genes operons P-type ATPases	Location	Homologues	Origin of the homologues	Putative function
zntB	MPL	Δ*czcB*	pMOL30	Efflux ?
zntC	MPL	*czcC*	pMOL30	OMP
zntI	MPL	*czcI*	pMOL30	Regulation
zntA	MPL	*zntA*	*E. coli*	P-type ATPase
czcP	pMOL30	*cadA*	pMOL30 (under control czcDRS)	P-type ATPase
$pbrR_2$	Chr. (RMGI-2(C))	$pbrR_1$	pMOL30	MerR-like Regulation
cadA	Chr. (RMGI-2(C))	*cadA*	*S. aureus*	P-type ATPase
$pbrC_2$	Chr. (RMGI-2(C))	$pbrC_1$	pMOL30	Lipoprotein signal peptidase
cupC	Chr.	–	–	Copper chaperone
cupA	Chr.	*copF*	pMOL30	P-Type ATPase
cupR	Chr.	*merR*	–	MerR-like regulator
copF	pMOL30	–	–	P-type ATPase [29]
copTKSRABCDIJGLH	pMOL30	–	–	
pbrA	pMOL30	–	–	P-type ATPase [11]
pbrTRBCD	pMOL30	–	–	
$fixI_1$	Chr. (RMGI-2(C))	*fixI*	*Bradyrhizobium* sp.	P-type ATPase
fixN, *fixO*, *cutE*, *armSR*	Chr. (RMGI-2(C))		*Bradyrhizobium* sp.	
$fixI_2$	Chr.	*fixI*	*Bradyrhizobium* sp.	P-type ATPase
fixGPONS	Chr.		*Bradyrhizobium* sp.	

[a] The first column contains the nomenclature we propose for the various P1-type ATPases and their adjacent genes. For each gene, the localization (pMOL30; Chr. for chromosome; MPL for megaplasmid) and closest homologue together with its origin in *C. metallidurans* or in other bacteria are shown. A putative function is also assigned to each protein.

