MARINE BIOTECHNOLOGY

Small Metallothionein MT-10 Genes in Coastal and Hydrothermal Mussels

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Abstract

Metallothioneins (MTs) are important proteins in the intracellular regulation of metals. In the Mytilidae family, which includes many economically important species, 2 major forms of MTs have been reported: MT-10 (10 kDa) and MT-20 (20 kDa). Many different MT-10 proteins have been isolated from the common species *Mytilus edulis*, which suggests that distinct MT-10 genes may occur in a single specimen. Some MT genes, involving 3 exons and 2 large introns, have been isolated in Mytilidae. Our aim was to determine whether intron-free forms of the MT-10 genes can exist, which could allow rapid transcription in response to exposure to metals. Our study focused on 2 species living under very different environmental conditions: *Mytilus edulis* (a coastal mussel) and *Bathymodiolus thermophilus* (a hydrothermal mussel). We report here the first description of small, intron-free MT-10 genes, possessing a correct open reading frame in these 2 species.

Key words: metallothionein — MT-10 — gene — Mytilus edulis — Bathymodiolus thermophilus

Introduction

Metallothioneins (MTs) constitute a family of ubiquitous cysteine-rich, low molecular weight proteins that are involved in intracellular regulatory mechanisms (Bauman et al., 1993; Kag, 1993; Roesijadi et al., 1997; Legras et al., 2000; Park et al., 2001; Wu et al., 2002) and play an antioxidant role (Viarengo et al., 1999, 2000; Cavaletto et al., 2002; Sato and Kondoh, 2002). The MTs have been divided into 3 classes according to the distribution of the Cys-Cys motif: Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys (where X is an amino acid other than cysteine). Mollusk MTs all belong to class I, as do mammalian and crustacean MTs (Kagi, 1993; Picinni et al., 1999). The spatial arrangement of these repetitive motifs seems to determine the metal-binding properties of the MT (George and Hodgson, 1995). The cysteine residues constitute 2 domains (α and β) (metal-thiolate clusters) that bind the metal ions (Kagi et al., 1984; Braun et al., 1992). If the intracellular concentrations of essential metals are too high, or toxic metals are present within the cell, then synthesis of the MT proteins is induced. These proteins bind the metal ions and convey them to the intracellular lysosomal system (George, 1983).

Many reports have suggested that MT concentrations could constitute a potential indicator of marine pollution by metals (Hennig, 1986; Cajaraville et al., 2000; Dallinger et al., 2000). In the Mytilidae and Ostreidae families, 2 major marine bivalve families, the use of metallothioneins has been investigated in several genera: *Mytilus* (Amiard-Triquet et al., 1998; Petrovic et al., 2001; Geret and Cosson, 2002), *Perumytilus* (Riveros et al., 2003), and *Crassostrea* (Geffard et al., 2002). Many studies have also attempted to characterize the protein structure and complementary DNA sequences of MTs, but few publications have focused on genomic information.

In the Mytilidae, 2 major forms have been reported: MT-10 and MT-20 (Frazier et al., 1985; Mackay et al., 1993; Barsyte et al., 1999; Ivankovic et al., 2002). High Zn and Cd concentrations induce the synthesis of MT-10 (monomeric form, 10 kDa), whereas the more specific MT-20 (dimeric form, 20 kDa) is induced by exposure to Cd (Frazier et al., 1985; Isani et al., 2000; Lemoine et al., 2000). In the *Mytilus* genus MT-10 and MT-20 contain 73 amino acids (21 cysteines) and 72 amino acids (23 cysteines), respectively (Mackay et al., 1993). The addi-

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tional cysteine residues in MT-20 could enhance its metal-chelating properties or create intermolecular linkages between the 2 monomeric subunits (Mackay et al., 1993). Several cDNA isoforms of MT-10 and MT-20 have been reported in a pool of *Mytilus edulis* individuals (Mackay et al., 1993; Barsyte et al., 1999). The technique used did not make it possible to find out whether these isoforms were alleloforms or distinct MTs genes.

