

## Heterologously Expressed Bacterial and Human Multidrug Resistance Proteins Confer Cadmium Resistance to *Escherichia coli*<sup>†</sup>

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**ABSTRACT:** The human *MDR1* gene is induced by cadmium exposure although no resistance to this metal is observed in human cells overexpressing *hMDR1*. To access the role of MDR proteins in cadmium resistance, human *MDR1*, *Lactococcus lactis* *lmrA*, and *Oenococcus oeni* *omrA* were expressed in an *Escherichia coli* *tolC* mutant strain which proved to be hypersensitive to cadmium. Both the human and bacterial MDR genes conferred cadmium resistance to *E. coli* up to 0.4 mM concentration. Protection was abolished by 100  $\mu$ M verapamil. Quantification of intracellular cadmium concentration by atomic absorption spectrometry showed a reduced cadmium accumulation in cells expressing the MDR genes. Inside-out membrane vesicles of *L. lactis* overexpressing *lmrA* displayed an ATP-dependent <sup>109</sup>Cd<sup>2+</sup> uptake that was stimulated by glutathione. An evolutionary model is discussed in which MDR proteins have evolved independently from an ancestor protein displaying both organic xenobiotic- and divalent metal-extrusion abilities.

Multidrug resistance (MDR) is one of the major causes of failure of chemotherapy employed for the treatment of cancer (1). Among others, MDR is caused by the overexpression of a 170 kDa membrane protein called MDR1 or P-glycoprotein (P-gp). MDR1 is a member of the ATP binding cassette (ABC) superfamily of transporters, whose functions include the ATP-dependent extrusion of amphiphilic compounds out of the cell (1). In addition to MDR1, other ABC transporters<sup>1</sup> have been implicated in MDR in mammalian cells such as the multidrug resistance associated protein, MRP1 (2, 3).

Like cancer cells, many organisms have the ability to develop resistance to a variety of toxic compounds through ATP-dependent extrusion mechanisms. Indeed, MDR also occurs in lower eukaryotes and bacteria. For instance, MDR1-like proteins have been identified in a variety of aquatic organisms (4) where they can account for the protection against a broad variety of toxic compounds such as xenobiotics and naturally occurring toxins, as well as endogenous toxic compounds. Such transporters can be relatively specific for a given substrate range or can handle a wide variety of structurally unrelated compounds. Among the xenobiotics, heavy metals pose a serious threat to the

environment. Several ABC transporters such as MRP1, *Leishmania* LtpgpA, and the yeast cadmium resistance factor 1 (YCF1) may take part in metal resistance. Indeed, these genuine xenobiotic pumps are also involved in heavy-metal detoxification: LtpgpA pumps out arsenite and antimony (5), YCF1 pumps out arsenite, and human MRP1 and YCF1 catalyze the extrusion of bis(glutathionato)cadmium conjugates (6, 7).

In our laboratory, we focus on the problem of long-term pollution in aquatic species by heavy metals such as cadmium and mercury. In particular, we study resistance mechanisms against metal contamination displayed by a biological model, the Asiatic clam *Corbicula fluminea*. In this bivalve, a gene encodes a multixenobiotic resistance protein (MXR) that is homologous to the mammalian P-gp. The MXR protein level was considerably elevated in the tissues of bivalves living in a cadmium-contaminated environment (8). Similar observations have been made in the mussel *Mytilus californianus* exposed to arsenite and cadmium (9). Moreover, *MRP1* is overexpressed in human heavy-metal-selected tumor cells (10), while *MDR1* is induced by cadmium and arsenite ions although no resistance to cadmium ions has been observed in cells overexpressing P-gp (11). These different examples suggest that ABC transporters of the MDR group are induced in the presence of metal contaminants. This raises the question as to whether these proteins are directly involved in cellular extrusion of metals in addition to their role as xenobiotic extruders.

Herein we have analyzed the role of MDR-like proteins in metallic resistance. Three transporters were tested: the human *hMDR1* and two bacterial ABC-type multidrug extrusion systems, *LmrA* and *OmrA*. *LmrA* is an ABC transporter of *Lactococcus lactis* (12) that is functionally and

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<sup>1</sup> Abbreviations: ABC transporter, ATP binding cassette transporter; Cd<sup>2+</sup>, cadmium; <sup>109</sup>Cd<sup>2+</sup>, radioactive cadmium; LB medium, Luria-Bertani broth medium; GSH, glutathione; AMP-PNP, adenosine 5'-[ $\beta$ , $\gamma$ -imino]triphosphate; OD<sub>660</sub>, optical density at 660 nm; MIC, minimal inhibitory concentration.

structurally related to the human MDR1 (13). It uses the free energy of ATP hydrolysis to drive the export of toxic compounds from the inner leaflet of the cytoplasmic membrane (14). Homologues of LmrA have been detected in other lactic acid bacteria, like the *Oenococcus oeni* *omrA* gene (15). OmrA confers protection to bacteria grown on high-salt medium. It also triggered bacterial resistance to sodium laurate, wine, and ethanol toxicity.

