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Molecular characterization, expression and antimicrobial activity of complement factor D in *Megalobrama amblycephala*

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ABSTRACT

Complement factor D (*Df*) is a serine protease, which can activate the alternative pathway by cleaving complement factor B, and involves in the innate defense against pathogens infection in teleost. In this study, we cloned, characterized the *Df* gene from blunt snout bream (*Megalobrama amblycephala*) (*Mamdf*), and examined its expression pattern and antimicrobial activity. The open reading frame (ORF) of *Mamdf* was 753 bp, encoding 250 amino acids with a molecular mass of 27.2 kDa. Mamdf consisted of a single serine protease trypsin superfamily domain, 3 substrate binding sites and 3 active sites, but no potential *N*-glycosylation site. Pairwise alignment showed that Mamdf shared the highest identity (94%) with grass carp (*Ctenopharyngodon idellus*). Phylogenetic analysis indicated that *Mamdf* and other vertebrate *Df* had a common ancestral origin. *Mamdf* structured with 4 introns and 5 exons. The *Mamdf* mRNA expressed relatively high at the intestine appearance stage during early development and constitutively expressed in various tissues with the highest expression in the kidney in healthy adults. After challenged with *Aeromonas hydrophila*, significant changes of *Mamdf* at both mRNA and protein levels in the kidney, spleen, liver and head-kidney were observed. The recombinant Mamdf protein showed antimicrobial activity against both gram-positive bacteria and gram-negative bacteria. The above results suggested the immune function of *Mamdf*, and would benefit further detailed *Df* function research in the immune process in teleost.

1. Introduction

The complement system is a central component of innate immunity and plays a crucial role in inflammation, phagocytosis, antibody production, clearance of foreign cells and molecules and killing of susceptible cells [1–3]. The complement system is composed of nearly 60 soluble and surface-bound proteins [4,5], and activated by one or a combination of 3 pathways, the classical, the alternative, and the lectin pathway [6]. It has been reported that the alternative pathway was established firstly during the evolution of the complement system of vertebrates, followed by the classical pathway [7–9].

In the alternative pathway of complement activation, the enzymatic reaction leading to the formation of the C3 convertase is catalyzed by the serine protease complement factor D (Df), which is a member of the trypsin family [10,11]. The central molecule C3 is spontaneously converted to the fluid phase C3 (H_2O), which is unstable and splits into C3a and C3b. Factor B is another complement serine protease, which is the only known natural substrate of factor D and can be catalyzed to Ba and Bb by factor D at the single Arg-Lys bond. Then Bb and C3b form the C3 convertase (C3bBb) complex, which forms a positive feedback loop to generate more C3b, and is stabilized by a positive regulator, properdin (factor P). Subsequently, stable C3 convertase in the alternative complement cascade leads to a formation of the membrane attack complex (MAC), which induces the cell death [3,6,12]. Therefore Df is absolutely required for alternative pathway activation, because it's the only enzyme that able to catalyze C3bBb formation [13,14]. In addition, earlier studies have shown that Df is a differentiation dependent serine protease secreted by adipocytes, and its expression is deficient in obese and diabetic disease in mice [15–17]. Furthermore, recent studies show that Df plays a role in maintaining β cell function and obviously stimulates insulin secretion in mice [18].

Almost one-third of all the proteases can be classified as serine protease [19], and most serine proteases contain the typical serine protease trypsin superfamily domain, which have similar tertiary structure deciding the function [20]. Little alteration in the serine protease structure will lead to changes in function, and this is the reason why serine protease have various physiology and immunology functions involving in almost all life processes such as growth,

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Table 1
Primers used in this study.

Primer name	Sequence (5'→3')	Usage
DF-ORF-F	CGGACATCAATCGTTCA	ORF amplification
DF-ORF-R	TTTTTTACTGGGTGGTT	ORF amplification
DF-28a-F	CCG**GAATTC**ATTACAGGAGGAAGTGAGGCT	Recombinant expression
DF-28a-R	CCG**CTCGAG**CTGGGTGGTTGTACTGTCAAT	Recombinant expression
DF-RT-PCR-F	GAGGAAGTGAGGCTGGTGC	qRT-PCR
DF-RT-PCR-R	AAATCAGGATGGCTGTAGACG	qRT-PCR
18S rRNA-F	CGGAGGTTCGAAGACGATCA	qRT-PCR
18S rRNA-R	GGGTCGGCATCGTTTACG	qRT-PCR

development, and metabolic [21]. Df is also a kind of serine protease, but there are few researches on its function.

The complement system is present in both vertebrates and a wide range of invertebrate species, emphasizing its importance in the innate immunity [22]. However, only few studies have been conducted on the function and expression pattern of the *Df* gene in teleost. The effects of *Df* on follicle contraction in brook trout (*Salvelinus fontinalis*) [23], the molecular and functional properties of carp (*Cyprinus carpio*) *Df* [24], Df/adipsin and kallikrein-like serine protease from the olive flounder (*Paralichthys olivaceus*) [25], the molecular characterization and expression analysis of *Df* in channel catfish (*Ictalurus punctatus*) [26], and the function and immune response of *Df* in rock bream (*Oplegnathus fasciatus*) have been reported previously [27]. Besides, antimicrobial function of other complement components has been proven in a minority of invertebrates, such as horseshoe crabs (*Tachypleus tridentatus*) [28,29], sea urchin (*Echinoidea*) [30], ascidians (*Pyrosomella verticilliata*) [31] and also in vertebrate tongue sole (*Cynoglossus semilaevis*) [32]. But these studies are very limited compared to those of mammals, and further study about the potential role that Df played in the fish innate immunity system are required.

Blunt snout bream (*Megalobrama amblycephala*) belongs to *Megalobrama*, Cyprinidae, and has been widely cultured in China as an important commercial fish in the last few decades. With the increase of enormous production, bacterial sepsis caused by *Aeromonas hydrophila* has become one of the most serious negative factors, causing serious economic losses [33–35]. In the present study, we aimed to characterize the *M. amblycephala Df* gene (*Mamdf*), determine its expression pattern during early development, in healthy tissues and after *A. hydrophila* infection, and explore the bacteriostatic functions of the recombinant Mamdf, in order to gain a better understanding of the role of *Mamdf* played in the innate immune system.

