Fish and Shellfish Immunology 89 (2019) 271–280

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Molecular identification and expression analysis of four Lysin motif (LysM) domain-containing proteins from turbot (*Scophthalmus maximus*)

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ARTICLE INFO

Keywords:
LysM
Turbot
Vibrio vulnificus
Antibacterial activity
Immune stimulation

ABSTRACT

Lysin motif (LysM) is involved in chitin, peptidoglycan and other structurally-related oligosaccharides recognition and binding, and it is important for the biological processes of responsing to bacterial and viral infections and pathogen defense. LysM is also a widely spread protein, ranging from prokaryotes to eukaryotes, including bacteria, plants and mammals. However, research of LysM in teleosts especially in marine fish was rarely scarce. In the present study, four novel LysM domain-containing proteins in turbot (*Scophthalmus maximus*), named as *SmLysMd1*, *SmLysMd2*, *SmLysMd3*, and *SmLysMd4*, were cloned and identified firstly. The full-length cDNA of *SmLysMd1* was 1235 bp with a 678 bp ORF, capable of encoding a peptide of 225 amino acids. The complete cDNA sequence of *SmLysMd2* was 1273 bp, and contained a 675 bp ORF, encoding a predicted protein of 224 amino acids. The full-length of *SmLysMd3* cDNA was 2132 bp, containing a ORF of 987 bp, with a ORF of encoding 328 amino acids. The full-length *SmLysMd4* cDNA was 1115 bp contained a 888 bp ORF, encoding 295 amino acids. And all the four predicated proteins contained a specific LYSM domain. Moreover, *SmLysMd1* and *SmLysMd2* belong to the intracellular non-secretory types, and *SmLysMd3* and *SmLysMd4* belong to the anchored transmembrane types. In addition, the four *SmLysMd* were ubiquitously expressed in all the examined tissues. Moreover, the *SmLysMds* levels were up-regulated in muscle and liver, and had a reduce tendency immediately in different degree following *Vibrio vulnificus* challenge, indicating that the turbot LysM could be participant in the immune responses to bacterial infections. The present result of LysM in turbot for the first time in a marine fish will provide foundation knowledge for the functions studies of LysM in immune responses. Further studies should be carried out to better understand their immune mechanism in turbot and other teleosts.

1. Introduction

As we all known, the innate immune system is the first line for organisms to defend against invading pathogens [1,2]. The most crucial step of onset immune responses is the recognition of non-self, which was regulated by pattern recognition receptors (PRRs) [2]. Meanwhile, the fact that the organisms could exactly recognize the pathogen-associated molecular patterns (PAMPs) on the surface of foreign intruders such as bacteria, fungi and viruses, is depending on the PRRs to conserved [3–6].

The lysin motif (LysM) is an ancient and widely spread protein, and it has also been reported in a wide spread of prokaryotes and eukaryotes, including almost all the plants and mammals [7–9]. Generally, the LysM comprises a small protein domain contained 40–60 amino acids with a wide range of complex domain architectures [10,11]. It is accepted that the LysM is involved in chitin, peptidoglycan and other structurally-related oligosaccharides recognition and binding, which is important for the biological processes of responsing to exogenetic bacterial or viral infections [12–14]. Actually, LysM domain is initially reported as the degrading enzymes of bacterial *Bacillus* phage Θ29 in its cell wall. And, it is also found in the peptidoglycan hydrolase of *Enterococcus faecalis* [15]. In fact, in the process of bacterial cell division, LysM domains are known in remodeling of cell wall peptidoglycans (PGN), and they are widely distributed in enzymes of bacteria. Thus, in prokaryotes, the LysM domains play an essential role in bacterial pathogenesis and symbiosis [11,15]. In plant, LysM-

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https://doi.org/10.1016/j.fsi.2019.03.074
Received 12 February 2019; Received in revised form 28 March 2019; Accepted 29 March 2019
Available online 30 March 2019

Table 1
Primers used in this research.

Primer	Sequence (5' – 3')
SmLysmd1-5′RACE-R	CCGACAGCACGGGGATGGACAAAG
SmLysmd2-5′RACE-R	GGACCTGGACCGAGGGAAGATGGG
SmLysmd3-5′RACE-R	GGCGAGGCAGATTTAAGAGGACCG
SmLysmd4-5′RACE-R	CATCGAGGTCCTCGCCGTCCGAGG
SmLysmd1-3′RACE-F	CACCAGGAGCGCTTCCGCGAACGA
SmLysmd2-3′RACE-F	GTCCCCGCCCAATGAGGACACCCA
SmLysmd3-3′RACE-F	CCATCATGGCATTCCTACCCAAGC
SmLysmd4-3′RACE-F	GTTTAAGAGGACAGGGCCTGACGA
SmLysmd1-RT-F	TAAACCAGTCCAAGCAGGCC
SmLysmd1-RT-R	CCCTGTAGAGGACCTGGGAA
SmLysmd2-RT-F	GTCACGATGGAGCAGGTCAA
SmLysmd2-RT-R	TACGTAGCGCTTCTCTGACG
SmLysmd3-RT-F	GGAGGATGGCGAGAACTACG
SmLysmd3-RT-R	GAGTCTCGGTAAGGACGCTG
SmLysmd4-RT-F	CTAGCGCAGATGGTCAGGTC
SmLysmd4-RT-R	TGTCGCCATCTTCCACCTTC
Smβ-Actin-RT-F	ATCGTGGGGCGCCCCAGGCACC
Smβ-Actin-RT-R	CTCCTTAATGTCACGCACGATTTC

containing proteins are shown to be implicated in perceiving PGN and chitin and have important role in its symbiosis and immunity [16–19]. In rice, the LYP4 and LYP6 proteins performed function in recognition of bacterial peptidoglycan and fungal chitin, and initiate the innate immune reaction [9,20]. In *Arabidopsis* [14], as well as in fern *Pteris ryukyuensis* [21] and horsetail *Equisetum arvense* [22], several types of Lysin motif proteins have been identified and shown to mediate the immune reaction to bacterial and fungal challenge [18,19]. Lately, the LysM domains are also present in animal and human beings. In *Caenorhabditis elegans*, several Lysin motifs contained in chitinases had been studied [11]. The Lysin motif from red swamp crayfish *Procambarus clarkia* was identified. And it is a putative peptidoglycan-binding domain-containing protein which is essential to the innate immune reaction against bacterial infection by recognizing different microorganisms [23]. Moreover, a novel LysM named *MjLPBP* was identified to take part in the innate immunity in kuruma shrimp *Marsupenaeus japonicas*, displaying that it could bound to peptidoglycans, lipopolysaccharide, lipoteichoic acid and chitin from bacterial cell wall and accelerate *V. anguillarum* clearance in vivo [13]. Meanwhile, in the marine mollusk *Crassostrea hongkongensis*, *ChLysM* exhibited a broad-spectrum antibacterial activity and a pattern recognition protein in bacterial growth-inhibiting activity during immune defense [24]. Two distinct sub-families of LysM in zebrafish were investigated and identified to be highly conserved across vertebrates [7].

