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Heterologous expression of bacterial and human multidrug resistance proteins protect *Escherichia coli* against mercury and zinc contamination

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Abstract

In order to determine the role of multidrug resistance proteins in mercury and zinc resistance, human *MDR1*, *Lactococcus lactis lmrA*, and *Oenococcus oeni omrA* genes were expressed in an *Escherichia coli tolC* mutant which is hypersensitive to metals. The three transporters conferred an increased mercury and zinc resistance to *E. coli* as compared to the control bacteria. This improved resistance correlated with a decreased zinc and mercury bioaccumulation. Indeed, quantification of intracellular metal concentrations by atomic absorption spectrometry (AAS) showed a 2.1-, 3-, and 5.1-fold decrease in zinc in cells expressing *hMDR1*, *omrA*, and *lmrA*, respectively, and a 2.7-, 7.5-, and 7.7-fold decrease in mercury in cells expressing *omrA*, *lmrA*, and *hMDR1*, respectively, as compared to the control bacteria. This means that hMDR1, LmrA, and OmrA proteins which are specialised in xenobiotic scavenging, their main known function, are nevertheless able to confer some resistance against metals. Our results show that the *tolC* mutated strain is well adapted to the study of MDR transporter activity and could be used to screen substrates and competitive hMDR1 inhibitors.

Abbreviations: AAS – atomic absorption spectroscopy; ABC transporter – ATP binding cassette transporter; Cd – cadmium; Hg – mercury; LB medium – Luria Bertani broth medium; GSH – glutathione; MDR – multidrug resistance; MIC – minimal inhibitory concentration; OD_{660} – optical density at 660 nm; Zn – zinc

Introduction

Multidrug resistance (MDR), a major obstacle to the effective chemotherapy of many human malignancies, is commonly associated with overexpression of a membrane glycoprotein called P-glycoprotein (P-gp). The human P-gp, hMDR1, is a 170 kDa plasma membrane protein that actively extrudes a wide variety of structurally unrelated compounds from the cell. MDR-type proteins belong to the ATP-binding cassette (ABC) transporter superfamily and function as ATP-dependent efflux pumps that prevent intracellular cytotoxic drug accumulation (Ambudkar *et al.* 1999).

Experimental evidence indicates that MDR-like proteins may be directly involved in cellular extrusion of metals in addition to their role as xenobiotic extruders. Indeed, the multidrug resistance-associated protein (MRP1), the *Leishmania* LtpgpA transporter, and the yeast cadmium resistance factor 1 (YCF1) are three genuine xenobiotic pumps also involved in heavy metal detoxification. LtpgpA pumps out arsenite and antimony (Dey *et al.* 1996). The YCF1 transporter catalyses the pumping of bis(glutathionato)

cadmium (Li *et al*. 1997) and the human MRP1 protein functionally complements the YCF1 factor (Tommasini *et al*. 1996). This means that a xenobiotic pump can also be a metal pump, provided that the metal ion forms a complex with glutathione. Moreover, *MRP1* is overexpressed in human heavy-metal-selected tumour cells (Vernhet *et al*. 1999), while *MDR1* is induced by cadmium and arsenite ions (Chin *et al*. 1990). The nematode *Caenorhabditis elegans* is hypersensitive to heavy metals when both the MRP homologue and a member of the P-gp gene family are deleted (Broeks *et al*. 1996). In the Asiatic clam *Corbicula fluminea*, we recently detected high concentrations of a P-gp homologue in the gills of bivalves living in a cadmium-contaminated environment. This protein was present in increased levels after chronic contamination by cadmium, zinc, mercury, copper, and uranium (Achard *et al*. 2004; Tran *et al*. 2005). Similar observations have been made in the mussel *Mytilus californianus* when exposed to arsenite and cadmium (Eufemia & Epel 2000).

Using *Escherichia coli* as a cellular tool, we have recently analysed the role of MDR-like proteins in metal resistance (Achard-Joris *et al*. 2005). Three transporters were tested: the human hMDR1 and two bacterial ABC-type multidrug extrusion systems, LmrA and OmrA. LmrA is an ABC transporter of *Lactoccocus lactis* (van Veen *et al*. 1996) that is functionally and structurally related to the human MDR1 (van Veen *et al*. 1998). OmrA is an LmrA homologue of the wine bacteria *Oenococcus oeni*. OmrA and LmrA conferred protection to bacteria grown on high salt medium. They also triggered bacterial resistance to sodium laurate, wine and ethanol toxicity (Bourdineaud *et al*. 2004). We have shown that both the human and bacterial MDR genes conferred cadmium resistance to *E. coli* up to 0.4 mM. Protection was abolished by 100 μM verapamil. Quantification of intracellular cadmium concentration showed a reduced cadmium accumulation in cells expressing the MDR genes; inside-out membrane vesicles of *L. lactis* overexpressing *lmrA* displayed an ATP-dependent ^{109}Cd(II) uptake that was stimulated by glutathione (Achard-Joris *et al*. 2005).

