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Complement regulatory protein genes in channel catfish and their involvement in disease defense response

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ABSTRACT

Complement system is one of the most important defense systems of innate immunity, which plays a crucial role in disease defense responses in channel catfish. However, inappropriate and excessive complement activation could lead to potential damage to the host cells. Therefore the complement system is controlled by a set of complement regulatory proteins to allow normal defensive functions, but prevent hazardous complement activation to host tissues. In this study, we identified nine complement regulatory protein genes from the channel catfish genome. Phylogenetic and syntenic analyses were conducted to determine their orthology relationships, supporting their correct annotation and potential functional inferences. The expression profiles of the complement regulatory protein genes were determined in channel catfish healthy tissues and after infection with the two main bacterial pathogens, *Edwardsiella ictaluri* and *Flavobacterium columnare*. The vast majority of complement regulatory protein genes were significantly regulated after bacterial infections, but interestingly were generally up-regulated after *E. ictaluri* infection while mostly down-regulated after *F. columnare* infection, suggesting a pathogen-specific pattern of regulation. Collectively, these findings suggested that complement regulatory protein genes may play complex roles in the host immune responses to bacterial pathogens in channel catfish.

1. Introduction

The complement system is a major antimicrobial defense system of innate immunity, providing vital host defense, especially early after infection before adaptive immunity is activated. It is crucial for phagocytes recruitment, clearance of invading pathogens, and elimination of altered cells. It serves as a bridge between innate immunity and adaptive immunity (Abbas et al., 2012; Carroll, 2004). The complement system can be activated by three major pathways: classical pathway, alternative pathway and lectin pathway. These three pathways merge to a common terminal pathway where membrane attack complex (MAC) is assembled, and MAC can lyse the invading pathogens directly. The complement system consists of more than 30 soluble and membrane-bound proteins, which interact with one another and form a highly regulated complement cascades (Abbas et al., 2012; Carroll and Sim, 2011; Mayilyan, 2012; Noris and Remuzzi, 2013). Although the complement system is under strict control, there are still potential risks of damaging the host cells. In order to maintain homeostasis of the organism, a set of complement regulatory proteins are responsible for avoiding excessive host responses after microbial infection.

Complement regulatory proteins, also known as complement regulators or complement inhibitors, can be categorized into two major classes: membrane-bound regulators and soluble regulators. Membrane-bound regulators mainly protect host cells from complement attacks in all three complement pathways and inactivate both C3 and C4 (Zipfel and Skerka, 2009). The membrane-bound regulators include complement component receptor 1 (CR1 or CD35), complement component receptor 2 (CR2 or CD21), membrane cofactor protein (MCP or CD46), dacay-accelerating factor (DAF

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or CD55) and protectin (CD59). Soluble regulators are more specific and control either the alternative, the classical or the lectin pathway (Zipfel and Skerka, 2009). They are needed to control the excessive activation in the fluid phase (Meri and Jarva, 2013). The soluble regulators include factor I (CFI), factor H (CFH) and factor H-related genes (CFHRs), carboxypeptidase N (CPN), complement component 1 inhibitor (C1INH), C4 binding protein α chain (C4BPα), clusterin (CLU, also named SP40-40 or apolipoprotein J), vitronectin (VTN, or S-protein), plasminogen (PLG) and the only known positive regulator properdin (CFP). Structurally some of the complement regulatory proteins belong to a family of proteins named regulator of complement activation (RCA), since they share a common tandem short consensus repeats (SCRs, also named CCP, complement control protein modules or sushi domain) which typically consist of 60–70 amino acids (HeineSuner et al., 1997; Norman et al., 1991). In humans, RCA genes are clustered on chromosome 1 (1q32), and can be categorized into two groups: RCA group 1 containing factor H and related genes, and RCA group 2 containing C4BPα, CR1, CR1L, CR2, MCP and DAF (Krushkal et al., 2000).

Complement regulatory proteins have been well studied in mammals (Zipfel and Skerka, 2009). Although not equally extensive, a number of studies of complement regulatory proteins were conducted with teleost fish. For instance, C1INH, CFP, CLU, VTN and CPN have been characterized in *Danio rerio* (Sun et al., 2013; Zhang et al., 2013b), *Oncorhynchus mykiss* (Chondrou et al., 2008; Londou et al., 2008; Marioli and Zarkadis, 2008; Papanastasiou et al., 2007; Wang and Secombes, 2003), *Oplegnathus fasciatus* (Godahewa et al., 2014), *Oreochromis niloticus* (He et al., 2013), *Pseudosciaena crocea* (Wei et al., 2010). Studies on RCA genes have also been conducted in teleosts. For example, the SBP1 gene was reported from barred sand bass (*Paralabrax nebulifer*) (Dahmen et al., 1994), and its related gene SBCRP-1 was also annotated (Zipfel et al., 1996). SBP1 gene was reported to have overlapped regulatory activities of mammalian factor H and C4BP (Kemper et al., 1998). Both RCA group 1 genes CFH and CFHL1-4 and RCA group 2 genes ZRC1 and ZRC2 have been reported from zebrafish (Sun et al., 2010; Wu et al., 2012). Furthermore, three membrane-bound complement regulatory protein isoforms gTecrem-1, gTecrem-2 and gTecrem-3 were identified in ginbuna crucian carp (*Carassius auratus langsdorfii*) (Nur et al., 2013). A CD46-like complement-regulatory membrane protein (cTecrem), an ortholog of a zebrafish RCA group 2 gene ZRC1, was cloned and characterized in common carp (Tsujikura et al., 2015). In channel catfish *(Ictalurus punctatus)*, the primary aquaculture species in the United States, complement factors Bf/C2 and Df (Zhou et al., 2012), and three complement regulatory protein genes, CD59, factor I, and C1INH have been reported (Abernathy et al., 2009; Yeh and Klesius, 2007), and C1INH was found to be significantly up-regulated at early stages after bacterial infection with *Edwardsiella ictaluri* (Li et al., 2014).

