**Analysis of apolipoprotein genes and their involvement in disease response of channel
catfish after bacterial infection**

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Abstract

Apolipoproteins are protein component of plasma lipoproteins. They exert crucial roles in lipoprotein metabolism and serve as enzyme cofactors, receptor ligands, and lipid transfer carriers in mammals. In teleosts, apolipoproteins are also involved in diverse processes including embryonic and ontogenic development, liver and digestive system organogenesis, and innate immunity. In this study, we identified a set of 19 apolipoprotein genes in channel catfish (*Ictalurus punctatus*). Phylogenetic analysis and syntenic analysis were conducted to determine their identities and evolutionary relationships. The expression signatures of apolipoproteins in channel catfish were determined in healthy tissues and after infections with two major bacterial pathogens, *Edwardsiella ictaluri* and *Flavobacterium columnare*. In healthy channel catfish, most apolipoprotein genes exhibited tissue-specific expression patterns in channel catfish. After ESC and columnaris infections, 5 and 7 apolipoprotein genes were differentially expressed respectively, which presented a pathogen-specific and time-dependent pattern of regulation. After ESC infection, three exchangeable apolipoproteins (apoA-IB, apoC-I, and apoE-B) were suppressed in catfish intestine, while two nonexchangeable apolipoproteins (apoB-A and apoB-B) were slightly up-regulated. After columnaris infection, apoB-B, apoD-B, and apoE-A were significantly down-regulated in catfish gill, while apoF, apoL-IV, apoO-like, and apo-14kDa showed significantly up-regulation. Taken together, these results suggested that apolipoprotein genes may play significant roles in innate immune responses to bacterial pathogens in channel catfish.

34 **Keywords:** Fish; Genome; Disease; Immune response; Innate immunity

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1. Introduction

Apolipoproteins, the structural components of various plasma lipoproteins, bind and transport lipids to various tissues through lymphatic and circulatory systems (Havel, 1975; Kondo et al., 2005). Most of apolipoproteins are mainly synthesized in liver, intestine, and adipose tissues, which interact with lipoproteins and form distinct lipoprotein complexes depending on apolipoprotein species (Mahley et al., 1984). Apolipoproteins are mainly grouped into two major types in vertebrates, exchangeable and non-exchangeable apolipoproteins. Exchangeable apolipoproteins (apoA, apoC and apoE classes) are low-molecular-weight proteins featured by repeated amphipathic alpha helix regions for reversible lipid and lipoprotein binding (Babin et al., 1999; Wang et al., 1997). Non-exchangeable apolipoproteins, mainly comprised of apoB class, contain amphipathic beta-strand domains as their lipid-associating motif and associate with lipid droplets irreversibly (Segrest et al., 1998). In addition to these two typical types of apolipoproteins, there are also atypical apolipoproteins, including apoD, apoF, apoH, apoL, apoM, apoN, and apoO classes. They have no similarities to exchangeable or non-exchangeable apolipoproteins, but are also associated with plasma lipoproteins and involved in lipid metabolism (Page et al., 2001; Xu and Dahlbäck, 1999).

Apolipoproteins exert crucial roles in regulating the metabolism of lipoproteins and serve as enzyme cofactors, receptor ligands, and lipid transfer carriers in mammals (Mahley et al., 1984). Notably, apolipoproteins are also involved in various metabolic states and immune responses (Hardardóttir et al., 1997; Van den Elzen et al., 2005). In mammals, many

apolipoprotein species have been reported to have roles in modulating the inflammatory response to a major pathogenic factor lipopolysaccharide (LPS), either by directly binding to LPS or by regulating the function of LPS-response cells (Berbée et al., 2010; Henning et al., 2011). In contrast to the situation in mammals, only two members in apoA class (apoA-I and apoA-II) were reported as potential immune modulators or antimicrobial proteins in teleost fish (Concha et al., 2004; Johnston et al., 2008; Villarroel et al., 2007). Largely, this is because apolipoprotein genes have not been systematically characterized in teleost, and information of their expression after bacterial infection in teleost fish is lacking.

Channel catfish is a dominant aquaculture species in the United States. The catfish industry continues to be threatened by bacterial pathogens such as *Edwardsiella ictaluri* (Li et al., 2012) and *Flavobacterium columnare* (Sun et al., 2012). In order to generate effective strategies for better managing fish diseases, it is of great importance to study the immune-related genes and their expression patterns during disease pathogenesis. In the present study, a complete set of 19 apolipoprotein genes were identified and characterized in channel catfish. Their expression profiles in healthy tissues and after infection with bacterial pathogens *E. ictaluri* and *F. columnare* were determined in channel catfish.

