The ABCs of the amphipod P-glycoprotein: Heterologous production of the Abcb1 protein of a model species *Eulimnogammarus verrucosus* (Amphipoda: Gammaridae) from Lake Baikal

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Abstract

The multixenobiotic resistance (MXR) mechanism has been demonstrated to be present in a wide range of species, including aquatic organisms. However, amphipods (Crustacea: Malacostraca: Amphipoda), which constitute a large order of arthropods, are extremely poorly studied in this regard. Information on MXR proteins in these animals would be highly relevant, as some amphipods are important models in ecotoxicology due to their roles in many freshwater environments, including the ancient Lake Baikal. In this work, we studied the diversity of ABC transporters in the available transcriptomes of over 60 endemic Baikal amphipods in comparison to other related species. This showed that most classes of ABC transporters are present in all analyzed species and that most Baikal amphipods detectably express no more than one complete ABCB full transporter. We also showed that these sequences were conservative across different species, and their phylogeny was congruent with the species phylogeny. Thus, we chose the *abcb1* gene from *Eulimnogammarus verrucosus*, a widespread species playing an important role in the lake ecosystem, to establish the first heterologous expression system for an amphipod Abcb1/P-glycoprotein based on the *Drosophila melanogaster* S2 cell line. The resulting stably transfected S2 cell line was expressing the *abcb1* of *E. verrucosus* about 1,000 times higher than the homologous fly genes, and the target protein, Abcb1, showed to confer a high MXR-related efflux activity. Our results indicate the suitability of the S2-based expression systems for the study of arthropod ABCB1 homologs.

Introduction

One of the key protective mechanisms in all studied representatives of the living world, including aquatic organisms, is the mechanism of multixenobiotic resistance (MXR), which is equivalent to the multidrug resistance observed in some cancer cell lines (Kurelec, 1992; Bard, 2000). This mechanism ensures the organisms's ability to withstand the toxic effects of a wide range of substances at the cellular level, including anthropogenic chemicals (Smital et al., 2004). The latter is especially important, given that most anthropogenic toxicants end their life cycle in water (Jeong et al., 2017). The major players in the MXR system are the ABC (ATP-binding

cassette) transporters (Kurelec, 1992; Bard, 2000; Jeong et al., 2017). In arthropods, the ABC superfamily of proteins can be grouped into nine subfamilies, from ABCA to ABCI (Denecke et al., 2021; Saier et al., 2021), and according to phylogenetic reconstruction, this superfamily is polyphyletic (Wang et al., 2009). A functional ABC transporter comprises two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). So-called full ABC transporters comprise two TMDs and two NBDs; half transporter proteins consist of one TMD and one NBD and become functional by forming homo- or heterodimers (Dermauwa & Leeuwenab, 2014).

The representatives of only some of these subfamilies have been implicated in ecotoxicological roles, namely ABCB full transporters (ABCB FTs, some of which are also called permeability glycoproteins, P-glycoproteins, or P-gps), ABCC and ABCG (the human ABCG2 is also called the breast cancer resistance protein, BCRP) (Epel et al., 2008; Ferreira et al., 2014; Dermauwa & Leeuwenab, 2014). In at least one case, ABCA transporters have also been found to act in insecticide detoxification (Kalsi & Palli, 2017).

However, compared to the mammalian ABC transporters, the knowledge of their function and role in MXR in aquatic invertebrate and vertebrate species is still scarce (Luckenbach et al., 2014; Jeong et al., 2017). Amphipods (Crustacea: Malacostraca: Amphipoda) constitute with about 10,000 species (Arfianti et al., 2018) a large order within arthropods, often dominate aquatic macroinvertebrate communities and are commonly used in ecotoxicology research (e.g., Kunz et al., 2010; Nyman et al., 2013; Weston et al., 2013; Podlesińska & Dąbrowska, 2019; Jakob et al., 2017; Shatilina et al., 2020; Švara et al., 2022). Importantly, amphipods form multiple flocks of closely related species in several water bodies, including the ancient and pristine Lake Baikal (Copilaș-Ciocianu and Sidorov, 2021). The diversity of ABC transporters in arthropods was recently explored; however, only two amphipod genomes were included in this study due to genomic resource scarcity (Denecke et al., 2021). In addition, the ABCB subfamily was specifically studied in some Baikal amphipod species, but only partial sequences could be identified (Pavlichenko et al. 2014). As a result, information on the proteins that provide MXR in amphipods in general, and Baikal amphipods in particular, is extremely limited.

Moreover, it is still unclear whether long independent evolution in high pristine waters with low amounts of xenobiotics in Lake Baikal could lead to partial loss of MXR function due to relaxed selection, and what it could mean for the future of these endemic species in the context of increasing anthropogenic pollution (Moore et al., 2009; Brown et al., 2021). Some mutations and polymorphisms in the human *ABCB1* gene are known to be directly associated with partial or complete loss of MXR-related functions (Kwan, Gros, 1998; Fellay et al., 2002; Fromm, 2002). In general, studies show that mutations at specific positions in intracellular loops of human ABCB1 (Currier et al., 1992; Loo, Clarke, 1994) and other ABC transporters (Cotten et al., 1996; Seibert

et al., 1996) are able to affect protein and membrane stability. Furthermore, mutations near transmembrane domains have been shown to affect substrate specificity (Choi et al., 1988; Loo, Clarke, 1994) by modulating xenobiotic binding (Safa et al., 1990) and alter drug-stimulated ATPase activity (Rao, 1995). Certain mutations at nucleotide binding sites affect the substrate specificity and xenobiotic resistance profile of the proteins (Shustik, 1995).

