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Grouper (*Epinephelus coioides*) Mpeg1s: Molecular identification, expression analysis, and antimicrobial activity

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ABSTRACT

Macrophage expressed gene 1 (Mpeg1) is a molecule that can form pores and destroy the cell membrane of invading pathogens. In this study, we identified two Mpeg1 isoforms from the orange-spotted grouper (*Epinephelus coioides*) and named them EcMpeg1a and EcMpeg1b. Predicted proteins of the two EcMpeg1s contained a signal peptide, a conserved membrane attack complex/perforin (MACPF) domain, a transmembrane segment, and an intracellular region. Sequence alignment demonstrated that two EcMpeg1 proteins share a high sequence identity with that of other teleosts. Tissue distribution analysis showed that EcMpeg1s were expressed in all tissues tested in healthy grouper, with the highest expression in the head kidney and spleen. After infection with the ciliate parasite *Cryptocaryon irritans*, expression of the two EcMpeg1s was significantly upregulated in the spleen and gills. Furthermore, the recombinant EcMpeg1a showed antiparasitic and antibacterial activity against Gram-negative and -positive bacteria, whereas EcMpeg1b had an inhibitory effect only against Gram-positive bacteria. These results indicated that EcMpeg1s play an important role in the host response against invading pathogens.

1. Introduction

Pore-forming molecules containing a membrane attack complex/perforin (MACPF) domain are indispensable in innate immune defense because they perforate and destroy the cytomembrane of invading pathogens [1]. To date, three kinds of pore-forming molecules have been identified: members of the complement system (C6, C7, C8α, C8β, and C9), perforin 1, and perforin 2 [2–4]. Among them, perforin 2 is encoded by macrophage expressed gene 1 (Mpeg1), which also contains the MACPF domain [5]. When cells are infected by bacteria, perforin 2 vesicles can fuse with the bacteria-containing endosomes, causing the MACPF domain to face the bacteria and form pores on their surface, making the bacteria susceptible to other bactericidal compounds such as lysozyme [6].

Mpeg1 was first identified in 1995 by differential screening of mouse peritoneal macrophage cDNA, and its expression was found to be restricted by macrophages [7]. Since then, the expression and functional characteristics of Mpeg1 have been widely studied. In one study, knockdown of perforin-like molecules resulted in unrestricted bacterial growth in mouse embryonic fibroblasts [8], and bacterial infection in perforin-2$^{-/-}$ mice caused rapid onset of acute disease symptoms [9]. Studies also have shown that Mpeg1 has bactericidal activity and can prevent replication of bacterial pathogens in cells [10]. The bactericidal activity of Mpeg1 in invertebrates, such as sponges, abalones, and oysters, also has been studied intensively. In the disk abalone (*Haliotis discus discus*), significant upregulation of Mpeg1 expression levels was observed in hemocytes and gill tissues after pathogen attacks [11]. Recombinant expression of the MACPF domain of the Pacific oyster (*Crassostrea gigas*) Mpeg1 exhibited significant antibacterial activity against both Gram-negative and -positive bacteria [12]. In vertebrates, Mpeg1 is also used as a specific marker for macrophages [13].

In zebrafish (*Danio rerio*), three Mpeg1 isoforms have been identified, and all of them contain a conserved MACPF domain and a

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Table 1
Bacterium used in this paper.

Name	Source	
Streptococcus iniae	Trachinotus ovatus	Gram-positive bacteria
Lactococcus garvieae	Channa argus♂ × Channa maculata♀	
Streptococcus agalactiae	Oreochromis niloticus	
Streptococcus agalactiae	Trachinotus ovatus	
Edwardsiella ictaluri	Pseudobagrus fulvidraco	Gram-negative bacteria
Edwardsiella tarda	Anguilla japonica	
Aeromonas veronii	Channa argus♂ × Channa maculata♀	
Aeromonas hydrophila	Mystus guttatus	
Vibrio mimicus	Pseudobagrus fulvidraco	
Vibrio alginolyticus	Hippocampus	

transmembrane domain. Benard et al. reported that Mpeg1 and Mpeg1.2 are expressed in macrophages, and the third Mpeg1 is predicted to be a pseudogene [14]. When infected with bacterial pathogens, the Mpeg1 and Mpeg1.2 genes of zebrafish showed differential regulation; however, their functional mechanisms remain unclear [14].

Orange-spotted grouper (*Epinephelus coioides*) are economically important marine fish in South China, and they are often infected by *Cryptocaryon irritans*, which caused high economic losses [15]. Some fish acquire protective immunity against subsequent parasitic attacks after infection with *C. irritans*, suggesting that the fish can produce specific antibodies [16]. However, the innate antiparasitic immune response of grouper is poorly understood. In the present study, we identified two unigenes encoding homologs of Mpeg1 in grouper and analyzed their expression pattern in healthy *E. coioides* and in specimens challenged with *C. irritans*. The antiparasitic activity and antimicrobial activity of the recombinant proteins were also examined.

2. Materials and methods

2.1. Fish, C. irritans, and bacteria

Orange-spotted groupers (12–15 cm) used in this study were purchased from the Marine Fisheries Development Center of Guangdong Province, China. They were maintained in a continuous flow seawater system at 25 °C for 2 weeks before processing. Fish were fed twice daily with a commercial feed.