Since a bis(glutathionato)-divalent metal complex may constitute an MDR-like protein substrate, it should be possible to use it as a competitive inhibitor of chemotherapeutic-drug pumping. For such a strategy it would be best to use the weakest of the harmful metallic species, for instance made up with zinc. We then decided to test whether MDR transporters could efficiently scavenge zinc compared to a much more toxic metal such as mercury. We here show that human and bacterial MDR genes could confer resistance against both mercury and zinc.

Materials and methods

Bacterial strain, growth conditions and plasmid constructions

The bacterial strain used was *E. coli* CS1562 ($F^{-}\lambda^{-}$ *tolC6*::Tn10 *his leu proA argT his thi galK lacY trpE nonmtl xyl ara rpsL sup*$^{+}$) (Austin *et al*. 1990). It was grown at 37 °C (or 28 °C when transformed with pSF and pSF-MDR) in LB medium (5 g l^{-1} yeast extract, 10 g l^{-1} bactotryptone, 10 g l^{-1} NaCl) supplemented with the appropriate antibiotic (ampicillin, kanamycin, or chloramphenicol).

The human gene (*hMDR1*) and two bacterial homologues, *lmrA* and *omrA*, of *L. lactis* (van Veen *et al*. 1998) and *O. oeni* (Bourdineaud *et al*. 2004), respectively, were expressed in *E. coli* strain CS1562 which is hypersensitive to drugs (Austin *et al*. 1990) and cadmium (Achard-Joris *et al*. 2005), due to a deficiency in the TolC transporter. Indeed, the 50% growth inhibitory cadmium concentration (IC_{50}) was shifted from 1.1 to 0.35 mM in *tolC*$^{+}$ and *tolC*$^{-}$ strains (Achard-Joris *et al*. 2005). As shown in Figure 1, the *tolC* mutant is also sensitive to zinc and mercury. The zinc IC_{50} value is shifted from 1.1 to 0.9 mM in *tolC*$^{+}$ and *tolC*$^{-}$ strains (panel A), and this of mercury is shifted from 13 to 11.5 μM in *tolC*$^{+}$ and *tolC*$^{-}$ strains (panel B).

Plasmids used are pSF and pSF-MDR which contains the *hMDR1* cDNA, both plasmids possessing an ampicillin resistance gene (Baum *et al*. 1995), pGK13 and pGKLmrA containing a chloramphenicol resistance gene (van Veen *et al*. 1996), and pCR-XL-TOPO (Invitrogen) and pJPB66 (pCR-XL-TOPO-based plasmid harbouring the *O. oeni omrA* gene) which contain a kanamycin resistance gene (Bourdineaud *et al*. 2004).

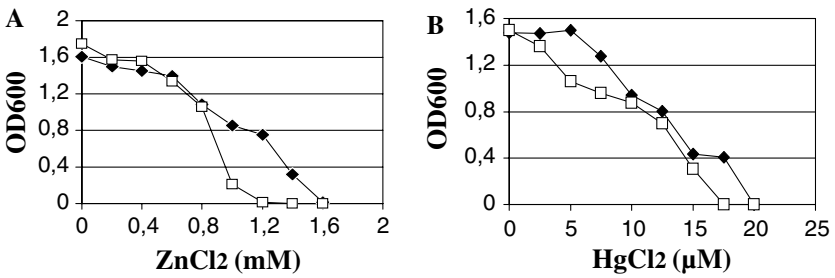

Figure 1. Zinc and mercury sensitivity of the *tolC* mutant. Overnight cultures of *E. coli tolC*$^-$ (open squares) and *tolC*$^+$ (JM109) (filled diamonds) were diluted to an OD_{600} of 0.01 into LB medium containing various concentrations of zinc (A) or mercury (B). Cell growth was monitored as the absorbance at 600 nm after 17 h of incubation at 37 °C. Measurements were done in triplicate. Two other *tolC*$^+$ strains (W3110, and BL21(DE3)) were tested and gave similar results for both panels A and B.

