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Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis

David Cánovas, Ildefonso Cases† and Víctor de Lorenzo*
Centro Nacional de Biotecnología – CSIC, Campus de Cantoblanco, 28049 Madrid, Spain.

Summary

The genome of *Pseudomonas putida* KT2440 encodes an unexpected capacity to tolerate heavy metals and metalloids. The availability of the complete chromosomal sequence allowed the categorization of 61 open reading frames likely to be involved in metal tolerance or homeostasis, plus seven more possibly involved in metal resistance mechanisms. Some systems appeared to be duplicated. These might perform redundant functions or be involved in tolerance to different metals. In total, *P. putida* was found to bear two systems for arsenic (*arsRBCH*), one for chromate (*chrA*), four to six systems for divalent cations (two *cadA* and two to four *czc* chemiosmotic antiporters), two systems for monovalent cations: *pacS*, *cusCBA* (plus one cryptic *silP* gene containing a frameshift mutation), two operons for Cu chelation (*copAB*), one metallothionein for metal(loid) binding, one system for Te/Se methylation (*tpmT*) and four ABC transporters for the uptake of essential Zn, Mn, Mo and Ni (one *nikABCDE*, two *znuACB* and one *mobABC*). Some of the metal-related clusters are located in gene islands with atypical genome signatures. The predicted capacity of *P. putida* to endure exposure to heavy metals is discussed from an evolutionary perspective.

Introduction

Pseudomonas putida is a ubiquitous saprophytic bacterium endowed with a remarkable adaptability to diverse environments. This soil microorganism has been studied extensively as an experimental model for the biodegradation of aromatic compounds (Timmis, 2002). Such an emphasis in the metabolism of unusual carbon sources has often overshadowed much of the research on heavy metal resistances in these bacteria. Heavy metal ions and metalloids are chemical species that can be very toxic to cells in a variety of ways, e.g. through binding to essential respiratory chain proteins, oxidative damage via the production of reactive oxygen species, DNA damage, etc. It can be anticipated that soil organisms are likely to bear systems to cope with toxic metals in their environment.

Although an excess of metals is generally toxic, some of them are essential to life in trace amounts (Cu, Mn, Zn, etc.). Cells need to maintain certain cytoplasmic concentrations of these metals if they are to meet physiological requirements. To this end, microorganisms use a number of mechanisms to maintain the correct equilibrium, including the uptake, chelation and extrusion of metals (for reviews, see Silver, 1996; Robinson *et al*., 2001). Many systems have already been identified in bacteria that involve metallothioneins, P-type ATPases, cation/proton antiporters and redox enzymes. Some systems, such as that encoded by the arsenic/antimony detoxification *ars* genes, are found in many microorganisms (for a review, see Rosen, 2002). However, others have only been identified in certain types of bacteria.

In this work, the genomic sequence of *P. putida* KT2440 (Nelson *et al*., 2002) was used to survey the organism's possible mechanisms of uptake, resistance to and homeostatic regulation of several metals and metalloids. This paper describes an unexpectedly large variety of genes predicted to be involved in metal(loid) homeostasis, tolerance and resistance.

Results and discussion

Metal homeostasis

Computer-assisted BLAST searches of the genome sequence identified a number of putative genes involved in the regulation, uptake, extrusion and chelation of metals in *P. putida*. Table 1 summarizes all the proteins found, and Fig. 1 shows their positions in the genome. A total of 61 ORFs (open reading frames) with a putative role in metal homeostasis and detoxification were found. Seven more ORFs were identified that could have a role in metal(loid) detoxification, although the analysis of their

Received 25 November, 2003; accepted 19 March, 2003. *For correspondence. E-mail vdlorenzo@cnb.uam.es; Tel. (+34) 91 585 4536; Fax (+34) 91 585 4506. †Present address: European Bioinformatics Institute, Cambridge, UK.

Table 1. Summary of the proteins found in *P. putida* KT2440 that might be involved in metal resistance and homeostasis.

ORF ID[a]	Genomic location	Orientation	Protein name	Metal	Family/domain[b]	Predicted role
PP0026	30431–31333	→	CzcD	Me^{2+}	CDF	Transport and regulation
PP0029	32893–33564	→	CzcR1	Me^{2+}	TC reg	Response activator
PP0030	33564–34979	→	CzcS1	Me^{2+}	TC reg	Response activator
PP0041	43530–45524	←	CadA1		P-type ATPase	Me^{2+} efflux
PP0043	46216–49374	←	CzcA1	Me^{2+}	RND	Me^{2+} efflux
PP0044	49402–50649	←	CzcB1	Me^{2+}	RND MFP/HlyD	Me^{2+} efflux
PP0045	50721–51989	←	CzcC1	Me^{2+}	OEP	Me^{2+} efflux
PP0046	52394–53734	→	PorD		Porin	Channel basic amino acids
PP0047	54227–54898	→	CzcR3	Me^{2+}	TC reg	Response activator
PP0117	123087–123866	←	ZnuB1	Zn	IM pore	Zn uptake
PP0118	123862–124632	←	ZnuC1	Zn	ATP-binding protein	Zn uptake
PP0119	124632–125036	←	Zur	Zn	Fur	Regulator
PP0120	125181–126086	→	ZnuA1	Zn	PBP	Zn uptake
PP0360	438427–439188	←	ModR	Mo	modE	Mo uptake regulation (?)
PP0585	683441–683848	←	PacR(CueR)	Cu/Ag	MerR	Transcriptional regulator
PP0586	683848–686244	←	PacS	Cu	P-type ATPase	Cu uptake
PP0588	686829–687023	←	PacZ(CopZ)	Cu	HMA	Activator
PP1437	1637356–1638735	←	CzcS2	Me^{2+}	TC reg	Sensor protein
PP1438	1638735–1639406	←	CzcR2	Me^{2+}	TC reg	Response activator
PP1870	2093311–2093958	→	TPMT	Te, Se		Te and Se methylation
PP1927	2174037–2174759	←	ArsH1			Unknown
PP1928	2174774–2175241[c]	←	ArsC1	As	ArsC	As(V) reduction
PP1929	2175259–2176539	←	ArsB1	As, Sb	ArsB	As(III), Sb(III) efflux
PP1930	2176572–2176925[c]	←	ArsR1	As, Sb	ArsR	Transcriptional repressor
PP2157	2464285–2465634	←	CopS2	Cu	TC reg	Response activator
PP2158	2465634–2466311	←	CopR2	Cu	TC reg	Sensor protein
PP2204	2509129–2510010	←	CopB2	Cu	OM protein	Cu chelation(?)
PP2205	2510006–2511727	←	CopA2	Cu	MultiCu oxidases	Cu chelation
PP2408	2751171–2752445	→	CzcC2	Me^{2+}	OEP	Cation efflux
PP2409	2752441–2753652	→	CzcB2	Me^{2+}	RND MFP/HlyD	Cation efflux
PP2410	2753672–2756815	→	CzcA2	Me^{2+}	RND	Cation efflux
PP2556	2903575–2904924	←	ChrA	Cr	ChrA	Chromate efflux
PP2715	3104850–3105548	←	ArsH2			Unknown
PP2716	3105565–3106032	←	ArsC2	As, Sb	ArsC	As(V) reduction
PP2717	3106064–3107344	←	ArsB2	As, Sb	ArsB	As(III), Sb(III) efflux
PP2718	3107369–3107713	←	ArsR2	As, Sb	ArsR	Transcriptional repressor
PP3262	3696753–3696974	→	MT	Wide	Metallothionein	Metal chelation
PP3341	3779046–3779459	←	NikR	Ni	CopG/NikR	Transcriptional regulator
PP3342	3779547–3781118	→	NikA	Ni	PBP	Ni uptake
PP3343	3781123–3782061	→	NikB	Ni	IM pore	Ni uptake
PP3344	3782061–3782903	→	NikC	Ni	IM pore	Ni uptake
PP3345	3782907–3783674	→	NikD	Ni	ATP-binding protein	Ni uptake
PP3346	3783674–3784492	→	NikE	Ni	ATP-binding protein	Ni uptake
PP3801	4329654–4330553	→	ZnuA2	Zn/Mn(?)	PBP	Zn/Mn(?) uptake
PP3802	4330553–4331296	→	ZnuC2	Zn/Mn(?)	ATP-binding protein	Zn/Mn(?) uptake
PP3803	4331299–4332195	→	ZnuB2	Zn/Mn(?)	IM pore	Zn/Mn(?) uptake
PP3828	4354041–4354796	→	ModA	Mo	PBP	Mo uptake
PP3829	4354799–4355476	→	ModB	Mo	IM pore	Mo uptake
PP3830	4355481–4356569	→	ModC	Mo	ATP-binding protein	Mo uptake
PP4730	5380198–5380599	←	Fur	Fe	Fur	Fe regulation
PP5139	5863892–5866141	←	CadA2	Cd/Zn(?)	P-type ATPase	Cd, Zn efflux
PP5140	5866225–5866665	→	CadR	Zn/Cd?	MerR	
PP5379	6130435–6131836[c]	←	CopB1	Cu	OM protein	Cu chelation (?)
PP5380	6131836–6133842	←	CopA1	Cu	MultiCu oxidases	Cu chelation
PP5383	6134976–6135650	→	CopR1	Ag/Cu(?)	TC reg	Response activator
PP5384	6135650–6137056	→	CopS1	Ag/Cu(?)	TC reg	Response activator
PP5385	6137536–6138786	→	CusC	Ag/Cu(?)	OEP	Ag,/Cu(?) efflux
PP5386	6138786–6140255	→	CusB	Ag/Cu(?)	RND MFP/HlyD	Ag,/Cu(?) efflux
PP5387	6140255–6143410	→	CusA	Ag/Cu(?)	RND	Ag,/Cu(?) efflux
PP5388	6143410–6143748	→	CusX			?
PP5389	6144773–6145429	←	CzcN		IM potential protein	?
PP5392	6146321–6147451	←	NirF(?)			Dehydrogenase?
PP5394[d]	6148147–6150675	←	SilP	Ag/Cu(?)	P-type ATPase	Ag,/Cu(?) efflux
PP1515[e]	1719368–1720006	←	TetR	Drug(?)	TetR	Transcriptional regulator
PP1516[e]	1720257–1721354	→	Mtrc2	Me^{2+}/drug	RND MFP/HlyD	Me^{2+}/drug efflux
PP1517[e]	1721364–1724426	→	CzcA5	Me^{2+}/drug	RND	Me^{2+}/drug efflux
PP3300[e]	3733309–3733920	→	TetR	Drug(?)	TetR	Transcriptional regulator