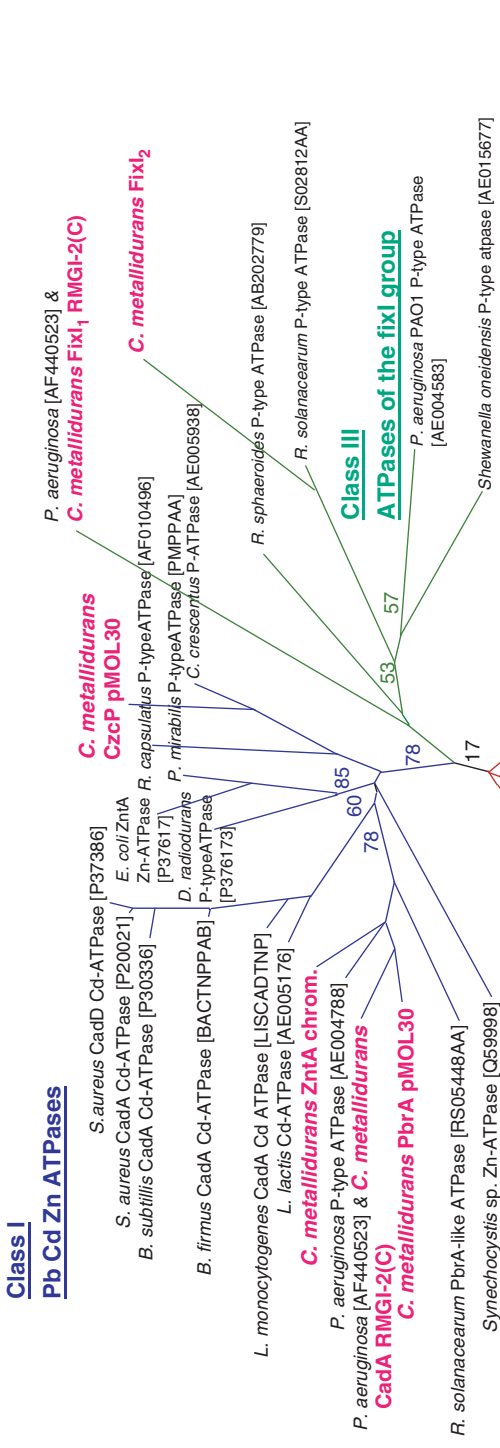

Figure 2. Phylogenetic representation of P1-ATPases. The phylogeny includes the eight P1-type ATPases found in *C. metallidurans* CH34 and shows three major branches: (1) class I P1-type ATPases involved in Cd(II), Zn(II), and Pb(II) efflux; (2) class II involved in Ag(I), Cu(II) efflux; (3) class III P1-type ATPases with unknown ionic specificity (adapted from [7]).

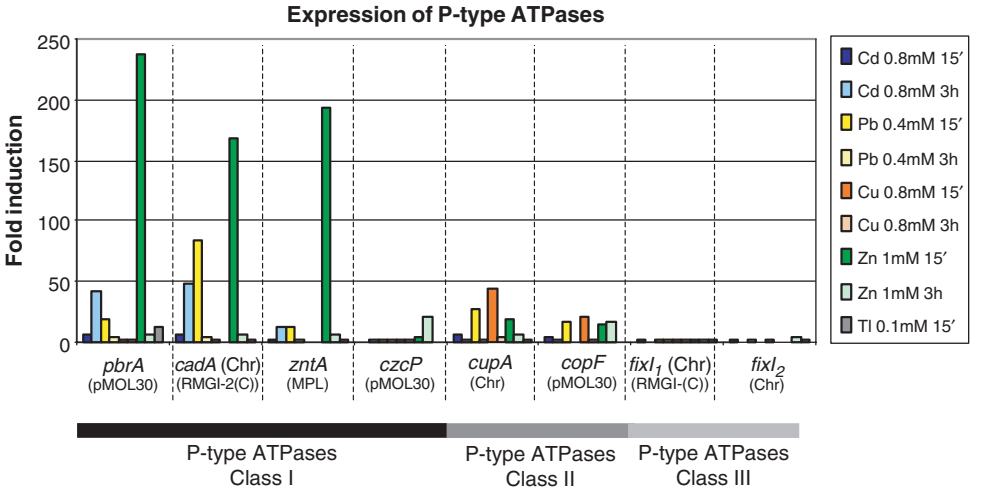

Figure 3. Expression analysis of the eight P1-ATPases of *C. metallidurans*. Expression analysis was performed by quantitative PCR on the eight P1-type ATPases found in the *C. metallidurans* CH34 genome. Induction with Cd(II) (0.8 mM), Pb(II) (0.4 mM), Zn(II) (1 mM), and Cu(II) (0.8 mM) was performed for 15 min or 3 h, and with Tl(I) (0.1 mM) for 15 min. Total RNA was extracted, and expression of all the genes encoding the eight P1-type ATPases was measured.

by five genes encoding cytochrome type-C oxidases (figure 1). The substrate specificities of those two ATPases are not yet known and could not be predicted with motif searches or sequence analysis.

3.2 *Expression analysis on the eight P-type ATPases*

Expression analysis using quantitative PCR was performed after 15 min or 3 h of induction with Cd(II) 0.8 mM, Pb(II) 0.4 mM, Cu(II) 0.8 mM, or Zn(II) 1 mM, respectively.

3.2.1 Class I P-ATPases. These show an increased level of expression after induction by zinc, lead, or cadmium (figure 3). ZntA, CadA, and PbrA were induced by zinc 193-, 237-, and 167-fold, respectively. Zinc appears to be the best metal inducer for the ATPases of this group. The expression levels of these three proteins are also highly induced by lead with an induction rate of 13, 85, and 18 times the basal level, respectively, and by cadmium with a respective 13-, 48-, and 42-fold induction. These three proteins surely play an essential role in the resistance of *C. metallidurans* to these metals. The CzcP ATPase shows only a slight induction (five times) after 3 h of exposure to zinc, while all the other ATPases show their maximum of induction after only 15 min and no (increased) induction after 3 h. For this first class of P-type ATPases, no significant induction by copper was observed. For the *zntA* gene, we also tested the expression of neighbouring genes. The corresponding data are reported below.