To test their ability to provide resistance to toxic metals, the proteins were expressed in *Escherichia coli*. All three systems provided cadmium resistance to *E. coli*. Transport assays with inside-out membrane vesicles isolated from *L. lactis* suggest an LmrA-mediated cadmium extrusion activity.

## EXPERIMENTAL PROCEDURES

**Reagents.** Cadmium chloride ( $\text{CdCl}_2$ ) was purchased from Merck; reduced glutathione, adenosine 5'-[ $\beta,\gamma$ -imino]triphosphate (AMP-PNP), nisin, and verapamil were from Sigma.  $^{109}\text{Cd}^{2+}$  was purchased from Amersham (Pharmacia, Amersham).

**Bacterial Strains, Growth Conditions, and Plasmid Constructions.** Bacterial strains used were *E. coli* CS1562 (F- $\lambda$ -*tolC6::Tn10 his leu proA argT his thi galK lacY trpE non mtl xyl ara rpsL sup<sup>+</sup>) (16), *E. coli* JM109 (e14<sup>-</sup>(McrA<sup>-</sup>) *recA1 endA1 gyrA96 thi-1 hsdR17* ( $r_K^-$   $m_K^+$ ) *supE44 relA1*  $\Delta$ (*lac-proAB*) [*F'* *traD36 proAB lacI<sup>n</sup>  $\Delta$ AM15*]), *E. coli* W3110 (F- $\lambda$ -*thyA36*), *E. coli* BL21(DE3) (B F<sup>-</sup> *dcm ompT hsdS*( $r_B^-$   $m_B^-$ ) *gal  $\lambda$*  (DE3)), and *L. lactis* NZ9000 ( $\Delta$ *lmrA*, MG1363 derivative, *pepN::nisRK*) (17). *E. coli* was grown at 37 °C (or 28 °C when transformed with pSF or pSF-MDR) in LB medium (5 g·L<sup>-1</sup> yeast extract, 10 g·L<sup>-1</sup> bactotryptone, 10 g·L<sup>-1</sup> NaCl) supplemented with the appropriate antibiotic (ampicillin, kanamycin, or chloramphenicol). *L. lactis* was grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose and 5  $\mu\text{g}\cdot\text{mL}^{-1}$  chloramphenicol.*

The human gene (*hMDR1*) and two bacterial homologues, *lmrA* and *omrA*, of *L. lactis* (12) and *O. oeni* (15), respectively, were expressed in *E. coli* strains JM109, W3110, and BL21 and in strain CS1562, which is hypersensitive to drugs (16) and cadmium (this work, Figure 1B) due to a deficiency in the TolC protein.

Plasmids used are pSF and pSF-MDR containing an ampicillin resistance gene (18), pGK13 and pGKLmrA containing a chloramphenicol resistance gene (12), and pCR-XL-TOPO (Invitrogen) and pJPB66 (pCR-XL-TOPO) which harbors the *O. oeni omrA* gene containing a kanamycin resistance gene (15).

For the isolation of inside-out membrane vesicles, *L. lactis* NZ9000 was transformed with either pNZ8048 (control) or pNHLmrA (overexpressing *lmrA*).

**Resistance Assays on Agar Media.** Overnight cultures with the appropriate antibiotic were adjusted to an OD<sub>660</sub> of 2. Then, serial 10-fold dilutions were performed, and 5  $\mu\text{L}$  of each dilution was spotted on the agar medium with or without cadmium, verapamil, or cadmium and verapamil. The plates were incubated overnight at 28 or 37 °C and photographed.

**Bacterial Survival and Metal Quantification.** Overnight cultures were diluted into fresh LB medium (1% v/v) containing the appropriate antibiotic and grown up to an OD<sub>660</sub> of 0.1. Each culture was divided in three: a control and two cultures containing 0.25 and 0.4 mM  $\text{CdCl}_2$ ,

