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Identification and characterisation of a multidrug resistance-related protein mRNA in the blue mussel *Mytilus edulis*

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ABSTRACT: Membrane-associated transport proteins were discovered in the 1970s in mammals and were considered to be expressed in response to chemotherapy during cancer treatment. Prominent members of this class of proteins are multidrug resistance-related or -associated proteins (MRPs). Besides their expression in cancer cells, MRPs are ubiquitously expressed in normal tissues and are active transporters of reduced glutathione, glucuronate and organic anions of toxicological relevance, either conjugated or unconjugated with sulphate. MRPs may also provide aquatic organisms with resistance to chemicals in a polluted environment by binding xenobiotics and excreting them from cells in an energy-dependent manner. The present study investigated expression of MRPs as part of the multixenobiotic resistance (MXR) system in the blue mussel *Mytilus edulis*. We isolated and characterised 2 putative *mrp* cDNA fragments, *mrp1* and *mrp2*, which showed 50 to 70% homology on the protein level with MRPs of other species. The *mrp1* fragment could not be linked with any mRNA in Northern blots of *M. edulis* tissues, whereas the *mrp2* fragment hybridised with an mRNA of approximately 4.6 kb. *Mrp2* showed tissue-specific expression patterns. Highest expression was found in digestive gland and gill tissue. Its expression could be induced 2-fold by the model carcinogen 2-acetylaminofluorene (AAF), whereas *mrp1* expression was unaffected. The cDNA fragment of the inducible form was then integrated into a multiplex PCR system for analysis of multixenobiotic resistance in the blue mussel, in concert with other detoxification and biotransformation genes.

KEY WORDS: Multixenobiotic resistance · MXR · Multidrug resistance-related protein · MRP · Multiplex PCR · *Mytilus edulis*

INTRODUCTION

Multidrug resistance-related proteins (MRPs) are members of the ATP-binding cassette (ABC) superfamily of transport proteins. This superfamily is among the largest and most widespread protein family known so far and its members are responsible for the transport of a wide variety of compounds across biological membranes, including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, drugs and other xenobiotics (Higgins 1992, Cole & Deeley 1998, Borst et al. 1999, 2000, Klein et al. 1999). MRPs are highly conserved among species from microorganisms to man. MRP-mediated xenobiotic transport processes have been extensively studied in mammals but far fewer data are available for marine organisms, despite the fact that the function and regulation of expression of xenobiotic transporters in marine organisms, and especially in marine invertebrates, are important issues in ecotoxicology.

After the discovery of P-glycoprotein (P-gp)-mediated drug resistance, it was widely believed that P-gp1, encoded by the multidrug resistance gene *mdr1*, is the exclusive transporter involved in multidrug resistance

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(MDR) (Roninson 1987). However, in the second half of the 1980s, evidence was provided showing that this was not the case (Baas et al. 1990, Nygren et al. 1991, Eijdems et al. 1992). Several cell lines were characterised that displayed a MDR phenotype without detectable expression of P-gp. One of these cell lines was H69AR, a doxorubicin-selected human tumour cell line expressing non-P-gp MDR (Slovak et al. 1993). MRP1 was discovered in 1992 by differential hybridisation of this cell line. The human cDNA encoding MRP1 was first cloned from a drug-selected lung cancer cell line, resistant to a variety of natural products that were used as chemotherapeutic agents (Cole et al. 1992). ABC transporters typically contain 2 types of structural domains: hydrophobic polytopic membrane-spanning domains (MSDs), and hydrophilic cytosolic nucleotide-binding domains (NBDs). MSDs are highly conserved among ABC (super)family members showing 30 to 40% identity, whereas NBDs generally show little sequence identity with one another, consistent with the notion that MSDs are the major determinants of substrate specificity of ABC transporters (Hipfner et al. 1999).

