Tissue distribution and functional characterization of mytimacin-4 in *Mytilus galloprovincialis*

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Antimicrobial peptides (AMPs) play fundamental roles in the innate immunity of invertebrates. Mytimacin-4 is a kind of AMP gene previously sequenced from *Mytilus galloprovincialis* based on an identified EST sequence in our lab. In the present study, the tissue distribution and antimicrobial activities of mytimacin-4 were further investigated. A qRT-PCR analysis revealed that mytimacin-4 transcripts were constitutively expressed in all of the tested tissues of *M. galloprovincialis*, with the highest expression level in the posterior adductor muscle. After challenge by *Vibrio anguillarum*, the expression level of mytimacin-4 gene was significantly increased at 24 h (*P* < 0.05) in the mantle and increased at 48 h (*P* < 0.05) in the posterior adductor muscle. This finding suggested that mytimacin-4 transcripts were inducible upon pathogen infection. A minimal inhibitory concentration (MIC) assay indicated that recombinant mytimacin-4 protein had potent antimicrobial activities against gram-positive and gram-negative bacteria. Among the tested microorganisms, mytimacin-4 protein exhibited strong inhibition activities against *Bacillus subtilis* and *Vibrio parahaemolyticus* with MICs of 0.315 μM and 0.62 μM, respectively. This study provides for the first time direct evidence of antimicrobial action of mytimacin-4 in *M. galloprovincialis*.

1. **Introduction**

It is well known that invertebrates rely on innate immunity to defend against opportunistic environmental pathogens (Bouallegui, 2019; Chen and He, 2019; Cooper, 2018). After the foreign microbes are detected by a series of pattern recognition receptors, immune effectors are stimulated and applied to kill the pathogens. Antimicrobial peptides (AMPs) are central immune effectors with broad-spectrum antimicrobial activities against bacteria, fungi, and viruses. They can initiate an immediate direct defense reaction as antibiotics and constitute the first line of the host defense against foreign pathogens (Bachère et al., 2004; Boman, 2003; Bulet et al., 2004; Volety et al., 1999). Additionally, AMPs can function as immune-modulators to enhance immunity, in contrast to the majority of conventional antibiotics (Balseiro et al., 2011). Therefore, AMPs are considered to be host-defense peptides and play vital functions in the survival of the invertebrate.

In mollusks, multiple AMPs have been identified (Adema et al., 2017; Gerdol et al., 2012; Gerdol and Venier, 2015; Gómez et al., 2017; Schmitt et al., 2016). Macins are a class of secreted and positively charged AMPs, which have been studied relatively rarely to date (Gerdol and Venier, 2015). In contrast to the majority of molluscan cysteine-rich AMPs, macins are not specifically expressed in the circulating cells, instead of the digestive gland, gill and mantle (Gerdol and Venier, 2015). Only one exception has been reported in the freshwater pearl oyster *Hyriopsis cumingii*, in which a theromacin-like protein was preferentially expressed in hemocytes (Xu et al., 2010). Macins perform effective defense activities against gram-positive bacteria (Schikorski et al., 2008; Tasiemski et al., 2004) and gram-negative bacteria (Jung et al., 2009).

As a kind of marine sentinel, the mussels *Mytilus galloprovincialis* are widely distributed along the coasts globally because of its wide temperature adaptability and resilience to biotic and abiotic stress. Although there are many kinds of anthropic pollutants, pathogens and parasites in the coastal marine environment, the mussel *M. galloprovincialis* appears to be highly tolerant to these adverse factors. This implies that the mussel has an effective immune system. In *M. galloprovincialis*, five macins (named mytimacins) have been reported. They are characterized according to the numbers of conserved cysteines, ranging from 8 to 12 (Gerdol and Venier, 2015). Sequence features and
expression profiles of mytimacin-1, mytimacin-2, mytimacin-3, and mytimacin-5 have already been clearly described (Gerdol et al., 2012), but there is no other information about mytimacin-4.

Mytimacin-4 was characterized from *M. galloprovincialis* in our lab and the sequence was deposited in the GenBank database under the accession No. JN935273. Based on a study from an evolutionary perspective, Gerdol et al. (2012) reported an identical sequence as a member of the immune effectors of *M. galloprovincialis* under the protein accession number CCCC15018. However, there has been no other detailed information about its tissue distribution or antimicrobial activity.

In the present study, the mussels were challenged with the gram-negative bacterium *Vibrio anguillarum* and the expression profiles of mytimacin-4 gene were investigated in different tissues. Then, the antimicrobial activities of recombinant mytimacin-4 protein were investigated. We addressed a gap in knowledge of mytimacin-4 protein in *M. galloprovincialis*, providing direct evidence for the first time that mytimacin-4 has antimicrobial activity and enriching our knowledge of the defense mechanisms and antimicrobial functions of the molluscan macin protein.

