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ORIGINAL PAPER

Lead(II) resistance in *Cupriavidus metallidurans* CH34: interplay between plasmid and chromosomally-located functions

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Abstract Proteome and transcriptome analysis, combined with mutagenesis, were used to better understand the response of *Cupriavidus metallidurans* CH34 against lead(II). Structural Pb(II)-resistance genes of the pMOL30-encoded *pbrUTRABCD* operon formed the major line of defense against Pb(II). However, several general stress response mechanisms under the control of alternative sigma factors such as σ24/*rpoK*, σ32/*rpoH* and σ28/*fliA* were also induced. In addition, the expression of the *pbrR*$_2$ *cadA pbrC*$_2$ operon of the CMGI-1 region and the chromosomally encoded *zntA* were clearly induced in the presence of Pb(II), although their respective gene products were not detected via proteomics. After inactivation of the *pbrA*, *pbrB* or *pbrD* genes, the expression of the *pbrR*$_2$ *cadA pbrC*$_2$ operon went up considerably. This points towards synergistic interactions between *pbrUTRABCD* and *pbrR*$_2$ *cadA pbrC*$_2$ to maintain a low intracellular Pb(II) concentration, where *pbrR*$_2$ *cadA pbrC*$_2$ gene functions can complement and compensate for the mutations in the *pbrA* and *pbrD* genes. This role of *zntA* and *cadA* to complement for the loss of *pbrA* was further confirmed by mutation analysis. The *pbrB*::Tn(Km2) mutation resulted in the most significant decrease of Pb(II) resistance, indicating that Pb(II) sequestration, avoiding re-entry of this toxic metal ion, forms a critical step in the *pbr*-encoded Pb(II) resistance mechanism.

Keywords *Cupriavidus metallidurans* CH34 · Lead (II) resistance · *pbrUTRABCD* operon · *zntA* · *pbrR*$_2$ *cadA pbrC*$_2$ · Gene expression

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Introduction

Lead(II) resistance have been reported for bacteria representing different genera. Pb(II) resistant *Arthrobacter* sp. and other firmicutes were isolated from the waste site of a factory of lead batteries (Trajanovska et al. 1997). *Bacillus megaterium* demonstrating intracellular cytoplasmic Pb(II) accumulation and *Pseudomonas marginalis* showing extracellular Pb(II) exclusion were isolated from lead contaminated soils (Roane 1999). Pb(II) resistant strains of

Staphylococcus aureus and *Citrobacter freundii* have been isolated that accumulated the metal as an intracellular lead-phosphate (Levinson and Mahler 1998), though the molecular mechanism of detoxification remains to be elucidated. Efflux of Pb(II) has also been reported for the CadA ATPase of the *S. aureus* and the ZntA ATPase of *Escherichia coli* (Rensing et al. 1998a, b).

Pb(II) resistance was early recognized to be located on pMOL30, one of the two plasmids found in *Cupriavidus metallidurans* CH34 (Diels et al. 1989), although it was only more recently described at the molecular level (Borremans et al. 2001). The Pb(II) resistance operon, *pbr*, of *C. metallidurans* CH34 is unusual in the way that it combines functions involved in uptake, efflux, and accumulation of Pb(II).

A MerR-like regulator, PbrR, controls Pb(II)-dependent transcription of the *pbr* operon. Two additional *pbrR*-like genes are found on the *C. metallidurans* CH34 chromosome 1, $pbrR_2$ (Rmet_2302; previously referred to as *pbrR*691) that is part of the $pbrR_2$ *cadA* $pbrC_2$ operon present in the CMGI-1 region (*C. metallidurans* genomic island 1), and $pbrR_3$ (Rmet_3456) (see also Fig. 1). Detailed characterization of $PbrR_2$ protein, which was overexpressed in *E. coli*, showed that this protein binds Pb(II) almost 1,000-fold selectively over other metal ions such as Hg(II), Cd(II), Zn(II), Co(II), Ni(II), Cu(I), and Ag(I) (Chen et al. 2005). This high degree of selectivety is based on the unique coordination chemistry of Pb(II), where the relativistic effect causes contraction of the 6s orbital of Pb(II). As a result, Pb(II) exhibits a stereochemically active lone electron pair that is resistant to engage in bonding to ligands (Claudio et al. 2003; Shimoni-Livny et al. 1998). Thus, in low coordinate Pb(II) complexes (3- or 4-coordinate), the lone electron pair is exposed and the Pb(II) center adopts a hemidirected geometry with all ligands clustered on one side of the metal (Claudio et al. 2003; Lewis and Cohen 2004; Shimoni-Livny et al. 1998). Chen et al. (2007) were able to show that it is the protein folding that enforces such a unique geometry, which enables $PbrR_2$ to selectively bind Pb(II) and exclude other soft metal ions. In this scenario other metal ions would stay in solution and avoid these high energetic penalties to enter the metal binding site in $PbrR_2$, whereas Pb(II) can adopt both geometries and could be preferentially recognized by $PbrR_2$ due to the chelate effect. A similar argument helped to explain the preferred binding of Zn(II) by zinc fingers (Berg and Godwin 1997; Lippard and Berg 1994).

The first gene downstream of the *pbr* operator/promoter on pMOL30, *pbrA*, encodes a P-type ATPase similar to ZntA (Brocklehurst et al. 1999). The PbrA Pb(II)-ATPase is phylogenetically grouped with the CadA-type Cd-ATPases and the ZntA Zn/Cd-ATPase, and they form a distinct group separate from the Cu/Ag-type ATPases (Borremans et al. 2001; Monchy et al. 2006b). In contrast to the *cad* and *znt* operons, which both appear to comprise a

