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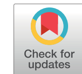
Gene profiling of antimicrobial peptides, complement factors and MHC molecules from the skin transcriptome of *Channa striatus* and its expression pattern during *Aeromonas hydrophila* infection



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ABSTRACT

Channa striatus is one of the economically important freshwater fish with high demand in Southeast Asia for its nutritional and medicinal values. The unique composition of skin mucus of murrel provides immunity against pathogens; however, they are susceptible to few bacterial pathogens especially *Aeromonas hydrophila*. Although few immune molecules such as antimicrobial peptides have already been identified from the murrel mucus, there is no report on the complete gene profile of the skin and mucosal immunity. Therefore, in this study we applied transcriptome approach to identify the mRNA sequences of various immune molecules such as antimicrobial peptides, complement factors and adaptive immune molecules from the skin tissue. Transcriptome wide search revealed unique mRNA sequences of 13 antimicrobial peptides, 11 complement components, 2 major histocompatibility complex proteins and its receptor, 6 butyrophilins, 2 leptins and its receptor. Brief bioinformatics analysis of the identified mRNA sequences and their respective putative protein sequences were performed to understand molecular information of those immune components. Further, we analysed the differential expression pattern of selected 13 mRNA sequences representing each immune group using qRT-PCR technique which highlighted the role of those genes during *A. hydrophila* challenge. Overall, this study revealed the complex immune response of murrel skin and the involvement of various innate and adaptive immune molecules against *A. hydrophila* infection.

1. Introduction

Channa striatus is a tropical, air breathing carnivorous fish which is commonly found in freshwater bodies of Asian countries especially India, Sri Lanka, China, Indonesia, Philippines, Vietnam and Thailand. In India, it is one of the most economically important species for its tasty flesh with nutritive and medicinal properties. The fish is consumed mainly to recover the lost energy after prolonged illness and to elevate wound healing process after surgeries. Reports suggested that murrel has a wide range of medicinal properties such as wound healing, antimicrobial property, antinociceptive property, osteoarthritic treatment, antioxidant property, cardiological treatment and neurological property [1].

Although epizootic ulcerative syndrome (EUS) is one of the common diseases found in *C. striatus*, they are also infected with various bacteria, fungi, virus and parasites. So far, various reports stated that murrel was susceptible to bacterial pathogens such as *Aeromonas hydrophila*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella salmonicida* [2]. However, the murrel skin protects the fish from those pathogens where the mucus plays a key role in protection. Murrel mucus has unique composition of immune molecules which highly contributes to the protective mechanisms of fish against pathogens [3,4]. Also, we have identified and reported various cytokines, antioxidant molecules, pattern recognition receptor (PRR) molecules such as lectins and few proteases, caspases and heat shock proteins from *C. striatus* and their differential expression pattern during *A. hydrophila* infection [5–7]. Rauta et al. [8] analysed the immunoglobulin (Ig) response in the serum of *C. striatus* to formalin-killed *A. hydrophila* antigen and implicated the

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role of Ig molecules during bacterial infection. However, the exact gene profile of antimicrobial peptides, complement components and adaptive immune molecules from the skin tissues of *C. striatus* during bacterial infection remain unclear. Therefore, it is necessary to determine the genetic profile of the key immune molecules from the skin which provides novel insights into the gene profiling of skin and mucosal immunity in *C. striatus*.

In this background, we performed transcriptome sequencing of skin tissue of healthy *C. striatus* using Illumina technology. Our primary interest was to identify the key immune molecules such as antimicrobial peptides and complement factors from the transcriptome library. Interestingly, we identified few other molecules such as MHC molecules, leptins and butyrophilins whose antibacterial roles were not reported earlier in fish. Further, we analysed the sequence and structural parameters of the identified genes and their proteins using bioinformatics tools. Also, we performed comparative qRT-PCR analysis to analyse the differential expression pattern of the selected potential genes between healthy and *A. hydrophila* challenged *C. striatus*.

2. Materials and methods

2.1. Animal and tissue collection

Healthy snakehead murrel, *C. striatus* (average weight 40 g) were purchased from a commercial aquaculture farm in Bhimavaram, Andhra Pradesh, India. Fishes were acclimatized in aerated water tanks (150 L) at 29 ± 2 °C for at least two weeks in the laboratory aquarium at SRM Institute of Science and Technology. A maximum of 10 fishes were maintained per tank during the experiment. Skin tissues from nine healthy individuals were collected, immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. RNA isolation and transcriptome construction

Total RNA was extracted from the isolated skin tissues and three sets of RNA pool were prepared using High Pure RNA Tissue kit (Roche Diagnostics GmbH, Germany). The integrity and quantity of RNA was confirmed by Bioanalyzer (Agilent). Based on the purity and integrity of the sample, one tissue pool was selected for RNAseq analysis. Approximately 5 μg of DNase-treated total RNA was used to construct the cDNA library by following the protocols of the Illumina TruSeq RNA Sample Preparation Kit (Illumina, USA). After necessary quantification and qualification, the library was sequenced using an Illumina HiSeq™ 2500 instrument with 100 bp paired-end (PE) reads. The reads generated by the Illumina sequencing platform were checked for quality parameters to obtain high quality transcripts using Fastq which includes parameters such as base quality score distributions, average base content per read and GC distribution. Cutadapt (https://github.com/marcelm/cutadapt) was used to remove the adapter sequences and all low quality (Q < 30) data were filtered out using Sickle (https://github.com/najoshi/sickle). The reads that are of length less than 30 bases were discarded and low quality bases were trimmed from 3′ and 5′ end. From the Trimmed paired-end reads, non-coding RNAs and mitochondrial RNAs were removed using Bowtie 2 (version 2.2.5) and Perlscripts. The reads were normalized and error-correction was performed using BBNorm, a kmer-based normalization tool for NGS reads. Finally, the cleaned reads were assembled using Trinity with default settings.