Metal IC_{50} determinations and choice of applied metal concentrations

IC_{50} of zinc and mercury were first determined in order to precise the useful metal concentrations to study a possible effect of hMDR1, LmrA, and OmrA transporters against metal contaminations. Overnight cultures of *E. coli* CS1562 (*tolC*$^-$) transformed with control or gene transporter-expressing plasmids were diluted to an OD_{660} of 0.06 into LB medium containing various concentrations of mercury (ranging from 0 to 30 μM) or zinc (ranging from 0 to 2 mM). Cell growth was monitored as the absorbance at 600 nm after 12 h of incubation at 37 °C (28 °C when bacteria were transformed with pSF or pSF-MDR). Measurements were done in triplicate. The IC_{50} were then inferred from the curves giving the OD_{600} *versus* metal concentrations as the metal concentrations corresponding to the bacterial mid-growth. From the values obtained in liquid media (Table 1), the chosen working metal concentrations were closed to or equal to the corresponding IC_{50} values. The chosen zinc concentrations were 0.65, 0.6, and 1 mM to study OmrA, hMDR1, and LmrA, respectively, and the chosen mercury concentrations were 9, 11.5, and 14 μM to study OmrA, hMDR1, and LmrA, respectively. On agar media the above selected concentrations were increased to take into account the binding of part of divalent metal ions on agar polysaccharides, and the volatility of mercury when added into hot melted agar media.

The above chosen concentrations near the IC_{50} values were pertinent since metal concentrations tested below these values resulted in hardly visible differential growths. For instance when zinc concentrations were chosen 20–30% below the IC_{50} values, only slight differential growths were observed (Figure 2, second row from left).

Resistance assays on agar media

Overnight cultures with the appropriate antibiotic were adjusted to an OD_{600} of 2. Next, serial 10-fold dilutions were performed and 5 μl of each dilution was spotted on the agar medium with or without mercury or zinc ($ZnCl_2$ and $HgCl_2$ were purchased from Merck). The plates were incubated overnight at 28 or 37 °C and photographed.

Bacterial survival quantification

Overnight cultures were diluted into fresh LB medium (1% v/v) containing the appropriate antibiotic, and grown up to an OD_{600} of 0.1. Each culture was divided into three: a control and two

Table 1. Zinc and mercury IC_{50} displayed by *E. coli tolC* mutant transformed with ABC transporter-encoding plasmids.

Transforming plasmid	Zinc IC_{50} (mM)	Mercury IC_{50} (μM)
pCR-XL-TOPO	0.55 ± 0.02	9 ± 0.4
pJPB66 (OmrA)	0.65 ± 0.03*	11.5 ± 0.5*
pSF	0.45 ± 0.02	11.5 ± 0.5
pSF-MDR1	0.6 ± 0.03*	13 ± 0.5*
pGK13	0.9 ± 0.04	12.5 ± 0.5
pGKLmrA	1.25 ± 0.05*	16 ± 0.7*

Asterisks are indicating significant differences between IC_{50} of control and MDR transporter-expressing bacteria. Significant differences between IC_{50} values were determined with the nonparametric Mann–Whitney *U* test (Statistica 5.1, StatSoft). Results were considered statistically significant at $P < 0.05$.

cultures containing either zinc or mercury at the indicated concentrations. Cultures were further incubated for 3 h at 37 °C (or 28 °C for *hMDR*-transformed bacteria and corresponding control). Bacterial survival was determined as follows: after 3 h, 100 μl of each culture was taken, serial 10-fold dilutions were plated on LB solid medium and colonies were counted after an overnight incubation. Survival ratios were calculated as: colony forming units (cfu) observed in presence of the metal to those observed without toxic compound.

Bioaccumulated metal quantification

Cell associated zinc levels were determined as follows: after 3 h of incubation, cells from 30 ml cultures were collected by centrifugation (15 min, 6000 g, 6 °C) and washed with a buffer containing 10 mM Tris–Cl pH 7.4, and 0.15 M NaCl. Pellets were destroyed by nitric acid [3 ml of pure HNO_3 – 65% (v/v)] in a pressurised medium (borosilicate glass tubes) at 100 °C for 3 h. Samples were diluted with 18 ml of ultrapure water (MilliQ plus), and zinc concentrations were determined by flame atomic absorption spectrometry (AAS) (Varian SpectrAA 220 FS). The detection limit was 5 μg of Zn l^{-1}.

Total mercury determination was carried out by flameless AAS directly on bacterial samples (Leco Ama 254). The detection limit was 0.01 nanograms of Hg. Results were expressed in nanograms of Zn or Hg per 10^9 bacteria.