Mussels of the genus *Mytilus* are among the most common marine molluscs and are an important element in the ecology of coastal waters. They are sessile filter feeders and effectively concentrate trace toxic substances. Therefore, mussels are now widely used as model organisms to study physiological, biochemical, genetic and toxicological responses to environmental changes, including pollution. We therefore selected the blue mussel *Mytilus edulis* to investigate transcriptional regulation of MDR-related detoxification. The proteins involved are the so-called phase 0 or phase III proteins (Koehler et al. 2004). These proteins can act as a first line of defence by directly clearing xenobiotics from the cytoplasm and/or the nucleus of cells (phase 0), alongside their capacity to remove components after conjugation by phase II enzymes (phase III reactions). Multixenobiotic resistance is a MDR-like system in marine invertebrates (McFadzen et al. 2000, Smital et al. 2000). Its relevance is shown by its potential to protect aquatic organisms from nuclear damage (Waldmann et al. 1995) and deleterious effects during cell division in embryonic development (Toomey & Epel 1993). The expression of multixenobiotic resistance proteins is inducible by exposure to toxic compounds (Minier & Moore 1996), and the levels of these proteins in organisms vary strongly in differently polluted sites (Minier et al. 1999). Accordingly, the multixenobiotic resistance phenotype may serve as an index of exposure to toxic compounds (Minier et al. 1999).

The relevance of the multixenobiotic resistance system for aquatic organisms is illustrated by the impact of multixenobiotic resistance inhibitors, the so-called chemosensitisers, that can inhibit multixenobiotic resistance-dependent defence mechanisms of marine organisms and so alter processes such as uptake, intracellular accumulation, response, synergism and toxicity of xenobiotics. For example, such inhibitors can enhance accumulation of carcinogenic aromatic amines in mussel (Smital & Kurelec 1998). This property of inhibiting the defence mechanisms of organisms against xenobiotics classifies multixenobiotic resistance inhibitors as highly hazardous environmental pollutants.

Recently, we identified mRNA sequences of some of the most prominent multixenobiotic resistance-related transporter genes, such as P-gp (*pgp*) and the major vault protein (*mvp*), in the blue mussel (Luedeking & Koehler 2002). From field sampling studies we know that *pgp* gene expression can be inhibited at polluted sites (A. Koehler et al. unpubl. data). The study of Lin et al. (2002) with knock-out mouse fibroblast cell lines suggested the existence of a compensatory mechanism in P-gp- and MRP-mediated resistance, by which the loss of one transporter can be functionally compensated by (over) expression of the other in the transport of substrates like calcein. As a consequence, the expression of P-gp and MRPs may be considered as a functional network. The study of the functioning of such a network may contribute to the improvement in our understanding of ecologically relevant issues. So far, the only available mRNA sequences of multixenobiotic resistance-related transporter genes in *Mytilus edulis* are *pgp* and *mvp*. Therefore, we applied degenerate primers in RT-PCR reactions to amplify defined fragments of a putative *mrp*-like gene of *M. edulis.* Using sequence information, we subsequently integrated an inducible form of *mrp* into our multiplex PCR system that is used to determine coordinated expression of multixenobiotic resistance- and biotransformation-related genes in *M. edulis*. RT-PCR techniques enable the analysis of regulation and/or inhibition of expression of these genes at the transcriptional level, in response to xenobiotics. The identification of 2 putative MRP isoforms in *M. edulis* may serve as the basis for further studies analysing whether and under which conditions a functional network of multixenobiotic resistance-related transporters may exist in marine invertebrates.

MATERIALS AND METHODS

Materials. For RT-PCR, antisense transcription was performed with 2 µg of total RNA, 50 units mMulv antisense transcriptase, 50 pmol oligo-dT primer and 1 mM each of dATP, dGTP, dCTP and dTTP (dNTPs) in a total volume of 20 µl, at 40°C for 60 min. Antisense tran-

scriptase mMulV, deoxynucleotides and *Taq* polymerase were purchased from Sigma. Oligo-dVT primers and specific primers for multiplex PCR were obtained from Life Technologies. Northern blot equipment was purchased from Roche. All other chemicals and supplies were of the purest grade available and were obtained from commercial sources.

Animal handling. Mussels (*Mytilus edulis*), which were approximately 5 cm in shell length, were collected in the neighbourhood of the German islands Sylt and Helgoland. They were then kept at 15°C in a current water flow system without feeding for a minimum of 3 and maximum of 10 d until RNA extraction.

Isolation of total RNA. The posterior adductor muscle, digestive gland, mantle and gills were isolated from the mussels and washed twice in sterile saline. Total RNA was prepared from all samples immediately after isolation with 1 ml of RNApure reagent (peqlab), according to the manufacturer's specification. An additional purification step with the High Pure RNA Tissue Kit (Roche) was necessary to remove remaining contamination of polysaccharides and DNA. Total RNA concentrations were determined by UV spectroscopy and RNA quality was checked by non-denaturing gel electrophoresis. Ratios of absorbance values at 230 nm and 260 nm and at 260 nm and 280 nm had to be at least 2.0 for good-quality RNA batches when determined with a Biophotometer (Eppendorf).