One of the most commonly exploited approaches to study the function of a protein is its heterologous expression in cell cultures. Such expression systems are in most cases much more easily manipulated than the source organism, have shorter generation times and can provide tunable protein levels (Moraes et al., 2012). Naturally, the first proteins explored in heterologous systems were human and mouse P-gps (ABCB1/Abcb1) (Evans et al., 1995; Lerner-Marmarosh et al., 1998). These proteins could be produced in the bacterium *Escherichia coli*, yeast *Saccharomyces cerevisiae* and insect/baculovirus system (Sf9) cells with varying degrees of success (Evans et al., 1995); the mouse protein was also purified from the yeast *Komagataella* (*Pichia*) *pastoris* (Lerner-Marmarosh et al., 1998). In addition, human ABCB6 was successfully expressed in the protozoan *Leishmania tarentolae* (Grebowski et al., 2016). Among other ABC transporter classes, the human transporter ABCG2 has been produced in the insect Sf9 cell–baculovirus system (Ozvegy et al., 2001) and even in *Lactococcus lactis* (Janvilisri et al., 2003). The Sf9-based system has also been used for the heterologous expression of P-gp from a protozoan parasite *Leishmania tropica* (Cortes-Selva et al., 2005). The same system was used to produce and study the Abcb4 protein from *Danio rerio* and confirm its role as a P-gp (Fischer et al., 2013). To assess the role of *Drosophila melanogaster* P-gp, the *Mdr65* and *Mdr49* genes were functionally expressed in Sf9 cells, and patch-clamp methods were used to assess the role of these proteins in the electrodiffusion movement of ATP (Bosch et al, 1996). Among other invertebrate species, the P-gp from a parasitic nematode *Haemonchus contortus* was expressed in the mammalian (porcine) cell line LLC-PK1 (Godoy et al., 2016). The P-gp proteins from sheep and pigs were successfully produced in canine MDCK cells (Zahner et al., 2010; Guo et al., 2016). Many plant ABC proteins have been successfully expressed in diverse heterologous expression systems: bacteria *E. coli* and *L. lactis*, yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, mammalian HeLa and HEK293 cells, insect Sf9 cells, *Xenopus* oocytes, and plant cell lines (Lefevre and Boutry, 2018). Overall, ABC transporters have been produced in various expression systems.

Here, we sought to characterize the ABC transporter repertoire of amphipods endemic to the highly pristine Lake Baikal and attempted to create an *in vitro* model for studying these proteins. To achieve this, we first analyzed the diversity of ABC transporters in transcriptomes of over 60 endemic Baikal amphipods in comparison to other related species. Based on the results of this

analysis, we chose the *abcb1* gene from *Eulimnogammarus verrucosus*, a widespread species playing an important role in the lake ecosystem, and successfully established the first proof-of-principle heterologous expression system for an amphipod ABC transporter. In this work, we used the S2 cells to create the heterologous system, as this cell line is easily transfectable (Park et al., 1999; Jongh et al., 2013), can be used to produce membrane proteins (Brillet et al., 2009), is convenient in experimental work, and, moreover, is phylogenetically close to the organism we are studying.

Materials and methods

Computational analysis of ABC sequences

The initial search for ABC protein sequences was performed with a local installation of ABC_scan (Denecke et al., 2021). ABC_scan is a pipeline to identify and classify ABC transporter sequences from genome assemblies, that was developed for non-model species and tested on a large number of arthropod genomes (Denecke et al., 2021). It was run on transcriptome sequences of amphipods and the cotton bollworm *Helicoverpa armigera* (Table S1). The raw RNA sequencing data were from published works (Carlini and Fong, 2017; Chen et al., 2019; Christie et al., 2018; Cogne et al., 2019; Collins et al.; 2017; Hunt et al., 2019; Jin et al., 2019; Kobayashi et al., 2018; Naumenko et al., 2017; O'Grady et al., 2016; Schwentner et al., 2018). The assemblies were performed with rnaSPAdes (Bushmanova et al., 2019) v3.13.1 and published earlier (Drozdova et al., 2021; https://doi.org/10.5061/dryad.fj6q573r9). The published assemblies of *H. armigera* were from (Xiong et al., 2015; Lomate et al., 2018; Yuan et al., 2018). In addition, one set of raw data, SRR4242253 from (Xu et al., 2016), was reassembled with rnaSPAdes v13.3.1. In each transcriptome assembly, the proteins were predicted with TransDecoder (Haas et al., 2013) v5.5.0 with the -single_best_only option enabled and then clustered with cd-hit with the similarity threshold of 0.95 (-c 0.95) to exclude nearly identical proteins. The resulting set of proteins was subjected to ABC_scan analysis with default parameters.

To build the phylogeny of the predicted ABCB full transporter (ABCB FT) sequences (Fig. 1), we chose the most reliable predicted ABCB FT sequences (henceforth complete ABCB FTs) as those having start and stop codons and longer than 1,000 amino acids with a custom script. These sequences were aligned with mafft (Katoh & Standley, 2013) v7.310, and the tree was built with IQ-TREE (Nguyen et al., 2015) v1.6.12. The best-fit model was chosen automatically with ModelFinder (Kalyaanamoorthy, 2017), and the topology was assessed using 1000 Shimodaira-Hasegawa approximate likelihood ratio test replicates with the -alrt 1000 option and approximate Bayes test with the -abayes option (Guindon et al., 2010; Anisimova et al., 2011). The tree (Fig. 1) was visualized with the ggtree (Yu et al., 2016; Yu et al., 2020) v3.2.1 and ggimage (Yu, 2022,

https://CRAN.R-project.org/package=ggimage) packages for the R programming environment (R Core Team, 2022) and finally edited with Inkscape (inkscape.org).

The most probable open reading frames (ORFs) on the transcripts were predicted with ATGpr (Salamov, AA., Nishikawa, T., Swindells, MB, https://atgpr.dbcls.jp/). The topology of Abcb1 proteins was visualized with the Protter web server (Omasits et al., 2014) and further modified with Inkscape (inkscape.org).