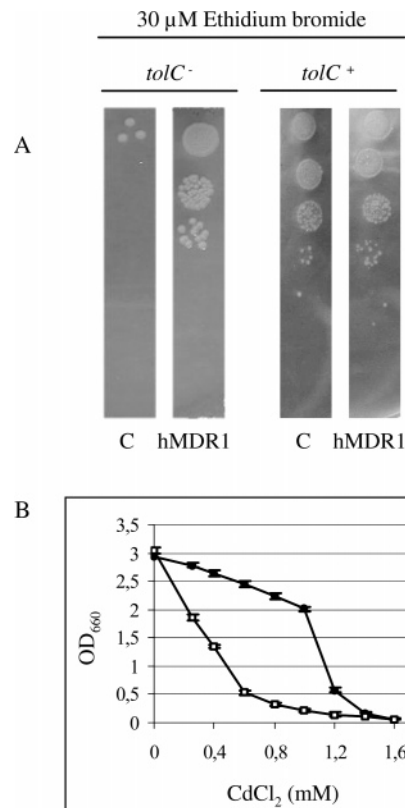


FIGURE 1: *E. coli tolC* mutant: a suitable strain to study xenobiotic and metal resistance conferred by expression of MDR transporters. (A) Effect of TolC on the ethidium bromide resistance introduced by expression of the hMDR1 transporter. *E. coli* CS1562 (*tolC*<sup>-</sup>) and JM109 (*tolC*<sup>+</sup>) were transformed with plasmids pSF (C) or pSF-MDR (hMDR1). An equivalent growth pattern was observed between control and hMDR1-expressing bacteria when the medium was devoid of ethidium bromide. (B) TolC is involved in cadmium resistance. Overnight cultures of *E. coli* CS1562 (*tolC*<sup>-</sup>) (open squares) and JM109 (*tolC*<sup>+</sup>) (filled circles) were diluted to an OD<sub>660</sub> of 0.06 into LB medium containing various concentrations of cadmium. Cell growth was monitored as the absorbance at 660 nm after 12 h of incubation at 37 °C. Measurements were done in triplicate. Several other *tolC*<sup>+</sup> strains [W3110 and BL21(DE3)] were tested with similar results for both panels A and B.

respectively. Cultures were further incubated for 3 h at 37 °C (or 28 °C for *hMDR*-transformed bacteria and the corresponding control). Bacterial survival was determined as follows: after 3 h, 100  $\mu\text{L}$  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 the colony-forming units (cfu) observed in the presence of the metal to that observed without toxic compound. Cell-associated cadmium levels were determined as follows: after 3 h of incubation, cells from 30 mL of culture were collected by centrifugation (15 min, 6000g, 6 °C) and washed with a buffer containing 10 mM Tris·HCl, pH 7.4, and 0.15 mM NaCl. Pellets were destructed by nitric acid [3 mL of pure  $\text{HNO}_3$ , 65% (v/v)] in a pressurized medium (borosilicate glass tubes) at 100 °C for 3 h. Samples were diluted with 18 mL of ultrapure water (MilliQ plus), and cadmium concentrations were determined by atomic absorption spectrometry with Zeeman correction, using a graphite tube atomizer (AAS, Thermoptec M6Solaar). The detection limit was 0.1  $\mu\text{g}$  of  $\text{Cd}\cdot\text{L}^{-1}$  ( $3 \times$  standard deviation of the reagent blanks). Results were expressed in nanograms of  $\text{Cd}^{2+}/10^6$  bacteria.

**Isolation of Inside-Out Membrane Vesicles from *L. lactis*.** Inside-out membrane vesicles were isolated from *L. lactis* NZ9000 ( $\Delta lmrA$ ) cells harboring either pNZ8048 or pNHLmrA. Cells were grown at 30 °C to an OD<sub>660</sub> of about 0.7, whereupon *lmrA* expression was induced for 1 h by addition of 0.5 ng·mL<sup>-1</sup> nisin (Sigma). Cells were cooled on ice for 30 min and harvested by centrifugation (6000g for 8 min) at 4 °C. All subsequent steps were carried out at the same temperature. Cells were washed twice in 1 L of ice-cold 100 mM HEPES–KOH, pH 7.4. Pellets were resuspended in 42 mL of the same buffer. *N*-Ethylmaleimide (NEM; Sigma), MgSO<sub>4</sub>, DNase I (Sigma), and lysozyme were added to a concentration of 1 mM, 10 mM, 0.1 mg·mL<sup>-1</sup>, and 10 mg·mL<sup>-1</sup>, respectively. The cell suspension was incubated for 45 min at 30 °C, cooled on ice, and lysed by two successive passages through a French press (20000 psi; Basic Z Model Cell Disrupter, Constant Cell Disruption Systems). The lysed cells were diluted to a volume of 70 mL with ice-cold HEPES–KOH, pH 7.4, and 1 mM NEM. Unbroken cells and debris were removed by centrifugations (13000g for 45 min followed by 25000g for 30 min, 4 °C), whereupon the membranes were collected from the supernatant by high-speed centrifugation (125000g for 1 h, 4 °C). Pellets were resuspended in 1.5 volumes of a buffer containing 50 mM HEPES–KOH, pH 7.4, 10% glycerol, and 1 mM NEM, frozen in small aliquots in liquid nitrogen, and stored at –80 °C.