Degenerate primers and PCR. After cDNA synthesis, 1 µl of the reaction mixture was used as template for subsequent PCR. In a total volume of 50 µl, 2.5 units of *Taq* polymerase and 0.2 mM of each of the dNTPs were used.

Multixenobiotic resistance gene-specific primer pairs were selected for highly conserved regions of the mRNA after sequence alignment of all so far known vertebrate and invertebrate species (Luedeking & Koehler 2002). The sequences of the primers used were as follows: *mrp1270* antisense TCI ACI GCI ACI ATR TTI GTY TC; *mrp1396* antisense GGR TCI AGR TTC ATI CG; *mrp1531* antisense GCR TCY TTI GCC ATI CC; *mrp1396* sense CGI ATG AAR CTI GAR CCI TT; *mrp1270* sense GAR ACI AAY ATI GTI GCI GT and *mrp730* sense GAY IGI ACI GAR ATI GGI GAR AAR GG. All primers were used at a final concentration of 2 µM.

Agarose gel electrophoresis (2% agarose, and TBE buffer consisting of 1.0 M Tris, 0.9 M boric acid, 0.01 M EDTA) was performed with 10 µl of each sample. After staining with ethidium bromide, bands of predicted sizes were isolated from the gels and cloned into the sequencing vector Topo 2.1 (Invitrogen). Both strands of at least 3 clones for every cDNA were sequenced. Sequencing of the successfully-cloned inserts was performed by GATC (Konstanz). Blast (sequence similarity) searches were performed using the FASTA3 internet service (version 3.3t07 Sept. 19, 2000) of the European Molecular Biology Laboratories (Pearson & Lipman 1988).

Multiplex PCR. For multiplex RT-PCR, the following primer pairs and concentrations were used: *mrp2* sense AAA GAC GGA CTG GAT CAC CA and antisense AAA TTG GTC GGT GAG TCG AA, 296 bp product, 0.2 µM each; *pgp* sense CAG AGG TTC TAT GAC CCA GAT GCA G and antisense GTT CTC ACT CTC AGA GTC TAA TGC AG, 381 bp product, 0.3 µM each; *topoisomerase II* sense CTT CTC TGA TAT GGA CAA ACA TAA GAT TCC and antisense GGA CTG TGG GAC AAC AGG ACA ATA C, 664 bp product, 0.2 µM each; *mvp* (*lrp*) sense ACA GGT TGT AAC TCC CTT G and antisense CTT CAT GAT GAC CTC GAC C, 818 bp product, 0.3 µM each; *gst-pi* sense GTT AGA GGC CGA GCT GAA GC and antisense GGC ATA TTC TTG ACG TGG TC C, 545 bp product, 0.1 µM each; *actin* sense CTG AGA GTA GCA CCA GAG G and antisense TAG CGC CGA TGA TGA CGC C, 429 bp product, 0.1 µM each; *hsp70* sense GAC TTG GGT GGT GGA AC and antisense GGC TAC AGC TTC ATC AGG G, 516 bp product, 0.1 µM each. Single-target PCR for *hsp70* and *mrp2* was performed with the same primers that were used in multiplex PCR. *Mrp1* primers were as follows: *mrp1* sense GGT GGC GGT TGA AAG AGT AA and antisense CCG AAA TAT AGC TTG GGA CAA, 0.2 µM each. Amplification was performed with 29 sequential cycles of 95°C for 45 s, 58°C for 60 s, 70°C for 60 s, followed by a final 2 min extension at 72°C. Equal aliquots of each PCR reaction were separated on a 2% agarose gel using TBE buffer, and gels were photographed following ethidium bromide staining. Gels were analysed with 1D image analysis software (Kodak Digital Science). After determination of tissue-specific expression in gills, mantle, digestive gland and muscle using primer concentrations of 0.1 µM, primer pair concentrations were modified in the subsequent experiments in order to obtain similar amplification of all genes in every PCR cycle.