The identification of several isoforms could be accounted for by the existence of several genes in individuals or by polymorphism within mussel populations. In Mytilidae recent genomic characterizations indicate that MT genes consist of 3 exons and 2 introns (Khoo and Patel, 1999; Ceratto et al., 2002). This typical genomic organization matches mammalian MT structure. In *Mytilus galloprovincialis* the MT-20 gene is 1865 bp in length (Ceratto et al., 2002). Khoo and Patel (1999) suggested that in *Perna viridis* 2 distinct MT sequences may correspond to 2 different metallothionein genes: MT-I1 and MT-I2, 1657 bp and 2045 bp, respectively. However, these 2 sequences encode the same protein. A large second intron has been reported in MT-I1 (1093 bp) and MT-I2 (1291 bp). Each individual mussel could possess different MT genes, characterized by distinct regulatory regions and induced by different factors. These MT genes could have specific structures that favor their rapid transcription in response to exposure to a specific metal or metals.

Our aim was to find out whether several different mussel MT genes exist. We analyzed the genomic MT information of 2 Mytilidae species, collected from very different environments: *Mytilus edulis* (the blue mussel) and *Bathymodiolus thermophilus* (a hydrothermal mussel). We investigated 2 questions. Do Mytilidae, like the Ostreidae (A. Tanguy, personal communication), have small MT genes? And if they do, could we identify any in 2 different mussel species living in very different environments?

Materials and Methods

Biological Samples. Two specimens of *Mytilus edulis* (blue mussel, a coastal species) were examined (individuals coded N1, and N2). They were collected in Normandy (France). One specimen of *Bathymodiolus thermophilus* (a hydrothermal mussel) was collected from a site 9°N in the Pacific Ocean in 1994 (oceanographic mission ''Hero''). These mussels were conserved at −70°C until required.

DNA Isolation, Polymerase Chain Reaction, and Sequencing. Genomic DNA was isolated and purified from the mussels using the classic phenol-chloroform protocol. Any messenger RNA trace was visualized during the DNA extractions. The metallothionein genes were amplified by polymerase chain reaction (PCR) from genomic DNA (100 ng template). PCR was performed using different primers, depending on the species being studied. The primers specific for the amplification of the MT genes of *Bathymodiolus thermophilus* were BathA (5′-CCTTGTAACTGTGTCG-3′) and BathB (5′-GCAGGAACAGGCAGG-3′), and those for *Mytilus edulis* were Bath01 (5′-GACCACTGAGACCACTAC-3′) and Bath06 (5′-GGAATAAGTTCGAGTATATTGTC-3′). These 2 pairs of oligonucleotide primers were defined from a multiple alignment of the mRNA MT sequences of *Mytilus edulis* (available in GenBank) and the mRNA MT-10 sequence of *Bathymodiolus thermophilus* (available in Denis et al., 2002). PCR was performed in a 25-μl reaction volume for 35 cycles: 1 minute at 94°C (denaturing phase), 1 minute at 49°C and 57°C (for BathA/BathB and Bath01/Bath06, respectively) (annealing phase), and 1 minute at 72°C (elongation phase). The short elongation phase was designed to permit the replication only of fragments up to 1000 bases. The amplification of MT genes with the typical structure (3 exons and 2 introns) can require an elongation phase of about 2.5 minutes in view of the lengths of the MT genes already identified in Mytilidae: *Perna viridis*, 1657 and 2045 bp (Khoo and Patel, 1999); *Mytilus galloprovincialis*, 1865 bp (Ceratto et al., 2002).

The PCR products were ligated to the pGEM-T Easy (Promega) plasmid vector and transfected into *Escherichia coli* JM-109 strain (Promega). The colonies containing MT inserts were confirmed by PCR using the BathA/BathB or Bath01/Bath06 primers. A second confirmation was carried out using a *B. thermophilus* MT-10 probe. The high sequence similarity of this probe means that it could be expected to hybridize with all the metallothionein variants. The selected clones were sequenced by GENOME Express Enterprise using the universal primers SP6 and T7.

Do* M. edulis *and* B. thermophilus *Contain Processed Pseudogenes? To find out whether there were any processed pseudogenes (reverse transcription events) in the individual mussels, PCRs were carried out using the primers BathA/oligo(dT) (24-mer) for *Bathymodiolus thermophilus* and Bath01/oligo (dT) (24-mer) for *Mytilus edulis*. The optimum melting temperature of oligo (dT) is 42°C. The PCR conditions were 35 cycles consisting of 1 minute at 94°C, 1 minute at between 37°C and 42°C and 1 minute at 72°C. The low annealing temperature facilitated PCR amplification with the forward

primers BathA or Bath01. The 1-minute elongation phase allowed the replication of only small fragments.

