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Polymorphism of metallothionein genes in the Pacific oyster *Crassostrea gigas* as a biomarker of response to metal exposure

ARNAUD TANGUY[1], ISABELLE BOUTET[2], FRANCOIS BONHOMME[3], PIERRE BOUDRY[4] and DARIO MORAGA[2]*

[1] Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, NJ 08349, USA
[2] Laboratoire des Sciences de l'Environnement Marin (LEMAR), UMR CNRS 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Place Nicolas Copernic, 29280 Plouzané, France
[3] Laboratoire Génome, Populations, Interactions UMR CNRS 5000, Université de Montpellier II, Place Eugène Bataillon, 34095 Montpellier, France
[4] Laboratoire de Génétique et Pathologie, IFREMER La Tremblade, 17390 La Tremblade, France

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Quantification of metallothioneins (MTs) is classically associated with a cellular response to heavy metal contamination and is used in the monitoring of disturbed ecosystems. Despite the characterization of several MT genes in marine bivalves, only a few genetic studies have used MT genes as potential biomarkers of pollution. The aim of this study was to assess whether MT gene polymorphism could be used to monitor exposure of the Pacific oyster *Crassostrea gigas* to heavy metals and to develop specific genetic markers for population genetic studies in relation to environmental stress. The polymorphism of two exons of the *C. gigas* MT gene *CgMT1* were studied using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) in both field populations exposed to various metals concentrations and in experimentally exposed populations. High frequencies of two SSCP types in exons 2 and 3 of the *CgMT1* gene have found to be significantly associated with tolerance to metals in experimental and field oyster populations. The use of *MT1* gene polymorphism in *C. gigas* as in the present study should therefore be of high ecological relevance. In conclusion, the analysis of the types in these two *CgMT1* gene exons, which can confer a greater tolerance to heavy metals, can constitute a good biomarker of effect of the presence of heavy metals in ecosystems.

Keywords: Metallothionein, polymorphism, type, heavy metals

Introduction

The metallothioneins (MTs) are small, cysteine-rich, heat-stable proteins that bind to metal ions through metal–thiolate bonds. They are involved in the cellular regulation of metabolically important metals (copper and zinc) and in the detoxification of non-essential metals (e.g. cadmium, mercury). MT genes have been identified in all major classes of invertebrates and vertebrates (Kägi 1993). They present similar characteristics in terms of a conserved tripartite gene structure and

* Corresponding author: Dario Moraga, Laboratoire des Sciences de l'Environnement Marin (LEMAR), UMR CNRS 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest Iroise, Place Nicolas Copernic, F-29280 Plouzané, France. Tel: (+33) 2 98 49 86 42; fax: (+33) 2 98 49 86 45; e-mail: Dario.Moraga@univ-brest.fr

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in the arrangement of cysteine residues in Cys-Cys, Cys-X-Cys or Cys-X-Y-Cys motifs (Hamer 1986), but some differences appear in terms of the amino acid sequence between vertebrates and invertebrates (Unger *et al.* 1991, Barsyte *et al.* 1999, Tanguy and Moraga, 2001). Several characteristics of MT genes have been studied, such as their ability to be amplified in the presence of heavy metals (Crawford *et al.* 1985, Mehra *et al.* 1990), their inducibility by different heavy metals, and their tissue-specific expression (Nemer *et al.* 1985, Freedman *et al.* 1993). In marine or freshwater species, the quantification of MTs, as proteins or mRNA, is often used as a biological tool for monitoring heavy metal contaminated ecosystems (Linde *et al.* 2001, Hamza-Chaffai *et al.* 2000, Butler and Roesijadi 2001). Similar studies were conducted in the Pacific oyster *Crassostrea gigas*, a sentinel species used in ecosystem monitoring, in which two genes coding for two distinct MTs were characterized (Tanguy and Moraga 2001, Tanguy *et al.* 2001) and a sensitive enzyme-linked immunosorbent assay (ELISA) was developed (Boutet *et al.* 2002). At present, however, only studies dealing with the expression of MT genes in the presence of heavy metals have been conducted, and no genetic data on the relationship between polymorphism of these genes and the ability of individuals to be more susceptible or resistant to heavy metals is available. Only a few studies of polymorphism in MT genes have been done in humans or mice using restriction fragment length polymorphism (RFLP) (Varshney *et al.* 1984, Bates and Mulley 1988, Watanabe *et al.* 1989). The sequence variability of the 3′ untranslated region in the Eastern oyster *Crassostrea virginica* MT cDNA has been correlated with the degree of metal pollution (Fuentes *et al.* 1994). Previously, we demonstrated the existence of variations in the sequences of the 5′ and 3′ untranslated regions of *C. gigas* MT (*CgMT*) genes and of variants in coding sequence (Tanguy *et al.* 2001). We also identified a particular MT isoform in *C. gigas* that presents an unique duplication of coding sequence leading to the formation of a protein that possesses a higher metal-binding capacity compared with other MTs (Tanguy and Moraga 2001). This novel protein could reflect an adaptive process to heavy metal resistance. A variety of techniques are available for the identification of single nucleotide polymorphisms (SNPs) in polymerase chain reaction (PCR) products such as RFLP analysis, single-strand conformation polymorphism (SSCP) analysis (Orita *et al.* 1989), heteroduplex analysis (White *et al.* 1992) and denaturing gradient gel electrophoresis (Myers *et al.* 1987). In this study, we used SSCP analysis, which has been reported to be able to detect more than 99% of point mutations in DNA molecules 100–300 bp in length (Orita *et al.* 1989, Hayashi 1991).