The catfish industry continues to suffer from infectious diseases. In particular, the enteric septicemia of catfish (ESC) caused by *E. ictaluri* and columnaris disease caused by *Flavobacterium columnare* are the two major bacterial diseases that cause huge economic losses. Understanding of immune-relevant functional genes and their expression in the course of bacterial disease is important for designing strategies for disease management. With the advances in genomic sciences, rapid progress has been made in characterization and analysis of expression of innate immune genes such as pathogen recognition receptors (Baoprasertkul et al., 2007a, 2006, 2007b; Rajendran et al., 2012a; Rajendran et al., 2012b; Sun et al., 2014; Zhang et al., 2013a), chemokines (Bao et al., 2006a; Peatman et al., 2006), antimicrobial peptides (Bao et al., 2005, 2006b; Wang et al., 2006a; Wang et al., 2006b; Xu et al., 2005), lysozymes (Wang et al., 2013), lectins (Takano et al., 2008; Thongda et al., 2014; Zhang et al., 2012), NOS genes (Yao et al., 2014a) and protease inhibitors (Li et al., 2014; Yao et al., 2014b). However, systematic analysis of complement regulatory protein genes has not been conducted in channel catfish. Here in this study, we identified and characterized nine complement regulatory protein genes, and analyzed their expression profiles after bacterial infections with *E. ictaluri* and *F. columnare*.

2. Materials and methods

2.1. Identification and sequence analysis of complement regulatory protein genes

The complement regulatory protein genes in catfish were identified by searching the RNA-seq database (Liu et al., 2012) and the whole genome sequence database of catfish (Lu et al., 2011), using all available sequences of complement regulatory protein genes from fish, human, mouse, chicken and frogs retrieved from the GenBank (NCBI) and Ensembl as queries. TBLASTN similarity searches were conducted against RNA-seq database to identify all complement regulatory protein gene-related sequences using a cutoff E-value of e-5. The retrieved sequences were further analyzed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) for the generation of coding sequences. To confirm the candidate complement regulatory protein gene sequences, BLASTN was conducted against the unpublished catfish whole genome sequence database with a cutoff E-value of e-10. The retrieved genome sequences were subjected to gene prediction by FGENESH program of MolQuest software version 2.4.3 using Fish model (Solovyev et al., 2006) and the resulted coding sequences were further confirmed by BLASTP against NCBI non-redundant (NR) protein sequence database.

2.2. Structural and phylogenetic analyses

Conserved domains of complement regulatory protein genes were identified using Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/) (Letunic et al., 2012; Schultz et al., 1998), and confirmed by conserved domain prediction from BLASTP. In order to further identify channel catfish complement regulatory protein genes, phylogenetic analysis was conducted using all the amino acid sequences of complement regulatory protein genes from channel catfish and selected vertebrate species retrieved from GenBank and Ensembl, including those from human, cattle, mouse, chicken, lizard, frog, and several fish species such as zebrafish, tilapia, medaka, fugu, stickleback, platyfish, green spotted puffer and Atlantic cod. Separate phylogenetic trees for different complement regulatory protein genes were conducted, additional phylogenetic trees were also constructed to compare the sequences of each tandem SCR domain between zebrafish ZRCs and catfish CRCs. Alignment of multiple protein sequences were performed using Muscle v3.8 (multiple sequence comparison by log-expectation) (Edgar, 2004a, b) with default parameters. The phylogenetic trees of complement regulatory protein genes and homologies among SCR domains of RCA group 2 genes were constructed using maximum likelihood method in MEGA 5.2 (Tamura et al., 2011). Bootstrap test of 1000 replications was conducted to evaluate the phylogenetic trees.

2.3. Syntenic analysis

When required, such as for the RCA group 2 genes where phylogenetic analysis alone was not sufficient to provide annotation for gene duplicates, syntenic analysis was also conducted to determine orthologous relationship. Shared syntenies were assessed based on comparisons of the genes, their order on the

genomic neighborhood that harbors RCA group 2 genes in channel catfish and several other vertebrates, including human, frog and zebrafish. In order to obtain the location information of channel catfish complement regulatory protein genes on chromosomes, all identified channel catfish RCA group 2 gene amino acid sequences were used as queries to search against channel catfish whole genome sequence database. The retrieved genome sequences were predicted by FGENESH of MolQuest v2.4.3 using Fish model (Solovyev et al., 2006) and BLASTP against NCBI NR database to identify the neighboring genes of RCA group 2 genes in channel catfish. Genomicus v72.01 (Louis et al., 2013; Muffato et al., 2010) and Ensembl genome database were utilized to construct the conserved syntenic regions harboring RCA group 2 genes for human, frog and zebrafish.

2.4. Bacterial challenges

All procedures involved in handling and treatment of fish during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to the initiation of the study. *E. ictaluri* and *F. columnare* challenges were conducted as previously described (Li et al., 2012; Sun et al., 2012). Briefly, fish were challenged in six 30-L aquaria with 3 control and 3 treatment groups for each challenge. The bacteria were first cultured from a single colony, re-isolated from a symptomatic fish and biochemically confirmed before being inoculated in Brain Heart Infusion broth (BHI) and incubated in a shaker incubator overnight. The concentrations of the bacteria were determined using colony forming unit (CFU) per ml by plating 10 μl of 10-fold serial dilutions onto BHI agar plates.

