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Short title: **Zebrafish ABCB transporters protect against metal contamination.**

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Abstract

ATP-binding cassette (ABC) proteins are efflux transporters and some of them are involved in xenobiotic detoxification. The involvement of four zebrafish ABC transporters in cadmium, zinc and mercury detoxification was characterised in a metal hypersensitive mutant of *Escherichia coli*. The *E. coli tolC* mutant expressing ABCB3 or ABCB7 transporters exhibited higher survival ratios and lower metal accumulation under metal exposure condition than controls. For instance, in presence of 8 and 10 µM of $HgCl_2$, the survival ratios of bacteria expressing ABCB3 were 4 and 6-times higher than control while mercury concentrations were 2.5 and 2-times lower than in control. This work provides new data on the function of zebrafish ABCB3 and ABCB7 transporters, and highlights their significance in metal detoxification.

1. Introduction

ATP-binding cassette (ABC) transporters are membrane proteins distributed among eight subfamilies A to G (Dean *et al.*, 2001) and H (Popovic *et al.*, 2010) based on structural arrangements and phylogenetic analyses. These efflux pumps carry various compounds across biological membrane including phospholipids, ions, peptides, steroids, polysaccharides amino acids, organic anions, bile acids, drugs and other xenobiotics. Mitochondria comprise up to four ABC systems, ABCB6, ABCB7/ATM1, ABCB10/MDL1, and ABCB8. These half-transporters, which assemble into homodimeric complexes, are involved in biogenesis of cytosolic iron–sulfur clusters, heme biosynthesis, iron homeostasis (Zutz *et al.*, 2009), protection against oxidative stress (Liesa *et al.*, 2012) and multidrug resistance (Elliott and Al-Hajj, 2009). Multidrug resistance is associated to overexpression of ABC transporters belonging to B (MDR1), C (MRP1 and MRP2) and G2 (BCRP) subfamilies (Leslie *et al.*, 2005). Some ABC transporters carry toxic compounds and their metabolites out of the cell through binding and hydrolyzing ATP and play an important role in detoxification.

ABC transporter genes have been identified in fish species (Liu *et al.*, 2013; Lončar *et al.*, 2010; Tutundjian *et al.*, 2002; Zucchi *et al.*, 2010). Transcriptional responses of ABC transporter genes belonging to B and/or C sub-families have been studied in zebrafish (*Danio rerio*) (Bresolin *et al.*, 2005), turbot (*Scophthalmus maximus*) (Tutundjian *et al.*, 2002), rainbow trout (*Oncorhynchus mykiss*) (Zaja *et al.*, 2008; Lončar *et al.*, 2010), red mullet (*Mullus barbatus*) (Sauerborn *et al.*, 2004), Nile tilapia (*Oreochromis niloticus*) (Costa *et al.*, 2012) and rock cod (*Trematomus bernacchii*) (Zucchi *et al.*, 2010). The identification of 50 ABC transporter genes was recently achieved by phylogenetic analyses in catfish (Liu *et al.*, 2013). Proteins of the B subfamily have been identified in rainbow trout (Sturm *et al.*, 2001), killifish (*Fundulus heteroclitus*) (Cooper *et al.*, 1999), turbot (Tutundjian *et al.*, 2002) and the rock cod (Zucchi *et al.*, 2010). Finally, mRNA and proteins of ABC transporter have also been identified and

characterised in fish cells (Zaja *et al.*, 2007, 2008, 2011). Transcriptional changes of ABCB1 and ABCC2 transporter genes have been detected in liver of rock cod after intraperitoneal injection of Cd, stimulating an acute exposure event (Zucchi *et al.*, 2010) and in PLHC-1 cells exposed to Hg, Cd, As and Cr (Della Torre *et al.*, 2012). The transcriptional response of *abcb1*, *abcc2*, and *abcg2* genes was modified in liver of killifish exposed to a marine site polluted by polycyclic aromatic hydrocarbons, as compared to fish from a clean environment (Paetzold *et al.*, 2009). The *abcb1* gene is absent in the zebrafish genome. Zebrafish ABCB4 and ABCB5 are structurally similar to mammalian ABCB1, and it has been shown that ABCB4 transporter, but not ABCB5, conferred resistance of embryos to ABCB1 substrates (Fischer *et al.*, 2013).

Four zebrafish ABC genes responding to metal exposure in adult zebrafish have been selected, and their cDNAs have been heterologously expressed in a model cell reactor, the bacterium *Escherichia coli*, in order to assess the possible resistance conferred by those zebrafish ABC transporters against cadmium, zinc and mercury contaminations. *E. coli* does not possess MDR, MRP or BCRP-like ABC transporter genes in its genome, so that any beneficial action against metal of zebrafish cDNAs expressed in that bacterium would indicate their potential protecting role in fish.