The aim of this study was to characterize new nuclear genetic markers in the Pacific oyster, *C. gigas,* and to study the polymorphism of selected genes in relation to environmental stress factors such as pollution by heavy metals. First, we characterized several mutations in exons 2 and 3 of *CgMT1* in field populations and compared their frequency to the degree of heavy metal contamination of these populations. We then validated the field data in controlled laboratory experiments. Here, we report on the existence of a significant correlation between the frequency distribution of two SSCP types in exons 2 and 3 of *CgMT1* and the resistance of oysters to heavy metals.

Materials and methods

Experimental design

The Pacific oysters, *C. gigas,* were sampled from a field population located at 'La Pointe du Chateau' (Bay of Brest, Brittany, France) and maintained in aerated filtered seawater for 2 weeks before beginning the experiment. Three groups of 150 individuals were then exposed to either 1 p.p.m. of cadmium (Cd^{2+}), 1 p.p.m. of copper (Cu^{2+}) or a mixture of 0.5 p.p.m. copper plus 0.5 p.p.m. of cadmium. A group of 50 oysters were also maintained in seawater without metal as a control. The seawater was aerated and changed every day, and the oysters were fed microalgae every 2 days. Mortality was determined every day, and the gills of the dead oysters were immediately sampled for DNA extraction.

Field collection

Seven field populations of *C. gigas* were sampled along the French Atlantic and Mediterranean coasts. The populations were characterized according to the degree of environmental contamination by heavy metals. The main physicochemical characteristics of the population sites studied are presented in figure 1. For each oyster sampled, the gill was harvested and its DNA was immediately extracted according to the protocol described below.

DNA extraction

Genomic DNA was extracted from oyster gills. About 100 mg of tissue sample was placed in extraction buffer (0.1 M NaCl, 0.02 M ethylene diamine tetra-acetic acid [EDTA], 0.3 M Tris, pH 8). Sodium dodecyl sulphate (SDS) and proteinase K were added at a final concentration of 0.6% and 0.1 mg ml^{-1}, respectively, and the mixture was incubated at 55°C until complete dissolution of tissue had occurred. NaCl was then added to a final concentration of 1.3 M, and the samples were homogenized before centrifugation at 3000 *g* at 20°C for 10 min. The supernatant was transferred to a new tube and two phenol/chloroform/isoamyl alcohol (25:24:1) extractions were performed. DNA was precipitated with absolute ethanol, recovered with a Pasteur pipette, dried and dissolved in 1 ml of TE buffer (composition = 10 mM Tris pH = 8, 1 mM EDTA).

PCR-SSCP analysis

Exon 2 of the *CgMT1* gene was amplified using the forward primer P1 (5′-TAACTGATCATTTTTTGTCAG-3′) and the reverse primer P2 (5′-TCAATCGATAGAAAATACTTAC-3′). Exon 3 of the *CgMT1* gene was amplified using the forward primer P3 (5′-ATCATTGATTTTTCTTTTGACAGG-3′) and the reverse primer P4 (5′-AGAATACATCCAGGAGAAAC-3′).

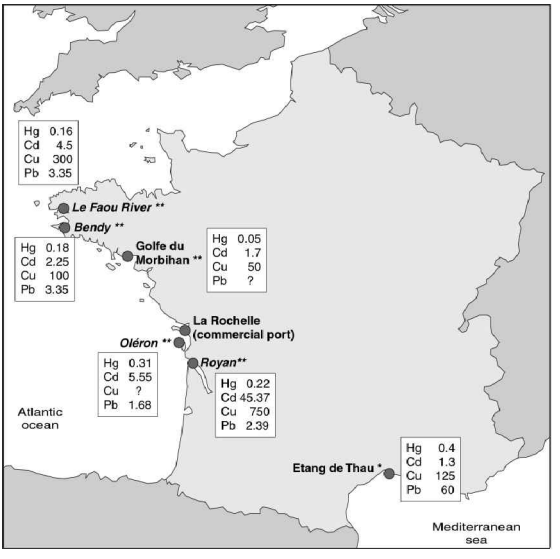

Figure 1. Oyster collection sites. Metal concentrations (*µg g^{-1} of sediment, **mg kg^{-1} dry weight tissue) were provided by RNO (National Observation Network, IFREMER, France).