As a main commercial marine fish species in China, turbot (*Scophthalmus maximus*) is widely cultured under artificial farming conditions [25,26]. However, in recent years, turbot industry is suffering from great challenge that many diseases including bacterial disease, exophthalmic disease and ascites disease have threaten turbot aquaculture industry, bringing extremely economic losses [27]. More and more researchers have done the effort to identify immune-related genes to govern mucosal immune, which plays crucial roles in pathogen-rich aquatic environment, for disease control and prevention [28,29]. In order to further understand and development turbot immunity system, we characterized four LysM domains, and analyzed their different expression patterns following *Vibrio vulnificus* infections. The present result of LysM in a marine fish for the first time will increase our knowledge in immune responses.

2. Materials and methods

2.1. Fish and bacterial challenge experiments

The turbot used in this experiment were obtained from a turbot culture farm in Laizhou (Shandong, China), approximately 14.6 g in body weight and 5.0 cm in body length. All the fish were acclimated in the laboratory condition under aerated flow-through water (20 ± 0.5 °C) for one week before the experiments. For tissue-specific expression analyzing, eight tissues of liver, spleen, kidney, brain, gill, intestine, skin and muscle were collected from healthy turbot. The samples were frozen in liquid nitrogen immediately and stored at −80 °C until RNA extraction.

In this study, the bacteria used was *V. vulnificus*, which was isolated from diseased fish and kept in our laboratory, were used to infect the healthy turbot. Before challenge, the bacteria were incubated to mid-logarithmic stage at 28 °C in LB broth medium, then collected by centrifugation and re-suspended in PBS. Then, all the fish were injected individually with 100 μl live *V. vulnificus* suspension with the final concentration of 1.0×10^6 CFU/ml during the immune challenge. Meanwhile, control fish were immersed with sterilized physiological saline under the identical protocols. The liver and muscle tissues were collected from six fish at 0, 3, 12, 24, 48 and 72 h after bacterial induction. All samples were frozen in liquid nitrogen immediately, and then stored at −80 °C freezer before RNA extraction.

2.2. Isolation of full-length SmLysMd1, SmLysMd2, SmLysMd3 and SmLysMd4

Firstly, partial cDNA fragments of the four turbot *SmLysMd* homolog were identified from transcriptome database. Then, the complete cDNA sequence of four genes were obtained using the Rapid amplification of the cDNA ends (RACE) method. 5′- and 3′-RACE-ready cDNA were synthesized from the total RNA using a SMART™ RACE cDNA amplification kit (Clontech, USA). The gene specific primers (Table 1) were designed based on the other corresponding sequences of conserved regions. Both the 5′ and 3′ RACE-PCR were performed using the 5′and 3′-Full RACE Kit (TaKaRa, China), according to the manufacturer's instructions. The PCR amplification procedure was denaturation at 94 °C for 5 min; 40 cycles of amplification at 94 °C for 30 s, 63 °C for 45 s, 72 °C for 45 s, an additional elongation at 72 °C for 5 min. Last, the PCR product was cloned into the pEASY-T1 vector (Trans, China) and sequenced.

2.3. Bioinformatics analysis

The ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder/) was used to identify the open reading frame. Sequence similarity analysis was performed using the BLAST program from the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Structure domains were predicted by Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Multiple sequence alignment was performed using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic tree was constructed using Molecular Evolution Genetics Analysis (MEGA) software version 7.0 under the neighbor-joining (NJ) analysis.

2.4. Quantitative real-time PCR analysis

Total RNA of each collected tissue was extracted using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA quality was measured on a nanodrop 2000 (Thermo, USA).