2. Material and methods

*2.1 * Selection of zebrafish genes encoding ABC transporters for heterologous expression in* tolC *mutant*

We selected them without any preconceived idea about their function, because 1/ the function of many ABC genes is only inferred from sequence alignment and not from thorough biochemical studies, and 2/ some ABC transporters were found to display various functions besides their historically discovered one. Indeed, 1/ the human cystic fibrosis transmembrane conductance regulator, CFTR, historically known to pump out chloride anions has recently been

shown to pump out glutathione (Gould *et al*., 2012); 2/ CFTR has also been demonstrated to conduct ATP movements (Reisin *et al*., 1994); 3/ Murine and drosophila P-gp homologues have been shown to serve as ATP-conducting channels in the plasma membrane (Abraham *et al*., 1993; Bosch *et al*., 1996); 4/ The bacterial P-gp homologue LmrA (van Veen *et al*., 1998) protected bacteria not only against sodium laurate toxicity, ethanol, and wine shock, but also against sodium chloride shock (Bourdineaud *et al*., 2004). It was further confirmed that indeed LmrA can pump out salt (Velamakanni *et al*., 2009); 5/ MDR1 protein also displays a chloride-channel activity and regulates the cell volume (Valverde *et al*., 1992; Valverde *et al*., 1996); 6/ Overexpression of CFTR leads to a multidrug resistance phenotype (Wei *et al*., 1995) and induction by antitumoral drugs of hMDR1 and hMRP1 complements CFTR function (Lallemand *et al*., 1997). 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* (Breuer *et al*., 1993). 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.

Therefore our selection was not guided by the supposed function of ABC transporters but rather by their pattern of expression in response to metals in zebrafish tissues (Bourdineaud *et al*., 2015). For instances *abcb4*, *abcb11b* and *abcc7* members were excluded because their level of expression was found too weak in gills (despite the fact *abcb4* is expressed in zebrafish embryo; Fischer *et al*., 2013). The expression of *abcb4*, *abcb10*, *abcb11b*, *abcc6a*, *abcc7* and *abcc12* members were also found too weak in brain and liver. The following genes were selected for heterologous expression in *E. coli*: *abcb3* because of its induction by cadmium exposure in liver; *abcb3l1* because of its induction by cadmium exposure in brain; *abcb7* because of its induction by zinc exposure in muscles; and *abcb8* because of its induction by cadmium exposure in gills and zinc exposure in muscles and gills (Bourdineaud *et al*., 2015). In addition, the *abcb3l1* gene

had been found up regulated in gills, muscles, digestive tract and brain of zebrafish after 3 days of exposure to the contaminated water of the Riou-Mort River (in the south west of France, the Lot River and one of its tributaries, the Riou-Mort, are polluted by Cd and Zn released by an old industrial Zn factory) (Orieux *et al.*, 2011). Besides, we found it relevant to include *abcb3* and *abcb3l1* genes because they actively transport peptides from the cytosol to the ER lumen and display structural similarities with other transporters. Therefore we thought that they might be involved in the transport of peptide-metal complexes. Another criterion of selection was the length of the expressed transporter: we limited its size to 715-744 amino-acids to avoid peptide folding problems frequently encountered in *E. coli* with big proteins. Indeed, only half-transporters are encountered in bacteria.

2.2 Heterologous expression of ABC transporters

Specific primer pairs were designed (Table 1). The Shine-Dalgarno sequence and a stop codon were added in the 5' and 3' moiety of forward and reverse primers, respectively. All transcripts were successfully amplified using the Expand enzyme "Expand Long Template PCR System" (Roche). PCR products were visualized on 1% agarose gel containing crystal violet in order to prevent DNA from UV exposure required when using ethidium bromide. DNA bands were extracted and purified using the "TOPO XL PCR Cloning" kit (Invitrogen). Their integrity was checked on 1% agarose gel. One µL vector was mixed with 4 µL of PCR products and incubated at room temperature for 5 min and transformed in TOP 10 *E. coli*. Bacteria were then spread on agarose plates containing kanamycine (50 µg/mL). PCRs were performed to check the size and the orientation of the insert using internal primers as well as plasmid primers (M13 and T7). Orientation of the inserts was opposed to *lacZ* promoter in plasmids containing ADNc of *abcb3*, *abcb8* and *abcb3l1*. This suggests that the overexpression of these transporter genes are toxic in *E. coli* and that only a moderate expression is compatible with bacterial life. Only *abcb7*

displayed both orientations. Moreover, *tolC* mutant containing *abcb7* cDNA under *lacZ* control exhibited a slower growth than that transformed with the *abcb7* cDNA in the reverse orientation. Thus, the latter construction was used to perform experiments. Therefore, the system of expression used in this study is deliberately devoid of promoter in order to reach a low abundance of transporter molecules within cells, in order to get rid off possible disturbance, toxicity and inclusion bodies formation. Plasmids were extracted using a lysis protocol (Berghammer and Auer, 1993), and transferred in mutant bacteria *E. coli tolC* CS1562 (F-*λ*- *tolC6*::Tn10 *his leu proA argT his thi galK lacY trpE non mtl xyl ara rpsL sup*$^{+}$) (Austin *et al.*, 1990) in order to study the potential resistance to metal exposure conferred by ABC transporters.

2.3 Inhibitory concentrations

Concentrations inhibiting growth at 50% (IC_{50}) were determined for zinc, mercury and cadmium in order to define the exposure concentrations to use regarding a potential influence of ABC transporters in metal detoxification. Overnight *tolC* bacterial cultures hosting control or cDNA-containing plasmid were diluted to an OD_{600} of 0.01 into LB medium containing kanamycin (50 µg/mL) and different concentrations of mercury (from 0 to 20 µM of $HgCl_2$), zinc (from 0 to 2 mM of $ZnCl_2$) and cadmium (from 0 to 2 mM of $CdCl_2$). After 16 h of incubation at 28°C cell growth was monitored by spectrometry (absorbance at 600 nm) and IC_{50} were calculated by plotting the observed optical density versus the concentrations of metals in the medium. At 50 % of growth inhibition, the corresponding concentration was chosen to give the IC_{50}. The mean IC_{50} was obtained from 3 independent plots. Selected from these IC_{50} values, working exposure concentrations were 0.55 and 0.8 mM for $ZnCl_2$, 8 and 10 µM for $HgCl_2$ and 0.4 and 0.6 mM for $CdCl_2$.