Northern blotting and transcript size determination. For Northern blot analysis, 10 µg of total RNA obtained from the different mussel tissues was separated by electrophoresis on a 0.8% agarose-formaldehyde gel in MOPS (3-[N-morpholino]propanesulphonic acid) buffer at 60 V and 50 mA for 2 h. RNA was then transferred to a positively-charged nylon membrane by capillary blotting overnight in 10× saline-sodium citrate (SSC). RNA was baked to the blot at 120°C for 30 min. After prehybridisation, a denatured digoxigenin (DIG)-labelled *mrp2* probe was added. Hybridisation was performed with PCR fragments at 40°C for 16 h. The hybridisation buffer contained

5× SSPE buffer (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.005 M EDTA), 50% formamid, 5× Denhardt's reagent (50 mg Ficoll, 50 mg polyvinylpyrrolidone [PVP], 50 mg RNase-free bovine serum albumin) and 0.5% sodium dodecyl sulphate (SDS). Detection was carried out with the DIG Detection Kit (Roche) according to the manufacturer's instructions. RNA I and II markers (Roche) were used to determine molecular weights. Labelled PCR fragments, amplified with the primers that were also used in multiplex PCR, gave acceptable results as probes in hybridisation procedures.

Exposure to 2-acetylaminofluorene (AAF). Mussels were kept in groups of 6 in 5 l seawater for 3 d at 15°C with constant oxygenation. Induction of gene expression was initiated with a single dose of 200 µM AAF. Since AAF is water insoluble, emulsions of AAF in vegetable oil were used. No lethal effects were observed for up to 10 d of incubation.

RESULTS

Identification of 2 *mrp* homologue fragments in *Mytilus edulis*

Various pairs of degenerate oligonucleotides were designed *in silico* to clone fragments of *mrp* gene homologues in *Mytilus edulis*. Conserved regions of the mRNA of *Rattus norvegicus*, *Homo sapiens*, *Drosophila melanogasta* and *Caenorhabditis elegans* *mrp* genes were taken as the basis for the primer sequences. These oligonucleotides were used to amplify sequences from *M. edulis* mantle tissue in RT-PCR. Mantle tissue was used instead of gill and digestive gland tissue to avoid contamination by nutrient particles such as plankton, and thus amplification of plankton RNA. The primer-annealing step of the PCR was performed at 45°C. As a consequence, a short first elongation step at 60°C was used to stabilise primer-template binding before final elongation at 70°C. Fragments of the predicted size containing the correct primer sequences and open reading frames were detected for 2 primer pair combinations: *mrp1531* antisense GCR TCY TTI GCC ATI CC and *mrp1396* sense CGI ATG AAR CTI GAR CCI TT, which produced a 414 bp fragment, referred to as *mrp2*; and *mrp1396* antisense GGR TCI AGR TTC ATI CG and *mrp1270* sense GAR ACI AAY ATI GTI GCI GT, which produced a 360 bp fragment, referred to as *mrp1*. Both fragments were isolated from the gel and cloned into the Topo 2.1 vector. Computer-based sequence analysis using the FASTA3 internet service revealed high sequence homologies with other species at the nucleotide level (data not shown). Homologies at the protein level are summarised in Table 1, and specified for the human MRP1 protein in Fig. 1. Sequence data were then submitted to GenBank: *mrp1* with accession number AF397142 and *mrp2* with accession number AF 397143.

Tissue distribution patterns of *mrp2* expression in *Mytilus edulis*

Based on the sequence information, specific nested primers for the *mrp1* and *mrp2* sequences were synthesised for use in multiplex PCR and to produce probes for Northern blot experiments. Only *mrp2* was used in multiplex PCR, where the resulting fragment appeared as a 296 bp band. Comparison of the amplification behaviour of the *mrp2* fragment alone and as part of the multiplex reaction shows that *mrp2* and the other fragments of the system did not affect each other when amplified in parallel (Fig. 2). Fig. 2 also shows the tissue-specific expression of *mrp2* in gill, mantle, digestive gland and posterior adductor muscle. Highest expression levels were found in the digestive gland. In the gill, expression was lower but strongly detectable. Lowest expression occurred in mantle and muscle at a similar level. Interestingly, similar tissue-specific expression profiles have been found for *pgp* and *gst-pi* (Luedeking & Koehler 2002).