Table 1. *cont.*

ORF ID[a]	Genomic location	Orientation	Protein name	Metal	Family/domain[b]	Predicted role
PP3301[e]	3734001–3735101	→	Mtrc1	Me^{2+}/drug	RND MFP/HlyD	Me^{2+}/drug efflux
PP3302[e]	3735101–3738190	→	CzcA3	Me^{2+}/drug	RND	Me^{2+}/drug efflux
PP5173[e]	5897828–5900878	←	CzcA4	Me^{2+}/drug	RND	Me^{2+}/drug efflux
PP5174[e]	5900878–5901942	←	MfpI	Me^{2+}/drug	RND MFP/HlyD	Me^{2+}/drug efflux
PP5175[e]	5901942–5903027	←	MfpII	Me^{2+}/drug	RND MFP/HlyD	Me^{2+}/drug efflux
PP1645[e]	1839164–1839514	←	ArsC3			Arsenate reductase

a. ORF ID are available at the TIGR web page.
b. TC reg, two-component regulators; RND, resistance–nodulation–cell division; MDF, membrane diffusion facilitator; CDF, cation diffusion facilitator; OEP, outer membrane efflux protein; IM, inner membrane; OM, outer membrane; PBP, periplasmic binding protein; HMA, heavy metal-associated domain. Question marks indicate cases in which *in silico* analysis is insufficient to identify the activity or the target metal.
c. Discrepancies between our predictions and the TIGR database were found in the starting ATG. Suggested genomic locations are shown.
d. Reported to have an authentic point mutation (http://www.tigr.org and http://www.ncbi.nlm.nih.gov/). Our ORF prediction is longer than the reported PP5394 (6148150–6150429).
e. These genes have unclear roles in metal(loid) resistance and homeostasis.

amino acid sequence was insufficient to assign them an unequivocal role. In addition, several ORFs without similarity to known proteins in the databases were found included in or surrounded by metal homeostasis-related operons.

Thirteen of the above 61 ORFs containing RND (resistance/nodulation/cell division) domains, and other associated ORFs containing HlyD (RND-associated membrane fusion proteins) and/or OEP (outer membrane efflux protein) domains were found arranged in 12 operons. Genes with such domains are known to provide cells with resistance to drugs or metals (Taghavi *et al*., 1997). Three out of these 12 operons (plus three likely but less clear gene clusters) could be related to metal detoxification. Twelve is a large number of efflux systems, only comparable to those found in *Pseudomonas aeruginosa* (10 multidrug plus two divalent cation efflux systems; Stover *et al*., 2000). In contrast, the number of predicted efflux systems in bacteria with genomes that have been sequenced is lower: four in *Escherichia coli*, one in *Bacillus subtilis* and none in *Mycobacterium tuberculosis* (Stover *et al*., 2000). Both *P. putida* and *P. aeruginosa* also showed four P-type ATPases that might be involved in soft metal transport, as well as one putative chromate transporter. BLAST searches showed other systems likely to be involved in the detoxification of copper (*copAB*) and arsenic (*ars*) in both organisms' genomes. These systems appear to be duplicated in *P. putida* but not in *P. aeruginosa*.

Although the metal-related systems are spread throughout the genome, a significant portion of the genes involved in resistance appear to be located around the origin of replication region (ORI). In contrast, metal uptake systems are located far from the ORI region (Fig. 1). In *E. coli*, the position of a kanamycin resistance gene close to the ORI region provides a higher level of kanamycin resistance than at other locations (Sousa *et al*., 1997). Similarly, in *P. putida*, the location of two large clusters of genes involved in metal resistance in the ORI region might result in increased tolerance. These genes mediate resistance to both monovalent and divalent cations, thus providing resistance to a wide spectrum of metals. Interestingly, the genes responsible for arsenic resistance, which are not located in the ORI region, are duplicated in the chromosome. This may compensate for the effect of location-mediated low levels of resistance. All these features suggest that *P. putida* is a bacterium habituated to exposure to heavy metals and metalloids in its environment.

Metal chelation

Metallothioneins (MT) are small cysteine-rich peptides with a high affinity for heavy metals. One such protein, identified in a *P. putida* strain isolated from a metal-polluted site (Higham *et al*., 1984), displays a high degree of homology to the *Synechococcus* SmtA (Robinson *et al*., 2001). The three-dimensional structure of the *Synechococcus* MT has been determined, and all the cysteine residues present in the peptide have been assigned to the chelation of metals. Alignment of the *Synechococcus* MT with the pseudomonad (*P. putida* KT2440 and *P. aeruginosa*) MTs showed the latter to have one or two cysteine residues more (one conserved at position 33) that could play a role in binding the metal (Robinson *et al*., 2001). All the cysteine and histidine residues in SmtA involved in metal binding are conserved in the pseudomonad MTs, except His-49. Actually, this residue is different in both pseudomonad MTs, being replaced by either methionine or aspartate. Pseudomonad MTs are longer than those of other bacteria, although the C-terminal tail lacks cysteine and histidine residues. A stoichiometry of 4:1 Zn to protein molecules has been reported for *Synechococcus* SmtA, in which both cysteine and histidine residues are involved in Zn co-ordination. This is different from mammalian MTs,