All PCR amplifications were performed in a robocycler (Stratagene, Amsterdam, The Netherlands) in a volume of 25 µl containing 1 × Taq polymerase buffer, 2 mM $MgCl_2$, 200 µM deoxynucleotides (dNTPs), 10 pmol of each primer, 0.5 units of Taq polymerase (Promega, Madison, Wisconsin, USA) and about 100 ng of total genomic DNA. After an initial 5 min denaturation at 94°C, 2 min annealing at 55°C and 40 s elongation at 72°C, 35 amplification cycles were performed as follows: 40 s at 72°C, 30 s at 94°C, 40 s at 55°C, with a final 10 min at 72°C. The PCR products were then added to a 30 µl of denaturing/loading buffer (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue and 0.25% xylene cyanol), heated for 5 min at 94°C, and rapidly chilled on ice to melt and retain single-strand DNA. After loading on a neutral 12% polyacrylamide gel (37.5:1, acrylamide:bisacrylamide), the samples were electrophoresed at constant voltage (120 V) in a 0.6 × TBE buffer (composition = 0.05 m Tris, 0.05 m Boric acid, 0.001 m EDTA) for 20 h at 4°C. After electrophoresis, the gels were stained by ethidium bromide and visualized under ultraviolet light. Single-strand DNA from the PCR products visualized on the gel as different conformation types obtained were gel-purified (Kit Geneclean III, Bio 101, Vista, California, USA) and inserted into the pGEM-T vector (Promega). The inserts of pGEM-T were manually sequenced by extension from both ends using T7 and Sp6 universal primers (T7 sequencing kit, Amersham Pharmacia Biotech, Uppsala, Sweden). Each type was sequenced from three different samples for confirmation when possible.

Statistical analysis

The frequency distributions of the types in the field and laboratory populations was analysed using an R × C test of independence (G-test) using the Williams correction. The correlation between type frequency and oyster survival time was tested using Cox's model (Cox and Oakes 1984) as developed in 'Survival Analysis' of the CSS Statistica (Statsoft, Tulsa, Oklahoma, USA).

Results

Field populations

Polymorphism of CgMT1 *exon 2.* PCR-SSCP performed on the second coding exon of the *CgMT1* gene allowed us to characterize seven different polymorphisms named, respectively, A, B, C, D, E, F and G, with types A, B and C being present in more than 83% of the individuals. The impossibility to discriminate homozygotes from heterozygotes on the gel conducted to analyse the profiles observed in terms of types. The pattern of each type is shown in figure 2A. Each was composed of one to three fragments on the acrylamide gel. A common fragment was observed for most of them that corresponds to the sequence of type A; this was used as the reference with which all other fragment sequences were compared. All the bands at the same migration level on the gel yielded the same sequence. The type frequency distribution in the field populations is presented in table 1. Among these types, four (types D, E, F and G) were characterized by a very low frequency and were only present in some populations. The application of a G-test on the frequency distribution of the exon 2 types between the populations showed a significant value ($G = 772.65$, $p < 0.001$), showing a non-homogeneous partitioning of the different types in these populations. Type C increased in frequency from the least to the most polluted populations, except in the La Rochelle population.

The sequences of the different fragments characterized as the SSCP types revealed that four of them (C, D, E and F) contain a fragment with a polymorphism resulting in a modification of the corresponding amino acid (figure 3A). Type C has a variation in the amino acids asparagine 46 and cysteine 47, which are changed to a lysine and a valine residue, respectively. Two other types (B and G) showed a modification in the third base of the codon that does not change the corresponding amino acid.

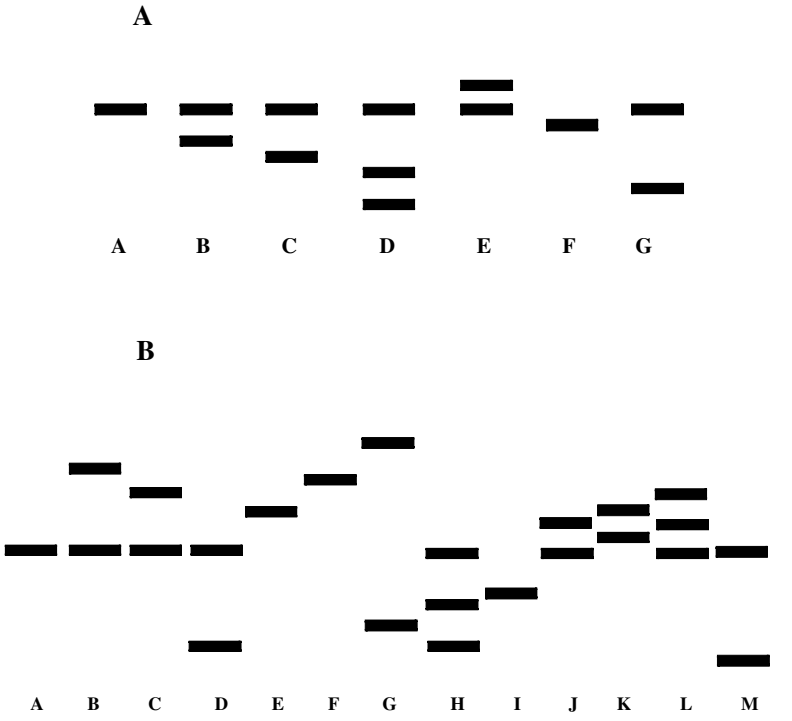

Figure 2. Representation of the PCR-SSCP profiles obtained for exon 2 (A) and exon 3 (B) of the MT gene in *C. gigas*. The names of the different types are indicated by the capital letters beneath them.

Table 1. Distribution of CgMT1 exon 2 type frequency in the field and experimental *C. gigas* populations. The number of individuals is indicated in parentheses. S: susceptible oysters. R: resistant oysters.

