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Abcb and Abcc transporter homologs are expressed and active in larvae and adults of zebra mussel and induced by chemical stress

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ABSTRACT

Multixenobiotic resistance (MXR) of aquatic invertebrates has so far been associated with cellular efflux activity mediated by P-glycoprotein (ABCB1) and MRP (multidrug resistance protein; ABCC) type ABC (ATP binding cassette) transporters. Expression and activity of an *abcb1*/Abcb1 homolog has been shown in eggs and larvae of the zebra mussel *Dreissena polymorpha*. Here we report identification of a partial cDNA sequence of an *abcc*/Abcc homolog from zebra mussel that is transcribed and active as a cellular efflux transporter in embryos and gill tissue of adult mussels. Transcript expression levels were comparatively low in eggs and sharply increased after fertilization, then maintaining high expression levels in 1 and 2 dpf (days post fertilization) larvae. MK571, a known inhibitor of mammalian ABCC transporters, blocks efflux of calcein-am in larvae and gill tissue as indicated by elevated calcein fluorescence; this indicates the presence of active Abcc protein in cells of the larvae and gills. Dacthal and mercury used as chemical stressors both induced expression of *abcb1* and *abcc* mRNAs in larvae; accordingly, assays with calcein-am and ABCB1 inhibitor reversin 205 and ABCC inhibitor MK571 indicated enhanced Abcb1 and Abcc efflux activities. Responses to chemicals were different in gills, where *abcb1* transcript abundances were enhanced in dacthal and mercury treatments, whereas *abcc* mRNA was only increased with mercury. Abcb1 and Abcc activities did not in all cases show increases that were according to respective mRNA levels; thus, Abcc activity was significantly higher with dacthal, whereas Abcb1 activity was unchanged with mercury. Our data indicate that *abcb1*/Abcb1 and *abcc*/Abcc transporters are expressed and active in larvae and adult stages of zebra mussel. Expression of both genes is induced as cellular stress response, but regulation appears to differ in larvae and tissue of adult stages.

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1. Introduction

Aquatic organisms are constantly exposed to complex mixtures of structurally diverse chemicals dissolved in the water and must avoid their potential toxic impact. The cellular multixenobiotic resistance (MXR) system represents a broad-scale defense mechanism protecting cells and organisms against both endogenous and environmental toxicants, including also anthropogenic chemicals (Epel et al., 2008). MXR is mediated by membrane transport proteins from the ABC (ATP binding cassette) protein family, which recognize a wide variety of potential xenobiotics as substrates, pumping them out of the cell in an energy dependent, ATP-driven process.

ABC transporters constitute a large protein family, subcategorized into sub-families ABCA-ABCH with a variety of functions (Dean, 2005). The transporters with MXR related functions are P-glycoprotein (P-gp, MDR1, ABCB1[1]) belonging to the ABCB subfamily, multidrug resistance associated proteins 1–5 (MRP1-5, ABCC1-5) from the ABCC subfamily, and breast cancer resistance protein (BCRP, ABCG2) from the ABCG subfamily (Leslie et al., 2005). The earlier studies associated MXR of aquatic invertebrates with Abcb1 homologs (Epel, 1998; Kurelec, 1992), and more recently with Abcc subfamily homologs, such as in embryos of sea urchin and gill tissue of marine mussels (Hamdoun et al., 2004; Luckenbach and Epel, 2008).

Induction of expression and activity of an *abcb1*/Abcb1 homolog by chemical and physical stressors has previously been shown in bivalves, indicating responsiveness of this gene as a general stress response (Eufemia and Epel, 2000; Lüdeking and Köhler, 2002, 2004). Various studies have provided evidence for cellular protection against environmental toxicants by MXR transporters in tissues (Britvic and Kurelec, 1999; Contardo-Jara et al., 2008; Smital

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[1] Our nomenclature for gene/protein names is: *abcc*/Abcc for bivalve and *ABCC*/ABCC for human.

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and Kurelec, 1997; Smital et al., 2003) and larvae of *D. polymorpha* (Faria et al., 2011) and MXR could be associated with an Abcb1 homolog (Faria et al., 2011; Tutundjian and Minier, 2007). In addition, there was indication for Abcc type efflux activity in *Dreissena* larvae as accumulation of calcein-am in the cells was increased in the presence of MK571, an inhibitor of ABCC transporters (Faria et al., 2011).

We here present a study where we identified a partial *abcc* type homolog from *D. polymorpha*, quantified constitutive transcript levels of *abcb1* and *abcc* homologs in larval stages and adult tissue and upon the impact of chemical stressors and assayed Abcb1 and Abcc type efflux activities with the proxy dye calcein-am and specific pharmacologic inhibitors.

We show that both *abcb1*/Abcb1 and *abcc*/Abcc type transporters are constitutively expressed and active in cells of larvae and tissue of adult mussels and that expression of both transporters is induced by chemicals.

2. Material and methods

2.1. Chemicals

Reversin 205, MK571, calcein-am (Ca-AM), mercury as $HgCl_2$, dacthal and serotonin creatinine sulphate monohydrate were purchased from Sigma–Aldrich (Steinheim, Germany). Acetone and DMSO (analytical grade) were obtained from Merck (Darmstadt, Germany).