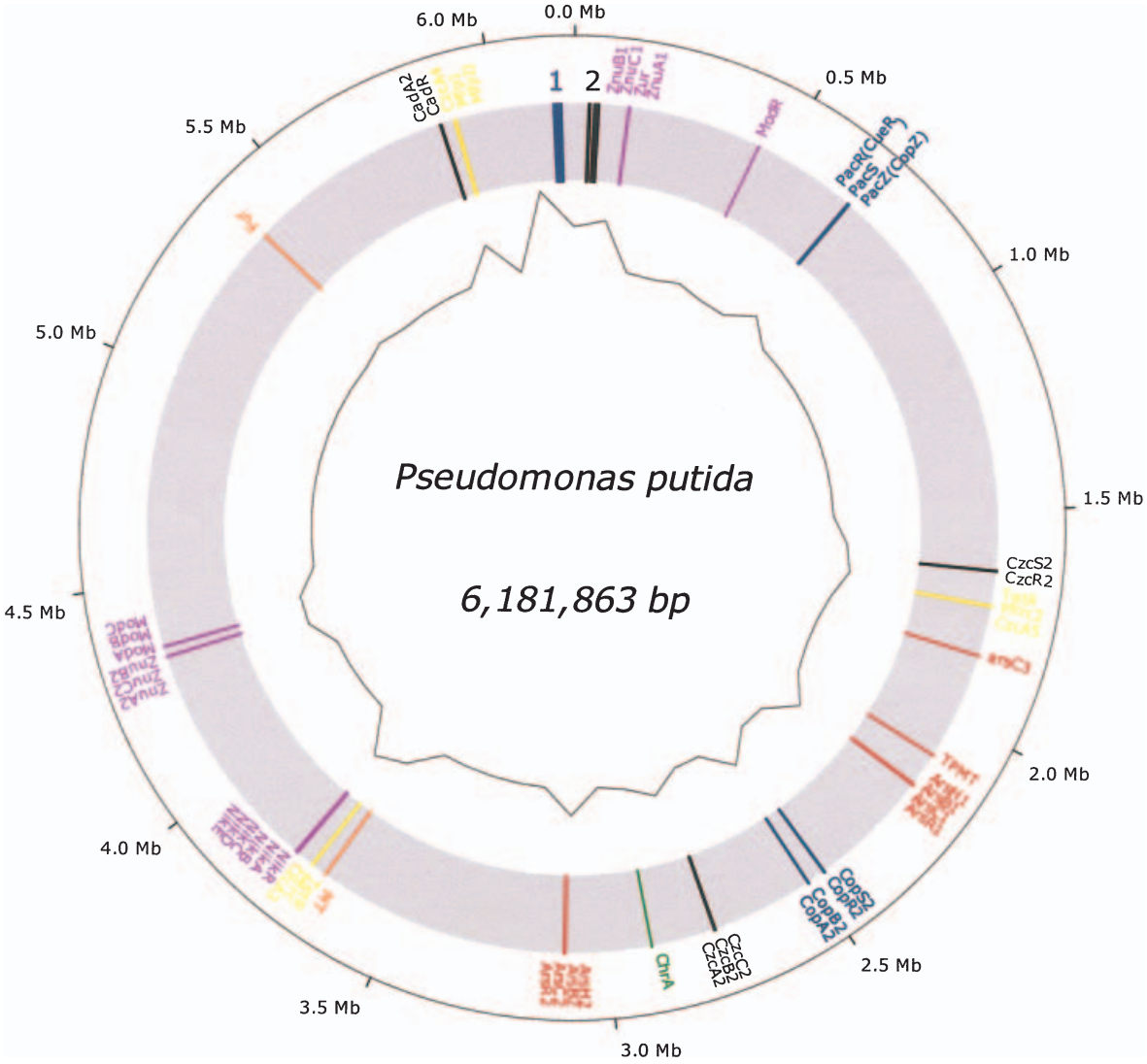


Fig. 1. Circular map of the complete genome of *P. putida* KT2440 showing the genes involved in metal resistance and homeostasis. The outer circle indicates the co-ordinates in Mb. The central circle indicates the position of the genes. The inner circle shows the density of genes putatively involved in metal(loid) homeostasis (number of genes per kb). Cluster 1 includes the following genes: *copAB1 copRS1 cusCBA cusX czcN nirF silP*. Cluster 2 corresponds to: *czcD czcRS1 cadA1 czcCBA1 porD czcR3*. The colour code indicates the metal substrate of the system and/or the function of the system: black, resistance to divalent cations; blue, homeostasis of monovalent cations; red, resistance to metalloids As or Se and TE; purple, uptake of cations Zn, Ni, Mo, Mn; green, resistance to Cr; yellow, systems belonging to the RND family that might be involved in metal resistance; orange, others.

in which only cysteine residues participate in the metal-binding pocket (Blindauer *et al.*, 2001; Robinson *et al.*, 2001). In *P. aeruginosa*, an MT-like protein seems to bind zinc with a stoichiometry of between 3:1 and 4:1 (Robinson *et al.*, 2001). In *Synechococcus*, the expression of SmtA is regulated by a member of the *ars* family, SmtB (Huckle *et al.*, 1993). In *P. putida* KT2440, this regulatory protein could not be found, nor was any possible regulator detected either upstream or downstream of the metallothionein gene. Another ORF (PP3261) was found upstream of the metallothionein gene overlapping one nucleotide of the sequence, but no information on its role could be obtained from the predicted amino acid composition.

The first well-studied copper resistance mechanism was reported in *Pseudomonas syringae* pv. *tomato* (for reviews, see Cervantes and Gutierrez-Corona, 1994; Cooksey, 1994). Four genes were identified: *copAB*$_{Psy}$ (subscript letters indicate the microorganisms to which the gene belongs) is required for full copper resistance, whereas *copCD*$_{Psy}$ might be involved in copper transport. CopA$_{Psy}$ and CopC$_{Psy}$ are periplasmic proteins responsible for the binding of Cu with a stoichiometry of 1:11 and 1:1 (polypeptide:Cu atoms) respectively. CopB$_{Psy}$ is an outer

membrane protein that binds additional copper. *P. putida* KT2440 has two copies of the *copAB*$_{Ppu}$ genes, although no *copCD* genes were found (Fig. 4B). Both CopA$_{Psy}$ and CopB$_{Psy}$ contain an MXXMXHXXM (MDH) motif repeated several times throughout the sequence (Cha and Cooksey, 1991). This motif also appears to be repeated in *P. putida* Cop proteins. Interestingly, in CopA1$_{Ppu}$, the eight-amino-acid motif is repeated 14 times, whereas it appears only four or five times in homologues from *P. syringae*, *P. putida* (CopA2$_{Ppu}$) and *E. coli*. A single copper binding site has been predicted in copper oxidases that is also conserved in CopA$_{Psy}$ homologues His-542, Cys-591, His-596 and Met-601 (Ouzounis and Sander, 1991). As these proteins only have one or two cysteines, it was postulated that the histidine residues must be involved in the binding of copper. CopA proteins from *P. syringae*, *E. coli* and the predicted ones from *P. aeruginosa* and *Ralstonia metallidurans* were found to contain between 20 and 24 histidine and 38–44 methionine residues, whereas CopA1$_{Ppu}$ and CopA2$_{Ppu}$ contained 30 and 24 histidines and 60 and 25 methionines respectively. The assumption of a linear correlation between the number of histidine (and maybe methionine) residues and the number of bound copper atoms suggests that CopA1$_{Ppu}$ can bind between 14 and 17 atoms of Cu per polypeptide, a value higher than that assigned to CopA$_{Psy}$.

As in CopA, CopB proteins have several repetitions of the MDH motif. These proteins have either zero or one cysteine residues and a number of histidines that varies (from six in *E. coli* PcoB to 22 in *P. putida* CopB1$_{Ppu}$). CopB$_{Psy}$ is associated with the outer membrane. Because of the presence of the MDH motif, it might bind copper as suggested by Cha and Cooksey (1991), and may be involved in copper transport across the membrane (Cervantes and Gutierrez-Corona, 1994; Cooksey, 1994). In the case of *copB1*$_{Ppu}$, a critical insertion of one nucleotide was detected in the reported sequence. This led to a frameshift and the annotation of a truncated protein. Whether this is the result of a sequencing or assembly error, or just an example of the enormous genome plasticity (i.e. potential for phase variation) of this microorganism remains to be studied. If the frameshift were disregarded, the resulting protein of CopB1$_{Ppu}$ is histidine rich (22 residues) and has nine repeats of an MDH-like motif, whereas the truncated reported protein would only have 13 histidine residues and four repeats of the motif. As in CopAs, CopB2$_{Ppu}$ has a lower number of histidine residues (10) and no MDH motif. Thus, it is questionable whether either system is really involved in copper homeostasis. If they are, the question arises as to why *P. putida* has maintained two systems for performing the same function when, from the predicted protein sequence, one would appear to be much less efficient. A further enigma is that, if *copB1*$_{Ppu}$ is non-functional, why/how has *copB2*$_{Ppu}$ been selected if, *in silico*, *copB1*$_{Ppu}$ (without the nucleotide insertion) is predicted as the more efficient version? Could it be that their proteins form 'homo-heterodimers' composed of CopA1$_{Ppu}$–CopA2$_{Ppu}$ and CopB1$_{Ppu}$–CopB2$_{Ppu}$? Other possibilities include one of the systems not being active.