Following the methods described by Dan et al. [17], *C. irritans* were propagated in our laboratory on pompano (*Trachinotus ovatus*).

Table 1 lists the bacteria used in this study. They were isolated, identified, and stored in our lab.

2.2. C. irritans challenge and sample collection

For the challenge trial, groupers were exposed to *C. irritans* at the dose of 20,000 theronts per fish [18]. To investigate the expression pattern of EcMpeg1s after *C. irritans* infection, the spleens and gills from three fish were collected at 0, 1, 2, and 3 days post infection, respectively, then immediately snap frozen in liquid nitrogen and stored at −80 °C. For analysis of tissue distribution patterns, the thymus, spleen, head kidney, gill, liver, and hindgut were sampled from three healthy groupers, immediately snap frozen in liquid nitrogen, and stored at −80 °C.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from the samples collected using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA integrity was assessed by electrophoresis on 1% agarose gel, and the purity was determined by OD260/280 analysis. Next, 1 μg of total RNA was used to synthesize the first strand cDNA using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, San Diego, CA, USA).

The cDNA for rapid amplification of cDNA ends (RACE) was synthesized from the total RNA of the spleen using the SMARTer™ RACE cDNA Amplification Kit (Clontech, San Francisco, CA, USA). All cDNA was stored at −20 °C until its use in subsequent experiments.

2.4. Amplification and sequence analysis of Mpeg1a and Mpeg1b

Based on the transcriptome database of grouper we obtained previously (unpublished), two unigenes were identified that both shared high sequence identity with the Mpeg1 of other fish, and we named them Mpeg1a and Mpeg1b. The unigene of Mpeg1a (3042 bp) contained a complete open reading frame (ORF), but Mpeg1b lacked part of the 5′ region. The RACE approach was used to obtain the 5’ region of Mpeg1b via the SMARTer™ RACE cDNA Amplification Kit using nested polymerase chain reaction (PCR). The first round PCR was amplified using the primers UPM and Mpeg1b rR1, and the second round PCR was amplified using NUP and Mpeg1b rR2 (Table 2). The PCR conditions were as follows: 35 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 1 min 30 s, then 72 °C for 7 min. Finally, the primers Mpeg1a F/R and Mpeg1b F/R were designed to confirm the ORF of Mpeg1a and Mpeg1b. All products then were inserted into the pEASY-Blunt Simple Cloning vector (TransGen Biotech, Beijing, China) and sequenced.

The amino acid sequences of the Mpeg1s were analyzed using NCBI

Table 2
Primers used in this study.

Name	Sequence (5′ to 3′)
Mpeg1a F1	TCCCACTCCTGTAAGTTGCGTCAC
Mpeg1a R1	GGAGGAACTTGGTTTGAACCTTATGTG
Mpeg1b rR1	CCTTGTTTGGTTGTTTTCAATCGCATC
Mpeg1b rR2	GCTTTGACGGAGGAACTTTCTGACG
Mpeg1b F1	ATGAAGGCGA
Mpeg1b R1	CTAAACCTCACCTTGC
Mpeg1a F2	GCCATGGCTGATATCGGATCCATCAGCCGCCCCACCAAC
Mpeg1a R2	GTGGTGGTGGTGGTGCTCGAGTTATTCACCACCTGTCATCTGATTC
Mpeg1b F2	GCCATGGCTGATATCGGATCCATCAGACACCCCACCAACTGG
Mpeg1b R2	GTGGTGGTGGTGGTGCTCGAGTTAGTCCTACGTATGATCTTGTTGATGTCC
Mpeg1a RT-F1	ATGACGAAAGATATCATTGTGGG
Mpeg1a RT-R1	TCTGGAATGAAGTTTGGGGGG
Mpeg1b RT-F1	GCACTATTGCATTTTTGAGGACC
Mpeg1b RT-R1	ACTGGAATGAAGTTTGGGGGA
β-actin RT-F1	TGCTGTCCCTGTATGCCTCT
β-actin RT-R1	CCTTGATGTCACGCACGAT

