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Microfibrillar-associated protein 4 (MFAP4) genes in catfish play a novel role in innate immune responses

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ABSTRACT

The lectin pathway of the complement system is characterized by two groups of soluble pattern recognition molecules, mannose-binding lectins (MBLs) and ficolins. These molecules recognize and bind carbohydrates in pathogens and activate complement leading to opsonization, leukocyte activation, and direct pathogen killing. While MBLs have been reported in many fish species, ficolins do not appear to be present in the teleost lineage, despite their importance in invertebrate and higher vertebrate innate immunity. A protein with a similar fibrinogen-like domain, microfibrillar-associated protein 4, MFAP4, is present in fish, albeit with no described immune function. We examined whether MFAP4 genes in fish may potentially act as pathogen receptors in the absence of ficolin. We isolated and characterized five MFAP4 genes from channel catfish. Linkage mapping and phylogenetic analysis indicated that at least three of the catfish MFAP4 genes are tightly clustered on a single chromosome, suggesting that they may have arisen through tandem duplication. Divergent, duplicated families of MFAP4 genes are also present in other teleost species. Expression analysis of the catfish MFAP4 transcripts revealed unique patterns of homeostatic expression among the genes in gill, spleen, skin, liver, and muscle. Expression of the five MFAP4 transcripts showed significant changes in expression as soon as 4 h after infection with either *Edwardsiella ictaluri* or *Flavobacterium columnare* with modulation of expression continuing up to 7 d following pathogen exposure. Several different tissues and gene-specific patterns were captured and transcript expression changes of >30-fold were observed over the course of the bacterial challenges. Our results suggest a novel role for MFAP4 in teleost immune responses.

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1. Introduction

The ability of lectins to act against pathogens by aggregating and opsonizing them has been well-established in a host of vertebrate and invertebrate species, including teleost fish (Endo et al., 2006). Further potency is afforded a subset of lectins through their association with the complement system. In the lectin pathway of the complement system, mannose-binding lectins (MBLs) and ficolins serve as pathogen recognition molecules, activating the complement cascade that is at the heart of many innate immune strategies. While a diverse group of lectins, including MBLs, have been described and their pathogen responses well-characterized in teleost fish and invertebrates (Vasta et al., 1999; Nakao et al., 2006), relatively little research has been conducted on ficolins or other fibrinogen-related proteins (FREPs, also FREDs) in these groups.

FREPs are a family of glycoproteins that encode a fibrinogen-like (FBG) domain in the C-terminal end but differ in the N-terminal region (Romero et al., 2010). FREP members include fibrinogen/fibrin, ficolins, angiopoietin, tenascins, tachylectins, fibroleukin, FIBCD1, and microfibrillar-associated protein 4 (Thomsen et al., 2010). Ficolins, with their proven abilities to detect pathogens, enhance phagocytosis, and activate complement, are the best characterized molecules of the family. In spite of the importance of ficolins in mammals, these molecules do not appear to be present in teleost fish (Garred et al., 2010). Interestingly, ficolins have been reported from amphibians, birds (Kakinuma et al., 2003; Garred et al., 2010) and several invertebrate species (Kenjo et al., 2001), although phylogenetic relationships remain unclear. The apparent absence of ficolin genes in some species groups has caused some to examine other FBG-containing members for their ability to mediate similar functions. The tachylectins, a unique

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Table 1
Primers used for the study of the five catfish MFAP4 genes.

Gene	Amplicon sizes (bp)	Primer	Forward primer (5′–3′)	Reverse primer (5′–3′)
MFAP4-1	157	For qRT-PCR	TTGTATGTGCGGCTCCTGCCAC	TCACTGTCCATCCACGGGCTGA
	729	For Southern blot	GCATCTGCTCCCCATCACTT	TGGCCCACACGTTACCAATA
	160	For linkage map	GTTTTTTCCTGCCACATT	TTATTCCCAAAGCCATTC
MFAP4-2	143	For qRT-PCR	GTTGTGTGTGCTCCCCCTGCTG	CCTGGACAGGTGTGTCTGCTGT
	675	For Southern blot	GGCACCTTAGGACATGGT	CCGATGGCGTAATAAGTG
	210	For linkage map	TTCCTTTCTGTGATTGTGAC	TAATATCGCTGTTGTTGATG
MFAP4-3	145	For qRT-PCR	ATGCTGCTGCTGTTCGTAGCACTG	CAGACCCAGCAGGGAAGATGGTGT
	879	For Southern blot	GACGATAATACAGACGAAACAGT	AATGCTTTGTGGTTATACAGAGT
	168	For linkage map	GATTTTAACTGGACTTTGATGT	GCATTATTATTATTGTTTCAGC
MFAP4-4	185	For qRT-PCR	CTGGGACTTGAGACAATTCATC	GCACCACCATCTTCAAAATCAG
	750	For Southern blot	TATGACTCTGAAATCCCTGGTT	TAAAGCCTTTCCAAGTTCTCC
	176	For linkage map	CAAATTCAGGCACAGAGAC	GAAAGACAACATAGAGGCA
MFAP4-5	163	For qRT-PCR	AACACGTCCCTGCCGATGGACT	CATGGCCTCCGGTTTCCAAGCA
	995	For Southern blot	ACACGAGAATTAGCACAGAAAGA	ACAGAGAGGATATACCACAGGAA
	151	For linkage map	AGTGCGTGTCACTTAGCC	GATCTTCCAGACCCATCC

lectin group from horseshoe crab (*Tachypleus trimentatus*) with a C-terminal fibrinogen domain, were shown to bind and agglutinate a variety of bacterial types (Gokudan et al., 1999). Recent studies in a range of invertebrate species have revealed large clusters of FREPs with tremendous coding diversity and immune responsiveness after pathogen challenge (Middha and Wang, 2008; Romero et al., 2010). A similar examination of alternative FREPs has not been carried out in fish species.

In previous research from our group on the innate immune responses of channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) to a Gram-negative bacterium (Peatman et al., 2007, 2008), we observed strong upregulation of FREP family member microfibrillar-associated protein 4 (MFAP4) by microarray analysis. Little is known about MFAP4 function. After its original discovery in porcine aorta (Kobayashi et al., 1989), it was found to be one of several genes deleted in connection with Smith-Magenis Syndrome (Zhao et al., 1995). MFAP4 was purified from bovine lung washings and found to bind the collagen domain of surfactant protein D (SP-D) as well as mannan (Lausen et al., 1999), while human recombinant MFAP4 was reported to bind the collagen domain of surfactant protein A (SP-A), suggesting that MFAP4 may play a role in inflammatory processes of the mammalian lung (Schlosser et al., 2006). Limited reports of additional functions of MFAP4 in aortic structure and function suggest that it is likely a multifunctional protein with different roles in several organ systems (Toyoshima et al., 2005). Structurally, MFAP4 possesses a characteristic FBG domain at the C-terminal end. Additionally, MFAP4 has an integrin-binding motif with a single cysteine residue and an Arg-Gly-Asp (RGD) sequence located at the N-terminal region in mammals (Schlosser et al., 2006). The RGD sequence motif is often associated with cell adhesion activity and is known to be a ligand motif for cell surface integrins (Ruoslahti, 1996).

Given our previous observation of MFAP4 upregulation during bacterial infection and the potential of FREPs to serve as pattern recognition molecules in early innate immune responses, here we set out to characterize MFAP4 in catfish and to determine immune responsiveness to pathogen challenge. We isolated and characterized five unique MFAP4 cDNAs from channel catfish. Expression of the five MFAP4 genes showed significant changes in transcript expression as soon as 4 h after infection with either *Edwardsiella ictaluri* or *Flavobacterium columnare* with modulation of expression continuing up to 7 d following pathogen exposure. Several different tissue and gene-specific patterns were captured and transcript expression changes of >30-fold were observed over the course of the bacterial challenges. Our results represent the first functional characterization of MFAP4 genes in fish and suggest a novel role for MFAP4 in teleost immune responses.