Transcript-size determination by Northern blotting

The fragment of *mrp2* amplified in the multiplex PCR reaction was used as a probe in Northern blot experiments. A single band of approximately 4.6 kb was detected in gill and digestive gland tissue preparations (Fig. 3). This appeared to be in agreement with

Table 1. *Mytilus edulis*. Percentage of homology at the protein level of MRP1 and MRP2 fragments with MRP forms from other species

Species	Protein	Percentage of homology
Fragment 1 (*mrp1*)		
Homo sapiens	MRP1	55.6
Homo sapiens	MRP2	55.2
Rattus norvegicus	MRP3	54.1
Homo sapiens	MRP3	51.8
Caenorhabditis elegans	MRP	53.3
Fragment 2 (*mrp2*)		
Homo sapiens	MRP1	71.7
Homo sapiens	MRP3	71.0
Rattus norvegicus	MRP3	71.0
Homo sapiens	MRP2	67.9
Caenorhabditis elegans	MRP	65.9

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VRLECVGNCIVLFAALFAVISRHSLSAGLVGLSVSYSLQVTTYLNWLVRMSSEMETNIVA   Majority
         1210      1220      1230      1240      1250      1260
---------+---------+---------+---------+---------+---------+
VRLECVGNCIVLFAALFAVISRHSLSAGLVGLSVSYSLQVTTYLNWLVRMSSEMETNIVA   MRP1 human
-----------------------------------------------------ETNMVA   MRP2 mussel

VERVKEYSDIEAEAELQIDETAPVSSNPQVGVVEFQNYSLRYRDGLSLVLKNITVTIEGG   Majority
         1270      1280      1290      1300      1310      1320
---------+---------+---------+---------+---------+---------+
VERLKEYSETEKEAPWQIQETAPPSSWPQVGRVEFRNYCLRYREDLDFVLRHINVTINGG   MRP1 human
VERVKEYTDIPAEAELYNDYKLPVNTN-QQGVIEFQQYSTRYRDGLSLVLKNITFKIEPG   MRP2 mussel

EKVGIVGRTGAGKSSLSLGLFRLIESATGEIIVDGIDIAKIGLHDLRSKVTVLPQDPVLF   Majority
         1330      1340      1350      1360      1370      1380
---------+---------+---------+---------+---------+---------+
EKVGIVGRTGAGKSSLTLGLFRINESAEGEIIIDGINIAKIGLHDLRFKITIIPQDPVLF   MRP1 human
EKVGIVGRTGAGKTSLSQAIFRLIEPTTGRIIVDGEDISMMGLHDCRSKVTVLPQ-----   MRP2 mussel

SGSLRMNLDPFSEYSDEDVWTALELAHLKAFVIGLKDGLDHECAEGGDNLSVGQRQLVCL   Majority
         1390      1400      1410      1420      1430      1440
---------+---------+---------+---------+---------+---------+
SGSLRMNLDPFSQYSDEEVWTSLELAHLKDFVSALPDKLDHECAEGGENLSVGQRQLVCL   MRP1 human
----RMKLEPFDEYSNEDIWTALNHAHLKAFVIGLKDGLDHHCSEGGDNLSVGQRQLICL   MRP1 mussel

ARALLRKTKILVLDEATAAVDLETDDLIQSTIRTEFADCTVLTIAHRLNTIMDYTRVIVL   Majority
         1450      1460      1470      1480      1490      1500
---------+---------+---------+---------+---------+---------+
ARALLRKTKILVLDEATAAVDLETDDLIQSTIRTQFEDCTVLTIAHRLNTIMDYTRVIVL   MRP1 human
ARALLRKTRILVLDEATAAVDLETDDLIQTTIRTEFADCTVLTIAHRLNTIMDYTRIMVL   MRP1 mussel

DKGEIQEFGAPSDLLLDKGLIFSMAKDAGLV                                 Majority
         1510      1520      1530
---------+---------+---------+-
DKGEIQEYGAPSDLLQQRGLFYSMAKDAGLV                                 MRP1 human
DCGQIMEFDSPTNLLLDKKSIF                                          MRP1 mussel
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Fig. 1. *Mytilus edulis*. Comparison of amino acid (aa) sequence encoded by 2 isolated mRNA fragments of putative MRP proteins with the human MRP1 protein sequence. Homologies are indicated in the consensus (majority). The *M. edulis* MRP2 fragment spans the region from aa 1255 to aa 1375 and the MRP1 fragment spans the region from aa 1385 to aa 1522. Sequence alignment was performed using the CLUSTALW software

