Multi-xenobiotic resistance (MXR) transporters and biotransformation enzymes in the blue mussel Mytilus edulis

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Regulation of expression of multi-xenobiotic resistance (MXR) genes by environmental factors in the blue mussel

*Mytilus edulis*

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ABSTRACT

Marine organisms and especially those living in tidal zones are confronted with dramatic changes in their environment on a daily and/or seasonal basis such as temperature fluctuations. In the present study, we investigated whether these parameters affect expression of multixenobiotic resistance (MXR)-related genes which serve as a first line of defense against a broad spectrum of natural and man-made toxicants. Expression of MXR-related genes seems to be an appropriate biomarker to determine hazardous effects of chemicals in contaminated marine habitats. An important issue with respect to the use of biomarkers in monitoring biological effects of pollutants is the interference of natural environmental factors in the expression of biomarkers, making interpretations rather difficult. In order to differentiate between pollution-induced stress and responses to natural environmental variations, we studied the effects of temperature, salinity and oxygen supply (anaerobiosis) on expression of MXR-related genes in gills and digestive gland of the blue mussel *M. edulis*. Changes in expression levels of P-glycoprotein (*pgp*), major vault protein (*mvp*), topoisomerase II (*topolII*), heat shock protein 70 (*hsp70*) but not of the multidrug resistance-related protein (*mrp2*) genes were found in laboratory experiments in relation to high but not low temperature, low salinity and anaerobiosis. These effects of environmental factors have to
be considered in sampling strategies for monitoring programmes to prevent false interpretation of results.

KEYWORDS: Multixenobiotic resistance (MXR), environmental parameter, P-glycoprotein (P-gp), major vault protein (MVP), multidrug resistance-related protein (MRP), heat shock protein 70 (HSP70), topoisomerase II (TOPOII), Mytilus edulis

INTRODUCTION

Mussels of the genus *Mytilus* are one of the most common marine molluscs and are important components of coastal ecosystems. In addition, they are commercially important for human food production by wild catches and aquafarming. Their distribution spans the moderately-tempered waters of the northern and southern hemisphere. Joergensen (1990) considers the mussel as an autonomous unit, incapable of regulating metabolic processes, so that temporal variations in physiological processes occur solely in direct response to environmental factors (Joergensen 1990). Yet, a compelling body of evidence indicates that mussels act as homeostatic systems, responding to the ‘direct’ effects of environmental changes by modulating their metabolism, physiology and/or morphology to adapt to changing conditions. Due to their capacity to adapt, mussels are able to colonise extreme habitats. Mussels are sessile filter feeders and thus effective concentrators of toxic substances. Therefore, mussels are now widely used as model organisms in physiological, biochemical, genetic and toxicological studies as well as indicator species in monitoring the effects of marine pollution. We have selected the blue mussel *Mytilus edulis* as a model to investigate the regulation of multixenobiotic resistance (MXR)-related detoxification processes at the level of gene expression (Luedeking & Koehler 2002). The sensitivity of physiological responses of mussels to environmental toxicants such as hydrocarbons (Donkin et al. 1989), copper (Davenport & Redpath 1984) and the antifouling paint tributyltin (Page & Widdows 1991) is high when compared to other aquatic animals.

Aim of the present study was to analyse to what extent natural environmental parameters affect expression of detoxification and biotransformation proteins and, thus, may interfere with the diagnostic and prognostic values of gene expression patterns in biomonitoring. Transcriptional regulation is reflected by phenotypic changes due to modulation of mRNA synthesis and hence changes in expression of individual proteins. Temporal variations in environmental factors, such as temperature, salinity and oxygen levels, induce changes in the physiology of bivalves. Adaptational adjustments help to maintain their physiology.
independently of environmental changes. These adjustments may include regulation of expression of biotransformation and detoxification genes in a direct or indirect way.

We have cloned and sequenced fragments of genes that encode proteins involved in the various phases of detoxification processes (Luedeking & Koehler 2002). In the present study, we investigated expression of the genes of P-glycoprotein (pgp), multidrug resistance-related protein (mrp2), major vault protein (mvp), heat shock protein 70 (hsp70) and topoisomerase II (topoII).