2.3. Transcriptome data analysis and bioinformatics characterisation

The assembled transcripts were annotated using translated BLAST (blastx) and Blast2Go PRO software by matching with complete UniProt database using blastx program and organism annotation. Gene Ontology (GO) terms [Molecular Function (MF), Cellular Component (CC), and Biological Process (BP)] for transcripts were mapped using Blast2Go. This study has been registered in EMBL database and all the full length sequences mentioned in this report have been submitted under accession number, PRJEB27485. Bioinformatics analysis was performed to understand the sequence parameters of the cDNA sequences and their respective protein sequences. Domain analysis was performed to understand the conserved domains located within each protein sequence using NCBI-Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignment was performed to determine the conserved amino acids between the homologous sequences using BioEdit sequence alignment editor (ver 7.2.6). I-Tasser online structure prediction tool (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to predict the three dimensional structure of selected immune proteins; and PyMOL (ver 0.99) was used to analyse the structural parameters of the predicted models. Transmembrane prediction was performed using MEMSAT ver 1.8 (http://www.sacs.ucsf.edu/cgi-bin/memsat.py).

2.4. Bacterial challenge and qRT-PCR

For bacterial challenge, the fish were injected intraperitoneally with *A. hydrophila* (5×10^6 CFU/mL) suspended in phosphate buffer saline (1× PBS) (100 μL/fish) while PBS injected fish were maintained as control. Skin tissue was dissected from the control and bacterial challenged fish after 24 h of challenge. Total RNA from the skin tissues were isolated using High Pure RNA Tissue kit (Roche Diagnostics GmbH, Germany) and cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol. qRT-PCR analysis was carried out using Roche Light Cycler instrument in 20 μL reaction volume containing 4 μL of cDNA from healthy and infected skin tissue, 10 μL of Fast SYBR®Green Master Mix, 0.5 μL of each primer (20 pmol/μL) and 5 μL dH_2O. The qRT-PCR cycle profile was 1 cycle of 95 °C for 10 s, followed by 35 cycles of 95 °C for 5 s, 58 °C for 10 s and 72°Cfor 20 s and finally 1 cycle of 95 °C for 15 s, 60 °C for 30 s and 95°Cfor 15 s. The same qRT-PCR cycle profile was used for the internal control gene, β-actin. The primers of the internal control gene were designed from the *C. striatus* β-actin sequence (Gen-Bank Accession No. EU570219). All the primers were designed using PrimerQuest tool of Integrated DNA technologies and purchased from Synergy Scientific Services, India (Table 1). Melting curve analysis was performed to confirm the specificity of the target genes. Finally, the data were analysed by $2^{-\Delta\Delta CT}$ method using the Light Cycler 96 software (Version 1.1.0.1320). The values are recorded as quantification cycle (Cq) values as mentioned in MIQE guidelines. All the analysis was performed in triplicates and the obtained data were subjected to statistical analysis, followed by an unpaired sample *t*-test. A significant difference was accepted at a P-value < 0.05.

3. Results and discussion

This is the first study to perform a transcriptome wide analysis in *C. striatus* to identify the antimicrobial peptides, complement factors and few adaptive immune molecules such as MHC molecules, leptins and butyrophilins. So far, the immune roles of leptins and butyrophilins have been reported with putative roles in adaptive immunity in higher vertebrates; however the immunological roles of those genes especially during bacterial infection have not yet been reported in fish. Also, there are no previous reports on the selected antimicrobial peptides and complement factors in *C. striatus*. Therefore, we identified 17 full length gene sequences (Table 2) encoding proteins with the above discussed functions from the transcriptome data and determined their expression pattern during *A. hydrophila* infection.

3.1. Antimicrobial peptides

In-depth analysis of skin transcriptome of *C. striatus* revealed 13

Table 1
List of gene expression primers used to analyse the differential expression pattern of selected immune genes during bacterial challenge.