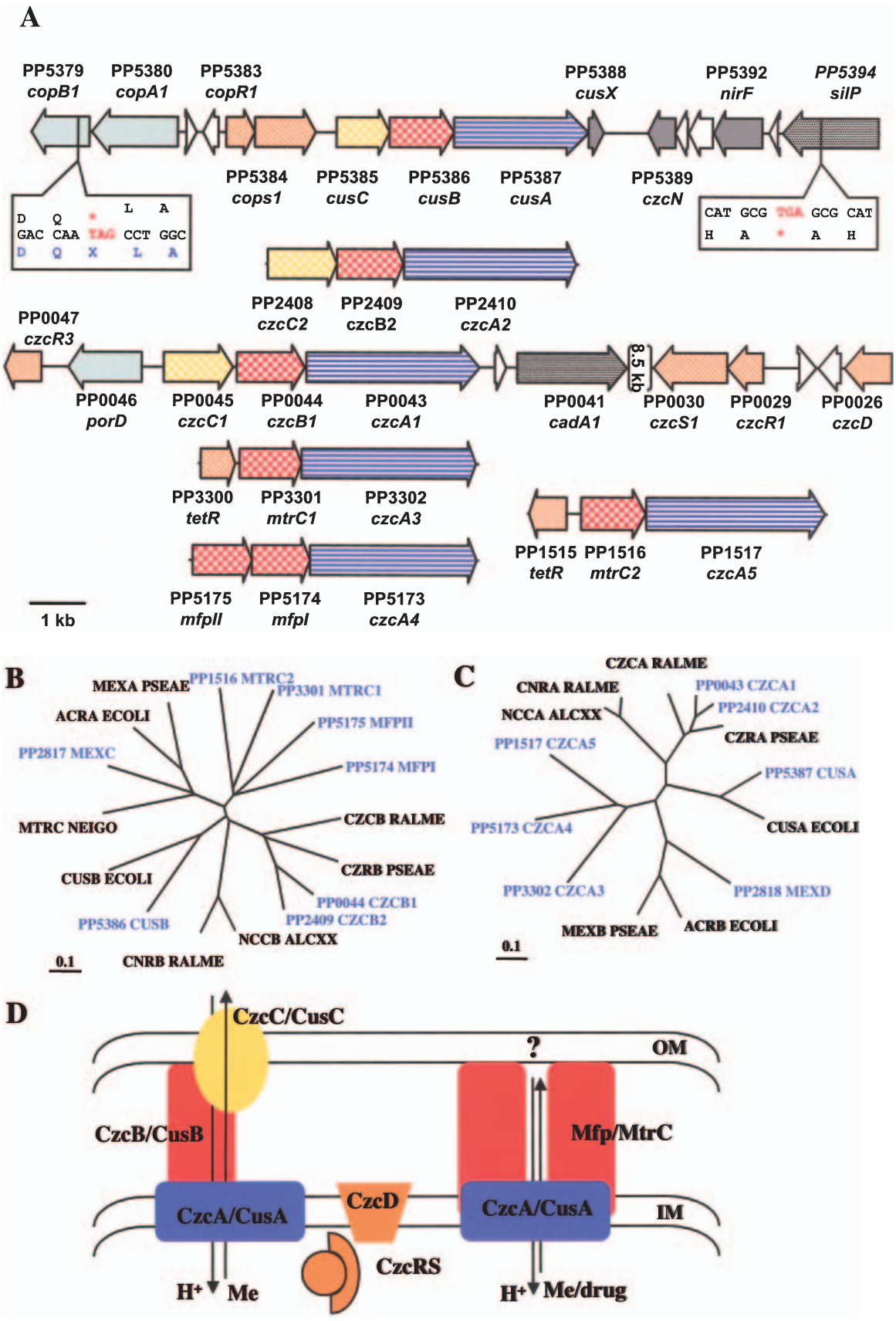
Interestingly, *copAB2*$_{Ppu}$ has a G+C content of 66%, whereas *copAB1*$_{Ppu}$ has 56–58%. The G+C content of the other *copAB* genes is 52% (*E. coli*), 59% (*P. syringae*), 59–60% (*R. metallidurans*) and 67–71% (*P. aeruginosa*), whereas the average G+C content of *P. putida* is 61.6%. These data suggest that at least one of the copies of the *copAB*$_{Ppu}$ genes might have been acquired by horizontal transfer. Further, ORFs 1, 2 and 3 of the ISPpu14 transposase (PP0275, PP0271 and PP1854) were found located 2.3 kb away from the copper cluster (named 1 in Fig. 1), and this cluster is located in a gene island with an atypical genome signature (G+C content, dinucleotide bias and variances in tetranucleotide frequencies; Weinel *et al*., 2002). This supports the hypothesis that *copAB*$_{Ppu}$ genes have been acquired by horizontal transfer.

Cop proteins could play an important role in Cu homeostasis by regulating the free Cu in the periplasm, which is susceptible to uptake by the P-type ATPase described below. As Cop systems can be overloaded under conditions of excess copper, *P. putida* possesses an auxiliary system (the *cus* gene cluster; see below) that could provide the cell with increased resistance. The Cus system transports Cu directly out of the cell through the inner and outer membrane without releasing the Cu into the periplasmic space. As shown in Fig, 2A, the *cus* genes lie adjacent to the *copAB1* genes, to the regulatory pair *copRS* and to *silP*. Interestingly, this genomic segment is arranged similarly to a region determining silver resistance in *Salmonella*, so it could also play some role in resistance to Ag cations (Fig. 2A; Gupta *et al*., 1999).

Systems with a role in metal uptake

In some metabolic pathways, certain metals are essential, and this requires specialized transport systems that can translocate them into the cell. In *P. putida*, some ABC transporters seem to be responsible for this. One of these, the *nikRABCDE* system, is responsible for the uptake of Ni. In *E. coli*, NikA is the periplasmic binding protein (PBP), NikB and NikC form a heterodimeric inner membrane pore for the translocation of the metal, and NikD and NikE are the heterodimeric ATP-binding proteins (Fig. 3C; Navarro *et al*., 1993). The organization of the genes in *P. putida* is different from that in *E. coli* (Navarro *et al*., 1993) but the same as in *Brucella suis*, where *nikR* is upstream of and transcribed divergently from *nikABCDE* (Jubier-Maurin *et al*., 2001).

There are two systems in *P. putida* KT2440 that are



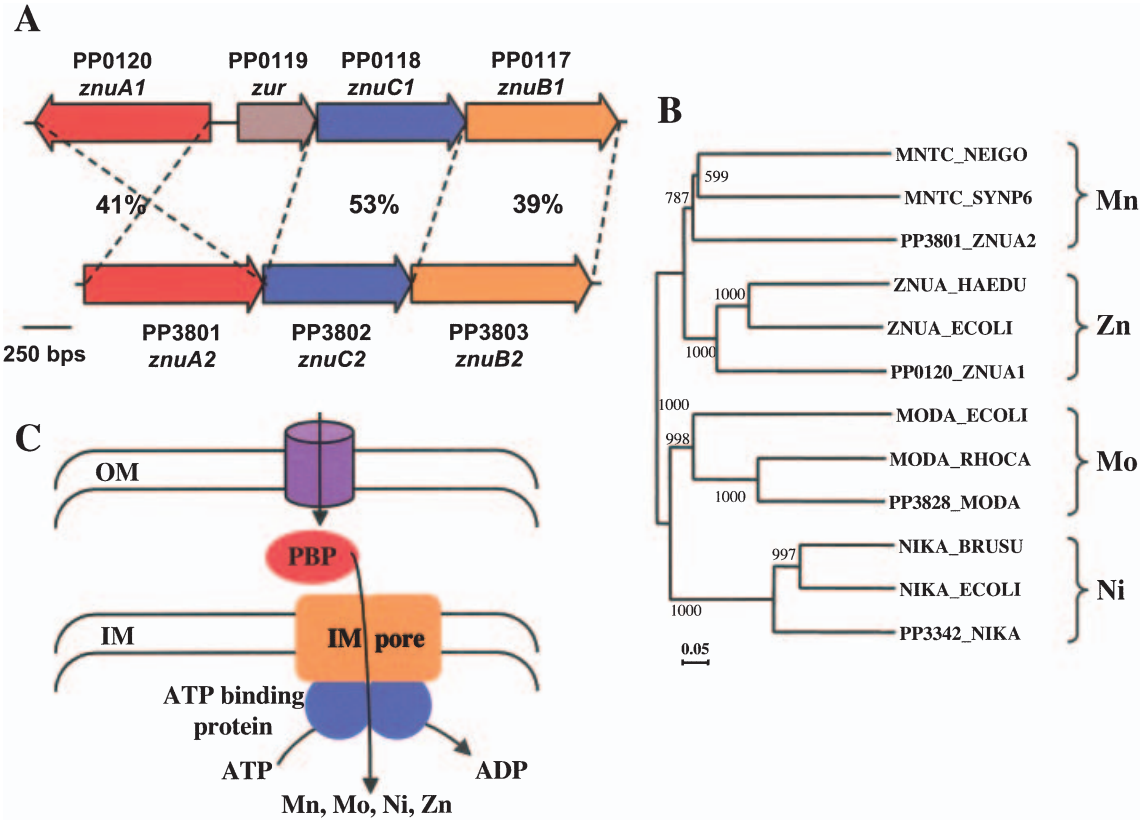


Fig. 3. ABC transporters are responsible for the uptake of some essential metals in *P. putida*.
A. Genetic organization of the two *znu* systems showing the degree of similarity between the proteins.
B. Phylogenetic analysis of the PBPs of the ABC transporters. Bootstrap values are indicated in the branch points. ECOLI, *E. coli*; NEIGO, *N. gonorrhoeae*; SYNP6, *Synechocystis* sp. strain PCC6803; HAEDU, *Haemophilus ducreyi*; RHOCA, *Rhodobacter capsulatus*; BRUSU, *Brucella suis*.
C. Schematic representation of the ABC transporters. PBP, periplasmic binding protein; OM, outer membrane; IM, inner membrane.