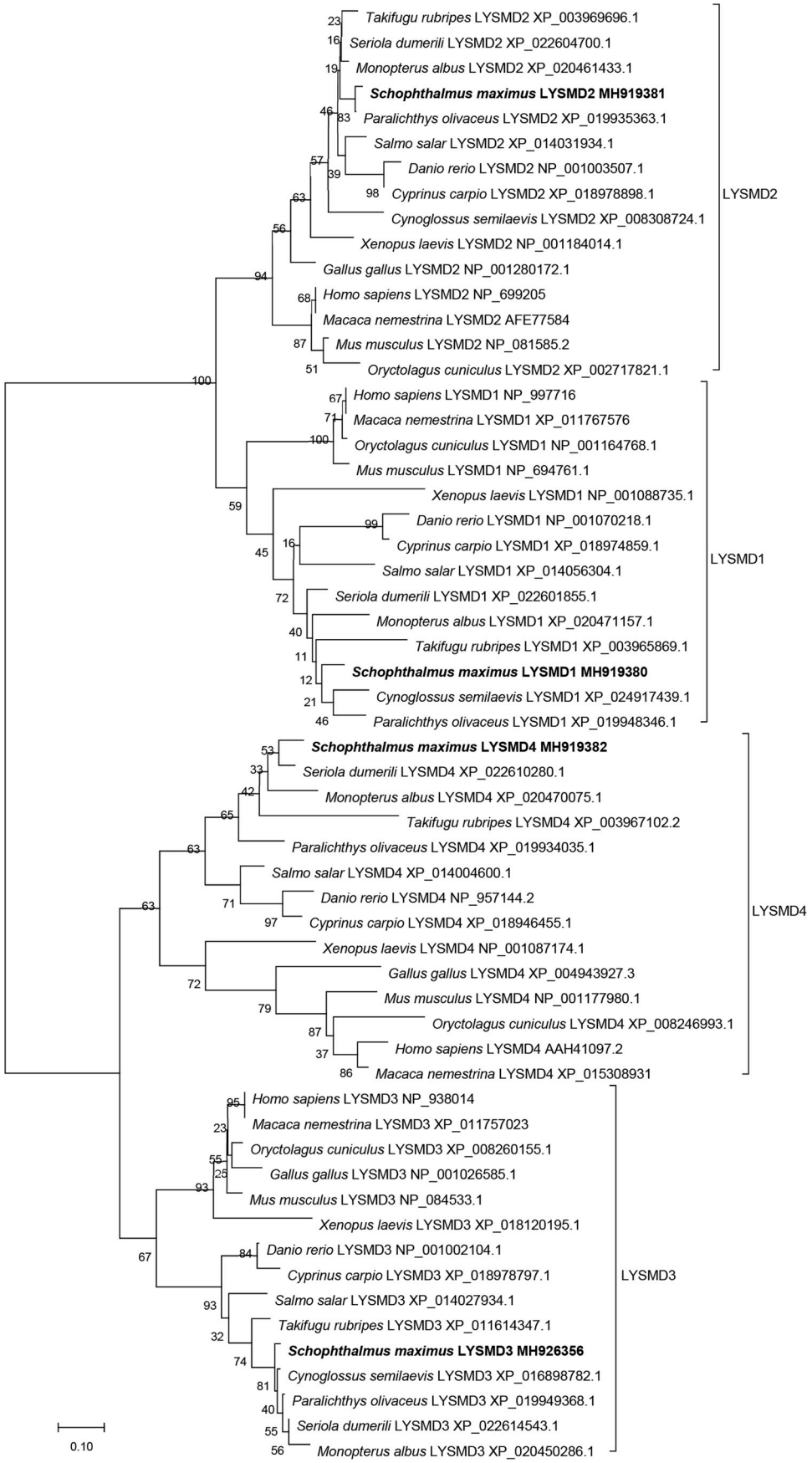

Fig. 1. Phylogenetic tree of deduced amino acid sequences for four *SmLysMds* from turbot and other vertebrates was constructed by MEGA4.1 with neighbor-joining method. The numbers adjacent to nodes indicate bootstrap percentage value for 1000 replicates (> 80%). The GenBank accession numbers of the sequences presented before the species name. The turbot LYSMD were Bold.

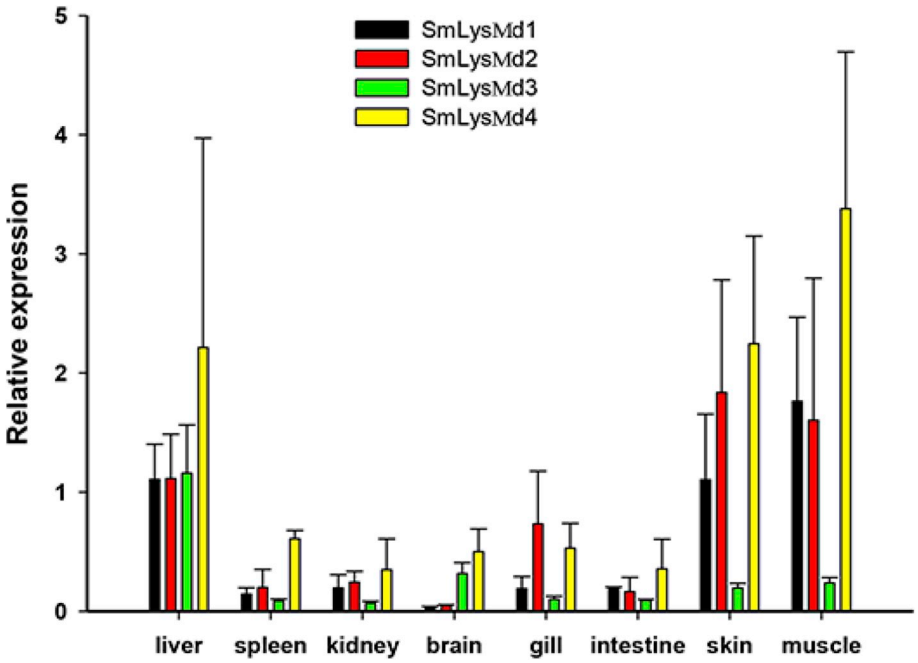

Fig. 2. The tissue distribution of four *SmLysMd* genes in turbot. *SmLysMd* expression levels in liver, spleen, kidney, brain, gill, blood, intestine, skin and muscle were determined by quantitative real-time PCR. The expression level of each *SmLysMd* in liver was set as 1. The relative abundance of four *SmLysMd* genes were expressed as mean ± SE (N = 3).

And, all the RNA samples had an A260/280 ratio greater than 1.8.

First strand cDNA was synthesized by PrimeScript™ RT reagent Kit (TaKaRa, China) following manufacturer's protocol.

Gene specific primers (Table 1) were designed based on the four turbot *LysMd* sequences. And turbot *β-Actin* gene was used as the reference gene. Quantitative real-time PCR was then performed on a CFX96 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), using SYBR Premix Ex Taq Kit (Takara Bio Inc.) according to the manufacturer's protocol. PCR was performed with 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 68 °C for 30 s. A dissociation curve was added at the end of each program to check the amplification specificity. All experiments were conducted with three replicates.

The results were analyzed according to the $2^{-\triangle\triangle Ct}$ method. Statistical analysis was performed by SPSS version 20.0 software (www-01.ibm.com/software/analytics/spss). Data for all the groups were analyzed using two-way analysis of variance (ANOVA). Significance was set at $P < 0.05$.

3. Result

3.1. Cloning and characterization of four LysMd genes

The full sequence of four *LysMd* genes were successfully obtained using 3′- and 5′-RACE respectively. The determined cDNA sequence of *SmLysMd1* (GenBank accession number MH919380) was 1235 bp, and contained a 678 bp ORF, capable of encoding a peptide of 225 amino acids (Supplementary Fig. 1A), with a predicted molecular mass of 24.22 kDa and a pI of 7.00. The complete cDNA sequence of *SmLysMd2* (GenBank accession number MH919381) gene was 1273 bp, and contained a 675 bp ORF, encoding a protein of 224 aa (Supplementary Fig. 1B). The predicted MW and pI of *SmLysMd2* are 24.13 kDa and 5.08, respectively. The full-length *SmLysMd3* (GenBank accession number MH926356) cDNA was 2132 bp, containing a ORF of 987 bp (Supplementary Fig. 1C). And, the ORF encoded 328 amino acids with a molecular mass of approximately 36.04 kDa and a pI of 6.27. The full-length *SmLysMd4* (GenBank accession number MH919382) cDNA was 1115 bp with a 888 bp ORF, encoding 295 amino acids (Supplementary Fig. 1D) with a molecular mass of approximately 33.86 kDa and a pI of 8.35. All the *SmLysMd1*, *SmLysMd2*, *SmLysMd3* and *SmLysMd4* contain a specific LYSM domain conserved.