2.4 Bacterial survival quantification

Overnight cultures were diluted into fresh LB medium (1% v/v) containing kanamycin and grown up to an OD_{600} of 0.1. Each culture was divided into seven: one control and six exposed to the targeted metal concentrations ($ZnCl_2$: 0.55 and 0.8 mM, $HgCl_2$: 8 and 10 µM and $CdCl_2$: 0.4 and 0.6 mM). Cultures were exposed during 3h at 28°C and 100 µL of each culture were used to perform serial 10-fold dilutions and each was spread on LB agar plates. Colonies were counted after overnight incubation. Survival rates are the ratio between the number of colonies after exposure to metals on those counted without exposure to metals.

2.5 Quantification of metal concentrations in bacteria

After 3 h of incubation as described previously, cells from 3 mL cultures were collected by centrifugation (5 min, 6000 g, 4 °C) and washed with a buffer containing 10 mM Tris-Cl pH 7.4 and 0.15 M NaCl. Pellets were mineralized in 3 mL of concentrated nitric acid (pure HNO_3 60%, v/v) at 100 °C for 3 h in a pressurized medium (borosilicate glass tube). Samples were diluted in 18 mL of ultrapure water (MilliQ plus) and analyzed by atomic absorption spectrophotometry. The Cd determinations were performed with an atomic absorption spectrophotometer (M6 Solaar AA, Thermo Elemental) equipped with a graphite tube atomizer (GF95 Graphite Furnace). The detection and quantification limits were 0.44 nmol Cd/L and 2.6 pmol Cd/10^9 bacterial cells. Zinc concentrations for water and digested tissue samples were determined by flame atomic absorption spectrophotometry (AAS) (Varian AA 220 FS). The detection and quantification limits were 0.15 µmol Zn/L and 0.9 nmol Zn/10^9 bacterial cells. The analytical methods were simultaneously validated for each sample series by the analysis of standard biological reference materials (TORT-2, lobster hepatopancreas; DOLT-2, dogfish liver; National Research Council of Canada, Ottawa). Total Hg concentration was determined by flameless AAS directly on bacterial samples (Leco Ama 254). The detection limit was 0.01 ng of Hg.

2.6 Statistical analyses

As the normality of the residuals wasn't validated, non-parametric tests were selected. After a significant Kruskall-Wallis test, the Mann-Whitney U test was used to evaluate significant difference between results from an exposed condition versus the corresponding control one for survival ratios, metal quantification and IC50 analyses (*: $p < 0.05$). Both tests were performed using R (version 3.0.2) coin package.

3. Results

3.1 ABC transporter proteins confer metal resistance to E. coli

The *tolC* mutant, which is hypersensitive to metals (Achard-Joris *et al.*, 2005), was transformed with a control plasmid or plasmids containing cDNA of ABC transporters *abcb3*, *abcb7*, *abcb3l1* and *abcb8*, and then tested for its resistance to metal exposure ($CdCl_2$, $ZnCl_2$ and $HgCl_2$). When transformed with plasmids containing *abcb3* and *abcb7* bacteria displayed a significant increase of 50% of growth inhibitory concentration (IC_{50}) values as compared to control bacteria for every metal (Table 2). In contrast, there were no differences in cell growth for bacteria containing *abcb3l1* or *abcb8* cDNAs as compared to control.

3.2 Expression of abcb3 and abcb7 cDNAs result in a lower metal accumulation in bacteria

E. coli tolC mutant expressing ABCB3 or ABCB7 transporters exhibited higher survival ratios and lower metal accumulations under metal exposure condition than controls (Table 2 and Figure 1). For instance, in presence of 8 and 10 µM of $HgCl_2$, the survival ratios of bacteria expressing ABCB3 were 4- and 6-times higher than control while mercury concentrations were 2.5- and 2-times lower than in control. The same pattern of resistance associated with a decreased accumulation of metal was observed for cadmium and zinc exposures as well as for bacteria

expressing ABCB7 (Table 2 and Figure 1). When *E. coli tolC* mutant expressed ABCB3l1 and ABCB8, survival ratios and metal accumulation levels were similar to control (Figure 1).