Study species and animal sampling

The object of this study was an endemic Baikal amphipod species, *Eulimnogammarus verrucosus* (Gerstfeldt, 1858). This stenobiotic species has a size of up to 45 mm (from telson to rostrum), it is widespread and dominates the benthic communities on the stony beaches of the littoral zone (Kravtsova et al, 2004). The preferred temperature of *E. verrucosus* adults has been determined experimentally to be around 5-6 °C (Timofeyev, Shatilina, 2007). This species may be the most studied representative of the Baikal endemic amphipod fauna (Bedulina et al, 2013, 2017; Rivarola-Duarte et al., 2014; Jakob et al., 2016; Drozdova et al., 2019; Dimova et al., 2018; Shchapova et al., 2019; Shchapova et al., 2021; Zolotovskaya et al., 2021; Lipaeva et al., 2022).

As *E. verrucosus* lives close to the coast, it can be especially influenced by anthropogenic factors, including water pollution by various xenobiotics, and therefore it can be used as a model species to study the effects of pollution on the amphipods of Lake Baikal.Indeed, the sensitivity of this species to some inorganic and organic substances has been explored (Timofeyev et al., 2006; Protopopova et al., 2014; Jakob et al., 2017; Protopopova et al., 2020). Within inorganic pollutants, the best studied in this regard is a model heavy metal, cadmium. The 24-hour 50 % lethal concentration (LC50) in *E. verrucosus* was estimated as about 2×10^{-5} M (Timofeyev et al., 2008; Protopopova et al., 2020). This value is very similar to the 24-hour LC50 value for a widespread species *Gammarus pulex* from Europe (Vellinger et al., 2012), which is widely used as a test species in ecotoxicology (Kunz et al., 2010), and lower than this value in a Holarctic species *G. lacustris* from the Baikal region (4.5×10^{-5} M; Timofeyev et al., 2008). The effects of some organic substances have also been studied. In particular, the addition of natural organic matters, when applied in concentrations that are higher than in Lake Baikal but environmentally realistic for other water bodies, were found to inhibit efflux transporter activity in *E. verrucosus* as effective as verapamil, a known inhibitor of ABC transporters (Timofeyev et al., 2006). Moreover, exposure to a synthetic humic compound HS150 caused a substantial decrease in the abundance of *abcb1* mRNA in this species (Protopopova et al., 2014).

Adult individuals of *E. verrucosus* were collected in May 2018 in Listvyanka (51° 52′14.07″N, 104° 49′41.78″E) with a hand net from 0.5–1 m. All animals used in this study belonged to the W barcoding species (Gurkov et al., 2019; Drozdova et al., 2022). The animals

were transferred to the laboratory in thermostatic boxes for acclimation for three weeks at 6 ± 1 °C in well-aerated Baikal water. During acclimation, the amphipods were fed *ad libitum* with dried and ground invertebrates and algae from their habitat, and water was exchanged every three to four days. No mortality was observed during acclimation. Before RNA isolation, the animals were snap-frozen in liquid nitrogen.

Cloning of *Evabcb1*

Total RNA was extracted from single individuals of *E. verrucosus* by mechanical homogenization in RNAzol RT reagent (MRC), chloroform-assisted phase separation, and alcohol precipitation. The samples were further treated with the RNase-free DNase (Thermo Scientific) and purified with CleanRNA Standard columns (Evrogen) according to the manufacturer's protocol. Two µg of total RNA were used for cDNA synthesis with Oligo(dT)20 primer (Biosset) and the SuperScript III reverse transcriptase (Life Technologies). Synthesis was performed using a C1000 Touch™ thermal cycler (Bio-Rad).

The primers used for *abcb1* amplification, verifying the inserts in the obtained constructs by PCR, and Sanger sequencing are given in Table S2. PCR reactions were performed using a C1000 Touch™ thermal cycler (Bio-Rad) with the Phusion Hot Start II DNA Polymerase (Thermo Scientific). The resulting PCR products were concentrated by ethanol precipitation if needed. The aliquots of each reaction were separated by electrophoresis on a 1% agarose gel in TAE buffer; the gels were stained with ethidium bromide and visualized under UV light. The bands of the expected size were excised, and DNA was purified using the GeneJET Gel Extraction Kit (Thermo Scientific).

The resulting *abcb1* PCR product from *E. verrucosus* was cloned into a piggyBac-based (pPB) transposon plasmid vector conferring blasticidin resistance (Pavlova et al., 2019). Four constructs were generated, containing only the *abcb1* ORF (pPB-pCoBlast-actin5c-abcb1); a slightly shorter ORF (pPB-pCoBlast-actin5c-abcb1(67-1411)); a construct encoding the *abcb1* fusion with the mScarlet-I (Bindels et al., 2016) fluorescent protein to facilitate visualization (pPB-pCoBlast-actin5c-abcb1-mScarlet-I); and a control construct to produce mScarlet-I alone (pPB-pCoBlast-actin5c-mScarlet-I) (Texts S2, S3, S4, and S5; Fig. 2). The expression of each target gene was controlled by the constitutive *Drosophila actin5c* (*Act5C*) promoter. All plasmids contained a blasticidin resistance cassette for selection in S2 cells. Cloning was performed via Gibson assembly using the Gibson Assembly Master Mix (New England Biolabs) or with conventional restriction cloning. Electrocompetent *Escherichia coli* TOP10 cells were used for plasmid amplification and maintenance. The resulting plasmids were isolated using the GeneJet Plasmid MiniPrep or MaxiPrep kit (Thermo Scientific) following the manufacturer's protocols. All plasmids were confirmed by Sanger sequencing.

Cell culture and transfection

Drosophila melanogaster Schneider line-2 (S2) cells of embryonic origin (Han, 1996) were cultured in Shields and Sang M3 insect medium (M3) (Sigma) containing 5% heat-inactivated fetal bovine serum (Gibco), 100 IU/ml penicillin, and 100 mg/ml streptomycin (HyClone) at 25°C and without CO_2 supply. Cells were split to a density of ~5×10^5 cells/ml every 3-4 days.