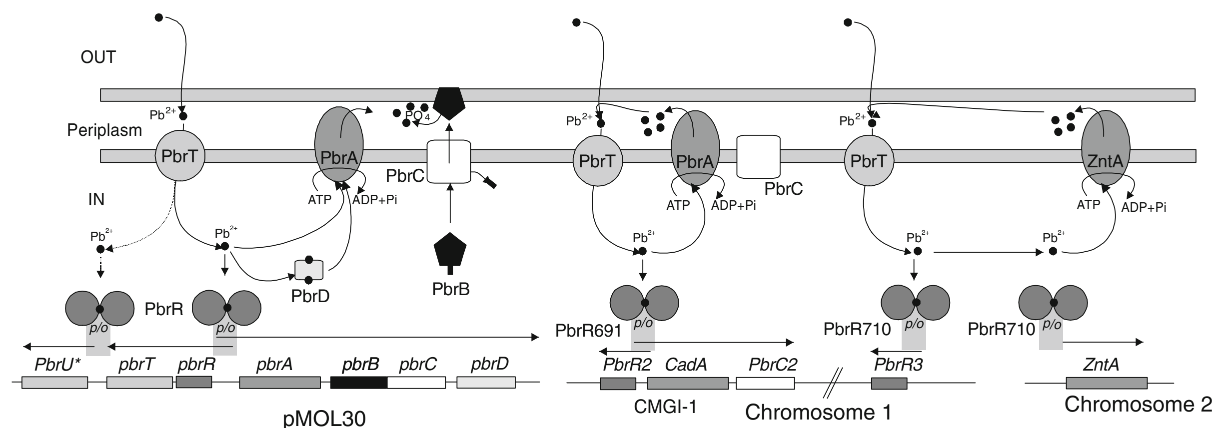

Fig. 1 Model for Pb(II) resistance in *C. metallidurans* CH34 and the interplay between plasmid and chromosomal located functions. The *pbrUTRABCD* operon is located on the pMOL30 plasmid, $pbrR_2$ *cadA* $pbrC_2$ and $pbrR_3$ on chromosome 1, and *zntA* on chromosome 2. The *asterisk* symbol after *pbrU** indicates that the gene has been inactivated due to an insertion of Tn*Cme2* at the 3′ end of the gene (insertion not shown)

regulatory gene plus an efflux-ATPase only, additional proteins are required for maximal Pb(II)-resistance in *C. metallidurans* CH34. These are PbrT, PbrB, PbrC and PbrD. Microarray studies showed that *pbrU*, located downstream of *pbrT*, was also induced by Pb(II) (Monchy et al. 2007). However, *pbrU* seems to be inactivated due to the insertion of Tn*Cme2*.

To understand the role of each of the structural *pbr* Pb(II) resistance genes, mutants were constructed and their Pb(II)-resistance phenotype was tested. In addition, proteome and transcriptome analysis were used to study the involvement of chromosomally located functions in the regulation of Pb(II)-resistance.

Materials and methods

Bacterial strains, plasmids, and media

Cupriavidus metallidurans and *E. coli* were grown in 869 medium (Mergeay et al. 1985) at 30 and 37°C, respectively. Antibiotic resistance was selected on media supplemented with 20 mg/l of tetracycline or 100 or 1,000 mg/l of kanamycin for *E. coli* and *C. metallidurans*, as appropriate.

The following *C. metallidurans* strains were used in this study: the wild-type strain CH34 (Pb(II)-resistant); the plasmid-free, Pb(II)-sensitive derivative AE104 (Mergeay et al. 1985); AE104 containing pMOL1027, a derivative of pLAFR3 in which the *pbr* operon of CH34 was cloned (Borremans et al. 2001); and AE104 containing derivatives of pMOL1027 with Tn(Km2) in different genes of the *pbr* operon. The sites and orientations of the Tn(Km2) insertions are given in Fig. 2. In addition the AE104 derivatives DN438 (*cadA*$^-$), DN439 (*zntA*$^-$) and DN440 (*cadA*$^-$, *zntA*$^-$) (Legatzki et al. 2003) were used to further examine the synergy between chromosomally encoded P-type ATPases and the *pbr* operon on pMOL1027.

A modified 284 mineral salt medium was used to grow *C. metallidurans* and its derivatives for proteomic, transcriptomic and phenotypic analysis of Pb(II) resistance. The modified medium differed from the original 284 medium (Mergeay et al. 1985) by supplementing it with 20 mM morpholinepropanesulfonic acid (MOPS)–NaOH (pH 7) instead of Tris–HCl, 0.95 mM beta-glycerol-phosphate instead of Na_2HPO_4, and 0.1% w/v gluconate as carbon source. These components were filter sterilized and added separately after the medium was autoclaved. For plating 1.5% (w/v) agar was added to the medium. To determine the minimal inhibitory concentration of Pb(II), final concentrations of 0.2, 0.3, 0.4, 0.5 mM $Pb(NO_3)_2$ were added to the medium.

Molecular cloning techniques, Tn(Km2) mutagenesis and sequencing

Standard molecular cloning techniques and plasmid DNA isolation from *E. coli* were performed as described (Sambrook et al. 1989). Electroporation was used to introduce plasmid DNA into *E. coli* and *C. metallidurans* (Taghavi et al. 1994). In vitro Tn(Km2) mutagenesis of the *pbr* operon on pMOL1027 was performed using the EZ::Tn^{TM} <Km> Insertion Kit (Epicentre Technologies,

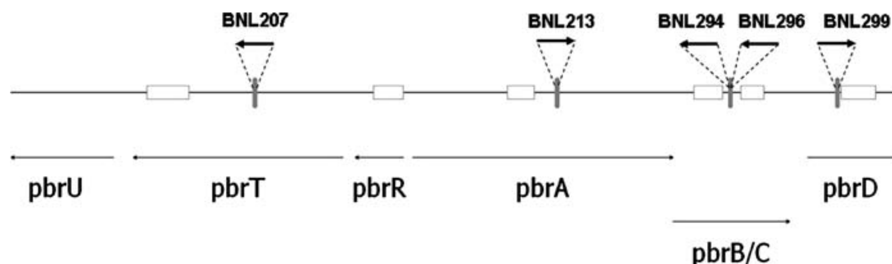

Fig. 2 Position and orientation of the Tn(Km2) insertions into the *pbrT*, *pbrA*, *pbrBC* and *pbrD* genes. The exact localization and orientation of the Tn(Km2) insertions was determined by sequencing. The direction of transcription for the *pbr* genes is indicated by an *arrow*, the localization of the regions amplified during the qPCR are indicated by *boxes*, and the Tn(Km2) orientation and insertion sites in the different strains (*pbrT* for BNL207, *pbrA* for BNL213, *pbrB* for BNL294, *pbrC* for BNL296, and *pbrD* for BNL299) are indicated by *bold arrows*. The *pbrU* gene is inactivated by the insertion of the Tn*Cme2* transposon

Madison, WI) according to the manufacturer's instructions. After electroporation into *E. coli* DH10B, transconjugants were selected for tetracycline and kanamycin resistance. Plasmids were isolated, and restriction site analysis with *Hin*dIII and *Eco*RI was used to identify pMOL1027 derivatives with Tn(Km2) insertions located in the *pbr* operon. The exact positions and orientations of the insertions were determined by sequencing using the KAN-2 FP-1 forward primer (5′-ACCTACAACAAAGCTCTCA TCAACC-3′) and the KAN-2 RP-1 reverse primer (5′-GCAATGTAACATCAGAGATTTTGAG-3′).