In comparison with other species, the four amino acid sequences of *SmLysMd* had high identity and similarity with *Paralichthys olivaceus*, *Larimichthys crocea*, *Seriola dumerili* and *Cynoglossus semilaevis*. Detailedly, *SmLysMd1* showed 64%, 54%, 64%, 56% identity and 70%, 61%, 74%, 62% similarity with *Paralichthys olivaceus*, *Larimichthys crocea*, *Seriola dumerili* and *Cynoglossus semilaevis* respectively. *SmLysMd2* shared highest identity of 85% with *Seriola dumerili,* followed by *Paralichthys olivaceus* with 83%, *Larimichthys crocea* with 80%, and *Cynoglossus semilaevis* with 75%, but the highest similarity was also found with *Paralichthys olivaceus* with 87%, followed by *Larimichthys crocea* with 85%. *SmLysMd3* showed the highest identity of 84% and the highest similarity of 90% with *Paralichthys olivaceus*. While *SmLysMd4* showed the highest identity of 85% and the highest similarity of 89% with *Seriola dumerili*. And *SmLysMd4* showed identity of 80% and with similarity of 85% with *Paralichthys olivaceus*.

Phylogenetic analyses showed that the LysMd1 and LysMd2 proteins cluster together in a branch, while LysMd3 clusters with LysMd4 to a different branch (Fig. 1). These two LysMd branches have a common nexus. Meanwhile, each SmLysMd protein clearly grouped into its own clade as expected, and the four fish LysMd homologues were separated and distant from the avian and mammalian clade.

3.2. Tissue distribution of four SmLysMds

The tissue distribution patterns of four *SmLysMds* were detected in turbot healthy tissues, including liver, spleen, kidney, brain, gill, intestine, skin and muscle, by real-time PCR method. Among all examined tissues, for each *SmLysMd*, the expression level of liver was used as the baseline for comparisons. The results showed that all the four *SmLysMds* were expressed in all examined turbot tissues exhibiting with distinct expression patterns (Fig. 2). For *SmLysMd1*, the highest expression level was appeared in muscle, followed by kidney and skin, with the

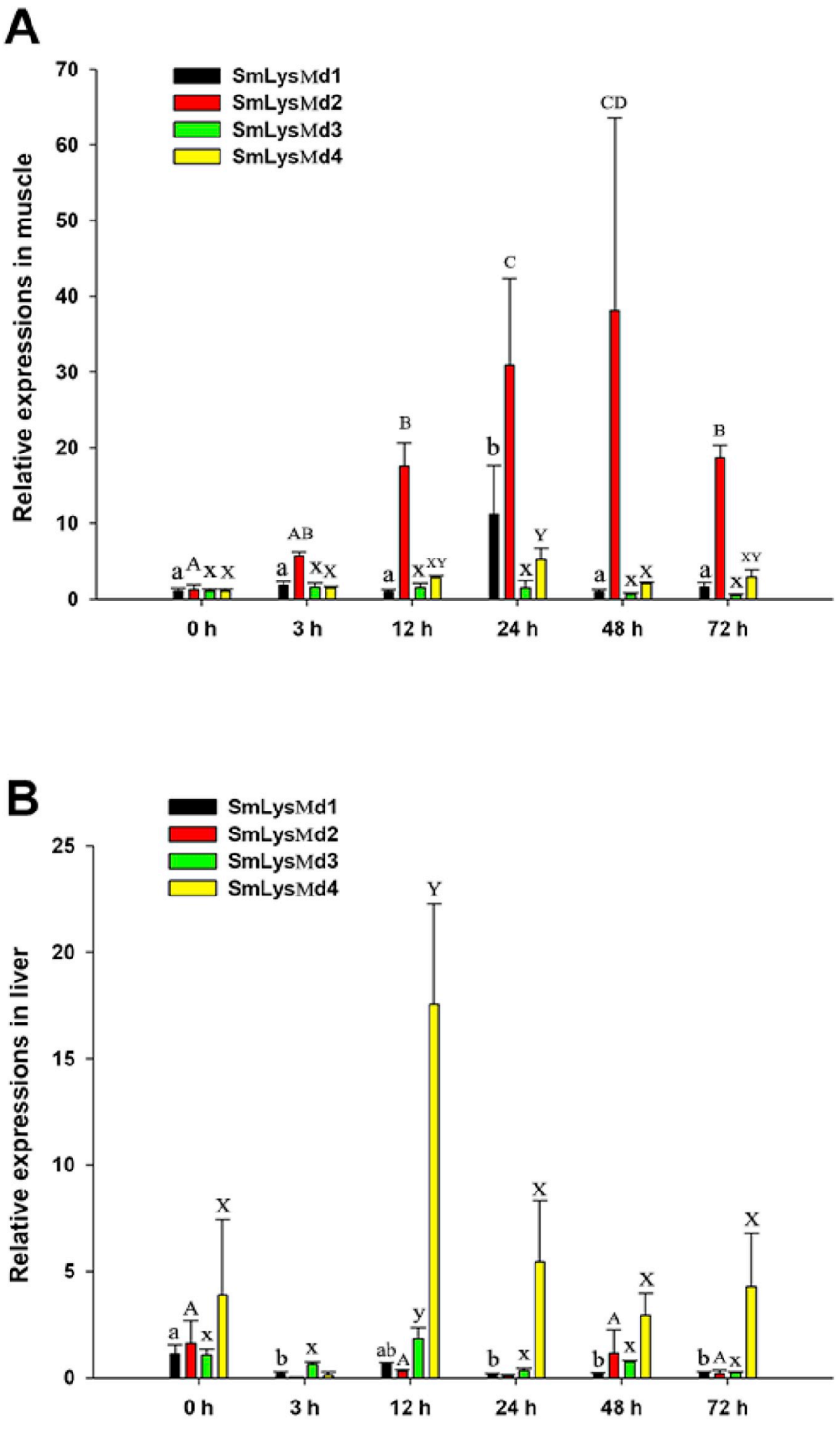

Fig. 3. Expression of four *SmLysMd* genes were upregulated in turbot after pathogen challenge. **A,** Four *SmLysMd* expression levels in muscle were determined by quantitative realtime PCR. The internal control was *β-Actin*. **B,** Four *SmLysMd* expression levels in muscle were determined by quantitative realtime PCR. The internal control was *β-Actin*. Different letters represent significant differences among different time-points ($P < 0.05$).

excessive low expressions in the other tissues. For *SmLysMd2*, the highest expression level was observed in skin, followed by muscle, liver and gill, while the expression level were extremely low in the kidney, spleen, intestine and brain. Then, for *SmLysMd3*, the highest expression level happened in liver, and the expression levels in the other tissues were not very different, but showed significantly lower than that in liver. At last for *SmLysMd4*, the highest expression level happened in muscle, and the expression levels in skin, liver, spleen, gill, brain, intestine and kidney were gradually decreased in order.

3.3. Expression profiles of four SmLysMds in muscle and liver following bacterial challenge

To further investigate the immune roles of four *SmLysMds* in turbot, the expression profiles of *SmLysMds* were investigated in muscle and liver in response to the challenges with *V. vulnificus*. In general, the *SmLysMds* levels were up-regulated in muscle and liver, then had a reduce tendency in different degree.