To generate cell lines stably expressing the Abcb1 from *E. verrucosus*, an S2 cell line was transfected with pPB plasmid constructs using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's instructions. The selection of transfected S2 cells was performed using 10 µg/mL of blasticidin S (Sigma) for two weeks.

Quantitative real-time PCR analysis

The expression level of transgenes was measured by reverse transcription followed by quantitative PCR (RT-qPCR). Total RNA was isolated using RNAzol RT reagent (MRC) according to the manufacturer's instructions. The resulting RNA was treated with DNase I (Thermo Fisher Scientific) and DpnI (NEB). Reverse transcription was performed with the RevertAid reverse transcriptase (Thermo Fisher Scientific) using 2 µg of total RNA in the presence of 2 U/µl of RNaseOut Recombinant RNase Inhibitor (Thermo Fisher Scientific). qPCR was carried out using the BioMaster HS-qPCR SYBR Blue (2×) reagent kit (Biolabmix). PCR amplification was performed using a BioRad C1000 Thermal cycler supplied with CFX96 Real-Time System according to the manufacturer's instructions. The primers are listed in Table S2.

Immunostaining

The intracellular localization of EvAbcb1 in transfected S2 cells was studied by fluorescent immunostaining. Approximately 2×10^6 stably transfected cells were centrifuged at 800 g for 5 min and washed in 3 ml of phosphate-buffered saline (PBS; Sigma). Then, 3 µl of Hoechst 33342 (10 mg/ml, H3570, Invitrogen) and acridine orange (C.I. 46005) (Roth) were added to the cells and incubated for 10 min. The cells were then pelleted by centrifugation, washed with PBS, and fixed for 10 min in 3 ml of 3.7% formaldehyde in PBS. All procedures were performed at room temperature.

Fixed cells were spun down by centrifugation (at 800 g for 5 min), resuspended in 500 µl of PBS and placed onto a clean slide using a Cytospin 4 cytocentrifuge (Thermo Fisher Scientific) at 900 rpm for 4 min. The slides were immersed in liquid nitrogen, washed in PBS, incubated in PBS with 0.1% saponin for 30 min, and then in PBS containing 3% BSA for 30 min. The slides were then immunostained using the following primary monoclonal antibodies, all diluted in PBS / saponin: mouse C219 (1:350, Thermo Fisher Scientific, MA1-26528; this antibody recognizes the internal, highly conserved amino acid sequences VQEALD and VQAALD, which are present in

many ABC transporters); mouse anti-Actin (1:100, Hybridoma Bank, JLa20) and mouse anti-Lamin (1:30, Developmental Studies Hybridoma Bank, ADL67.10). These primary antibodies were detected by incubation for 1 h with anti-Mouse Alexa Fluor 488 (1:300, IgG2a, Invitrogen, A11029) and anti-Mouse Alexa Fluor 488 (1:300, IgM, Invitrogen, A10667) secondary antibodies. Slides were mounted in Vectashield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) or in ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) to stain DNA and reduce fluorescence fading. Images of fixed cells were obtained on a Zeiss LSM 710 confocal microscope using a plan-apo 63×/1.40 oil lens and the ZEN 2012 software.

Live cell imaging

Before microscopic analysis, live S2 cells were transferred onto glass-bottomed culture dishes (Invitrogen A-7816) treated with 0.25 mg/mL of concanavalin A (Sigma–Aldrich, C0412) for 2 h to favor cell attachment to the substratum. Half of a µl of 10 mg/ml Hoechst 33342 was added to 500 µl of the cell suspension. Observations were made after 10 min of incubation of the culture dishes at room temperature. Images of living cells were obtained on a Zeiss LSM 710 confocal microscope using a plan-apo 63×/1.40 oil lens and the ZEN 2012 software.

Western blot analysis

Transfected and control S2 cells were harvested by centrifugation at 200 g for 5 min at room temperature, washed with PBS, and centrifuged again. Then we homogenized and lysed S2 cell pellets in RIPA buffer (Sigma, R0278) containing 1x Halt protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, 1861282). The lysates were clarified by centrifugation at 15 000 g for 15 min at 4 °C, and the protein extracts were normalized using the DC Protein Assay (Bio-Rad, 5000116). Before SDS-PAGE analysis and subsequent immunoblotting, each normalized sample was mixed with an equal volume of (2x) Laemmli buffer and incubated for 5 min at 95 °C. The primary antibodies were mouse monoclonal anti-Lamin Dm0 (1:300; Developmental Studies Hybridoma Bank, ADL67.10), and mouse monoclonal C219 (1:3500, Thermo Fisher Scientific, MA1-26528). They were detected using HRP-conjugated goat anti-mouse (1:3500; IgG, Life Technology, G-21040) secondary antibody. Images were captured using an Amersham Imager 600 System (GE Healthcare).

Hoechst 33342 efflux assay

To analyze the functional activity of the EvAbcb1, 11×10^4 stably transfected cells were transferred on the µ-dish (35 mm, IBIDI) treated with 0.1 mg/ml poly-D-lysine (Sigma, P1149), where they were incubated for two days at 25 °C. Experiments were performed with the addition of verapamil (Sigma, V4629) or cyclosporin A (LC Laboratories, 59865-13-3), as well as without adding an inhibitor. Thus, at the next step, 4 µl of the inhibitor or DMSO control (AppliChem,

A3672, 0100) was added to 800 µl of the cell suspension, and the dish was incubated at 25 °C for 10 min. The medium was removed from the dish, the cells were washed one time with PBS. After that, 1 µl of 0.5 mM Hoechst 33342 in 1 ml of fresh PBS was added to the cell suspension. After 10 min of incubation at 25 °C, the culture dishes were observed. Images of living cells were obtained with a Leica DM4000/6000 M fluorescence microscope (Leica Microsystems) using Filters A (ex 340-380, DC 400, EM LP 425), L5 (ex 440-520, DC 505, EM LP 497-557), N2.1 (EX 515-560, DC 580, EM LP 590), and HCX PL FLUOTAR 63× / 1.25 OIL lens. The same settings for image acquisition and processing have been applied for all samples to allow comparison of the fluorescence intensities between different samples.