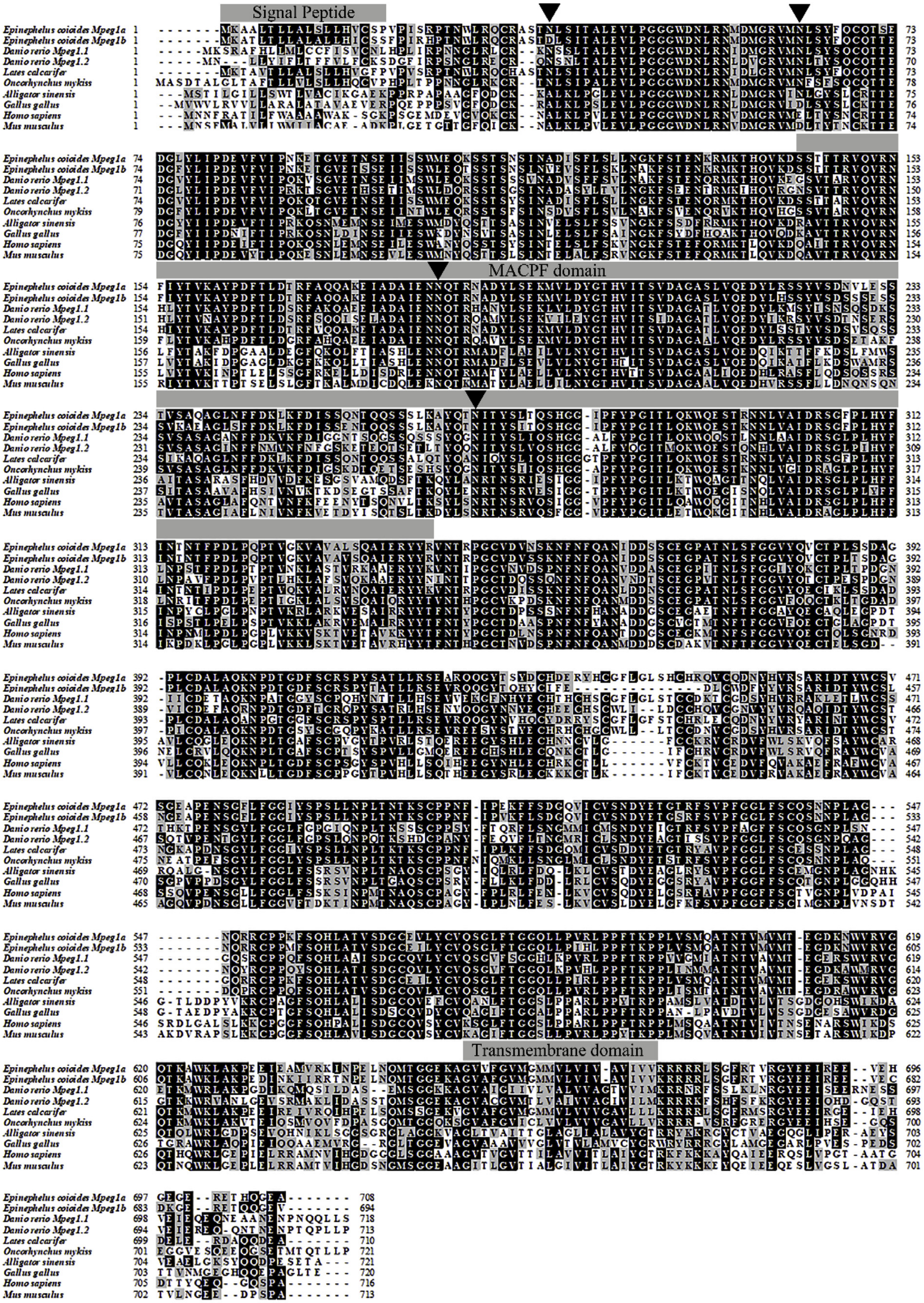

Fig. 1. Sequence alignment of EcMpeg1a and EcMpeg1b. The signal peptide, MACPF domain, and C-terminal transmembrane segment of EcMpeg1a and EcMpeg1b are indicated by black letters on a gray background. The black arrow points to the predicted glycosylation site.

Table 3
Amino acid identity (%) between EcMpeg1s and Mpeg1s of other species.

Species	Full length		MACPF domain	
	EcMpeg1a	EcMpeg1b	EcMpeg1a	EcMpeg1b
EcMpeg1a	100	90	100	94
EcMpeg1b	90	100	94	100
Lates calcarifer	84	81	87	87
Paralichthys olivaceus	80	80	85	84
Larimichthys crocea	83	81	87	86
Takifugu rubripes	77	75	79	80
Oncorhynchus mykiss	72	73	77	77
Salmo salar	72	72	76	77
Danio rerio 1.1	65	64	73	74
Danio rerio 1.2	63	62	66	67
Alligator sinensis	49	49	53	56
Gallus gallus	48	48	50	52
Homo sapiens	50	49	53	56
Mus musculus	49	48	49	51
Haliotis discus discus	35	36	35	37
Crassostrea gigas	34	34	29	32

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNAMAN version 4.13. The ORF was identified using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide was predicted using the tool Signal P (http://www.cds.dtu.dk/services/SignaiP). The SMART program (http://smart.emblheidelberg.de/) was used to analyze the protein structure. Transmembrane helices were predicted by the TMHMM Server (http://cbs.dtu.dk/services/TMHMM). The N-glycosylation sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Amino acid identity between EcMpeg1a, EcMpeg1b, and Mpeg1s of other species were analyzed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). A neighbor-joining phylogenetic tree was constructed using MEGA version 5.0.

2.5. Real-time PCR

Real-time PCR was used to investigate the expression pattern of EcMpeg1a and EcMpeg1b in various healthy tissues and in *C. irritans*-infected tissues. Gene-specific qPCR primers of Mpeg1a RT-F1/RT-R1 and Mpeg1b RT-F1/RT-R1 (Table 2) were designed using Beacon Designer, and β-actin F/R primers were used as a reference. Real-time PCR was performed on an ABI Quant Studio 5 device (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 μl, containing 5 μl of SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 0.3 μl of each primer (10 mM), 1 μl of cDNA, and 3.4 μl of double distilled water. The PCR conditions were as follows: 95 °C for 10 min and 40 amplification cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. All samples were analyzed in triplicate. The mRNA expression of the EcMpeg1s compared to the reference gene was calculated using the $2^{-\Delta\Delta Ct}$ method [19].