For muscle (Fig. 3A), three *SmLysMd* genes (*SmLysMd1*, *SmLysMd3*, *SmLysMd4*) reached to the highest levels at 24 h, while the most expression of *SmLysMd2* was at 48 h. Specifically, the *SmLysMd1* was significantly up-regulated to 10.30 fold at 24 h. And the *SmLysMd3* was 1.36 fold and the *SmLysMd4* was the 4.89 fold at 24 h separately. As for *SmLysMd2*, it was 30.68 fold at 48 h.

However, for liver (Fig. 3B), the expression patterns of *SmLysMd3*, *SmLysMd4* exhibit the same tendency as that in muscle, and reached to the highest level at 12 h (1.70 and 4.51 fold, respectively). As for *SmLysMd1* and *SmLysMd2*, the expression reached to the highest level immediately after bacterial challenge at 0 h, and significantly down-regulated with 3 h-72 h. Meanwhile, the *SmLysMd1* was 0.17 fold at 3 h, 0.54 fold at 12 h, 0.14 fold at 24 h, 0.20 fold at 48 h, 0.24 fold at 72 h, and the *SmLysMd2* was 0.02 fold at 3 h, 0.19 fold at 12 h, 0.07 fold at 24 h, 0.72 fold at 48 h, 0.13 fold at 72 h, respectively.

4. Discussion

LysM is a ubiquitous protein module in the organism, and it is known as the function of a PGN binding domain and plays the crucial role in the innate immunity. In this study, we identified four *LysMd* genes, named *SmLysMd1*, *SmLysMd2*, *SmLysMd3* and *SmLysMd4,* from the turbot, for the first time in a marine fish. And we also compared the four predicted protein structure and investigated the tissue expression signatures, including expression patterns under different healthy tissue and upon activation with bacteria of *V. vulnificus* in immune tissues.

Before this study, there is only a few researches that investigated of *LysMd* in teleost species, especially in marine fish. However, there were many sequences of the lysM proteins from animals searched in NCBI. Generally speaking, lysM 1 and 2 like proteins were identified as the intracellular non-secretory proteins, and lysM 3 and 4 like protein were some membrane anchored transmembrane proteins [12,23,24]. Multiple Alignment (Fig. 4) showed that all the four *SmLysMds*, as the novel members of the LysM, had a conserved LysM motif with a 45 amino acid long residue, which was identified as the LysM protein super family, and might participate in antibacterial immunity of turbot. In turbot, the LysM domain of *SmLysMd1* was at 43–87 amino acid, the *SmLysMd2* at 70–114, the *SmLysMd3* at 69–113, the *SmLysMd4* at 74–118. Generally, LysMd proteins could be classed into three types, which were the secreted proteins type, the membrane proteins type, and the intracellular non-secreted proteins type [10,11]. Kuruma shrimp [13] and Hong Kong oyster [24] LYSMD belonged to LysM and putative PGN binding domain-containing protein 3 and were identified to the type of outer membrane proteins. While, red swamp crayfish [13] LysM belonged to

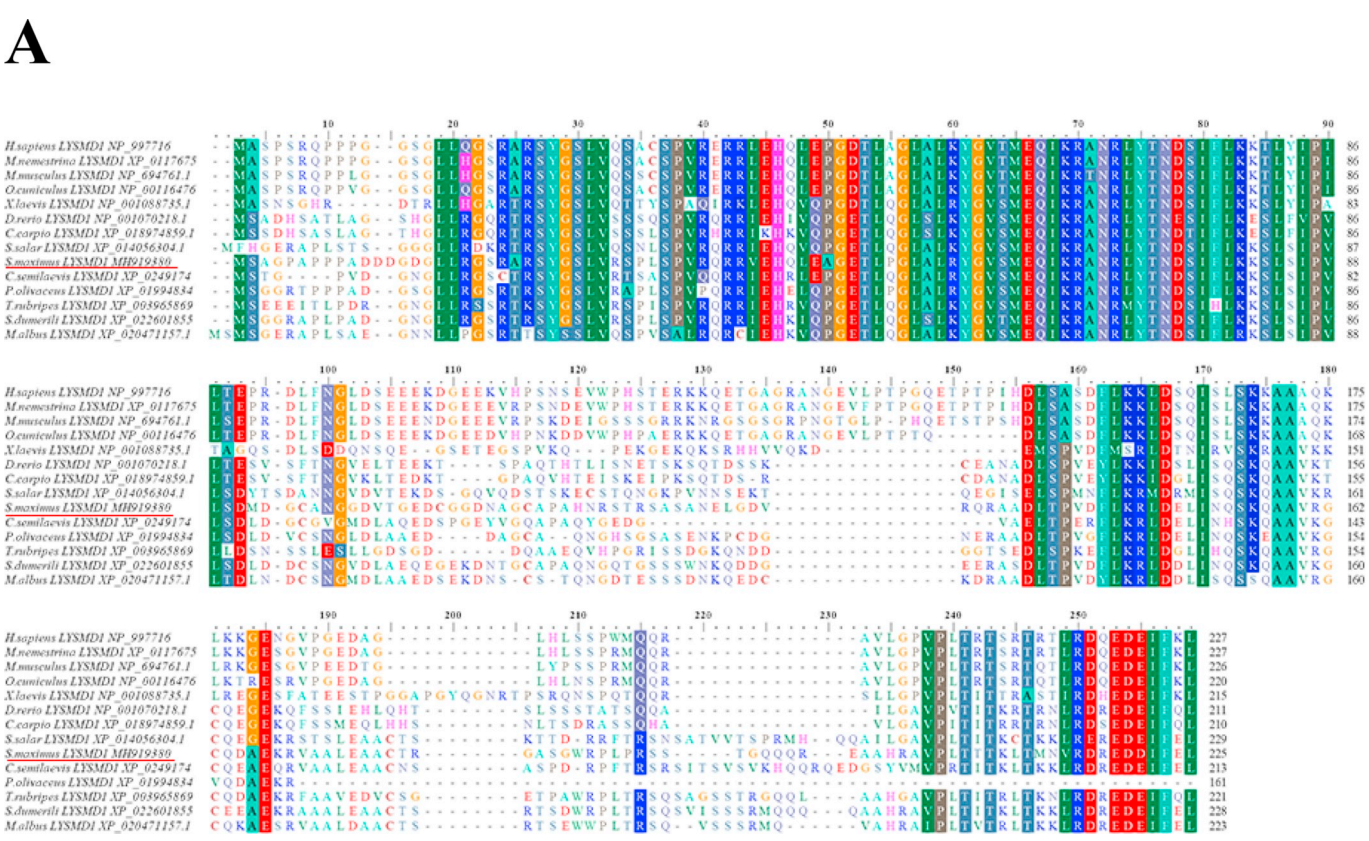

Fig. 4. Multiple Alignment of the deduced amino acid sequences of (A) *SmLysMd1*, (B) *SmLysMd2*, (C) *SmLysMd3* and (D) *SmLysMd4* from turbot and several other different animals.