3.2.2 Class II P-ATPases. These are mainly induced by copper (figure 3). CupA is induced 45 times by copper, but also to a lesser extent by lead (28 times) and zinc (18 times) after 15 min of induction. CopF shows a similar induction profile with a major induction of 22 times by copper, but also an induction by lead (18 times) and zinc (14 times). CopF and CupA are classified in the group of copper efflux P-type ATPases, but because they are both also induced by zinc, cadmium, and lead, the possibility that they may be involved in resistance to those metals as well cannot be excluded.

3.2.3 Class III P-ATPases. These display sequence similarities to the FixI protein of *Bradyrhizobium* sp. (figure 2) but did not show any significant metal induction by any of the heavy metals tested (figure 3). These proteins are probably involved in other cation effluxes and may not be involved in heavy-metal resistance in *C. metallidurans*.

3.3 *Expression analysis in response to thallium*

We also investigated whether thallium, to which *C. metallidurans* CH34 shows resistance via an as yet unknown mechanism, would induce P-type ATPase expression. Thallium is located close to lead in the table of elements and has a predominant valence of one, this contrary to all the other heavy-metal ions tested before. A culture of *C. metallidurans* was induced with a concentration of 0.1 mM thallium for 15 min. Only *pbrA* showed a significant response with a 12-fold induction (figure 3), which is in the same order as the induction by Pb(II), but which is much lower than that for Zn(II) and Cd(II). PbrA is normally involved in lead resistance [11]. This result suggests that this P-type ATPase could also be involved in the response to Tl(I). Appropriate experiments have to be designed to test if Tl(I) could be a substrate of PbrA.

3.4 *znt cluster: expression analysis*

An expression analysis performed on each of the genes belonging to the cluster *zntB zntCI zntA* showed a main induction by zinc and a slight induction by cadmium. The other metals tested (Pb(II) or Cu(II)) did not show any induction of the *zntB–zntCI* genes for the concentration and time ranges used. In this cluster of genes, *zntC* was induced the most by Zn(II) (up to 240 times), while the other genes of this cluster showed a considerably lower induction rate (22 times for the *zntI* and 15 times for *zntB*). Thus, the response to Zn(II) observed for this group of genes supports the proposed *znt* nomenclature.

3.5 *Phenotypic analysis of the* C. metallidurans zntA *mutants*

The motif CXXEE was found in all *C. metallidurans* P-type ATPases belonging to the Zn/Cd/Pb type of transporters (PbrA, ZntA, and CadA). This motif appeared to be present as part of a fully conserved MDCXXEEXLXR motif, which could be repeated up to three times in the protein sequence (as is the case in CadA). The chromosomal ZntA harboured both a CXXEE and a histidine-rich motif. The peculiar histidine-rich region is composed of 16 histidines in a 35-amino-acid-long peptide located in the ZntA NH_2-terminal part.

Table 2. Minimal inhibitory concentration (MIC) for *C. metallidurans* CH34 and its derivatives.[a]

	zntA$^+$ Czc$^-$ (mM)	*zntA*$^-$ Czc$^-$ (mM)	*zntA*$^+$ *czc*$^+$ (pMOL30 and pMOL28) (mM)	*zntA*$^-$ *czc*$^+$ (pMOL30 and pMOL28) (mM)
Zinc	0.4	0.2	6.4	3.2
Cadmium	0.4	0.4	3.2	0.8
Thallium	0.1	0.1	0.4	0.2
Bismuth	0.4	0.2	0.1	0.1
Lead	0.8	0.8	1	~1
Copper	0.6	0.6	1.2	1.2