Gene	Primer Code	Sequence
Piscidin	*Cs*PISC1 F1	CAGAGGATGAAGTGTACTGTGG
	*Cs*PISC1 R2	CAACTTGCCAACATGAAGGG
Hepcidin	*Cs*HAMP F1	GGCCTGACACTCATGACAAA
	*Cs*HAMP R2	CTGCAACTGCAATGCTGAAC
Tachykinin-1	*Cs*TAC1 F1	CAGCAACCAAATTCAGGATGG
	*Cs*TAC1 R2	TCAGACCGATGAACTGATGTG
LEAP-2	*Cs*LEAP2 F1	AACCATTTGGAGCCTACTGC
	*Cs*LEAP2 R2	TGTGTCCGGTGACATCTAGT
Lysozyme C	*Cs*LyzC F1	GTCATGAGGAGTCTGGTGTTTC
	*Cs*LyzC R2	CCATGCCGTGACTCTTCAAA
Complement Factor Properdin	*Cs*CFP F1	GCTCTGTTGGTTCTGGTTCT
	*Cs*CFP R2	CAGTCATCTTCATCCACCTCAC
Complement component 4	*Cs*C4 F1	CCGTCTGATTGCCTACTTCTAC
	*Cs*C4 R2	CATTGATCCCTGACATCTACCC
Complement c1qC	*Cs*C1qC F1	CACCAACATCGAAGGAGACTAC
	*Cs*C1qC R2	GTCGTCTAAAGAGGCGTGAAA
MHC Class 1 alpha antigen	*Cs*HLA1 F1	CTGGAAACAGAGACATGGATCG
	*Cs*HLA1 R2	AGGACAGATCTGGGTGAGATAG
MHC class II invariant chain	*Cs*HLADR F1	CCACAGGATTGGTTCCTACAA
	*Cs*HLADR R2	ATCCACACACCAGCAGAAG
Butyrophilin 2	*Cs*BTNL2 F1	GCGGTGAGATGACAAACAAAC
	*Cs*BTNL2 R2	CTTTCCAGCATCGAACAACATC
Leptin A	*Cs*LEPA F1	CCGGTAGAAGTGGTGAAGATG
	*Cs*LEPA R2	GGGACCTGGAAGTCTTTGTT
Leptin receptor	*Cs*LEPR F1	GAAGAAACTGGGACCTCACTAC
	*Cs*LEPR R2	CCTGATTCCTCTCAGCAACTAC
β-actin	*Cs*ßACT F1	TCTTCCAGCCTTCCTTCCTTGGTA
	*Cs*ßACT R2	GACGTCGCACTTCATGATGCTGTT

sequences which encoded proteins containing domains that are associated with antimicrobial functions. The identified proteins ranged from multifunctional, high molecular weight proteins such as lysozyme to low molecular weight, short antimicrobial peptides such as piscidin (Table 2). In total, there are five short antimicrobial peptides including piscidin isoforms (1 and 4), hepcidin, tachykinin-1, liver associated antimicrobial peptide 2 (LEAP-2) and thymosin beta-12. Other classical antimicrobial proteins include lysozyme C and lysozyme G, lipopolysaccharide-binding bactericidal permeability-increasing protein. This implies the involvement of wide range of host antimicrobial peptides against bacterial infections. It is also to be noted that proteins such as lysozyme are highly conserved with other fish homologs whereas, short peptides such as piscidin and thymosine are highly variable with limited number of conserved residues (Fig. 1). This showed the evolutionary conservation and variation pattern of antimicrobial peptides encoding genes in the fish immune system. We have discussed the sequence parameters and expression pattern of each antimicrobial protein in the following section.

Piscidins constitute a family of cationic antimicrobial peptides that are reported with membrane attacking and toroidal pore formation activity in teleosts [9]. Piscidins are known for its diversity and there are many isoforms of piscidins reported from the same organism. Fernandes et al. [10] reported the diversity of piscidins in Atlantic cod stating that the diversification of piscidins might be required to cope with the diversity of rapidly evolving pathogens. In our study, we have identified two isoforms of piscidin comprising 70 amino acids which are highly conserved in the N-terminal region whereas, the C-terminal is completely different between and within other teleost homologs (Fig. 1A) however structural analysis showed both the isoforms of *C. striatus* piscidins shared similar conformation with three distinct alpha helices (Fig. 1B). The α-helical structure is responsible for the amphipathic character of Piscidin which is involved in permeabilization of the bacterial membrane [9]. Sequence analysis showed that both piscidins belonged to pleurocidin family (pfam08107) of antimicrobial peptides which are generally found in the skin mucous secretions of fish. Differential expression analysis showed that piscidin (*Cs*PISC1) was highly up-regulated in skin tissue during *A. hydrophila* challenge (Fig. 2). It is also to be noted that piscidin showed highest level of up regulation after 24 h of bacterial challenge than other tested genes. This is obvious because the major role of these AMPs is in skin mucous against bacterial pathogens especially Gram negative bacterial lipid membrane [11].

Hepcidin is a multifunctional peptide found in teleost with antimicrobial as well as iron homeostasis functions. Jiang et al. [12] reported that stimulation of zebrafish with *A. hydrophila* DNA results in increased level of hepcidin protein and decrease in iron concentration in serum. This highlights the role of hepcidin in innate immune system during bacterial infection. In this study, we identified a full length hepcidin mRNA sequence encoding 89 amino acids which was 97% similar to its homolog from *Channa maculate* highlighting its conserved pattern between closely related organisms. Conserved domain analysis showed that the *Cs*HAMP protein belongs to Hepcidin superfamily (pfam06446) of AMPs. There are two forms of HAMP in fish namely HAMP1 and HAMP2 where *Cs*HAMP belongs to HAMP1 class of hepcidin. Hepcidin protein is a cysteine-rich protein that mainly expressed in the liver, however during bacterial and fungal infections they are secreted by various tissues including skin [13]. We observed that the expression of hepcidin gene was significantly up-regulated during *A. hydrophila* challenge (Fig. 2) in the skin of *C. striatus* which confirm its putative role in protection of fish skin from bacterial attack.

