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Polymorphism of mytilin B mRNA is not translated into mature peptide

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ABSTRACT

Diversity of mRNAs from mytilin B, one of the five mytilins identified in the Mediterranean mussel, *Mytilus galloprovincialis*, has been investigated from circulating hemocytes. One mussel expressed simultaneously two to ten different mytilin B mRNAs as observed in denaturing gradient gel electrophoresis (DGGE), defining 10 individual DGGE patterns (named A to J) within the mussels from Messina, Sicily (Italy). Three patterns accounted for 79% of the individuals whereas other patterns were found in only 2–7% of the 57 analyzed mussels. Base mutations were observed at specific locations, mainly within COOH-terminus and 3′UTR, leading to 36 nucleotide sequence variants and 21 different coding sequences (cds) segregating in two different clusters. Most of the base mutations were silent, and the number of pro-peptide variants was restricted to four. Finally, as the two amino acid replacements occurred within COOH-terminus, mature peptide from mytilin B appeared unique. Multiple sequencing of partial mytilin B gene from one mussel revealed that one to four randomly distributed mutation points occurred within intron-3. Only one sequence out of the 91 analyzed contained 16 mutation points. In addition, this sequence was the only one containing four out of the six mutation points occurring within exon-4, that code for most of the COOH-terminus domain, including the unique amino acid replacement. Statistical tests for neutrality indicated negative selection pressure on signal and mature peptide domains, but possible positive selection pressure for COOH-terminus domain.

1. Introduction

In invertebrates, hemocytes are the primary immune cells engaged in several innate immune reactions including phagocytosis, clotting, encapsulation, and the synthesis of several immune-related molecules. Among these molecules are the antimicrobial peptides (AMP) characterized by their extremely heterogenic structures. More than 2300 different AMPs were reported and are mainly available through three databases: Antimicrobial Sequence Database (AMSDb, http://www.bbcm.univ.trieste.it/~tossi/amsdb.html) with 1000 entries, Antimicrobial Peptide database (APD, http://aps.unmc.edu/AP/main.php) with 1008 records, and Antimic (http://www.ambis.org.sg/ppt/Manisha.ppt) with 1788 entries. Based on primary structure and consensus cysteine array, four AMP families have been identified in the mussels, *Mytilus edulis* (Charlet et al., 1996) and *Mytilus galloprovincialis* (Hubert et al., 1996; Mitta et al., 1999): defensin, mytilin, myticin and mytimycin. More recently, similar AMPs have been discovered in the bay mussel, *Mytilus trossulus* (GenBank AY730626), the Eastern oyster, *Crassostrea virginica* (Cunningham et al., 2006; Seo et al., 2005), the Manila clam, *Ruditapes philippinarum* (Kang et al., 2006), the Pacific oyster, *Crassostrea gigas* (Gonzalez et al., 2007; Gueguen et al., 2006b), the bay scallop *Argopecten irradians irradians* (Song et al., 2006; Zhao et al., 2007) and finally the carpet-shell clam, *Ruditapes decussatus* (Gestal et al., 2007).

Mytilin is composed of five different sequences, referred to as isoforms (Charlet et al., 1996; Mitta et al., 2000b). From one gene copy per genome, mytilin B mRNA is translated into pro-peptide and is matured inside hemocyte granules (Mitta et al., 2000a). The 3D structure of mytilin B has been established (Roch et al., 2008) revealing unexpected similarity to *M. galloprovincialis* defensin MGD-1 (Yang et al., 2000) considering primary amino acid sequences. In unchallenged *M. galloprovincialis* circulating hemocytes, mytilin B mRNA was evaluated as ten times less abundant than myticin B mRNA (Cellura et al., 2007). Myticin C appeared extremely polymorphic as 74 variants were identified with nucleotide mutations inducing amino acid replacements located on the entire pro-peptide sequence, i.e. the signal peptide, the mature myticin C and the COOH-terminus (Pallavicini et al., 2008). Individual sequences of myticin C are unique for each

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mussel and only animal belonging to the same family shared myticin C sequences, suggesting that its expression is probably a familial character (Costa et al., 2009). Even more extended diversity was reported for penaeidins from different shrimp species, not only within individuals from the same species but also within a single shrimp (Gueguen et al., 2006a).

Denaturing gradient gel electrophoresis (DGGE) is an electrophoresis separation method based on differences in melting behaviour of double stranded DNA fragments (Fisher and Lerman, 1979). It is one of the most efficient and widely applied methods for detection of nucleotide differences through an increasing gradient of the denaturant formamide and urea (Abrams and Stanton, 1992; Lerman and Beldjord, 1998). DGGE has been applied to different biological purposes, from genetic mutations (Sheffield et al., 1989) to bacterial diversity (Muyzer et al., 1993; Van der Gucht et al., 2005) and genetic polymorphism (Ge et al., 1999). Sensitivity of DGGE allows to detect one single base substitution by adding a 40-base pair GC rich sequence (called GC clam) to the forward specific PCR primer used in amplification (Sheffield et al., 1989).

Here, we applied DGGE technology to demonstrate the diversity of *M. galloprovincialis* mytilin B mRNAs segregating in ten individual patterns in untreated mussels. Random cloning and sequencing of multiple PCR-generated mytilin B fragments established the relationships between patterns and nucleotide sequences, point mutations and deduced pro-peptide sequences. In addition, multiple random cloning and sequencing of partial intron-3-exon-4 fragment, analyzed with several tests for neutrality, suggested negative selection pressure for signal and mature peptide domains, but possible positive selection pressure for COOH-terminus domain.