[a] The MIC values for zinc, cadmium, thallium, and bismuth were determined for the *C. metallidurans* CH34 wild-type strain (*zntA*$^+$ *czc*$^+$) and its corresponding mutant strain AE2710 (*zntA*$^-$ *czc*$^+$), and for the plasmid-free derivative strain AE104 (*zntA*$^+$ Czc$^-$) and it corresponding mutant strain AE2716 (*zntA*$^-$ Czc$^-$). Bacterial cultures were diluted appropriately, and 20 µL droplets of dilutions were deposited in triplicate on Petri dishes containing TRIS minimal medium supplemented with various metal concentrations. The MIC values were determined by counting colonies that appeared after 5 days' growth at 30°C. NB: Czc$^-$ indicates the phenotype of sensitivity to Cd(II), Zn(II), Co(II) that is observed in the absence of plasmid pMOL30 (carrying the *czc* cluster).

Because of its particular stucture, insertional mutagenesis was conducted on ZntA to investigate the metal resistance mechanism provided by this protein. Insertional *zntA* mutants were constructed both in the wild type strain and in its plasmid-free derivative AE104 (leading to *zntA* mutant strains AE2710 and AE2716, respectively). Although ZntA is involved in the resistance to zinc, the *czc* operon located on plasmid pMOL30 also confers a high resistance to zinc and could partially mask the zinc-related phenotype of *zntA*. Therefore, *zntA* mutants were constructed in both genetic backgrounds and viable counts were performed after exposure to various concentrations of zinc, cadmium, thallium, lead and bismuth.

3.5.1 Response to Zn(II). The *zntA*$^-$ Czc$^+$ mutant AE2710 appeared 16 times more resistant to zinc, with an MIC of 3.2 mM than its plasmid-free counterpart, strain AE2716 (*zntA*$^-$, Czc$^-$) with an MIC of 0.2 mM. Strain AE2710 is also clearly more sensitive to zinc (twice) than the CH34 wild-type strain (*zntA*$^+$, Czc$^+$), with an MIC of 6.4 mM (table 2). This indicates that both *zntA* and *czc* genes are required for full expression of the zinc resistance phenotype and detoxification, which confirms previous observations [19]. As would be expected for an ATPase, ZntA appears to be involved mainly in cytoplasmic detoxification.

3.5.2 Response to Cd(II). As far as the resistance to cadmium is concerned, the maximum detoxification is observed with the presence of active ZntA and Czc (MIC 3.2 mM). In the *zntA*$^-$ Czc$^+$ derivative, the MIC decreases four times (MIC 0.8 mM), but the resistance remains higher than that observed in the plasmid-free derivative of *C. metallidurans* CH34 (MIC 0.4 mM) (table 2). However, no cadmium resistance phenotype is observed with *zntA* alone (although Cd(II) induced *zntA*). A role for *zntA* in the cadmium detoxification is observed only in the Czc$^+$ background (strain 2710). This is very different to what is observed with zinc and suggests the importance of the periplasmic compartment for effective detoxification of Cd(II).

3.5.3 Response to Pb(II). In the presence of lead, the wild-type strain CH34 has an MIC of 1 mM (table 2). A slight decrease of this MIC was observed for its corresponding *zntA* mutant, similar to that observed by Legatzki *et al*. [19]. For the plasmid-free derivative strain, the MIC is 0.8 mM, but no significant difference is observed for its *zntA* mutant. After streaking the bacteria on increased lead concentrations, we observed a confluent growth of the bacteria in the first streaks (where the bacteria density is the highest). Here, there is likely a mass effect that is followed not by a progressive decrease in the density of colonies along the further streaks, but rather by a sharp lethal effect. The bacteria are probably pumping in the metal and dying, leading to a decrease in the toxic metal concentration per bacteria, allowing the neighbouring bacteria a better chance of survival. Such a 'protective' effect would quickly disappear when the bacterial concentration decreased.