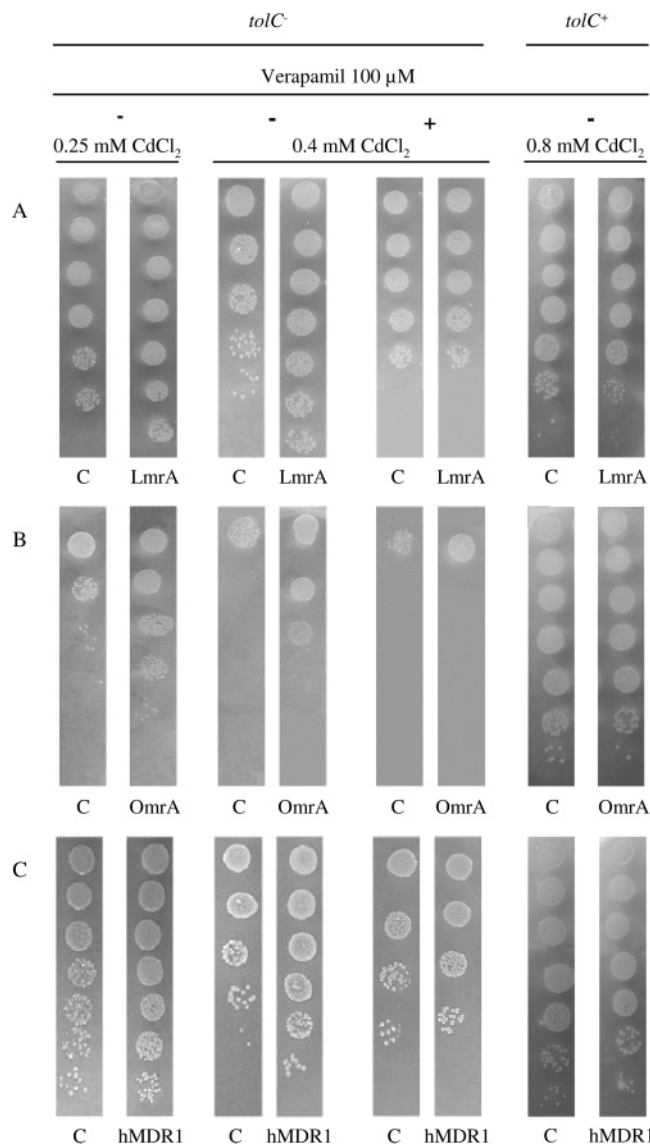
Protein was assayed according to the method of Lowry (DC Protein Assay, Bio-Rad), using bovine serum albumin as a standard.

**Transport Assays with <sup>109</sup>Cd<sup>2+</sup>.** Membrane vesicles were added to 1.5 mL of 50 mM HEPES–KOH, pH 7.4, 2 mM MgSO<sub>4</sub>, 8.5 mM NaCl, 5 mM phosphocreatine, 0.1 mg/mL creatine kinase, and 50  $\mu$ M <sup>109</sup>Cd<sup>2+</sup> (9.08  $\mu$ Ci· $\mu$ mol<sup>-1</sup> specific activity) in a 5 mL plastic tube to a final concentration of 0.5 mg of protein/mL. After 1 min incubation at 30 °C, transport was initiated by the addition of 2 mM ATP. When indicated, 650  $\mu$ M reduced glutathione and/or 2 mM AMP-PNP instead of ATP were (was) included in the reaction mixture. The transport was monitored over 14 min, and at each time point, 200  $\mu$ L of the reaction mixture was passed over cellulose nitrate filters (0.2  $\mu$ m pore size; BA 83 Schleicher and Schuell) using a vacuum manifold (Trivac B; Leybold AG, Koln, Germany) under a pressure of 400 millibar. Filters were washed twice with 2 mL of ice-cold 0.1 M LiCl and assayed for radioactivity using a liquid scintillation analyzer, Tri-carb 2000 CA.

Uptake rates were determined by means of a linear regression model. In the statistical analysis, a probability of  $P < 0.05$  was considered significant.

## RESULTS

***hMDR1*, *LmrA*, and *OmrA* Proteins Protect Bacteria against Metal Stress.** The difference in in vivo metal ion susceptibility between *E. coli* cells harboring the *hMDR1*-, *lmrA*-, or *omrA*-containing plasmids and bacterial cells harboring a control plasmid was first studied on solid media. *hMDR1*, *lmrA*, and *omrA* expression in a *tolC*<sup>-</sup> background resulted in an increased resistance to cadmium chloride which was manifested by the colony formation at the highest dilution in the presence of 0.4 mM CdCl<sub>2</sub> relative to control.



**FIGURE 2:** Heterologously expressed ABC MDR transporters in *E. coli* confer protection against cadmium toxicity. The *E. coli* CS1562 (*tolC*<sup>-</sup>) and JM109 (*tolC*<sup>+</sup>) strains were transformed with *omrA*-, *hMDR1*-, and *lmrA*-harboring plasmids and the relevant control plasmids. (A) pGK13 (C) or pGKLmrA (LmrA). (B) pCR-XL-TOPO (C) or pJPB66 (OmrA). (C) pSF (C) or pSF-MDR (hMDR1). An equivalent growth pattern was observed between control bacteria and transporter-expressing bacteria when the medium was devoid of CdCl<sub>2</sub>, while verapamil (100  $\mu$ M) alone exerted no influence on bacterial growth. Several other *tolC*<sup>+</sup> strains [W3110 and BL21(DE3)] were tested and gave similar results.

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  $\mu$ M concentration (19). In the presence of verapamil all resistance was abolished (Figure 2), suggesting a MDR-dependent resistance mechanism. This resistance phenotype against Cd<sup>2+</sup> conferred by these ABC transporters could only be observed in a *tolC*<sup>-</sup> background. In a *tolC*<sup>+</sup> strain, which resists higher levels of Cd<sup>2+</sup> concentrations than a *tolC* mutant (Figure 1B), expression of the ABC transporters did not afford an additional Cd<sup>2+</sup> resistance (Figure 2).