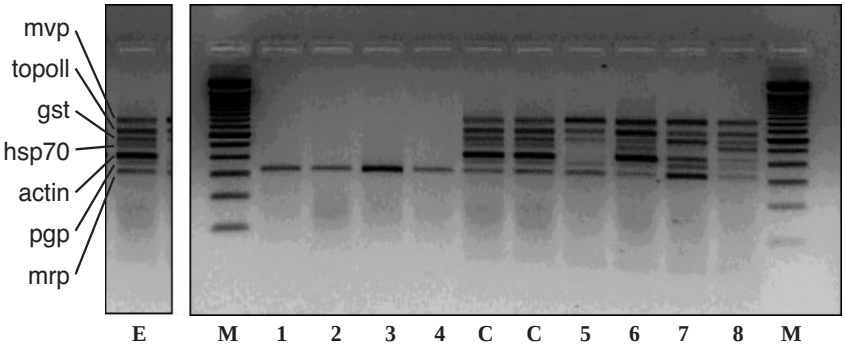

Fig. 2. *Mytilus edulis*. Multiplex PCR of 6 multixenobiotic resistance- and biotransformation-related genes in tissues from 8 pooled individuals. E: display of multiplex genes; M: 100-bp markers; C: expression patterns in 2 mantle samples from 1 individual; 1, 2, 3, 4: *mrp2* expression in gill tissue, mantle tissue, digestive gland, and in the posterior adductor muscle, respectively; 5, 6, 7, 8: multiplex PCR expression pattern in gill tissue, mantle tissue, digestive gland, and in the posterior adductor muscle, respectively

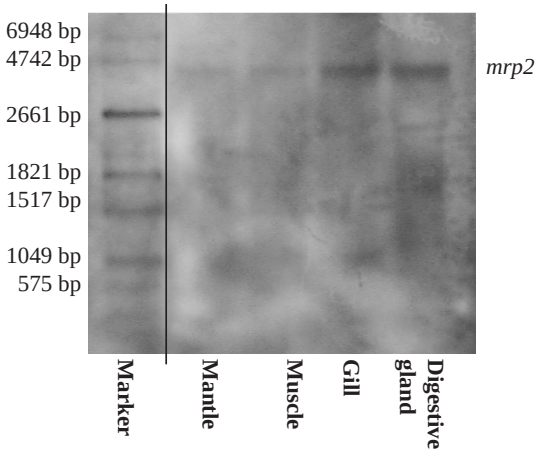

Fig. 3. *Mytilus edulis*. *Mrp2* mRNA size determination in tissues using Northern blot analysis. Marker: DIG-labelled RNA molecular weight marker

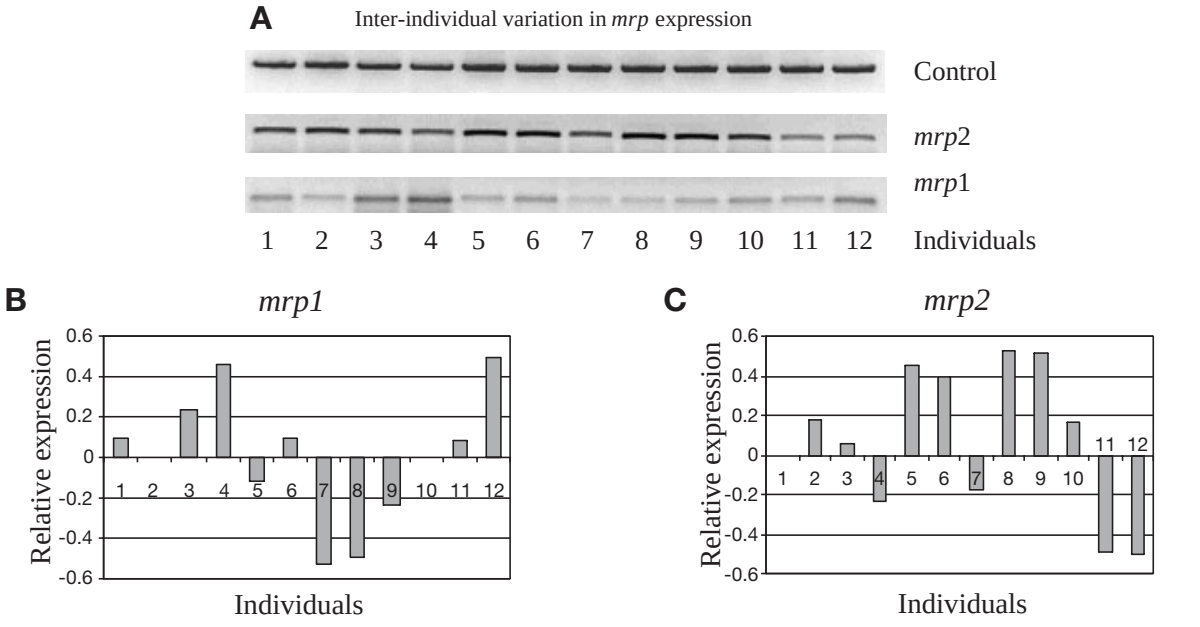

Fig. 4. *Mytilus edulis*. (A) Inter-individual variation in *mrp1* and *mrp2* expression in gill tissue of 12 unrelated individuals. Control: *hsp70* expression in gills of the same individuals. Variation in (B) *mrp1* and (C) *mrp2* expression shown as deviations from the average band intensity value