4. Discussion

cDNA of four transporter genes belonging to the sub-families ABCB and ABCC were expressed in *tolC* mutant, which is hypersensitive to metals (Achard-Joris *et al.*, 2005; Achard-Joris and Bourdineaud, 2006). In the present study, we used this mutant as a simple reactor in which transporters were expressed in order to assess their ability to confer resistance to *E. coli* when exposed to cadmium, zinc and mercury. Previous studies evidenced that human MDR1 and two bacterial homologs, LmrA and OmrA, protected *tolC* mutant against Cd, Zn and Hg (Achard-Joris *et al.*, 2005; Achard-Joris and Bourdineaud, 2006). ABCB3 and ABCB7 expression induced a survival increase varying between + 13 % and + 58 % and a decrease in metal concentration ranging between - 50 % to - 150 %. Numerous data in the literature, including those using a bacterial system like in the present work, are giving differences in the same range when dealing with the involvement of ATPase transporters in metal or xenobiotic detoxification. Indeed, 1/ The mortality of zebrafish embryos caused by 10 and 20 µM vincristine was increased from 6.5 to 26 % (+ 19.5 %) and from 29 to 39 % (+ 10 %) upon addition of 5 µM of the ABC transporters inhibitor cyclosporine A, respectively (Fischer *et al.*, 2013); 2/ When using morpholino compounds to knock-down *abcb4* gene, the mortality in presence of 2 µM vinblastine was increased from 51 % in controls up to 74 % in targeted embryos (+ 23 %) (Fischer *et al.*, 2013); 3/ The addition of the MRP proteins inhibitor MK-571 to canine kidney cells (expressing MRP1 and MRP2) resulted in a decreased survival ratio from 71 down to 47 % (- 24 %) and from 54 down to 27 % (- 27 %), during exposure to 20 and 40 µM of $HgCl_2$, respectively. The intracellular mercury simultaneously increased of from 1.26 up to 1.66 µg/mg protein (+ 32 %) when cells were exposed to 40 µM of $HgCl_2$ (Aleo *et al.*, 2005); 4/ The overexpression of *abcc1*

(*mrp1*) protected zebrafish embryos from acute toxicity of cadmium (100 µM) from 26 up to 41 % of survival (+ 15 %), mercury (3 µM) from 33 up to 52 % (+ 19 %), and arsenic (2 mM) from 45 up to 61 % (+ 16 %) (Long *et al.*, 2011a); 5/ The overexpression of *abcc2* (*mrp2*) decreased lead accumulation in zebrafish embryos exposed to 5 µM Pb from 145 to 87 nmol/g dry weight (- 40 %) (Long *et al.*, 2011b); 6/ The overexpression of *abcc5* (*mrp5*) protected zebrafish embryos from acute toxicity of cadmium (100 µM) from 58 up to 77 % of survival (+ 19 %) (Long *et al.*, 2011c); 7/ The IC_{50} value for cadmium on wild-type zebrafish fibroblast cells was 71 ± 4 µM and 105 ± 7 µM for Cd-resistant cells overexpressing *abcc2* and *abcc4* genes (+ 49 %) (Long *et al.*, 2011d); 8/ The expression of the PbtA transporter from *Achromobacter xylosoxidans* in *E. coli* decreased the accumulation of Cd, Pb and Zn (37, 13 and 20 %, respectively). The IC_{50} value for zinc increased from 78 to 134 µM (+ 72 %) upon expression of *pbtA* gene in *E. coli* (Hložková *et al.*, 2013); 9/ The differences in IC_{50} values between a *zntA* mutant of *Thermus thermophilus* and a wild-type strain overexpressing ZntA were 33, 25 and 38 % after exposure to cadmium, copper and zinc (Schurig-Briccio and Gennis, 2012); 10/ The overexpression of the CzrB transporter from *T. thermophilus* in *E. coli* was associated with increased IC_{50} values from 1.6 up to 2.5 mM (+ 56 %) for zinc and 0.7 up to 0.9 mM (+ 28 %) for cadmium. The intracellular zinc concentration decreased from 75 down to 35 mg/g dry weight cells (- 53 %) after 1 h of incubation with 5 mM Zn (Spada *et al.*, 2002); 11/ *Arabidopsis thaliana* plants expressing the *E. coli* ZntA transporter displayed resistance to 70 µM cadmium with increased fresh weight from 22 up to 38 mg/plant (+ 73 %), increased chlorophyll content from 16 to 21 mg/ml of plant extract (+ 31 %), and decreased cadmium concentration from 620 down to 355 nmol/mg dw (- 42 %) (Lee *et al.*, 2003); 12/ The expression of the rice heavy-metal ATPase OsHMA9 in the *E. coli* *copA* mutant led to an increased IC_{50} value fom 1.6 up to 3 mM $CuSO_4$ (+ 87 %). Mutant plants knocked-out for *oshma9* gene featured a greater sensitivity towards 300 µM copper with a plant height decreased from 11 down to 8 cm (- 27 %) and a fresh weight decreased from 110 down to

80 mg/plant (- 27 %) (Lee *et al.*, 2007). To conclude, our bacterial *tolC*$^{-}$ system is a relevant one to figure out the possible involvement of half ABC transporters in metal resistance.