***hMDR1*, *lmrA*, and *omrA* Expression Results in a Lowered Metal Concentration in Bacteria.** Atomic absorption spectrometry was used to quantify the cadmium level associated with Cd-exposed *E. coli tolC*<sup>-</sup> cells that were transformed



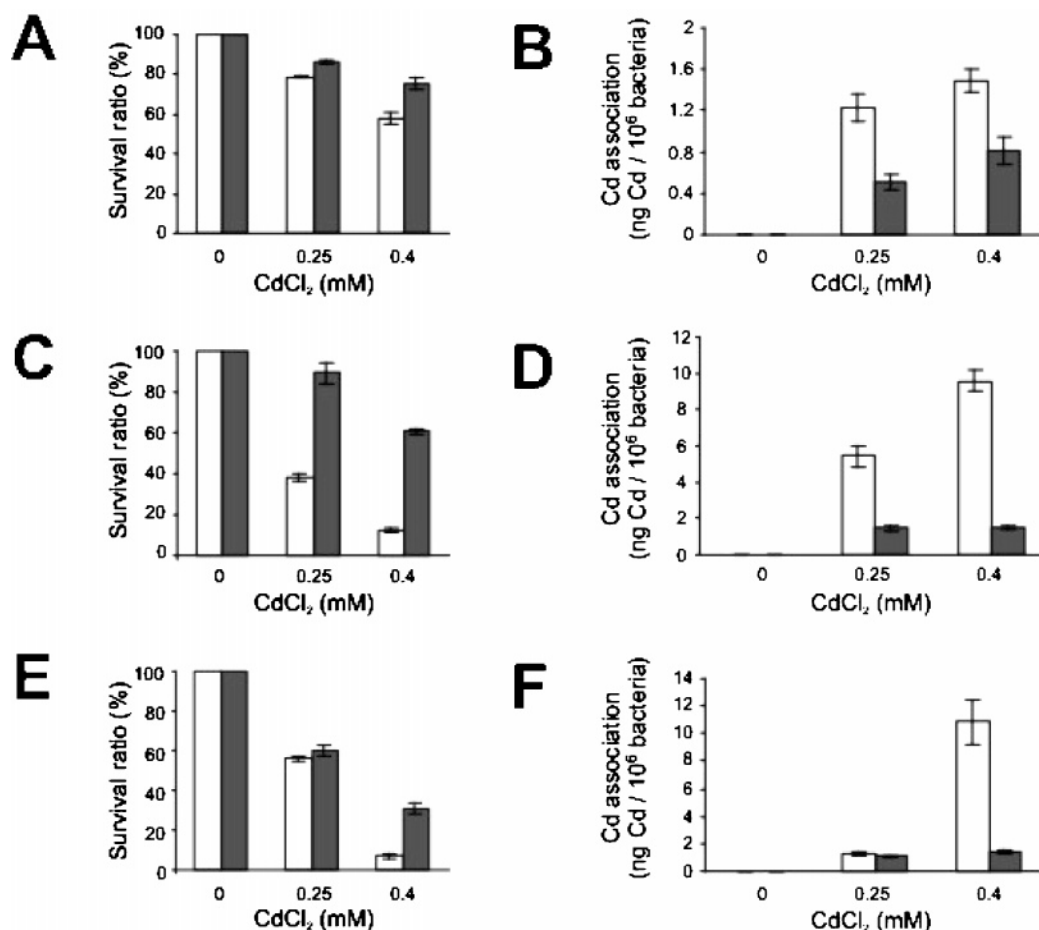


FIGURE 3: Heterologously expressed ABC MDR transporters in the *E. coli tolC* mutant reduce the cell-associated level of cadmium. *E. coli* CS1562 (*tolC* mutant) was transformed with control plasmids and *lmrA*-, *omrA*-, and *hMDR1*-containing plasmids. After 3 h of incubation with the indicated amount of cadmium, the bacterial cultures were either serially diluted and plated or used for metal quantification. Colonies were counted, and the survival ratios were calculated as the colony-forming units observed at a given concentration of cadmium to that observed without added toxic compound (A, C, E). Cell-associated cadmium concentrations were determined by atomic absorption spectrometry, and results were expressed in ng of Cd<sup>2+</sup>/10<sup>6</sup> bacteria (B, D, F). The number of bacteria per digested pellet was determined by cell enumeration. (A, B) Bacteria were transformed with either pGK13 or pGKLmrA. (C, D) Bacteria were transformed with either pCR-XL-TOPO or pJPB66 (containing the *omrA* gene). (E, F) Bacteria were transformed with either pSF or pSF-MDR. White bars stand for survival ratio or Cd<sup>2+</sup> associated with control bacteria and black ones for survival ratio or Cd<sup>2+</sup> associated with bacteria expressing one of the ABC transporters.