the average size found in other species (data not shown). Tissue-specific detection of *mrp2* in Northern blots (Fig. 3) are in line with the results of the tissue-specific RT-PCR experiments (Fig. 2). No signal for *mrp1* was detectable under the experimental conditions applied.

Inter-individual variation of *mrp1* and *mrp2* expression

We investigated inter-individual variation in *mrp1* and *mrp2* expression in a mussel population under laboratory conditions. Expression of both genes varied by approximately 50% around the mean value (Fig. 4).

Induction of *mrp2* expression

Inducible forms of detoxification genes are particularly relevant for toxicogenomics. As a putative inducer of *mrp*, we tested the model carcinogenic compound AAF which induces MRP1 expression in primary human hepatocytes (Kauffmann et al. 1997, Schrenk et al. 2001). After incubation for 3 d in seawater containing 200 µM AAF, *mrp2* expression in gill tissue showed an average 2-fold increase, compared with that of control animals (Fig. 5). This level of induction was higher than inter-individual variations. No change in the expression level of *mrp1* was detectable (data not shown).

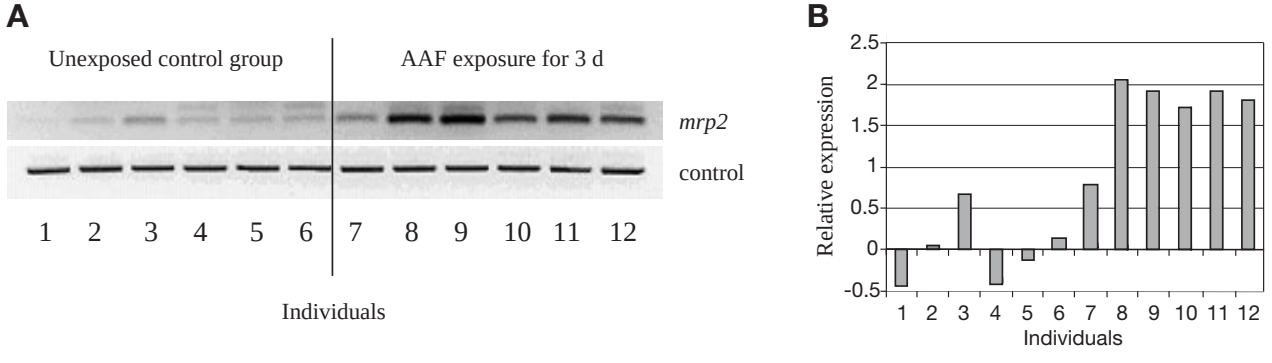

Fig. 5. *Mytilus edulis*. (A) Induction of *mrp2* expression in gill tissue after 3 d exposure to 200 µM AAF. (B) *Mrp2* gene expression shown as deviations from the average band intensity value of *mvp* control gene expression

DISCUSSION

When endogenous and xenobiotic lipophilic substances enter cells, oxidation, conjugation with GSH or alternative anionic groups, and ATP-dependent excretion by a member of the MRP family is of vital importance for detoxification and cellular homeostasis (Sharma et al. 2003). Conjugate-transporting members of the MRP-family with related sequences and similar functions have been detected recently. In addition to several MRP isoforms (MRP1-6) and orthologues in mammals (human, rat, rabbit, mouse), MRP family members have been identified in the nematode *Caenorhabditis elegans* (Broeks et al. 1996), the yeast *Saccharomyces cerevisiae* (Szczypka et al. 1994), and the plant *Arabidopsis thaliana* (Lu et al. 1997, Marin et al. 1998). These conjugate-export pumps play a significant role in detoxification, drug resistance, and, because of the roles of MRP1 and MRP2 in the export of glutathione disulfides, in the defence against oxidative stress. Most *in vitro* data indicate that MRP has a role in the removal of toxins conjugated to GSH, glucuronide or sulphate from cells (Suzuki et al. 1997, Hipfner et al. 1999). However, some unmodified toxins and drugs have also been shown to be transported by MRP1, but their transport requires the presence of GSH. Substrates for MRPs include lipid peroxidation products, herbicides, tobacco-specific nitrosamines, mycotoxins, heavy metals, natural compounds and antifolate anti-cancer agents (Loe et al. 1998, Evers et al. 2000, Renes et al. 2000).