	Field populations							Experimental populations			
								Cadmium		Copper-cadmium	
Type	Gulf of Morbihan (96)	Bendy (96)	Faou River (96)	Oleron island (72)	Thau Lake (96)	La Rochelle (72)	Royan (72)	S (76)	R (52)	S (92)	R (45)
A	0.823	0.804	0.756	0.853	0.755	0.797	0.763	0.776	0.711	0.811	0.721
B	0.104	0.087	0.054	0.027	0.088	0.140	0.055	0.065	0.115	0.011	0.022
C	0.031	0.065	0.108	0.095	0.111	0.062	0.125	0.092	0.135	0.144	0.209
D	0.031	0.032	0.027	0.013	0.022	–	0.028	–	–	–	–
E	–	–	–	–	0.022	–	0.014	0.013	–	0.022	–
F	–	–	–	0.013	–	–	0.014	–	–	–	–
G	0.01	0.011	0.054	–	–	–	–	0.039	0.039	0.011	0.044

Polymorphism of CgMT1 *exon 3*. As described for exon 2, the PCR-SSCP results from the third coding exon of the *CgMT1* gene were analysed and characterized into 13 different types, named from A to M (figure 2B). The type frequency distribution in the field populations is presented in table 2. Among these types, only two (type A and B) were present at a high frequency, between 65 and 80% in these populations.

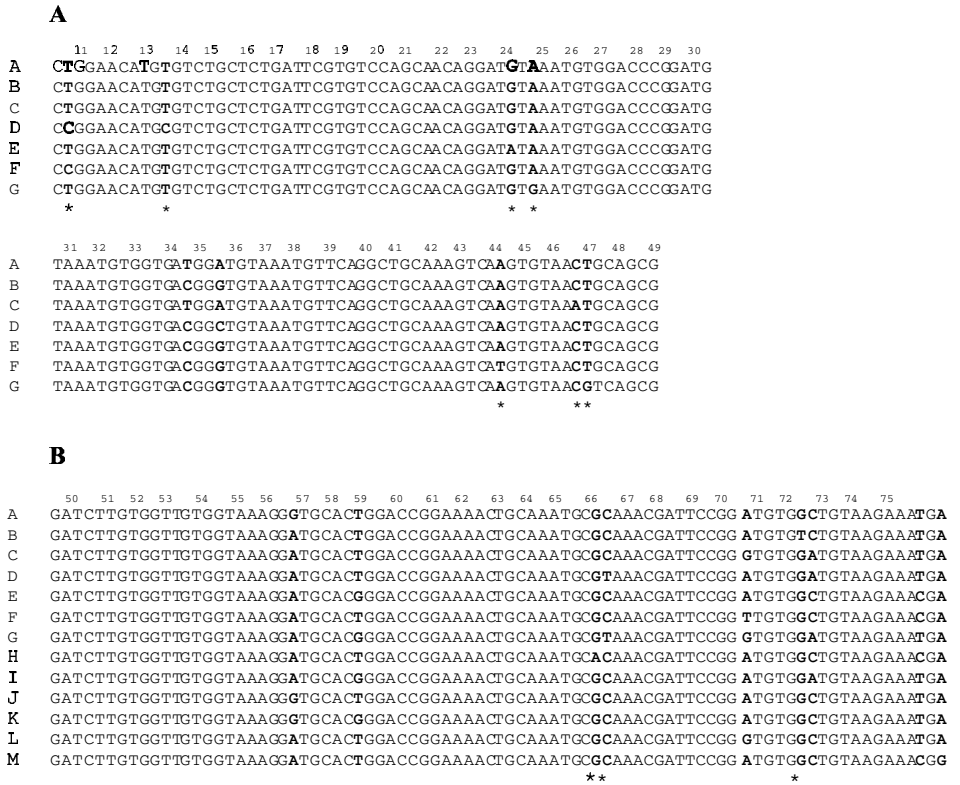

Figure 3. Sequences of *CgMT1* exon 2 types (A) and of CgMT1 exon 3 types (B). Nucleic base substitutions are shown in bold, and modified amino acids are indicated by asterisks. The corresponding number of each amino acid in the *CgMT1* gene sequence is indicated at the top of the figure in line with the first base of the corresponding codon.

Table 2. Distribution of CgMT1 exon 3 type frequency in the field and experimental *C. gigas* populations. The number of individuals is indicated in parentheses. S: susceptible oysters. R: resistant oysters.

	Field populations							Experimental populations			
								Cadmium		Copper-cadmium	
Type	Gulf of Morbihan (96)	Bendy (96)	Faou River (96)	Oleron island (72)	Thau Lake (96)	La Rochelle (72)	Royan (72)	S (76)	R (52)	S (92)	R (45)
A	0.698	0.703	0.632	0.507	0.568	0.513	0.567	0.652	0.490	0.728	0.605
B	0.138	0.175	0.195	0.145	0.227	0.257	0.390	0.202	0.347	0.197	0.373
C	0.032	–	0.034	0.098	0.068	0.040	–	–	–	–	–
D	0.011	0.027	0.046	0.028	0.022	–	–	0.039	0.038	0.022	–
E	-	0.054	–	–	–	0.014	0.013	0.052	0.038	0.011	–
F	0.010	–	–	–	–	–	–	–	–	–	–
G	0.011	–	0.046	0.067	0.022	0.068	–	–	–	–	–
H	-	–	0.014	–	–	0.040	0.013	–	–	–	–
I	0.021	–	0.023	0.014	0.068	0.040	–	–	–	–	–
J	-	0.054	0.018	0.067	0.022	0.014	0.014	0.026	0.019	–	0.022
K	0.056	–	–	0.056	–	0.014	–	–	–	–	–
L	0.021	0.041	–	–	–	–	–	0.013	0.057	0.033	–
M	-	–	–	0.014	–	–	–	–	–	–	–

Comparison of the frequency distribution of the types in the field populations yielded a significant value ($G = 623.2$, $p < 0.001$), indicating a non-homogeneous distribution of the SSCP types in these populations. The frequency of type B increased from the least contaminated populations to the most exposed populations, whereas the frequency of type A showed an inverse trend.