The number of ABCB3 and ABCB7 molecules expressed per bacterial cell was estimated taking the *zwf* gene as a reference (encoding the glucose-6-phosphate dehydrogenase, G6PDH). The related specific primers have already been used and published (Kabir and Shimizu, 2003). Under non-exposed growth we found relative expressions of 0.29 ± 0.05 and 0.27 ± 0.04 for *abcb3* and *abcb7* cDNAs, respectively (n = 4, ± SD; a Roche LightCycler apparatus was used for qPCR analysis). The abundance of the *zwf* gene product, G6PDH, has been quantified to be 883 molecules per cell (Ishihama et al., 2008). If we accept the assumption according to which the rate of translation of the *zwf* RNA is equal to that of the *abcb3* and *abcb7* RNAs and the proteins half-lives similar, then the number of transporter molecules per cell is 256 ± 44 for ABCB3 and 238 ± 35 for ABCB7. We found herein that the expression of ABCB7 was responsible of the reduction of cadmium concentration from 41 down to 22 nmol/10^9 cells, making 114 x 10^{14} Cd ions pumped by 238 x 10^9 ABCB7 molecules for 3 hours yielding 4.4 ± 0.7 Cd ions/s/molecule. This is in accordance with ATP hydrolysis activity of metal ATPases such as *E. coli* ZntA (3.9 ATP/s/molecule; Sharma *et al.*, 2000), *T. thermophilus* Zn^{2+}/Cd^{2+}-ATPase (2.4 ATP/s/molecule; Schurig-Briccio and Gennis, 2012) and even the human MDR1 (4.9 ATP/s/molecule; Loo *et al.*, 2012). These results strongly suggest that ABCB3 and ABCB7 are involved in metal detoxification. Additionally, inductions of *abcb3* gene in liver of fish exposed to cadmium and *abcb7* gene in muscles of fish exposed to zinc corroborate their involvement in protection (Bourdineaud *et al.*, 2015). The present study characterised for the first time the function of ABCB3 and ABCB7 transporters in metal detoxification. Indeed, these zebrafish proteins were classified among the ABCB subfamily on the basis of sequence alignment with counterparts from other species, but no data related to their genuine biochemical properties were known. Bacteria are lacking intracellular membranes whereas in eukaryotic cells, ABCB half transporters are

known to localise within such intracellular membranes questioning the role of those transporters in reducing cellular metal concentration. ABCB7 might well contribute to mitochondria detoxification by pumping divalent metals from the matrix out in the cytoplasm. Indeed, when cells are facing metal contamination, mitochondria are primary targets due to the developed transmembrane potential of around 120 mV. This creates a thermodynamic driving force attracting divalent metals within the negatively charged matrix so important that the theoretical ratio between divalent metal concentration within the matrix over the one in the cytoplasm at the Nernst equilibrium is equal to e^k (with $k = 2F\Delta\Psi/RT$) giving a value of 8100. ABCB3 transporter equivalent to human transporters located within the endoplasmic reticulum and involved in peptide transport might as well be implicated in the transport of peptide-metal complexes, whenever a peptide containing a cysteinyl residue bound to a divalent metal ion is processed.

To summarise, the belonging of *abcb3* an *abcb7* genes to the ABC superfamily could only be deduced from sequence alignments, and our study introduces new information in the field of ABC transporters and sheds light on a new function of ABCB3 and ABCB7 zebrafish transporters, which was up to now ignored by protein data banks and literature.

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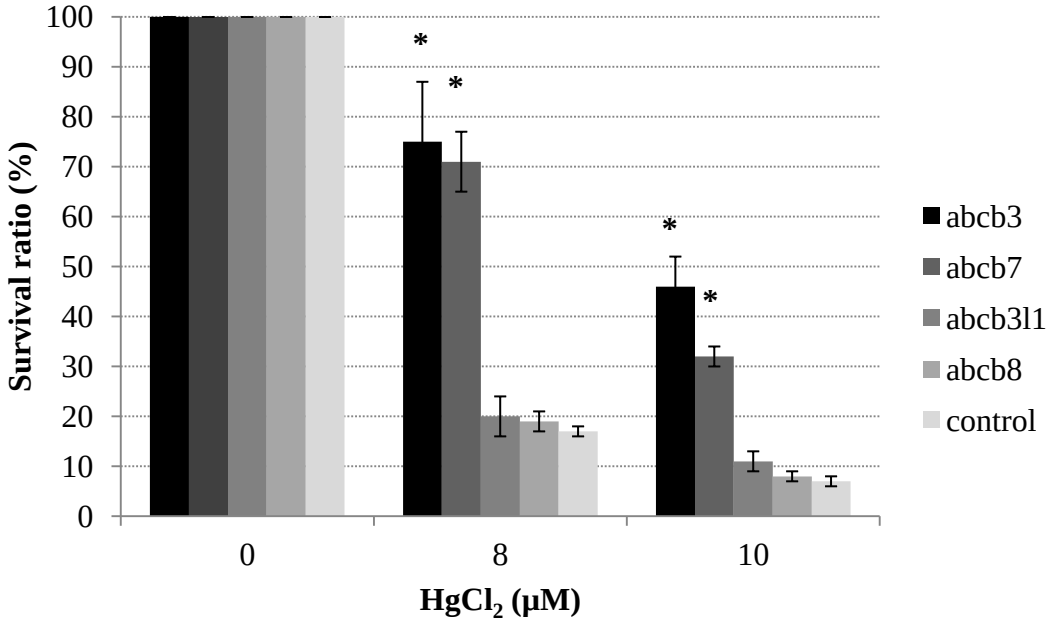
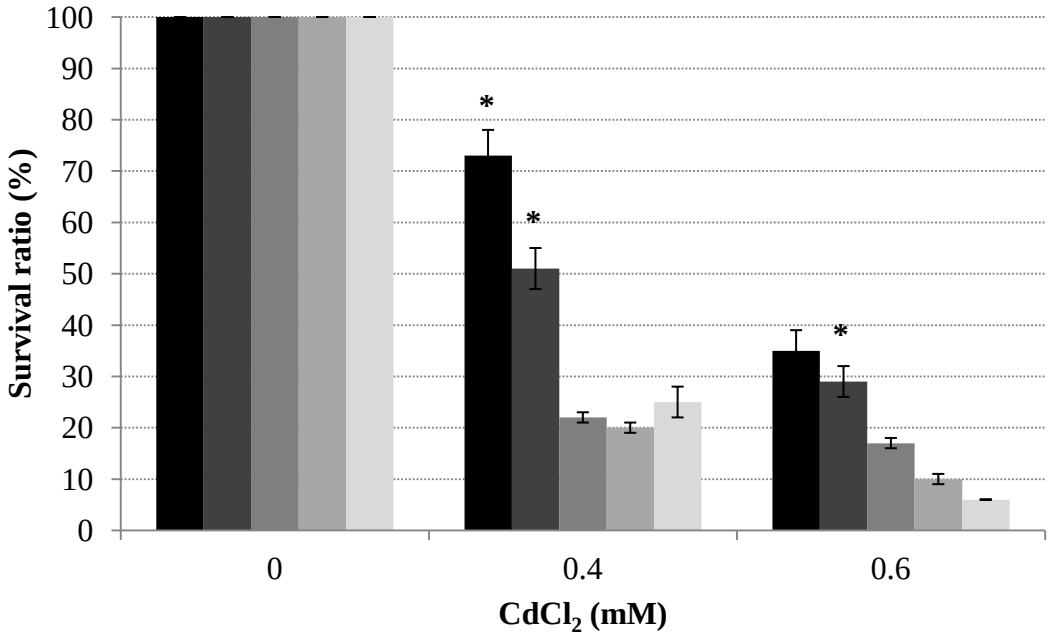
Figure 1. Heterologously expressed ABCB3 and ABCB7 transporters protect *E. coli tolC* mutant against metals and are associated with a lower cell level of cadmium. *E. coli tolC* mutant was transformed with control plasmid or plasmids containing *abcb3*, *abcb3l1*, *abcb7*, and *abcb8* cDNAs. After 3 hours of incubation at 28°C with the indicated concentrations of $HgCl_2$ (A, D), $CdCl_2$ (B, E) and $ZnCl_2$ (C, F), the bacterial cultures were diluted and spread on agar plates in order to determine the survival ratio (A, B, C). The remaining cultures were used to quantify cell-associated metals (D, E, F).