Tachykinin peptides belong to a large group with numerous members which are reported with antimicrobial activity from the skin and gut of amphibians. In mammals, they are present in the central nervous

Table 2
List of full length immune genes encoding antimicrobial peptides, complement components, MHC molecules and immunoglobulins identified from the transcriptome.

Accession Number	Protein	Identical organism	Similarity (%)	Length
LS974203	Piscidin	*Epinephelus bruneus*	87	256
LS974204	Piscidin-4	*Epinephelus coioides*	66	353
LS974205	Hepcidin	*Channa maculata*	97	541
LS974206	Tachykinin-1	*Gasterosteus aculeatus*	94	658
LS974207	Thymosin beta-12	*Lateolabrax japonicus*	97	1075
LS974208	Lysozyme G	*Channa striatus*	100	705
LS974209	Complement Factor D	*Oplegnathus fasciatus*	89	1153
LS974210	Complement component 1S	*Oplegnathus fasciatus*	83	2287
LS974211	Complement component 4	*Oplegnathus fasciatus*	82	5612
LS974212	Complement C1qB	*Oplegnathus fasciatus*	80	1163
LS974213	Complement C1qC	*Oplegnathus fasciatus*	74	1193
LS974214	Complement Factor Properdin	*Aphyosemion striatum*	81	1744
LS974215	MHC Class 1 alpha antigen	*Trachinotus ovatus*	84	1579
LS974216	MHC class II invariant chain	*Sinirca chuatsi*	85	1278
LS974217	Butyrophilin 2	*Larimichthys crocea*	67	1170
LS974310	Leptin A	*Sinirca chuatsi*	95	704
LS974311	Leptin receptor	*Scomber japonicus*	83	4697

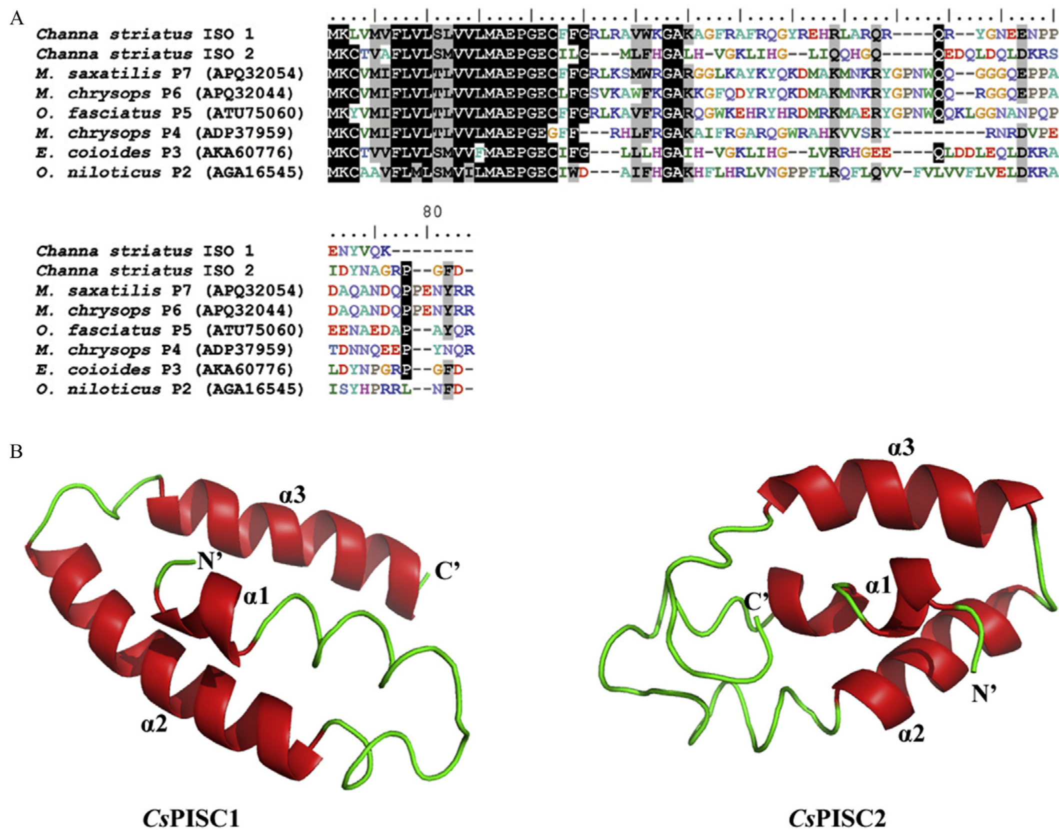

Fig. 1. Sequence and structural analysis of two isoforms of *Cs*PISC. A) Multiple sequence alignment of two isoforms of *Cs*PISC and six other homologs of piscidin showing the highly conserved N′ region and non-conserved C′ region between different piscidins from different fish species. B) Three dimensional structures of two isoforms of *Cs*PISC showing the similar structural conformation with three conserved α helices.