2. Materials and methods

2.1 Gene identification and sequence analysis

TBLASTN was conducted against catfish transcriptome databases (Li et al., 2012; Lu et al., 2011; Sun et al., 2012) using apolipoprotein amino acid sequences in human (*Homo*

sapiens), zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), and tilapia (*Oreochromis niloticus*) retrieved from the Ensembl (<http://www.ensembl.org>), Uniprot (<http://www.uniprot.org/>), and NCBI (<http://www.ncbi.nlm.nih.gov/>) as queries, with a cutoff E-value of $1e^{-5}$. Raw sequences were then aligned by Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) to remove the duplicates and generate a unique set of sequences. Open reading frames (ORF) were identified in ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and further validated by BLASTP against NCBI non-redundant (nr) protein database. BLASTN was conducted against the reference genome sequence of channel catfish (Liu et al., 2016) to assure sequence accuracy with a cutoff E-value of $1e^{-10}$. The genes from retrieved sequences were predicted by FGENESH program of Molquest software (Softberry Int.) and were further confirmed by BLASTP against non-redundant (nr) protein database. Sequence analysis and secondary structure prediction were performed by PredictProtein (<https://www.predictprotein.org/>).

2.2 Phylogenetic and syntenic analysis

Phylogenetic analysis was conducted using amino acid sequences of apolipoprotein genes from channel catfish and selected vertebrate species retrieved from NCBI, Uniprot, and Ensembl, including mammals (*Homo sapiens*, *Mus musculus*), bird (*Gallus gallus*), amphibian (*Xenopus tropicalis*), and teleost fish (*Danio rerio*, *Ictalurus punctatus*). Multiple protein sequences were aligned by MUSCLE with default parameters (Edgar, 2004). The phylogenetic trees were constructed using maximum likelihood method in MEGA 6 (Tamura et al., 2013). JTT (Jones-Taylor-Thornton) + I (invariant sites) + G (gamma distribution for

modeling rate heterogeneity) model was selected according to the alignment results (Darriba et al., 2011). Gaps were removed by complete deletion and bootstrapping with 1000 replications were conducted to evaluate the overall phylogenetic tree.

Syntenic analysis was conducted by comparing genome regions that harbor apolipoprotein genes in channel catfish with other vertebrates. The neighboring genes of the catfish apolipoprotein genes were identified from channel catfish scaffolds by FGENESH program in Molquest software (Softberry Int.) and were further confirmed by BLASTP against non-redundant (nr) database. Ensembl and Genomicus (Louis et al., 2015) databases were used to determine the conserved syntenic pattern of apolipoprotein genes among various vertebrates.

2.3 Expression analysis of apolipoprotein genes in healthy channel catfish tissues

The expression patterns of apolipoprotein genes were determined in channel catfish by quantitative real-time PCR (qPCR). All procedures involved in handling and treatment of catfish were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC). After euthanized with MS-222 (200 mg/L), eight tissues (skin, spleen, head kidney, liver, gill, trunk kidney, intestine, muscle) from nine healthy catfish (Marion strain) (three fish each for three replicate samples) were pooled together for each tissue. Total RNA was extracted using RNeasy Plus Universal Mini kit (Qiagen, USA). The RNA concentration and integrity were measured using Nanodrop 2000 Spectrophotometer (NanoDrop Technologies, USA). A uniform quantity of DNA-free RNA was used to synthesize first-strand cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). All

the cDNA products were then diluted to 250 ng/μL. The qPCR was performed using SYBR Green PCR Master Mix on a CFX96 real-time PCR detection system (Bio-Rad, USA). The cycling conditions of qPCR were denaturation, 95 °C/30 s, 40 cycles of 95 °C/5 s, 60 °C/5 s, and 72 °C/5 s. The 18s rRNA gene was used as an internal reference gene (Small et al., 2008). All gene specific primers were designed by Primer3 software (Rozen and Skaletsky, 1999) and listed in [Appendix: Supplementary table S1](#). The specificity of primers was assessed by aligning with the channel catfish reference genome sequence (Liu et al., 2016) using BLASTN with E-value of $1e^{-10}$. The melting curve analysis was performed to demonstrate that each primer set amplified a single product. The expression levels of each gene were expressed relative to those of 18S rRNA in each sample using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Tissue with lowest C_t value of each gene was used as the reference for normalization of the relative expression of apolipoprotein genes such that all the values are greater than 1. The data of real-time PCR were expressed as the standard error of the mean (SEM). Two-tailed Student's t test was used for the significance test of the gene expression levels. P -value < 0.05 was considered statistically significant. The qPCR was repeated in triplicate runs to confirm expression patterns.