The validity of the analytical method was checked periodically by means of two biological reference materials (Tort-2: Lobster hepatopancreas; Dolt-2: Dogfish liver – NRC-CNRC, Ottawa, Canada) (Andrès *et al*. 1999).

Results

hMDR1, LmrA and OmrA proteins are conferring mercury and zinc resistance to E. coli

When transformed with the transporter-encoding plasmids, all the IC_{50} showed slight but significant increased values as compared to control bacteria (Table 1). The difference in *in vivo* metal ion susceptibility between *E. coli* cells harbouring *hMDR1*- or *lmrA*- or *omrA*-containing plasmids and bacterial cells harbouring a control plasmid was then studied on solid media. *hMDR1, omrA, and lmrA* expression resulted in an increased resistance to both zinc and mercury chloride which was manifested by colony formation at the highest dilution relative to control in the presence of 0.6, 0.65, and 1.25 mM $ZnCl_2$, respectively, and in the presence of 14.5, 13, and 19.5 μM $HgCl_2$, respectively, (Figure 2). Verapamil is a well-known inhibitor of MDR-type transporters. It has been shown that verapamil alone exerted no influence on bacterial growth at 100 μM concentration (Tisa *et al*. 2000). In the presence of verapamil all resistance against $ZnCl_2$ and $HgCl_2$ was abolished for the three transporters, as exemplified by the case of LmrA (Figure 3).

hMDR1, lmrA, and omrA expression results in a lowered metal concentrations in bacteria

Atomic absorption spectrometry was used to quantify Zn and Hg levels associated with Zn- and Hg-exposed *E. coli* cells that were transformed with either *omrA*-, *hMDR1*-, *lmrA*-containing plasmids or a control plasmid.

When the culture medium contained 0.6 to 1 mM of $ZnCl_2$, a marked decrease in cell-associated zinc was observed in cells expressing *hMDR1*, *lmrA*, or *omrA* as compared to the control (Figure 4B, D, F). A 3-, 2.1-, and 5.1-fold decrease in cell-associated zinc was observed in cells expressing *omrA*, *hMDR1*, or *lmrA*, respectively. This reduction in cell-associated zinc correlated with the 14, 19, and 21% increases in survival observed in the cells expressing *omrA*, *hMDR1*, and *lmrA*, respectively (Figure 4A, C, E).

When the culture medium contained micromolar amounts of $HgCl_2$, a marked decrease in cell-associated Hg was observed in cells expressing *omrA*, *hMDR1*, or *lmrA*, as compared to the control (Figure 5B, D, F). A 2.7-, 7.7-, and 7.5-fold decrease in cell-associated mercury was observed in cells expressing *omrA*, *hMDR1*, or *lmrA*, respectively. This reduction in cell-associated mercury correlated with the 21, 46, and 51% increases in survival observed in the cells expressing *omrA*, *hMDR1*, or *lmrA*, respectively (Figure 5A, C, E). Some differences in bacterial mercury and zinc accumulations were observed among the control cells (bacteria transformed with empty vectors, Figures 4 and 5). However, these differences likely reflect differences in growth