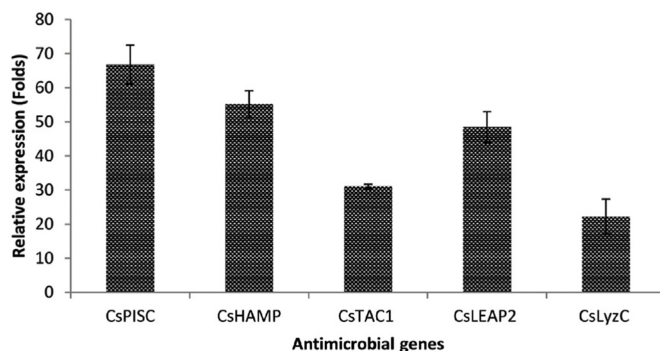

Fig. 2. Gene expression modulation of murrel antimicrobial peptides. The relative expression of five different genes encoding antimicrobial peptides, Piscidin, Hepcidin, Tachykinin, LEAP2 and Lysozyme C in 24 h post bacterial challenge from *C. striatus*. Data shown is the mean ± SD, n = 3.

system and gut with multiple functions. Although the neurological function of tachykinin in mammals are well characterised, the immunological roles especially during bacterial infection in fish is poorly understood. Sequence analysis of *Cs*TAC1 showed that the protein comprised of 115 amino acids with repeated Pro, Arg, Lys residues which is one of the unique feature of tachykinin. Simmaco et al. [14] reported the bioactivity of tachykinin like short peptides from the skin of frog which includes substance P which is one of the potential antimicrobial substances. *Cs*TAC1 also exhibited a similar sequence, ^{57}RKPRPHQFIGLM^{68} which shared high homology with the substance P, thus revealing the possibility of the involvement of *Cs*TAC1 in antibacterial immune mechanism. However, the exact role of the peptide sequence in *C. striatus* has to be elucidated in detail. Further, the gene expression results also clearly indicated the presence of tachykinin 1 in the skin of *C. striatus* and the up-regulation pattern showed the immune role of the protein against bacterial pathogens (Fig. 2).

Liver-expressed antimicrobial peptide 2 (LEAP-2) is another important antimicrobial peptide found in vertebrate species, first identified in human blood [15]. LEAP 2 although primarily expressed in liver, it is also expressed in various other tissues such as gill, skin, muscle, spleen, blood, head kidney, heart, brain and intestine in teleosts [16]. In *C. striatus*, a partial sequence encoding LEAP 2 was identified from the skin transcriptome which encoded for 31 residues at the C-terminal region of the LEAP protein. During bacterial infection, the expression of LEAP2 genes increased in most of the tissues except brain representing the ubiquitous nature and potential of this antimicrobial peptide [17]. Similarly, we observed an up-regulation pattern of *Cs*LEAP2 gene in *C. striatus* skin post *A. hydrophila* challenge, thus confirming the immunological role of LEAP-2 peptide during bacterial infection (Fig. 2).

Lysozyme is a major membranolytic enzyme produced by a wide range of organisms starting from bacteria, bacteriophages, fungi, plants and animals which specifically hydrolyse the 1, 4-beta-linkages between N-acetyl-*d*-glucosamine (NAG) and N-acetylmuramic acid (NAM) in peptidoglycan heteropolymers of prokaryotic cell walls [18]. In our study, two types of lysozyme were identified from the transcriptome of *C. striatus*, chicken type (LyzC) and goose type (LyzG). Both the group of lysozyme differ in amino acid sequence, structure, molecular weight as well as enzymatic mechanism [19]. In our earlier study [20], we reported the antibacterial role of *Cs*LyzG and its gene expression modulation during bacterial and fungal challenges. Therefore, in this study, we analysed the role of LyzC during *A. hydrophila* challenge which showed that *Cs*LyzC also gets up-regulated during bacterial challenge (Fig. 2).

Lipopolysaccharide binding proteins are also known as bactericidal

permeability increasing (BPI) protein which is another major class of antimicrobial peptide that function in a co-ordinated manner to facilitate an integrated host response to invading Gram-negative bacteria [21]. In *C. striatus*, we have identified 21 partial sequences encoding this class of proteins. Although the presence of multiple isoforms of BPI genes was identified, none of the sequence was full length and we did not include them for gene expression analysis as well as further characterisation of these genes is required to understand the molecular level information and their putative roles in antibacterial mechanism.

3.2. Complement proteins

It is a known fact that complement system is an important component of innate immune system in vertebrates with the involvement of around 35 individual proteins including activators and effectors which finally lyse foreign cells and opsonise foreign organisms for destruction by phagocytes. Although many studies reported the involvement of complement system in fish immune system [22], the components of fish complement system and the genes associated with those proteins and their modulation of expression pattern during bacterial infection remains unclear. In this study, we have identified several mRNA sequences encoding multiple complement proteins such as sub components of c1q B and C, c1q receptor, c1s which are the key components of classical pathway and c4 which is an important component of lectin-mediated/classical pathways and complement factors such as factor H, factor D and properdin which are involved in the alternative pathway (Fig. 3). Gene expression analysis was performed to understand the expression modulation of key components from each pathway (i.e) properdin, an activator component of alternative pathway, c4 from lectin mediated pathway and c1qC from classical pathway. Our results revealed that properdin and c4 are weakly activated whereas c1qC showed high level of up-regulation. This depicts that the complement system also plays an important role in *C. striatus* to combat the bacterial pathogen, *A. hydrophila* notably through classical pathway after 24 h post challenge (Fig. 4). Lu et al. [23] reported a similar expression pattern of complement components during *Aeromonas* infection in Zebra fish skin where properdin and c4 showed weaker up regulation after 24 h but significant expression modulation after 36 h post challenge whereas c1qC was highly expressed in both time points. This clearly indicated the significant role of classical pathway during *Aeromonas* infection in fish however other complement pathways were also involved in the defense mechanism.