2.6. Expression and purification of recombinant EcMpeg1a and EcMpeg1b

The extracellular regions without signal peptides (61–1950 bp for EcMpeg1a and 61–1717 bp for EcMpeg1b) were amplified with specific primers Mpeg1a F2/R2 and Mpeg1b F2/R2 (Table 2), and then inserted into the expression vector pET-32a. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells, and the positive clone was cultured at 37 °C. When the optical density (OD) reached 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of the recombinant protein for 4 h at 37 °C. The recombinant proteins rEcMpeg1a and rEcMpeg1b were purified using Ni Resin FF (GenScript, Nanjing, China) according to the manufacturer's specifications. The purified proteins were detected by 10% SDS-PAGE and then dialyzed in dialysis bags in gradient urea solution (6 M, 4 M, 2.5 M, 2 M, 1 M, 0.5 M, 0 M) and lastly in phosphate buffered saline (PBS) at 4 °C. The concentration of the purified proteins was determined using the BCA Protein Assay Kit (CWBIO, Beijing, China), and the proteins were then stored at −80 °C.

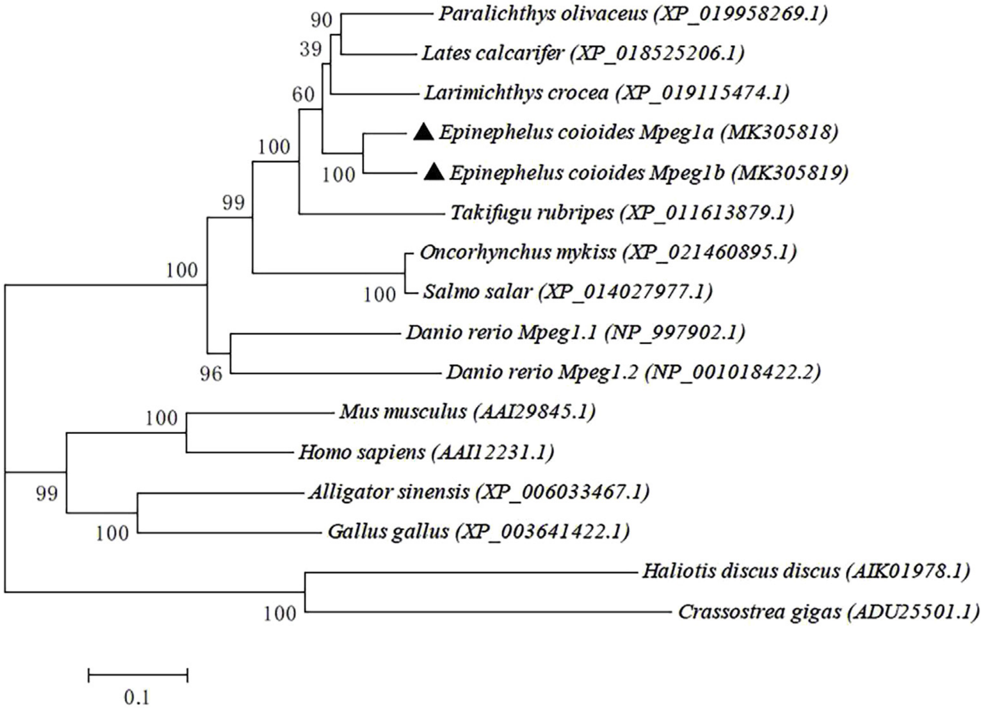

Fig. 2. The phylogenetic tree was constructed using the neighbor-joining method. Node values represent percent bootstrap confidence derived from 1000 replicates. The scale bar is 0.1. GenBank accession numbers are listed in brackets.