Pb(II) specific proteomics

A culture was grown overnight in modified 284 medium at 30°C and shaken at 120 rpm on an orbital shaker. 0.5 ml of the culture was used to inoculate a set of flasks containing 25 ml of modified 284 medium supplemented with 0.4 mM $Pb(NO_3)_2$) or with no metal cation added (control). Aliquots at log phase (OD600 nm = 0.3) and plateau phase (OD600 nm = 0.7) were collected and centrifuged at room temperature at 6,000 rpm for 10 min. Pellets were stored frozen at −80°C immediately after centrifugation. Protein separation via 2-D electrophoresis, electroblotting and N-terminal amino acid microsequence analysis, sample preparation for MS analyses and MALDI-MS were performed as described previously (Noel-Georis et al. 2004). Searches for protein identity from sequence data were performed using the TBLASTP algorithm (Altschul et al. 1990) on the *C. metallidurans* CH34 genomic database.

Microarray analyses of heavy metal resistance gene expression

The *C. metallidurans* CH34 genome array (Monchy et al. 2007) was used to study Pb(II)-specific changes of gene expression as described previously. Liquid cultures were grown at 30°C in flasks with 10 ml of modified 284 medium and shaken at 120 rpm until an OD_{660} of 0.3 was reached (early exponential phase). Ten ml culture samples were transferred into Falcon tubes, supplemented with 0.4 mM $Pb(NO_3)_2$ and further incubated at 30°C for 30 min before centrifugation. Induction and RNA extraction were performed in duplicate using two independent cultures. RNA extraction, cDNA synthesis, labeling, hybridization, and scanning and analysis of the microarray data was performed as previous (Monchy et al. 2007). Array data were deposited at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE7272.

Measurements of Pb(II) mediated gene induction by real time quantitative RT-PCR

Liquid cultures were grown and induced as described in the microarray section, with 0.4 and 0.6 mM of $Pb(NO_3)_2$ for 30 min. Total RNA was extracted from control and induced cultures, and converted into cDNA as previously described (Monchy et al. 2007). Quantitative PCR was then conducted on the resulting cDNA using specific primer sets (see supplementary data Table 1). Quantitative PCR was also conducted on *pbr*::Tn(Km2) mutants. The 16S rRNA and *lysA* genes were used as controls for constitutive gene expression. All quantitative PCR reactions were performed in triplicate and, each time, a standard deviation was calculated. Quantitative PCR was performed with SYBR Green (iQ SYBR Green Supermix; Bio-Rad, USA), while fluorescence was continuously monitored by the sequence detection system iCycler (Bio-Rad, USA). Data were analyzed by normalization against 16S rRNA and *lysA* levels as internal controls. Calculation of fold induction was done comparing to the control condition without added Pb(II).

Results

Genes involved in Pb(II) resistance

During the initial study of the *pbr* Pb(II)-resistance determinant the following structural resistance genes were identified in addition to the transcriptional regulator *pbrR* (Borremans et al. 2001): *pbrT* encodes a Pb(II)-uptake protein, PbrT, which consists of a cytochrome domain (a.a. 1–276) and a transmembrane metal transport domain (a.a. 386–632). Expression of PbrT in the absence of PbrABCD results in Pb(II)-hypersensitivity, probably due to increased Pb(II)-uptake into the cytoplasm. A homolog of PbrT, CopT, was found in the *C. metallidurans* CH34 *cop* operon. Interestingly, CopT lacks the metal transport domain (Monchy et al. 2006a).

pbrA, encoding a P-type Pb(II)-efflux ATPase

pbrB and *pbrC*, which encode for a prolipoprotein and prolipoprotein signal peptidase, respectively. Based on homology, PbrC is probably an aspartic peptidase of the MEROPS family A08 (EC3.4.23.36) (Rawlings et al. 2008). Members of this peptidase family release signal peptides from bacterial membrane prolipoproteins, and specifically recognize and hydrolyze the consensus aminoacid sequence -Xaa-Yaa-Zaa-l-(S,diacylglyceryl)Cys-, in which Xaa is hydrophobic (preferably Leu), and Yaa (preferably Ala or Ser) and Zaa (preferably Gly or Ala) have small, neutral side chains. PbrB contains the sequence Ile-Ala-Ser-Cys as the most likely site for its processing by PbrC. PbrB belongs to the PAP2-like proteins (Neuwald 1997), a super-family of histidine phosphatases and vanadium haloperoxidases, including type 2 phosphatidic acid phosphatase or lipid phosphate phosphatase (LPP), glucose-6-phosphatase, phosphatidylglycerophosphatase B and bacterial acid phosphatase, and several other mostly uncharacterized subfamilies. Several members of this superfamily have been predicted to be transmembrane proteins. Based on its close similarity with the phosphatases we hypothesize that PbrB plays a role in the precipitation of Pb(II) under the form of Pb-phosphate in the periplasm, thus preventing its re-entry in the cell.

Initially, *pbrB* and *pbrC* were thought of as two separate genes. However, analysis of the revised *pbr* sequence revealed that *pbrBC* form one transcriptional unit. A similar organization of *pbrBC* was since found in *Acidovorax* sp. JS42 12J (gb:ABM41468.1), *Shewanella frigidimarina* NCIMB 400 (gb:ABI73294.1), *Alcaligenes faecalis* (gb:AAS45113.1), *Comamonas testosteroni* KF-1 (gb:EAV17075.1), *Klebsiella pneumoniae* CG43 (gb:AAR07818.1), *Ralstonia pickettii* 12J (gb:ACD26951.1) and others.

pbrD, encoding a putative Pb(II)-sequestration protein

PbrD is not required for Pb(II)-resistance (Borremans et al. 2001), but cells lacking PbrD show a decreased accumulation of Pb(II) compared to wild-type cells, and this accumulation may protect against free exported Pb(II) and a putative futile cycle of Pb(II)-uptake and export. To date, no homologues of PbrD have been identified on other genomes.

Analysis of the *C. metallidurans* CH34 Pb(II)-induced proteome

After induction of strain CH34 with 0.4 mM $Pb(NO_3)_2$, the PbrT, PbrA, PbrB, PbrC and PbrD proteins were all identified by 2-D gel electrophoresis using the protocols described for the global analysis of the *C. metallidurans* CH34 proteome (Noel-Georis et al. 2004). Protein identification was carried out via N-terminal amino acid sequencing, matrix assisted laser desorption/ionization-time of flightmass spectrometry (MALDITOF-MS) and tandem mass spectrometry. Three isoforms of the PbrA protein were found that had identical MALDI-TOF-MS patterns; all other proteins migrated as single spots in the 2D gel (Fig. 3), including PbrB and PbrC, which points towards post-transcriptional or post-translational processing of the mRNA or fusion protein, respectively. The PbrR protein could not be identified, which makes sense as *pbrR* was never found to be highly induced in the presence of Pb(II). Interestingly, under these conditions, we have yet to identify the specific induction of other potential