Phylogenetic Analysis. The MT sequences obtained from *Mytilus edulis* and *Bathymodiolus thermophilus* were identified using the BLAST program (Altschul et al., 1997). A multiple alignment between the MT sequences of Mytilidae was carried out to identify the nucleotide differences. The Pileup module of the GCG software (Genetics Computer Group) and the Genedoc program (Nicholas and Nicholas, 1997) were used for the multiple alignment. Phylogenetic trees were constructed using the Phylip package (Felsentein, 1993) by the neighbor-joining method (Jukes and Cantor genetic distance). The bootstrap option was used (1000 replicates). Two trees were established: one using the complete sequences, including the insertion and deletion regions (indels), and the other based on the sequences without the indel regions. The MT mRNA sequence of *Perna viridis* (accession number AF036904) constituted the outgroup of the phylogenetic trees.

Results

***Putative Active Intron-free MT-10 Genes of* Mytilus edulis.** Amplification of the MTs from *M. edulis* (coastal blue mussel) DNA revealed 2 distinct MT genes with differing nucleotide sequence lengths (198 and 222 bp, respectively, from the start [ATG] to the stop [TGA] codons) in specimen N2, but only one MT gene (198 bp) in N1. The 2 MT genes were characterized by the absence of an intron and the presence of a correct open reading frame (ORF). Multiple alignment of these sequences (with or without the indel positions) and the MT-10 and MT-20 cDNA available in GenBank was performed using the BLAST program. This analysis revealed that small MT genes were closer to MT-10 than to MT-20 sequences of *M edulis*. These 2 small MTs were named MT-10A (222 bp in length) and MT-10B (198 bp in length), respectively. The most obvious difference between these 2 small MT-10 genes is the large deletion in the region corresponding to exon 2 of the gene. However, many other polymorphic sites are observable in the regions corresponding to exon 2 and exon 3 (Figure 1).

MT-10 A (222 bp) also had 3 CGG nucleotides (positions 119–121), that were not observed in other MT-10 isoforms. These additional nucleotides correspond to a duplication of the neighboring codon (Figure 1). If correctly incorporated in the ORF this insertion induces one additional amino acid glycine (G) in the protein (at site 41). Comparison with the protein sequences available in GenBank revealed the similarity between MT-10A (without the extra G) and the MT-10 IV protein found in *Mytilus edulis* (Figure 2). Two putative alleloforms of MT-10A were observed in the N2 mussel, MT-10A(1) and MT-10A(2) respectively. These 2 probable alleloforms displayed 3 nucleotide polymorphism sites (Figure 1), which induced one distinct amino acid in position 4 serine (S) instead of proline (P) (Figure 2).

MT-10B (198 bp) was characterized by a large deletion (site 84–107), 2 short insertions (139–144 and 189–191), and several silent mutations (35, 47, 52, 108, 138, 150, 169, 170, 175, 197, and 219) (Figure 1). This MT-10B gene possessed an ORF, and the translation revealed a protein sequence (66 amino acids, including 19 cysteines at the expected positions) identified by the BLAST program as the MT-10 protein (Figure 2). The large nucleotide deletion resulted in the elimination of a polypeptide core (protein sites 34–41) incorporating 2 cysteines. Two putative alleloforms of MT-10B were observed in the N2 mussel and coded MT-10B(1)/MT-10B(2), whereas only MT-10B(1) was observed in the N1 mussel.

The EMBL accession numbers for our sequences MT-10A(1), AJ577124, MT-10A(2), AJ577125; MT-10B (1); AJ577126; and MT-10B(2), AJ577127.

***Putative Active, Intron-free MT-10 Gene in* Bathymodiolus thermophilus.** Metallothionein ampli fication of *Bathymodiolus thermophilus* (hydrothermal mussel) yielded a partial MT sequence corresponding to a portion of the MT-10 mRNA sequence (from bases 10 to 233) of *M. edulis*. Amplification of these MT genes revealed a duplication (CAG) at position 155, a correct ORF, and no an intron. It appeared to be closer to MT-10 than to MT-20 (*Mytilus edulis*), and was designated the MT-10Bt gene. Two sequences were obtained, MT-10Bt(1) and MT-10Bt(2). These probable alleles displayed 3 polymorphic sites (positions 39, 197, and 218) (Figure 1). The level of similarity between the mRNA MT-10 sequence of *B. thermophilus* (Denis et al, 2002) and MT-10Bt (1) or MT-10Bt(2) was 99% or 99.5%. The nucleotide substitutions observed in the 2 alleles do not change the protein information (Figure 2). The EMBL accession numbers for our sequences are as follows: MT-10Bt(1), AJ577128; and MT-10Bt(2), AJ577129.