2. Materials and methods

2.1. Identification and sequencing of MFAP4 cDNAs

Zebrafish MFAP4 (NP_998054) was used as a query to search cDNAs encoding MFAP4 from channel catfish expressed sequence tags (ESTs) using *tblastn*. Contigs were assembled using Vector NTI 10.0 (Invitrogen, Carlsbad, CA) to identify clones that potentially contain full open reading frames (ORFs). These clones were resequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols with modifications (Xu et al., 2006) on an ABI 3130XL automated DNA sequencer (Applied Biosystems). Assembly and comparison of existing transcripts with *de novo* sequences allowed high certainty about transcript identity and sequence accuracy.

2.2. Sequence analysis

The MFAP4 amino acid sequences were either identified by simple key-word searches; or with *blastp* searches using zebrafish MFAP4 amino acid sequences to query the NCBI non-redundant (nr) amino acid sequence database (http://www.ncbi.nlm.nih.gov/BLAST). Protein sequences retrieved from the public database were used for ORF and domain searches; alignment; and phylogenetic reconstruction.

ORFs were predicted using Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and signal peptides and fibrinogen-like domain (FBG) were identified by the NCBI conserved domain feature of *blastp* (http://www.ncbi.nlm.nih.gov/BLAST) and by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de).

2.3. Phylogenetic analysis

Sequences of MFAP4: microfibrillar-associated protein 4, FCN: ficolin, FB: fibrinogen, TNR: tenascin R, FIBCD1: fibrinogen c domain containing 1, ANGPT: angiopoietin of human, mouse, frog, zebrafish and ascidian (*Ciona intestinalis*) retrieved from databases were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). The complete list of species, gene names and accession numbers are listed in Supplementary Table 1. Phylogenetic trees were constructed using the neighbour-joining (NJ) method based on the deduced full-length amino acid sequences with 10,000 bootstrapping replications within the Molecular Evolutionary Genetics Analysis

Table 2
Characteristics of the catfish MFAP4 cDNAs.

Gene name	Accession no.	Open reading frame (AA)	Fibrinogen domain (AA)	Signal peptide (AA)
MFAP4-1	FD277724	246	216	22
MFAP4-2	FD290690	248	220	17
MFAP4-3	FD303964	255	199	11
MFAP4-4	FD344009	240	216	16
	FD344010			
MFAP4-5	FD309065	264	230	19

(MEGA 4.0) package (Tamura et al., 2007). Data were analyzed using Poisson's correction, and gaps were removed by complete deletion.

2.4. Mapping of MFAP4 genes on the linkage map

To locate the MFAP4 genes on the linkage map of catfish, we searched catfish genomic DNA contigs from our ongoing whole genome sequencing project using MFAP4 cDNA sequences as queries by *blastn*. Microsatellites were then identified from matching contigs. Microsatellite primers of five MFAP4 genes were designed based on microsatellite sequences (Table 1). Progeny of a specific backcross family, F1-2 × Ch-6 were used for constructing a linkage map (Kucuktas et al., 2009). A tailed primer protocol (Boutin-Ganache et al., 2001) was used to amplify microsatellite alleles. The PCR conditions used followed Kucuktas et al. (2009). Genotypes were called manually and PCR were repeated in cases where polymorphism was detected but could not be genotyped. Polymorphic markers were assigned to the linkage groups using JoinMap 4.0 (Kyazma B.V., Wageningen, Netherlands).

2.5. Determination of genomic copy number

To determine the genomic copy number of MFAP4 genes in channel catfish, Southern blot analysis was conducted as previously described (Liu et al., 2010). Briefly, blood genomic DNA was isolated from three individual adult channel catfish and 10 μg was digested with 30 U of the restriction endonuclease *Eco*RI, *Hin*dIII or *Pst*I with NEBuffer *Eco*RI, NEBuffer 2, NEBuffer 3, respectively (New England Biolabs, Beverly, MA) in a 25 μL reaction at 37 °C. The digested DNA samples were electrophoresed on a 0.8% agarose gel with 1× TE buffer by using a λ DNA marker. The gel was submerged in 0.25 N HCl for 15 min, then in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) and neutralization buffer (0.5 M Tris–HCl, 3.0 M NaCl, pH 7.0) for 30 min, respectively. The DNA was transferred to an Immobilon

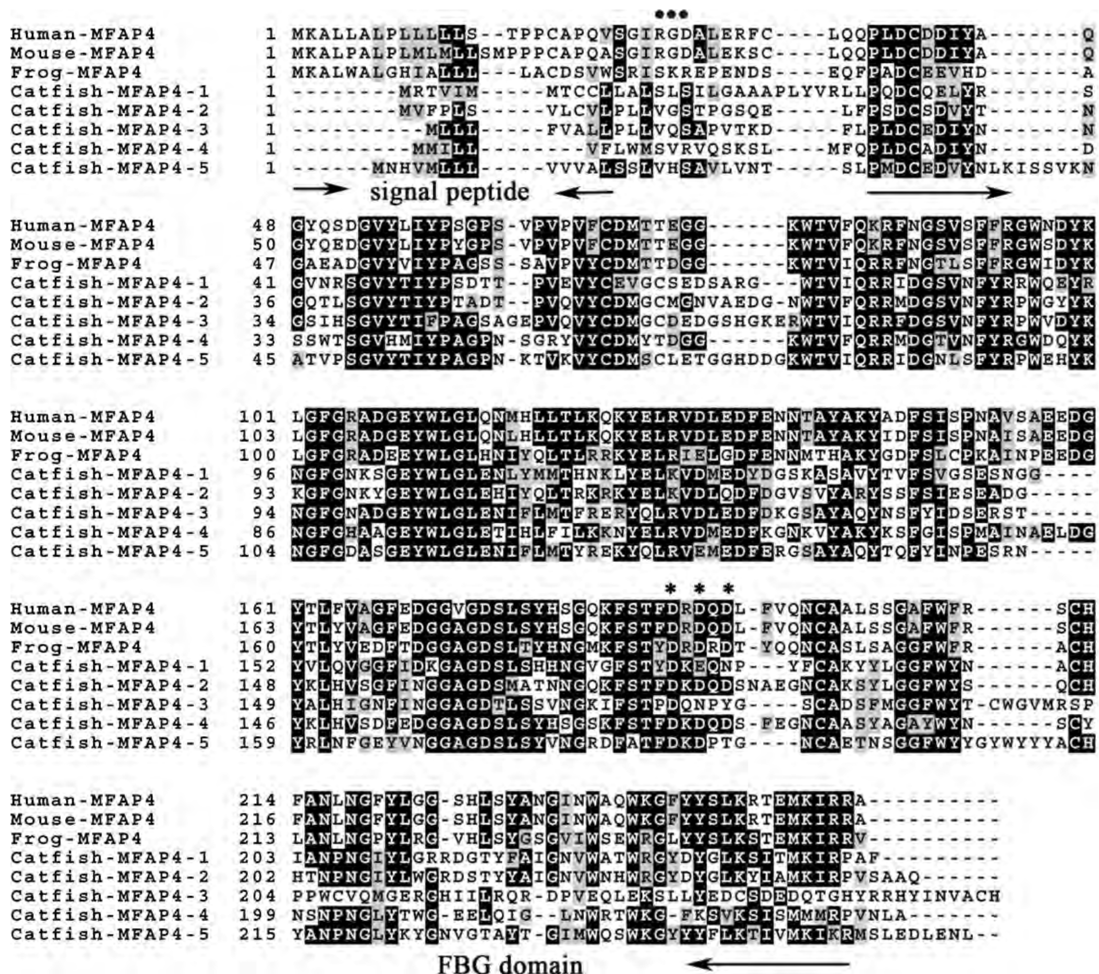


Fig. 1. Alignment of amino acid sequences of MFAP4 genes for human, mouse, frog and channel catfish. The conserved and identical residues are represented by black shading, and conservative substitutions are represented by grey shading. The region of the signal peptide and fibrinogen (FBG) domain, which were predicted by the SMART program, are indicted by arrows. The Ca^{2+}-binding sites of MFAP4 are indicated by stars. The RGD (Arg-Gly-Asp) sites for human and mouse are indicated by dark spots.