For ESC infection, 200 ml bacterial culture with a concentration of 4×10^8 CFH/ml was added into the treatment tanks with the water flow turned off. Aquaria were randomly divided into three sampling time points: 4 h, 24 h and 3 d post-infection for both control and treatment groups. Intestine and skin tissues from 30 fish (3 replicates of 10 fish each) were collected at each time point from both control and treatment tanks, after being euthanized with MS-222 (300 mg/L). The same tissues of 10 fish were pooled, flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Fish for *F. columnare* challenges were also randomly divided into six aquaria and *F. columnare* bacteria with a concentration of 3×10^6 CFU/ml were added into the treatment aquaria for 2 h. Gill and skin were collected and pooled from 18 fish at each time point: 4 h, 24 h, 48 h (3 replicates of 6 fish each). Samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.5. RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted using the Trizol reagents (Invitrogen) following the manufacturer's protocol. First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Quanta BioSciences) according to the manufacturer's instructions. All the cDNA products were adjusted to 250 ng/μl for quantitative real-time PCR reaction using the PerfeCTa® SYBR® Green FastMix® Reaction Mixes on CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The primers used in quantitative real-time PCR were listed in Table 1 and the house-keeping gene 18S rRNA was used as the internal reference gene based on the previous catfish gene expression studies (Small et al., 2008). The thermal cycling profile consisted of an initial denaturation at 95 °C for 30s, 40 cycles of denaturation at 94 °C for 5s and an appropriate extension temperature at 58 °C for 5s, and 72 °C for 5s followed by dissociation curve analysis to verify the specificity of amplified products.

Relative fold change for each sample and 18S were calculated in Relative Expression Software Tool (REST) version 2009 based on the cycle threshold (Ct) values generated by quantitative real-time PCR (Pfaffl et al., 2002). The statistical analysis with REST is based on an efficiency corrected mathematical model, which calculates the relative expression ratio based on real-time PCR efficiency and the crossing point deviation of the investigated sample versus a control. For the analysis of gene expression in healthy fish tissues, Ct values of each complement regulatory protein gene in intestine were used as control group, 18S rRNA was used as reference for normalization of the relative expression, and the relative expression level of other tissues were then calculated based on the control group using REST software (Pfaffl et al., 2002). For the analysis of gene expression following each bacterial infection, expression differences of complement regulatory protein genes were assessed between control and treatment groups at different time points using REST software (Pfaffl et al., 2002). Expression differences were considered significant when p-value < 0.05. A no template control was used on all plates. Quantitative real-time PCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

Table 1
List of primer sequences used in this study.

Gene name	Primer name	Primer sequence (5′–3′)
CFP	CFP-F	CTTCGCCTCCCCATTCTATT
	CFP-R	CTGCCCAGGAGAAAATACCA
CLU	CLU-F	ATCAGTTCAGCTGGGTGTCC
	CLU-R	CATTTCTGGCTCGTCAAACA
CPN1	CPN1-F	GGAATATGCATGGGAACGAG
	CPN1-R	AGCCATCTGGATTCATGGAG
CPN2	CPN2-F	GTTTGGTCAGGTTTCCCAGA
	CPN2-R	GTGCCAGTGTTTCTCTGCAA
CRC1	CRC1-F	GGGGAAACAGGGTTCTCACT
	CRC1-R	GATCGGTCCACTGTTGTCCT
CRC2	CRC2-F	AGACGGAATTTCGTTTGGTG
	CRC2-R	TGTTATACCGGGAGGCTTTG
PLG	PLG-F	AGCTCACTGCCTTGAACGAT
	PLG-R	AAGCGATGTCTGTTCCTGCT
VTNa	VTNa-F	TCCCCTGAAGGCATAGAGTG
	VTNa-R	ACAATGACCCTGCCTTCATC
VTNb	VTNb-F	TGCACTTGTCCTCTCCACTG
	VTNb-R	CACGGCGCTGTAGTTCATTA
18S rRNA	18S-F	GAGAAACGGCTACCACATCC
	18S-R	GATACGCTCATTCCGATTACAG

3. Results

3.1. Identification of channel catfish complement regulatory protein genes

A total of nine complement regulatory protein genes were identified in channel catfish including properdin (CFP), vitronectin a (VTNa), vitronectin b (VTNb), carboxypeptidase N polypeptide 1 (CPN1), carboxypeptidase N polypeptide 2 (CPN2), clusterin (CLU), plasminogen (PLG), and two catfish RCA group 2 genes (named CRC1 and CRC2). The characteristics of these genes including the sizes of the transcripts, coding sequences, and accession numbers were summarized in Table 2. Functional domains of the nine complement regulatory protein genes in channel catfish were listed in Table 3. Compared with the domains within relevant genes in human and zebrafish, the catfish genes shared very similar domain architecture to those in the human and zebrafish. For instance, the CFP gene in human, zebrafish and catfish all harbored one signal peptide SP domain and six thrombospondin type 1 repeats TSP1. Similarly, the domain architecture among human, zebrafish and catfish were conserved with CLU, CPN1, PLG, and VTN. However, with CPN2, the catfish gene contained the same domain structure

Table 2
Characteristics of the complement regulatory protein genes identified from channel catfish.