highly homologous to *E. coli znuACB*. *znuACB1*$_{Ppu}$ is organized differently from *E. coli*, in which the regulatory protein *zur* is located elsewhere in the genome (Patzer and Hantke, 1998). In *P. putida*, *zur*$_{Ppu}$ is located between *znuA1*$_{Ppu}$ and *znuC1*$_{Ppu}$ and is transcribed in the same orientation as *znuCB1*$_{Ppu}$ (Fig. 3A). The other system found in *P. putida*, *znuACB2*, not only displays similarity to *znu*$_{Eco}$ but also has the same degree of similarity to the Mn transporters (Bartsevich and Pakrasi, 1996). Similarity searches revealed no other Mn systems in the genome. Consequently, *znuACB2* could be involved in Mn uptake or even Mn and Zn uptake. The degree of similarity between the *znuACB1*$_{Ppu}$ and *znuACB2*$_{Ppu}$ genes is 41%, 53% and 39% respectively (Fig. 3A). However, although in one of the operons, *znuA*$_{Ppu}$ is transcribed divergently from *znuCB*$_{Ppu}$, in the other, all the genes are transcribed in the same orientation (Fig. 3A). Phylogenetic reconstruction of the PBPs involved in metal uptake showed that *znuA2*$_{Ppu}$ clusters with the manganese PBP of *Synechocystis*, whereas *znuA1*$_{Ppu}$ clusters with the zinc PBP from enterobacteria (Fig. 3B). The fact that *znuA2*$_{Ppu}$ is phylogenetically closer to the Mn transporters suggests that *znuACB2*$_{Ppu}$ might be a Mn uptake system rather than a zinc transporter. However, it may be that it transports

Fig. 2. Members of the RND family of metal resistance determinants.
A. Genetic organization of the five clusters identified that could play a role in metal homeostasis. Point mutations/discrepancies leading to frameshifts (*copB1*) or early stop codons (*silP*) are shown in boxes. The 8.5 kb region between *cadA1* and *czcS1* contains ORFs with no matches to proteins of known function. Cluster 1 shown in Fig. 1 corresponds to *copAB1 copRS1 cusCBA cusX czcN nirF silP* and cluster 2 corresponds to *czcD czcRS1 cadA1 czcCBA1 porD czcR3*.
B. Phylogenetic reconstruction of the RND-associated membrane fusion proteins. *P. putida* proteins are labelled in blue. ECOLI, *E. coli*; PSEAE, *P. aeruginosa*; RALME, *R. metallidurans*; NEIGO, *Neisseria gonorrhoeae*; ALCXX, *Alcaligenes xylosoxidans*.
C. Phylogenetic reconstruction of the RND proteins. Note that the clustering is identical in both trees.
D. Schematic representation of the metal resistance mediated by the *czc*/*cus* determinant. OM, outer membrane; IM, inner membrane.

both Zn and Mn. Koski and Golding (2001) reported that the best hit in a BLAST search does not always correlate with the closest phylogenetic neighbour, and that this usually happens for genes with few homologues (Koski and Golding, 2001). There are few known homologues of *znuA2*$_{Ppu}$. Although phylogenetic proximity cannot be relied upon to assign the biological function of a protein, it can provide hints on the role of the protein. In this respect, it is also worth noting that a BLAST search for MntH in *P. putida* suggested the absence of any homologues of this protein in this bacterium. This is in striking contrast to the two MntH variants found in *P. aeruginosa*.

Another ABC metal transporter found in *P. putida* KT2440 seems to constitute a Mo uptake system. This was named *modABC*$_{Ppu}$ according to the nomenclature used with *E. coli*, where ModA$_{Eco}$ is the periplasmic binding protein, ModB$_{Eco}$ the inner membrane permease and ModC$_{Eco}$ the ATP binding protein (Fig. 3C; Maupin-Furlow *et al.*, 1995). Besides these three genes, other organisms also have a fourth ORF, *modD*, of unknown function (Maupin-Furlow *et al.*, 1995). *modD* mutants of *Rhodobacter capsulatus* require four times greater concentrations of Mo than the wild type in alternative nitrogenase assays (Wang *et al.*, 1993). However, no *modD* homologue was found in *P. putida*. *modR*$_{Ppu}$ is also involved in the uptake of Mo, but is located far from the corresponding *modABC*$_{Ppu}$ genes in the *P. putida* genome. The fact that it displays a similar degree of similarity (around 40%) to both *E. coli* ModE and *R. capsulatus* MopA and MopB makes it difficult to assign a putative role for *P. putida* ModE in the transport of Mo. ModE$_{Eco}$ dimerizes after Mo binding and then becomes competent for DNA binding, acting as a transcriptional repressor (Maupin-Furlow *et al.*, 1995). MopA$_{Rca}$ and MopB$_{Rca}$ are molybdenum-pterin-binding proteins involved in the transport of Mo (Wang *et al.*, 1993). A domain analysis of the predicted protein showed ModE$_{Ppu}$ to have an HTH_9 domain (helix–turn–helix) in the N-terminal region responsible for DNA binding, plus one TOBE domain (transport-associated OB) in the central region of the peptide (which seems to be involved in the binding of small solutes such as Mo or sulphate). This is similar to that found in ModE$_{Eco}$, so the former could be involved in the regulation of Mo transport. MopA$_{Rca}$ and MopB$_{Rca}$ proteins also each have one HTH_9 domain and two TOBE domains.

Soft metal P-type ATPases

The P-type ATPases are a family of transporters that mediate the uptake and extrusion of both soft and hard metals (Fig. 4B). Soft metals are ionic species of elements that are chemically soft Lewis acids, as opposed to the hard Lewis acids of groups I and II elements such as Na^+ and Ca^{2+} (Gatti *et al.*, 2000). There are four DNA segments with the potential to encode soft metal P-type ATPases in *P. putida*. SilP$_{Ppu}$ and PacS$_{Ppu}$, appear to be involved in the transport of monovalent cations (Cu and/or Ag), whereas CadA1$_{Ppu}$ and CadA2$_{Ppu}$ could play a role in the detoxification of divalent metals from the cytoplasm. All four ATPases have the conserved motifs DKTGT necessary for phosphorylation during the catalytic cycle,

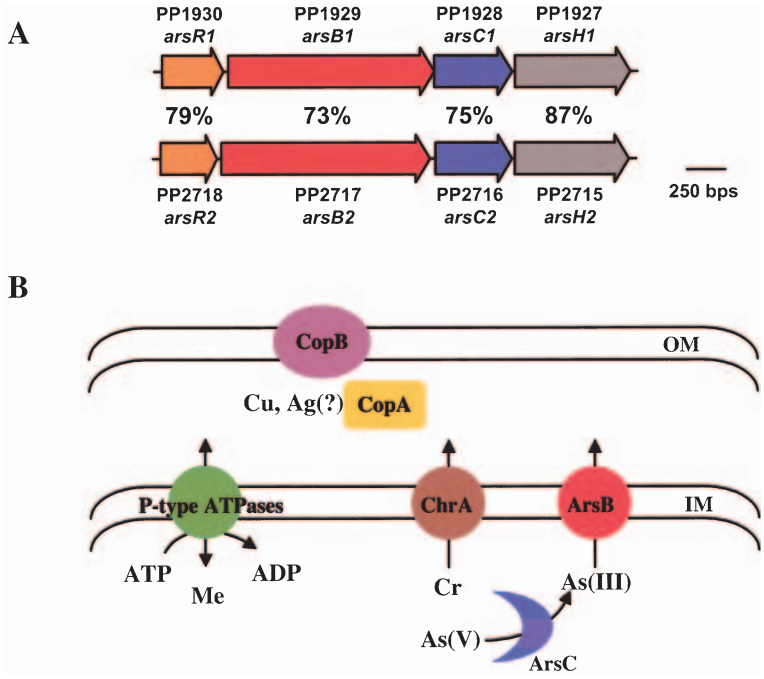


Fig. 4. Representative metal-related systems in *P. putida*.
A. Genetic organization of the *ars* genes showing the degree of similarity between the proteins.
B. Systems responsible for metal(loid) resistance/uptake are chelation of Cu by CopA and CopB, efflux of Cr by ChrA, reduction of arsenate to arsenite by ArsC and subsequent extrusion of arsenite by ArsB, and transport of monovalent and divalent cations by P-type ATPases (SilP, PacS, CadAs). OM, outer membrane; IM, inner membrane.