with either *hMDR1*-, *lmrA*-, *omrA*-containing plasmids or a control plasmid. When the culture medium contained 0.25 or 0.4 mM CdCl<sub>2</sub>, a marked decrease in cell-associated Cd<sup>2+</sup> was observed in cells expressing *hMDR1*, *lmrA*, or *omrA* as compared to the control (Figure 3A,C,E). At 0.4 mM CdCl<sub>2</sub>, an 8-, 2-, and 6-fold decrease in cell-associated Cd<sup>2+</sup> was observed with cells expressing *hMDR1*, *lmrA*, and *omrA*, respectively. This reduction in cell-associated cadmium correlated with the 4.4-, 1.3-, and 5-fold increases in survival observed in the cells expressing *hMDR1*, *lmrA*, and *omrA*, respectively (Figure 3B,D,F). Some differences in cadmium accumulation were observed among the control cells (bacteria transformed with empty vectors, Figure 3). However, these differences likely reflect differences in growth temperature (in the case of pSF transformed bacteria) or plasmid copy number (high for pCR-XL-TOPO and low for pGK13). These data suggest that MDR1, LmrA, and OmrA provide cadmium resistance to cells through metal pumping.

**LmrA-Mediated Cadmium Transport.** The increased resistance of cells to cadmium upon expression of MDR transporters is most likely due to an increased cellular extrusion of the cadmium. To establish a direct biochemical

demonstration of the ability of the MDR transporter to extrude cadmium, radioactive cadmium transport experiments were conducted with inside-out vesicles derived from *L. lactis* cells expressing LmrA. The cadmium concentration used in these experiments was set to 50  $\mu$ M to facilitate the detection of the transport activity. This concentration is in the range of xenobiotic concentrations used in LmrA mechanistic studies (20). Control membrane vesicles were prepared from *L. lactis* NZ9000 ( $\Delta$ *lmrA*) cells harboring the control plasmid pNZ8048. Before the addition of ATP, nonspecific cadmium adsorption was observed with the membrane vesicles. This behavior is characteristic of divalent metal/membrane interaction, since we could also observe Cd<sup>2+</sup> binding on dead or alive *E. coli* cells within seconds using a cadmium-specific electrode (data not shown).

<sup>109</sup>Cd<sup>2+</sup> transport was initiated by the addition of 2 mM ATP. LmrA-containing inside-out vesicles accumulated more cadmium than the control membranes (Figure 4A). This difference was calculated to be  $30 \pm 7$  pmol of Cd $\cdot$ min<sup>-1</sup>·(mg of protein)<sup>-1</sup> ( $P < 0.05$ ). To test if cadmium accumulation is dependent on the hydrolysis of ATP, the same experiment was performed with the nonhydrolyzable ATP analogue,

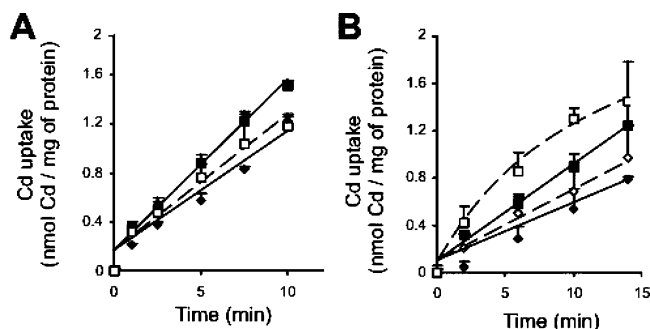


FIGURE 4: (A) LmrA-mediated ATP-dependent  $^{109}\text{Cd}^{2+}$  uptake in inside-out membrane vesicles. The uptake of  $^{109}\text{Cd}^{2+}$  by inside-out vesicles derived from cells that overexpressed LmrA (squares) or that were devoid of LmrA (diamonds) was measured in the presence of ATP (filled square/diamond, solid line) or AMP-PNP (open square, dotted line). Values shown are the means with the indicated standard deviation ( $n = 3$ ).  $^{109}\text{Cd}^{2+}$  was added at time  $-1$ , and reactions were started at time 0 by the addition of ATP or AMP-PNP. At time 0, the cadmium level was set to 0 to avoid background related to a nonspecific binding of cadmium to the membrane vesicles. (B) LmrA-catalyzed uptake of  $^{109}\text{Cd}^{2+}$  is stimulated by glutathione. The uptake of  $^{109}\text{Cd}^{2+}$  by inside-out vesicles derived from cells that overexpressed LmrA (filled and open squares) or control vesicles that were devoid of LmrA (filled and open diamonds) was measured in the presence of ATP in the presence (open symbols, thick line) or absence (filled symbols, thin line) of GSH. Values shown are means  $\pm$  standard deviation ( $n = 3$ ).  $^{109}\text{Cd}^{2+}$  and GSH were added 1 min before starting the reaction by the addition of ATP. At time 0, the cadmium level was set to 0 to avoid background related to a nonspecific binding of cadmium to the membrane vesicles. The transport activity was defined as the amount of cadmium taken up during the first 2 min.

AMP-PNP. Again, the difference of  $^{109}\text{Cd}^{2+}$  uptake kinetics by LmrA-containing vesicles with ATP compared to AMP-PNP was  $30 \pm 10$  pmol of  $\text{Cd} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$  ( $P < 0.05$ ). Moreover, no significant difference of  $\text{Cd}^{2+}$  uptake kinetics was observed between the LmrA-containing vesicles in the presence of AMP-PNP as compared to the control membranes with ATP. Taken together, these data suggest that LmrA mediates a low transport activity of  $\text{Cd}^{2+}$ .