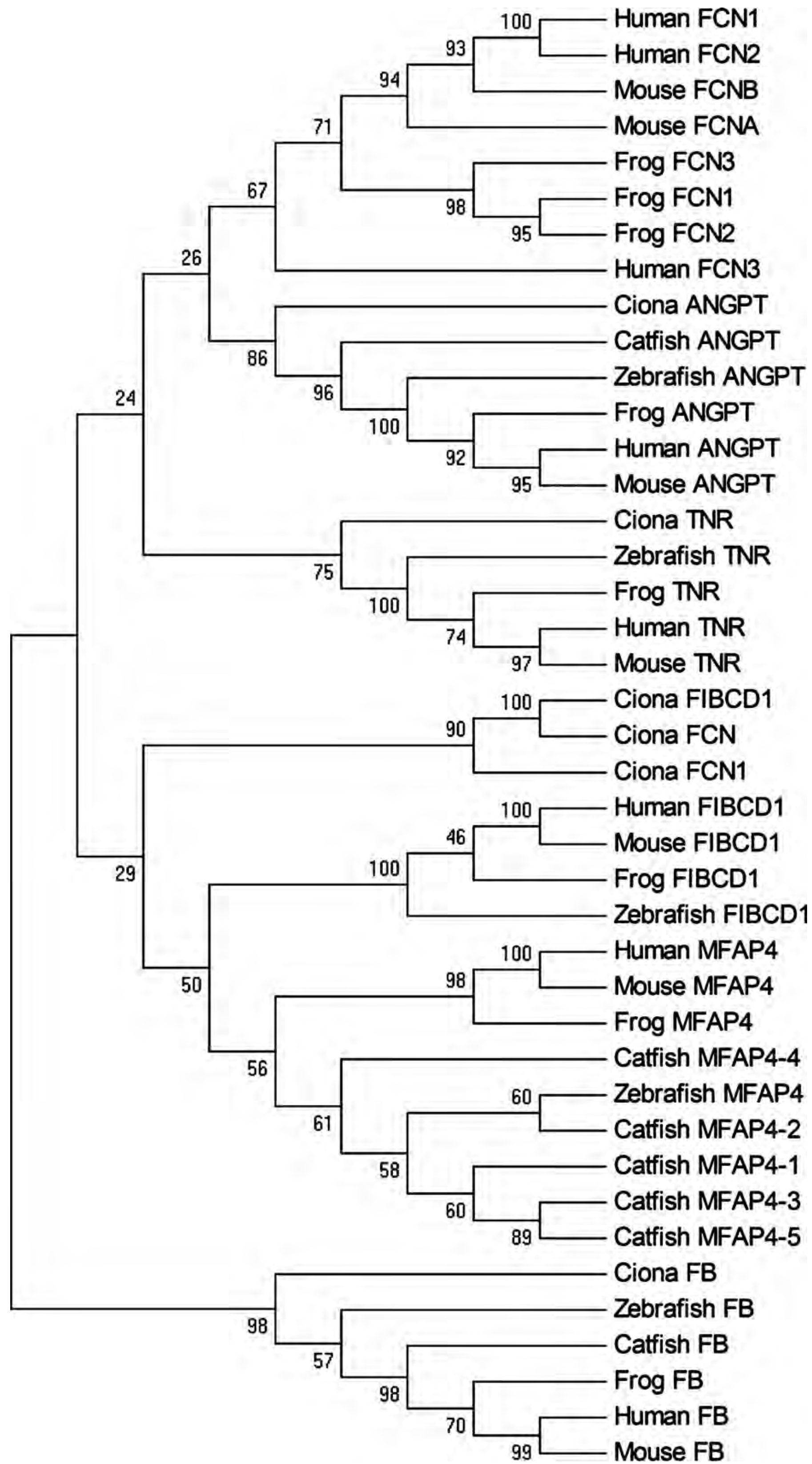


Fig. 2. Phylogenetic tree of microfibrillar-associated protein 4 (MFAP4), ficolin (FCN), fibrinogen (FB), tenascin R (TNR), fibrinogen c domain containing 1 (FIBCD1), angiopoietin (ANGPT). The phylogenetic tree was constructed based on ClustalW-generated multiple sequence alignment of amino acid sequences using the neighbour-joining method within the MEGA 4 package. The topological stability of the neighbour-joining trees was evaluated by 10,000 bootstrapping replications, and the bootstrapping percentage values are indicated by numbers at the nodes.

Table 3
Similarity of the five MFAP4 genes of channel catfish. Shading indicates greater levels of sequence similarities. Similarities below the diagonal were based on the entire coding region while similarities above the diagonal were based on the FBG domain.

	MFAP4-1	MFAP4-2	MFAP4-3	MFAP4-4	MFAP4-5
MFAP4-1		60	46	46	48
MFAP4-2	55		50	55	51
MFAP4-3	39	45		45	55
MFAP4-4	42	52	40		49
MFAP4-5	45	48	49	47	

positively charged nylon membrane (Millipore, Bedford, MA) by capillary transfer for 18 h using 20× SSC buffer. The DNA was fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA) and the auto-crosslink setting. The membrane was hybridized with cDNA probes amplified using primers listed in Table 1. After pre-hybridization for 2 h with 100 μg/ml salmon sperm DNA (Sigma) and hybridization with a ^{32}P-dCTP labeled probe at 63 °C for 16 h in a hybridization oven, the membrane was washed twice at 60 °C with wash buffer 1 (2× SSC, 0.1% SDS buffer) and one time with wash buffer 2 (0.5× SSC, 0.1% SDS buffer) at 60 °C and exposed to X-ray film overnight at −80 °C.

2.6. *E. ictaluri* and *F. columnare* challenges

All experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of Auburn University under PRN 2008-1386. In the first challenge, channel catfish, with an average body weight of 6.1 g and average body length of 9.5 cm, kept at 27 °C in a flow-through system utilizing heated, dechlorinated municipal water were used for the challenge. Fish were treated in each of two groups: (1) control group (phosphate-buffered saline, 100 μL PBS injected); (2) *E. ictaluri* challenged group (injection). To inoculate bacteria for the challenge, a single colony of *E. ictaluri* was isolated and cultured in BHI broth at 28 °C overnight. The bacterial culture was diluted with PBS (pH 7.4), and 1×10^5 CFU of bacteria in 100 μL PBS were injected intraperitoneally into the channel catfish. Injections were carried out under anesthesia using tricaine methanesulphonate (MS 222) at 100 mg L^{-1} using a 26 gauge needle.