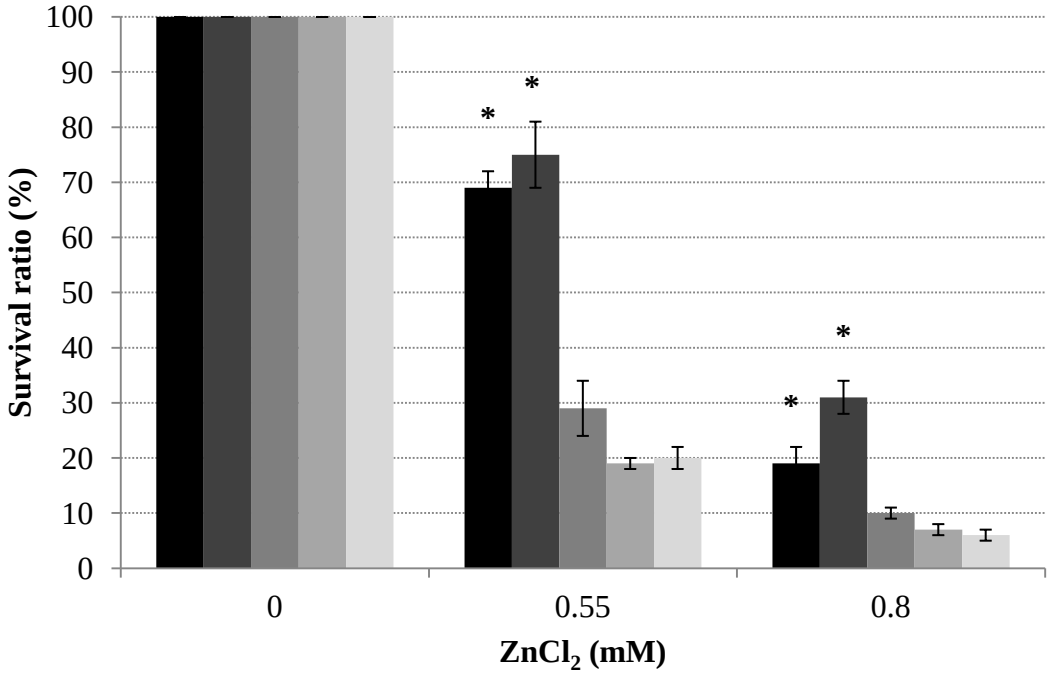
A


100
90
80
70
60
50
40
30
20
10
0
Survival ratio (%)
*
*
*
*
0
8
10
HgCl2 (µM)
abcb3
abcb7
abcb3l1
abcb8
control