The transport assays were carried out in the absence of glutathione, cysteine, or other molecules that may form a thiolate complex with  $\text{Cd}^{2+}$ . Nevertheless, the assay contains ATP to drive the LmrA function. ATP may also function as a cadmium ion vehicle as ABC transporters such as CFTR or hMDR1 have been shown to pump  $\text{Mg-ATP}$  (21, 22). YCF1-catalyzed cadmium extrusion is dependent on glutathione (GSH) and more precisely on the bis(glutathionato)-cadmium complex (6). Therefore, we addressed the possible influence of GSH on LmrA-mediated cadmium transport.  $^{109}\text{Cd}^{2+}$  transport by LmrA containing inside-out vesicles was measured in the presence of ATP with or without  $625 \mu\text{M}$  GSH and compared with control vesicles (Figure 4B). In the absence and presence of glutathione, the difference in transport activity between the LmrA and control membranes was  $28 \pm 7$  and  $124 \pm 42$  pmol of  $\text{Cd} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ , respectively ( $P < 0.05$ ). This demonstrates that glutathione stimulates the LmrA-mediated cadmium transport activity by more than 4-fold.

## DISCUSSION

Here we report on the ability of the heterologous expression of human *MDR1* and the bacterial *lmrA* and *omrA* genes

to confer resistance in *E. coli* to cadmium. This study reveals a novel function of these proteins and provides a mechanistic explanation for the observation that rat *mdr1* overexpression protects kidney cells against cadmium-mediated apoptosis (23). Surprisingly, overexpression of human P-gp in renal carcinoma cells does not result in an increased resistance to cadmium (11). However, in these cells the activity of P-gp might have been masked by the activity of MRP proteins. Indeed, the nematode *Caenorhabditis elegans* becomes hypersensitive to heavy metals when both the MRP homologue and a member of the P-gp gene family are deleted (24).

We noted that the MDR protein mediated cadmium resistance was only evident in an *E. coli* strain lacking the outer membrane protein TolC. Loss of TolC is known to increase sensitivity to detergents and drugs, but it was unknown that a *tolC* mutation also results in an increased  $\text{Cd}^{2+}$  sensitivity. It has been suggested that residual metal resistance in a strain disrupted in both *zntA*, a P-type zinc ATPase, and *zitB*, a zinc/ $\text{H}^+$  antiporter of the major facilitator superfamily, is caused by additional factors or systems (25). Our studies suggest that one of these factors is TolC (and possibly associated inner membrane transporters such as AcrAB) since the MIC for  $\text{Cd}^{2+}$  was shifted from 1.1 to 0.35 mM in *tolC*<sup>+</sup> and *tolC*<sup>−</sup> strains, respectively. Alternatively, the pleiotropic effects displayed by the *tolC* mutant could explain its increased sensitivity. Resistance to  $\text{Cd}^{2+}$  and to ethidium bromide conferred by MDR transporters was only observed in a *tolC*<sup>−</sup> genetic background (Figure 1A). This loss of a detectable MDR function in the *tolC*<sup>+</sup> strains suggests that TolC impedes or masks the ability of these heterologously expressed MDR proteins to resist Cd. The latter may be due to the presence of functional and efficient endogenous cadmium scavenging systems in the TolC-containing strain.

The present observed cadmium resistance associated with the three exogenous ABC protein expressions is unlikely the result of an interaction between these proteins and endogenous *E. coli* metal transporters such as the P-type ATPase ZntA (26). First, the observed bacterial resistance is abolished by verapamil, while verapamil is not a known inhibitor of P-type ATPase. Furthermore, verapamil inhibition of ZntA would lead to a lack of growth at high  $\text{Cd}^{2+}$  concentrations [MIC 0.03 mM (27)] whereas growth in the presence of verapamil was observed at 0.4 mM  $\text{CdCl}_2$ . In addition, at the concentration used, verapamil exerted no effect on the colony formation of the control *E. coli* strains on solid media, demonstrating that the observed reversal of metal resistance is not due to inhibition of an endogenous *E. coli* system but rather to the inhibition of LmrA, OmrA, and hMDR1. Second, the LmrA activity is independent from accessory proteins (20), and murine and human MDR1 genes have previously been expressed functionally in *E. coli* without requirement of an endogenous system or factor (28, 29).