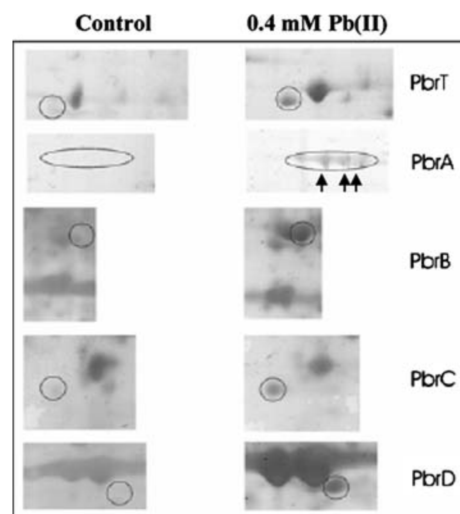

Fig. 3 Identification of Pb(II)-resistance proteins in *C. metallidurans* CH34 by comparative two dimensional gel electrophoresis. The figure shows isolated sectors of gels that contain Pbr proteins (*circled*) extracted from *C. metallidurans* CH34 overnight cultures grown in the absence (control) or in the presence of 0.4 mM $Pb(NO_3)_2$. The three isoforms of PbrA are indicated by *arrows*

Pb(II)-resistance proteins, such as those encoded by the *pbrR*$_2$ *cadA pbrC*$_2$ (chromosome 1) and the *znt*Δ*BCIA* (chromosome 2) operons, which both contain P1-type metal efflux ATPases (*cadA* and *zntA*, respectively) closely related to the PbrA Pb(II)-efflux ATPase (Mergeay et al. 2003).

pbr operon mutants and Pb(II)-resistance phenotype analysis

The *pbr* operon was previously cloned in cosmid pLAFR3, resulting in pMOL1027 (Borremans et al. 2001). Efforts to construct mutants concentrated on the structural resistance genes of the *pbr* operon, whose expression was dependent on the presence of Pb(II). The EZ::Tn(Km2) in vitro transposition system was used to construct a library of *pbr*::Tn(Km2) mutants in pMOL1027. Approximately 400 insertion mutants were obtained, for which restriction endonuclease site analysis with *Hin*dIII and *Eco*RI showed that 89 were located within the *pbr* region. The presumed positions of the transposon insertions as well as their orientation were confirmed by sequence analysis. With the exception of the *pbrR*, insertion mutants were obtained in all genes of the *pbr* operon (Fig. 2).

Representative pMOL1027-*pbr* mutants for the different structural genes were analyzed for their Pb(II)-resistance phenotype in AE104, and derivatives of AE104 with inactivated *cadA* (DN438), *zntA* (DN439) or both genes (DN440) (Legatzki et al. 2003). As controls the wild type strain *C. metallidurans* CH34 and the plasmid free Pb(II)-sensitive derivative AE104 were used. The results are presented in Table 1. The wild type strain *C. metallidurans* CH34 and AE104 (pMOL1027) are unable to grow when challenged with a lead concentration above 0.5 and 0.4 mM, respectively.

Inactivation of *pbrT* on pMOL1027 resulted in an increased of Pb(II) resistance to a MIC value above that of AE104 (pMOL1027) and similar to that observed for the wild type strain CH34. This increase in MIC value, most likely the result of decreased Pb(II) uptake, was also observed for BNL819 (*cadA*$^-$) and BNL831 (*zntA*$^-$), but not for strain BNL843 (*cadA*$^-$ *zntA*$^-$).

Inactivation of *pbrA* on pMOL1027 (strain BNL213), tested in AE104 or in a genetic background with inactivated *cadA* (strain BNL821) or *zntA* (strain BNL833), had no major effect on the Pb(II) resistance phenotype (MIC value of 0.4 mM). This result suggests complementation between the different P-type ATPases for Pb(II) efflux. This complementation is further confirmed by the observed descrease in Pb(II) resistance, with a MIC value of 0.3 mM, when all three P-type ATPases are inactivated (strain BNL845).

An insertion of Tn(Km2) at the 3′ end of *pbrB* on pMOL1027 (strain BNL294) resulted in Pb(II)-sensitivity with a MIC value of 0.2 mM similar to that observed for the plasmid free, Pb(II)-sensitive strain AE104. Interestingly, this MIC value goes up to 0.3–0.4 mM when the *cadA* and/or *zntA* genes are inactivated.

A Tn(Km2) insertion in the 5′ end of *pbrC* (strain BNL296) also resulted in a decrease of MIC value to 0.3 mM, while Tn(Km2) insertions further downstream in *pbrC* did not result in a Pb(II) sensitive phenotype (data not shown). Therefore, the post-transcriptonal or post-translational maturation of *pbrBC* may play a role in Pb(II) resistance. In addition, the Pb(II) resistance levels of the *pbrC* mutant could be increased by inactivation of the *cadA* gene located on the CMGI-1, and might reflect a polar effect on the activity of the downstream *pbrC*$_2$ gene, thus compensating the loss of *pbrC*.

As previously reported, inactivation of *pbrD* (strain BNL299) did not affect Pb(II)-resistance (Borremans et al. 2001). Similarly, inactivation of *pbrD* together with *cadA* (strain BNL829), *zntA* (strain BNL841) or both P-type ATPases (strain BNL853) did not significantly affect the Pb(II) resistance phenotype.

The *C. metallidurans* CH34 Pb(II)-induced transcriptome

A sensitive analysis of the transcriptome of *C. metallidurans* CH34 and its *pbr*-Tn(Km2) mutants, using a combination of microarray analysis and quantitative RT-PCR with gene specific primers, was used complementary to the analysis of the Pb(II)-induced proteome.

Microarray analysis of the CH34 transcriptome showed that after 30 min the *pbrUTRABCD* locus was induced by the presence of 0.4 mM Pb(II) and 0.5 mM Cd(II) (Monchy et al. 2007). The *pbrC* and *pbrD* genes were also slightly induced in the presence

Table 1 Resistance phenotype of the *C. metallidurans* CH34 and derivatives grown in the presence of increased lead concentrations