2. Material and methods

2.1. Mussels and RNA extraction

Adult mussels, *M. galloprovincialis*, were collected from "Pantano piccolo" lagoon of Messina, Sicily (Italy) by the "Associazione ZOE". They were acclimated for two days in the laboratory of the Palermo University in a flow-through system of oxygenated sea water at 20 °C. Hemolymph from individual mussels was collected from the posterior adductor muscle using a 1 ml syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution buffer (27 mM sodium citrate, 115 mM glucose, 18 mM EDTA and 336 mM NaCl in distilled water, pH 7.0). Hemocytes were pelleted by 10 min centrifugation at 800 × *g* and 4 °C, carefully resuspended in 500 μl RNALater (Sigma) and stored at 4 °C until used. After removal of RNALater by 10 min centrifugation at 12,000 × *g* and 4 °C, total RNA was extracted with 1 ml Trizol Reagent (Invitrogen Life Technologies) and resuspended in 20 μl of Tris-EDTA buffer (TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA). RNA concentration was measured with spectrophotometer ND-1000 (NanoDrop Technologies, USA) and stored at −80 °C until use.

2.2. RT-PCR for DGGE and electrophoresis

Reverse transcription was performed at 45 °C for 30 min, followed by denaturizing at 94 °C for 10 min with the One Step RT-PCR kit (Invitrogen Life Technologies) according to the manufacturer's instructions using GC clamp forward primer Ml-F2 (GC-Ml-F2): 5′CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGTGCTGACTATCTGAATCATACACATA3′, and reverse Ml-R2: 5′GTATAATGTCAAACAGAACGGGTC3′.

Thereafter, the PCR program included: 30 cycles of denaturing at 94 °C for 45 s, primers annealing at 60 °C for 1 min and elongation at 68 °C for 2 min, and a final extension at 68 °C for 7 min. Uniqueness and expected size of amplicons were checked by 1% agarose gel electrophoresis. Polymorphism of amplicons was then analyzed by DGGE on a Vertical Electrophoresis DCODE system (Bio-Rad) with 6% (w/v) polyacrylamide gels and denaturizing gradient from 20% to 80%. The 100% denaturizing solution was composed of 7 M urea and 40% formamide (v/v). Samples were prepared by adding 7 μl of 6× loading dye solution (Fermentas; 0.03% bromophenol blue, 0.03% xylen cyanol, 60% glycerol, 60 mM EDTA in 10 mM Tris–HCl pH 7.6) to 7 μl of PCR amplicon. DGGE was performed in Tris-acetate-EDTA buffer (TAE: 40 mM Tris-base, pH 7.4 with HCl, 20 mM glacial acetic acid, 1 mM EDTA) at 60 °C during 16 h at 80 V constant voltage. Gels were stained for about 30 min in the dark with 3 μl concentrated 10,000× SybrGold (Molecular Probes) diluted in 30 ml TAE, and washed 15 min in TAE. They were photographed using the UV Imager Gel Doc XR (BioRad) using SYBR filter. Band profiles were analyzed on the basis of densitometry calculated for each lane through AlphaView gel acquisition software (Alpha Innotech).

2.3. RT-PCR and cDNA cloning

Reverse primer Ml-R2 was as above. Forward primer Ml-F2 was the specific part of the above GC-Ml-F2: 5′TGCTGACTATCTGAATCATACACATA3′. First strend cDNA was synthesized using the one-step Access RT-PCR kit (Promega) according to the manufacturer's instructions performed on 50–100 ng of total RNA from 10 selected mussels corresponding to the 10 patterns observed in DGGE. Briefly, RT was performed at 45 °C for 45 min, followed by denaturizing at 94 °C for 2 min. Thereafter, the PCR program included: 40 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 1 min and elongation at 68 °C for 1 min, and a final extension at 68 °C for 7 min. Existence of expected size amplicons at 421 bp was monitored by 1% agarose gel electrophoresis. For each profile, amplicons were cloned according to the TOPO TA Cloning kit (Invitrogen Life Technologies) in the plasmid pCR 2.1 TOPO. White *E. coli* colonies were individually transferred to deep agar containing Luria-Bertoni medium and 50 μg/ml kanamycin, in 96 well microtiter plates and sent to Agowa GmbH (Berlin, Germany) for sequencing using M13 universal primers. Each clone was double strand sequenced and the released sequences corrected accordingly.

2.4. Analysis of cDNA and deduced amino acid sequences

Several adjustments and comparisons have been made: (i) the nucleotides from up-stream and down-stream the primers, including the primer sequences, were removed, giving identical lengths of 371 bp, (ii) for each of the 10 DGGE patterns, all the sequences were aligned using Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and clustered according to nucleotide sequences, (iii) UTRs were removed and cds compared using Multalin, (iv) the different cds were translated into pro-peptides (http://www.expasy.ch/tools/dna.html) and (v) the resulting amino acid sequences compared using Multalin. The evolutionary relationships were inferred using the Neighbor-Joining algorithm MEGA-4 (http://www.megasoftware.net) (Tamura et al., 2007) considering the different complete cds (312 nucleotides) and adding the mytilin B sequence from GenBank (AF162336). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Predictable structures of pro-peptides were established using Phyre software available at http://www.sbg.bio.ic.ac.uk/phyre/. Isoelectric points were calculated by http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html.