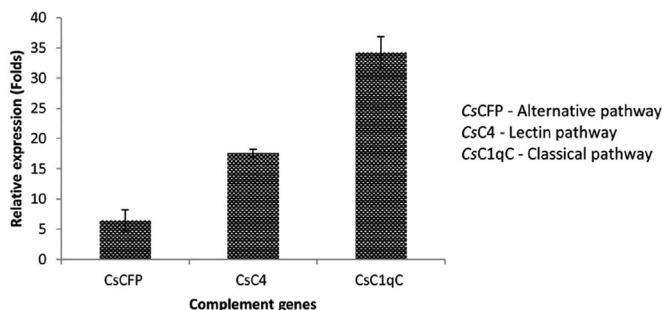

Fig. 4. *A. hydrophila* induced modulation of complement genes in *C. striatus*. The relative expression (in folds) of candidate genes of each pathway of complement system such as c1qC (classical), component 4 (lectin-mediated) and properdin (alternative) in 24 h post bacterial challenge from *C. striatus*. Data shown is the mean ± SD, n = 3.

3.3. Major histocompatibility complex (MHC)

T-cell mediated immune response require MHC presentation especially by antigen presenting cells which further activates the

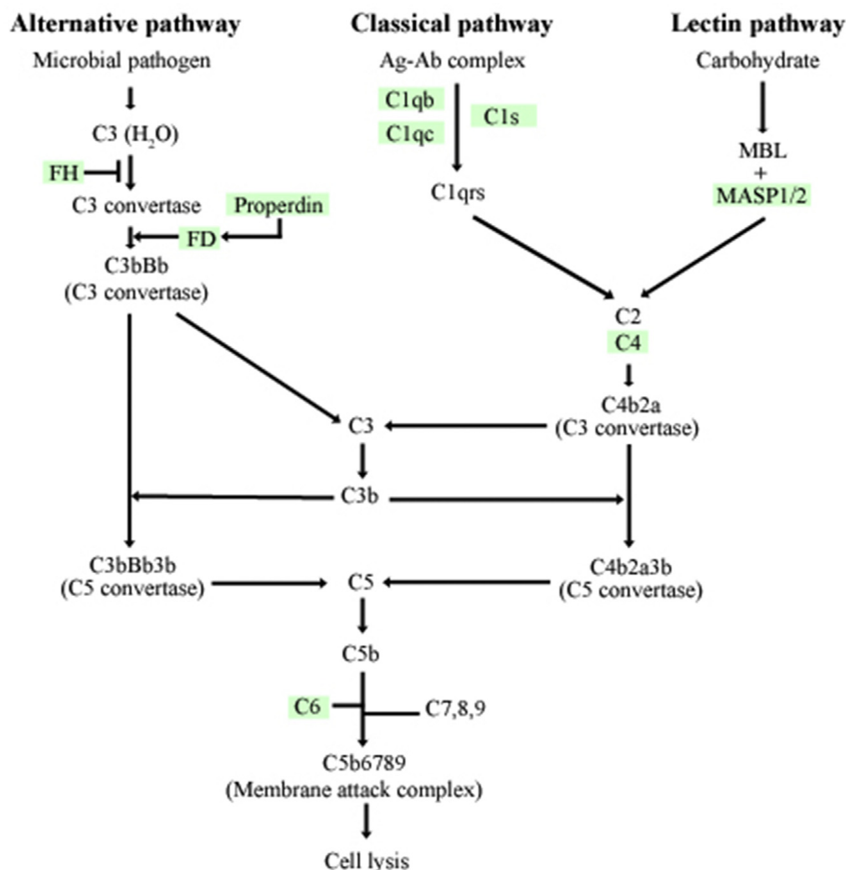

Fig. 3. Road map of Complement components in *C. striatus*. Based on the complement components identified from the skin transcriptome of *C. striatus*, the involvement of those components in three categories of complement pathways such as classical, lectin and alternative pathways were highlighted in green in standard complement pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