Pb(II) concentration (mM)							
Strain			0	0.2	0.3	0.4	0.5
CH34			+++	+++	+++	+	+/−
AE104			+++	+++	+	−	−
pMOL1027			+++	+++	+++	+	−
DN438			+++	+++	+	−	−
DN439			+++	+++	+	−	−
DN440			+++	+++	+	−	−
pMOL1027 mut.	Chr. mut.	Strain no.	0	0.2	0.3	0.4	0.5
$pbrT^{-}$	–	207	+++	+++	+++	++/−	−
	Δ*cadA*	819	+++	+++	+++	++	+/−
	Δ*zntA*	831	+++	+++	+++	++/−	+/−
	Δ*cadA*, Δ*zntA*	843	+++	+++	+++	+	−
$pbrA^{-}$	–	213	+++	+++	+++	++	−
	Δ*cadA*	821	+++	+++	+++	+/−	−
	Δ*zntA*	833	+++	+++	+++	+	−
	Δ*cadA*, Δ*zntA*	845	+++	+++	+	−	−
$pbrB^{-}$	–	294	+++	++	−	−	−
	Δ*cadA*	825	+++	+++	+	−	−
	Δ*zntA*	837	+++	+++	+	+/−	−
	Δ*cadA*, Δ*zntA*	847	+++	+++	+	+/−	−
$pbrC^{-}$	–	296	+++	+++	+	−	−
	Δ*cadA*	827	+++	+++	++	+/−	−
	Δ*zntA*	839	+++	+++	+	−	−
	Δ*cadA*, Δ*zntA*	851	+++	+++	+++	+/−	−
$pbrD^{-}$	–	299	+++	+++	+++	++	−
	Δ*cadA*	829	+++	+++	+++/−	+	−
	Δ*zntA*	841	+++	+++	+++/−	+	−
	Δ*cadA*, Δ*zntA*	853	+++	+++	+++	++	−

The MIC (minimal inhibitory concentration) was determined on Petri dishes supplemented with 0, 0.2, 0.3, 0.4, and 0.5 mM of Pb(II). The mutations in the *pbr* genes present on pMOL1027 are indicated. The different *pbr* mutants obtained for pMOL1027 were tested in AE104 ("−"; no chromosomal mutation) and derivatives of AE104 with inactivated *cadA* (DN438), *zntA* (DN439) or both (DN442) (Legatzki et al. 2003). Strain numbers indicate the BNL-name given to each strain. None of the strains were able to grow in the presence of a lead concentration higher than 0.6 mM. The number of "+" symbols indicates the comparative level of grows observed for each of the strains; the symbol "−" indicates that not growth was observed

of 0.8 mM Zn(II), while no significant induction of *pbrR* was observed for any of the metal cations tested (Monchy et al. 2007). The presence of Pb(II) resulted, in addition, in the induction of the $pbrR_3$ regulator encoding gene, the $pbrR_2$ *cadA* $pbrC_2$ operon of CMGI-1, the *zntA* P-ATPase encoding gene, the *cupRAC* operon (involved in copper efflux) and the *ars* locus (involved in arsenite–arsenate resistance). Other metal resistance genes carried by the plasmids pMOL28 and pMOL30 were shown the be upregulated in presence of Pb(II) (Monchy et al. 2007). This general response can be explained by an induction of several regulatory networks controled by the sigma factors σ24/*rpoK* (2.35 times induced), σ32/*rpoH* (2.31 times induced), and σ28/*fliA* (2.09 times induced). The *fecR* regulator, located next to *rpoK* and which inactivates *rpoK* under iron limiting conditions, was also upregulated 2.51 times in presence of Pb(II). This resulted in the increased expression of several genes involved in iron storage

(ferritin), FeS cluster biosynthesis (involved in the mobilization of Fe and S atoms from storage sources in case of iron limitation), and of a putative ferredoxin. The metabolism of glutathione, which in its reduced form is able to bind Pb(II), was also induced by Pb(II) with the upregulation of a glutathione reductase (3.1 times), a glutathione S-transferase (2.1 times) and a gamma-glutamyl-transferase (2.3 times). Many other genes were up-regulated in the presence of Pb(II), such as transporters from the major facilitator super (MFS) family and the ABC families, including a potassium transporter and an ammonium transporter; genes involved in the metabolism of poly-hydroxybutyrate (PHB), several regulators, as well as genes with still unknown functions. Interestingly, eight genes located on transposon Tn*Cme1* (present in two copies on chromosome 2 and one copy on chromosome 1), which is suspected to be involved in Tl(I) and Cs(I) resistance, were upregulated in presence of Pb(II). The nitrate assimilative pathways was also found to be up-regulated in presence of Pb(II), with overexpression of the periplasmic nitrate reductase complex (*napA* 4.3 times and *napB* 2.8 times), the glutamine synthetase (2.2 times) and the D-amino acid dehydrogenase *dadA* (5.5 times). This induction is not surprising as lead was added to the medium as $Pb(NO_3)_2$.

Quantitative RT-PCR on the lead induced transcriptome of CH34 showed that in the early hours after the addition of 0.4 mM Pb(II), the transcription of the chromosomally located *pbrR*$_2$ *cadA pbrC*$_2$ operon and *zntA* went up. Induction levels were comparable to those determined by microarray analysis (see Table 2). Quantitative RT-PCR was further used on the *pbr*::Tn(Km2) mutants to confirm complementarities between the *pbr* Pb(II)-resistance operon and chromosomal functions with a putative role in Pb(II)-resistance (Table 3). A comparison between the Pb(II)-induced transcriptome of CH34 and AE104 in the presence of the sub-lethal concentration of 0.4 mM Pb(II) showed similar levels of induction for the *cadA pbrC*$_2$ genes and *zntA* in CH34, AE104, and AE104 containing pMOL1027. The induction levels of *pbr* genes present on pMOL1027 were much lower than those observed for the wild type strain CH34 where the *pbr* operon is present on pMOL30. However, the absolute values for the *pbr* gene expression levels after Pb(II) induction were comparable for both strains (data not shown). Thus, the basic uninduced expression levels for *pbr* on pMOL1027 were much higher, most likely due to the increased copy number of this plasmid.

For the *pbrT*::Tn(Km2) mutant BNL207 the induction of *pbrT* in the presence of Pb(II) was similar to that observed for the wild type strain CH34. This could be due to the downstream location of Tn(Km2) compared to the quantitative Q-RT-PCR amplified region (Fig. 2), resulting in no modification of mRNA transcript production (intact promoter). Furthermore, inactivation of *pbrT* by Tn(Km2) at the end of the cytochrome domain had no major effects on expression of the other *pbr* genes, with the exception of *pbrD* (Table 3).

Inactivation of *pbrA* (strain BNL213) resulted in a significant increase in expression of the *cadA pbrC*$_2$ genes, as well as that of the inactivated *pbrA* gene. This expression is higher than observed for AE104, and could be attributed to the presence of a functional *pbrT* gene, whose activity resulted in increased levels of Pb(II) uptake. To compensate and maintain a lower intracellular Pb(II) concentration, the bacteria supposedly has to increase expression *pbrR*$_2$ *cadA pbrC*$_2$.