The resulting images were analyzed in the ImageJ (Fiji) program following the method of determining the cell fluorescence level using fluorescent microscopy images (Fitzpatrick, 2014). The experiments were performed in two replicates for each concentration of each inhibitor. Statistical analysis of the data and preparation of illustrative material were carried out in the R programming environment (R Core Team, 2022) using the ggplot2 (Wickham, 2016) and ggsignif (Ahlmann-Eltze and Patil, 2021) packages. To compare values in different groups, we used the non-parametric Mann-Whitney criterion with Holm's correction for multiple comparisons where applicable. To compare different treatment conditions with the control, we used the Kruskal-Wallis test with Dunn's post-hoc test with Holm's correction.

Results and Discussion

The diversity of ABC transporter sequences in amphipod transcriptomes

Recently, Denecke et al. (2021) published a pipeline intended to explore the diversity of ABC transporters, ABC_scan, and successfully applied it to many arthropod groups. However, the small number of published amphipod genome assemblies makes drawing conclusions about ABC transporters in Amphipoda quite difficult. To perform a little step towards filling this cavern, we used the transcriptome data as input to ABC_scan. To check if the results of searching a genome and a transcriptome for ABC transporters would be comparable, we ran ABC_scan on several transcriptome assemblies of *Helicoverpa armigera*, the genome of which was analyzed for ABC sequences earlier (Denecke et al., 2021). Indeed, the results were convincingly similar (Table S1). Thus, we applied ABC_scan to a collection of various amphipod transcriptomes (see Materials and Methods) reassembled with rnaSPAdes (Bushmanova et al., 2019), which we earlier found to be more suitable than Trinity (Haas et al., 2013) for phylogenetics-related purposes (Drozdova et al., 2021). Unfortunately, the quality of the assemblies did not allow us to compare the overall numbers of ABC transporters or their subclasses between species, as BUSCO completeness and the number of unique ABC transporter sequences were strongly correlated (Fig. S1).

However, this analysis allowed us to conclude that all ABC transporter classes are present in most amphipod species (Fig. 1A for high-quality transcriptome assemblies, i.e. those with >85% BUSCO completeness; Table S1 for all data). Then, we turned to ABCB full transporters, which are implicated in detoxification and are also the best studied group, and explored such sequences recovered from transcriptome assemblies. Since most Baikal amphipods had only one ABCB FT-encoding gene, the *abcb1* gene, as we suggest denoting it, it is most probably the only such gene expressed under normal conditions in these species. In some species (for example, in a deep-water scavenger *Ommatogammarus flavus*), we also found additional fragmented sequences, which were most similar to ABCB FTs. These sequences may represent other paralogs with low (potentially condition-specific) expression. However, a deeper insight into the number of *abcb* paralogs in different amphipod groups would require comparison of transcriptomes in similar conditions or full genome assemblies, which are available for only a few species to date. Nevertheless, this analysis provided sequence data for further study.

We then selected the contigs encoding the complete ABCB FT sequences (> 1,000 amino acids; start and stop codons present) to be able to design primers for further amplification. All studied amphipod assemblies had no or only one such sequence. The obtained tree of predicted complete ABCB FT protein sequences showed very high conservation of these sequences within Baikal amphipods and even with some other related non-Baikal species of the genus *Gammarus* (Fig. S2 for all found sequences and Fig. 1B for those in high-quality transcriptome assemblies). This similarity suggests that any of these sequences could serve as good model to explore the properties of ABCB FTs in Baikal amphipods.

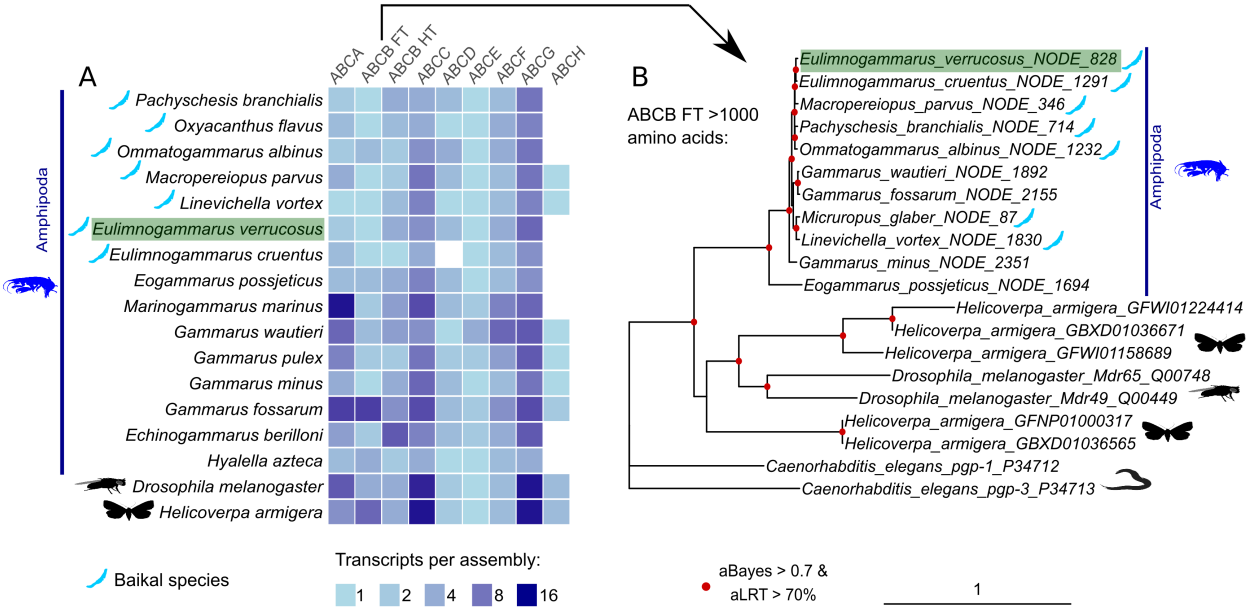

Fig. 1. The diversity of ABC transporters in selected amphipod species and the phylogeny of complete ABCB FTs shows their high conservation. (A) The number of transcripts of each ABC