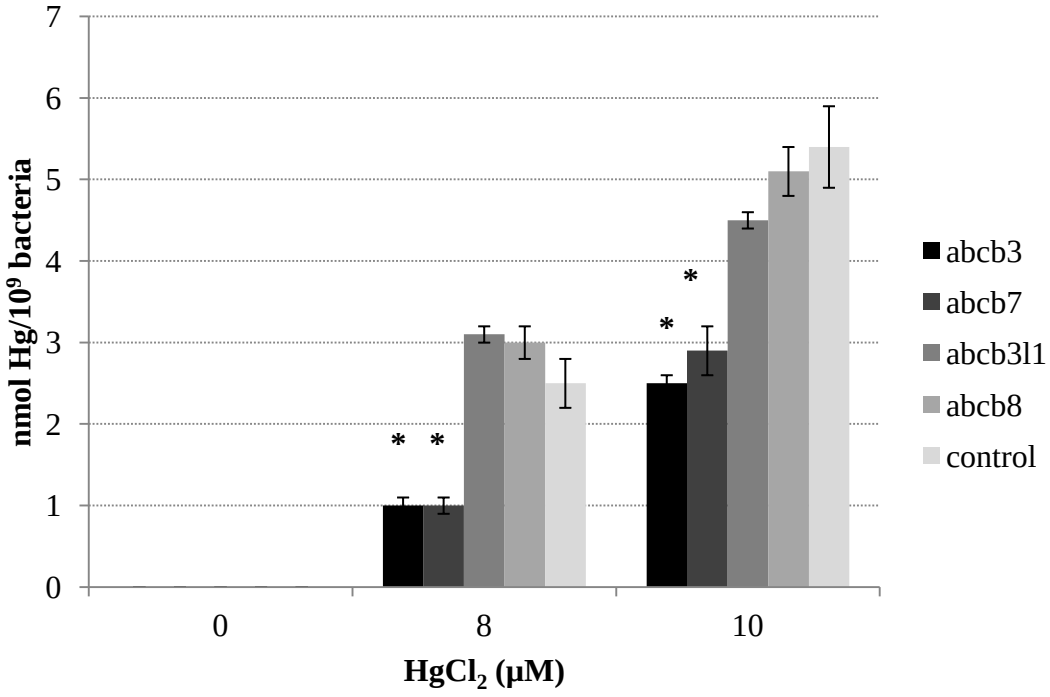
B


100
90
80
70
60
50
40
30
20
10
0
Survival ratio (%)
*
*
*
0
0.4
0.6
CdCl2 (mM)

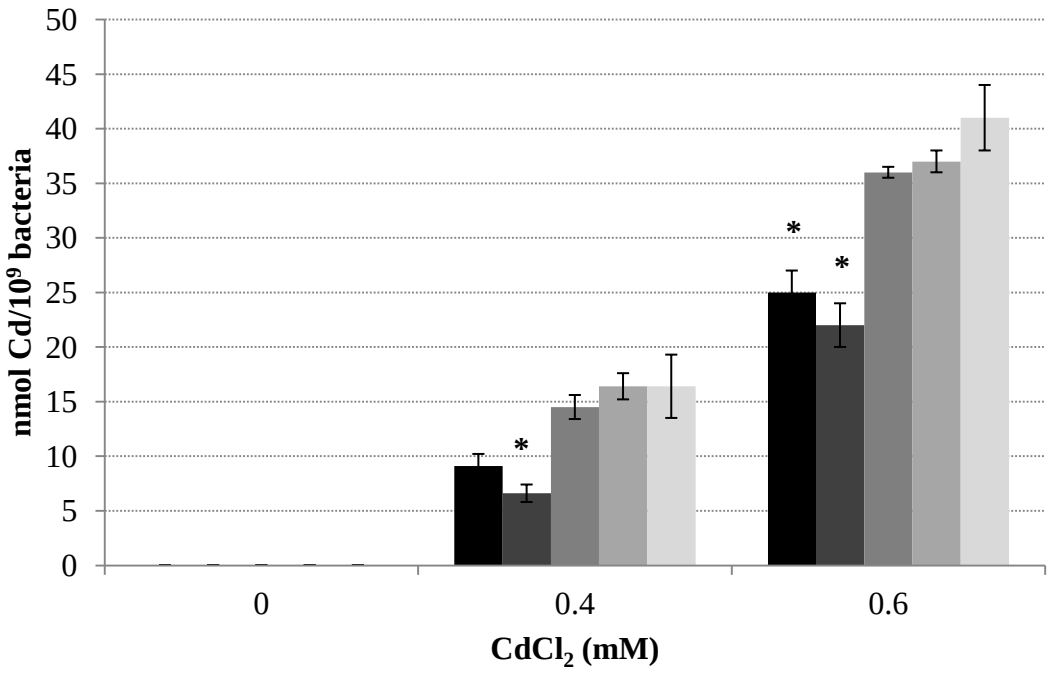
C


100
90
80
70
60
50
40
30
20
10
0
Survival ratio (%)
*
*
*
*
0
0.55
0.8
ZnCl2 (mM)

D


7
6
5
4
3
2
1
0
nmol Hg/10^9 bacteria
* *
*
*
0
8
10
HgCl2 (µM)
abcb3
abcb7
abcb3l1
abcb8
control

E


50
45
40
35
30
25
20
15
10
5
0
nmol Cd/10^9 bacteria
*
*
*
0
0.4
0.6
CdCl2 (mM)