In the second challenge, healthy catfish with a mean weight of 10.6 g were used. The fish were divided into two groups: (1) control group (without bacteria); (2) *F. columnare* challenge group (immersed with the dose of 3 ml of 10^7 CFU ml^{-1} of *F. columnare* for 1 h). To inoculate bacteria for the challenge, a single colony of *F. columnare* was isolated and cultured in BHI broth at 28 °C overnight. Fish used in this study were euthanized by MS 222 exposure at 300 mg L^{-1} before dissection. To determine MFAP4 gene expression in various healthy catfish tissues, samples of 11 tissues including brain, gill, heart, head kidney, liver, stomach, intestine, spleen, trunk kidney, skin and muscle from control channel catfish were isolated, pooled, and flash-frozen in liquid nitrogen. Tissues were homogenized under liquid nitrogen using a mortar and pestle, and stored at −80 °C until RNA extraction. Similarly, the spleen and gill tissues from 45 fish (three pools of 15 fish each) at 4 h, 24 h, 3 d and 7 d after *E. ictaluri* treatment and from 24 fish (three pools of 8 fish each) at 4 h, 24 h and 48 h after *F. columnare* treatment were isolated for RNA extraction, respectively. Corresponding, uninfected control samples were taken at each time interval.

2.7. Quantitative real-time PCR analysis

Total RNA was extracted using the RNeasy Plus kit (Qiagen) following manufacturer's instructions with modifications previously described (Liu et al., 2010). The RNeasy kit included a genomic DNA elimination column. The RNA was pooled (500 ng from each sample), first strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's protocol, and all cDNA samples were stored at −20 °C prior to use in real-time PCR assays. Quantitative real-time PCR (qRT-PCR) analysis was conducted using 11 healthy tissues from three pools of 15 fish each, and spleen and gill tissues from the bacterial challenges. Primers were designed using the Primer 5.0 software and listed in Table 1. In order to select optimum primer pairs, a melting curve analysis was carried out. Optimal primer pairs were selected based on their amplification specificity by melting curve analysis. Real-time PCR was performed using the CFX Real Time PCR Detection System (Bio-Rad) in 10 μl reactions containing the following components: 250 ng of cDNA, 5 μl SYBR-Green Mix (Bio-Rad), and 5 pmol of each primer. The real-time PCR profile was as follows: one cycle of 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 58 °C for 5 s, followed by 65–95 °C, 5 s/step. In addition, we checked the amplicons by agarose gel with a 100 bp ladder in order to confirm the correct amplicon sizes and that a single product was amplified.

All statistical analyses were based on MFAP4 gene expression levels normalized by 18S rRNA (forward primer 5′GATACGCTCATTCCGATTACAG3′; reverse primer 5′GAGAAACGGCTACCACATCC3′). The triplicate fluorescence intensities of the control and treatment products for each gene,

Table 4
Pairwise similarities among five MFAP4 genes of channel catfish and MFAP4 and ficolin genes of other species as specified. Shading indicates high levels of similarity.

Species	Catfish MFAP4-1	Catfish MFAP4-2	Catfish MFAP4-3	Catfish MFAP4-4	Catfish MFAP4-5
Homo sapiens MFAP4	42	45	36	51	42
Mus musculus MFAP4	41	45	33	53	42
Xenopus laevis MFAP4	39	44	32	45	43
Danio rerio MFAP4	51	58	40	50	46
Homo sapiens FCN1	36	40	32	42	30
Homo sapiens FCN2	32	33	27	34	25
Homo sapiens FCN3	36	40	31	39	30
Mus musculus FCNA	35	39	29	37	27
Mus musculus FCNB	39	42	31	39	34
Xenopus laevis FCN1	34	36	30	35	32
Xenopus laevis FCN2	36	37	32	37	34
Xenopus laevis FCN3	32	35	32	38	31
Ciona intestinalis FCN	38	46	27	39	35

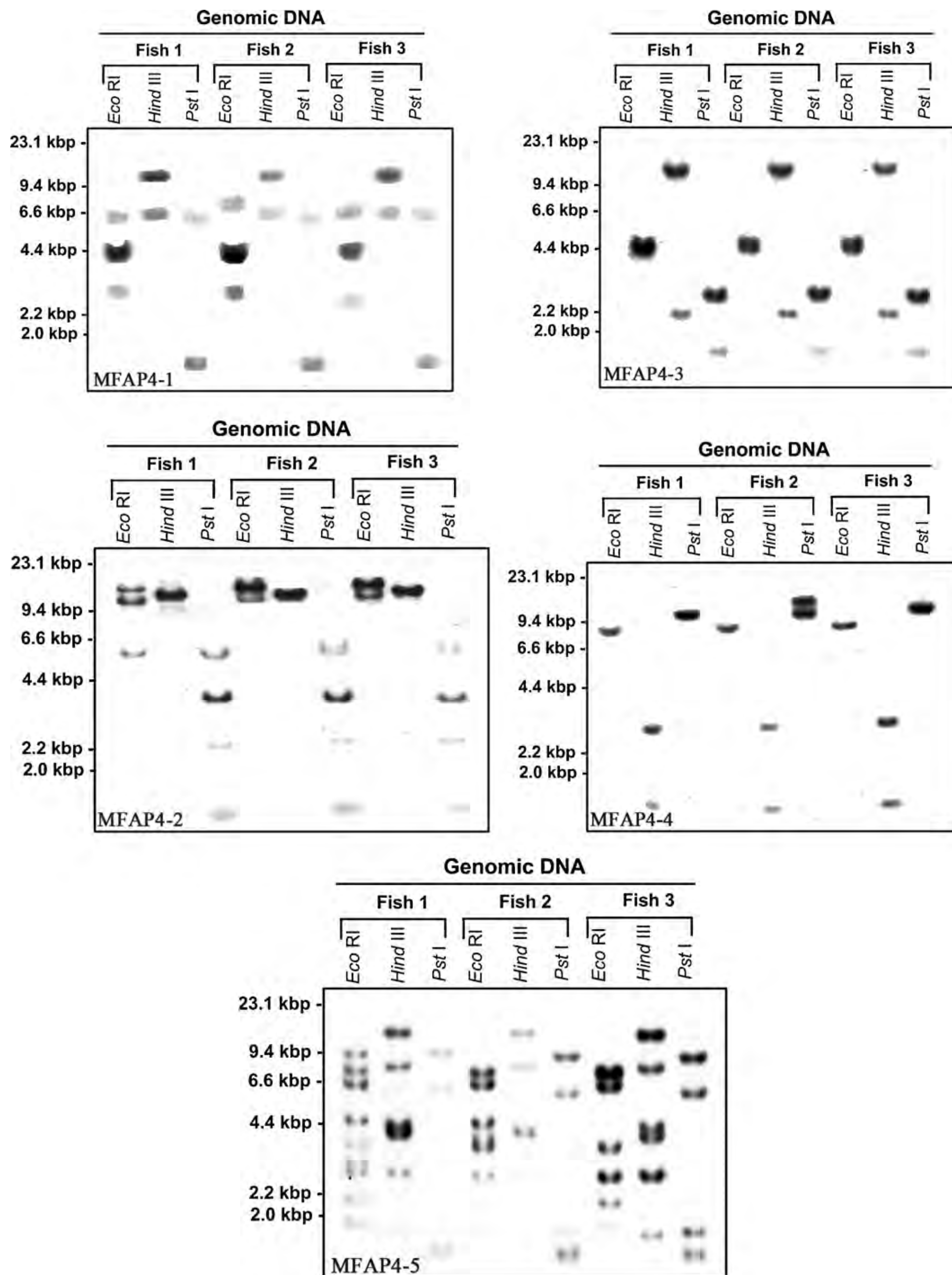


Fig. 3. Southern blot analysis of the catfish MFAP4 genes for the determination of genomic copy numbers. DNA from three individual channel catfish, fish 1, fish 2, and fish 3, was digested with *Eco*RI, *Hind*III, and *Pst*I. Molecular weight (kb) standards are indicated on the left margin of each blot. Genes are specified at the lower left corner of each blot.