Phylogenetic Analysis. The 2 trees produced using the neighbor-joining method (Jukes and Cantor genetic distance) with or without the indel regions observed in genomic sequences (MT-10A, MT-10B, and MT-10Bt), displayed the same typology (Fig-

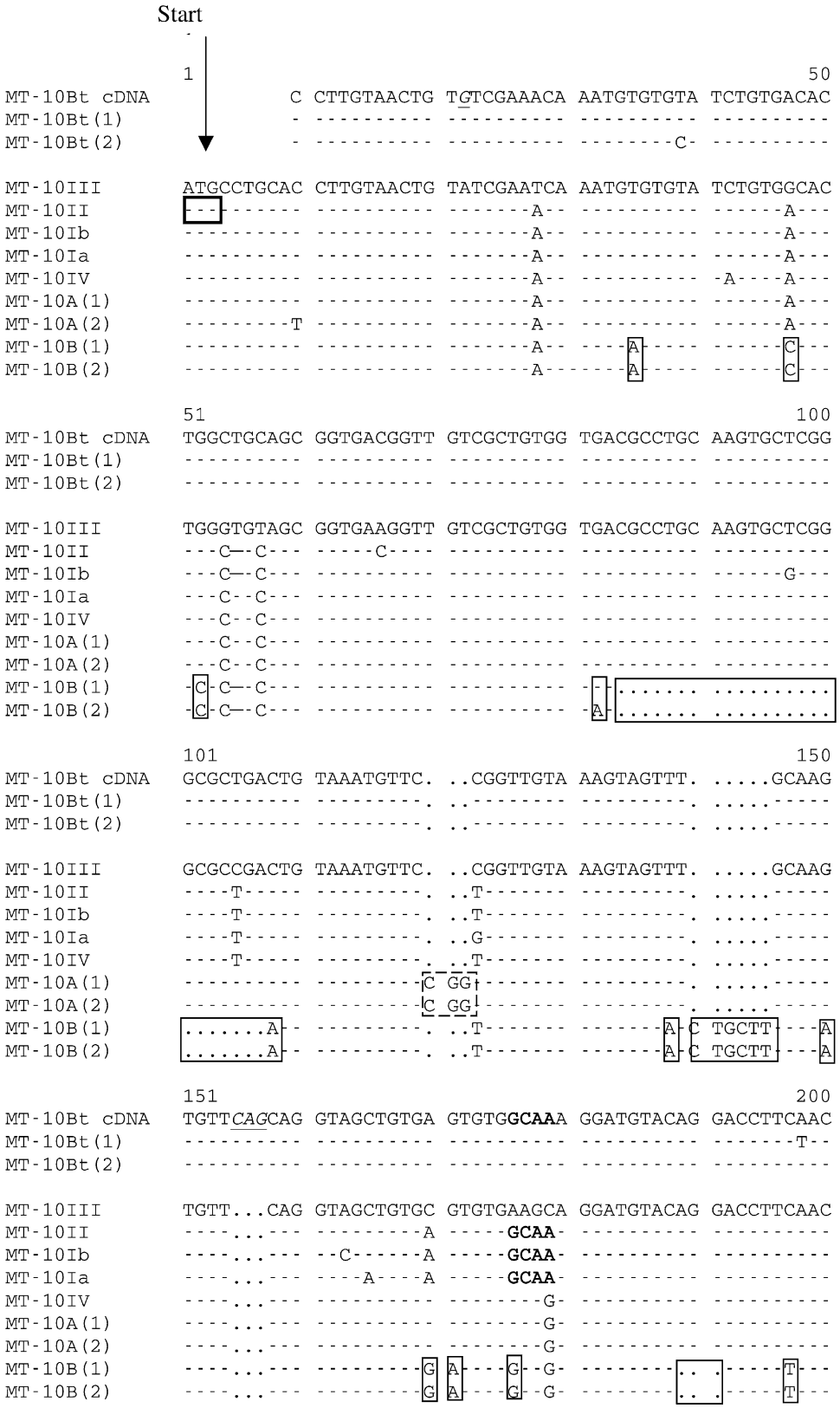

Fig. 1. Multiple alignments of MT-10 cDNA metallothionein available in GenBank with small MT-10 genes isolated from *Mytilus edulis* (MT-10A and MT-10B) and *Bathymodiolus thermophilus* (MT-10Bt). The GenBank accession numbers of the cDNA sequences are as follows: MT-10III (AJ005454), MT-10II (AJ005453), MT-10Ib (AJ005452), MT-10Ia (AJ005451), MT-10IV (AJ007506). Dots indicate deletions. The motif corresponding to MT-10I and MT-10II is shown in bold face letters. The specific insertion and deletion sites for MT-10A and MT-10B (the MT-10 genes of *Mytilus edulis*) are enclosed with dashed and solid boxes, respectively. The specific sites of the MT-10Bt sequences in *Bathymodiolus thermophilus* are indicated by italics and underlining.

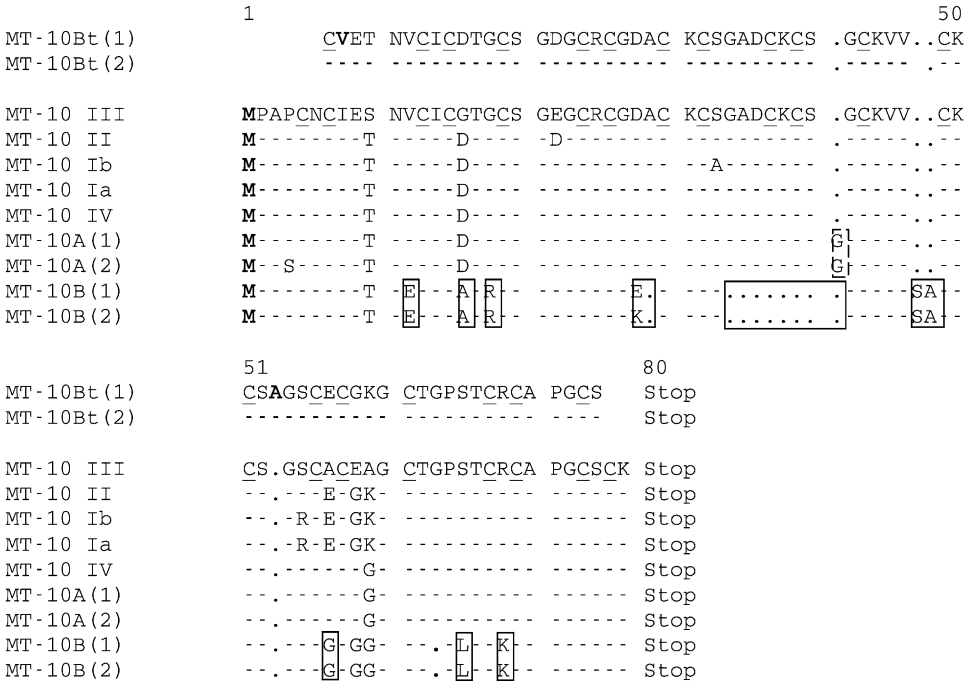

Fig. 2. Multiple alignments of a protein sequence obtained *in silico* (bioinformatic approach) from the MT-10 nucleic sequence (showed in the Figure 1) obtained from *Mytitus edulis* and *Bathymodiolus thermophilus*. The GenBank accession numbers of the cDNA sequences are as follows: MT-10III (AJ005454), MT-10II (AJ005453), MT-10Ib (AJ005452), MT-10Ia (AJ005451), and MT-10IV (AJ007506). The cysteine residues characteristic of metallothioneins are underlined. Dots indicate deletions. The specific mutation sites for MT-10A and MT-10B are enclosed by dashed and solid boxes, respectively. The specific sites for the MT-10Bt(1) and MT-10Bt(2) sequences in *Bathymodiolus thermophilus* are indicated in bold face letters.

ure 3). These trees presented 2 groups with robust branches: the first consisted of the MT-10 sequences, and the second of the MT-20 sequences. Within the MT-10 group, the *Bathymodiolus* MT-10Bt sequences were similar to the MT-10 cDNA of *B. thermophilus* and similar to the MT-10I and MT-10II isoforms of *M. edulis*. The small MT-10A sequences were similar to MT-10IV and MT-10III, whereas the MT-10B isoform was quite distinct.