plus the GCGXNDXP motif required for ATP binding. The CPC amino acid stretch, located in a region of hydrophobic residues, is related to the transport of transition metals across the membrane (Silver and Walderhaug, 1992). Another characteristic motif found in transition metal pumps is the phosphatase domain TGES, which was found to be retained in *P. putida* pumps with little modification of the monovalent cation transporters $SilP_{Ppu}$ (TGEP) and $PacS_{Ppu}$ (SGES). The Kyte–Doolittle hydropathy of the two $CadA_{Ppu}$ proteins and the $PacS_{Ppu}$ are similar to that of $CadA_{Hpy}$ (Melchers *et al*., 1999). Strikingly, $SilP_{Ppu}$ could have 10 transmembrane regions according to its hydropathy profile, which would resemble the hard metal P-type ATPase MgtB of *Salmonella typhimurium* (Smith *et al*., 1993). In contrast to $CadA2_{Ppu}$ and $PacS_{Ppu}$, $CadA1_{Ppu}$ and $SilP_{Ppu}$ did not contain the HMA motif (heavy metal-associated domain).

$SilP_{Ppu}$ deserves a separate comment. The corresponding genomic segment is located in a large gene cluster probably involved in Ag resistance and/or Cu homeostasis (Fig. 2A). Yet, the corresponding DNA sequence was reported to contain a frameshift mutation (http://www.tigr.org and http://www.ncbi.nlm.nih.gov, TIGR PP5394). This causes an early stop codon within the putative gene. However, a reframed gene sequence would recreate all the conserved motifs typical of P-type ATPases. A large Ag/Cu-related genomic segment could encode a P-type ATPase ($SilP_{Ppu}$), a three polypeptide cation/proton antiporter ($CusCBA_{Ppu}$), as well as two metal-binding proteins ($CopAB1_{Ppu}$), and it could all be regulated by a two-component regulatory system ($CopSR1_{Ppu}$; Fig. 2A). This organization resembles the *sil* determinant found in the hospital burn ward *Salmonella* plasmid pMG101 (Gupta *et al*., 1999). It is intriguing that the two cases of mutations (*silP* and *copB1*; see above) leading to truncated or early stop codons were found in proteins determined by this cluster. As such a large metal-related determinant contains atypical genome signatures (Weinel *et al*., 2002) and it is adjacent to a 5 kb region containing transposases, it is likely that the whole segment was acquired by horizontal gene transfer. Mutations could have then occurred in proteins of the cluster with activities redundant to those already present in a separate genomic location, for example *pacS* and *copB2*. Another interesting feature is that this cluster lies relatively close to a second gene grouping involved in divalent cation export (Fig. 1; described below).

The alternative copper P-type ATPase, $pacS_{Ppu}$, is flanked by two regulatory genes: $copZ_{Ppu}$ (upstream of *pacS*, encoding a putative activator) and $cueR_{Ppu}$ (downstream, determining a regulator belonging to the *merR* family). Yet, the *copY* gene, encoding the aporepressor released by *copZ* when bound to Cu ions in *Enterococus*, was not found (Strausak and Solioz, 1997; Cobine *et al*., 1999).

Two CadA homologues were found in the *P. putida* genome. $cadA2_{Ppu}$ is transcribed divergently from its putative regulator CadR, and $cadA1_{Ppu}$ is located in the middle of the *czc* region (Fig. 2A). $cadRA_{Ppu06909}$ has already been reported for *P. putida* strain 06909, a rhizosphere bacterium (Lee *et al*., 2001), and shows a high degree of similarity to the *P. putida* KT2440 *cadA* genes. A salient feature of the sequence of $CadA2_{Ppu}$ is that it contains a histidine-rich motif in its N-terminal region that resembles the HEHKHDHHAH fragment of the P-type ATPase of *P. putida* 06909, although it is longer: HEHKHPHDHAHGD DDHGHAAHGH. Although $CadA2_{Ppu}$ has a long hydrophilic N-terminal region, $CadA1_{Ppu}$ has a short hydrophilic N-terminal region containing a shorter histidine-rich motif: HEGHSHEH. None of the three pseudomonad CadA proteins have an ion-binding motif (CxxC) in their N-terminal region. $CadA_{Ppu06909}$ is responsible for resistance to Cd and partial resistance to Zn (Lee *et al*., 2001).

Extrusion of metals

To avoid the toxic effects of metals, bacteria frequently activate extrusion mechanisms. This constitutes the mechanism of resistance to chromate, which is based on active efflux driven by the membrane potential (Alvarez *et al*., 1999). The gene responsible, *chrA*, is also found in *P. putida*. In contrast to *R. metallidurans*, no *chrB* has been found in the genomes of *P. aeruginosa* or *P. putida*. In *R. metallidurans*, ChrB appears to be associated with the inner membrane and is thought to play a role in the regulation of the system. In *Pseudomonas*, the absence of a putative transcriptional regulator could mean that *chrA* is constitutively expressed.

Arsenic resistance is generally mediated by the *arsRBC* genes, three genes usually found in most bacteria. *arsR* is a transcriptional repressor that responds to As(III) and Sb(III). ArsB is a secondary transporter that extrudes both As(III) and Sb(III) using the proton motive force and is sufficient for arsenite and antimony resistance. In some cases, an ATPase (ArsA) is also found with ArsB providing the energy for the translocation of the metalloids and increasing the level of arsenic resistance (Rosen, 2002). In *P. putida*, only *arsB* was found. *arsC* encodes an arsenic reductase responsible for the transformation of As(V) into As(III), the arsenic form transported out of the cell. Two copies of an *arsRBCH* operon are found in the chromosome of *P. putida* (Fig. 4A). In order to determine whether the second copy of the genes came from a duplication or a horizontal transfer, the G+C content, the BLAST best hit in the database (Eisen, 2000) and their closest phylogenetic neighbours (Koski and Golding, 2001) were compared with each other. The reality is that the G+C

content and sequences are very similar in both copies of *ars* genes. But the *ars1* cluster is located in a region with atypical genome signatures (Weinel *et al*., 2002). Therefore, it is still unclear how the *ars1* cluster was acquired.

The distinct gene named *arsH* was also found in both copies of the genomic ars_{Ppu} operons. *arsH* was originally identified in the *ars* cluster of a Tn*2502* transposon (belonging to the virulent plasmid pYV of *Yersinia enterocolitica*, Neyt *et al*., 1997), and in *Thiobacillus ferrooxidans* (Butcher *et al*., 2000). In *Y. enterocolitica*, *arsH* appears to be necessary for arsenic resistance (Neyt *et al*., 1997). Both $ArsH1_{Ppu}$ and $ArsH2_{Ppu}$ were highly similar to their counterparts in *Y. enterocolitica* and *T. ferrooxidans* (over 74%). Although *Pseudomonas fluorescens* strain MSP3 lacks an *arsH* gene (Prithivirajsingh *et al*., 2001), *P. aeruginosa* has an *arsH* homologue (87% amino acid similarity) located downstream of *arsC*. The genetic organization of the *ars* operon in *P. putida*, *Y. enterocolitica* and *T. ferrooxidans* is different. $arsH_{Ppu}$ is placed downstream of $arsC_{Ppu}$ and transcribed in the same orientation, whereas *Yersinia* $arsH_{pYV}$ is located upstream of $arsR_{pYV}$ and transcribed divergently. In *T. ferrooxidans*, $arsH_{Tfe}$ is placed downstream of $arsB_{Tfe}$, and both are transcribed in the opposite orientation to $arsRC_{Tfe}$. Besides, $ArsH1_{Ppu}$ (241 amino acids) and $ArsH2_{Ppu}$ (233 amino acids) show a less pronounced but still significant homology to plant NADH oxidoreductases (up to 44% in a distinct segment of 190 amino acids) and *B. subtilis* azoreductase (47% in a distinct segment of 182 amino acids). Neyt *et al*. (1997) suggested that $ArsH_{pYV}$ might be a transcriptional activator because a plasmid containing the genes $arsRBC_{pYV}$ without $arsH_{pYV}$ in *Y. enterocolitica* did not cause an increase in arsenic (arsenate and arsenite) resistance.

In addition to the two copies of the *ars* genes discussed so far, an additional ORF (designated by TIGR as *arsC1*, PP1645, and renamed in Table 1 as *arsC3*) could be involved in arsenic tolerance. Yet, such a third *arsC* gene has far less similarity to the other two *arsC* genes than they have to one another. Moreover, this ORF is in an isolated chromosomal context that gives no further evidence for a role in arsenic tolerance.