2. Materials and methods

2.1. Fish collection and bacterial challenge

Healthy blunt snout bream were obtained from the Tuanfeng breeding base of Huazhong Agricultural University (Hubei, China). The fish were acclimatized at 25 ± 1 °C for 2 weeks, feeding with a commercial pellet diet before experiment. Ten tissue samples including the liver, spleen, kidney, head-kidney, heart, gill, muscle, blood, brain and intestine were collected from 6 adult blunt snout bream (weight 500 ± 10 g), respectively.

Fertilized eggs by artificial insemination from 3 parental blunt snout bream were cultured at 25 ± 1 °C with stable water flow. By microscopic observation (Supplemental Fig.1), 10 developmental stages including fertilized egg, blastula stage, body segment appearance stage, hatching, eyeball pigment stage, air bladder formation stage, intestine appearance stage, 1, 7 and 10 dph (days post-hatching) were collected for expression analysis of *Mamdf* during early development.

Bacterial challenges were performed as described by Zhou et al. [34]. Briefly, 300 healthy juvenile blunt snout bream (30 ± 5 g) were randomly divided into control and challenge group, and injected intraperitoneally with 100 μL A. *hydrophila* (1.0×10^7 CFU/mL) in the challenge group and with 100 μL PBS in the control. Four tissue samples including the liver, spleen, kidney and head-kidney from a total of 3 pools of 7 individuals each were collected separately from each group at 0 h, 4 h, 24 h, 3 d and 5 d post infection.

The fish were anesthetized with 100 mg/L MS-222 before sampling and dissected on ice. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C for further analysis.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The quality and integrity of total RNA were determined by using the 1.0% agarose gel and NanoDrop 2000 (Thermo Scientific, Delaware, USA), respectively. The first-strand cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) following the manufacturer's protocol, and then stored at −20 °C for next use.

2.3. Sequence analysis

The genomic DNA sequence of *Mamdf* was obtained from the blunt snout bream genome database [46], using tBLASTn and the zebrafish *Df* mRNA sequences as subject, then the ORF sequence of *Mamdf* was identified by molecular cloning methods (Primers listed in Table 1).

All other sequences used in this study were obtained from GenBank (https://www.ncbi.nlm.nih.gov/). The amino acid sequences of Mamdf were deduced by the Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and the characteristic domain was predicted using the conserved domains search program (https://www.ncbi.nlm.nih.gov/cdd/) at NCBI. The physicochemical parameters of Mamdf were calculated using Protparam (https://web.expasy.org/protparam/). Sequence similarities were identified by the Blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments of the amino acid sequence of Mamdf with those of other species were performed using DNAMAN software. The phylogenetic tree was constructed based on the amino acid sequences using the neighbor-joining (NJ) method in MEGA 6.06. The signal peptide was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and the *N*-glycosylation sites were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The secondary and tertiary structure of the predicted Mamdf protein was predicted by Phyr2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.4. Quantitative real-time PCR (qRT-PCR) analysis

The expression patterns of the *Mamdf* mRNA during early

development, in healthy tissues and post *A. hydrophila* infection were analyzed using qRT-PCR. The qRT-PCR was conducted on a 7300 RT-PCR system (Applied Biosystems, USA), and the final volume of the reaction mixture was 20 μL, with 1 μL cDNA template, 10 μL SYBR Green master mix (TaKaRa, China), 0.8 μL of each primer (10 mM) and 7.4 μL nuclease-free water. The qRT-PCR cycling was 95 °C for 30 s, followed by 40 repeat cycles at 95 °C for 5 s, 60 °C for 20 s, 72 °C for 20 s, and the melting curve temperature ranged from 55 to 99 °C. For each cDNA sample, reactions were performed in triplicate. The relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method [47]. The *18S rRNA* gene was used as an internal reference. All primers were designed using the Primer Premier 5.0 software and listed in Table 1.

2.5. Production and purification of the recombinant Mamdf protein

In order to construct the recombinant plasmid, sequences encoding the mature Df peptide was amplified by PCR with primers listed in Table 1. The PCR products were digested with *Eco*RIand *Xho*Iand cloned into the pET28a expression vector. The recombinant plasmid was transformed into the *Escherichia coli* Rosetta (DE3) competent cells, and then cultured in LB broth containing kan^+ (50 μg/mL) at 37 °C to mid-log phase, the expression of recombinant protein was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) with a final concentration of 0.05 mM. After further incubation at 37 °C for 6 h, the bacterial cell were harvested by centrifugation at 6000*g* at 4 °C for 20 min, re-suspended in buffer I (pH 8.0) consisting of 20 mM Tris-HCl and 500 mM NaCl, and then sonicated on ice. After centrifugation at 12000*g* at 4 °C for 20 min, the pellets collected were re-suspended in buffer II (pH 8.0) consisting of 20 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA and 0.5% TritonX-100, and then centrifuged at 12000 g at 4 °C for 20 min. The pellets were washed with buffer III (pH 8.0) consisting of 20 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA and 1 M urea, and then with buffer IV (pH 8.0) consisting of 20 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA and 2 M urea. Subsequently, the pellets were solubilized with buffer V (pH 8.0) consisting of 20 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA and 8 M urea. After centrifugation, the recombinant Mamdf protein was purified using Ni-Agarose His-tagged protein purification Kit (CoWin Biosciences) according to the manufacturer's instructions and samples were subjected to 12% SDS-PAGE along with protein marker. Then the purified recombinant protein was dialyzed for further experiment.