Proteins involved in detoxification processes may be divided into three or four types or 'phases', respectively. Phase I biotransformation is mainly performed by members of the cytochrome P450 (CYP450) family. Chemical reduction of lipophilic xenobiotics by CYP450 results in either more polar metabolites, that can be excreted directly, or chemically more reactive molecules which are then better substrates for phase II enzymes. Phase II enzymes catalyse conjugation of xenobiotics. In M. edulis, glutathione S-transferases (GSTs) play a major role in phase II reactions. GSTs are a family of enzymes that are mainly cytosolic and catalyse a range of conjugation reactions between reduced glutathione (GSH) and electrophilic substrates. P-glycoprotein (P-gp), multidrug resistance-related protein (MRP), two ATP-driven membrane pumps, and the lung resistance protein (LRP) which is the major vault protein (MVP) are members of the phase III system and are involved in excretion of conjugated metabolites. However, these transporters can also act as a first line of defense referred to as phase 0 by preventing intracellular accumulation of toxicants by direct binding and excretion of xenobiotics. Phase 0 and phase III transporters have been identified at first in cancer cells, where they provide resistance to a broad spectrum of structurally- and functionally-unrelated drugs, thus causing the phenomenon known as multidrug resistance (MDR) (Endicott & Ling 1989). MXR is a MDR-like system which has been identified in marine invertebrates (McFadzen et al. 2000, Smital et al. 2000). The relevance of MXR has been shown by its potential to protect aquatic organisms from DNA damage (Waldmann et al. 1995) and during embryonal development (Toomey & Epel 1993). Expression of MXR proteins is inducible by toxic compounds (Minier & Moore 1996) and levels of MXR proteins vary strongly between organisms living in differentially-polluted sites (Minier et al. 1999). Accordingly, the MXR phenotype may serve as an index of exposure to toxic compounds (Minier et al. 1999).

Besides these biotransformation and elimination systems, repair mechanisms such as the heat shock protein 70 (HSP70) molecular chaperone can induce higher tolerance to general stress and exposure to xenobiotics (Tedengren et al. 1999). Therefore, induction of HSP70 is often
used as a general marker in studies of stress-related effects. Changes in mitotic activity is a valuable index of the physiological status of individual organisms because reduction in cell proliferation may be caused by exposure to xenobiotics or insufficient nutrient supply. Therefore, we assayed expression of topoisomerase II (TOPOII) as marker of cell proliferation (Heck & Earnshaw 1986). TOPOII is an ubiquitously-expressed nuclear protein required for cell division. The primary role of TOPOII is to disentangle (decatenate) intertwined chromosomes during anaphase to allow chromosome segregation prior to cell division. Besides, it plays an important role in transcription and probably DNA repair (Withoff et al. 1996).

In the present study, effects of physiological adaptation to environmental changes on gene expression has been investigated as a baseline for the interpretation of gene expression patterns of biomarkers of exposure to pollutants.

MATERIAL AND METHODS

Tissue preparation.

Mussels (Mytilus edulis) of approx. 5 cm in shell length were collected near the German island of Sylt. Mussels were maintained in seawater (salinity 32 ppt) at 15°C for 3 days prior to use without feeding. Six immature mussels were kept under each of the environmental conditions tested. Anaerobic conditions were established by exposure of mussels to air for 24 h at 15°C. Temperature experiments were performed in 10 l seawater basins by gradually changing the temperature from 15°C to 4°C or 30°C, respectively, during a period of 12 h to avoid any heat shock. After 12 h, the desired temperature was reached in both basins. Salinity experiments were performed in 20 l seawater basins with salinity adjusted to 16 ppt as low salinity and 45 ppt as high salinity. Low salinity was obtained by dilution of seawater with doubly-distilled sterile filtered water whereas high salinity was obtained by the addition of sodium chloride. Mussels were kept for 24 h at the selected salinities. Control animals were maintained at 15°C in a steady-flow seawater basin. None of the parameters was lethal to any of the individuals analysed. Individuals were numbered from 1 to 36. Individuals 1-6: control animals; 7-12: 4°C ambient temperature; 13-18: 30°C ambient temperature; 19-24: anaerobiosis; 25-30: low salinity; 31-36: high salinity.