The sequences of the types revealed that six of them (B, D, E, F, G and M) show a polymorphism resulting in a modification of the corresponding amino acid (figure 3B), the sequence of type A being used as a reference. Type B has a variation in the amino acid glycine 72 that changes it to a valine residue. The other types (C, H, I, J, K and L) have a modification in the third base of the codon that does not change the corresponding amino acid.

Experimental populations

Survival curves. Survival curves over time and as a function of cadmium or cadmium-copper mixture concentration were constructed based on the monitoring of oyster mortality. Two different groups – 'susceptible', which included oysters that died during the experiment, and 'resistant', which represented those oysters still alive at the end of the experiment, were identified. The control population, which was not exposed to heavy metals, did not exhibit any mortality, suggesting that the observed mortality was attributable to the presence of the contaminants. The survival curves obtained from both 1 p.p.m. of cadmium exposure and the mixture of 0.5 p.p.m. of cadmium and 0.5 p.p.m. of copper exposure reveal that 59% and 67% of the oysters, respectively, died after about 40 days of exposure. No mortality was observed in oysters exposed to 1 p.p.m. of copper.

Polymorphism of CgMT1 *exon 2.* Comparison of the frequency distributions of the different types between the susceptible and the resistant groups of oysters (table 1) generated a significant G-test value for both exposure regimes (cadmium: $G = 159.6$, $p < 0.001$; cadmium-copper: $G = 4.6$, $p < 0.1$), indicating there is a non-homogeneous distribution of the types among the two groups. The frequency of type C increased in resistant oysters in the two contamination experiments with exposure to either cadmium or the cadmium-copper mixture. Cox's statistical model was used to detect the occurrence of oyster type associated with either susceptibility or resistance to heavy metals by testing whether survival time differed between the types. However, this model generated no significant results for type C of the *CgMT1* exon 2 in both the cadmium and the cadmium-copper exposure experiments ($\chi^2 = 1.406$, $p = 0.244$ and $\chi^2 = 1.393$, $p = 0.166$, respectively).

Polymorphism of CgMT1 *exon 3.* Comparison of the frequency distributions of the different types between the susceptible and the resistant groups of oysters (table 2) generated a significant value for both experiments (cadmium: $G = 6.1$, $p < 0.05$; cadmium-copper: $G = 8.72$, $p < 0.05$), indicating a non-homogeneous distribution of the types among the two groups. The frequency of type B was significantly higher in resistant oysters in the two contamination experiments, while type A frequency showed an inverse trend. Cox's model generated significant results for type B of exon 3 of *CgMT1* in both the cadmium and

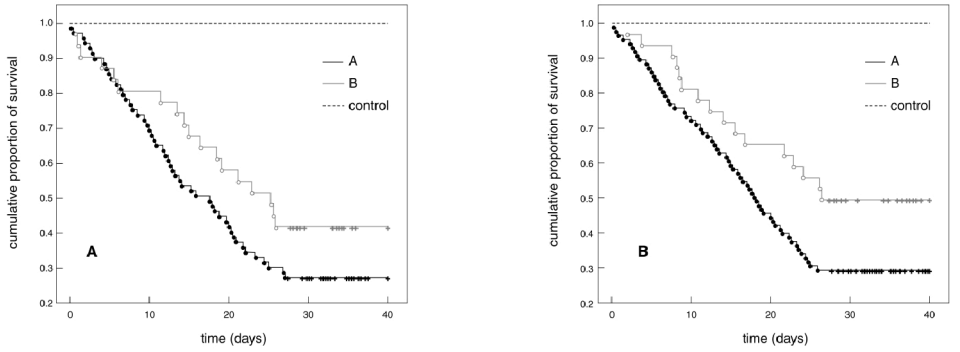

Figure 4. Cox's model cumulative percentage survival curves of the main exon 3 types (A, black line and B, grey line) in the cadmium (A) and copper-cadmium (B) contamination experiments.

the cadmium-copper exposure experiments ($\chi^2 = 1.823$, $p = 0.034$ and $\chi^2 = 1.92$, $p = 0.0256$, respectively) (figure 4).

Discussion

The SSCP techniques used allowed us to demonstrate the existence of polymorphism in the coding sequence of MT genes in the Pacific oyster *C. gigas*. Although a high number of types were detected in the two exons studied, only five were widely distributed in terms of frequency. Moreover, among these five types, only two (type C for exon 2 and type B for exon 3) presented a variation that resulted in a modification of the corresponding amino acid. These results show a low level of polymorphism in the coding sequences of MT genes in *C. gigas*. This study was also conducted on exon 3 of the *CgMT2* gene, showing a case of sequence duplication but no polymorphism in the first 24 samples analysed in each population (data not shown). This very weak level of polymorphism in *CgMT2* could be partially explained by a possible recent appearance of this particular gene in the *C. gigas* genome, the sequence of *CgMT2* being identical to the type A sequences of *CgMT1* for both exon 2 and 3.