2. Materials and methods

2.1. Sequence analysis of mytimacin-4 cDNA

The complete nucleotide sequences of mytimacin-4 cDNA are shown in Fig. 1A. In order to clearly understand the relationship of different mytimacins in *M. galloprovincialis* and other related macin proteins, seven macins were used to analyze their similarities. These sequences included five mytimacins in *M. galloprovincialis* (mytimacin-1, -2, -3, -4, and -5), hydramacin-1 in cnidian *Hydra magnipapillata* and macin in *Achatina fulica*. Similarity analysis was performed by the BLAST program (Altschul et al., 1990) at the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/blast/).

2.2. Mollusks cultivation and Vibrio challenge

*M. galloprovincialis* mussels (7.0 ± 0.5 cm in length) were purchased from the Yanda market of Yantai City. They were allowed to acclimate in aerated seawater (20 ± 2 °C, 32 psu) in plastic tanks for two weeks and were fed with *Ischyrosis galbana*. To investigate the tissue distribution of mytimacin-4 gene, six tissue types (including hemocytes, hepatopancreas, gill, posterior adductor muscle, mantle, and gonad) of seven mussels were collected and quantified by real-time fluorescent quantitative PCR reaction. The mussels were then divided into control and infected groups, each with 150 individuals. In the *Vibrio* challenged group, *M. galloprovincialis* were injected with 40 μL of *Vibrio anguillarum* (OD<sub>600</sub> = 0.4) into the posterior adductor muscle using a syringe needle. For controls, 40 μL of filtered sterile seawater was injected into the posterior adductor muscle of the control mussels. Based on results of tissue distribution, the tissue with abundant mytimacin-4 transcripts, the adductor muscle, was selected to investigate the mRNA expression profile at 0, 24 and 48 h post *Vibrio* challenge. In addition, mantle tissue was also collected due to its important function in immunity against infection by bacteria and viruses (Zhang et al., 2018). At each time-point, five individual clams were collected in the control and *Vibrio*-challenged groups, respectively.

2.3. Quantitative analysis of mytimacin-4 mRNA expression

Total RNA from different tissues was extracted with TRIzol Reagent (Invitrogen). To synthesize cDNA from RNA by reverse transcription, a total volume of 25.0 μL reaction mixture containing 4.0 μL total RNA, 5.0 μL M-MLV reverse transcriptase (Promega), 5.0 μL dNTP and 2.0 μL oligo-dT primer ([5'-GGCCAGCGTGCAGTACTAGTAC(T)_16(A/C/G)-3']) was incubated at 42 °C for 1 h according to the manufacturer's instructions.

The SYBR Green RT-PCR assay was conducted to determine the expression levels of mytimacin-4 mRNA in an ABI 7500 Fast Sequence Detection System. A pair of specific primers, P1 (5'-TTGGTGATTGCTGGATGAC-3') and P2 (5'-GGTCCATAGGAAATCCGAGAC-3'), was used to amplify a product of 184 bp of *Mytimacin-4*. The β-actin gene (GenBank NO. AF157491) was used as the reference gene, with two specific primers (P3: 5'-TCAACGGCGCTTCCTTCG-3', P4: 5'-GTCCGCAA TACCTGGGAA-3') to amplify a 252 bp fragment of the actin gene. The real-time PCR amplifications were carried out in a total volume of 50.0 μL, containing 25.0 μL of 2 × SYBR Green Supermix (Applied Biosystems), 15.0 μL of diluted cDNA, 1.0 μL of ROX Reference Dye II and 1.0 μL of each primer. The PCR program was as follows: 50 °C for 2 min, 95 °C for 5 min followed by 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. To confirm that only one PCR product was amplified and detected, a dissociation curve analysis of amplification products was performed at the end of each PCR. Then, the PCR data were analyzed by the ABI 7500 SDS software (Applied Biosystems), with the baseline set automatically to maintain consistency. All of the expression levels of mytimacin-4 relative to β-actin were analyzed by the comparative CT method (2^-ΔΔCT) (Livak and Schmittgen, 2001), and given in terms of means ± S.E. (n = 7 for tissue distribution analysis, 5 in *Vibrio* challenge experiment). The data were analyzed by one-way ANOVA followed by Least-significant difference (LSD) analysis to describe the differences among different groups, with a P value less than 0.05 considered to be a statistically significant difference.