2.6. Bacteriostatic activity analysis of the recombinant Mamdf protein

The storage concentration of the recombinant Mamdf protein was 1.0 mg/mL. The gram-negative bacteria (*A. hydrophila* and *E. coli*) and gram-positive bacteria (*Staphylococcus aureus*) from the department of aquatic animal medicine, HZAU were identified using molecular identification method. A single colony of each kind of bacteria was isolated and cultured in LB broth at 37 °C. When the OD_{600} of the culture reached 0.8–1.0 (approximately 1–2 × 10^8 CFU/mL), it was diluted 100 times to obtain a bacteria solution of 1–2 × 10^6 CFU/mL. The recombinant Mamdf protein was added to the medium with a final concentration of 500 μg/mL, cultured at 180 rpm at 37 °C and the absorbance was measured at 600 nm at different time points by using NanoDrop 2000. Set ampicillin or kanamycin as the positive control and phosphate buffer solution (PBS) as the negative control (three parallels in each group).

2.7. Western blotting analysis

The rabbit polyclonal antibody against Mamdf was prepared using the purified recombinant Mamdf protein by ABclonal technology (Wuhan, China).

Western blotting analysis was performed as described by Ding et al. [33]. Briefly, 30 μg sample proteins were subjected to 12% SDS-PAGE gel and then transferred onto polyvinylidene difluoride (PVDF) membranes using Trans-Blot (BioRad, Berkeley, CA, USA) for 45 min at 200 mA, then the PVDF membranes was blocked with 5% (w/v) skim milk power in TBST (20 mM Tris-base, 150 mM Nacl, 0.08% Tween-20, pH 7.4) for 2 h at room temperature, and after that the membrane was washed for 3 times (5 min each) in TBST. The membranes were then incubated with anti-Mamdf polyclonal antibody (1:1000) and anti-β-actin antibody (Biodragon, Beijing, China; 1:2000 dilution) overnight at 4 °C, respectively. Then the membrane were incubated with IRDye 800 CW goat anti-rabbit IgG (H + L) (LICOR, Boca Raton, FL, USA; 1:10000 dilution) for 1 h at room temperature, with 3 times 10-min washes after each incubation. The membrane was finally scanned using the Odyssey CLx Infrared Imaging System (LICOR).

2.8. Statistical analysis

All data in this study were presented as mean ± SE. Statistical significance was assessed by one-way analysis of variance (one-way ANOVA) using SPSS 16.0. Multiple comparisons were presented using Duncan's multiple-range tests. Significant difference was defined at $P < 0.05$, and extremely significant difference was defined at $P < 0.01$.

3. Results

3.1. Molecular characterization of Mamdf

Based on sequence comparison with the blunt snout bream genome by local BLAST analysis, we identified a putative *Mamdf* ORF of 753 bp (Supplemental Fig.2). The deduced Mamdf protein contained 250 amino acid residues with a calculated molecular weight of 27.2 kDa, a signal peptide of 20 amino acid (1–20), and a predicted theoretical isoelectric point (*pI*) of 6.65. The aliphatic index was 71.04, and the grand average of hydropathicity (GRAVY) was −0.342. The instability index (II) was computed to be 39.65, indicating that the Mamdf protein was stable. In addition, no potential *N*-glycosylation site was observed. The Mamdf secondary structure included 4 alpha helix (13%), 16 beta turn (40%) and one TM helix (6%) (Supplemental Fig.3). As shown in Supplemental Fig.4, the predicted Mamdf protein tertiary structure was similar to that of the prophenoloxidase activating factor.

3.2. Sequence alignment and homology analysis

The result of pairwise alignment (Supplemental Table 1) showed that Df was highly conserved in both fish and mammals. The amino acids sequence of Mamdf shared the highest identity (94%) with Df of grass carp, followed by Df of zebrafish (*Danio rerio*) (84%) and channel catfish (68%), and the lowest identities with Df of rainbow trout (*Oncorhynchus mykiss*) (42%) and Atlantic salmon (*Salmo salar*) (38%).

ClustalW multiple sequence alignment analysis (Fig. 1) showed that the serine protease trypsin superfamily domain was strictly conserved, and all the 3 substrate binding sites and 3 active sites were entirely conserved among all the sequences analyzed, suggesting their importance among different species.

Phylogenetic analysis (Fig. 2) was constructed based on Df amino acids sequences from 18 taxa by using the neighbor-joining method in MEGA 6.06. As shown in Fig. 2, there were 2 major distinct clades, fish and other vertebrate. Nevertheless, rainbow trout and Atlantic salmon were divided into the clade of other vertebrate, suggesting that rainbow