2.2. Origin and maintenance of adult zebra mussels, egg production and maintenance of larvae

Sexually mature zebra mussels (*Dreissena polymorpha*) were collected in shallow water (0.5–1 m depth) in the Mequinenza reservoir/Ebro River (NE, Spain) from May to August 2010. Within 3 h of collection, animals were transported in local water in aerated 10 L plastic containers to the lab. The animals were then rinsed and placed in glass aquaria in a density of 0.5 L per individual and maintained in ASTM hard water (ASTM, 1999) at >90% oxygen saturation at 20 °C and at a 14 h:10 h/light:dark photoperiod.

Prior to spawning mussels were acclimated for at least 1 d. Spawning of mussels was induced by exposure of animals to 10^{-3} M serotonin creatinine sulphate monohydrate for 15 min. For obtaining gametes, at least three males and at least five females were then placed in separate beakers with 200 mL of clean ASTM hard water. Spawning occurred within 15–30 min in males and within 1–3 h in females. About 10^6 eggs from the pool of females were transferred to 200 mL ASTM hard water and fertilized with the sperm contained in 1 mL of the water from the beaker with the males. Only gametes that were released by mussels into the serotonin-free water were used for fertilization (Faria et al., 2011). Larvae were cultured in 9 L ASTM hard water in 10 L glass bottles under constant oxygenation (>90% saturation), temperature (20 °C) and photoperiod (14 h:10 h/light: dark) without food.

2.3. Identification of a partial abcc-like cDNA sequence from D. polymorpha

For designing primers for RT-PCR of a partial cDNA of an unknown *D. polymorpha abcc* transporter conserved sequence stretches of molluscan *abcc* transporter cDNAs were identified from a clustal alignment of *abcc* sequences from *Cyphoma gibbosum* (EU487192), *Mytilus californianus* (EF521415) and *Tritonia hamnerorum* (*abcc1* – EU487194; *abcc2* – EU487195). The primer pair used was ACR GAR ATT GGW GAA AAG GG (forward) and TDG TBA CNC AYG GMV TYV ABT (reverse).

RT-PCR was performed using a MiniCycler™ (MJ Research) with total RNA isolated from mussel gills as described below. PCR conditions were 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 55 °C and 72 °C for 30 s each, and a final step at 72 °C for 10 min.

The PCR product was cloned into vector pTZ57R/T from the InsTAclone™ PCR Cloning Kit (Fermentas) and propagated using X Blue competent cells. Sequencing of DNA was performed on a 3730 DNA Analyzer (Applied Biosystems). Homology of the obtained putative *abcc* transporter cDNA with *abcc* transporter sequences from other organisms was confirmed using NCBI (National Center for Biotechnology Information, Bethesda, MD, US) blastx and the sequence was deposited on GenBank (accession no. HM448029). For phylogenetic analyses of the sequence alignments were performed with a range of Abcc and Abcb1 sequences from various organisms using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Based on the alignment percent sequence identities were determined and a phylogenetic tree was generated using BioEdit v. 7.0 software (Hall, 1999).

2.4. RNA extraction and qRT-PCR analysis

Total RNA was isolated from eggs/larvae and gill tissue of adult animals using Trizol reagent® (Invitrogen™). The RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE) and the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Quantities from 1 μg to 100 ng of DNAse I-treated RNA (Ambion®) were retro-transcribed to cDNA using the First Strand cDNA Synthesis Kit (Roche Applied Science®) and stored at −20 °C. The amounts of cDNA used for quantitative real-time PCR (qRT-PCR) corresponded to 10 ng and 12 ng of the original RNA preparation for larvae and for gills, respectively. qRT-PCR was performed with a LightCycler® 480 Real-Time PCR System using LightCycler® 480 SYBR Green I Master (Roche Applied Science®). Primers were designed with Primer Express software and the sequences were MRP_fw GTA TCA ACC TGT CCG GTG GG, MRP_re TTG TTG TAC ACC GCC CTG G; S3_fw CAG TGT GAG TCC CTG AGA TAC AAG, S3_re AAC TTC ATG GAC TTG GCT CTC TG. Sequences of amplicons were confirmed by sequencing using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) and by comparing them to the corresponding references in GenBank (Table 1) using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Relative mRNA abundance values were calculated from the second derivative maximum of their respective amplification curve (Cp, values from triplicate assays). Cp values obtained for the target genes (TG), *abcc* and *abcb1*, were compared to the corresponding values of the reference gene *ribosomal protein s3* (Navarro et al., 2011) to obtain ΔCp values (ΔCp = Cp ref-CpTG). PCR efficiency values for reference and target genes were calculated using the equation described by Pfaffl (2002) and assumed to be close to 100% from these calculations. To facilitate reading graphs, the mRNA abundances were represented as mRNA copies of target gene per 1000 copies of the reference gene (‰ of reference gene, $1000 \times 2^{\Delta Cp}$).

Constitutive transcript expression levels of the *abcc* homolog were determined in freshly fertilized eggs, in embryos 2 h post fertilization and in 1 d and 2 d trochophora larvae. Induction of mRNA expression of *abcb1* and *abcc* genes by chemicals was quantified in 1 d trochophora larvae and in gills of adult mussels upon 24 h chemical exposures.

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