RNA extraction was performed after homogenisation with a rotor homogeniser. MMulv reverse transcriptase and desoxynucleotides were purchased from Peqlab (Erlangen, Germany) and Taq polymerase (RedTaq) from Sigma (Taufkirchen, Germany). Oligo-dT- primers and specific primers for multiplex PCR were obtained from Life Technologies
Isolation of total RNA.

The digestive gland and gills were isolated from the mussels and total RNA was prepared from all samples immediately after isolation using 1 ml of RNAPure reagent (Peqlab, Erlangen, Germany) according to the manufacturer's specifications. An additional purification step with the High Pure RNA Tissue Kit (Roche, Mannheim, Germany) was performed to remove any remaining contamination of polysaccharides and to improve RNA quality. The amount of total RNA was determined by UV spectroscopy. Contamination with polysaccharides or proteins was undetectable on the basis of absorbance values at 260, 280 and 320 nm. Integrity of RNA was investigated on 1.5% agarose gels.

RT-PCR.

The amount of isolated total RNA was measured by UV spectroscopy at 260 nm and by non-denaturing gel electrophoresis in comparison with molecular weight markers. Subsequently, the total RNA concentration was adjusted to 0.5 µg/µl followed by an additional quantification control using non-denaturing gel electrophoresis. For RT-PCR, reverse transcription was performed with 1 µg of DNase-treated total RNA with 20 U mMulv reverse transcriptase, 50 pmol oligo-dT primer and 1 mM of dATP, dGTP, dCTP and dTTP each in a total volume of 30 µl.

PCR samples (10 µl) were analysed at cycle 26, 28 and 30 by gel electrophoresis to ensure that all mRNAs investigated were still in the exponential phase of amplification. Amplification was performed for 28 sequential cycles at 95°C for 30 sec, at 60°C for 60 sec and at 70 °C for 60 sec followed by a final 3-min extension at 72 °C. Equal aliquots of each PCR reaction were separated on 2% agarose gels using Tris-borate buffer containing 1.0 M Tris, 0.9 M boric acid and 0.01 M EDTA and photographed after ethidium bromide staining. Gels were analysed with ID Image analysis software (Kodak Digital Science, Jähnsdorf, Germany). For semi-quantitative RT-PCR, the following primer pairs were used: *mrp2* sense AAA GAC GGA CTG GAT CAC CA and antisense AAA TTG GTC GGT GAG TCG AA, 296 bp, concentrations in PCR, 0.1 µM each; *pgp* sense CAG AGG TTC TAT GAC CCA GAT GCA G and antisense GTT CTC ACT CTC ACT CAG, 381 bp, concentrations in PCR, 0.1 µM each; *topoll* sense CTT CTC TGA TAT GGA CAA ACA TAA GAT TCC and antisense GGA CTG TGG GAC AAC AGG ACA ATA C, 664 bp,
concentrations in PCR, 0.2 μM each; \textit{mvp (lrp)} sense ACA GGT TGT AAC TCC CTT G and antisense CTT CAT GAT GAC CTC GAC C, 818 bp, concentrations in PCR, 0.1 μM each; \textit{hsp70} sense GAC TTG GGT GGT GGA AC and antisense GGC TAC AGC TTC ATC AGG G, 516 bp, concentrations in the PCR, 0.1 μM each.

We selected genes that did not express a difference between control and test experiments as internal controls in the gels. Histograms were made semiquantitative on the basis of net band intensities and intensities were calculated as deviation from the average value of control animals.

\section*{RESULTS}

The effects of various natural parameters on expression modulation of selected genes are summarised in Table 1. Expression of \textit{pgp, mvp, hsp70} and \textit{topoll} genes was altered by environmental parameters at the transcriptional level. \textit{Mrp2} gene expression was not affected by any of the natural environmental parameters investigated.