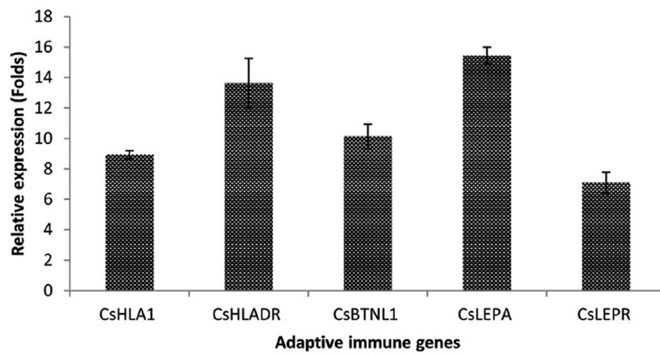

Fig. 5. Gene expression of adaptive immune genes during bacterial infection. The relative expression of various adaptive immune genes such as MHC-1α, MHC-II invariant chain, leptin a, leptin receptor and butyrophilin after *A. hydrophila* challenge (24 h p.i). Data shown is the mean ± SD, n = 3.

downstream cytotoxic function such as phagocytosis which provides innate immune cells with a mechanism to take up and destroy pathogenic bacteria, apoptotic cells and other large particles. Grimholt [24] classified the MHC molecules in teleosts where he stated that fish MHC molecules have been classified into various lineages and several of these non-classical lineages are only present in some teleost and the number of genes within each lineage greatly varies between teleost species. The MHC-I processing pathway is mainly involved in the presentation of endogenous antigens which are expressed in wide range of cells whereas MHC-II are professional antigen presenting cells which are expressed in limited cells especially B lymphocytes [25]. From the transcriptome data, we identified various genes encoding proteins such as alpha and beta subunits of MHC class I and class II molecules, MHC class II transactivator and MHC class II regulatory factor RFX1. So far, the role of MHC molecules in teleosts during bacterial encounter is not clear, therefore, we took the effort to analyse the modulation of MHC class I-α and MHC class II invariant chain during bacterial infection in *C. striatus* and we observed a weaker up-regulation pattern of both MHC molecules (Fig. 5). Romagnoli and Germain [26] demonstrated that the CLIP region of invariant chain plays a critical role in the structure folding and peptide occupancy in MHC-II molecules [27]. Liu et al. [28] reported that bacterial invasion in human activates intracellular MHC-II molecules which in turn promote TLR-triggered innate immune responses thus highlighting the role of MHC-II invariant chain during bacterial infection. However, the involvement of these MHC molecules during earlier stage of bacterial challenge was limited; hence there is a minimal level of up-regulation during bacterial infection in *C. striatus.*

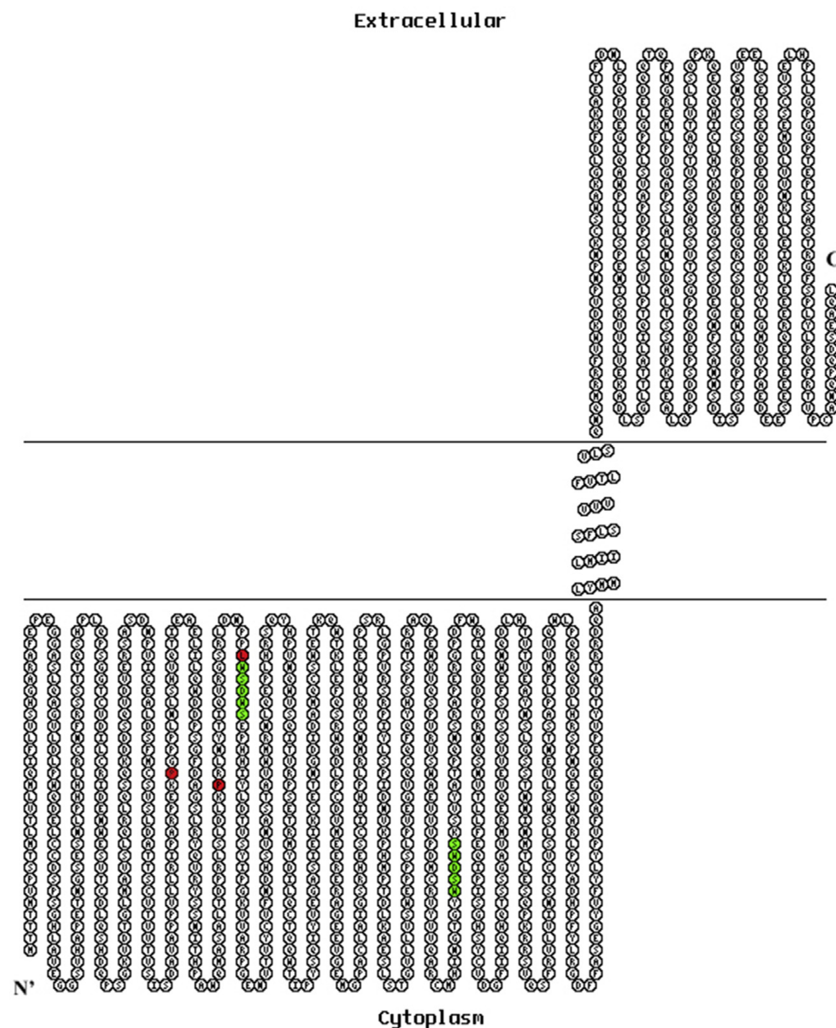

Fig. 6. Transmembrane region of leptin receptor. The transmembrane region of leptin receptor showing the helical region between 799 and 820 amino acids located in the membrane region with N-terminal in the cytoplasmic region and C-terminal in the extracellular space. In cytoplasmic region, red colour indicates residues involved in intradomain contacts and green colour indicates WSxWS cytokine receptor motif involved in cytokine binding function. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