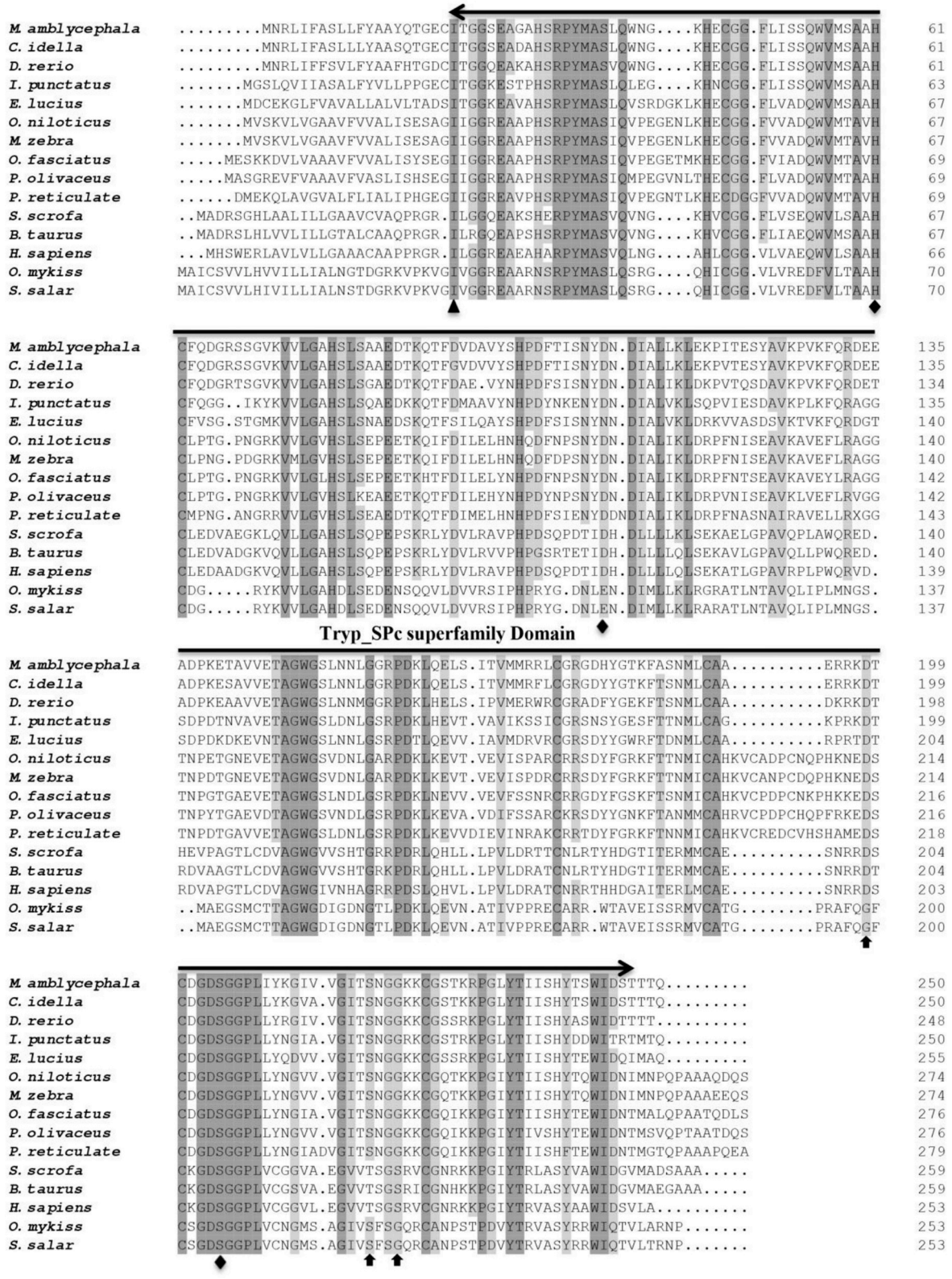

Fig. 1. Multiple sequence alignment of complement factor D from blunt snout bream and other fish. Gaps are indicated by dots. Identical and similar residues are shaded in gray and light gray, respectively. The serine protease trypsin superfamily domain is marked with a black double-headed arrow. The cleavage site is marked with a black triangle. Active sites are marked with a black rhombus, and substrate binding sites are marked by a downward arrow.

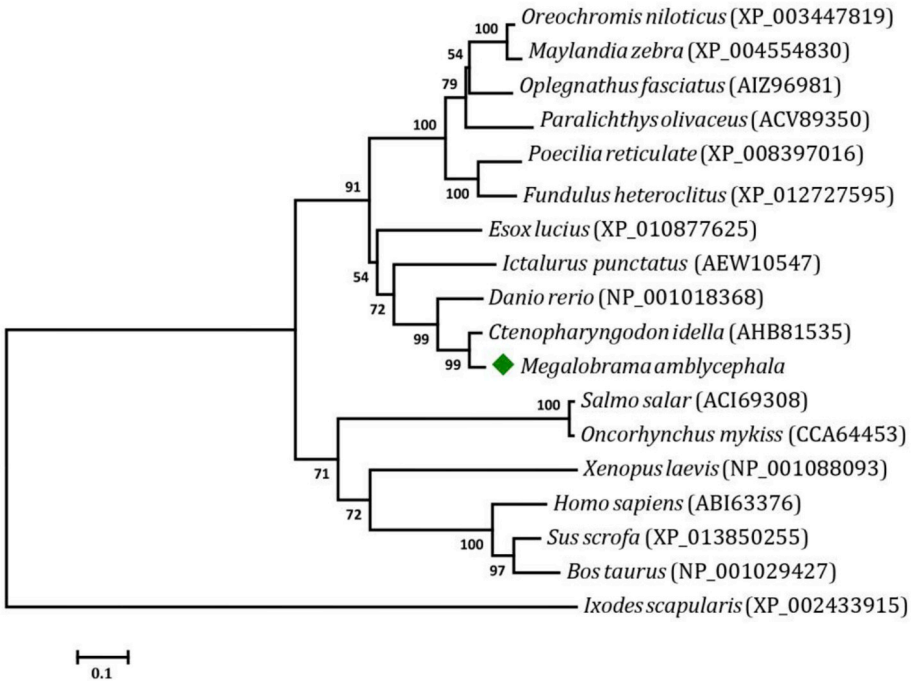

Fig. 2. Phylogenetic analysis of Mamdf and other vertebrates complement factor D. The tree was generated using amino acid multiple alignments and the neighbor-joining method within the MEGA6.06 program. Numbers at tree nodes indicate percent bootstrap confidence values derived from 1000 replications. The scale bar is 0.1, which refers to percentage of divergence. The GenBank accession numbers are shown within brackets next to each species.