as measured by crossing-point (C_t) values, were compared and converted to fold differences by the relative quantification method (Pfaffl et al., 2002) using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment groups were assessed for statistical significance ($p < 0.05$) using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S rRNA gene in the same samples. Expression levels of 18S rRNA were constant between all samples. Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

3. Results

3.1. Identification of MFAP4-like gene sequences

Five MFAP4-like genes were identified in channel catfish using *tblastn* with zebrafish MFAP4 gene as the query to search against catfish EST sequences. Further sequence analysis indicated that the coding regions of these five catfish MFAP4-like genes ranged from 240 to 264 amino acids in length. All five genes contained a signal peptide (11–22 amino acids) and a conserved fibrinogen-like (FBG) domain (199–230 amino acids) (Table 2). However, unlike mammalian MFAP4 genes, the catfish MFAP4 genes do not contain an

RGD sequence (Fig. 1). All analyzed MFAP4 genes lack the collagen-like region characteristic of ficolin proteins. Herein, these five genes will be referred to as MFAP4-1, MFAP4-2, MFAP4-3, MFAP4-4, and MFAP4-5.

Sequence analysis indicated low similarities among the five MFAP4 genes from catfish, ranging from 39% to 55% amino acid identities within the open reading frame, and 45–60% amino acid identities within the FBG domain (Table 3). Similarly, the catfish MFAP4 genes had low sequence similarities with MFAP4 genes from other species, ranging from 32% to 58% sequence similarities (Table 4). MFAP4-2 showed somewhat higher similarity to a MFAP4 gene of zebrafish (58%); whereas catfish MFAP4-4 had higher similarity to MFAP4 genes of mammals (51–53%) (Table 4). Comparison of the catfish MFAP4 genes to ficolin revealed uniformly lower similarity levels than seen with MFAP4, with catfish MFAP4-2 and MFAP4-4 most similar to ficolin sequences.

3.2. Phylogenetic analysis of MFAP4 with other FREP proteins

To better understand the evolutionary relationships between MFAP4 and other FREP proteins, we conducted phylogenetic analysis using MFAP4, ficolin (FCN), fibrinogen (FB), tenascin R (TNR), fibrinogen c domain containing 1 (FIBCD1) and angiopoietin (ANGPT) amino acid sequences from a range of vertebrate and invertebrate organisms. Interestingly, no MFAP4-like sequences were revealed in searches of invertebrate genomes. Mammalian, amphibian, and teleost fish MFAP4 sequences were consistently grouped together with moderate bootstrap support, suggesting orthology between these proteins. Consistent with amino acid similarities, catfish MFAP4-4 was more closely related to mammalian and amphibian MFAP4, and catfish MFAP4-2 clustered with zebrafish MFAP4 (Fig. 2).

Less support was given to the relationship of MFAP4 with other FREP proteins. MFAP4 proteins appear related to FIBCD1 proteins and predicted ficolin-like sequences from *Ciona intestinalis*, albeit with marginal bootstrapping support. Notably, the vertebrate ficolins formed a distinct, well-supported clade separate of the invertebrate ficolin sequences. The absence of MFAP4 in invertebrates and ficolin in fish, along with clear species-specific duplications, presents a difficult puzzle for resolving a clear evolutionary history of FREP proteins from invertebrates to mammals.

3.3. Genomic copy number and location of MFAP4 genes

The genomic copy numbers of the catfish MFAP4 genes were assessed using genomic Southern blot analysis. As shown in Fig. 3, MFAP4-1, MFAP4-2 and MFAP4-4 likely have a single copy in the genome, whereas two copies of MFAP4-3 and multiple copies of MFAP4-5 may exist in the catfish genome. Although two or more bands were detected from the Southern blot of MFAP4-1, internal restriction sites existed to account for the additional bands. Similarly, for MFAP4-2, internal restriction sites for *Pst*I were detected, but no internal sites for *EcoR*I and *Hind*III, fitting the observed Southern blot banding patterns. However, individual variation was detected. For instance, the first individual fish had three *EcoR*I bands, apparently due to sequence variations of this individual. Catfish MFAP4-3 likely has two copies in the catfish genome because there were no internal restriction sites for *Hind*III or *Pst*I restriction enzymes, but two bands were detected with either restriction enzyme. One copy of MFAP4-4 likely exists in the catfish genome, with only internal restriction sites for *Hind*III detected, fitting the observed Southern blot banding patterns. However, individual variation was detected in bands resulting from *Pst*I digestion. MFAP4-5 probably has multiple copies, likely 4 copies, in the catfish genome because multiple bands of large segments were detected that cannot be explained by the internal restriction sites within the

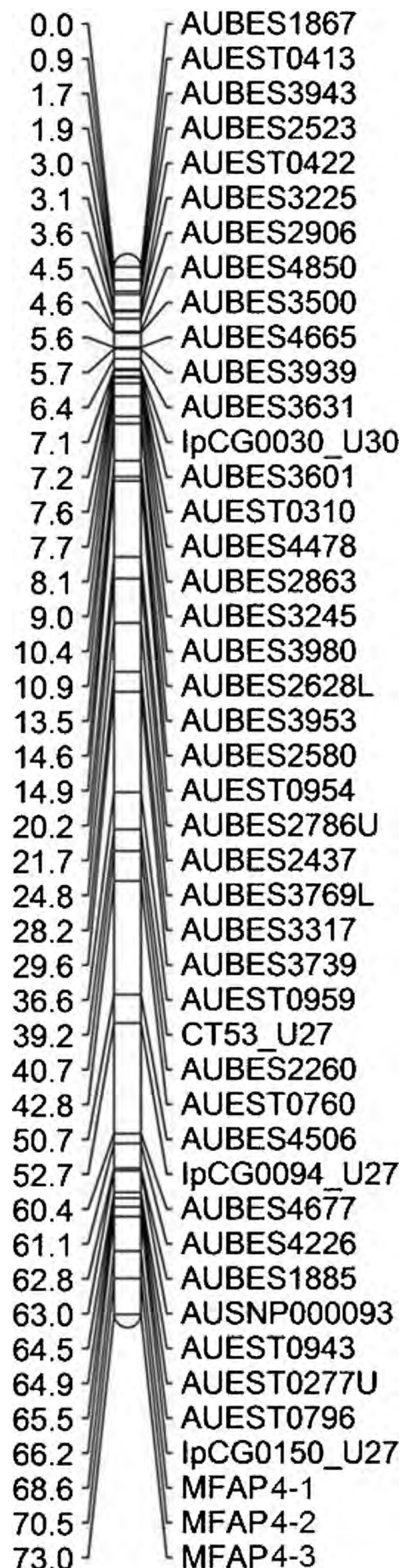


Fig. 4. The location of MFAP4-1, MFAP4-2 and MFAP4-3 on linkage group 8 of the catfish genetic linkage map.