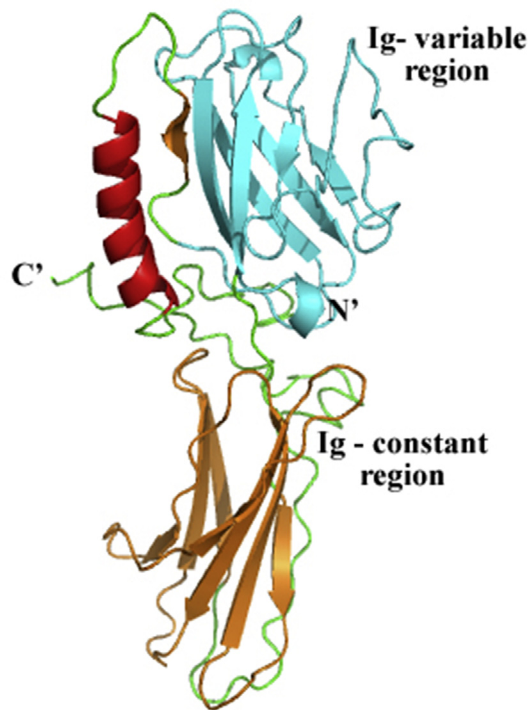

Fig. 7. Three-dimensional structure of butyrophilin. The three dimensional structure of butyrophilin 2 with two distinct immunoglobulin domains with one constant (orange colour) at the C-terminal and one variable domain (cyan colour) at the N-terminal. The helix is highlighted in red colour and coil in green colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Leptin and its receptors

Leptin is a cytokine produced by a wide range of cells which interplays among appetite, metabolism and energy storage. Apart from those functions, they are also involved in mucosal immunity. They are mainly expressed in adipose tissue and its secretion is strongly upregulated during skin injury. Leptins exert anti-inflammatory effects by mediating innate response and in contrast stimulate T-lymphocytes mediated pro-inflammatory response thus suggesting a rather complex role for leptin in immune-mediated inflammatory conditions in humans [29]. Although multiple roles of fish leptins in food intake, fat metabolism and reproduction have been reported [30], the exact roles of these leptins in fish immune system are still unclear. In this study, we identified two full length sequences encoding leptin A and leptin receptors from the transcriptome of *C. striatus*. Transmembrane analysis of the leptin receptor revealed that the receptor is a transmembrane protein with a single transmembrane region (799–820) of the 1153 length protein (Fig. 6). Upon binding of the extracellular receptor fragment with the leptins, the long intracellular tail of the leptin receptor mediates the activation of signalling cascades of various pathways such as JAK2, STAT3 which is crucial in wound healing and energy metabolism during microbial infections [31,32]. Especially, two WSxWS motifs present in the intracellular region of leptin receptor plays a major role in cytokine binding which is responsible for the activation of further downstream signalling and the proline residues P^{208} and P^{271} are involved in the intradomain interactions. Moreover, the differential gene expression analysis revealed that leptin and its receptor genes were significantly up-regulated during bacterial infection stating their roles in preventing the bacterial infection in murrel skin (Fig. 5). The structural analysis and differential expression pattern together highlighted the immunological role of leptins and its receptors during bacterial infection and its involvement in wound healing.

3.5. Butyrophilins

Butyrophilins belonging to the immunoglobulin superfamily are one of the recently identified immune regulators which are reported to be present in wide range of immune cells such as lymphocytes, dendritic cells, monocytes, macrophages, neutrophils and eosinophils in human; however, their immune roles in fish are still unclear. Therefore, we identified different isoforms of butyrophilin 1 (A1), butyrophilin 2 (A23), butyrophilin 3 (A2) and butyrophilin 3 (A3) from the skin transcriptome of *C. striatus*. Among the identified butyrophilin isoforms, *Cs*BTNL2 was selected for sequence characterisation and gene expression analysis. *Cs*BTNL2 contained two conserved regions: a variable type immunoglobulin domain (Ig-V) located between 48 and 136 amino acids and a constant type immunoglobulin domain (Ig-C) of antigen receptor CD80 between 171 and 230 residues in the *Cs*BTNL2 sequence (Fig. 7). Gene expression analysis showed that *Cs*BTNL2 was up-regulated during bacterial challenge revealing that the gene plays a key role in bacterial challenge (Fig. 5). Xia et al. [33] reported that butyrophilin protein over-expressed in LPS-challenged Asian Seabass which suggest that this immunoglobulin protein plays a major role in adaptive immunity-mediated expulsion of bacterial pathogens. Therefore, this report acclaims the immunological role of butyrophilin genes during bacterial infection in teleost however further protein level studies are required to confirm the exact functions of these proteins during bacterial infections.

4. Conclusion

This study delivers important information about the mRNA sequences of various antimicrobial peptides, complement components, MHC molecules, butyrophilins, leptins and its receptors in *C. striatus* and their involvement during *A. hydrophila* challenge thus highlighting their involvement in the defense mechanism. Based on this study, the genes of specific interest can be studied further to characterise them at molecular level which might give novel insights into the bacterial resistance mechanism in fish.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.09.061.

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