Discussion

Are the Small MT-10 Genes Processed Pseudogenes or Putative Active Genes? The few MT genes that have been isolated from bivalves all contain 3 exons and 2 introns (*Crassostrea gigas*, Tanguy et al, 2001; *Mytilus galloprovmcialis*, Ceratto et al., 2002; *Perna viridis*, Khoo and Patel, 1999), and this structure is typical of the MTs found in other organisms such as birds, fish, mammals, and rice (Fernando and Andrews, 1989; Hung et al., 1991; Wenming et al., 1998; Guo et al., 2003). The expression studies demonstrated of high induction metallothionein genes of following metal exposure (Cousins, 1979; Cherian and Nordberg, 1983; Karin and Richards, 1984; Lemoine et al., 2000; Tanguy and Moraga, 2001; Patchun et al., 2004). We tried to determine whether the cosmopolitan coastal family Mytilidae (bivalves commonly chosen as sentinel organisms for detection of metal pollution) has others MT-10 genes that can be rapidly transcribed when the mussels are exposed to various metals. We confirmed the existence of intron-free MT-10 genes in *Mytilus edulis* and *Bathymodiolus thermophilus*. In *Mytilus edulis* we identified 2 distinct MT-10 genes (MT-10A and MT-10B, respectively, with putative alleles), characterized by an ORF and any intron sequence. Our investigations in *Bathymodiolus thermophilus* (a hydrothermal mussel) also revealed a distinctive MT-10 gene possessing an ORF and no intron. The MT-10Bt gene (2 putative alleles isolated) is very similar to the mRNA MT-10 of *B. thermophilus*.

We suggest 2 hypotheses that could account for the existence of these distinctive intron-free MT genes: they could be active gene; or they could be recent pseudogenes, resulting from reverse transcription.

On the one hand, the small MT genes found in *M. edulis* (MT-10A) and *B. thermophilus* (MT-10Bt) display a high degree of similarity to the MT-10 cDNA sequences. Thus these small MT-10 genes possess an ORF, and their cysteines, which confer

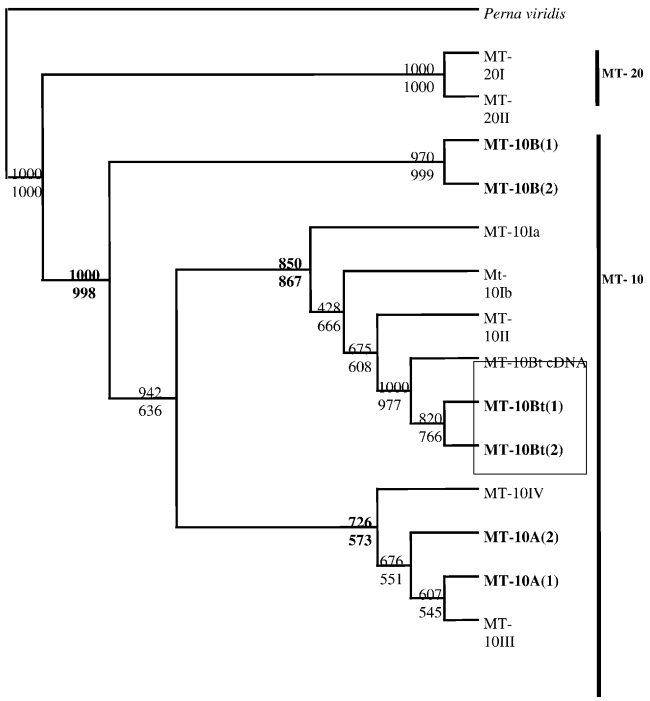

Fig. 3. Phylogenetic tree based on metallothionein cDNA and genomic sequences isolated from *Mytilus edulis* and *Bathymodiolus thermophilus*, using the neighbor-joining method with 1000 replicates (bootstrap) (top value, analysis including insertions and deletions (indels); bottom value, analysis without indel sites). (Outgroup: mRNA MTs of *Perna viridis*; AF036904). GenBank accession numbers: MT-10Ia, AJ005451; MT-10Ib, AJ005452; MT-10II, AJ005453; MT-10III, AJ005454; MT-10IV, AJ007506; MT-10A(1), AJ577124; MT-10A(2), AJ577125; MT-10B(1), AJ577126; MT-10B(2), AJ577127; MT-10Bt(1), (AJ577128); and MT-10Bt(2), AJ577129.