Gene name	Gene symbol	mRNA (bp)	CDS (aa)	Accession number
Properdin	CFP	1986	446	JT407929
Clusterin	CLU	1741	471	JT212553
Carboxypeptidase N polypeptide 1	CPN1	2489	450	JT412086
Carboxypeptidase N polypeptide 2	CPN2	2141	534	JT415830
RCA group 2 gene 1	CRC1	1057	316*	JT485772
RCA group 2 gene 2	CRC2	872	245*	JT319628
Plasminogen	PLG	2812	842	JT412020
Vitronectin a	VTNa	3896	492	JT407664
Vitronectin b	VTNb	2469	465	JT347360

Asterisk (*) indicated partial sequences.

Table 3
Domain architecture of complement regulatory protein genes in human, zebrafish and channel catfish.

	Human	Zebrafish	Channel catfish
CFP	SP-6TSP1	SP-6TSP1	SP-6TSP1
CLU	SP-CLb-CLa	SP-CLb-CLa	SP-CLb-CLa
CPN	SP-Zn_pept (CPN1)	Zn_pept (CPN1)	SP-Zn_pept (CPN1)
	SP-LRRNT-13LRR-LRRCT (CPN2)	LRRNT-9LRR-LRRCT (CPN2)	SP-LRRNT-13LRR-LRRCT (CPN2)
RCA group 2 genes	8SCR (C4BPα)	SP-5SCR-TM (ZRC1)	SP-4SCR-TM (CRC1)
	SP-30SCR-TM (CR1)	5SCR (ZRC2)	SP-4SCR (CRC2)
	SP-8SCR (CR1L)		
	SP-16SCR-TM (CR2)		
	SP-4SCR-TM (MCP)		
	SP-4SCR (DAF)		
PLG	SP-PAN_AP-5KR-Tryp_SPc	SP-PAN_AP-5KR-Tryp_SPc	SP-PAN_AP-5KR-Tryp_SPc
VTN	SP-SO-4HX	SP-SO-4HX (VTNa)	SP-SO-4HX (VTNa)
		SP-SO-4HX (VTNb)	SP-SO-4HX (VTNb)

The abbreviations of the functional domains were SP, signal peptide; TSP1, thrombospondin type 1 repeats; SO, somatomedin B domain; HX, hemopexin-like domain; Zn_pept, Zn_pept domain; LRRNT, leucine rich repeat N-terminal domain; LRR, leucine-rich repeats; LRRCT, leucine rich repeat C-terminal domain; CLb, clusterin beta chain; CLa, clusterin alpha chain; PAN_AP, divergent subfamily of APPLE domains; KR, kringle domain; Tryp_SPc, trypsin-like serine protease; SCR, short consensus repeats; TM, transmembrane region.

as that of the human gene, with one signal peptide SP, one leucine-rich repeat N-terminal domain LRRNT, 13 leucine-rich repeats LRR, and one leucine-rich repeat C-terminal domain LRRCT. In zebrafish, however, there was no signal peptide domain, and there were only 9 LRR rather than 13LRR. With RCA group 2 genes, the catfish CRC1 and CRC2 genes each had 4 short consensus repeats, but the zebrafish ZRC1 and ZRC2 genes each had 5 short consensus repeats (Table 3). Although the orthologies had not been established for RCA group 2 genes among human, zebrafish and catfish, the similar domains were present within genes in the RCA group 2 locus in human, as compared to those in zebrafish and catfish, in spite of differences in the number of short consensus repeat domain SCR (Table 3).

3.2. Phylogenetic analysis and gene annotation

Phylogenetic analysis was conducted to annotate the complement regulatory protein genes in catfish. Phylogenetic analysis for CFP, PLG, CPN1, CPN2, CLU, VTNa, and VTNb genes in channel catfish fell into proper clades as expected from the phylogenetic relationship with various organisms (Supplementary Figs. S1–4; Fig. 1). As shown in Fig. 1, the VTN genes were duplicated in the catfish genome as were in other fish genomes. Each of the VTN gene had evolved in separate sister clade, suggesting their origin as a consequence of the teleost-specific whole genome duplication. In contrast, the CRC1 and CRC2 genes were clustered together within each species as ZRCs in zebrafish and ARCs in frog (Fig. 2). In addition, CRC1 and CRC2 resembled each other with identity of 58%, suggesting that they were derived as duplicates within each species.

For the analysis of RCA group 2 genes, SCR domains were used for zebrafish ZRC1 and ZRC2 and catfish CRC1 and CRC2 genes. As shown in Fig. 3, the SCR domains in catfish fell into four distinct clades, A, B, C, and D, with the same pattern as in zebrafish (Wu et al., 2012). Thus, in spite of the number of domains being different in different species, the basic domain architecture was the same, with four basic domain components A, B, C, and D (Fig. 3).

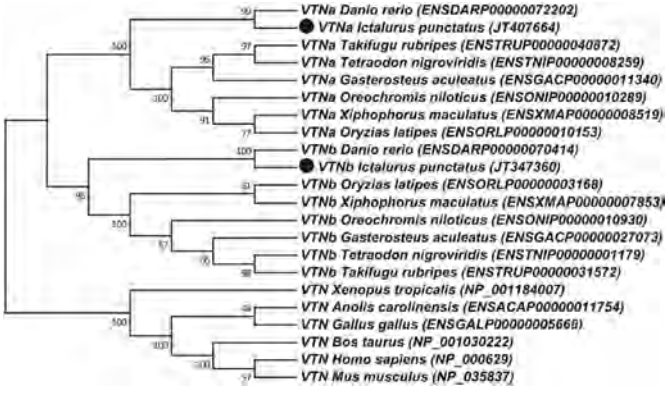

Fig. 1. Phylogenetic analysis of VTN genes. The phylogenetic tree was constructed based on amino acid sequences from selected fish species, birds and mammals using maximum likelihood method with 1,000 bootstrap replications in MEGA 5.2 software. The accession numbers were indicated following the species names. Dark dots indicated channel catfish VTN genes. Number on each node represented bootstrap probability.