2.4. Recombinant expression of mytimacin-4 protein

The cDNA of mature mytimacin-4 with restriction sites was amplified by the primers P5 (F: 5'-CATATGCGAAATGTGATTGGTGATTGCTGGATGAGTC-3') and P6 (5'-CTCGAGTTAGTGGTGGTGTTGGAACGCAACACTAGT-3'), where NdI and XhoI sites were underlined and 6-His tag was boxed. The PCR was performed as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min. The PCR product was purified, inserted into a pMD18-T vector and transformed into competent cells of *Escherichia coli* TOP10. The subcloned plasmid identified by PCR was digested with NdI and XhoI, gel-purified and cloned into an expression vector pET-21a (+), which was also digested with NdI and XhoI and gel-purified. A recombinant plasmid was obtained. The peptides were expressed in *Escherichia coli* BL21 (DE3) pLysS, purified by a Ni<sup>2+</sup> chelating sepharose column, and refolded in gradient urea-TBS glycerol buffer (10% glycerol, 2 mmol/L reduced glutathione, 0.2 mmol/L oxide glutathione, 50 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0) as described by Wei et al. (2018). The final concentration of the expressed protein was determined using a BCA assay (Smith et al., 1985).

2.5. Antimicrobial activity

Three species of gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*, and *Micrococcus luteus*) and six species of gram-negative bacteria (*Enterobacter cloacae, Proteus mirabilis, Enterobacter aerogenes, Vibrio parahaemolyticus, Vibrio anguillarum and Pseudomonas putida*) were cultured at their optimal conditions (Table 1) and used to assay the antimicrobial activities of mytimacin-4 protein by a minimum inhibitory concentration (MIC) assay.

Briefly, the bacteria were cultured overnight until the OD<sub>600</sub> value reached approximately 1.0 and then diluted 1000-fold in broth. In sterile 96-well microtiter plates, each well was filled with 100 μL of sterilized broth. Then, mytimacin-4 protein was serially two-fold diluted from the first well to the tenth well, with the final concentrations of 1.24, 0.62, 0.315, 0.155, 0.078, 0.039, 0.019, 0.001, 0.0005, 0.0002 μM, respectively. Then, 5.0 μL of the bacteria suspension were added to the gradient diluted mytimacin-4 protein. Only bacteria and broth were added to the eleventh and twelfth wells, respectively, and
used as the positive and negative controls. After culturing for 24 h at optimum conditions for each species (Table 1), the plates were scanned at 600 nm and the absorbance was read by a microplate reader (Tecan Infinite M200, Switzerland). There were three replicate tests for the MIC assay.

3. Results and discussion

3.1. Sequence features of mytimacin-4 cDNA

The complete nucleotide sequence of mytimacin-4 cDNA and its deduced amino acid sequence is shown in Fig. 1A. The signal peptide is underlined. The eight conserved cysteine residues are marked with * and the additional cysteine residue is marked with #. The protein sequences are mytimacin-1 (CCC15015.1), mytimacin-2 (CCC15016.1), mytimacin-3 (CCC15017.1), mytimacin-4 (AFC37170.1), mytimacin-5 (AHG59340.1) in M. Galloprovincialis, hydramacin-1 chain A in Hydra magnipapillata (PDB: 2K35_A), macin-AF in Achatina fulica (AFR36920.1).

Fig. 1. (A) Nucleotide sequence of mytimacin-4 cDNA from Mytilus galloprovincialis and its deduced amino acid sequence. The signal peptide is boxed and the eight conserved cysteine residues are marked with *. Two additional cysteine residues are marked with #. The protein sequences are mytimacin-1 (CCC15015.1), mytimacin-2 (CCC15016.1), mytimacin-3 (CCC15017.1), mytimacin-4 (AFC37170.1), mytimacin-5 (AHG59340.1) in M. Galloprovincialis, hydramacin-1 chain A in Hydra magnipapillata (PDB: 2K35_A), macin-AF in Achatina fulica (AFR36920.1).
mature protein sequence. Its predicted molecular mass was 8.88 KD, with a theoretical pl of 8.38.

An alignment of all of the mytimacin proteins in *M. galloprovincialis* with Hydramacin-1 from *H. magnipapillata* (Jung et al., 2009) and macin-AF in *A. fulica* (Zhong et al., 2013) is shown in Fig. 1B. Mytimacin-4 exhibited high identities to the selected protein sequences, with 76.2% identity to mytimacin-1, 66.0% to macin-AF, 32.4% to mytimacin-2 and 28.4% to mytimacin-3. Eight cysteine residues (Cys) were found to be conserved in all of the selected macin proteins, while there were two additional Cys conserved in mytimacin-1, -4, -5 and macin-AF. According to Gerdol et al. (2012), the two additional cysteines of mytimacin-4 engaged in the formation of the fifth disulfide bond at its C-terminus, like the theromacins of the segmented worm *Theromyzon tessulatum* (Tasiemski et al., 2004) and of the mussels *H. cumingii* (Xu et al., 2010).

### 3.2. Expression profiles of mytimacin-4 in different tissues

Mytimacin-4 was found to be widely expressed in all of the tested tissues, including the hemocytes, hepatopancreas, gill, gonad, posterior adductor muscle and mantle in the healthy *M. galloprovincialis* (Fig. 2). The lowest expression level of *mytimacin-4* was detected in the gill, and the highest level was detected in the posterior adductor muscle.