The results obtained in laboratory experiments were similar for oysters contaminated by either cadmium or a mixture of cadmium and copper, but no mortality was observed for copper concentration alone. These results confirm the higher toxicity of cadmium compared with copper and suggest that the presence of high concentrations of cadmium in field populations probably have a stronger selective effect on the oyster population survival than do high concentrations of copper. Moreover, the concentrations of cadmium used (1 p.p.m., and 0.5 p.p.m. with 0.5 p.p.m. of copper) had a similar selective effect on the same types for both exon 2 and 3 of the *CgMT1* gene.

When the field oyster populations were classified according to an increasing gradient of pollution by heavy metals (cadmium specifically), we observed a weak increase in the frequency of type C in exon 2 and a strong increase in the frequency of type B in exon 3 in the most polluted populations. The results obtained in the field populations agree with those obtained under experimental conditions. This allows us to consider these two types, especially type B in *CgMT1* exon 3, as

appropriate genetic indicators for the monitoring of heavy metal contaminated ecosystems.

In this study, we established that type C for the *CgMT1* exon 2 and type B for *CgMT1* exon 3 are characteristic of resistant oysters, but only few oysters possess both two types. This result could be explained by the existence of more than two loci encoding for the *CgMT1* gene in the genome, so that amplified types contain, in fact, the exon sequences of several MT genes. This also suggests that type C in exon 2 could belong to one locus and type B in exon 3 could belong to another locus. The fact that we were able to characterize from one to three bands for the same exon, depending on the oyster, confirms the possibility of a co-amplification of several loci for the MT gene. The impossibility of attributing the different alleles to one locus makes it difficult to analyses the results in terms of genotypes. Similar results were observed by Fulton *et al*. (2001), but in this study the authors assigned genotypes to different SSCP profiles: homozygotes were represented by one or two bands and heterozygotes by two or three bands on the gel. They also observed the existence of many null alleles that complicated the analysis of the SSCP results and could explain the variation in the SSCP profiles they observed. Similar interpretations could partially explain our SSCP profiles. For these reasons, we chose to evaluate the data in terms of types that were reproducible among individuals and that could be analysed statistically. Our results can be easily explained by the multigenic character of the MT family, as shown in other species (Karin *et al*. 1984, Andersen *et al*. 1987, Peterson *et al*. 1988) and in this species (Tanguy and Moraga 2001, Tanguy *et al*. 2001). Moreover, MT genes are known to be able to be amplified in the genome in response to heavy metal stress. Studies done on cadmium-resistant mouse cells showed an increase of the *MT-I* gene copy number (Beach and Palmiter 1981, Gick and McCarty 1982, Mayo and Palmiter 1982) and a case of MT gene duplication has also been described in natural populations of *Drosophila melanogaster* living in a cadmium-polluted environment (Maroni *et al*. 1987). More recently, we showed the existence of at least two loci of another MT gene in *C. gigas* (Tanguy and Moraga 2001) whose sequences are similar, especially in the flanking sequences of the second exon, making the selective amplification of the exon for each MT gene impossible. All these data confirm the possible existence of several loci for *CgMT1*, and this could partly explain our results and also the impossibility of characterizing a genotype for each exon.

The two types that seem to be selected by cadmium show the presence of a mutation resulting in a modification of the corresponding amino acid that is neighbour to a cysteine residue. The importance of the physical characteristics of the amino acid involved in the mutation has been previously demonstrated in *Neurospora crassa* (Cismowski and Huang 1991) and in mammal models (Cismowski *et al*. 1991, Chernaik and Huang 1991). The replacement of the cysteine residue by any amino acid except histidine can alter the structural, functional and stochiometric properties of the MT (Romeyer *et al*. 1990, Cismowski and Huang 1991). In a recent study, Muñoz *et al*. (2000) investigated the influence of the position of the cysteine residues and the steric and electrostatic effects of neighbouring amino acids on the folding and stability of the MT cluster in the lobster. The differences observed in the structure and the reactivity of the MTs demonstrated that the requirements for the formation of a stable cadmium cluster are more stringent than simply the sequential positions of the cysteines

along the peptide chain and must include interactions involving neighbouring, non-cysteine amino acids. In the case of type C in exon 2 of the *CgMT1* gene, the residue asparagine is characterized by the presence of an amide (CO) motif that imposes a particular spatial conformation to the neighbouring residue. In type C, this residue is replaced by the basic amino acid lysine that contains no amine motif, allowing free rotation of the neighbouring amino acid. In exon 3 of *CgMT1*, the small amino acid glycine is replaced by the bigger hydrophobic amino acid valine. Though no information is available on the modifications caused by this kind of substitution, it is possible that they affect the three-dimensional structure of MTs, resulting in different metal-binding properties in the corresponding proteins.

