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Characterization and expression of a *Bathymodiolus sp*. metallothionein gene

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Introduction

Metallothioneins are ubiquitous, cysteine-rich proteins that are able to bind metal cations such as zinc and copper (essentials elements) or cadmium, mercury and silver (toxic elements). Three classes of metallothioneins are known. The first class was discovered in mammals (Kagi & Vallee, 1960) and is defined on the basis of its cysteine positions. The metallothioneins that have been analysed from bivalves also belong to this first class. Two isoforms with distinct functional roles have been observed in this taxon (Tanguy, 2000 ; Mackay et al., 1993). They correspond to two proteins known as MT-10 (with a molecular mass of 10kDa) and MT-20 (with a molecular mass of 20 kDa). The expression of the metallothionein genes is postulated to be affected by high concentrations of metals in the environment. In this study, we isolated a metallothionein-complementary DNA from *Bathymodiolus* sp. by means of a reverse transcription polymerase chain reaction amplification (RT-PCR). This cDNA codes for a MT-10 isoform. It was used as probe for dot blot hybridizations to determine the level of MT mRNA in mantle and gill tissues from individual mussels exposed to an environment containing high concentrations of metals in the laboratory.

Material and methods

Extraction of RNA
Total RNA was extracted from mantle and gill tissues as described by Chomczynski & Sanchi (1987) after being frozen and pulverized in liquid nitrogen. The concentration of RNA was determined by measuring the absorbance at 260 nm.

Cloning and sequencing of the complementary DNA
The cDNA was obtained by heating 5 µg of total RNA extracted from *Bathymodiolus* sp. gills, using 80 pmol oligo(dT) primer, 200 u M-MLV reverse transcriptase and 25 u RNAsin according to the manufacturers protocol (Promega). The specific amplification was performed by PCR, using one 15-mer degenerated primer (5'-CTT GCA A/GG/CA/T A/GCA GCC-3') and one 18-mer degenerated primer (5'-CCA/T TGC/T AAC TGC/T A/GTC/T GAA/G-3'), and with *Taq* DNA polymerase according to the manufacturers protocol (Promega). The primer sequences were obtained by alignment of Mytilidae metallothionein databank sequences. The PCR program began with 1 min at 94 °C, followed by 30 cycles (1-min at 94 °C, 30 s at 55 °C and 1 min at 72 °C), and ended with one 10-min cycle at 72 °C. The RT-PCR product was purified on a 0.7% low melting point agarose gel and extracted using the Ultra-free DA kit (Millipore). The DNA fragment was ligated with the pGEM-T vector system (Promega) and used to transform *Escherichia coli* bacteria (strain TG1). Inserts included in the plasmid were sequenced.

Quantification of MT RNA
Bathymodiolus sp. were exposed to 1000 µg l^{-1} Zn, 40 µg l^{-1} Cu, 200 µg l^{-1} Cd, 20 µg l^{-1} Hg and 20 µg l^{-1} Ag at atmospheric pressure for 42 h. Five mussels were exposed to each metal cation and 5 mussels were kept as control. Excised mantle and gill tissues were frozen in liquid nitrogen until used for total RNA extractions. After estimating the RNA concentrations, 10 µg of each sample was blotted on a nylon membrane using a Bio-Dot

Microfiltration Apparatus (BioRad). A random priming protocol was used to prepare radioactively-labeled MT cDNA as the probe. Three hours of prehybridization and 20 hours of hybridization in 50% formamide buffer at 42 °C (Sambrook et al., 1989) were required. The membrane was washed in 2 X SSC 0.1% SDS at 25 °C for 5 min, then at 50 °C for 30 min (twice), 2 X SSC 0.5% SDS at 55 °C for 30 min, and ending with 0.1 X SSC at 25 °C for 5 min. Quantitative hybridization levels were performed with an Instant Imager (Packard). We used Mann-Whitney test to compare the means of hybridization levels. The film was exposed at -70 °C for 13 days.

Results

cDNA sequencing

Using the RT-PCR protocol, a discrete band approximately 200 bp in length was examined. It was cloned and sequenced, and yielded a DNA fragment 213 bp in length (Fig. 1). The Clustalw program of Infobiogene was used to align the nucleotide sequence with those in the databanks. The findings confirmed that the cloned cDNA fragment was indeed a metallothionein cDNA fragment. The 71 amino acid long sequence was deduced from the nucleotide sequence and corresponds to a MT-10 metallothionein sequence. This putative protein is characterized by a molecular weight of 13 KDa which in the same order as the MT-10 metallothionein molecular weight.

MT expression

The expression of the MT-10 mRNA gene was detected in both mantle and gill tissues (Fig. 4), but the expression pattern varied according to the tissue (Table 1). The weakest expression was detected in the mantle tissues and the strongest in the gills, showing that this tissue is a major site of MT transcription. The standard errors were very high, probably due to individual variations. In both tissues, the Mann-Whitney test showed that the expression patterns for the different metal cations did not differ significantly.