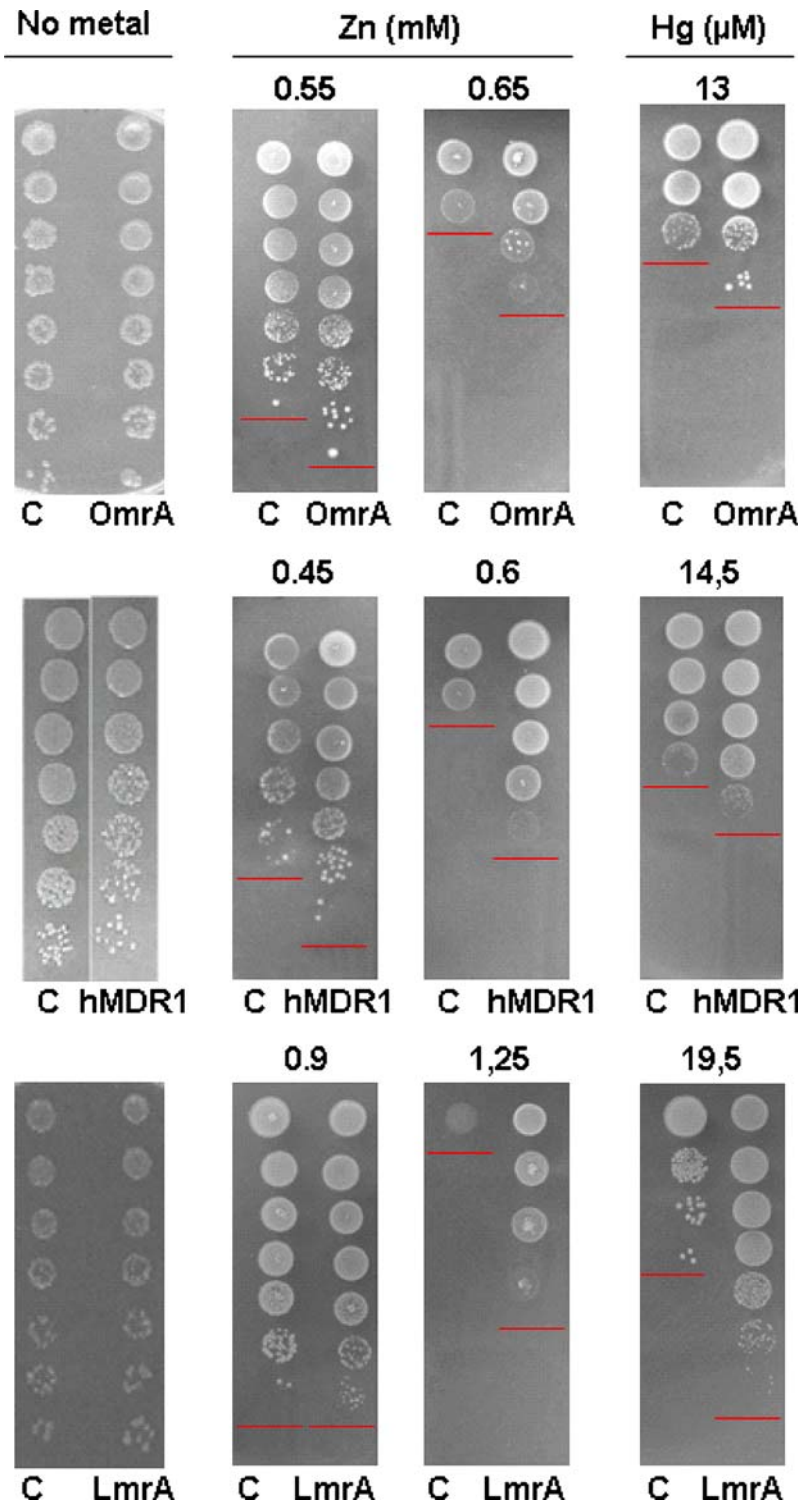

Figure 2. Heterologously expressed ABC MDR transporters in *E. coli* confer protection against zinc and mercury chloride toxicity. The *E. coli* CS1562 *tolC* mutant was transformed with pJPB66 (*omrA*), pSF-MDR (*hMDR1*), and pGKLmrA (*lmrA*), and the relevant control plasmids (C) pCR-XL-TOPO, pSF, and pGK13, respectively. The media were containing the indicated concentration of $ZnCl_2$ or $HgCl_2$. The bars are indicating the highest dilution giving rise to bacterial growth.

temperature (in the case of pSF transformed bacteria) or plasmid copy number (high for pCR-XL-TOPO and low for pGK13).

Discussion

Although we used *E. coli* as a cell reactor within which express heterologous MDR transporters and did not intend to study the natural resistance of this bacterium to metals, our results nevertheless show that besides ZntA, a P-type ATPase (Sharma *et al.* 2000), and ZitB, a member of the cation diffusion facilitator (CDF) family (Grass *et al.* 2001), TolC is also involved in metal resistance. TolC spans the entire periplasmic space and the outer membrane, and the opening of the entrance of TolC tunnel is triggered when TolC recruits substrate-loaded inner membrane ATPase or H^+-antiporter complex such as AcrB. This type of molecular system connecting an outer membrane protein with the periplasmic face of an inner membrane protein belonging to the resistance-nodulation-cell division (RND) protein family is known to intervene in the export of metallic cations in many prokaryotes (Nies 2003). The substrate binding domains of hMDR1 has been shown to occur at interfaces formed by transmembrane (TM) segments 3 and 11 on one hand and TM segments 5 and 8 on the other hand. Noteworthy, this substrate binding at domain interfaces is shared by LmrA and AcrB (Pleban *et al.* 2005). This last remark may help to explain the common efficiency of xenobiotic scavenging mediated by these transporters in *E. coli*.