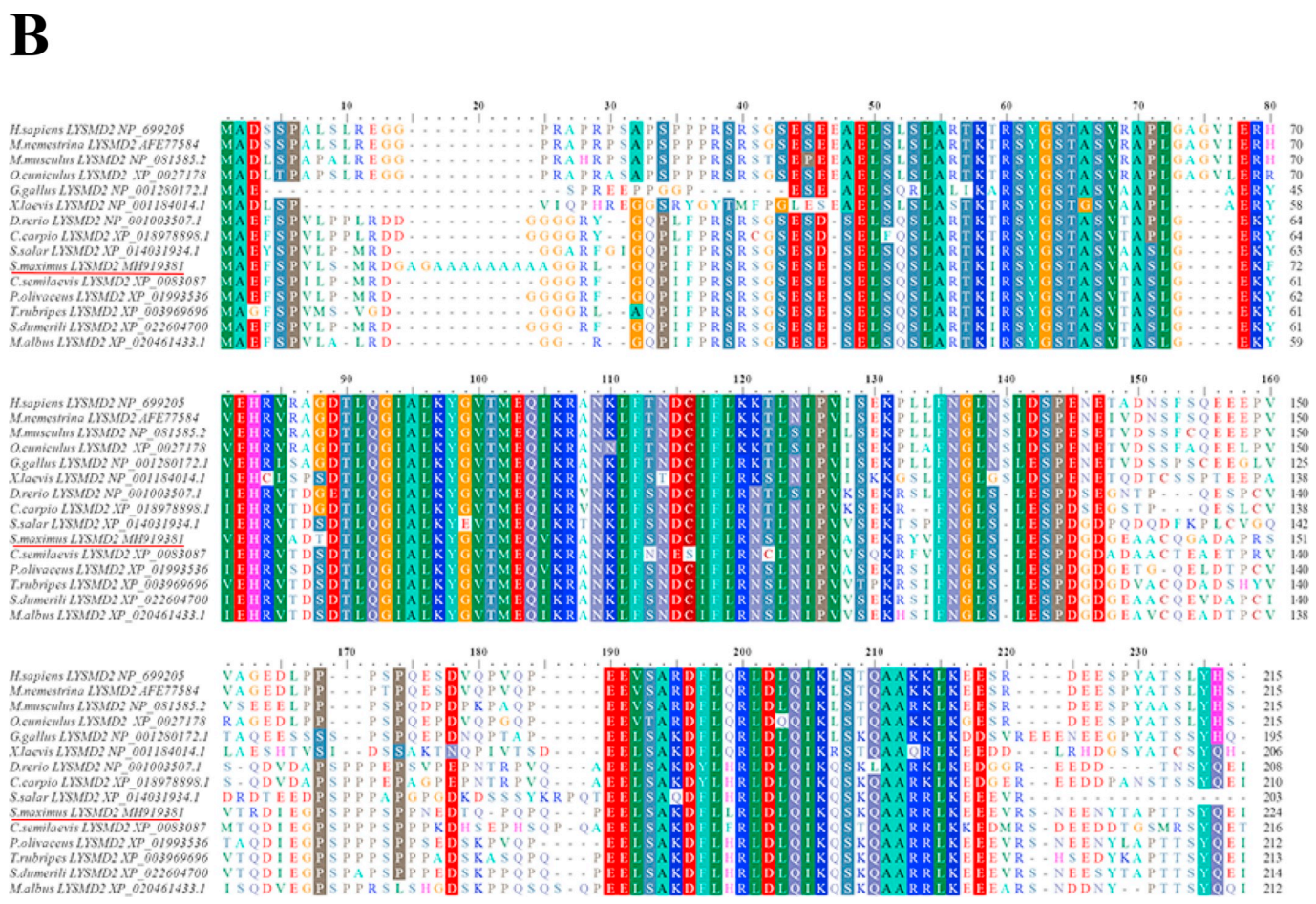

Fig. 4. (*continued*)

the type of intracellular non-secretory proteins, and was perceived as the lysM and putative peptidoglycan-binding domain containing protein2 like family. Concurrently, the results shown in Fig. 5 revealed that *SmLysMd1* and *SmLysMd2* belong to the type of intracellular non-secretory proteins, and *SmLysMd3* and *SmLysMd4* belong to the type of anchored transmembrane, located in the 228–250 and 209–231 amino acid, respectively.

Subsequent phylogenetic analysis indicated that the turbot LysM family were considerably conserved gene. And the four *SmLysMd* isoforms clustered within the teleost counterparts and fell into the corresponding *LysMd* branch separately, suggesting their similar function with other species. In addition, phylogenetic analysis also showed that, all the *LysMd* isoforms clustered in two major branches, the *LysMd1* and *LysMd2* branch and the *LysMd3* and *LysMd4* branch, suggesting that *LysMd1* had close evolutionary relationship with *LysMd2*, and *LysMd3* had close evolutionary relationship with *LysMd4*. Taken the protein structure together, all the results indicated that the type of *LysMd1* and *LysMd2* were co-evolutionary and had the similar function. Meanwhile, the type of *LysMd3* and *LysMd4* were also co-evolutionary and had the similar function.

In this study, four *SmLysMd* transcripts were widely expressed in all tested tissues of healthy turbot, exhibiting that the turbot LysM had an important function in maintaining the basal physiological process. And the results were consistent with the invertebrate, such as kuruma shrimp of *MjLPBP*, belonging to the lysM 3 like family, Hong Kong oyster of *ChLysM* as the lysM 3 like family and red swamp crayfish of *PcLysM,* defined as the lysM 2 like family [13,23,24]. However, the expression patterns of the four *SmLysMd* showed extremely different trend. As shown in Fig. 2, the highest expression levels of *SmLysMd1* happened in muscle, liver and skin. In contrast, the highest expression levels of *SmLysMd2* happened in skin, muscle and liver, and the highest expression levels of *SmLysMd3* happened in liver, brain and muscle. Meanwhile, the highest expression levels of *SmLysMd4* were highly expressed in muscle, skin and liver. Thus, in different tissues, the *SmLysMd* expression showed different patterns, suggesting different type of *SmLysMd* genes might play different role in the immunity process in specific tissue.