transporter class found in all high-quality amphipod assemblies (BUSCO completeness > 85%). ABCB FT, ABCB full transporter; ABCB HT, ABCB half transporters. (B) The phylogeny of complete ABCB FTs longer than 1000 amino acids found in these assemblies. A maximum likelihood tree of amino-acid sequences is shown; the tree scale features the number of substitutions per site. aBayes, the approximate Bayes test; aLRT, the approximate likelihood ratio test. Sequence identifiers containing “NODE” correspond to the names of the transcripts in the assemblies available at https://doi.org/10.5061/dryad.fj6q573r9; sequence identifiers starting with “G” are NCBI GenBank accession numbers; and those starting with “P” or “Q” and accompanied with gene names are taken from UniProt.

Within the amphipods from Lake Baikal, we chose the species *E. verrucosus*. It is very abundant in the littoral, well-studied and relatively sensitive to organic and inorganic pollutants (see Materials and methods). It is important to note that *E. verrucosus* tends to escape non-optimal conditions, e.g. increasing water temperatures, by migrating to greater depths rather than by adjusting its physiology (Jakob et al., 2016). As the phylogenetic analysis showed high similarity between ABCB transporter sequences from all studied Baikal amphipods (Fig. 1), we further used the *abcb1* of *E. verrucosus* as a representative example of a Baikal amphipod full ABCB transporter. In the *E. verrucosus* transcriptome assembly, we found a 4824-nucleotide-long transcript that contained a complete open reading frame (4236 bp) encoding a 1411-amino acid-long protein. We will therefore call this protein EvAbcb1. Its predicted structure was typical for ABC transporters and contained two transmembrane domains and two nucleotide-binding domains (Fig. 2).

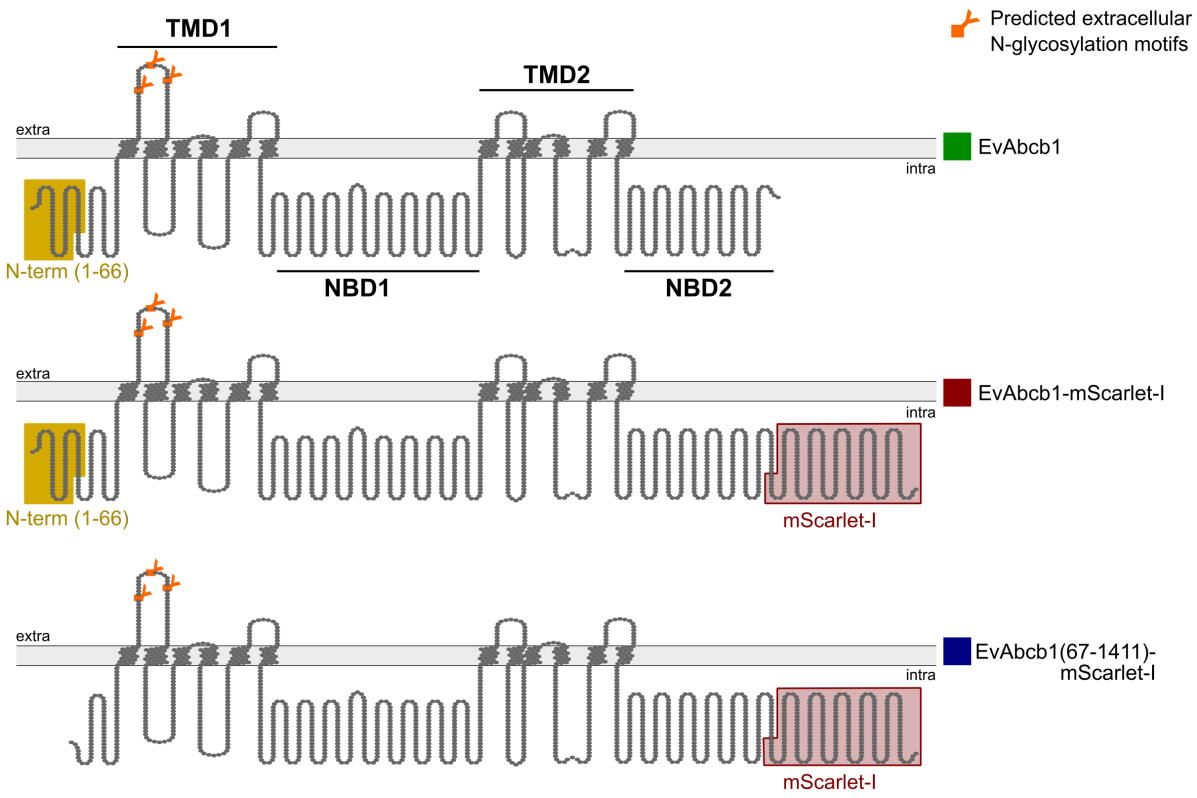

Fig. 2. Secondary structure of EvAbcb1 as predicted with Protter (Omasits et al., 2013) and a schematic showing the difference between the expression constructs with *Evabcb1* used in this work.

Interestingly, the 148-amino acid sequence preceding the first transmembrane domain was slightly longer than in well-studied invertebrate ABCB FTs, such as the *D. melanogaster* Mdr49 (1-43) or Mdr65 (1-65) or P-gp from the oyster *Crassostrea ariakensis* (1-92 according to (Xu et al., 2014)). To check for the most probable translation start site, we predicted it with ATGpr; indeed, the most reliable ORF was the longest one. It was also true for the ABCB FT sequences of other amphipods (Text S1) and thus it might be a feature of amphipods. As it was unclear whether this part of the protein plays a particular role in its function or stability, we generated an additional construct with a truncated N-terminus (hereafter Evabcb1(67-1411)-mScarlet-I; see below).