Our work demonstrated a selective effect of cadmium on a non-enzymatic protein that is assumed to be involved in both the regulation of cellular metals and the detoxification of heavy metals. As has been reported, MTs are able to bind most of the metallic ions (Waalkes and Klaassen 1984, Kägi and Kojima 1987). Therefore, the selective effect observed on particular types could be the same for other metals besides cadmium. MTs also have a role in the transfer of essential metallic ions to metalloproteins such as carbonic anhydrase, aldolase, alkaline phosphatase and thermolysine, which are involved in cellular division (Udom and Brady 1980, Compere and Palmiter 1981, Crawford *et al.* 1985). They also play a crucial part in zinc metabolism that involves some metalloenzymes important for nucleic acid transcription, such as DNA polymerases (Slater *et al.* 1971). An alteration of the MT structure could have important consequences for some physiological processes. If we assume that modifications of the coding sequence can have implications for the properties of MTs as previously described, then it is possible that type C in exon 2 and type B in exon 3 could encode for more efficient proteins.

Our results have demonstrated the existence of an exonic polymorphism in the *C. gigas* MT genes that could be related to greater resistance to heavy metals, these results being concordant in both experimentally contaminated and field populations. Even if analysis of this polymorphism in terms of genotypes is probably impossible due to the multigenic character of this gene family, the characterization of reproducible types allowed us to determine the possible selection by heavy metals of some alleles of the MT family in *C. gigas* and reflect a selective process on functional genes. The genetic indicators characterized will be used in further studies for the monitoring of disturbed ecosystems using *C. gigas* as a sentinel species.

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References

Andersen, R. D., Taplitz, S. J., Birren, B. W., Bristol, G. and Herschman, H. R. 1987, Rat metallothionein multigene family. *Experientia Supplementum*, **52**, 373–384

Barsyte, D., White, K. N. and Lovejoy, D. A. 1999, Cloning and characterization of metallothionein cDNAs in the mussel *Mytilus edulis* L. digestive gland. *Comparative Biochemistry and Physiology: Part C, Pharmacology, Toxicology and Endocrinology*, **122**, 287–296.

Bates, L. J. and Mulley, J. C. 1988, A BamHI RFLP at MT2A on human chromosome 16. *Nucleic Acids Research*, **16**, 9071.

Beach, L. R. and Palmiter, R. D. 1981, Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proceedings of the National Academy of Sciences of the United States of America*, **78**, 2110–2114.

Boutet, I., Tanguy A., Auffret, M., Riso, R. and Moraga, D. 2002, Immunochemical quantification of metallothioneins in marine molluscs: characterisation of a metal exposure bioindicator. *Environmental Toxicology and Chemistry*, **21**, 1009–1014.

Butler, R. A. and Roesijadi, G. 2001, Quantitative reverse transcription polymerase chain reaction of a molluscan metallothionein mRNA. *Aquatic Toxicology*, **54**, 59–67.

Chernaik, M. L. and Huang, P. C. 1991, Differential effect of cysteine to serine substitutions in metallothionein on cadmium resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 3024–3028.

Cismowski, M. J. and Huang, P. C. 1991, Effect of cysteine replacements at positions 13 and 50 on metallothionein structure. *Biochemistry*, **30**, 6626–6632.

Cismowski, M. J., Narula, S. S., Armitage, I. M., Chernaik, M. L. and Huang, P. C. 1991, Mutation of invariant cysteines of mammalian metallothionein alters metal binding capacity, cadmium resistance and Cd-113 NMR spectrum. *Journal of Biological Chemistry*, **26**, 24390–24397.

Compere, S. J. and Palmiter, R. D. 1981, DNA methylation controls the inducibility of the mouse metallothionein-I gene lymphoid cells. *Cell*, **25**, 233–240.

Cox, D. R. and Oakes, D. (editors) 1984, *Analysis of Survival Data* (London: Chapman & Hall).

Crawford, B. D., Enger, M. D., Griffith, B. B., Hanners, J. L., Longmire, J. L., Munk, A. C., Stallings, R. L., Tesmer, J. G. and Walters, R. A. 1985, Coordinate amplification of metallothionein I and II genes in cadmium-resistant Chinese hamster cells: implications for mechanisms regulating metallothionein gene expression. *Molecular and Cellular Biology*, **5**, 320–329.

Freedman, J. H., Slice, L. W., Dixon, D., Fire, A. and Rubin, C. S. 1993, The novel metallothionein genes of *Caenorhabditis elegans*. Structural organization and inducible, cell-specific expression. *Journal of Biological Chemistry*, **268**, 2554–2564.

Fuentes, M. E., Unger, M. E. and Roesijadi, G. 1994, Individual variability in the 3′ untranslated region of metallothionein mRNAs in a natural population of the mollusc *Crassostrea virginica*. *Molecular Marine Biology and Biotechnology*, **3**, 141–148.

Fulton, R. E., Salaseck, M. L., DuTeau, N. M. and Black, W. C. 2001, SSCP analysis of cDNA markers provides a dense linkage map of the *Aedes aegypti* genome. *Genetics*, **158**, 715–726.

Gick, G. G. and McCarty, K. S. 1982, Amplification of the metallothionein-I gene in cadmium- and zinc-resistant Chinese hamster ovary cells. *Journal of Biological Chemistry*, **257**, 9049–9053.

Hamer, D. H. 1986, Metallothioneins. *Annual Review of Biochemistry*, **55**, 913–951.