Subsequently, the transcription levels of *SmLysMd* in the muscle and liver which had the relatively abundant expression and were thought to play a crucial role in the immunological reaction, were analyzed after *V. vulnificus* challenge. Although different expression patterns under different type of *SmLysMd* were observed, four *SmLysMd* transcriptions were totally up-regulated in muscle and liver (Fig. 3). Consistently, significant up-regulations of *LysM* transcript expression have also been found in red swamp crayfish with the type of lysM 2, kuruma shrimp and Hong Kong oyster with the type of lysM 3 after *V. alginolyticus* and *S. haemolyticus* challenge [24]. Thus, it was speculated that *SmLysMds* should be involved in turbot defense against bacterial infection, and the *SmLysMd2* and *SmLysMd3* may be play the more important roles in immunity process. Thus, more researches should been done to confirm it. Until now, besides our research, there was only zebrafish *LysMd* identified in teleost [7]. However, there was no significant expression

C

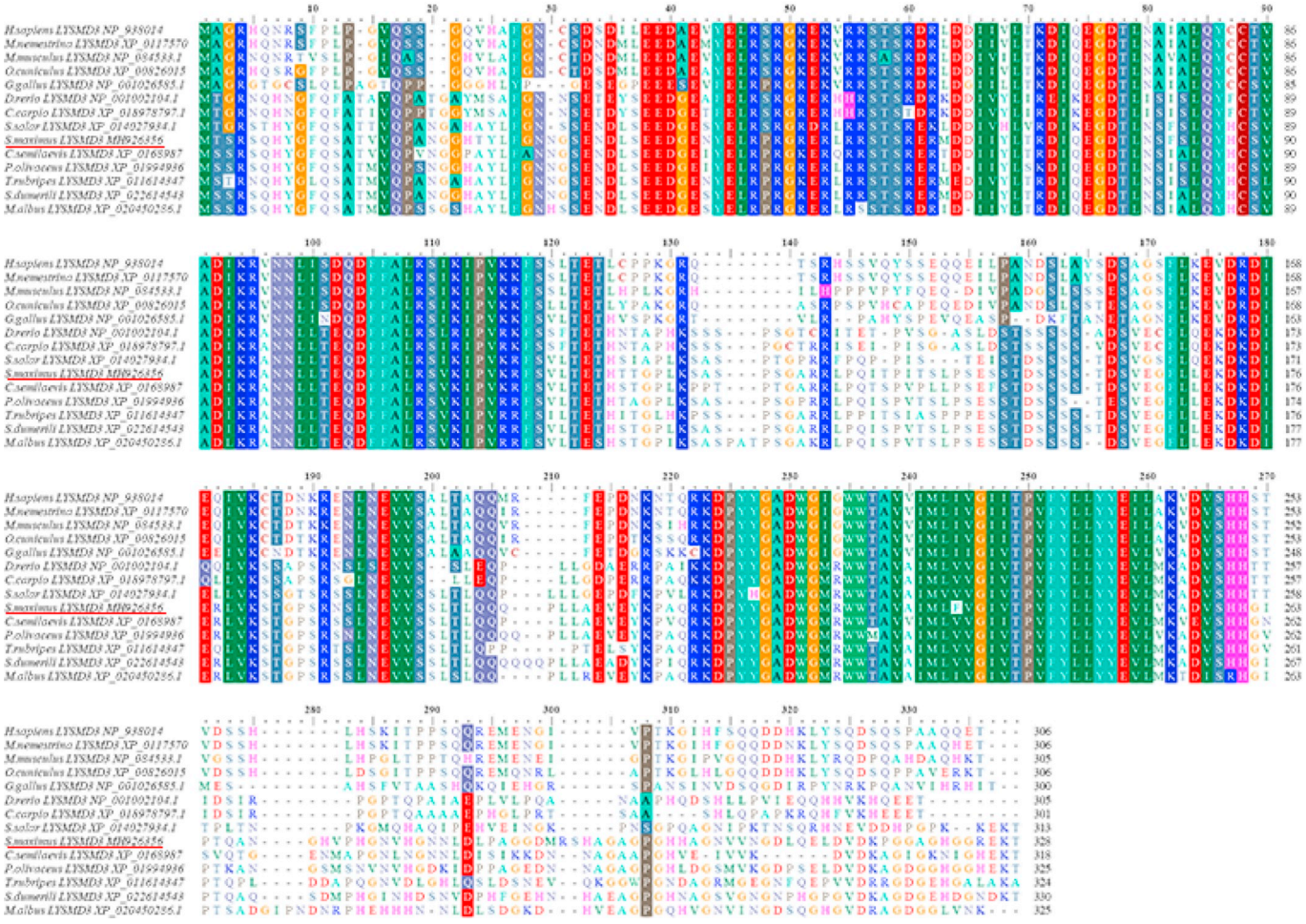

Fig. 4. (*continued*)

change of zebrafish *LysMd* encoding gene in response to *Salmonella* and *Mycobacterium* challenge in zebrafish [7,30]. Probably, *LysMd* could play different roles under different infection process exhibiting differential expression patterns. Indeed, Hong Kong oyster *LysM* showed highest expression in the gills and hemocytes and was confirmed to contribute to immune responses [24]. Therefore, further investigation should be done to characterize the subtle roles of *LysMd* for disease resistance selection. In addition, in zebrafish, it has been confirmed that *LysMd* might facilitate the development and functioning of brain, even nervous system. What's more, the LysM was provided resistance to oxidation by oxidative damage and apoptosis [31]. It could be seen, the LysM functioned in many physiology process associated with some disease. According to these results, more function of *LysMd* in turbot in controlling important biological processes should been studied in future.

Previous studies have indicated that LysM could bind different types of PGN and chitin-like compounds in bacteria and eukaryotes. And it also has been functioned in fungal infection through the immune response [8,21,32–34]. Researches in plants also showed that LysM were crucial component in innate immunity as PRRs for recognition of PGN and chitin [20,35]. Meanwhile, in *Arabidopsis thaliana*, LYP2 and LYP3 are involved in PGN recognition and play essential roles in immunity to bacterial infection [11,14]. And in the experiment of *Arabidopsis thaliana* vitro assays, the Lysin motif receptor-like kinase named CERK1 was verified to bind chitin directly [16,17,36]. In addition, recombinant invertebrate LysM could bind to PGN even at extremely low concentrations [24]. Crayfish LysM gene silencing could induce defense reaction after the *V. anguillarum* challenging, suggesting the LysM was also responded to AMPs to innate the immune signal pathways [23]. As well as in mice, the mutant of LysM protein could affect the spreading of bacteria after infection [37,38]. On the other hand, in bacteria, it is well known that PGN, as an indispensable cell wall molecule, could elicit defense responses [39]. Thus, more investigations about turbot LysM binding to PGN on various invading bacteria surface should be done in future.