\textit{Pgp} gene expression in digestive gland was inhibited by low osmolality (16 ppt) in comparison to control osmolality of 32 ppt after 24 h of exposure (Fig. 1). High osmolality seawater (45 ppt) and control osmolality (32 ppt) induced high inter-individual variability. Expression of \textit{pgp} in all individuals at high osmolality varied above the mean of the control values although the increase was not statistically significant in comparison to controls.

Anaerobiosis due to air exposure for 24 h induced increased gene expression of \textit{pgp} by a factor 2 in digestive gland (Fig. 2). Inter-individual differences in \textit{pgp} expression was high in control animals whereas air exposure lead to a more uniform and elevated expression pattern with low inter-individual variability. Changes in \textit{pgp} gene expression were not detectable in gill tissue under the experimental conditions.

\textit{Mvp} gene expression was affected in digestive gland following anaerobiosis. Its expression was distinctly increased in 5 individuals. Data were not available of mussel number 19. Inter-individual differences were rather low both in control mussels and mussels exposed to anaerobiosis as is shown in Fig. 3. Changes in \textit{mvp} gene expression were not detected in gill tissue under the experimental conditions.

The gradual temperature increase (not heat shock) from 15°C to 30°C over a period of 12 h clearly induced an increase in \textit{hsp70} expression in gill tissue (Fig. 4). Expression of \textit{hsp70} was not affected in digestive gland under the experimental conditions.

\textit{Topoll} expression was affected in digestive gland following a change in osmolality. Low salinity (16 ppt) resulted in inhibition of \textit{topoll} gene expression (Fig. 5) except for one
individual that even showed a somewhat higher expression level than control animals. Changes in topoll gene expression were not detected in gill tissue under the experimental conditions.

Mrp2 gene expression showed a unique expression pattern with low inter-individual variability that was not affected by natural environmental parameters in both gills and digestive gland.

**DISCUSSION**

*M. edulis* lives in tidal and shallow waters which are characterised by large fluctuations in temperature, salinity and the availability of oxygen. The present study demonstrates the relevance of taking into consideration the effects of natural environmental factors on regulation of gene expression in mussels when expression levels of these genes are used as biomarkers of pollution. When interfering natural environmental parameters have been characterised, sampling strategy and animal handling have to be rigidly controlled to circumvent effects of these factors.

Natural environmental parameters may affect regulation of transcription of genes of phase 0 or phase III detoxification proteins, their endogenous functions such as osmoregulation or excretion of metabolic products, their protective value and thereby the resistance of mussels against (anthropogenic) pollution. This can be due to either a primary effect such as activation of receptors or a secondary effect such as changes in metabolism like reduced ATP supply or pH shifts.

Mussels are facultatively anaerobic. They live either aerobically or anaerobically but prefer to use oxygen respiration when it is available. Mytilidae are also called euryoxic, because they tolerate a wide range of oxygen levels, including total anaerobiosis. The LD$_{50}$ in the absence of oxygen is 35 days for *M. edulis* at 10°C (Theede et al. 1969). Fluctuations in oxygen supply may induce an anaerobic type of metabolism. In sessile bivalves, ATP consumption under anaerobic conditions is less than 10% of resting aerobic rates (Widdows et al. 1979). In the present study, we show effects of anaerobiosis on expression patterns of *pgp* and *mvp*. Anaerobiosis causes induction of expression of both genes in the digestive gland. Moreover, inter-individual variability is reduced by anaerobiosis. Reduced inter-individual variability is also found after exposure to high concentrations of xenobiotics (Luedeking et al. submitted). Therefore, we recommend a strategy to sample mussels at high tide and thus with an aerobic metabolism or even better to sample subtidal mussel populations for biomonitoring to circumvent false-positive results. In the context of endogenous functions of *pgp* and *mvp*,
these transporters may be overexpressed as a result of increasing concentrations of metabolic end-products in tissues and the shell interspace of mussels during shell closure. These mechanisms protect mussel tissues against toxic effects of lipophilic compounds and bacterial end-products in analogy with the blood-brain barrier where phase 0 proteins are expressed at high levels. Temperature has a direct effect on metabolic rates in poikilotherms, so that compensatory adaptation is important for maintaining ATP production within thermally-unstable environments. *M. edulis* has a sustained thermal tolerance limit of approx. 29°C (Widdows 1976, Almada-Villela et al. 1982).