In addition to other transporters and biotransformation systems, MRP transmembrane transporters may serve as essential proteins that allow *Mytilus edulis* to survive and successfully reproduce in a polluted environment. However, little information is available regarding substrate or inhibitor specificity of multixenobiotic resistance proteins in general, and MRP proteins in particular, in marine invertebrates. Therefore, differentiation of the transporter classes by their activity is difficult. In the present study, we aimed to prove the existence of putative MRP transmembrane transporters in *M. edulis* at the mRNA level. With the use of degenerate primers, we demonstrated the existence of 2 fragments of putative *mrp* mRNAs. The *mrp1* and *mrp2* cDNA fragments appeared to be located in the predicted regions of closely-related genes from other species (Cole et al. 1992, Broeks et al. 1996, Taniguchi et al. 1996).

Our first amplification strategy for *mrp* was to amplify the region spanning the sequence that is now divided into the 2 parts *mrp1* and *mrp2*. This strategy did not amplify a fragment of the predicted size. Therefore, the sequence was split into 2 parts. This was achieved with 2 new primers that were designed to bind to a conserved internal sequence and to amplify upstream and downstream from that sequence. This resulted in 2 fragments of the predicted sizes, named *mrp1* and *mrp2* on the basis of their sequences. Since *mrp1* and *mrp2* are consecutively located within the predicted cDNA sequence (Fig. 1), we asked whether they belong to the same mRNA or are parts of different isoforms. Amplification of the entire sequence using the outer primers of *mrp1* and *mrp2* appeared to be impossible. We therefore conclude that the 2 fragments belong to different members of the *mrp* gene family. In support of this conclusion, we found that *mrp1* and *mrp2* expression ratios differ within individuals (Fig. 4). This indicates that expression is regulated independently. Further support for our hypothesis comes from our finding that only *mrp2* is inducible by AAF, whereas *mrp1* expression seems to be unaffected. In further experiments, DNA libraries can be screened with *mrp1* and *mrp2* probes to further characterise the identified isoforms with regard to their substrate specificity and their relation to MRPs of other species.

Northern blotting experiments indicated a single weak band of the predicted size for *mrp2*, whereas no bands were detectable for *mrp1*. The absence of a defined band in the case of *mrp1* may be due to low expression levels in *Mytilus edulis* tissues, as was also indicated by the RT-PCR experiments. Because the detection of *mrp1* in Northern blot experiments failed, proof beyond doubt of its membership of the MRP protein family was not possible. Therefore, only *mrp2* was added to the multiplex PCR for further studies of regulatory processes in detoxification and biotransformation in *M. edulis*.

The multiplex PCR was used to investigate tissue-specific expression of *mrp2*, which appeared to be highest in digestive gland and gills. This underlines its predicted function as a membrane-spanning export pump like P-gp, which shows similar tissue-specific expression patterns in *Mytilus edulis*.

The discovery of a mRNA fragment of the *mrp* gene family, expression of which can be induced, together with the identification of *pgp* and *mvp* mRNAs in a marine invertebrate may cast light on the complex regulation of multixenobiotic resistance-related detoxification processes in these animals. For example, efflux of fluorescent dyes from cells under experimental conditions has always been considered to be a consequence of P-gp activity, but other transporters such as MRPs may be involved as well. Clams freshly collected from a polluted Rhine river site were found to accumulate 41% less vincristine than control clams held in clean aquaria for 6 wk. Western blot analysis detected no differences in P-gp protein levels, suggesting that other transport systems were responsible for the efflux of vincristine (Kurelec et al. 1996). Addition-

ally, discrimination between P-gp- and MRP-mediated resistance will be of special interest with respect to chemosensitisers. Inhibition of P-gp or MRP alone may be tolerable for aquatic organisms, but inhibition of both defence mechanisms may lead to enhanced sensitivity to toxic agents (Lin et al. 2002).

In conclusion, we were able to demonstrate the existence of MRPs in the blue mussel, but further work is necessary to understand the complex function, regulation and substrate specificity of multixenobiotic resistance mechanisms in marine invertebrates.

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