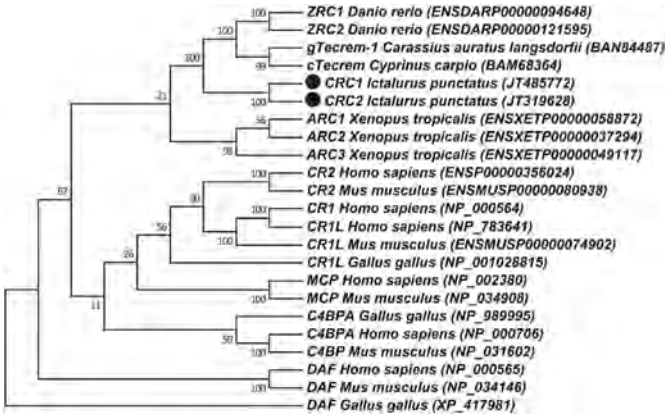

Fig. 2. Phylogenetic analysis of RCA group 2 genes. The phylogenetic tree was constructed based on amino acid sequences from zebrafish, frog, chicken and mammals using maximum likelihood method with 1000 bootstrap replications in MEGA 5.2 software. The accession numbers were indicated following the species names. Dark dots indicated channel catfish CRC genes. Number on each node represented bootstrap probability.

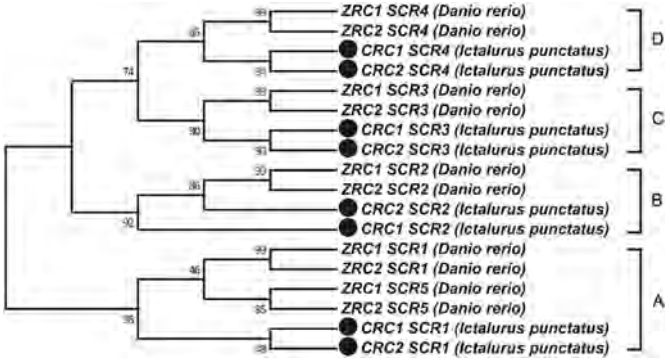

Fig. 3. Phylogenetic analysis of SCR domains from zebrafish ZRCs and channel catfish CRCs for individual SCR comparison. The phylogenetic tree was constructed based on each SCR amino acid sequence of ZRCs and CRCs using maximum likelihood method with 1000 bootstrap replications in MEGA 5.2 software. Four clades were formed and termed A, B, C, D followed zebrafish. Dark dots indicated each channel catfish SCRs. Number on each node represented bootstrap probability.

3.3. Syntenic analysis of catfish CRCs

Syntenic analysis was conducted to provide additional evidence for the orthologies of CRC genes. In human and frog, RCA group 2 genes were closely linked with PFKFB2 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2) gene, a maker gene for RCA group 2 loci, while in zebrafish, they were found closely linked with PFKFB1 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1) gene, 17 Mb downstream from zebrafish PFKFB2A. Catfish CRCs were found 3.2 Mb downstream of the PFKFB2A and closely linked with PFKFB1. As shown in Fig. 4, the catfish CRC genes were located between MAGI3 and PFKFB1 in the genome, which were highly conserved with that of zebrafish. In addition, catfish CRCs were also associated with MAGI3, PTPN22, ATP6AP1B, GDI1 and ITIH6. Therefore, the syntenic analysis supported the phylogenetic analysis. In addition, the CRC1 and CRC2 were tandem duplicates, as in the case of ZRC1 and ZRC2 in zebrafish (Fig. 4).

3.4. Expression of complement regulatory protein genes in healthy tissues

Tissue expression of the complement regulatory protein genes was studied using quantitative real-time PCR in six tissues of healthy catfish including gill, head kidney, intestine, liver, skin, and spleen. As shown in Fig. 5, nine complement regulatory protein genes were expressed in all six tissues but exhibited different tissue expression profiles. CFP, VTNa, CPN2 and PLG were all expressed highest in the liver, especially for CFP, VTNa and PLG with extremely high fold change, while the expression of these four genes in other tissues were relatively low. VTNb expression showed the highest level in skin followed by that in the liver. CPN1 was most highly expressed in spleen and liver. CLU and CRC2 were both expressed highly in spleen and skin, while CRC1 was also expressed at the highest level in spleen but followed by that in the gill.

3.5. Expression of complement regulatory protein genes after bacterial infection

Expression of the nine complement regulatory protein genes were determined after *E. ictaluri* and *F. columnare* bacterial infections in three mucosal tissues, gill, skin and intestine, at the time points of 4 h, 24 h and 3 d for the *E. ictaluri* challenge, and 4 h, 24 h and 48 h for the *F. columnare* challenge.