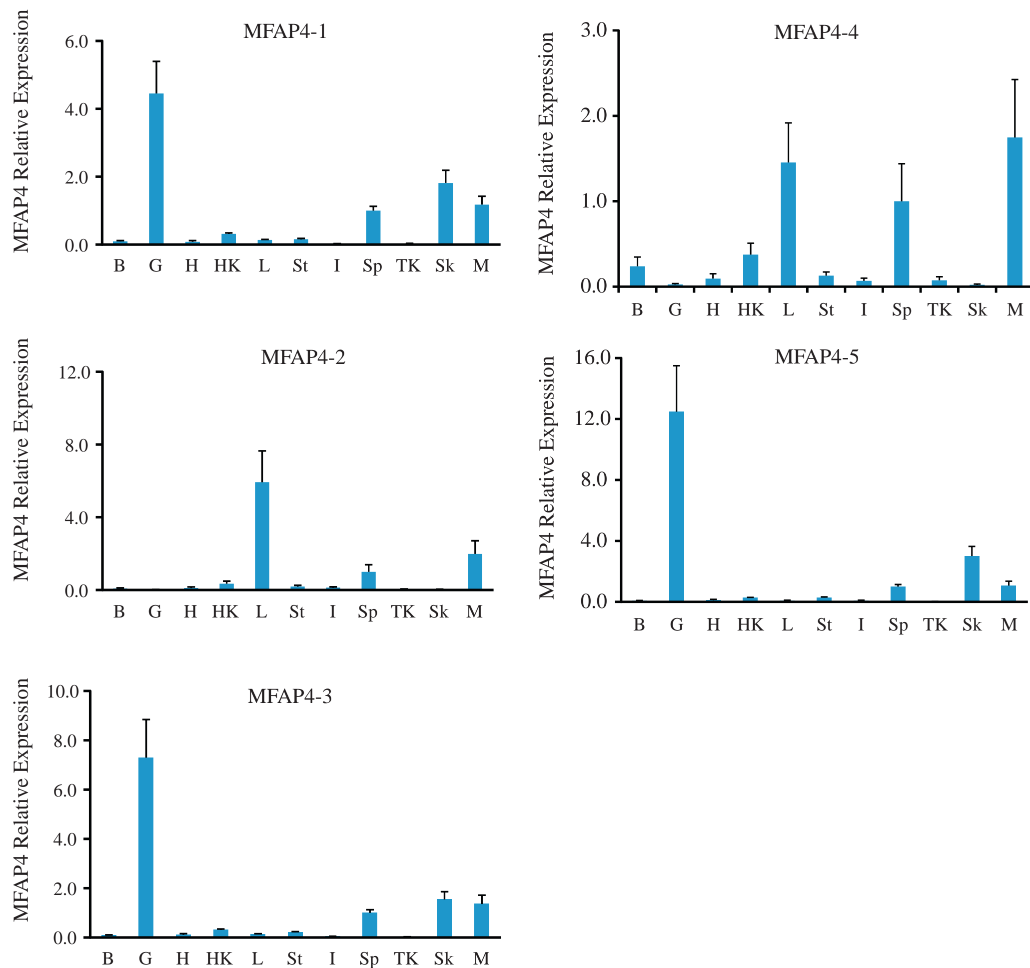


Fig. 5. Tissue expression of the five catfish MFAP4 genes as determined by real time quantitative RT-PCR in 11 healthy tissues of channel catfish. The tissues are as the following from left to right: B: brain, G: gill, H: heart, HK: head-kidney, L: liver, St: stomach, I: intestine, Sp: spleen, TK: trunk-kidney, Sk: skin, M: muscle. Gene expression levels of all tissues are expressed relative to that in the spleen.

gene. Analysis of restriction sites within the genomic sequences of MFAP4-5 using WatCut software (http://watcut.uwaterloo.ca) indicated the presence of one internal *Eco*RI site, one internal *Hin*dIII site, but no internal site for *Pst*I within the gene. With this restriction site distribution in the gene, a four-copy gene would generate a restriction pattern of eight bands, eight bands, and four bands for *Eco*RI, *Hin*dIII, and *Pst*I respectively, consistent with the pattern observed with the genomic Southern blot analysis (Fig. 3).

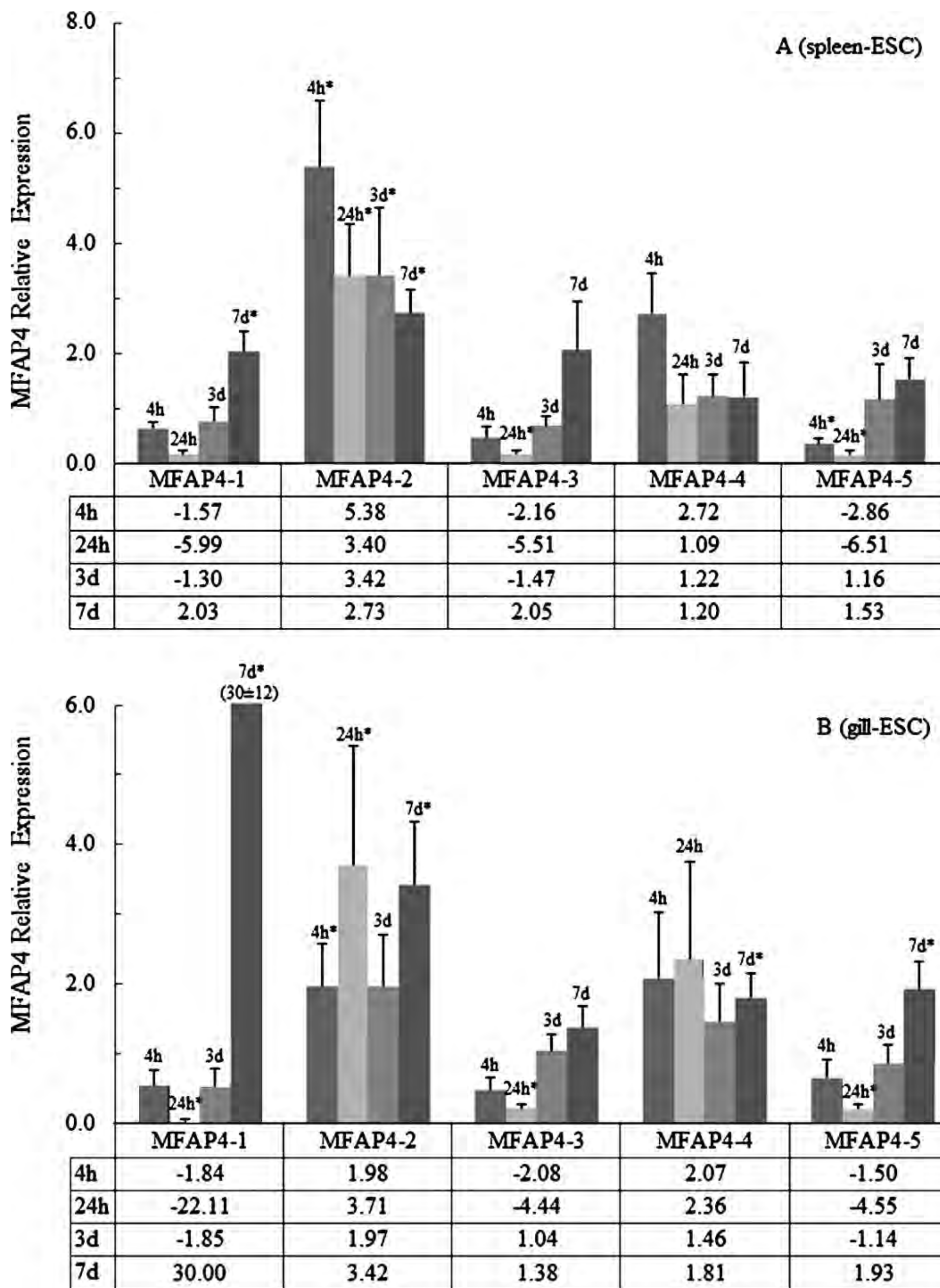
Microsatellites were identified from all five catfish MFAP4 genes. However, genotyping analysis revealed polymorphism of the microsatellite loci with MFAP4-1, MFAP4-2 and MFAP4-3, but not with MFAP4-4 and MFAP4-5. Segregation of the polymorphic microsatellites within the three MFAP4 loci allowed them to be mapped. All the three MFAP4 genes were mapped to a single genomic location in genetic linkage group 8 (Fig. 4), suggesting the evolution of these genes from tandem duplications.