Inactivation of the *pbrB* phosphatase region (strain BNL294) is hypothesized to result in a decrease in periplasmic lead sequestration. The consequent increase in Pb(II) reentry after efflux is expected to result in elevated levels of induction of other structural resistance genes. Indeed, increased induction is observed in strain BNL294 (Table 3) for *pbrA* and the *cadA pbrC*$_2$ genes. Downstream transcription of *pbrCD* is interrupted due to the insertion of Tn(Km2) that disrupts these genes from their promoter. In strain BNL296 the expression of *pbrC* and *pbrD* (polar effect) is inactivated by the Tn(Km2) insertion into the *pbrBC* junction. An expression pattern similar to strain BNL294 is obtained with stain BNL296, except that the level of *cadA pbrC*$_2$ transcription was lower and comparable to that observed for AE104. The *pbr* gene expression of strain BNL296 in presence of Pb(II) is the lowest from all the tested strains.

Inactivation of *pbrD* (strain BNL299) resulted in a very significant increase in *pbr* and *cadA pbrC*$_2$ induction levels, and even induction of *zntA*. This strongly supports the hypothesis that PbrD plays a role as a Pb(II)-chaperone, sequestering to decrease

Table 2 Expression level, measured by quantitative RT-PCR and microarray analysis, of the *C. metallidurans* CH34 *pbr*, $pbrR_2$ *cadA* $pbrC_2$, *znt* and *cup* operons in the presence of Pb(II), Zn(II) and Cd(II)

Gene	Q-RT-PCR		Microarrays		
	Pb (0.4 mM)	Pb (0.6 mM)	Pb (0.4 mM)	Zn (0.8 mM)	Cd (0.5 mM)
*pbrU*b	ND	ND	3.7 ± 0.4	1.5 ± 0.3	2.9 ± 0.5
*pbrU*a	ND	ND	3.3 ± 0.3	1 ± 0.5	2.6 ± 0.4
pbrT	1.1 ± 0.1	1.4 ± 0.1	2.1 ± 0.4	1.7 ± 0.5	5.2 ± 0.2
pbrR	1.5 ± 0.7	1.2 ± 0.6	−1.7 ± 0.2	1.0 ± 0.03	−1.1 ± 0.1
pbrA	5.4 ± 0.6	7.9 ± 0.7	7.3 ± 1.2	1.9 ± 0.7	5.0 ± 0.2
pbrB	4.0 ± 0.4	5.6 ± 1.0	4.1 ± 0.6	2.0 ± 0.5	4.3 ± 0.1
pbrC	3.3 ± 0.3	3.5 ± 0.7	ND	ND	ND
pbrD	3.5 ± 1.0	7.2 ± 1.2	3.0 ± 0.5	2.2 ± 0.4	5.0 ± 0.1
$pbrR_2$	ND	ND	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
cadA	1.9 ± 0.5	2.7 ± 0.6	3.9 ± 0.6	3.6 ± 0.6	3.3 ± 0.1
$pbrC_2$	5.3 ± 0.5	6.6 ± 1.1	2.0 ± 0.4	2.7 ± 0.6	3.6 ± 0.2
$pbrR_3$	ND	ND	5.7 ± 1.1	1.2 ± 0.5	2.0 ± 0.2
ΔzntB	ND	ND	1.1 ± 0.07	3.0 ± 0.9	3.1 ± 0.3
zntC	ND	ND	−1.4 ± 0.2	11.1 ± 0.9	1.4 ± 0.3
zntI	ND	ND	1.0 ± 0.6	3.2 ± 0.9	6.3 ± 0.3
zntA	2.9 ± 1.3	6.3 ± 1.4	2.7 ± 0.7	5.4 ± 0.8	7.2 ± 0.02
cupC	ND	ND	3.8 ± 0.7	3.1 ± 0.6	2.3 ± 0.1
cupA	ND	ND	9.4 ± 1.8	2.0 ± 0.5	2.8 ± 0.3
cupR	ND	ND	4.9 ± 1.3	1.4 ± 0.3	1.1 ± 0.02

Cultures of *C. metallidurans* CH34 (the Pb(II)-resistant wild-type strain) were grown for 30 min in the presence of 0.4 and 0.6 mM $Pb(NO_3)_2$, 0.8 mM $Zn(SO_4)$ or 0.5 mM $CdCl_2$ for microarrays analysis and in the presence of 0.4 and 0.6 mM $Pb(NO_3)_2$ for quantitative RT-PCR analysis. The total RNA was isolated from the harvested cells and used for transcriptomic analysis. The RNA used for Quantitative PCR and microarrays experiments came from independent cultures

For qPCR, specific primers were design for the *pbrTRABCD*, *cadA*, $pbrC_2$ and *zntA* genes (see supplementary data Table 1). Expression of *lysA* (encoding a protein involved in lysine biosynthesis) was used as an internal control to normalize gene expression. The values represent the mean of the fold induction, with respect to the unchallenged culture, and are mean ± standard deviation performed in triplicate ($N = 3$)

For the microarray analysis of gene expression, the value represents the mean of the fold induction calculated from two technical replicates on the same chip, and two biological replicates from independent cultures

ND not done

the free cytoplasmic levels of Pb(II), and subsequently transporting Pb(II) to the efflux ATPases for further periplasmic processing.

Discussion

The main line of defence against Pb(II) by *C. metallidurans* CH34 is the pMOL30 encoded *pbr* Pb(II) resistance operon. This was confirmed by several complementary approches. Analysis of the Pb(II)-induced proteome of *C. metallidurans* CH34 by 2-D gel electrophoresis allowed the identification of the PbrT, PbrA, PbrB, PbrC, and PbrD proteins, indicating that these proteins are abundant and defend the cell against Pb(II). Transcriptome analysis using quantitative RT-PCR and microarrays also revealed that the *pbr* operon represented the most induced system under conditions of Pb(II) exposure. In addition, several general stress response mechanisms under the control of alternative sigma factors such as σ24/*rpoK*, σ32/*rpoH* and σ28/*fliA* were induced. Recently, *rpoK* was shown to by upregulated in presence of 0.3 mM of Zn(II), Cu(II) or Ni(II) and also under iron depletion in strain AE104 (Grosse et al. 2007). The microarray data also link a response

Table 3 Transcription analysis of the *pbr* operon and related genes for *C. metallidurans* CH34 and its derivatives after induction with 0.4 mM of Pb(II)