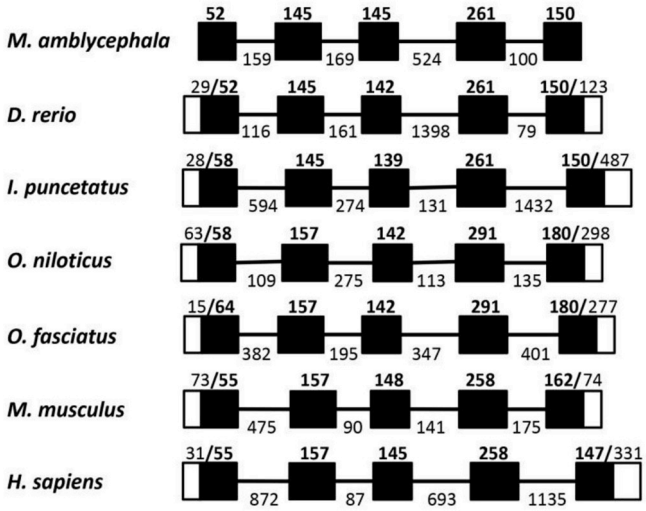

Fig. 3. Comparison of the gene organization and intron/exon sizes between *Mamdf* and the selected vertebrate complement factor D. Numbers in bold and black boxes indicate exons, normal number and lines joining the boxes indicate introns, and the 5′-and 3′- untranslated regions are denoted with empty boxes and normal number. The gene IDs of the complement factor D are as follow: *D. rerio*: 553553, *I. puncetatus*: 100862744, *O. niloticus*: 100692083, *O. fasciatus*: 100692083, *M. musculus*: 11537, *H. sapiens*: 1675.

trout and Atlantic salmon were more evolved teleost. In addition, among the fish clade, Mamdf was firstly clustered with the grass carp Df, indicating the closest relationship between them.

3.3. Gene structure of Mamdf

Firstly, we obtained the genomic DNA sequence and mRNA sequence of the *Df* gene from other vertebrates in GenBank. Then the genomic structure of *Df* was determined by aligning its mRNA sequence with the genomic sequence. The results showed that *Mamdf* was divided into 5 exons and 4 introns (Fig. 3), with the 5′- and 3′- ends of all introns confirmed to the AG-GT rule for splicing. The numbers of *Df* exons and introns were identical in all the species considered in the analysis. In addition, blunt snout bream, zebrafish, and channel catfish were similar in exon length, tilapia (*Oreochromis niloticus*) and rock bream were similar in exon length, and mouse (*Mus musculus*) and human (*Homo sapiens*) were similar in exon length too. But all the introns length was dramatically different.

3.4. Expression of Mamdf during early development and in different adult tissues

The *Mamdf* mRNA expression profiles during early development and in different adult tissues were analyzed by qRT-PCR with gene specific primers. As shown in Fig. 4A, the *Mamdf* mRNA remained at a very low level before hatching and increased to the highest level at intestine appearance stage, then decreased to a low level. In healthy adult blunt snout bream, the expression of *Mamdf* was detected in all the 10 tissues tested, including the liver, spleen, kidney, head-kidney, brain, gill, muscle, heart, intestine and blood, but the expression were distinctly different among various tissues. As shown in Fig. 4B, the highest level of the *Mamdf* transcript was detected in the kidney, moderate level were detected in the brain, gill, muscle and spleen, and low expression were detected in the intestine and blood.

3.5. Expression of Mamdf post bacterial infection

After challenged with *A. hydrophila*, the *Mamdf* expression showed different changes in various tissues including the liver, spleen, kidney and head-kidney. As shown in Fig. 5, the *Mamdf* mRNA was up-regulated in all the 4 tissues tested. In the liver, the expression level was initially up-regulated about 3.7 fold at 4 h, and then decreased to normal level at 5 d. In the spleen, the expression firstly decreased at 4 h, then increased to about 2.7 fold at 24 h, and remained at a high level at 3 d and 5 d. In the kidney, the *Mamdf* was rapidly up-regulated about 16 fold at 4 h, and then decreased at 24 h. In the head-kidney, the expression level showed a distinct rising at 4 h, and recovered to a low level at 5 d.

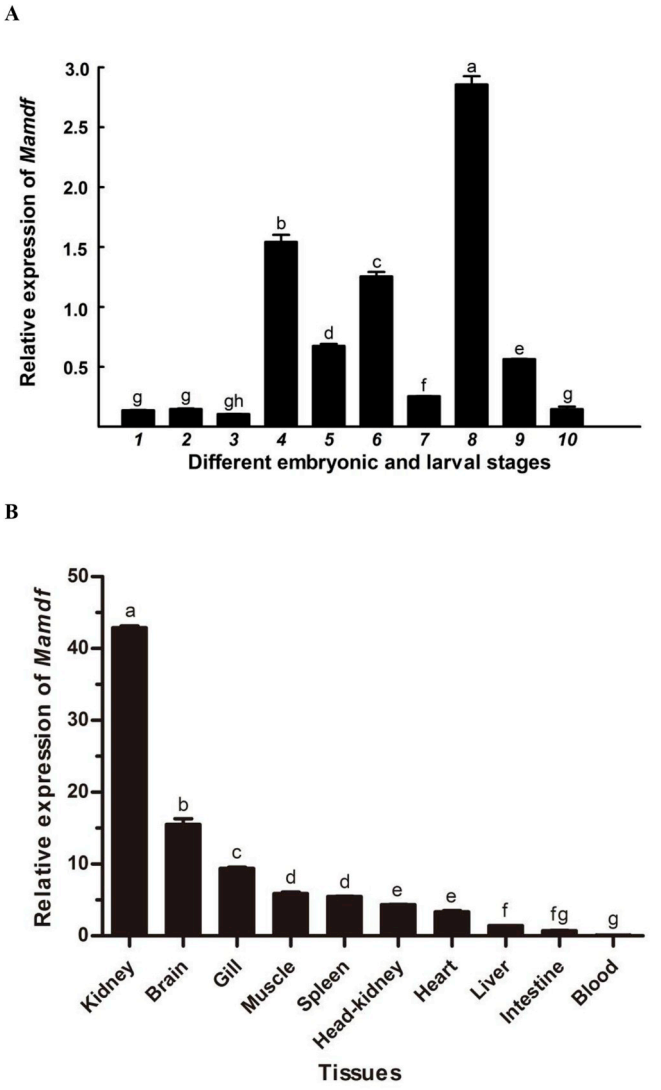

Fig. 4. Relative expression of the *Mamdf* mRNA by qRT-PCR during early developmental stages (A) and in different tissues of healthy adult blunt snout bream (B). The *18S rRNA* was used as the internal reference. Different letters above the pillars mean a significant difference ($P < 0.05$). The bars represent the standard deviation (n = 3). 1: fertilized egg; 2: blastula stage; 3: body segment appearance stage; 4: hatching; 5: eyeball pigment stage; 6: 1 dph; 7: air bladder formation stage; 8: intestine appearance stage; 9: 7 dph; 10: 10 dph. dph: days post hatching.