Hamza-Chaffai, A., Amiard, J. C., Pellerin, J., Joux, L. and Berthet, B. 2000, The potential use of metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal exposure. *Comparative Biochemistry and Physiology: Part C, Pharmacology, Toxicology and Endocrinology*, **127**, 185–197.

Hayashi, K. 1991, PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods and Applications*, **1**, 34–38.

Kägi, J. H. R. and Kojima, Y. 1987, Chemistry and biochemistry of metallothionein. *Experientia Supplementum*, **52**, 25–61.

Kägi, J. H. R. 1993, Evolution, structure and chemical activity of class I metallothioneins: an overview. In *Metallothionein III: Biological Roles and Medical Implications*, edited by K. T. Suzuki, N. Imura and M. Kimura (Basel: Birkhauser Verlag), pp. 29–55.

Karin, M., Eddy, R., Henry, M., Haley, L., Byers, M. G. and Shows, T. 1984, Human metallothionein genes are clustered on chromosome 16. *Proceedings of the National Academy of Sciences of the United States of America*, **81**, 5494–5498.

Linde, A. R., Sanchez-Galan, S., Valles-Mota, P. and Garcia-Vazquez, E. 2001, Metallothioneins as bioindicator of freshwater metal pollution: European eel and brown trout. *Ecotoxicology and Environmental Safety*, **49**, 60–63.

Maroni, G., Wise, J., Young, J. E. and Otto, E., 1987, Metallothionein genes duplications and metal tolerance in natural populations of *Drosophila Melanogaster*. *Genetics*, **117**, 739–744.

Mayo, K. E. and Palmiter, R. D. 1982, Glucocorticoid regulation of the mouse metallothionein genes is selectively lost following amplification of the gene. *Journal of Biological Chemistry*, **257**, 3061–3067.

Mehra, R. K., Garey, J. R. and Winge, D. R. 1990, Selective and tandem amplification of a member of the metallothionein gene family in *Candida glabrata*. *Journal of Biological Chemistry*, **265**, 6369–6375.

Muñoz, A., Petering, D. H. and Shaw, C. F. III. 2000, The requirements of stable metallothionein clusters examined using synthetic lobster domains. *Marine Environmental Research*, **50**, 93–97.

Myers, R. M., Maniatis, T. and Lerman, L. S. 1987, Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods in Enzymology*, **155**, 501–527.

Nemer, M., Wilkinson, D. G. and Travaglini, E. C. 1985, Primary differentiation and ectoderm-specific gene expression in the animalized sea urchin embryo. *Developmental Biology*, **109**, 418–427.

Orita, M., Iwahana, H., Kanazawa, H., Kayashi, K. and Sekiya, T. 1989, Detection of polymorphisms in human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 2766–2770.

Peterson, M. G., Hannan, F. and Mercer, J. F. B. 1988, The sheep metallothionein gene family. Structure, sequence and evolutionary relationship of five linked genes. *European Journal of Biochemistry*, **174**, 417–424.

Romeyer, F. M., Jacobs, F. A. and Brousseau, R. 1990, Expression of *Neurospora crassa* metallothionein and its variants in *Escherichia coli*. *Applied and Environmental Microbiology*, **56**, 2748–2754.

Slater, J. D., Mildvan, A. S. and Loeb, L. A. 1971, Zinc in DNA polymerases. *Biochemical and Biophysical Research Communications*, **44**, 37–43.

Tanguy, A. and Moraga, D. 2001, Cloning and characterization of a gene coding for a novel metallothionein in the Pacific oyster *Crassostrea gigas* (CgMT2): a case of adaptive response to metal-induced stress? *Gene*, **273**, 123–130.

Tanguy, A., Mura, C. and Moraga, D. 2001, Cloning of a metallothionein gene and characterization of two other cDNA sequences of the Pacific oyster *Crassostrea gigas*. *Aquatic Toxicology*, **55**, 35–47.

Udom, A. O. and Brady, F. O. 1980, Reactivation in vitro of zinc-requiring apo-enzymes by rat liver zinc-thionein. *Biochemical Journal*, **187**, 329–335.

Unger, M. E., Chen, T. T., Fenselau, C. C., Murphy, C. M., Vestling, M. M. and Roesijadi, G. 1991, Primary structure of a molluscan metallothionein deduced from molecular cloning and tandem mass spectrometry. *Biochimica et Biophysica Acta*, **1074**, 371–377.

Varshney, U. Hoar, D. I., Starozik, D. and Gedamu, L. 1984, A frequent restriction fragment length polymorphism in the human metallothionein-II processed gene region is evolutionarily conserved. *Molecular Biology and Medicine*, **2**, 193–206.

Waalkes, M. P. and Klaassen, C. D. 1984, Relative in vitro affinity of hepatic metallothionein for metals. *Toxicology Letters*, **20**, 33–39.

Watanabe, T., Shimizu, A., Ohno, K., Masaki, S. and Kondo, K. 1989, Restriction fragment length variations and chromosome mapping of two mouse metallothionein genes, Mt-1 and Mt-2. *Biochemical Genetics*, **27**, 689–697.

White, M. B., Carvalho, M., Derse, D., O'Brien, S. J. and Dean, M. 1992, Detecting single base substitutions as heteroduplex polymorphisms. *Genomics*, **12**, 301–306.