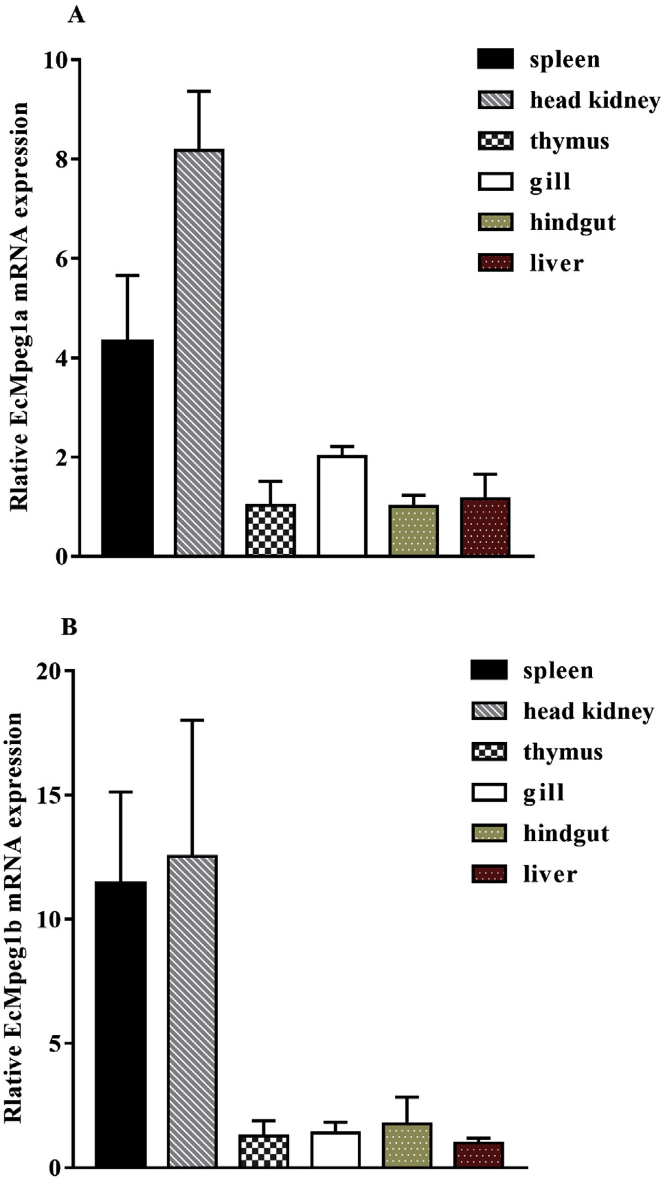

Fig. 3. The expression pattern of EcMpeg1a (A) and EcMpeg1b (B) in the spleen, head kidney, thymus, gill, hindgut, and liver of healthy grouper. The mRNA expression levels were normalized to transcripts of β-actin, and the data are shown as means ± standard error (N = 3).

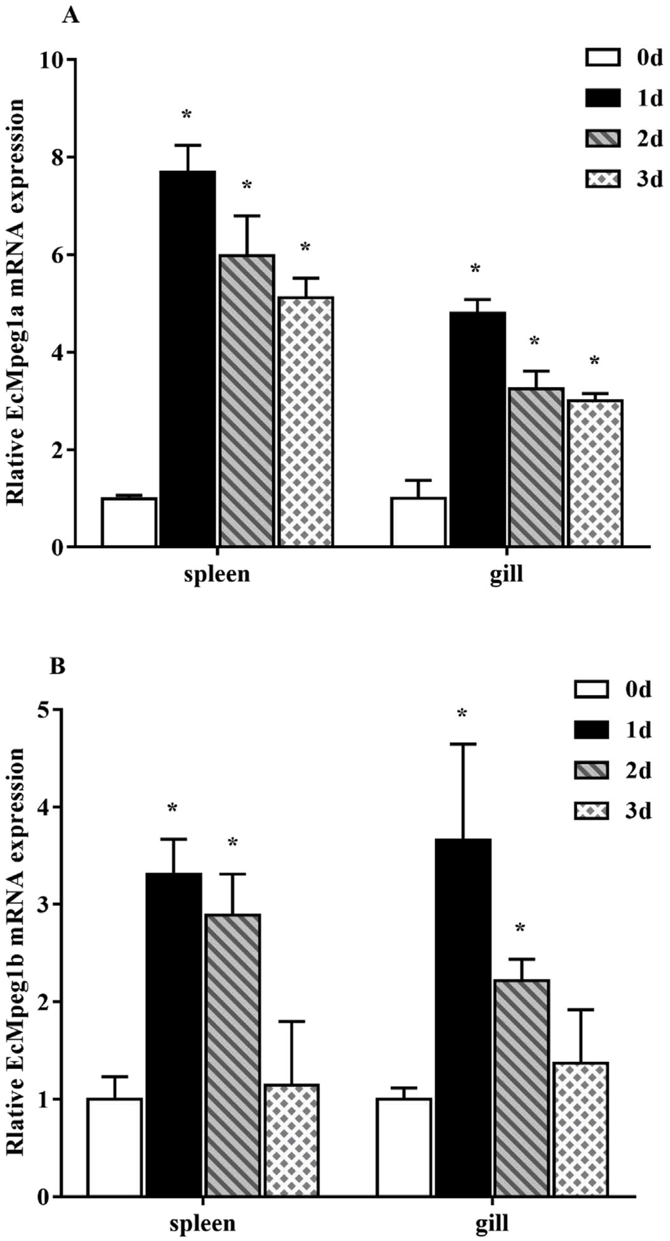

Fig. 4. Analysis of the relative expression of EcMpeg1a (A) and EcMpeg1b (B) in the spleen and gills at different time points post-*C. irritans* infection. Significant expression differences of these two genes between day 0 and *C. irritans*-challenged groups at days 1–3 are indicated with an asterisk ($P < 0.05$).

2.7. Antiparasitic activities of recombinant EcMpeg1a and EcMpeg1b

The antiparasitic activities of recombinant EcMpeg1a and EcMpeg1b were determined as described previously with minor modifications [20]. In brief, 100 μl of purified rEcMpeg1a (156 μg/μL) or rEcMpeg1b (160 μg/μL) were added to 96-well plates; PBS and purified pET-32a served as the blank and negative control, respectively. Next, 100 μl of seawater containing about 200 live theronts were added to each well and incubated for 1 h at 28 °C. The samples were observed under a light microscope (Mshot, Guangzhou, China).

2.8. Antibacterial activities of recombinant EcMpeg1a and EcMpeg1b

Four Gram-positive bacteria and six Gram-negative bacteria were used for the antibacterial activity test, which followed the method described by Luo et al. [21]. All bacteria were inoculated individually into 1 mL of brain heart infusion broth (BHI) at 28 °C until the OD reached 0.5. Next, 50 μl of the bacterial suspension was mixed with 50 μl of purified rEcMpeg1a (156 μg/μL) or rEcMpeg1b (160 μg/μL) for 3 h in 96-well plates. PBS and purified pET-32a were added to separate samples and served as the blank and negative control, respectively. Finally, 150 μl of the BHI medium was added and cultured for 30 min, and the absorbed value was determined at OD_{600} using a microplate reader (Thermo Fisher Scientific). The assay was performed in three independent experiments. The growth rate was obtained by multiplying the OD value by 100%.