Discussion

cDNA sequence

All the 213 bp sequenced are included in the translated sequence of MT-10, and no untranslated sequence is present. Comparing the cDNA metallothionein of *Bathymodiolus* sp. with MT-10 from other bivalves allows us to conclude that there are important similarities between the MT-10s in this group (Fig. 2). If the putative protein is placed alongside bivalve

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CCT TGT AAC TGT GTC GAA ACA AAT GTG TGT ATC TGT GAC ACT GGC TGC
 P   C   N   C   V   E   T   N   V   C   I   C   D   T   G   C

AGC GGT GAC GGT TGT CGC TGT GGT GAC GCC TGC AAG TGC TCG GGC GCT
 S   G   D   G   C   R   C   G   D   A   C   K   C   S   G   A

GAC TGT AAA TGT TCC GGT TGT AAA GTA GTT TGC AAG TGT TCA GCA GGT
 D   C   K   C   S   G   C   K   V   V   C   K   C   S   A   G

AGC TGT GAG TGT GGC AAA GGA TGT ACA GGA CCT TCA ACG TGT AGA TGT
 S   C   E   C   G   K   G   C   T   G   P   S   T   C   R   C

GCA CCT GGC TGT TCC TGC AAG
 A   P   G   C   S   C   K
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Figure 1. Nucleotide sequence of a MT-10 gene of *Bathymodiolus* sp. The sequences underlined are the primer sequences used for the PCR experiment. The italic letters correspond to the hypothetical amino acid sequence deduced from it.

Table 1. Expression levels of hybridization of the mRNA extracted from *Bathymodiolus sp*. with MT-10 cDNA probe in counts per minute (cpm).

Lane 1 : four controls, two tissues were extracted: Mantle (Mt) and Gill (Gl). Lanes 2 to 6 : exposed mussels, four metals tested, silver (lane 2), cadmium (lane 3), copper (lane 4), mercury (lane 5), or zinc (lane 6), for 42h at atmospheric pressure. Two tissues were extracted : Mantle (Mt) and Gill (Gl). X : No specimen.

	Gill	Mantle	Gill	Mantle	Gill	Mantle	Gill	Mantle
1	188	105	447	117	491	105	505	98
Ag	302	143	X	120	337	141	506	131
Cd	452	193	354	109	443	153	487	127
Cu	512	202	437	169	532	141	162	104
Hg	429	192	163	195	X	206	X	X
Zn	468	142	431	131	416	141	185	171

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a.  -------CCTTGTAACTGTGTCGAAACAAATGTGTGTATCTGTGACACTGGCTGCAGCGG
b.  GCCTGCACCTTGTAACTGTATCGAAACAAATGTGTGTATCTGTGACACTGGCTGCAGCGG
c.  GCCCAGCCCTTGTAATTGCATTGAAACACAAGTCTGTATCTGTGGTACTGGGTGCAGCGG
           ******** **  * ****** * ** **********  ***** ********

a.  TGACGGTTGTCGCTGTGGTGACGCCTGCAAGTGCTCGGGCGCT---GACTGTAAATGTTC
b.  TGACGGTTGTCGCTGTGGTGACGCCTGCAAGTGCTCGGGCGCT---GACTGTAAATGTTC
c.  AGAAGGTTGTCGTTGTGGTGACGCGTGCAAATGTAGCAGTGGTTGTGGTTGTGGATGTTC
     ** ******** ********** ***** **     * * *   *  ***  ******

a.  CGGTTGTAAAGTAGTTTGCAAGTGTTCAGCAGGTAGC-TGTGAGTGTGGCAAAGGATGTA
b.  TGGTTGTAAAGTAGTTTGCAAGTGTTCAG---GTAGC-TGTGAGTGTGGCAAAGGATGTA
c.  AGGGTGTAAAGTCGTGTGCAAATGT-CAGCCAGGAGAGTGTGCATGTGGCAAGCAATGTA
     ** ******** ** ***** *** ***   * **  ****  ********   *****

a.  CAGGACCTTCAACGTGTAGATGTGCACCTGGCTGTTCCTGCAA
b.  CAGGACCTTCAACGTGTAAATGTGCACCTGGCTGTTCCTGCAA
c.  CGGGACCAGACACCTGTAAATGTGACTCCAGTTGTTCGTGCAA
    * *****    ** **** *****   *  * ***** *****
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Figure 2. Nucleotide alignement of MT-10 gene of Mytilidae. a. our sequence of metallothionein cDNA ; b. sequence of *Mytilus edulis* metallothionein cDNA ; c. sequence of *Perna viridis* metallothionein cDNA.

metallothioneins, this highights the fact that the cystein amino acids are in exactly the same position (Fig. 3). As in the *Mytilus edulis* Linnaeus, 1758 metallothionein, they are included in eight C-X-C and one C-X-X-C patterns. As in many other bivalvs, no C-C pattern is observed ; this pattern is characteristic of a part of the protein called the α domain, defined in the mammalian metallothioneins and which is involved in binding metal cations. This brings into question the existence of this α domain in the bivalve MT-10 and consequently, the involvement of these MT-10's in the detoxication processes.