Phenotype	Strains							
	CH34 Wild type	AE104 Plasmid free	pMOL1027 Cosmid *pbr*	BNL207 $pbrT^-$	BNL213 $pbrA^-$	BNL294 $pbrB^-$	BNL296 $pbrC^-$	BNL299 $pbrD^-$
pbrT	3.7 ± 3.4	NR	9.6 ± 5.2	3.3 ± 1.6	−3.7 ± 0.1	5.5 ± 0.3	12.3 ± 15.8	1.49 ± 0.13
pbrR	−3.0 ± 0.3	NR	4.7 ± 2.7	−1.4 ± 1.0	−5.6 ± 0.04	2.1 ± 0.2	4.8 ± 1.1	−1.68 ± 0.60
pbrA	189.9 ± 55.8	NR	7.4 ± 3.3	9.9 ± 0.6	29.5 ± 33.0	17.8 ± 1.4	8.2 ± 11.3	34.38 ± 11.27
pbrB	88.8 ± 13.2	NR	2.1 ± 2.0	3.0 ± 1.2	−1.2 ± 0.1	4.8 ± 3.3	3.5 ± 2.1	6.99 ± 0.81
pbrC	521.9 ± 77.0	NR	13.5 ± 11.7	6.9 ± 4.3	−3.0 ± 0.02	−2.2 ± 0.1	−1.2 ± 0.6	69.51 ± 6.54
pbrD	253.1 ± 73.1	NR	4.7 ± 3.5	23.1 ± 7.1	−1.3 ± 0.4	−1.3 ± 0.2	1.1 ± 0.1	1.34 ± 0.06
cadA	25.3 ± 6.4	37.5 ± 18.5	38.6 ± 16.5	15.2 ± 1.7	80.4 ± 22.2	56.9 ± 13.0	20.0 ± 24.8	101.23 ± 6.45
$pbrC_2$	11.7 ± 1.2	11.1 ± 4.2	13.1 ± 0.7	7.7 ± 8.2	57.1 ± 13.9	53.5 ± 40.2	4.9 ± 4.5	56.91 ± 2.51
zntA	3.1 ± 0.5	2.1 ± 0.5	1.3 ± 0.1	4.1 ± 0.5	3.3 ± 0.2	3.7 ± 0.5	1.7 ± 0.7	10.31 ± 0.33

Cultures of *C. metallidurans* were grown for approximately 12 h in the presence of 0.4 mM $Pb(NO_3)_2$, until reaching an OD_{660} of 1.0, after which cells were harvested, their total RNA was isolated and used for Q-PCR analysis. The strains tested were the following: the wild-type strain CH34 (Pb(II)-resistant); the plasmid-free, Pb(II)-sensitive derivative AE104; AE104 containing pMOL1027, a derivative of pLAFR3 in which the *pbr* operon of CH34 was cloned; and AE104 containing derivatives of pMOL1027 with Tn(Km2) in different genes of the *pbr* operon [the sites and orientations of the Tn(Km2) insertions are given in Fig. 2]: BNL207: *pbrT*::Tn(Km2); BNL213: *pbrA*::Tn(Km2); BNL294: *pbrB*::Tn(Km2); BNL296: *pbrC*::Tn(Km2); and BNL299: *pbrD*::Tn(Km2). Expression of *lysA* (encoding a protein involved in lysine biosynthesis) was used as an internal control to normalize gene expression. Results are given in fold induction with respect to the unchallenged culture and are mean ± standard deviation ($N = 3$)

NR not relevant

to Pb(II) stress and iron limitation, and point to a role for the *fecR* regulator. Transcription of this regulator, located next to *rpoK* and which inactivates *rpoK* under iron limiting conditions, was upregulated under conditions of Pb(II), as were genes involved in iron homeostasis. Furthermore, the metabolism of glutathione, which is able to bind and detoxify Pb(II) was also induced by Pb(II).

Genes involved in the metabolism of PHB were also over expressed in presence of Pb(II). PHB is a carbon polymer employed by bacteria as a form of energy storage. It is produced in response to physiological stress (Ayub et al. 2004), which is probably the case in presence of Pb(II).

Although not the main mechanism of defense against Pb(II), transcriptomic expression of the $pbrR_2$ *cadA* $pbrC_2$ operon located on chromosome 1 and *zntA* located on chromosome 2 were clearly induced in the presence of Pb(II), although we did not detect their respective gene products via proteomics. The single and more noticeable the double deletion of *cadA* and *zntA* affected Pb(II) resistance, especially when *pbrA* was inactivated. When comparing their Pb(II)-induced expression levels for the wild-type strain CH34, the plasmid free derivative AE104, and AE104 containing pMOL1027, no major differences were observed. However, in the *pbr*::Tn(Km2) mutant strains with inactivated *pbrA*, *pbrB* or *pbrD* genes, the expression of the $pbrR_2$ *cadA* $pbrC_2$ operon went up considerably, probably in response to an increase of intracellular Pb(II) levels. This confirms a synergistic interaction between *pbrU-TRABCD* and $pbrR_2$ *cadA* $pbrC_2$ to maintain reduced intracellular Pb(II) concentrations, where $pbrR_2$ *cadA* $pbrC_2$ gene functions can complement and compensate for the mutations in the *pbrA* and *pbrD* genes. As a result, no significant decreases in MIC values for Pb(II) are observed with these mutants. As expected, expression of the $pbrR_2$ *cadA* $pbrC_2$ and *zntA* genes was also induced by Cd(II) and Zn(II), since both genes were previously reported to have a significant effect on Cd(II) and Zn(II) resistance (Legatzki et al. 2003). Thus, the acquisition of CMGI-1, on which the $pbrR_2$ *cadA* $pbrC_2$ operon is located, clearly improves the versatility of *C. metallidurans* CH34 towards metals. Acquisition of genomic insertions might reflect a second approach by CH34, complementary to plasmid encoded heavy metal resistance systems,

for niche adaptation to elevated levels of heavy metals.

The Tn(Km2) mutation affecting *pbrB* resulted in the most important decrease in Pb(II) resistance to levels comparable with the MIC value of the plasmid free Pb(II) sensitive strain AE104. This indicates that external Pb(II) sequestration, preventing its re-entry, forms a critical step in the *pbr*-encoded Pb(II) resistance mechanism. In addition, we observe that when *pbrB*, *cadA* and/or *zntA* are mutated, the Pb(II) MIC value increases. This can be explained via a decrease in Pb(II)-cytoplasmic efflux, compensated by intracellular Pb(II) sequestration (trough glutathione). Under these conditions, partially losing the ability to efflux Pb(II) is an advantage for the cell as compared to a continuous Pb(II) efflux (energy costly) and the toxic accumulation of free Pb(II) (not sequestrated by PbrB) in the periplasm, where it can inversely damage membrane proteins. The opposite is observed when all three P-type ATPases are mutated (strain BNL845), a situation that results in hyperaccumulation and Pb(II) toxicity in the cytoplasm. This result also confirms on the phenotype level the synergistic interactions between the PbrA, CadA and ZntA P-type ATPases for Pb(II) efflux.