The expression profile after ESC infection was examined in two key mucosal tissues for pathogen adhesion, intestine and skin. As shown in Fig. 6, expression of most complement regulatory protein genes was mostly up-regulated after ESC infection in both skin and intestine, but with slightly different regulation patterns. In the skin, seven of the nine complement regulatory protein genes, VTNb, CPN1, CPN2, PLG, CLU, CRC1, and CRC2, were all up-regulated, at least during one time point after infection. Of these PLG and CLU

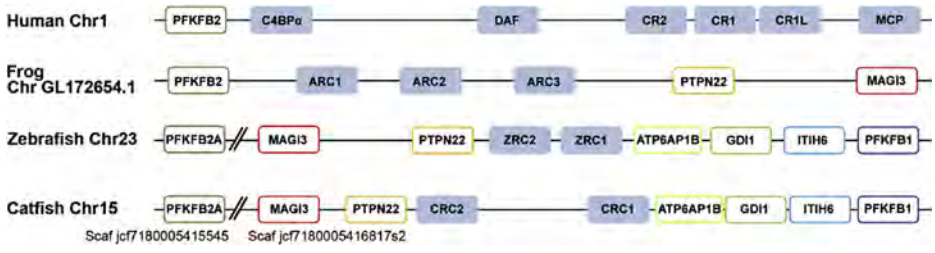

Fig. 4. Syntenic analysis of RCA group 2 genes from channel catfish, zebrafish, frog and human. The RCA group 2 genes were highlighted in blue color. The abbreviations are PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; PFKFB2A, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2a; MAGI3, membrane associated guanylate kinase, WW and PDZ domain containing 3; PTPN22, protein tyrosine phosphatase non-receptor type 22; ATP6AP1B, ATPase H+ transporting lysosomal accessory protein 1; GDI1, GDP dissociation inhibitor 1; ITIH6, inter-alpha-trypsin inhibitor heavy chain family member 6; PFKFB1, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1; C4BPα, complement component 4 binding protein alpha; DAF, decay accelerating factor; CR2, complement component (3d/Epstein Barr virus) receptor 2; CR1, complement component (3b/4b) receptor 1 (Knops blood group); CR1L, complement component (3b/4b) receptor 1-like; MCP, membrane cofactor protein; ARC1, amphibian RCA protein 1; ARC2, amphibian RCA protein 2; ARC3, amphibian RCA protein 3; ZRC1, zebrafish RCA group 2 gene 1; ZRC2, zebrafish RCA group 2 gene 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

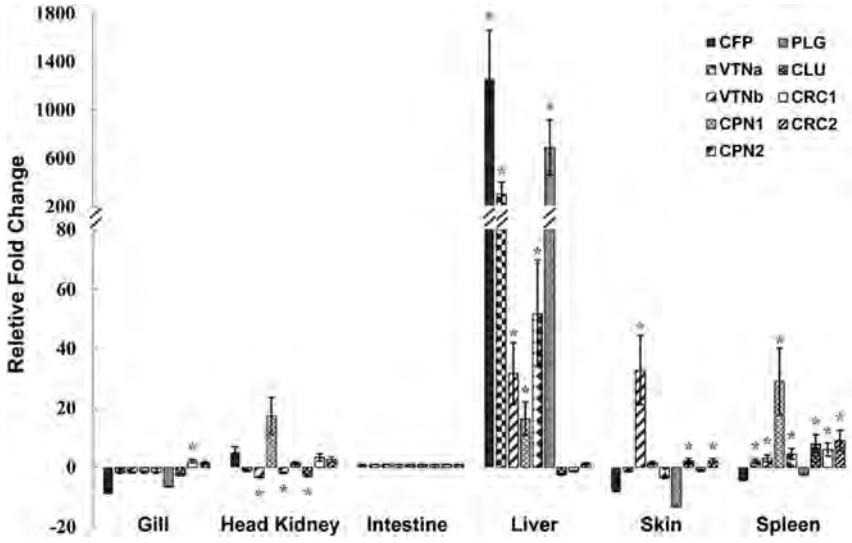

Fig. 5. Tissue expression analysis of complement regulatory protein genes in healthy channel catfish by quantitative real-time PCR. Relative expression values were calculated using intestine expression value as reference (set as 1) and normalized by the expression of 18S rRNA reference gene.

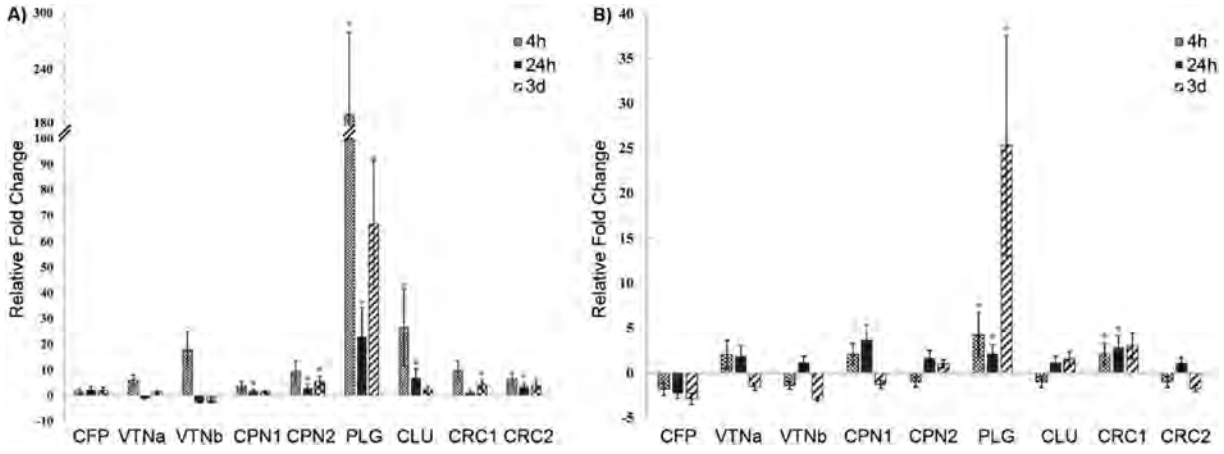

Fig. 6. Expression of complement regulatory protein genes following *Edwardsiella ictaluri* infection. Quantitative real-time PCR was used for the expression analysis in key mucosal tissues: skin (A) and intestine (B) at the time points of 4 h, 24 h and 3 d post-infection. The expression level of complement regulatory protein genes was presented as mean ± SE of fold change after *E. ictaluri* challenge to the control as normalized with 18S rRNA. Asterisks indicated statistical significant difference ($p < 0.05$).

were highly induced, especially with PLG that were up-regulated 192-fold. In the intestine, however, only PLG and CRC1 were significantly up-regulated, but the remaining seven genes were not significantly induced.