In our experiments, we used a continuous but slow decrease of temperature from 15°C to 4°C and increase from 15°C to 30°C over a period of 12 h in order to simulate natural fluctuations more closely than heat shocks would do. Eufemia (2000) reported higher P-gp levels and activity following heat shock in *M. californianus*. In our experiments, only *hsp70* gene expression was increased in gill tissue at elevated temperatures whereas expression of the other genes investigated was not affected. Gene expression in digestive gland was not affected at all by temperature. At 4°C, expression of all genes investigated was not affected in gills and digestive gland. The results underline the high tolerance of *M. edulis* towards temperature fluctuations. For seasonal biomonitoring, we recommend a temperature range of 4-15°C which is the most common temperature range under field conditions in the North Sea and North Atlantic. Our results indicate that phase 0 and phase III genes are not very much involved in temperature-related stress responses.

Changes in external salinity disrupt the steady-state balance in cells between influx and efflux of water and ions. In order to avoid associated changes in cell volume, mussels and other bivalves respond immediately to fluctuations in external salinity by closing the shell and maintain intracellular iso-osmosis by adjusting intracellular concentrations of ions, amino acids and other small molecules to maintain the volume of cells relatively constant. Almada-Villela (1984) reported strongly reduced shell growth due to exposure to low salinity of 16 ppt for a period of up to a month in comparison with 32 ppt, whereas exposure to 22 ppt caused only minor reduction in growth rate. On the other hand, *M. edulis* is able to adapt well to low salinities in the longer term (in the order of weeks) (Bohle 1972, Almada-Villela 1984). Almada-Villela (1984) also found that the growth rate of shells of individuals exposed to 13 ppt recovered after one month from almost zero to over 80% of the rate of control animals in 32 ppt. The effect of low salinity on growth rates is reflected in our study by the finding that *topoll* expression is reduced by low salinity to nearly zero in 5 out of 6 individuals. The data may also reflect the fact that mussels from the Baltic Sea with salinities of 7 ppt grow slower.
and reach a maximum size that is only one-third of that of mussels in the North Sea (32 ppt). The small size of Baltic sea mussels is likely a plastic trait caused by salinity stress, since Baltic Sea mussels grow to a large size when transplanted to the North Sea (Kautsky 1982, Kautsky et al. 1990).

The changes in expression of \textit{pgp} caused by low salinity but not so much by high salinity need further investigation to answer the question to which extent changes in \textit{pgp} expression may be interpreted as a physiological response or an active regulation of cell volume control. The finding that only \textit{topoII} and \textit{pgp} gene expression is reduced by low salinities suggests that this expression pattern is not due to a passive metabolic event such as reduction in transcription rate caused by reduced ATP levels. Recent studies suggest that in addition to the elimination of toxicants, P-gp is also involved in transport of endogenous molecules such as phospholipids and steroids, as well as cell-volume control (Ernest & Bello-Reuss 1998, Stieger & Meier 1998, Frijters et al. 1999, Gupta et al. 2000). The cystic fibrosis transporter (CFTR), an ABC transporter chloride channel that is also involved in the transport of phospholipids and steroids and cell volume control and Mdr1 (P-gp) show complementary tissue distribution patterns \textit{in vivo}. This phenomenon has been used as evidence that CFTR and P-gp may have similar roles in epithelial cells (Trezise et al. 1992). Studies on \textit{cftr}-knockout mice have demonstrated that a 4-fold decrease in intestinal \textit{cftr} expression in homozygote knockouts is accompanied by a four-fold increase in intestinal \textit{mdrl} mRNA levels. These findings indicate a role of P-gp in cell volume control (Trezise et al. 1997). Whether this is also the case in mussels has to be investigated in further studies.