Bacterial resistance to cadmium effected by LmrA, OmrA, and hMDR1 is also unlikely to be due to a physicochemical modification of the inner membrane which may in turn decrease the efficiency of the divalent metal ion uptake system ZupT (30). First, such an influence on ZupT activity would have a strong impact on bacterial systems requiring an optimal supply in  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  and thus would affect

cell activity or growth. We found no difference between the growth curves of bacteria expressing or devoid of heterologously expressed MDR-like transporters (data not shown). Second, it has already been shown that the expression of the murine *MDR1* gene in *E. coli* is not accompanied by a dissipation of the protonmotive force as lactose transport by LacY remained fully functional (28). Also, *hMDR1* does not affect the levels of the protonmotive force when expressed in mammalian cells (31). Finally, the cellular ATP levels, as measured by a luminescent assay, were not affected in the transformed bacteria (data not shown), which shows that the energy status of the cell is not significantly influenced. Third, contrary to previous adopted strategies to express the human and murine *MDR1* genes in *E. coli* (28, 29), we did not use a strong *E. coli* promoter to drive the expression of *lmrA*-, *omrA*-, and *hMDR1* genes in order to avoid membrane perturbation by high-level protein expression. Finally, the sodium dodecyl sulfate sensitivity of MDR-expressing bacteria was similar to that of control cells, indicating no major alterations of the cell envelope and membrane (data not shown).

Quantification of the cadmium level associated with cadmium-exposed *tolC*<sup>-</sup> bacteria (Figure 3) showed that cells expressing one of the three transporters contained 2 to 8 times less cadmium than control cells. This strongly suggests that the ABC proteins extrude Cd–glutathione complexes and, in the absence of glutathione, Cd–ATP complexes. Direct evidence for cadmium transport was obtained with membrane vesicles derived from *L. lactis*, despite an important LmrA nonspecific Cd<sup>2+</sup> binding. This high background might be due to the presence of the CadA-like P-type ATPase YuiA. In the absence of glutathione, LmrA-containing membranes showed a cadmium uptake rate of 28 pmol·min<sup>-1</sup>·(mg of protein)<sup>-1</sup> above that of control membranes. This transport rate is in the same order as previously shown for LmrA-catalyzed transport of daunomycin into inside-out *E. coli* membrane vesicles [7.8 pmol·min<sup>-1</sup>·(mg of protein)<sup>-1</sup>] (12). When glutathione was added to the medium, a more than 4-fold increase in the rate of cadmium transport was observed up to 124 pmol·min<sup>-1</sup>·(mg of protein)<sup>-1</sup>. Although much higher rates of cadmium transport have been reported for the yeast YCF1 (6) in the presence of glutathione [3.3 nmol·mg<sup>-1</sup>·(mg of protein)<sup>-1</sup>], it is difficult to directly compare these rates as they have been determined in vastly different experimental systems. Unlike YCF1, cadmium detoxification is likely not the primary function of LmrA. Among the superfamily of ABC transporters several systems seem to have evolved to function as efficient metal ion detoxification devices, such as MRP1, LtpgpA, and YCF1, while others have retained this activity with only a lower efficiency.

A weak ATP-dependent LmrA-mediated Cd<sup>2+</sup> transport activity occurred without added glutathione. Under those conditions, Cd<sup>2+</sup> may be complexed with ATP (32) which was present in large excess. Murine and *Drosophila* P-gp homologues have been shown to serve as ATP-conducting channels in the plasma membrane (21, 33). The cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that belongs to the ABC superfamily, has also been demonstrated to conduct ATP movements (22). Finally, the ZntA ATPase accumulates Cd<sup>2+</sup> in inside-out membrane vesicles 5-fold more efficiently in the presence of cysteine

than in its absence (26). Cd<sup>2+</sup>, Hg<sup>2+</sup>, and Zn<sup>2+</sup> form complexes with thiolates with affinities that are several orders of magnitude greater than complexes with ATP (26). This may explain the increased LmrA-mediated Cd<sup>2+</sup> transport activity in the presence of glutathione.

A high, nonspecific Cd<sup>2+</sup> binding and transport activity of *E. coli* membrane vesicles precluded an accurate analysis of OmrA- and hMDR1-mediated <sup>109</sup>Cd transport. However, given the structural and functional relatedness of OmrA and hMDR1 with LmrA, and the observation that these systems render *E. coli* cells more resistant to Cd<sup>2+</sup> with a concomitant decrease in intracellular cadmium concentrations, it is likely that OmrA and hMDR1 also extrude Cd<sup>2+</sup>. These systems have more in common. First, both LmrA and OmrA were shown to protect bacteria against sodium laurate toxicity and ethanol and wine shocks (15). Second, both CFTR and MDR proteins share ATP- and chloride-channel activity (21, 22, 34). Third, CFTR is able to release glutathione into airway surface fluid (35) and then displays functional similarity with the ABC proteins MRP1, MRP2, and YCF1 (2, 3, 6). Fourth, overexpression of CFTR leads to a multidrug resistance phenotype (36). And vice versa, induction by antitumoral drugs of *hMDR1* and *hMRP1* complements CFTR function (37). These results raise the possibility that ABC transporters may be interchanged, as outlined by the complementary patterns of expression of the *CFTR* and *hMDR1* genes observed in vivo (38). The common functional features in ABC transporters are likely to be explained by an early evolutionary event. ABC genes may have evolved separately and acquired a more specialized activity, while maintaining their general function with low efficiency. For instance, hMDR1, LmrA, and OmrA proteins are specialized in xenobiotic scavenging but still are able to confer some resistance against metals. In the future, it would be valuable to determine the selectivity of such proteins among heavy metals.

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