The genome of *P. putida* also harbours several putative chemiosmotic antiporter efflux systems. These antiporter devices are three-component systems involved in drug or metal efflux and include proteins belonging to the RND (resistance, nodulation, cell division) family of integral membrane proteins (Fig. 2D; Taghavi *et al*., 1997). Antiporter efflux systems may confer resistance to several metals, and they are known in many bacteria. The best known archetype is the *czcCBA* system of *R. metallidurans*, which confers resistance to Cd, Zn and Co. The CzcA protein of this organism is associated with the inner membrane and appears to have 12 transmembrane domains (Taghavi *et al*., 1997). CzcC belongs to the OEP family (outer membrane efflux protein) that functions as an auxiliary element for the export of metals. Outer membrane efflux proteins have a typical trimeric assembly: an internal channel shaped by the association of a total of 12 β-sheets. This channel is extended into the periplasm by α-helices, which associate and form a long conduit. CzcB has the HlyD domain in which the N-terminus is anchored to the inner membrane. Neither CzcB nor CzcC appears to be necessary for resistance to metals, but they may increase the efficiency and specificity of the main component of the system, CzcA (Taghavi *et al*., 1997). CzcA would thus be responsible for the translocation of metals across the plasma membrane, whereas CzcCB would avoid their release into the periplasmic space and export them out of the cell. The organization of the *czc*-like systems is generally conserved in all organisms studied, although regulatory proteins may be located in a variety of orientations with respect to the core *czcCBA* genes. Other chemiosmotic antiporter efflux systems include the *cnrCBA* (resistance to Co and Ni) of *R. metallidurans* (Taghavi *et al*., 1997), *nccCBA* (Ni, Co, low Cd) of *Alcaligenes xylosoxidans* (Schmidt and Schlegel, 1994), *cusCBA* (Ag, Cu) of *E. coli* (Franke *et al*., 2001; Outten *et al*., 2001a) and *czrCBA* (Cd, Zn) of *P. aeruginosa* (Hassan *et al*., 1999). *czrA* (Q93SR9) and a portion of *czrB* (Q93SS0) have fortuitously been found in *P. putida* strain P111.

In *P. putida* KT2440, at least five operons were found with enough similarity to chemiosmotic antiporter efflux systems to propose a similar function (Fig. 2A). One of these systems, *czcCBA2*, is extraordinary in that it appears as an isolated cluster in the chromosome (Fig. 1), so no sequences of regulatory proteins were found adjacent to the *czcCBA2* genes. Two more operons of this type (*czcCBA1* and *cusCBA*) were identified near the origin of replication (roughly spanning co-ordinates 6130k to 55k; Fig. 1). *czc/cus*-related regulatory proteins were generally associated with cognate genes. Interestingly, both clusters were located in regions with atypical genome signatures, suggesting that they originated in horizontal transfer events.

Besides the *czcCBA1*, *czcCBA2* and *cusCBA* operons, three more gene clusters of the *P. putida* genome (*tetR–mtrC1–czcA3*, *mfpII–mfpI–czcA4* and *tetR–mtrC2–czcA5*) may also encode chemiosmotic antiporter efflux systems. This is because their CzcA components belong to the RND family and have a considerable similarity to the CzcA protein (41% for CzcA3, 42% for CzcA4 and 39% for CzcA5) of *R. metallidurans*. The *mfpII mfpI* genes, the *mtrC1* gene and the *mtrC2* gene bear HlyD domains, which describe proteins with the N-terminal module anchored to the inner membrane (RND membrane fusion protein; Fig. 2A). The BLAST best hit for both CzcA3 and

CzcA4 is the CzcA protein of *R. metallidurans*, and the BLAST best hit for CzcA5 is the nodulation protein NolG of *Sinorhizobium meliloti*. However, in the absence of experimental data, we cannot rule out the possibility that these gene clusters are also (or instead) involved in drug resistance or other cellular processes. The metal resistance genes of the RND family share a strong similarity with drug efflux systems. Upstream of *mtrC1–czcA3* and *mtrC2–czcA5*, transcriptional regulators belonging to the TetR family were found (Fig. 2A), which, to the best of our knowledge, have no known counterparts in other systems for metal tolerance.

In an effort to identify (or at least to propose) the metal specificity pattern of each chemiosmotic antiporter system, two phylogenetic trees were constructed with the different proteins of the RND family (Fig. 2C) or their corresponding RND-associated membrane fusion proteins (Fig. 2B) involved in metal and drug resistance in *P. putida* and other microorganisms. $CzcA1_{Ppu}$ and $CzcA2_{Ppu}$ were phylogenetically closest to the heavy metal resistance determinants $CzcA_{Rme}$ (conferring tolerance to Cd, Zn and Co) and $CzrA_{Pae}$ (conferring tolerance to Cd and Zn). Thus, $CzcA1_{Ppu}$ and $CzcA2_{Ppu}$ could mediate resistance to these divalent cations. $CusA_{Ppu}$ also clustered together with the heavy metal resistance genes, but was phylogenetically closer to *E. coli* CusA, which has been related to silver (Franke *et al*., 2001) and copper (Outten *et al*., 2001a) resistances. In addition, $cusCBA_{Ppu}$ is located in the Ag/Cu cluster, suggesting that it could mediate the extrusion of both silver and copper. CzcA3, CzcA4 and CzcA5 clustered together, and they are nearly as far from the heavy metal resistance genes as from the drug resistance genes. The phylogenetic tree derived from the amino acid sequence of the RND-associated membrane fusion proteins shows a similar clustering (Fig. 2B). Thus, this type of analysis could not distinguish whether CzcA3, CzcA4 and CzcA5 are involved in heavy metal or drug resistance.

Regulatory proteins

As bacterial genome size increases, the number of regulatory genes and the complexity of regulatory networks also seems to increase (Stover *et al*., 2000). At least 19 proteins appear to be capable of mediating regulation of metal homeostasis in *P. putida*. These regulatory proteins were found spread throughout the genome. Although most of the regulators map adjacent to the genes that they regulate, others appear to be isolated in the chromosome.

Two *arsR* genes with a high degree of similarity (79%) to one another appeared as a result of the duplication of the arsenic resistance genes, discussed above. Both copies contain the arsenic-binding motif CVC overlapping a helix–turn–helix domain involved in binding to the DNA. In *E. coli*, $arsR_{Eco}$ is a transcriptional repressor. Curiously, $arsR_{Eco}$ contains a third Cys residue involved in the binding of arsenic, which is not required for the activation of transcription (Shi *et al*., 1996).

$CadR_{Ppu}$ and $CueR_{Ppu}$ belong to the MerR family of transcriptional regulators (Lee *et al*., 2001; Stoyanov *et al*., 2001). In *P. putida* 06909, CadR responds to Cd and represses its own expression, but is not required for the regulation of $CadA_{Ppu06909}$. However, $CadA_{Ppu06909}$ is induced by Cd, Zn and Pb (Lee *et al*., 2001). Other putative regulators (for example, *czcRS*; see below) that could respond to divalent cations might act *in trans* to provide further regulation of the $PcadA_{Ppu}$ promoter. In *E. coli*, CueR is required for the transcriptional activation of *copA* (a Cu-exporting ATPase; see above). $CueR_{Eco}$ responds to Cu and Ag ions (Stoyanov *et al*., 2001).

In addition to $CueR_{Ppu}$, $CopZ_{Ppu}$ is also found adjacent to a Cu P-type ATPase. In *Enterococcus hirae*, $CopZ_{Ehi}$ is a transcriptional activator. When Cu is present in the medium, it is bound to $CopZ_{Ehi}$, which transfers copper ions to the aporepressor $CopY_{Ehi}$, releasing $CopY_{Ehi}$ from the promoter DNA (Strausak and Solioz, 1997; Cobine *et al*., 1999; 2002). Although a CopZ-like protein is present in *P. putida* KT2440, BLAST searches did not reveal a $CopY_{Ehi}$-like protein. DNA-binding domains could not be found in $CopZ_{Ppu}$, which would indicate a role as a transcriptional regulator. Two possibilities are that there is either a different protein with the same activity as CopY or a different mechanism for the regulation of transcription by $CopZ_{Ppu}$ in *P. putida*.