The pattern of expression of the nine complement regulatory protein genes after columnaris infection was drastically different from that after ESC infection. Instead of up-regulation, most of the nine genes were down-regulated in both tested mucosal tissues: skin and gill (Fig. 7). Of the nine complement regulatory protein genes, five genes, CFP, VTNa, VTNb, CPN2, and PLG, were significantly down-regulated, with exception of PLG gene at 48 h after infection in the skin.

4. Discussion

The complement system is an old but key element of innate immunity, protecting individuals from invading pathogens. Previous studies reported that the complement system in teleost could be activated through all three pathways as in the mammals (Boshra et al., 2006; Nakao et al., 2011). However, inappropriate and excessive activation could lead to the potential damage to the host cells. Therefore complement regulatory proteins are important to prevent tissue destruction caused by uncontrolled complement activation, thereby maintaining the homeostasis in the organism. Given the crucial roles of complement regulatory proteins in innate immunity, we identified nine complement regulatory protein genes in channel catfish, and examined their expression profiles in healthy tissues and following infection in order to determine their involvement in the innate immune responses.

The annotation of the complement regulatory protein genes was relatively straightforward. For RCA group 2 genes, tandem duplications existed in frog, zebrafish and catfish (Fig. 2). In the phylogenetic tree, the CRCs were together and formed an independent clade, which resembled ARCs and ZRCs, suggesting that the CRCs went through a duplication event to form RCA group 2 gene cluster as ARCs and ZRCs (Oshiumi et al., 2009; Wu et al., 2012). The duplication event might happen after teleost split from the common ancestor of vertebrates to fulfill species-specific functional requirements. The SCR domains of each CRC genes were divided into four groups (Fig. 3): SCR-A, B, C and D, resembling those SCRs

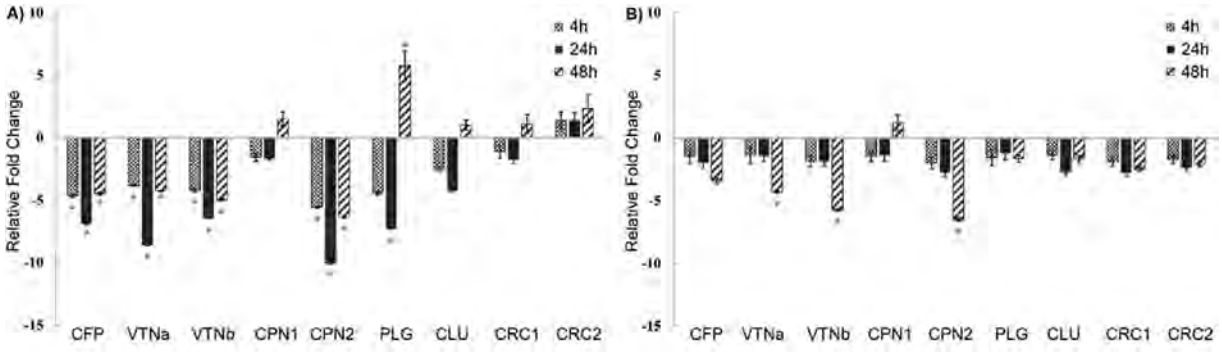

Fig. 7. Expression of complement regulatory protein genes following *Flavobacterium columnare* infection. Quantitative real-time PCR was used for the expression analysis in key mucosal tissues: skin (A) and gill (B) at the time points of 4 h, 24 h and 48 h post-infection. The expression level of complement regulatory protein genes was presented as mean ± SE of fold change after *F. columnare* challenge to the control as normalized with 18S rRNA. Asterisks indicated statistical significant difference ($p < 0.05$).

in zebrafish and frog RCA group 2 genes (Oshiumi et al., 2009; Wu et al., 2012). Oshiumi et al. (2009) suggested that the prototype of the RCA locus contains SCR domain combination of A, B, C, and D, then Wu et al. (2012) confirmed this combination presented in zebrafish. The observation in this study would add more information to the existing knowledge of the origin of the RCA group 2 cluster.

The complement regulatory protein genes have been well studied in mammals (Lindahl et al., 2000; Miwa and Song, 2001; Sjoberg et al., 2009; Zipfel and Skerka, 2009), but only some of these have been characterized in teleost fish (Nakao et al., 2011; Zhang et al., 2013b). Many complement regulatory protein genes in teleost fish were reported to possess extrahepatic expression while most mammalian complement regulatory protein genes were primarily expressed in the liver, such as zebrafish CD59 and CFP (Sun et al., 2013; Zhang et al., 2013b), channel catfish complement factor I, CD59 (Abernathy et al., 2009; Yeh and Klesius, 2007), large yellow croaker C1INH (Wei et al., 2010), rainbow trout CD59 (Papanastasiou et al., 2007) and rock bream CPN1 (Godahewa et al., 2014). In the present study, nine complement regulatory protein genes all showed similar extrahepatic expression, suggesting that complement regulatory protein genes in teleost fish might have distinct roles to maintain homeostasis.