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a.  MPAPCNCVETNVCICDTGCSGDGCRCGDACKCSG-ADCKCSGCKVVCKCSAGSCECGKGC
b.  MPAPCNCIETNVCICDTGCSGDGCRCGDACKCSG-ADCKCSGCKVVCKCSG-SCECGKGC
c.  MPSPCNCIETQVCICGTGCSGEGCRCGDACKCSSGCGCGCSGCKVVCKCQPGECACGKQC
    **:***:**:****.*****:**********. ..* **********.  .* *** *

a.  TGPSTCRCAPGCSCK
b.  TGPSTCKCAPGCSCK
c.  TGPDTCKCDSSCSCK
    ***.**:* ..****
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Figure 3. Amino acid sequences of MT-10 proteins of Mytilidae. a. our sequence of metallothionein protein ; b. sequence of one *Mytilus edulis* metallothionein protein ; c. sequence of one *Perna viridis* metallothionein protein. The cysteine, the C-X-C and C-X-X-C patterns are bold letters.

Expression of MT

MT-10 RNA is synthesized in the two tissues, particularly in the gills, and in all environmental conditions. The gills seem to be the major site for metal uptake, as in other bivalves (Viarengo et al., 1985). This is consistent with the basal expression of these genes, which are thought to be involved in essential metal homeostasis (Roesijadi, 1994 ; Cousin, 1998).

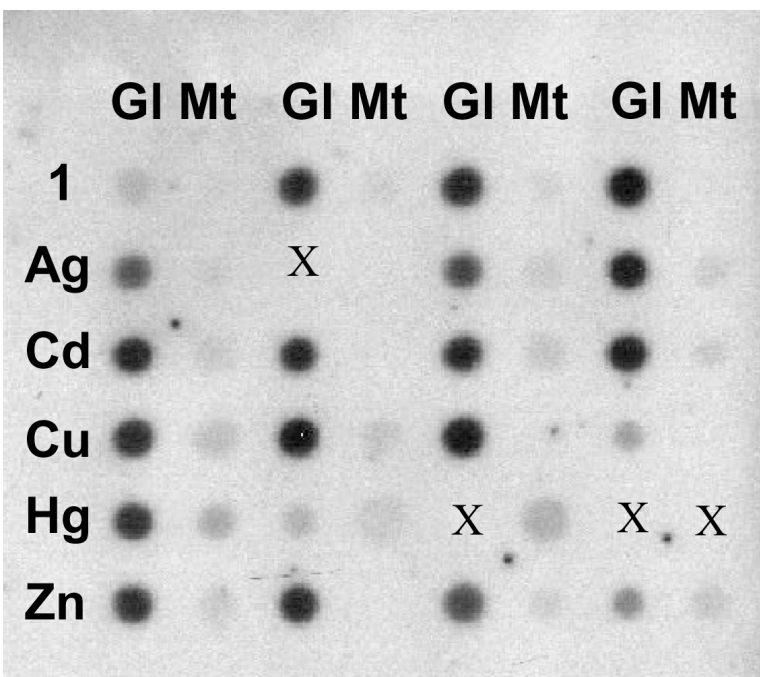

Figure 4. Autoradiogram of dot blot hybridization of total RNA extracted from *Bathymodiolus sp.*. Lane 1 : four controls, two tissues were extracted: Mantle (Mt) and Gill (Gl). Lanes 2 to 6 : exposed mussels, five metals were tested , silver (lane 2), cadmium (lane 3), copper (lane 4), mercury (lane 5), or zinc (lane 6), for 42h at atmospheric pressure. Two tissues were extracted: Mantle (Mt) and Gill (Gl). X : No specimen. The probe used was the MT-10 cDNA fragment

The experimental protocol used with other species showed that the MT-10 probe does not hybridize with MT-20 mRNA (Lemoine et al., 2000). The use of the MT-10 probe limits this experiment to the estimation of MT-10 expression and doesn't allow the estimation of all the metallothionein gene expression. If the same structural differences characterized the *Bathymodiolus* MT-10 and MT-20 metallothionein genes as those of *Mytilus edulis*, we can assume that we have analysed only the MT-10 gene expression. If we accept this assumption, which is contrary to results observed in *M. edulis* for similar metal concentrations (Lemoine et al., 2000), no increase of the synthesis was observed. These findings suggest that a MT-20 probe is required to analyse the differential implication of the metallothionein genes in detoxification processes. It is possible that the mechanisms of metal detoxification differ when the coastal and hydrothermal organisms are compared.

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