Resistance assays on liquid and solid media clearly demonstrate that the human *MDR1*, and the bacterial *lmrA* and *omrA* genes confer resistance to mercury and zinc chloride when heterologously expressed in *E. coli tolC* mutant. The same transporters also are displaying resistance toward cadmium chloride (Achard-Joris *et al.* 2005) and none of them could offer resistance to copper sulfate (data not shown), so that there is apparently no differential metal specificity among them. Verapamil, a competitive inhibitor of MDR protein, is abolishing metal resistance conferred by the three transporters, and this metal resistance is exactly correlated with the decrease of cell-associated metal, making it likely that MDR transporters protect bacteria through metal pumping as was demonstrated for cadmium in the case of LmrA (Achard-Joris *et al.* 2005). These data are in keeping with the observations that rat *mdr1* overexpression protects kidney cells against cadmium-mediated apoptosis (Thévenod *et al.* 2000) and that the nematode *C. elegans* becomes hypersensitive to heavy metals when both the MRP homologue and a member of the MDR gene family are deleted (Broeks *et al.* 1996). Although

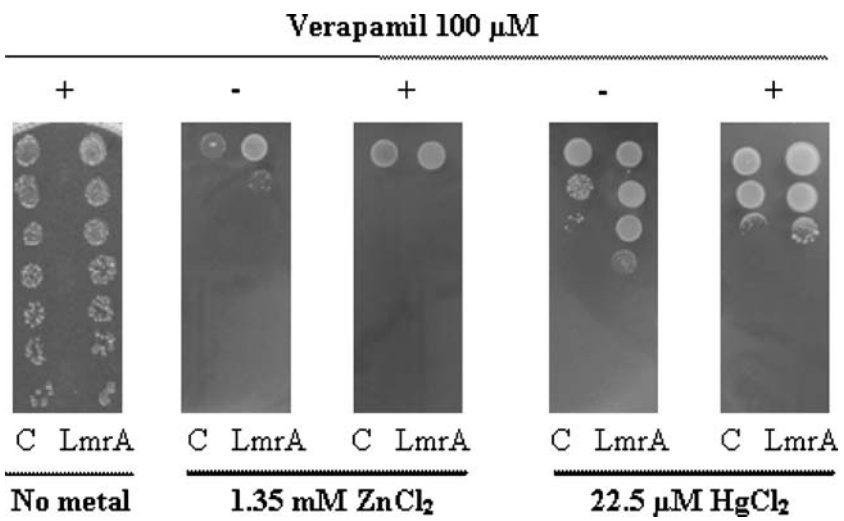

Figure 3. Verapamil abolishes the bacterial protection conferred by *lmrA* expression against zinc and mercury chloride. The *E. coli* CS1562 *tolC* mutant was transformed with pGKLmrA (*lmrA*), and the relevant control plasmids pGK13 (C). The media were containing the indicated concentration of $ZnCl_2$ or $HgCl_2$. Where indicated 100 μM verapamil was added to the media.

metal detoxification is likely not the primary function of MDR proteins, they nevertheless can explain metal resistance of eukaryotic organisms by adding their contribution to that of CDF family transporter such as ZnT-1 (Palmiter & Findley 1995). Our results raise the possibility to circumvent the failure of chemotherapy employed for the treatment of cancer by using an organic zinc species as a competitive inhibitor of chemotherapeutic-drug pumping. Such a compound could be generated in the cell by formation of bis(glutathionato)-zinc from zinc and glutathione, and the combined use of cadmium to saturate metallothioneins as already described in a cancer prevention case (Waalkes *et al*. 1991).

One may say that the zinc and mercury resistance conferred by MDR transporters would have been much more easily observed in a *zntA*::Km and *zntA*::Km Δ*zitB* mutated strains, since ZntA and ZitB are detoxifying *E. coli* by pumping