2.9. Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics version 22. The results of real-time PCR were analyzed using the Student's *t*-test ($P < 0.05$) and the antibacterial activities were analyzed using Duncan's test ($P < 0.05$).

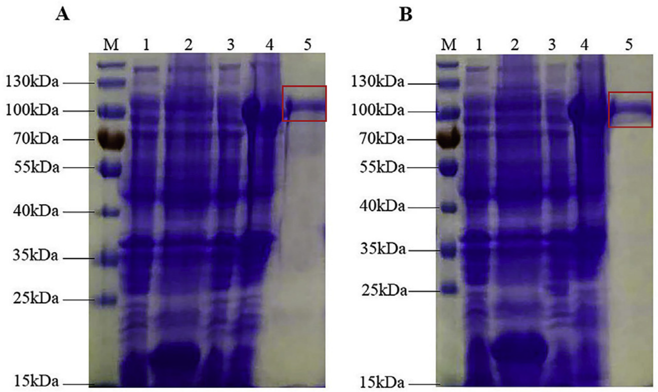

Fig. 5. SDS-PAGE analysis of the recombinant EcMpeg1a (A) and EcMpeg1b (B) proteins. Lanes M through 5 indicate: Protein marker, non-induced pET-32a, induced pET-32a, non-induced recombinant protein, induced recombinant protein, and purified recombinant protein.

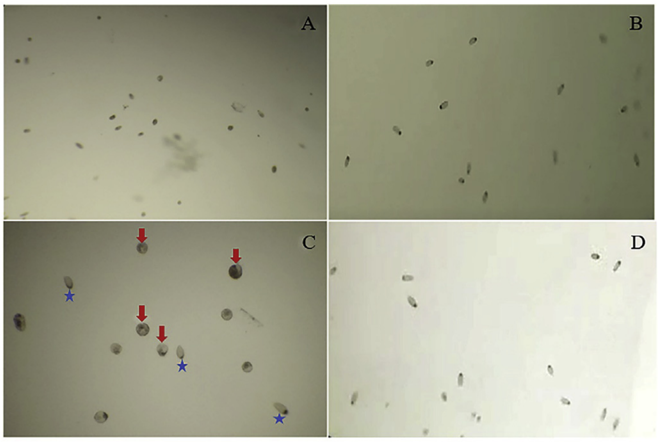

Fig. 6. Antiparasitic activities of recombinant EcMpeg1a and EcMpeg1b. A–D show results for the PBS negative control group, pET-32a group, rEcMpeg1a group, and rEcMpeg1b group, respectively. The red arrowhead points to a dead theront, and the blue asterisk indicates a theront that it is still alive. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Characteristics and phylogenetic analysis of EcMpeg1a and EcMpeg1b

The full-length ORFs of EcMpeg1a and EcMpeg1b were obtained and uploaded to GenBank with accession numbers MK305818 and MK305819. The ORF of EcMpeg1a was 2127 bp long, encoded 708 amino acid residues, and had a theoretical isoelectric point (pI) of 6.16 and a calculated molecular mass (Mw) of 78.61 kDa. The ORF of EcMpeg1b was 2085 bp in length, encoded 694 amino acids, and had pI and Mw values of 5.81 and 77.18 kDa, respectively. Both deduced mature polypeptides contained a signal peptide, a conserved MACPF domain, a C-terminal transmembrane segment, and an intracellular region (Fig. 1). Four (N-36, N-63, N-184, N-268) and two (N-63, N-184) glycosylation sites were predicted for EcMpeg1a and EcMpeg1b, respectively.

Sequence alignment showed that EcMpeg1a and EcMpeg1b had high sequence identity with the Mpeg1 of other teleost fish (62–84%); the highest identity was with *Lates calcarifer* (Table 3). The identities between the EcMpeg1s and those of mammals and invertebrates were 48–50% and 34–36%, respectively. The identity between EcMpeg1a and EcMpeg1b was as high as 90%. Additionally, the MACPF domains of EcMpeg1a and EcMpeg1b were similar to those of other teleosts (66–87%).

The neighbor-joining phylogenetic tree constructed using MEGA mainly divided the Mpeg1 sequences into three branches (Fig. 2). The Mpeg1 from bony fish together with the EcMpeg1s clustered into a single branch. Among them, the two EcMpeg1s, *P. olivaceus*, *L. calcarifer*, and *L. crocea* clustered together as a small branch. The Mpeg1 of mammals, birds, and reptiles clustered on the second branch, and those of invertebrates clustered on the third branch. The results of the phylogenetic analysis are consistent with the results of amino acid sequence identity.

3.2. Gene expression pattern in healthy E. coioides

Real-time PCR analysis revealed the tissue-specific expression pattern of EcMpeg1a and EcMpeg1b in six tissues (Fig. 3). Expression profiles of EcMpeg1a and EcMpeg1b mRNA were detected in all six tissues (thymus, spleen, head kidney, gill, liver, and hindgut), but expression levels were highest in the immune organs (head kidney and spleen).