Stable expression of *Evabcb1* in the *Drosophila* S2 cell line

The *E. verrucosus abcb1* ORF was amplified from cDNA and cloned into the pPB-pCoBlast plasmid vector (Pavlova et al., 2019) under the control of a strong constitutive *actin5c* promoter from *D. melanogaster*. In addition, we generated an analogous construct for the production of ABCB1-mScarlet-I fusion for easier visualization and an analogous mScarlet-I construct to use as a control. These constructs were individually and stably transposed into *D. melanogaster* S2 cells.

To check the functional properties of the expression system, we studied (i) the intracellular distribution of the proteins of interest, EvAbcb1, EvAbcb1-mScarlet-I, and EvAbcb1(67-

1411)-mScarlet-I, with immunostaining, (ii) the amount of the *abcb1* transcript with RT-qPCR and (iii) the amount of the Abcb1 proteins with western blotting. We found that at least the mScarlet-I-tagged protein was clearly localized at the cytoplasmic membrane (Fig. 3 B). The heterogeneous *abcb1* gene was expressed at much higher levels (at least 1000-fold higher) than the native *Drosophila Mdr49* and *Mdr65* genes encoding the fruit fly *abcb* homologs. The third endogenous fly *abcb*, *Mdr50*, had no detectable expression in any of the samples (Fig. 3 E). Finally, at the protein level, EvAbcb1 and Abcb1-mScarlet-I proteins formed smears on a polyacrylamide gel, which is typical for glycosylated proteins (Greer, Ivey, 2007); the levels of the two proteins were similar. As expected, the EvAbcb1-mScarlet-I migrated slower than EvAbcb1 due to the presence of the mScarlet-I tag. The predicted molecular weights of the two proteins were 154 and 182 kDa, respectively. The N-terminal-truncated protein (EvAbcb1(67-1411)-mScarlet-I) was virtually indetectable on western blots and only produced very faint fluorescence (Fig. 3 D). These data suggest that the N terminus of the EvAbcb1 protein is important for its stability.

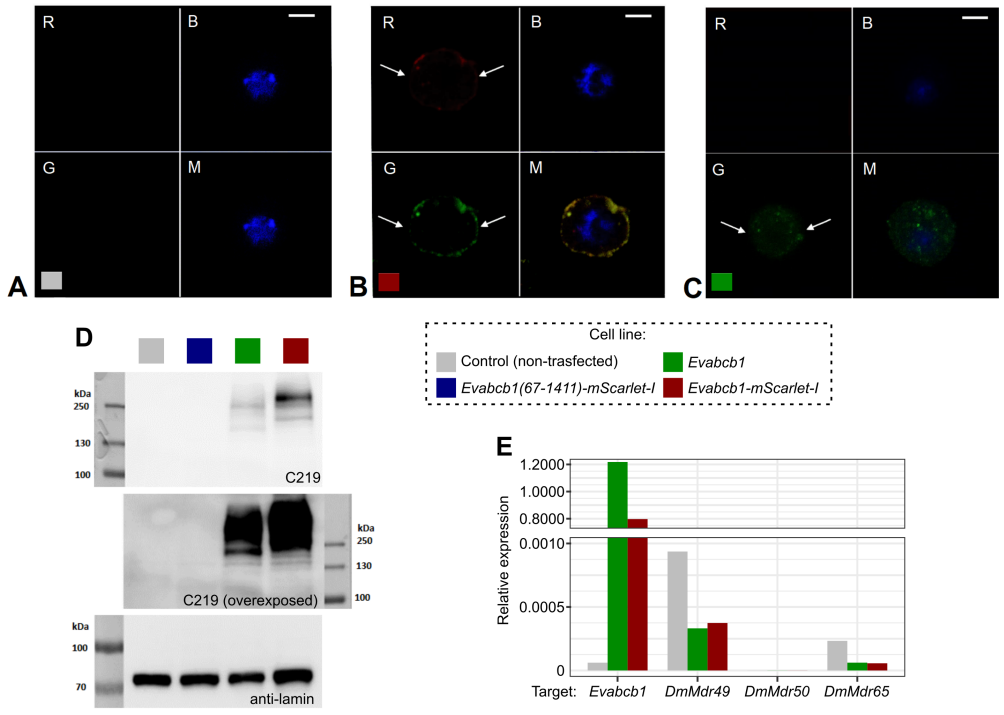

Fig 3. Quality control of the stably transfected cells obtained. (A-C) Localization of transgenic EvAbcb1 at the cell membrane is shown by fluorescent immunostaining of fixed wild-type S2 cells (A) compared to the cells expressing EvAbcb1-mScarlet-I (red channel, R) (B) or untagged EvAbcb1 (C) proteins with antibodies specific to P-gp, which were detected using secondary antibodies conjugated with Alexa Fluor 488 (green channel, G). The green signal of the antibody coincides with the mScarlet-I signal in (B), confirming the specificity of immunostaining. DAPI (blue channel, B) was used to detect DNA. Scale bar, 5 μm. M, merged images. (D) Western blotting analysis. For detection, we used an antibody against Abcb1, and for

loading control, we used an antibody against *Drosophila* Lamin Dm0. (E) The expression level of *abcb1* is higher than that of *Mdr49*, *Mdr50*, and *Mdr65* (native *D. melanogaster* MDR genes) in stably transfected cells of the EvAbcb1 and EvAbcb1-mScarlet-I lines as detected by RT-qPCR analysis.