In summary, we conclude that environmental parameters has to be considered in gene-expression studies. The question to what degree endogenous functions and their influence on gene regulation may interfere with the transport abilities of the investigated proteins for contaminants needs further investigation.

ACKNOWLEDGEMENTS

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Table 1. Effects of natural environmental parameters on gene expression of *pgp*, *mvp*, *topoII*, *hsp70*, and *mrp2* in gills and digestive gland of *M. edulis* in comparison to controls: low (4°C) and high (30°C) temperatures in comparison with control temperature (15°C), anaerobiosis, high (45 ppt) and low (16 ppt) osmolarity versus control osmolality (32 ppt). --, No alterations; ↑, elevated expression, ↓, decreased expression and ↑?, not significant, in gill/digestive gland, respectively.
Fig. 1

A

<table>
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<th>Low osmolality</th>
<th>High osmolality</th>
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<tbody>
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<td></td>
<td>Digestive gland</td>
</tr>
<tr>
<td>Control</td>
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25 26 27 28 29 30 31 32 33 34

Individuals

B

Fig. 2

A

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<th>Anaerobiosis</th>
<th>Digestive gland</th>
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<tr>
<td>Control</td>
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1 2 3 4 5 6 19 20 21 22 23 24

Individuals

B

85
Fig. 3

A  

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<td>Digestive gland</td>
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Mvp

Control

1 2 3 4 5 6 19 20 21 22 23 24

Individuals

B

Individuals

Fig. 4

A  

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</table>

Hsp70

Control

1 2 3 4 5 6 13 14 15 16 17 18

Individuals

B

Individuals

86
Fig. 5

A  

TopoII

Control  

Low osmolarity

Digestive gland

Digestive gland

1  2  3  4  5  6  25  26  27  28

Individuals

B

Individuals
Legends

Fig. 1. Expression of *pgp* in digestive gland of mussels and the effect of osmolarity of the seawater. A. Bands of *pgp* in mussels 25-30 that were exposed to low osmolarity sea water and mussels 31-36 that were exposed to high osmolarity sea water. For *pgp* expression at control osmolarity of 32 ppt see Fig. 2. *Mvp* expression of each individual was used as internal control. B. *Pgp* gene expression in digestive gland expressed semi-quantitatively as deviation from the average value band intensity of control animals incubated in control sea water.

Fig. 2. Expression of *pgp* in digestive gland of mussels and the effects of anaerobiosis. A. Bands of *pgp* in mussels 1-6 that were used as control mussels kept at 15°C with current water flow and mussels 19-24 that were exposed to anaerobiosis by air exposure. *Mrp2* expression of each individual was used as internal control. B. *Pgp* gene expression in digestive gland expressed semi-quantitatively as deviation from the average value band intensity of control animals.

Fig. 3. Expression of *mvp* in digestive gland of mussels and the effect of anaerobiosis. Data of individual no. 19 are missing. A. Bands of *mvp* in mussels 1-6 that were used as control mussels kept at 15°C with current water flow and mussels 19-24 that were exposed to anaerobiosis by air exposure. *Hsp70* gene expression of the same individuals was used as internal control. B. *Mvp* gene expression in digestive gland expressed semi-quantitatively as deviation from the average value band intensity of control animals.

Fig. 4. Expression of *hsp70* in gills of mussels and the effect of an elevated environmental temperature. A. Bands of *hsp70* in mussels 1-6 that were used as control mussels kept at 15°C with current water flow and mussels 13-18 that were exposed to a steady temperature increase from 15°C to 30°C. *Mrp2* expression of each individual was used as internal control. B. *Hsp70* gene expression in gills expressed semi-quantitatively as deviation from the average value band intensity of control animals.

Fig. 5. Expression of *topoll* in digestive gland of mussels and the effect of low osmolarity. A. Bands of *topoll* in mussels 1-6 that were used as control mussels at 15°C with current water flow and mussels 25-30 that were exposed to low osmolarity. *Mvp* gene expression of each
individual was used as internal control. B. *TopoII* gene expression in digestive gland expressed semi-quantitatively as deviation from the average value band intensity of control animals.