To make the initial assessment of the potential roles of channel catfish complement regulatory protein genes in mucosal immune responses, expression profiles of the nine genes were conducted after two major bacterial infection, *E. ictaluri* and *F. columnare*. Previous studies reported that skin and intestine were considered to be the critical point of pathogen entry for *E. ictaluri* (Baldwin and Newton, 1993; Menanteau-Ledouble et al., 2011; Skirpstunas and Baldwin, 2002), while for *F. columnare*, it primarily infected catfish through gill and skin (Declercq et al., 2013; Sun et al., 2012). Therefore, complement regulatory protein gene expression profiles were determined following infection in these mucosal tissues in this study.

Most complement regulatory protein genes were up-regulated following ESC infection in skin and intestine, but slightly different expression patterns were observed between the two tissues. Notably, PLG were remarkably up-regulated in both tested tissues at all time-points, but it was induced the highest level at the early stage (4 h) in the skin while reached the highest expression three days after infection in the intestine, suggesting PLG may play distinct roles across different tissues. Mammalian PLG is reported to inhibit both alternative and classical pathway by binding and cleaving both C3 and C5 into small fragments (Barthel et al., 2012). PLG is also well known to play a central role in coagulation system. It is the precursor of plasmin, the active form plasmin could dissolve preformed fibrin clots and degrade extracellular matrix (ECM) (Lahteenmaki et al., 2005). Both coagulation and complement cascades help maintain homeostasis. Based on microarray expression profiling previously conducted on channel catfish, PLG gene was found 2.1-fold significantly up-regulated in liver after three days infected with *E. ictaluri* (as compared to uninfected fish) (Peatman et al., 2007). Similar result was shown in ayu (Li et al., 2011), suppressive subtractive hybridization (SSH) was used to identify differentially expressed genes in liver of ayu after infection with *Listonella anguillarum*, PLG was observed significantly up-regulated during infection. These observations were consistent with our result in channel catfish following ESC infection, indicating the importance of PLG to immune defense. In a study conducted by Seiffert et al. (1994), mice plasma VTN level increased 2–3-fold within 16 h after induction of intraperitoneal endotoxin administration. Moreover, VTN levels in heart, lung, brain were not significantly changed by endotoxin, suggesting VTN were expressed in a tissue-specific fashion in mice (Seiffert et al., 1994). Similar acute and tissue-specific expression pattern after ESC infection were also observed in this work, where VTNb was significantly up-regulated 4 h post-infection in skin, but not being significant in intestine. Mammalian CPN is a crucial protein that can cleaves and reduces the biological activities of the complement anaphylatoxins C3a and C5a by cleaving the C-terminal arginine in all three major complement activation pathways. CPN is a tetramer composed of two large subunits CPN1 and two small subunits CPN2. The small subunit CPN1 is known to contain the enzymatic active sites while the large subunit CPN2 protects the protein from degradation or filtration from the bloodstream (Matthews et al., 2004). Expression of channel catfish CPN1 was significantly induced at 24 h post-infection, while CPN2 was induced at both 24 h and 3 d post-infection in skin. Similarly, rock bream CPN1 showed significant elevation from 3 h to 48 h post-infection in response to five immune regulators, including pathogenic bacteria (*Streptococcusiniae, Edwardsiellatarda*), rock bream iridovirus (RBIV), the immune modulators polyinosinic:polycytidylic acid (poly I:C) and lipopolysaccharide (LPS) (Godahewa et al., 2014).

The most striking finding of this study is the drastic differences in expression profiles of complement regulatory protein genes after infection with different bacterial pathogens. In contrast to the significantly induced expression after *E. ictaluri* infection, the expression of complement regulatory protein genes after *F. columnare* infection were generally down-regulated. Both skin and gill exhibited a pattern of down-regulation across most of the complement regulatory protein genes following *F. columnare* challenge. Previous studies have reported that some complement regulatory protein genes were down-regulated in several different

cell types in response to infection (Thurman and Renner, 2011). MCP, a member of RCA group 2 gene in human, can also serve as a receptor for pathogenic piliated *Neisseria gonorrhoeae*. The expression of surface levels of MCP were highly reduced after exposure of cervical epithelial cells to piliated *Neisseria* (Gill et al., 2003). While in this work, the CRCs were mostly down-regulated but not being statistically significant. Thurman and Renner (2011) suggested that modulation of complement regulatory activities on cell surface might be adaptive responses to different environmental challenges. Increased complement regulation can protect the cells from bystander injury, while decreased complement regulation may limit the spread of infection by fostering local complement activation better suited for the elimination of pathogens (Thurman and Renner, 2011). In addition, even though *F. columnare* and *E. ictaluri* are both Gram-negative bacteria, *F. columnare* causes acute to chronic infections and typically affects external tissues gills and skin with few to no internal lesions, while *E. ictaluri* also occurs in acute and chronic forms but mainly entries through intestine and other mucosal epithelial sites, causing damage to the internal organ. Therefore different pathogenesis might also be the reason causing the distinct expression patterns after the two bacterial infections. Further work is needed to elucidate the role of complement regulatory protein genes after different bacterial infections in various tissues to improve the understanding of disease pathogenesis.

In summary, nine complement regulatory protein genes were identified and characterized in channel catfish. The vast majority of complement regulatory protein genes were significantly regulated after bacterial infections, suggesting their involvement in the host immune response to bacterial infections. Different pathogenesis of the two bacteria and different adaptive responses of the host indicated the pathogen-specific pattern of regulation. The distinct expression patterns revealed in this study should set the foundation for future functional analysis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2015.06.002.

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