We also detected expression of the Mamdf protein in the liver, spleen, kidney and head-kidney after bacterial infection by Western blotting. As shown in Fig. 5, the Mamdf bands at approximately 27.2 kDa were observed in the liver, spleen, kidney and head-kidney. The change trend was similar to that at the mRNA level.

3.6. Expression, purification and bacteriostatic activity of the recombinant Mamdf protein

Expression of the recombinant Mamdf protein was induced with IPTG for 6 h at 37 °C in the *E. coli* (DE3), and detected by SDS-PAGE. The predicted weight of the recombinant Mamdf was 29.9 kDa including a tag of about 5 kDa. As shown in Fig. 6, the detected recombinant protein band was consistent with the predicted molecular weight. Compared to the empty vector and before induction, the recombinant Mamdf showed higher expression and mainly expressed in the pellet. The recombinant Mamdf was then purified using affinity chromatography.

To examine whether the recombinant Mamdf possesses the ability of bacteriostatic activity, we performed the bacteriostatic assay with gram-negative bacteria (*A. hydrophila* and *E. coli*) and gram-positive bacteria (*S. aureus*). As shown in Fig. 7, the recombinant protein was active against the 3 kinds of bacteria. The growth of *A. hydrophila* and *E. coli* was slightly suppressed at 2 h and the growth of *S. aureus* was inhibited at 4 h, respectively. After 4 h, the recombinant Mamdf showed significant bacteriostatic effects compared with the control.

4. Discussion

In the present study, we identified and characterized the *Df* gene in *M. amblycephala*. The *Mamdf* ORF was 753 bp, encoding 250 amino acids, with a predicted molecular weight of 27.2 kDa, similar to Df of channel catfish [26], olive flounder [25] and rock bream [27]. Domain analysis indicated that Mamdf consists of a single serine protease trypsin superfamily domain, 3 substrate binding sites and 3 active sites, which are entirely conserved among vertebrate, suggesting their importance for gene function. *N*-Linked protein glycosylation is a common posttranslational protein modification in eukaryotes involved in many biological processes [36]. But no potential *N*-glycosylation site was observed in Mamdf, similar to Df of grass carp, zebrafish, porcine [37] and human [38], but different from the channel catfish Df with 2 *N*-glycosylation sites and olive flounder Df with 3 *N*-glycosylation sites [25,26], indicating that Df may possess different functions in different species. The Mamdf secondary structure included 4 alpha helix and 16 beta turn, similar to the rock bream Df with 4 alpha helix and 14 beta turn [27]. It was determined in the present study that the *Mamdf* gene structured with 4 introns and 5 exons. Apparently, the teleost *Df* and mammal *Df* present a relatively stable gene structure, such as the number of introns and exons, and the length of exons, however, the length of introns are distinctly different. The tertiary structure of Mamdf is similar to the prophenoloxidase activating factor, and both of them belong to the family of serine protease, which mediate the proteolytic cascades of embryonic development and immune response in invertebrates [39], indicating that Mamdf may have more complex functions.

Phylogenetic analysis showed that there were 2 major clusters, fish and other vertebrate Df, which originated from the same root, indicating that these Df have a common ancestral origin. Interestingly, the rainbow trout and Atlantic salmon Df differed from fish Df but clustered with other vertebrates Df, suggesting a different evolution rate between them and other fish Df members, and similar clustering pattern was previously observed in a study of olive flounder Df [25]. It is suggested that Df of rainbow trout and Atlantic salmon were more evolved during the evolutionary process.

Few studies about the expression of *Df* during early developmental stages in teleost have been carried out. In the present study, the *Mamdf* mRNA was firstly detected at the fertilized egg stage, which might indicate that *Mamdf* was maternally inherited [52]. Similarly, the existence of immune related maternal mRNAs has also been reported previously in teleost. For example, maternal C3 mRNA is detected in unfertilized eggs and young embryos in carp [50], and mannose receptor (MR) is reported to express in the yolk sac at fertilized eggs stage implying its important role in the process of macrophage exercise function in blunt snout bream [51]. After hatching, larval fish will be exposed to the complicated water environment and they will not be protected by the egg membrane [53]. In order to adapt to changes in the environment, the body will produce a series of immune responses. The *Mamdf* mRNA was significantly up-regulated after hatching suggesting that it may involve in some immune-related activities. The