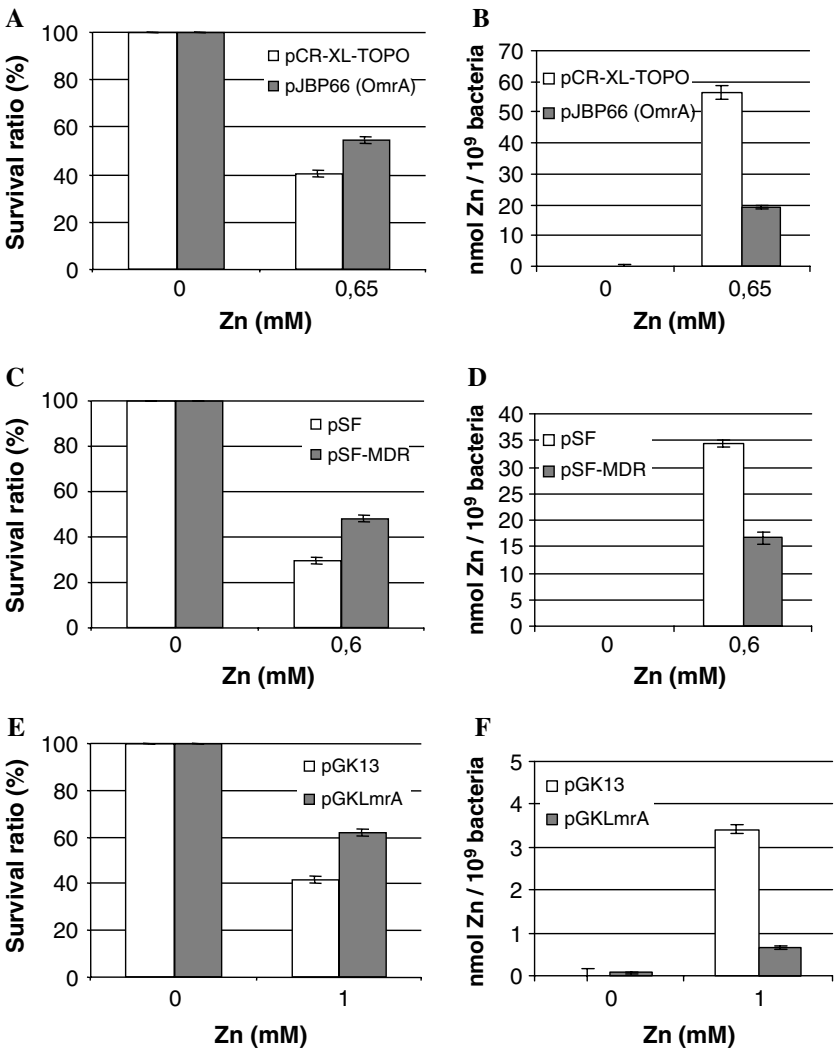

Figure 4. Heterologously expressed ABC MDR transporters in *E. coli tolC* mutant reduce the cell associated level of zinc chloride. *E. coli* CS1562 (*tolC* mutant) was transformed with control plasmids and *lmrA*-, *omrA*-, and *hMDR1*-containing plasmids. After 3 h of incubation with the indicated amount of zinc chloride, the bacterial cultures were either serially diluted and plated, or used for metal quantification. Colonies were counted and survival ratios were calculated as the colony forming units observed at a given concentration of zinc to those observed without added toxic compound (A, C, E). Cell-associated metal concentrations were determined by atomic absorption spectrometry and results were expressed in nanomoles of Zn per 10^9 bacteria (B, D, F). The number of bacteria per digested pellet was determined by cell numeration.

divalent Pb, Cd, and Zn in the case of ZntA, and specifically divalent Zn in the case of ZitB (Sharma *et al*. 2000; Grass *et al*. 2001). However, when we tested the mutated strains RW3110 *zntA*::Km and GG48 *zntA*::Km Δ*zitB*::Cm (Grass *et al*. 2001), we could not observe any protective effect of MDR transporters. We also noted that the MDR protein mediated metal resistance (cadmium, zinc, mercury) was only evident in a *tolC* mutated strain. The same held true even for the ethidium bromide resistance conferred by MDR transporters, and this has previously been discussed (Achard-Joris *et al*. 2005). A plausible explanation for the reason why the absence of TolC is necessary to observe an effect of MDR transporters against Hg and Zn might lay in the respective affinities of ZntA, TolC/AcrAB, and MDR transporters for metals. ZntA is a high affinity system for Zn(II) and Cd(II) since the reported K_m for ZntA ATPase activity are 5.2 μM for Zn and 3.8 μM for Cd (Hou *et al*. 2001). CzcA is a member of the RND family related to AcrB and is part of the CzcCBA system