their metal-binding capacity, are correctly positioned relative to the *M. edulis* cDNA MT sequence. Despite these deletions and insertions, the MT-10B small genes (in *Mytilus edulis*) also display a correct ORF and correct putative sites for the cysteines. These characteristics led us to think that they could be active genes. The observation of putative active, small MT genes is not exclusive to the *Mytilus* and *Bathymodiolus* genera. An unusual intron-free MT gene (MT-3) has been characterized in *Crassostrea gigas* (Mollusca, Ostreidae) (A. Tanguy, personal communication). This particular MT gene has an ORF, a polyadenylation signal, and a correct 3′ untranslated region. In other species several minor forms of MT genes have been isolated that deviate from the habitual tripartite structure. In the *Drosophila* genus (Insecta), 2 active genes, MTn and MTo, have been identified. They are characterized by their distinctive structure, including only one small intron (265 bp and 61 bp, respectively) (Maroni et al., 1986; Stephan et al., 1994; Bonneton and Wegnez, 1995). In *Crassostrea gigas*, another atypical MT gene, CgMT2, which has an organization characterized by an exon duplication, has been isolated and this also seems to be an active gene (Tanguy and Moraga, 2001; Tanguy et al., 2001).

Metallic intoxication induced the expression of many genes other than metallothioneins, such as cytosolic O-acetylserine (thiol) 1yase, decarboxylase, OSISPAP1, Phytochelatin synthases, and proline-rich-protein (PvSR1to PvSR7) genes in plants or the cysteine-rich protein (CRP) gene in yeast (Tuan-Yao et al., 1998; Clemens et al., 1999; Dominguez-Solis et al., 2001; Tschuschke et al., 2002; Fujimori and Ohta, 2003; Mukhopadhyay et al., 2004). PvSR2, PvSR5, and OSISPAP1 genes confer metal resistance in plants, and they do not possess any introns in the coding region (Zhang et al., 2001; Mukhopadhyay et al., 2004). PvSR2 is a specific heavy-metal-responsive gene that might play an important role in resistance to the damage caused by several metals such as mercury, arsenic, and cadmium (Zhang et al., 2001). In addition, the PvSR2 protein cannot be metallothionein, as no cysteine domains were observed in the sequence. The other PvSR genes encode proteins that interact in different metabolic responses. Indeed, the PvSRT1 gene encodes a proline-rich protein; PvSR3, a dehydrin protein; PvSR4, a pathogenesis-related protein; PvSR5, a polyubiquitin; PvSR6, a DNA-like protein; and PvSR7, a new Hg-C12 protein in the plant *Phaseolus vulgaris* (Tuan-Yao et al., 1998). The OSISPAP1 gene, also displaying an intronless structure, encodes a zinc-finger protein, which is essential to the intracellular regulation of metal. Thus the identification of intronless MTs genes could be another example of genetic adaptations, to control rapidly rising metal levels in the organisms.

On the other hand, many of the small, intron-free MT genes have been shown to be pseudogenes inactivated as a result of mutations. These pseudogenes are characterized by alterations of the coding sequence with one or several stop codons or repeated sequences (Karin and Richards, 1984; Schmidt et al., 1985; Andersen et al., 1986; Peterson et al., 1988; Tam et al., 1988). Walker and Gedamu (1990) have shown that there is a human MT pseudogene that displays numerous substitutions and repetitive elements upstream from the second exon, even though it conserves the cysteine residues in the protein information. If the small MT-10 genes present in *M. edulis* and *B. thermophilus* are recent pseudogenes, then one could expect to observe several mutations randomly located in the genomic sequence, whereas in fact we observed that these 3 MT genes all have an ORF and correctly positioned cysteines. Our PCR

test to amplify any processed pseudogene present using Bath01/oligodT and BathA/oligodT for *Mytilus edulis* and *Bathymodiolus thermophilus*, respectively, revealed no amplification. This result could suggest that any poly (A) is localized at the 3′ extremity of these small MT-10 genes (MT-10 A, MT-10B, and MT-10Bt).

MT-10 genes characterized by this tripartite organization or having a shorter structure could coexist in the same individual. Our data constitute the first description of putative active, intron-free MT genes in mussel. We are currently attempting to determine the flanking regions of these 3 genes in order to find out whether they have the regulatory regions characteristic of an active gene.

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