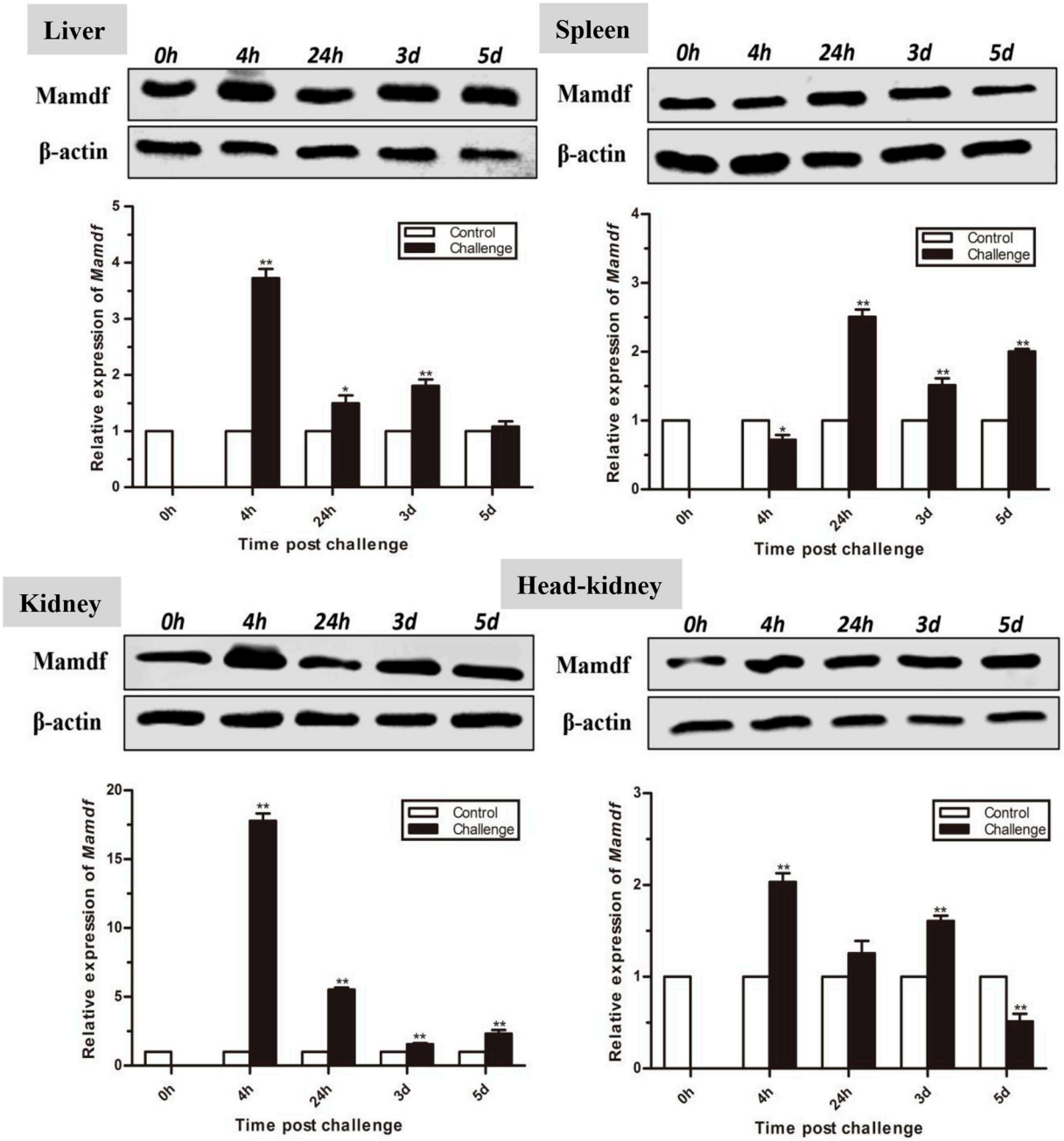

Fig. 5. Expression of the *M. amblycephala* complement factor D in the liver, spleen, kidney and head-kidney after *A. hydrophila* infection by Western blotting and qRT-PCR. Values of qRT-PCR were described as mean ± SE (n = 3 pools, with 7 fish per pool). The protein samples from 3 pools with a total of 21 fish was mixed and used for Western blotting analysis. Statistically significant up-regulation or down-regulation of gene expression are denoted with '*' ($P < 0.05$) and '**' ($P < 0.01$), respectively.

Mamdf mRNA peaked at the intestine appearance stage and declined gradually after that at 7–10 dph, indicating that the intestine execute main immunization function before the immune organs are formed [40]. These facts suggest that *Mamdf* plays an important role during early development, particularly in larval fish after hatching. Besides, the *Mamdf* mRNA was constitutively expressed in different tissues examined, with the highest expression appeared in the kidney. In previous studies, the expression patterns of *Df* in other fish are similar to that of *Mamdf*, expressed mainly in the immune-related tissues, such as the kidney, spleen, liver and gills [23,25–27], which may illustrate the vital role of *Df* played in the immune system in various fish species. Besides, previous studies show that the *Df* is highly expressed in the human [38] and porcine fat [37], suggesting that the adipose tissue plays a role in the biological immune system [41,42].

In this study, both the *Mamdf* mRNA and protein were distinctly up-regulated in the immune-related tissues after *A. hydrophila* challenge. In blunt snout bream in the present study, the *Mamdf* mRNA in the kidney (16 fold) and liver (3.7 fold) both peaked at 4 h. In olive flounder, after *Streptococcus iniae* infection, the *Df* mRNA in the kidney and liver peaked at 1 h (1.45 fold) and at 12 h (5.9 fold), respectively [25]. While in *Edwardsiella ictaluri* infected channel catfish, the *Df* mRNA peaked at 3 d (1.65 fold) and at 24 h (1.21 fold) in the kidney and liver, respectively [26]. As we know, the kidney and liver are major immune organs in teleost, the above expression patterns imply an important role of *Df* played in the innate immune system. In the spleen, after bacterial infection, the *Df* expression decreased at 4 h in blunt snout bream. Similarly, the *Df* expression decreased to some extent at some time points in other teleost, from 3 h to 6 h in olive flounder, from 3 d to 7 d in

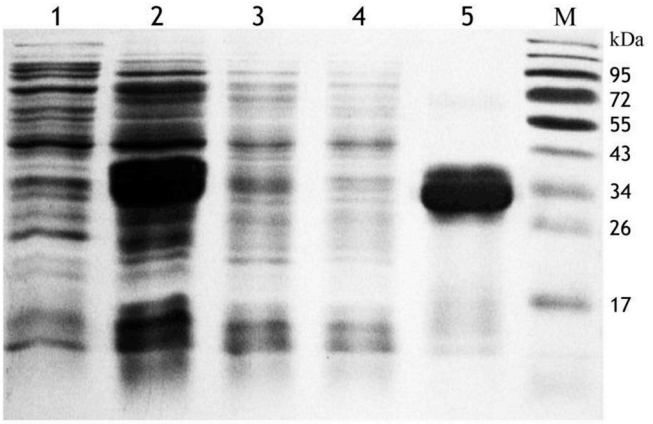

Fig. 6. Expression of the recombinant Mamdf protein in *E. coli* Rosetta (DE3) by SDS-PAGE. 1: supernatant after IPTG induction; 2: pellet after IPTG induction; 3: pellet prior to IPTG induction; 4: Empty vector pellet after IPTG induction; 5: purified protein elution; M: Protein marker.