Regarding iron/zinc uptake regulation, two proteins were found belonging to the Fur family. The first protein, *zur*, is probably involved in the regulation of Zn uptake. Zur is located within the *znuACB1* genes. The stop codon of *zur* overlaps with the ATG of *znuC1*, so there is no intergenic region. Thus, *zur* is probably co-transcribed with *znuCB1*. In *E. coli*, zur_{Eco} is located away from the $znuABC_{Eco}$ genes, and acts as a repressor when it binds Zn (Patzer and Hantke, 2000). Recent reports have shown that Zur_{Eco} binds two atoms of Zn per monomer of polypeptide, being the most sensitive metalloprotein known to date (Outten *et al*., 2001b). The second Fur-like ORF showed a high degree of similarity to the Fur proteins of other pseudomonads (98% *P. putida* WCS358 and 94% *P. aeruginosa*), and it is almost certainly involved in the regulation of iron uptake. The iron regulons of pseudomonads involve not only the Fur proteins but also dedicated sigma factors, extra activators and perhaps some small regulatory RNAs. This issue is also related to metal homeostasis and has been reviewed recently (Cornelis and Matthijs, 2002).

nikR is the transcriptional regulator of the *nik* operon. It belongs to the ribbon–helix–helix (beta–alpha–alpha) family of transcriptional factors (Chivers and Sauer, 1999).

In *E. coli*, when Ni is in excess, $NikR_{Eco}$ represses the transcription of the uptake system (Chivers and Sauer, 2000).

Several ORFs of *P. putida* (*czcRS1*, *czcR3*, *czcRS2*, *copRS1* and *copRS2*) showed a high degree of similarity to heavy metal regulators belonging to the two-component regulatory systems. *czcRS1*, *czcR3* and *copRS1* were found in the large heavy metal clusters shown in Fig. 2A. The other two systems (*czcRS2* and *copRS2*) were found at other loci in the genome (Fig. 1). This type of regulatory system consists of a sensing protein responsible for the phosphorylation and subsequent activation of the regulatory component. The latter is involved in the binding of DNA and activation of transcription (Stock *et al*., 2000). Interestingly, one *czcR* protein was not found to have its corresponding sensing component. As described above, *P. putida* seems to have several *czc* detoxification genes. A CzcS-sensing protein might detect the presence of heavy metals and amplify the signal transduction pathway by phosphorylating two different *czcR* products. Another possibility is that *czcR3* is the result of a partial duplication of a regulatory system that it is not active in *P. putida*.

Other proteins located in the metal clusters

A BLAST search against the genome of *P. putida* indicated the absence of *mer* homologues in this bacterium (including Hg reductase, transporter and organomercurial lyase).

An ORF of 377 amino acids (PP5392) was found in the copper/silver cluster that showed a significant similarity to a protein antigen containing tandem repeats of the Archaeum *Methanosarcina mazei* (Mayerhofer *et al*., 1995) and to the NirF protein of *P. aeruginosa* (39% similarity in a segment of 292 amino acids; Kawasaki *et al*., 1997). In *P. aeruginosa*, NirF is required for the biosynthesis of the haem group of nitrite reductase, and the gene is located in the *nir* operon. It could also have some dehydrogenase activity (Kawasaki *et al*., 1997). The role of ORF PP5392 in copper homeostasis is unclear. Other genes found in the copper cluster (*czcN*, PP5389; and *cusX*, PP5388) included ORFs homologous to *czcN* and *cusX* (Fig. 2A). $CzcN_{Pput}$ (219 amino acids) showed very low similarity to CzcN and NccN from *R. metallidurans* and *Alcaligenes xylosoxidans* (49% in a segment of 92 amino acids and 50% in a segment of 91 amino acids respectively) and to the yeast O-methyl transferase MAM4 (48% in a segment of 79 amino acids). The hydropathy profile suggests that it is a transmembrane protein. $CusX_{Ppu}$ (113 amino acids) displayed low similarity to CusX/YlcC from *E. coli* (59% in a segment of 68 amino acids). No role has been proposed for this protein so far (Franke *et al*., 2001).

In the second large heavy metal cluster (*czcCBA–cadA*), a *czcD*-like ORF (called CzcD, PP0026) and several ORFs (PorD, PP0046 and various small ORFs) with potential roles in heavy metal detoxification were also found (Fig. 2A and D). CzcD of *R. metallidurans* might be a membrane-spanning protein involved in heavy metal sensing. $CzcD_{Rme}$ belongs to the CDF family (cation diffuser family) and confers partial resistance to cobalt, zinc and cadmium by reducing the accumulation of cations. Besides, it seems to repress the transcription of $czcCBA_{Rme}$ (Anton *et al*., 1999). PorD is a porin that allows basic amino acids to cross the membrane (Siehnel *et al*., 1990; Huang *et al*., 1992). Several PorD homologues were found throughout the *P. putida* genome. PorD was placed in the heavy metal resistance determinant, which might be involved in the translocation of heavy metal cations across the outer membrane. The other small ORFs found within the *czc* cluster displayed no significant similarities to proteins in the databases (Fig. 2).

Concluding remarks

The availability of the genomic sequence of *P. putida* allowed many molecular mechanisms leading to metal(loid) homeostasis in this soil bacterium to be inferred. The most striking finding was the detection of duplicated systems. One example is the case of the chemiosmotic antiporter efflux systems, which include metal and drug detoxification systems. Two of these systems (*czcCBA1* and *czcCBA2*) could be involved in the detoxification of different divalent metals and/or display overlapping specificities, as is the case in *R. metallidurans* (*czc* for Cd, Zn and Co; and *ncc* for Co and Ni resistance). Further, the *cusCBA* operon (which is also a chemiosmotic antiporter efflux system) located in the copper/silver cluster is probably involved in the detoxification of monovalent cations. However, the difference between *R. metallidurans* and *P. putida* is that the *czcCBA* determinants are found in two megaplasmids in the former, whereas in the latter they are located in the chromosome. Three more gene clusters of this type (CzcA3, CzcA4 and CzcA5), with an unclear role, were also found in the genome. Two of these resistance determinants found in *P. putida* (*czcCBA1* and *cusCBA*) are located in regions with atypical genome signatures. In addition to the chemiosmotic antiporter efflux systems, two *cadA* homologues were found in the genome that could mediate further metal resistance. CadA2 was already reported to be involved in Cd resistance (Lee *et al*., 2001).

Two highly homologous *ars* operons were found in different locations in the genome. The *ars* determinants are very specific for arsenic and antimony (Silver, 1996). Therefore, it is likely that they have the same function in the cell. It is interesting that most of the heavy metal

resistance genes, with the exception of the *ars* operons and *chrA*, are located around the ORI region (Fig. 1). This segment of DNA constitutes a region with a high expression rate. Could it be that *P. putida* has upgraded the expression level by duplicating the gene content of the arsenic resistance operons? In fact, the presence of two very similar operons suggests that each may be preferentially active over different ranges of exposure to the metalloid. It is not clear whether *ars1* resulted in horizontal gene transfer, as discussed above.

These enticing hypotheses need to be tested experimentally. Further work needs to be carried out to identify the real substrates and inducers of the systems described in this paper. Inducers need not necessarily be the substrates for transporters. This implies that studying the metal interactions at the genomic level must include an extensive mutagenic analysis of all the systems involved if the specific substrates are to be determined. Surveying the regulation of all these genes by transcription profiling with DNA microarrays and proteomic analyses would also be quite informative.

Pseudomonas putida has been a pivotal instrument for studies on the bioremediation of soils contaminated with xenobiotic compounds. The findings of the present study suggest that *P. putida* is also adapted to thrive in environments with metal(loid) contamination. Owing to its easy laboratory manipulation, its innate robustness in the environment and the fact that tools for its genetic manipulation are already available, this strain may have also considerable potential as an agent for environmental decontamination of metals.

Experimental procedures

The complete sequence of the genome of *P. putida* KT2440 is available at the TIGR and NCBI (http://www.tigr.org and the http://www.ncbi.nlm.nih.gov accession number AE015451) websites as described by Nelson *et al*. (2002). For the identification of proteins, the BLAST program (Altschul *et al*., 1990) was used with a database containing the annotated sequence downloaded from the NCBI website in a dedicated server in our laboratory. BLAST searches were conducted with functionally characterized proteins of other microorganisms. ORFs identified in the *P. putida* genome were BLAST searched back against the SWISSPROT/TrEMBL database. Only functionally characterized proteins in the database were taken into account for the assignment of functions to the proteins encoded by genes in the *P. putida* genome. When discrepancies were found in the predicted ORFs, the DNA sequences translated in all six reading frames were studied. Hydropathy profiles were generated with the Kyte–Doolittle algorithm (Kyte and Doolittle, 1982). Alignments and phylogenetic analyses were performed with CLUSTALW 1.8 software (available at http://www.ebi.ac.uk/FTP/index.html). Trees were bootstrapped 1000 times to ensure the reliability of each branch point. The trees were drawn using NJPLOT software (available at http://www.ebi.ac.uk/FTP/index.html).

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