3.3. Expression profiles in C. irritans-challenged grouper

To investigate the immune response of EcMpeg1a and EcMpeg1b to *C. irritans* infection, real-time PCR was used to analyze the expression profiles of each EcMpeg1 in the spleen and gills at different time points. The expression level of EcMpeg1a was significantly upregulated relative to the control in spleen and gill tissue at 1–3 days after *C. irritans* challenge, and it peaked at day 1, when it was ~7-fold higher in spleen and 5-fold higher in gill tissue (Fig. 4). The expression level of EcMpeg1b was sharply upregulated at days 1 and 2 after infection, and it peaked at day 1, when it was ~3-fold higher in spleen and 4-fold higher in gill tissue.

3.4. Expression of rEcMpeg1a and rEcMpeg1b

Two recombinant plasmids (rEcMpeg1a and rEcMpeg1b) were successfully transformed into *E. coli*. After induction by IPTG and purification by Ni Resin FF, we obtained two proteins with a relative molecular weight of approximately 77–79 kDa, which were the size of predicted rEcMpeg1a and rEcMpeg1b (Fig. 5). However, there was no corresponding band in the bacteria non-induced by IPTG, and there was only one ~17 kDa polypeptide in the pET-32a transformed bacteria.

3.5. Antiparasitic activities of rEcMpeg1a and rEcMpeg1b

Using the experimental model of the existing *C. irritans* in our laboratory, we performed bioassays of recombinant EcMpeg1a and EcMpeg1b against *C. irritans* (Fig. 6). Within 1 h of the addition of PBS or pET-32a, no significant changes in the morphology and movement of theronts were detected; the theronts swam fast and had a fusiform shape throughout the process. However, after the addition of purified rEcMpeg1a protein, most of the theronts moved slower, the shape gradually changed from fusiform to circular, and ultimately they stopped moving. In contrast, compared with the EcMpeg1a groups, there was no significant change in theront morphology and movement speed in the group treated with rEcMpeg1b.

3.6. Antibacterial activities of recombinant EcMpeg1a and EcMpeg1b

Four Gram-positive and six Gram-negative bacteria were used to analyze the antibacterial activities of the recombinant EcMpeg1a and EcMpeg1b (Fig. 7). pET-32a promoted the growth of *S. iniae* and *V. alginolyticus*, inhibited *S. agalactiae*, and had no significant effect on the other bacteria. Compared with PBS or pET-32a, the rEcMpeg1a protein significantly decreased growth of both Gram-negative and Gram-positive bacterial strains. In contrast, rEcMpeg1b had an inhibitory effect

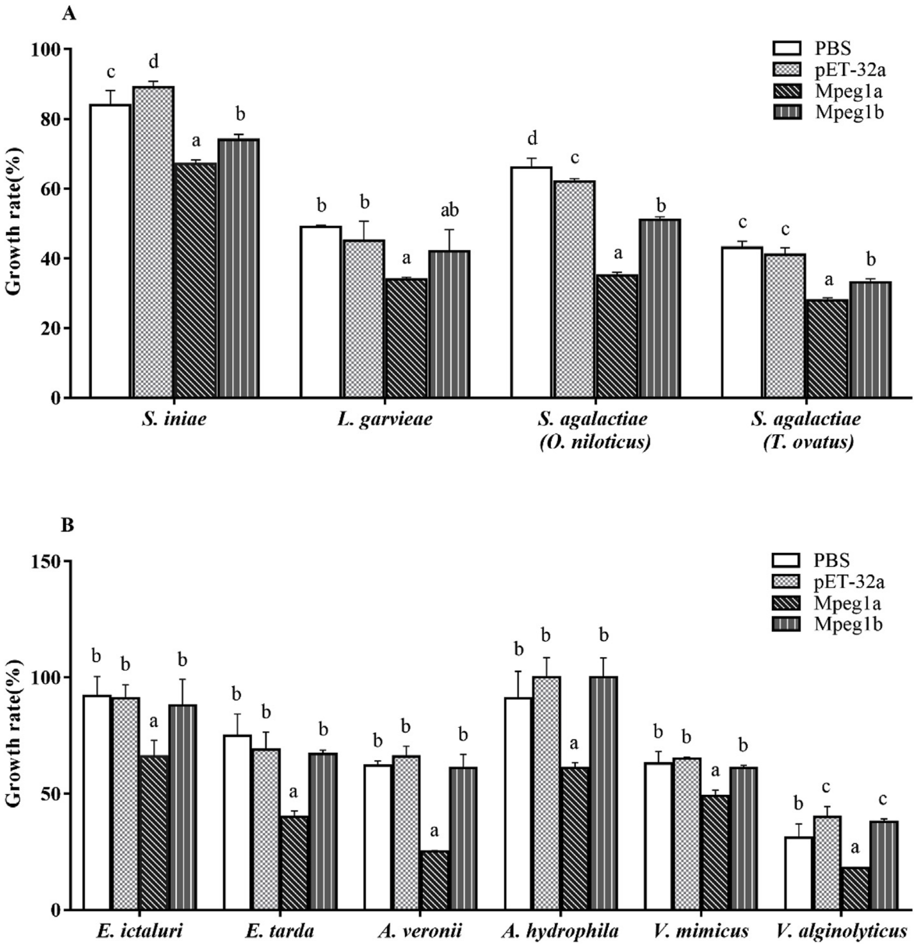

Fig. 7. (A) Growth rates of the following Gram-positive bacterial strains: *S. iniae*, *L. garvieae*, *S. agalactiae* (*O. niloticus*), *S. agalactiae* (*T. ovatus*) and (B) growth rates of the following Gram-negative bacterial strains: *E. ictaluri*, *E. tarda*, *A. veronii*, *A. hydrophila*, *V. mimicus*, *V. alginolyticus*. The bars represent the means ± standard error (N = 3). Significant differences between different groups are indicated with a, b, c, and d ($P < 0.05$).

only on Gram-positive bacterial strains. It also promoted growth of Gram-negative *V. alginolyticus* when compared with PBS treatment, but it showed no obvious antimicrobial activity when compared with pET-32a treatment.