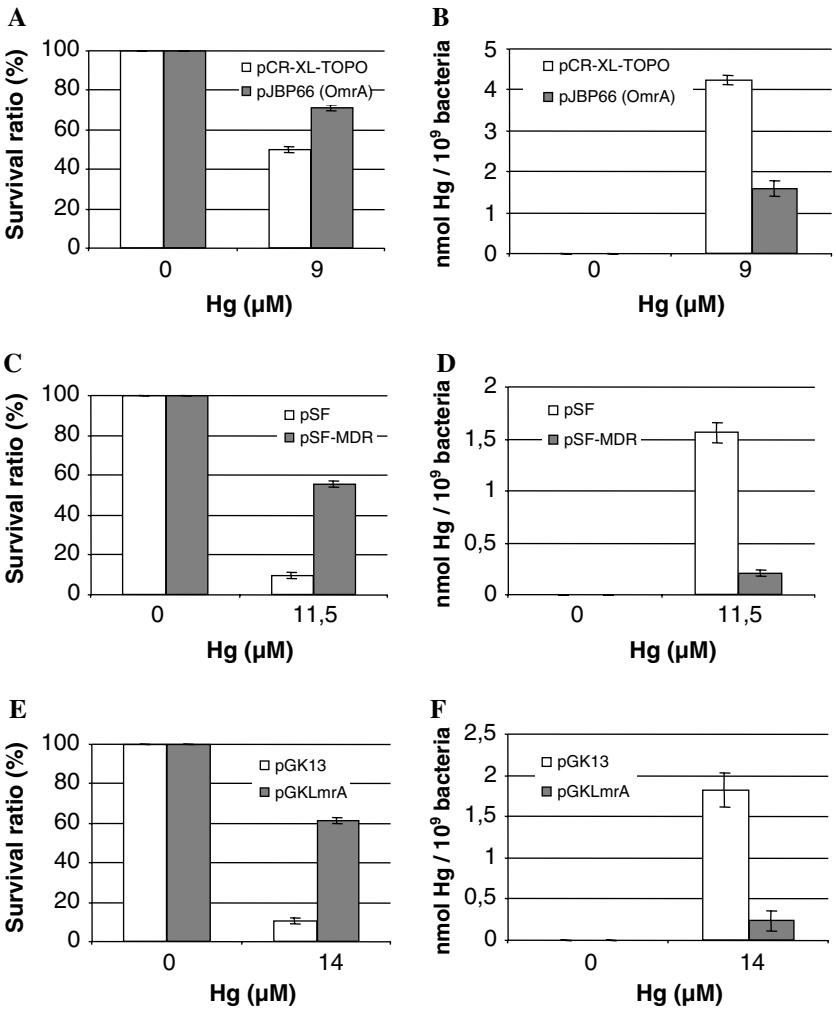

Figure 5. Heterologously expressed ABC MDR transporters in *E. coli tolC* mutant reduce the cell associated level of mercury chloride. *E. coli* CS1562 (*tolC* mutant) was transformed with control plasmids and *lmrA*-, *omrA*-, and *hMDR1*-containing plasmids. After 3 h of incubation with the indicated amount of mercury chloride, the bacterial cultures were either serially diluted and plated, or used for metal quantification. Colonies were counted and survival ratios were calculated as the colony forming units observed at a given concentration of mercury chloride to those observed without added toxic compound (A, C, E). Cell-associated metal concentrations were determined by atomic absorption spectrometry and results were expressed in nanomoles of Hg per 10^9 bacteria (B, D, F). The number of bacteria per digested pellet was determined by cell numeration.

which is efficiently decreasing cytoplasmic Cd and Zn concentrations in *Cupriavidus metallidurans* (formerly *Ralstonia metallidurans*). And yet, the K_m of CzcA for Zn and Cd is 6.6 and 7.7 mM, respectively, although CzcA transport was performed without the other proteins of the transport-complex CzcB and CzcC (Goldberg *et al.* 1999). Thus, compared to ZntA, CzcCBA is a low affinity system for metals. Most probably, the K_m of TolC/AcrAB and MDR transporters for metals are in the range or higher of this of CzCBA, because these systems, unlike ZntA and CzcCBA, are not primarily dedicated to metal scavenging but rather to organic xenobiotic pumping. Therefore, at high levels of metals, such as those we are using in this study, ZntA is functioning at V_{max}, so that its scavenging capacities are overwhelmed. In a *tolC* mutant, the function of the MDR transporters can then be unmasked at high metal concentration. However, in a Δ*zntA* Δ*zitB* strain, it is impossible to reach metal concentrations near the K_m of MDR transporters for these metals because this doubly mutated strain, or even the single Δ*zntA* mutant, is hypersensitive to metals and die before. Therefore, the *tolC* mutated strain is well adapted to the study of MDR transporters and could be used to screen competitive or dead-end hMDR1 inhibitors.

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