3.5.4 Response to Cu(II). In the presence of copper, the wild-type strain ($zntA^+$ Czc^+) has an MIC of 1.2 mM, and the plasmid-free derivative strain ($zntA^+$ Czc^-) has an MIC of 0.6 mM. In both strains, no difference in the MIC is observed when *zntA* is mutated (table 2).

3.5.5 Response to Tl(I). The wild-type strain has an MIC for Tl(I) four times higher than that of the plasmid free derivated strain (table 2). Interestingly enough, derivative strains containing either pMOL28 or pMOL30 also confer the same slight resistance to Tl(I) as the wild type (results not shown). Despite the fact that no significant difference is observed regarding the resistance of the plasmid-free derivative strain and its corresponding *zntA* mutant, a clear decrease (twice) of the MIC is observed between the wild-type strain CH34 and its *zntA* mutant (table 2). This points to a situation quite similar to that observed with Cd(II): ZntA is involved in resistance to Tl(I) but requires the presence of a determinant carried by either pMOL30 or pMOL28. Apart from the *mer* Hg(II)-resistance determinants, the only mechanisms shared by pMOL28 and pMOL30 are RND/CDF detoxification systems: *czc* in pMOL30 or *cnr* in pMOL28 (where there is no gene encoding for any P1-ATPase). Alternatively, as *pbrA* of pMOL30 is also induced by Tl(I), this P-ATPase may also play a role in the detoxification of Tl(I).

3.5.6 Response to Bi(III). This metal shows surprising results (table 2):

(1) The presence of plasmids clearly increased the sensitivity to this metal, irrespective of the status of *zntA*.
(2) In the plasmid-free derivative, the mutation in *zntA* increased the sensitivity to this metal, thus implicating ZntA in the response to Bi(III). It is one of the very first phenotypes linked to bismuth up to now.

The plasmids do not seem to carry resistance to Bi(III) and even seem to interfere with a chromosomal tolerance to this cation. The *znt* operon encodes a likely functional CzcC-like protein (an outer membrane protein) that could be adequately involved to achieve some detoxification of Bi(III), as observed in the wild-type strain (but also possibly Zn(II)) although we cannot exclude the participation of ZntB (the apparently truncated CzcB-like protein) in this mechanism. In the case of Bi(III), the presence

Table 3. Putative adjacent regulator for metal induced P1-type ATPases.[a]

P1-ATPase	Adjacent regulator	Metal regulator inducer	Metal inducer of the P-type ATPase				
			Zn(II)	Cd(II)	Cu(II)	Pb(II)	Tl(I)
ZntA	ZntI	Zn(II)	+	+	−	+	−
CadA	$PbrR_2$	Pb(II) [29]	+	+	−	+	−
PbrA	$PbrR_1$	Pb(II)	+	+	−	+	+
CzcP	No	−	+	−	−	−	−
CupA	CupR	nd	+	−	+	+	−
CopF	Unknown	Unknown	+	−	+	+	−

[a] For each of the P-Type ATPases shown to be induced in the metal condition, the putative adjacent regulator is given. The metal inducer of both the putative regulator and the P-ATPase is indicated. nd: not done.

of plasmid-encoded proteins clearly antagonizes the slight but significant detoxification effect of ZntA.

4. Discussion

P1-type ATPases constitute an efficient detoxification system, especially to extrude metals from thiolates complexes that could not be extruded by the plasmid-borne RND/CDF detoxification systems, such as *czc* [19]. *C. metallidurans* CH34 possesses eight P1-type ATPases, six of them being induced by one or more of the following metals Cu(II), Cd(II), Pb(II), Zn(II), and Tl(I). Those P1-type ATPases detoxify the cytoplasm from heavy metals such as Cd(II), Zn(II), Pb(II), and Cu(II). They seem to be involved also in the response to Tl(I) and Bi(III), although, up to now, there has been no evidence to suggest that efflux may be involved. The trans-membrane domain of the P1-type ATPase confers its metal selectivity [32, 33], but it is well established that the amino-terminal domains of P1-ATPases show a much larger ion-specificity than the ATPases themselves. This resistance mechanism may act in synergy with chromosomal or plasmid-borne RND/CDF detoxification systems, such as the pMOL30 *czcCBA* gene cluster involved in Cd(II), Zn(II), and Co(II) resistance [7], as was shown with *zntA* mutants. The presence of the P1-type ATPases increases the plasmid-borne resistance levels and allows a slight but decisive adaptation advantage in a metal-rich environment.