4. Discussion

Although Mpeg1 has been widely studied in humans, its functional properties are poorly known in teleost fish. In this study, two homologs of Mpeg1 from the orange-spotted grouper (EcMpeg1a and EcMpeg1b) were cloned, their sequences were determined, and phylogenetic analysis was performed. These two sequences contain a signal peptide, a conserved MACPF domain, a C-terminal transmembrane segment, and an intracellular region. The MACPF domain of Mpeg1 in grouper is highly conserved, as it was very similar to Mpeg1s from mammalian species and other teleosts. This domain is predicted to play a key role in defense against viral and bacterial pathogens [22], which suggests that grouper Mpeg1s may also be critical for immune responses. The neighbor-joining phylogenetic tree divided the Mpeg1 sequences into three branches, and Mpeg1s from bony fish together with EcMpeg1s clustered into a single branch. EcMpeg1a and EcMpeg1b had the highest identity with *L. calcarifer* (81–84%) and showed 48–50% identity with mammals and 34–36% identity with invertebrates. The presence of the conserved domains in these two Mpeg1 proteins as well as results of phylogenetic and sequence identity analyses indicate their evolutionary homology with Mpeg1 of other species. Polygenic phenomena have also been found in other species. For example, three Mpeg1 transcripts were found in zebrafish, although one was shown to be a pseudogene [14]. Both Mpeg1 and Mpeg1.2 of zebrafish are transmembrane forms, but they exhibit differential regulation during zebrafish embryo infection with bacterial pathogens. In addition, two Mpeg1 sequences were found in the caenogastropod *Littorina littorea*; one lacked the transmembrane helix, and the sensitivity of the two differed after infection with pathogens. In this study, we found two isoforms of Mpeg1s in grouper, and both contained a conserved MACPF domain. However, they revealed different effects on antiparasitic and antibacterial activities in-vitro.

To investigate the possible physiological functions of Mpeg1 in grouper, we studied the mRNA levels in different tissues. EcMpeg1a and EcMpeg1b had the same expression pattern; they were expressed in all examined tissues, and the highest expression was detected in the immune system organs (head kidney followed by spleen). The most important immune organs of fish are the head kidney, spleen, and thymus [23,24], as they are the main sites for the development, differentiation, maturation, settlement, and proliferation of immune cells and the generation of immune responses. The high expression of EcMpeg1a and EcMpeg1b in the immune system organs indicates that they are involved in protecting organisms from environmental stressors and disease.

We also explored the expression pattern of EcMpeg1s in grouper tissues challenged with *C. irritans* infection. After infection, both EcMpeg1a and EcMpeg1b were significantly upregulated and peaked at day 1. At day 3 the expression of EcMpeg1a was still significantly increased, but that of EcMpeg1b was not. The upregulation of EcMpeg1s after *C. irritans* challenge suggests that that may play a role in the process of parasite elimination. Upregulation of Mpeg1 expression also has been reported in small abalone (*Haliotis diversicolor supertexta*) [25] and *L. littorea* [26] after infection with bacteria. Mpeg1 is a macrophage-specific marker, and the increased expression of EcMpeg1s may indicate the migration of macrophages to the immune tissues. However, this premise requires further research.

After determining that EcMpeg1 expression was upregulated by exposure to *C. irritans*, we next tested whether the EcMpeg1s had direct antiparasitic activities. Recombinant EcMpeg1a inhibited the activity of theronts; the theronts stopped moving, became round, and died. However, rEcMpeg1b had no antiparasitic activity.

Some reports have indicated that Mpeg1 has antibacterial activities. For example, in sponges, the Mpeg1 molecule acts as an executive protein and is involved in the elimination of Gram-negative bacteria [27]. The Mpeg1 of *C. gigas* [13] exhibited significant antibacterial activities against both Gram-negative and -positive bacteria. These results suggest that Mpeg1 may have different antibacterial activities in different species. In our study, both EcMpeg1s had antibacterial activity but were effective over different bacterial spectra. rEcMpeg1a exhibited antibacterial activity against both Gram-negative and -positive bacteria, whereas rEcMpeg1b only had an inhibitory effect against Gram-positive bacteria. For Gram-negative bacteria, however, EcMpeg1a had stronger antimicrobial activity than EcMpeg1b (Fig. 7). EcMpeg1b may retain part of its perforin function, but it may play a more important role in immune regulation or simply serve as a macrophage-specific marker.

In conclusion, two Mpeg1-like molecules were identified from orange-spotted grouper. They had a conserved structure and were distributed in all examined tissues of healthy grouper. Furthermore, the expression of EcMpeg1a and EcMpeg1b was upregulated after *C. irritans* infection. EcMpeg1a had antiparasitic and antibacterial activities; EcMpeg1b had an inhibitory effect only against Gram-positive bacteria.

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