channel catfish and at 3 h in rock bream [25–27]. In addition, *Mamdf* in the head-kidney was about 2-fold up-regulated at 4 h, but decreased at 5 d. The head-kidney is the major lymphoid and hematopoietic organ in adult fish and it has been proved that bacterial antigen results in the mobilisation of head-kidney leucocytes to inflamed sites in gilthead seabream [54], indicating its importance in immune response. Df is significantly up-regulated among various fish species, but the peaking time points and levels are not entirely the same, suggesting that the expression of *Df* is modulated by a complex mechanism. At the same time, why and how the *Df* is down-regulated at some time points in some tissues in some species remains unclear, which may partially due to the complex immune response of the body, depending on the host condition such as taxa, ages, sizes and healthy situation, the kinds, intensity of the stimulation and many other factors. It is a very interesting aspect that undoubtedly needs further study in more detail in the future.

Complement factors have been confirmed to have proteolytic activity and antimicrobial activity [24,27,32,43]. The recombinant Df protein has been proven to exhibit substantial proteolytic activity in rock bream and carp [24,27]. In addition, a previous report shows that C3b and C4b can covalently bind microbes and render bacteria susceptible to the killing of phagocytes [43], and another research shows that Ba probably inhibit replication of *Edwardsiella tarda*, *Pseudomonas fluorescens* and *Vibrio harveyi* [32]. However, there are few researches about whether the recombinant Df protein has antimicrobial activity. In the present study, the recombinant Mamdf protein showed antimicrobial activity against all tested microbes, and the inhibitory effect on gram-negative bacteria was greater than that against gram-positive bacteria. Based on the previous studies [32,43,48,49], we suppose that the Mamdf protein may function by dissolving bacterial cell wall or affecting cell membrane permeability to kill or inhibit replication of the bacteria. Previous studies show that the serine protease trypsin superfamily domain may partially reflect the functions of the whole Df protein [20,44,45]. Whether the domain of Mamdf plays an immune role in antimicrobial process require further study.

In conclusion, the *M. amblycephala Df* gene was obtained and characterized in the present study. Genome structure and phylogenetic analysis indicated that *Df* was conserved among vertebrates. The *Mamdf* mRNA was highest expressed in the kidney in healthy adults and at the hatching stage during early development. Additionally, *Mamdf* was significant induced at both mRNA and protein levels post infection of *A. hydrophila*, suggesting an important role it played in immune response against bacterial infection. Furthermore, bacteriostatic activity of the recombinant Mamdf protein was observed. However, details of the potential functional mechanisms of Df require further in-depth study.

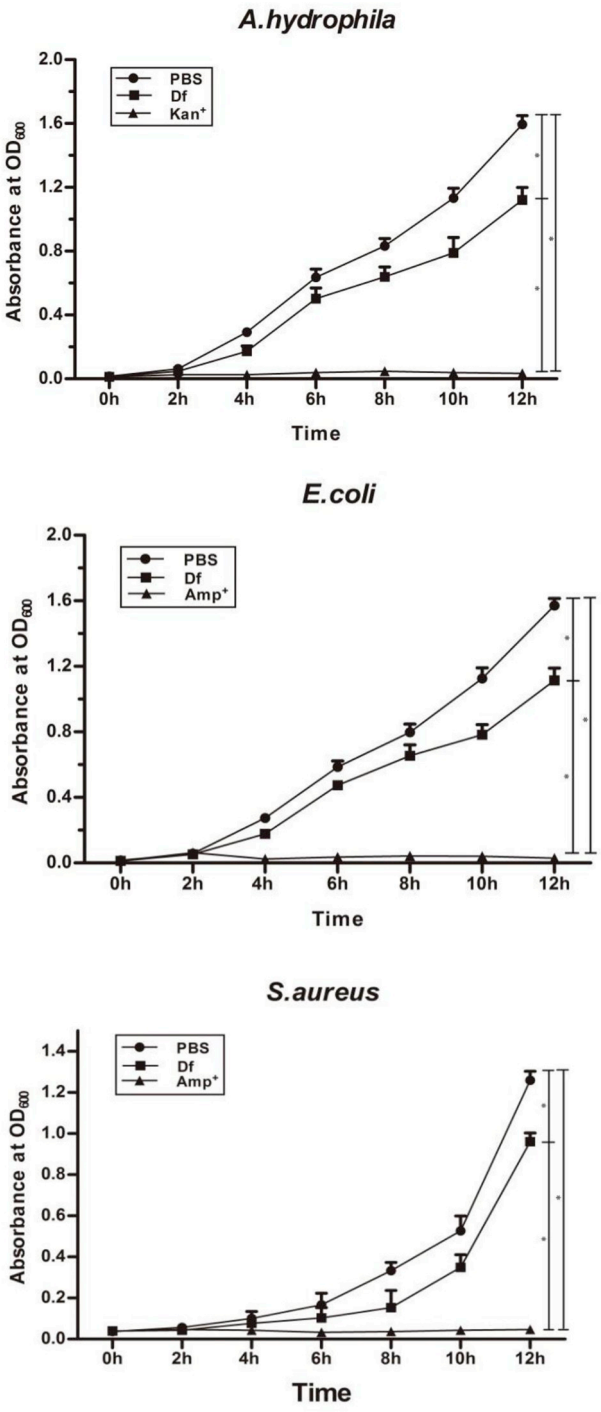

Fig. 7. The bacteriostatic activity of the recombinant *Mamdf* protein against gram-negative bacteria (*A. hydrophila* and *E. coli*) and gram-positive bacteria (*S. aureus*). Absorbance at OD_{600} is measured to determine the cell density of 3 kinds of bacteria at different time points. Error bars represent standard error (SE; n = 3). The asterisk indicates a statistical significance difference between every two treatments ($P < 0.05$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.031.

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