The regulators for the P-type ATPases usually belong to the MerR or ArsR-SmtB family of proteins [34]. The MerR-type regulators exert a negative control in the absence of metal by binding as dimers on the promoter of the P1-type ATPase gene [35]. In the presence of their specific heavy-metal ions, the MerR-type regulators will undergo a conformational change, which results in distortion of the promoter-operator region DNA followed by transcription of the P1-ATPase. In *C. metallidurans*, merR-like regulator genes adjacent to P-ATPases genes were identified for *pbrA*, *cupA*, and *cadA*, and were tentatively called $pbrR_1$, *cupR*, and $pbrR_2$ respectively (table 3). The *copF* gene lies inside the complex *cop* locus, and there have been no real hints about its regulation until now. The 'lonely' *czcP* gene was suggested to belong to the pMOL30 *czc* regulon and to be under the control of the *czcDRSE* gene cluster (D. Nies, pers. comm.).

In the case of *zntA*, *zntI*, which is part of the *znt* operon, could be a possible regulator. This gene is homologous to *czcI*, which belongs to the *czc* gene cluster and is involved in *czc* regulation. Other regulators acting *in trans* may also exist and interplay with a *cis*-acting regulator to induce the expression of P1-type ATPases. The protein encoded by *zntI* is a possible regulator of *zntA*, but *zntI* is induced mostly by Zn(II) and slightly by Cd(II), while *zntA* expression is also significantly induced in the presence of Pb(II) (figure 3) (table 3). In addition, no difference in Cd(II) resistance phenotype is observed with the *zntA* mutant in the absence of pMOL30. Thus, a possible *trans*-acting regulator, such as CzcI, could be located in the pMOL30 *czc* cluster. Another possible candidate for trans regulation is the chromosomally located PbrR710B, belonging to the family of MerR-like regulators (D. van der Lelie, pers. comm.). *In vitro* studies showed that PbrR710B, which has no structural resistance genes located in its proximity, is able to bind to the ZntA promoter (this promoter, with the sequence TTGACCCTGTA GCGACTAAAGGGTCTTCAAT, has all the features of a MerR-recognized promoter, including the internal symmetry via GACCCTgTAG and its inverse complement) and activate its transcription in the presence of Hg(II), but not in the presence of Zn(II), Cd(II), and Pb(II) (C. He and D. van der Lelie, pers. comm.). Therefore, we suggest that ZntA is under the control of several putative individual regulons: ZntI, PbrR710B, and a pMOL30 encoded regulator (presumably located in the *czc* cluster), where each regulon is specific for certain heavy metals (table 3).

A second example of double regulation is given by the expression of *cadA*. The gene $pbrR_2$, previously referred to as *pbr*R691 [29], is encoding a MerR-like regulator involved in the regulation of CadA (table 3). $PbrR_2$ binds Pb(II) very specifically, and *in vitro* studies show that it activates the gene encoding for CadA [29]. However, this P1-ATPase is also induced to high expression levels by Cd(II) and Zn(II), both metals that are not specifically recognized by the $PbrR_2$ protein (figure 3). An additional regulator, acting *in trans*, is probably involved in this induction.

The fact that some metal-resistance genes are induced via different regulation by a diversity of metals that are not substrates, might make sense from a geo-biological point of view, as heavy metals are often present together at variable concentration in ores and in waste. This may be of relevance for samples containing abundant essential metals (Zn, Cu, Mn, Co, . . .) and low but toxic concentrations of non-essential very toxic metals (Hg, Cd, Tl, Ag, . . .).

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