5. Conclusion

In this study, four turbot *LysMd* were identified and characterized as a novel member of the LysM and putative PGN-binding domain-

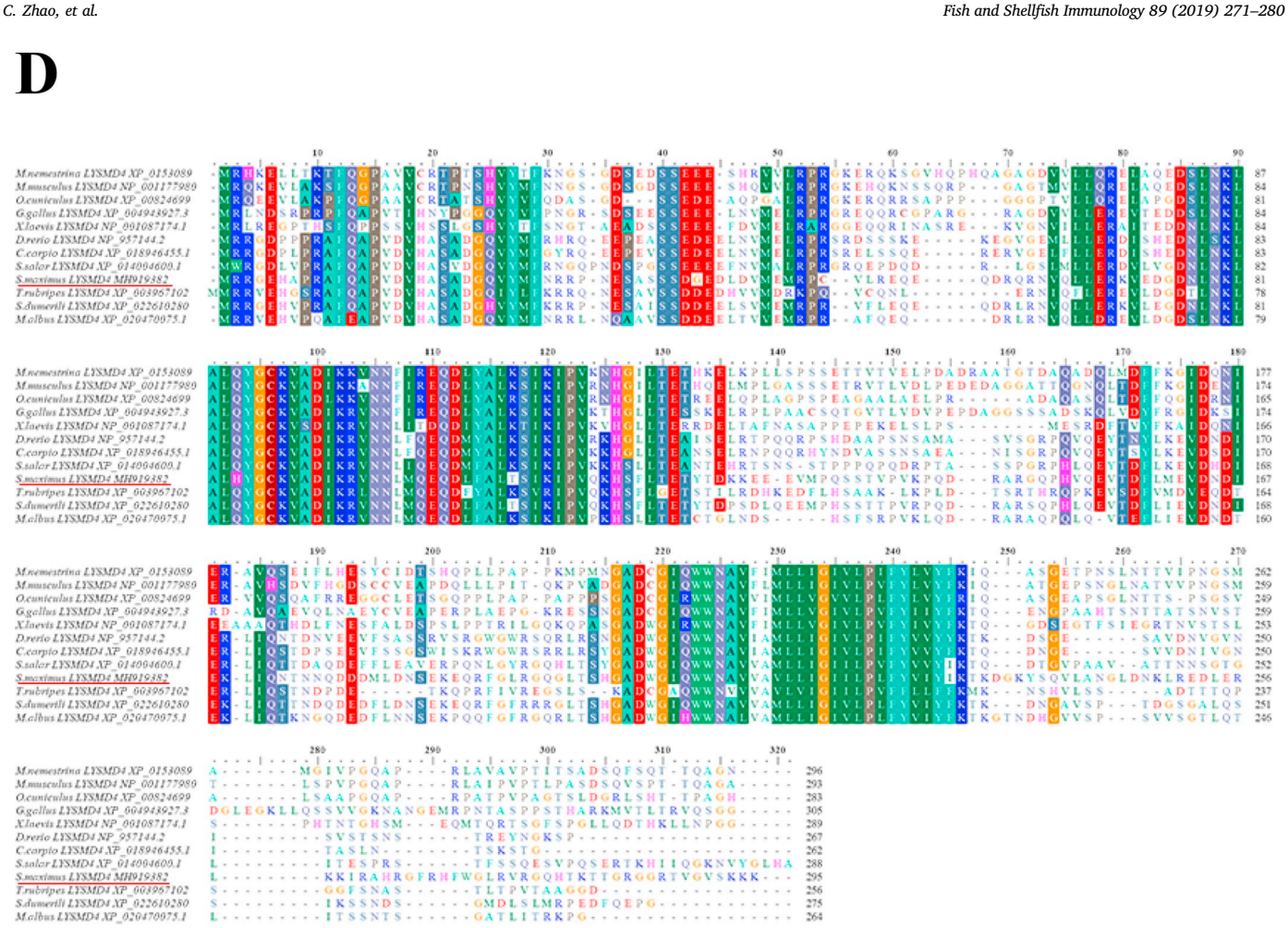

Fig. 4. (*continued*)

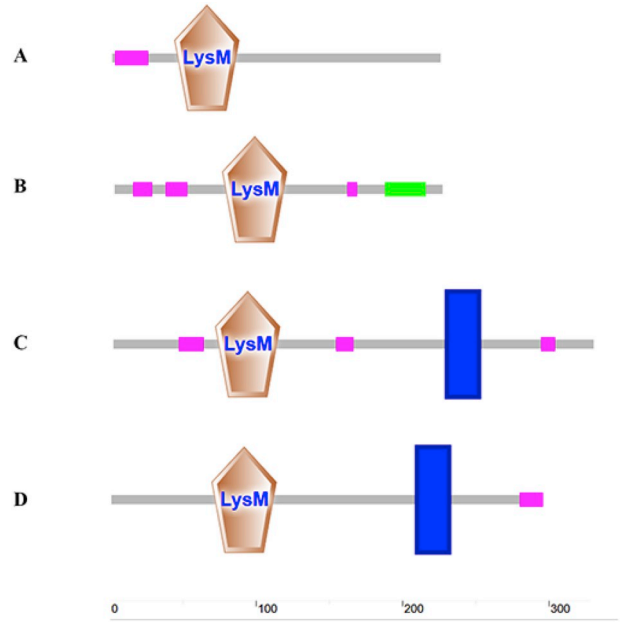

Fig. 5. Sequence analysis of four predicated *SmLysMds*. A, Predicted domains of *SmLysMd1*. B, Predicted domains of *SmLysMd2*. C, Predicted domains of *SmLysMd3*. D, Predicted domains of *SmLysMd4*. LysM indicated the Lysin motif, and the blue part was the transmembrane region. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

containing-like protein family. Moreover, *SmLysMd* transcriptions were ubiquitously expressed in all tested tissues, and analyzed their expression levels after *V. vulnificus* challenge. All the results suggested that the *LysMd* could be participant in the immune responses to bacterial infections. Our findings should provide foundation knowledge for the functions studies of *LysM* in immunity.

Acknowledgement

This study was supported by theAdvanced Talents Foundation of QAU (6651118016); Key Research and Development Program of Shandong Province (2016GSF115026, 2018YFJH0703); Major Agricultural Applied Technological Innovation Projects of Shandong Province; National Natural Science Foundation of China (No. 31802319, 31572644); Natural Science Foundation of Shandong Province (Grant No. ZR2018BC053); National Key R&D Program of China (2018YFD0900301); “First class fishery discipline” programme in Shandong Province.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.074.

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