The tissue distribution pattern of mytimacin-4 was not only different from most of the AMPs expressed in the molluscan hemocytes, such as defensins (Zhang et al., 2015), mytilins (Tanguy et al., 2018), mytinic (Mitta et al., 1999; Tanguy et al., 2018), and mytimycins (Sonthi et al., 2011), but also from other mytimacins in mussel. According to Gerdol et al. (2012), mytimacin-1 was constitutively expressed in all of the tested tissues, with higher expression levels in the digestive gland and adductor muscle than that in the hemocytes. Mytimacin-2 and mytimacin-3 showed specific localizations to the gill and mantle, respectively (Gerdol et al., 2012). Therefore, it can be concluded that *mytimacin-4* exhibits a different expression pattern from other macins, and hemocytes are not the definitive tissue for the most abundant mytimacin expression.

The higher expression level of *mytimacin-4* in the adductor muscle suggests that it is important for the tissue immunity of *M. galloprovincialis*. It is believed that the involvement of antimicrobial peptides in immune response relied on their strategic locations at interfaces between the organism and external environment (Tasiemski et al., 2004). In the leech *T. tessulatum*, two AMP genes (theromacin and theromyzin) were detected in the intestinal epithelial cells and in the epidermis. The mRNA expression levels were significantly enhanced after blood meal ingestion and upon bacterial challenge, which suggested that AMPs played important roles in epithelial defense (Tasiemski et al., 2004). Recently, macin proteins have been also found to be related to tissue regeneration (Tasiemski and Salzet, 2017) and meal ingestion (Ding et al., 2019). In mollusks, the adductor muscle is responsible for locomotion, water pumping and respiratory movements (Kier, 1988), and is covered with a layer of epithelial cells. The higher expression level of *mytimacin-4* in adductor muscle might relate to strategic immunity in the epithelial cells of the mussel adductor or other functions such as food capture by water pumping, a hypothesis that needs further investigation. Taking into account the different tissue distribution profiles of *mytimacin-4* in this study and another four mytimacin variants from Gerdol et al. (2012), we infer that the five mytimacins allow the mussel *M. galloprovincialis* to defend against environmental pathogens synergistically, aided by the different antimicrobial spectrums.

After challenge by *V. anguillarum*, the expression levels of *mytimacin-4* in the mantle tissue increased significantly at 24 h (*P* < 0.05) and returned to a normal level at 48 h post injection compared with the control (Fig. 3A). However, in the adductor muscle, *mytimacin-4* expression did not increase at 24 h but was found to be significantly higher at 48 h (*P* < 0.05) (Fig. 3B). This finding indicated that *mytimacin-4* was inducible upon *Vibrio* challenge and that the mantle tissue responded more quickly than the adductor muscle. For other invertebrate AMP genes, such as antilipopolysaccharide factor (ALF), mytichitin-A and penaradin 5, microbial challenge induced significant increases followed by a gradual return to their normal expression levels within 48 h (Oh et al., 2018; Supungul et al., 2004; Wu et al., 2019), which were similar to the expression profiles of *mytimacin-4* in the mantle.
lethal vibriosis in shellfish aquaculture and V. parahaemolyticus is a severe pathogen to marine invertebrates as well as humans (Kim and Lee, 2014; Park et al., 2018), but their DNA homology is only 30% (Anderson and Ordal, 1972). The different MICs of mytimacin-4 on the two pathogenic Vibrios are probably due to their large genetic difference. Accordingly, lower MIC of mytimacin-4 on V. parahaemolyticus suggested its stronger antimicrobial effect on this pathogen.

We still have limited information about the antimicrobial mechanism of mytimacin-4. Hydramacin-1, one of the invertebrate macins proteins, has been reported to promote the aggregation of bacteria and to kill bacteria through a series of steps, including forming electron-dense contacts, changing the bacterial cell morphology and permeabilizing the bacterial membrane (Jung et al., 2009). In order to characterize the antimicrobial mechanism of mytimacin-4, further work is required to investigate the detailed interactions between the mytimacin-4 protein and different bacteria. In addition, mytimacin-4 is structurally similar to mytimacin-1 in terms of the number of cysteine residues, but it is not clear whether the antimicrobial activities are similar or different. Do different numbers of cysteine residues lead to different antimicrobial spectrums? This is another interesting topic for study of the five mytimacin proteins in M. galloprovincialis.

In conclusion, the present study investigated the tissue distribution and antimicrobial functions of mytimacin-4 in M. galloprovincialis. Special tissue distribution and effective antimicrobial effects provide direct evidence of specific immune roles of mytimacin-4 in the mussel M. galloprovincialis.

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