F

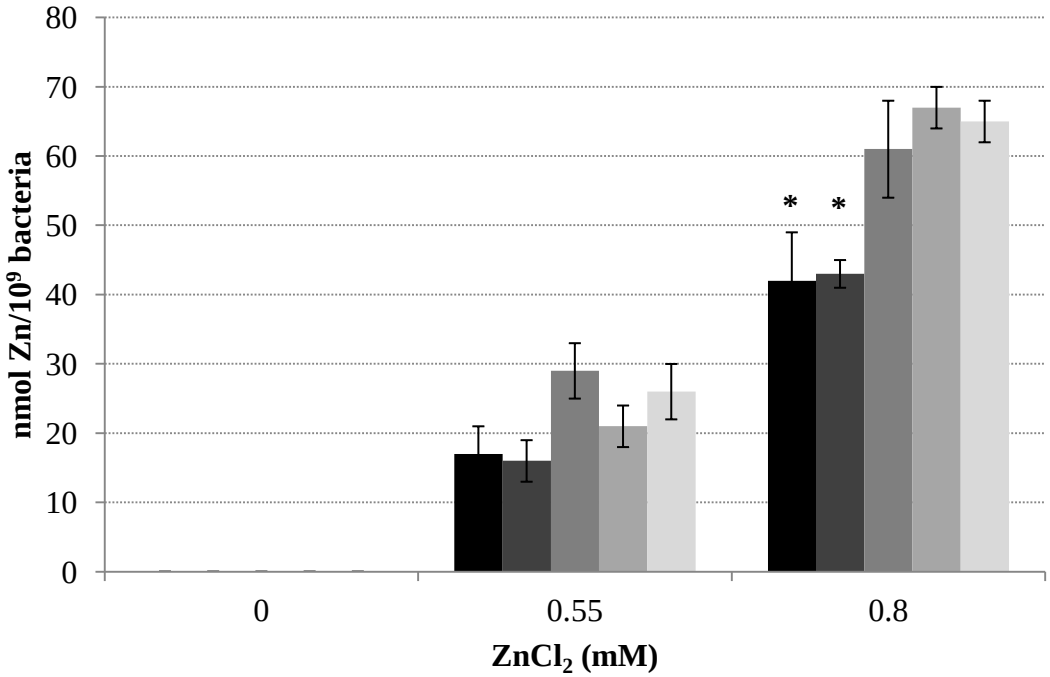

80
70
60
50
40
30
20
10
0
nmol Zn/10^9 bacteria
*
*
0
0.55
0.8
ZnCl2 (mM)

Table 1. Primer sequences used to amplify cDNA sequences of zebrafish genes to express in *Escherichia coli*. Shine-Dalgarno sequences are in bold and underlined. Stop codons are in bold and italics. Start codons are in blue italics.

Gene	Protein	Amplicon size	Primer sequences
abcb3	ABCB3	2187 bp	[a]AAC**AGGAGG**TTTAAG*ATG*GATTCAGATCAGG
			[b]CGTAC***TTA***AGTCGCCTGTTGCG
abcb3l1	TAP2	2178 bp	[a]AGTT**AGGAGG**CGCACC*ATG*CGGAAGGTTTTG
			[b]GGC***CTA***CTGCGTTTTTACGGTA
abcb7	ABCB7	2232 bp	[a]TCAACC**AGGAGG**TTCAGC*ATG*GCGCCGCTCTT
			[b]GGAC***TCA***GCACGAGCAGTTCC
abcb8	ABCB8	2145 bp	[a]TCTGAA**AGGAGG**TGAAAC*ATG*TTTCATTTTGCACG
			[b]TATT***TTA***TTTATGTCCATTAGATCG

[a]forward primer. [b]reverse primer.

Table 2. Median inhibiting concentrations (IC_{50}) of Zn, Hg and Cd in *tolC* mutant transformed with plasmids containing ABC transporter cDNA (mean ± SEM; $n = 3$, *: $p < 0.05$).

ADNc	Protein	IC_{50}		
		$ZnCl_2$ (mM)	$HgCl_2$ (µM)	$CdCl_2$ (mM)
control	none	0.63 ± 0.04	6.6 ± 0.4	0.59 ± 0.01
abcb3	ABCB3	* 0.96 ± 0.03	* 9.5 ± 0.3	* 0.82 ± 0.02
abcb3l1	TAP2	0.60 ± 0.03	7.1 ± 0.3	0.68 ± 0.04
abcb7	ABCB7	* 0.82 ± 0.06	* 9.7 ± 0.3	* 0.81 ± 0.02
abcb8	ABCB8	0.43 ± 0.03	6.0 ± 0.6	0.64 ± 0.04

Table 3. ABCB3 and ABCB7 transporters allow metal resistance in *tolC* mutant by decreasing the amount of bioaccumulated metals (mean ± SEM, $n = 3$, *: $p < 0.05$).

		Metal accumulated (nmol/10^9 bacteria)			Survival ratio (%)		
Metal	Concentration	ABCB3	ABCB7	Control	ABCB3	ABCB7	Control
$HgCl_2$	8 μM	* 1.0 ± 0.1	* 1.0 ± 0.1	2.5 ± 0.3	* 75 ± 12	* 71 ± 6	17 ± 1
	10 μM	* 2.5 ± 0.1	* 2.9 ± 0.3	5.4 ± 0.5	* 46 ± 6	* 32 ± 2	7 ± 1
$CdCl_2$	0.4 mM	9.1 ± 1.1	* 6.6 ± 0.8	16.4 ± 2.9	* 73 ± 5	* 51 ± 4	25 ± 3
	0.6 mM	* 25 ± 2	* 22 ± 2	41 ± 3	* 35 ± 4	* 29 ± 3	6 ± 0.1
$ZnCl_2$	0.55 mM	17 ± 4	16 ± 3	26 ± 4	* 69 ± 3	* 75 ± 6	20 ± 2
	0.8 mM	* 42 ± 7	* 43 ± 2	65 ± 3	* 19 ± 3	* 31 ± 3	6 ± 1