3.4. Tissue expression of MFAP4 genes

Quantitative real-time PCR was used to determine tissue distribution of MFAP4 gene expression in channel catfish. As shown in Fig. 5, MFAP4 genes were widely expressed in all 11 tissues tested (brain, gill, head kidney, heart, liver, stomach, intestine, spleen, trunk kidney, skin and muscle). A comparison of the expression profiles of the five genes, however, revealed an interesting pattern. Expression patterns of MFAP4-1, MFAP4-3 and MFAP4-5 were highly similar, with the highest expression in the gill tissue, followed by skin, spleen, and muscle tissues. Similarly, expression patterns of MFAP4-2 and MFAP4-4 were highly similar, with highest expression observed in the liver, muscle and spleen tissues.

3.5. Expression of MFAP4 after infection of *E. ictaluri*

In order to determine if the catfish MFAP4 genes are involved in responses to disease infection with the catfish pathogen and Gram-negative intracellular bacterium *E. ictaluri*, qRT-PCR analysis was conducted to determine the expression patterns of MFAP4 genes in infected spleen (Fig. 6A) and gill (Fig. 6B) tissues. In the spleen, MFAP4-1, MFAP4-3 and MFAP4-5 genes had highly similar expression patterns, as in the case of expression in uninfected tissues. Transcript expression of these genes was suppressed initially after infection at 4 h and 24 h after challenge, but up-regulated at 7 d after challenge. Strongest suppression (>5-fold) was observed at 24 h. MFAP4-2 and MFAP4-4 genes also had similar expression patterns after infection. The MFAP4-2 gene was significantly up-regulated, especially at 4 h after infection (Fig. 6A). Similar transcript expression patterns of the five catfish MFAP4 genes were observed in gill tissue. MFAP4-1, MFAP4-2, MFAP4-4 and MFAP4-5 expression was significantly up-regulated at 7 d after infection, especially MFAP4-1 expression (30-fold). Significant suppression of gene expression was again observed in MFAP4-1, MFAP4-3 and MFAP4-5 at 24 h in gill (Fig. 6B).



	MFAP4-1	MFAP4-2	MFAP4-3	MFAP4-4	MFAP4-5
4h	-1.57	5.38	-2.16	2.72	-2.86
24h	-5.99	3.40	-5.51	1.09	-6.51
3d	-1.30	3.42	-1.47	1.22	1.16
7d	2.03	2.73	2.05	1.20	1.53

	MFAP4-1	MFAP4-2	MFAP4-3	MFAP4-4	MFAP4-5
4h	-1.84	1.98	-2.08	2.07	-1.50
24h	-22.11	3.71	-4.44	2.36	-4.55
3d	-1.85	1.97	1.04	1.46	-1.14
7d	30.00	3.42	1.38	1.81	1.93

Fig. 6. Gene expression of the five MFAP4 genes as determined by real time quantitative RT-PCR at 4 h, 24 h, 3 d and 7 d after *Edwardsiella ictaluri* infection in the spleen tissue (A) and gill tissue (B) of catfish. Relative MFAP4 expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Asterisks indicate statistical significance at the level of $p < 0.05$ relative to appropriate control.

3.6. Expression of MFAP4 after infection of F. columnare

The transcript expression of the five MFAP4 genes after bacterial challenge with *F. columnare* in spleen (Fig. 7A) and gill (Fig. 7B) tissues was also examined using qRT-PCR. All MFAP4 genes were significantly up-regulated at 24 h after columnaris infection in spleen tissue. MFAP4-2 showed the most sensitive transcript responsiveness to infection, increasing expression 12.76-fold at 24 h, before suppressing expression 37.79-fold at 48 h (Fig. 7A). In gill, MFAP4-1, MFAP4-3 and MFAP4-5 were significantly up-regulated at 4 h after infection, with MFAP4-1 showing the largest expression changes over the course of the infection (Fig. 7B).

4. Discussion

A remarkable array of innate immune strategies have been revealed in recent years by large-scale sequencing in invertebrate and primitive vertebrate species (Hibino et al., 2006; Huang et al., 2008; Kasamatsu et al., 2010; Romero et al., 2010). Tracing the maintenance, loss and/or adaptation of these strategies through vertebrate evolution can highlight conserved immune elements