The absence of *pbrD* is not indispensable for Pb(II) resistance (Borremans et al. 2001), and can be compensated by increased expression levels of *pbrA*, *cadA* and *zntA*. Inactivation of *pbrD*, although not resulting in an increased Pb-sensitivity, is hypothesized to result in an increase of "free" internal Pb levels, which in turn will result in much higher levels of induction. As PbrD is not playing its Pb(II)-chaperone role, the bacteria will have to increase the PbrA, CadA, and ZntA levels to increase the function for these ATPases to bind Pb(II) without PbrD as a carrier.

So far we have not been able to identify a role for the *pbrU* gene in Pb(II) resistance, which encodes an MFS protein. This gene, which is located downstream of *pbrT*, is inactivated in *C. metallidurans* CH34 by the insertion of the Tn*Cme2* transposon, resulting into two separate ORF fragments (*pbrU*a and *pbrU*b). Expression of *pbrU*a is still upregulated in the presence of Pb(II). Upstream of *pbrU* a sequence was identified similar to that found in the region of the P_{pbrA} promoter (Monchy et al. 2007), which might point to the existence of a common mechanism to control their expression. In addition to *pbrU*, eight MFS encoding genes were found to become upregulated in the presence of Pb(II). One, flanking the *ars* operon, is possibly involved in the resistance to arsenite/arsenate and could represent a novel arsenic resistance gene. The most overexpressed MFS gene is located inside a cluster of siderophore biosynthesis genes. Even if the genes from this cluster were not induced in the presence of lead, the cross response between lead and iron should be noted. The other Pb(II)-induced MFS might compensate for the loss of *pbrU* and might point to a need for increased uptake of nutrients as part of a general response to detoxify heavy metals.

In this study, most of *C. metallidurans* CH34 Pb(II) resistance genes were identified. Interestingly, Pb(II)-resistance (MIC of 0.3 mM) similar to the MIC value for strain AE104 is still observed for strain DN440 (pbr^- $cadA^-$ $zntA^-$). The CupA P-type copper efflux ATPase is a good candidate to explain the residual Pb(II)-resistance, since *cupA* is highly expressed in the presence of Pb(II) (Table 2; Monchy et al. 2006b). Further studies will be necessary to confirm a role of *cupA* in Pb-resistance.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Ayub ND, Pettinari MJ, Ruiz JA, Lopez NI (2004) A polyhydroxybutyrate-producing *Pseudomonas* sp. isolated from antarctic environments with high stress resistance. Curr Microbiol 49:170–174

Berg JM, Godwin HA (1997) Lessons from zinc-binding peptides. Annu Rev Biophys Biomol Struct 26:357–371

Borremans B, Hobman JL, Provoost A, Brown NL, van der Lelie D (2001) Cloning and functional analysis of the pbr lead resistance determinant of *Ralstonia metallidurans* CH34. J Bacteriol 183:5651–5658

Brocklehurst KR, Hobman JL, Lawley B, Blank L, Marshall SJ, Brown NL, Morby AP (1999) ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of zntA in *Escherichia coli*. Mol Microbiol 31:893–902

Chen P, Greenberg B, Taghavi S, Romano C, van der Lelie D, He CA (2005) An exceptionally selective lead(II)-regulatory protein from *Ralstonia metallidurans*: development of a fluorescent lead(II) probe. Angew Chem Int Ed 44:2715–2719

Chen PR, Wasinger EC, Zhao J, van der Lelie D, Chen LX, He C (2007) Spectroscopic insights into lead(II) coordination by the selective lead(II)-binding protein PbrR691. J Am Chem Soc 129:12350–12351

Claudio ES, Godwin HA, Magyar JS (2003) Fundamental coordination chemistry, environmental chemistry, and biochemistry of lead(II). Prog Inorg Chem 51:1–144

Diels L, Sadouk A, Mergeay M (1989) Large plasmids governing multiple resistance to heavy metals: a genetic approach. Toxicol Environ Chem 23:79–89

Grosse C, Friedrich S, Nies DH (2007) Contribution of extracytoplasmic function sigma factors to transition metal homeostasis in *Cupriavidus metallidurans* strain CH34. J Mol Microbiol Biotechnol 12:227–240

Legatzki A, Grass G, Anton A, Rensing C, Nies DH (2003) Interplay of the czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. J Bacteriol 185:4354–4361

Levinson HS, Mahler I (1998) Phosphatase activity and lead resistance in *Citrobacter freundii* and *Staphylococcus aureus*. FEMS Microbiol Lett 161:135–138

Lewis JA, Cohen SM (2004) Addressing lead toxicity: complexation of lead(II) with thiopyrone and hydroxypyridinethione O,S mixed chelators. Inorg Chem 43:6534–6536

Lippard SJ, Berg JM (1994) Principles of bioinorganic chemistry. University Science Books, Mill Valley, CA

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Vangijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162:328–334

Mergeay M, Monchy S, Vallaeys T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D, Wattiez R (2003) *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. FEMS Microbiol Rev 27:385–410

Monchy S, Benotmane MA, Wattiez R, van Aelst S, Auquier V, Borremans B, Mergeay M, Taghavi S, van der Lelie D, Vallaeys T (2006a) Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in *Cupriavidus metallidurans* strain CH34. Microbiology 152:1765–1776

Monchy S, Vallaeys T, Bossus A, Mergeay M (2006b) Metal transport ATPase genes from *Cupriavidus metallidurans* CH34: a transcriptomic approach. Int J Environ Anal Chem 86:677–692

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

Neuwald AF (1997) An unexpected structural relationship between integral membrane phosphatases and soluble haloperoxidases. Protein Sci 6:1764–1767

Noel-Georis I, Vallaeys T, Chauvaux R, Monchy S, Falmagne P, Mergeay M, Wattiez R (2004) Global analysis of the *Ralstonia metallidurans* proteome: prelude for the large-scale study of heavy metal response. Proteomics 4: 151–179

Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ (2008) MEROPS: the peptidase database. Nucleic Acids Res 36:D320–D325

Rensing C, Mitra B, Rosen BP (1998a) A Zn(II)-translocating P-type ATPase from *Proteus mirabilis*. Biochem Cell Biol 76:787–790

Rensing C, Sun Y, Mitra B, Rosen BP (1998b) Pb(II)-translocating P-type ATPases. J Biol Chem 273:32614–32617

Roane TM (1999) Lead resistance in two bacterial isolates from heavy metal-contaminated soils. Microb Ecol 37:218–224

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, New York

Shimoni-Livny L, Glusker JP, Bock CW (1998) Lone pair functionality in divalent lead compounds. Inorg Chem 37: 1853–1867

Taghavi S, van der Lelie D, Mergeay M (1994) Electroporation of *Alcaligenes eutrophus* with (mega) plasmids and genomic DNA fragments. Appl Environ Microbiol 60:3585–3591

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