Finally, we checked the functional activity of the protein using Hoechst 33342 (Fig. S2), a dye known to be a human ABCB1 substrate (Müller et al., 2007; Subramanian et al., 2018). At the same time, the relative concentration of EvAbcb1 was monitored via the mScarlet-I fluorescence in the case of the fusion construct. We observed that untagged EvAbcb1 pumped out the dye very efficiently, as there were very few cells with Hoechst 33342 fluorescence (Fig. 4 A, B), and was partially inhibited by cyclosporin A and verapamil (Fig. 4 A, C). At the same time, mScarlet-I-tagged EvAbcb1 also effluxed the dye, even though less efficiently (Fig. 4 A, B), and was fully inhibited by cyclosporin A and verapamil (Fig. 4 C, right panel).

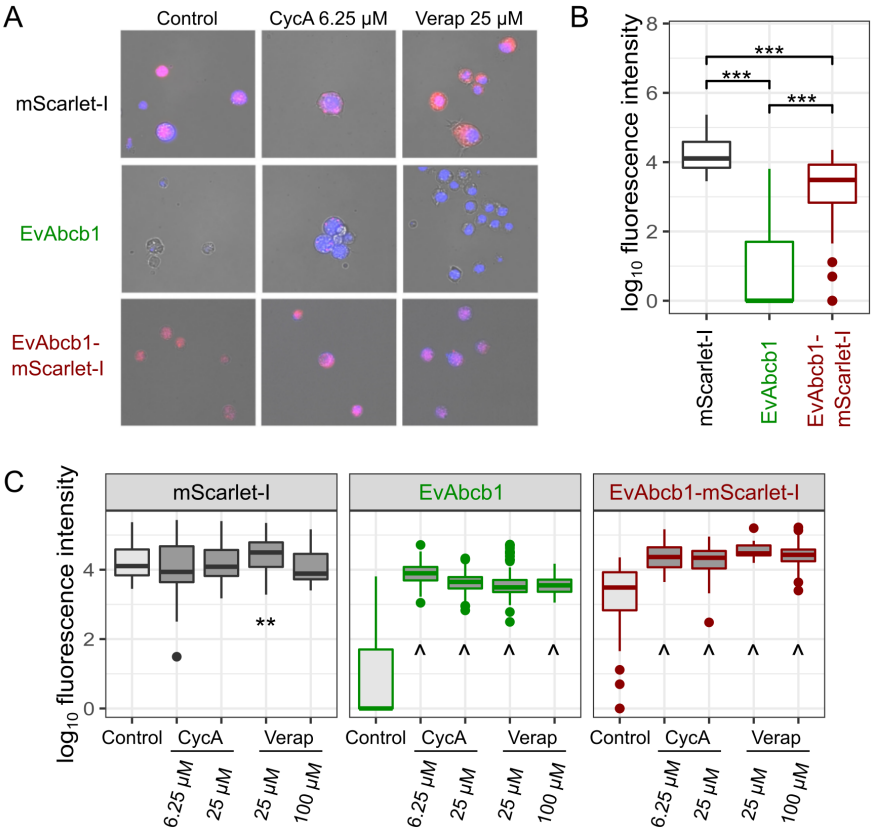

Fig 4. Efflux of the Hoechst 33342 dye by stably transfected S2 cells producing mScarlet-I, EvAbcb1, and EvAbcb1-mScarlet-I. (A) Representative photographs of S2 cells stably transfected with the corresponding constructs (rows), stained with Hoechst 33342 and treated with the indicated inhibitors (columns). Shown is the overlay of the blue and red channels (Hoechst 33342 and mScarlet-I signals, respectively). (B-C) Quantitative analysis of Hoechst 33342 fluorescence (inversely related to efflux activity). Note that the vertical axis is in base 10 logarithmic scale. (B)

Efflux activity in cells expressing untagged EvAbcb1 is higher than in those expressing EvAbcb1-mScarlet-I, but both transgenic cell lines exhibit significant efflux activity compared to the control line expressing mScarlet. ***, $p < 0.001$ (Mann-Whitney U-test with Holm's correction for multiple comparisons). (C) Efflux activity of both EvAbcb1 and EvAbcb1-mScarlet-I proteins is blocked by known transporter inhibitors. CycA, cyclosporin A; Verap, verapamil. **, $p < 0.01$; ^, $p < 0.0001$ (Dunn's test with Holm's correction for multiple comparisons). The raw data are available from Table S3.

Summary

In this work, we analyzed the diversity of ABC transporters in Baikal endemic amphipods and related species based on the available transcriptome assemblies. We found that most ABC transporter classes are present in all analyzed species, and most Baikal species express at most one ABCB full transporter at detectable levels under normal conditions. Moreover, the ABCB protein sequences were conservative across different species. It is important to note that all predicted complete ABCB full transporters of amphipods have longer N-termini preceding the first transmembrane domains compared to many well-studied sequences of this family from other invertebrates. Based on these results, we chose an endemic Baikal amphipod species *Eulimnogammarus verrucosus*, which is very abundant in the littoral and relatively well-studied, as the model for studying the features of its Abcb1.

For that, we cloned the *abcb1* gene from *E. verrucosus* and established the first heterologous expression system for an amphipod ABC transporter using *D. melanogaster* S2 cell line. Our results indicate the suitability of this system for studying invertebrate Abcb1 homologs, as we could detect the expression of the target gene at a much higher level than the expression of the homologous fly genes, and the target protein demonstrated high efflux activity. Interestingly, the N-terminal part of the protein (absent from the well-studied related proteins from other invertebrate species) turned out to be important for its stability. This result highlights the importance of studying this group of proteins in non-model species. It is also important to note that the cell line producing the protein with the C-terminal mScarlet-I tag had decreased efflux activity. Thus, tagging should be used with caution.

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Data availability

Annotated sequences of the obtained plasmids are available in the Supplementary materials (Texts S2–S5) and from NCBI GenBank (**OQ123895**–**OQ123898**). The code used for data analysis is available at GitHub (https://github.com/drozdovapb/code_chunks/tree/master/Evabcb1_Pgp_production).

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