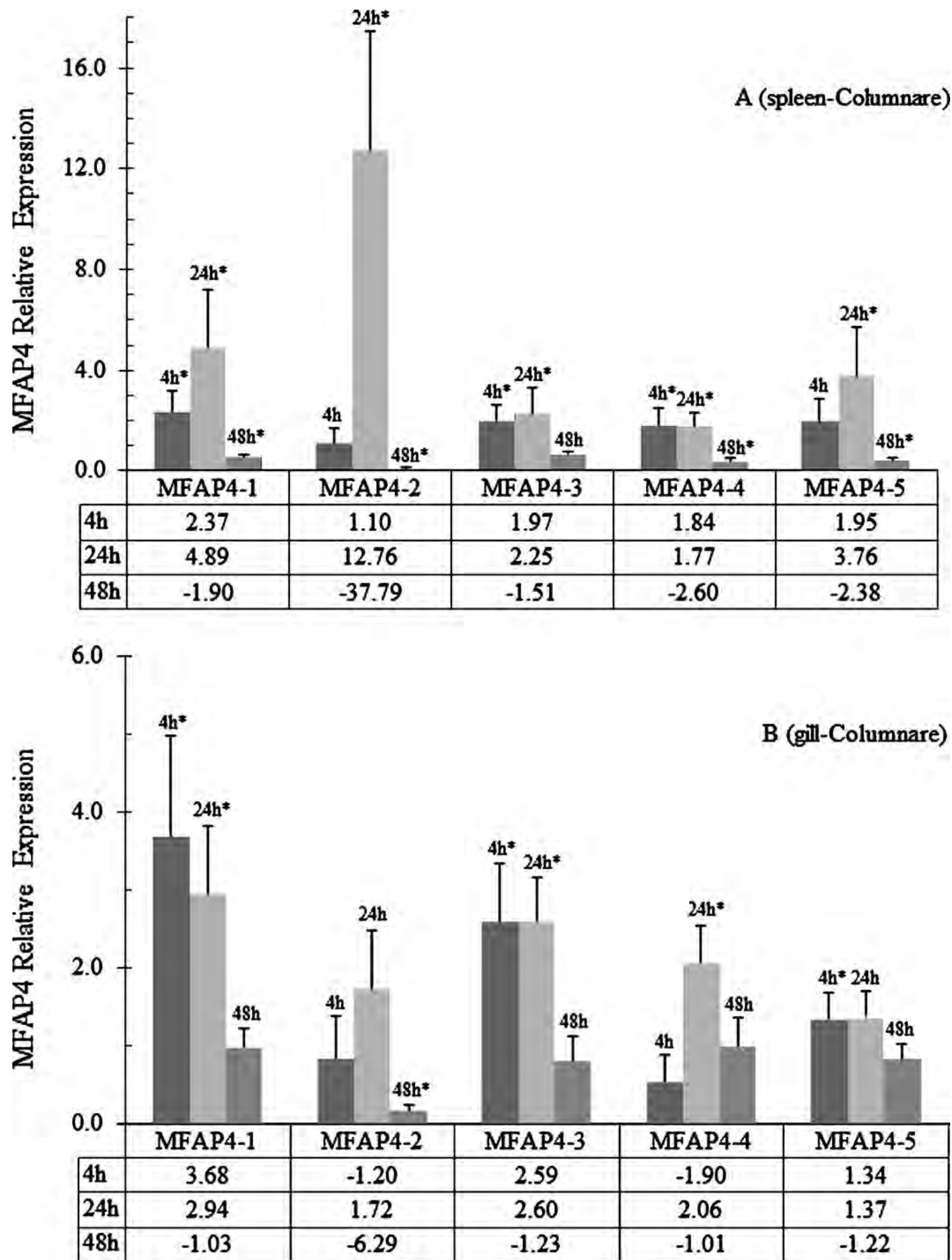


Fig. 7. Gene expression of the five MFAP4 genes as determined by real time quantitative RT-PCR at 4 h, 24 h and 48 h after *Flavobacterium columnare* infection in the spleen tissue (A) and gill tissue (B) of catfish. Relative MFAP4 expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (8 fish each) ± SE. Asterisks indicate statistical significance at the level of $p < 0.05$ relative to appropriate control.

that may be overlooked in single species studies. Study of the FREP family in catfish has highlighted a little-studied protein, MFAP4, with a conserved fibrinogen-like domain and a putative role in the first line of immune defense. Here, we have identified five catfish MFAP4 genes. Southern blot analysis and linkage mapping indicated that additional MFAP4 gene copies likely exist in the catfish genome, and that they may exist in clusters of tandem duplicates, similar to FREPs reported in invertebrate species. Transcript expression of catfish MFAP4 genes was modulated by exposure to two common bacterial pathogens of catfish, *E. ictaluri* and *F. columnare*. Analysis of expression in healthy and infected tissues indicated subfunctionalization of the MFAP4 genes in tissue specificity and pathogen response and potentially coordinated expression between groups of MFAP4 genes.

Little is known about MFAP4 gene function in mammalian species beyond early reports of collagen and mannan-binding activity in coordination with surfactant proteins (Lausen et al., 1999). Interestingly, while surfactant proteins have been reported in fish by Western blot (Sullivan et al., 1998), no clear sequence orthologs have been identified in fish or invertebrates. Likely, early protein blotting experiments were detecting fish MBL-like proteins, the probable ancestors of surfactant proteins (Hughes, 2007). This

raises the question whether MFAP4 may bind to fish MBLs to mediate immune responses either independent of or via the complement cascade.

While no direct studies of MFAP4 have been conducted outside mammals, a recent microarray study cast additional light on MFAP4 function in teleosts. Zakrzewska et al. (2010) reported that MFAP4 is expressed specifically in monocytes in zebrafish embryos and can serve as a robust marker for that cell type. Of note, ficolin-1 (or M-ficolin) in humans is expressed primarily on the surface and in the cytoplasm of circulating monocytes (Teh et al., 2000). Expression is down-regulated in differentiated macrophages and immature dendritic cells (Hashimoto et al., 1999), but can be dramatically reinduced with exposure to bacterial products such as LPS (Frankenberger et al., 2008). Further work is needed to determine whether monocyte recruitment, maturation, and re-stimulation can account for observed patterns of induction and repression of catfish MFAP4 gene expression following bacterial infection.

Determination of orthologous relationships between members of families of innate immune genes can be very difficult (Peatman and Liu, 2007). Duplications and divergence of these families often occur at accelerated rates in response to new pathogen environments. The MFAP4 genes of catfish share homologous structures with mammalian and amphibian MFAP4 sequences and are grouped together by phylogenetic analysis. While MFAP4 genes are represented by a single copy in mammals, catfish appear to have greater than five MFAP4 sequences. Zebrafish have 13 MFAP4 genes clustered at the end of chromosome 1, in a potentially similar arrangement as that indicated by preliminary linkage mapping in catfish. Phylogenetic analysis of zebrafish and catfish MFAP4 genes revealed a pattern indicative of rapid gene duplication following species divergence, with no clear orthologs identified between individual genes of the two species (data not shown). We also did not identify a clear ortholog to MFAP4 in invertebrate species, although some homology exists between invertebrate ficolin sequences and fish MFAP4. Similarly, while ficolins are clearly present in higher vertebrates, our searches did not identify any FBG-containing genes in fish with the prerequisite N-terminus collagen-like domain characteristic of ficolins. The relationship between invertebrate and vertebrate ficolins has never been clear based on phylogenetics (Garred et al., 2010). Based on existing sequences, it appears that prior to the teleost divergence, a diverse group of FREP proteins was present, including some with collagen-like domains. Following the divergence of teleosts from higher vertebrates, ficolin-like FREP genes were lost, and independent duplications of MFAP4 genes occurred in teleost species. Ficolin genes and MFAP4 were maintained in higher vertebrates, with independent tandem duplications of ficolin genes arising in several species (Garred et al., 2010) and MFAP4 showing higher conservation as a single copy gene. Subsequent rapid duplication and divergence of FREPs in invertebrate species have further obscured ancestral FREPs.

The functions of the FBG domain of FREP proteins are not fully understood. However, recent studies of novel FREP genes in invertebrates (Romero et al., 2010) and of FIBCD1 (Schlosser et al., 2006; Thomsen et al., 2010) have led to a consensus that the FBG domain can function in recognition of carbohydrates and their derivatives on the surface of microorganisms. In contrast to ficolins, surfactant proteins, and mannose-binding lectins, MFAP4 genes lack collagen-like domains. This may have several consequences for the function of MFAP4. The collagen-like sequence is known to enable the assembly of identical polypeptide chains into trimeric structures to maximize ligand binding through increased avidity. However, recent work in FIBCD1 demonstrated that these FREP proteins are capable of oligomerization independent of a collagen-like region, suggesting that alternative residues may facilitate formation of tetramers (Thomsen et al., 2010). Additional studies are needed to examine the potential of MFAP4 to recognize and bind microorganisms or their derivatives and the potential of MFAP4 to oligomerize. Also, in light of seemingly coordinated expression of groups of catfish MFAP4 genes, studies should determine whether MFAP4 transcripts resulting from different genes can form heterogeneous complexes to increase pathogen recognition capacity and sensitivity.

The lack of a collagen-like domain in MFAP4 also suggests that these proteins may not be involved in the complement cascade via traditional interaction with MBL-associated serine proteases (Matsushita et al., 2000). However, further work is needed to determine whether MFAP4 in fish interacts with MBLs to mediate its functions, or, like other soluble defense molecules, it can function independently through opsonization and enhancement of phagocytosis. One candidate receptor for soluble MFAP4 may be calreticulin (Liu et al., 2011), which has been reported to bind ficolin (Lacroix et al., 2009) and is upregulated in catfish along with MFAP4 following infection (Peatman et al., 2007, 2008).

In conclusion, we have described here the characterization of five unique MFAP4 genes from catfish. While MFAP4 is a member of the functionally important FREP family, little is known about its role in vertebrate immunity. Fish, apparently lacking ficolin genes, have variable numbers of MFAP4 genes which may mediate similar roles during the innate immune response. We observed rapid and sensitive responses of catfish MFAP4 genes to infection with two bacterial pathogens, with intriguing, coordinated patterns of expression seen between gene subsets. Further studies are needed to elucidate the functions of individual MFAP4 genes in regards to pathogen recognition, binding, and their larger immune context.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.01.002.

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