2.4 Expression analysis of apolipoprotein genes following E. ictaluri and F. columnare infections

Meta-analysis of RNA-Seq data was conducted using our previous RNA-Seq datasets in response to *E. ictaluri* (SRP009069) and *F. columnare* infection (SRP012586). The detailed procedures were described in our previous studies (Li et al., 2012; Sun et al., 2012). The

annotated 26,661 protein-coding genes containing the 19 catfish apolipoprotein genes was used as reference sequences for mapping analysis using CLC Genomics Workbench software package (V5.5.2). At least 95% of sequence identity and a maximum of two mismatches were allowed. Reads per kilobase of exon model per million mapped reads (RPKM) (Brockman et al., 2008) was calculated as original expression value. After normalization of the RPKM, fold changes were calculated using proportions-based Kal's test (Kal et al., 1999) to determine the differential expression pattern between control and treatment groups with FDR corrected p-value < 0.05. Transcripts with fold change value of larger than 1.5 and aligned read number larger than 5 were considered as differently expressed genes.

3. Results and discussion

3.1 Identification and annotation of apolipoprotein genes in channel catfish

A total of 19 apolipoprotein genes were identified in channel catfish, including apoA-IA, apoA-IB, apoA-IV, apoB-A, apoB-B, apoC-I, apoC-II, apoD-A, apoD-B, apoE-A, apoE-B, apoF, apoL-I, apoL-IV, apoL-VI, apoM, apoO, apoO-like, and apo-14kDa. The lengths of cDNA of apolipoprotein genes range from 594 to 12,587 bp, and the lengths of encoded protein sequences vary from 87 to 3,865 amino acids ([Appendix: Supplementary table S2](#)). The annotation of apolipoprotein genes were fairly explicit based on the phylogenetic and syntenic analysis. As shown in the overall phylogenetic tree (Fig. 1A) and detailed phylogenetic analysis for each class of apolipoprotein genes ([Appendix: Supplemental Figure S1-S10](#)), most catfish apolipoprotein genes were clustered into the clades with their counterparts in zebrafish, and then grouped with orthologous genes in tetrapods, except

apoC-II, apoD-A, apoE-B, apoL-I, apoO-like, and apo-14kDa. For these five genes, syntenic analysis was conducted that provided strong evidence for their annotation and orthologies, as they all were located in the same conserved syntenic blocks with similar neighboring genes (Fig. 1B). Interestingly, five apolipoprotein genes [apoA-IV (apoa4), apoC-I (apoc1), apoC-II (apoc2), apoE-B (apoeb), and apo-14kDa (apo14kda)] in catfish are tandemly arranged in the same scaffold. As human exchangeable apolipoproteins (apoA, apoC, and apoE classes) have the same genomic structure that probably evolved from a common ancestor (Li et al., 1988), the tandem arrangement of four exchangeable apolipoproteins and apo14-kda indicate that they were likely derived from tandem gene duplications (Korbel et al., 2008). Particularly, the fish-lineage specific apolipoprotein apo-14kDa appears to share similar genomic arrangement and neighboring genes as compared with apoA-II in zebrafish, indicating that apo-14kDa might be homologue to apoA-II. This is consistent with a previous study (Choudhury et al., 2009), which stated that fish apo-14kDa was homologue of mammalian apoA-II and termed as apoA-II-like protein.

3.2 Copy numbers of apolipoprotein genes

The analysis of copy numbers of apolipoprotein genes across a broad spectrum of species should provide insights into evolution of apolipoprotein genes. Overall, the numbers of apolipoprotein genes vary greatly among organisms (Fig. 1C). The duplicated catfish apolipoprotein genes (apoA-I, apoB, apoD, and apoE) are likely derived from the teleost-specific whole genome duplication or independent tandem duplication events (Hoegg et al., 2004; Thornton and DeSalle, 2000). Moreover, apoB and apoD in several other teleost

fish (zebrafish, medaka, and tilapia) generate more than 2 paralogous copies, indicating various levels of gene duplication for apolipoprotein genes in teleost fish. In the case of apoO, zebrafish, medaka, and tilapia have two paralogous copies (apoO-A, apoO-B), while only one copy is present in channel catfish, perhaps due to loss of this gene after whole genome duplication in teleosts (Hoegg et al., 2004). Four apolipoproteins, apoA-V, apoC-III, apoC-IV, and apoN, were not found in any of the teleost genomes.

3.3 Tissue expression of apolipoprotein genes in channel catfish

Most apolipoprotein genes exhibited tissue-specific expression patterns in channel catfish. Exchangeable apolipoprotein genes (apoA, apoC, and apoE classes) were expressed at high levels in catfish intestine and skin ([Appendix: Supplemental Figure S11](#)). Two copies of apoA-I showed quite distinct tissue expression profiles. ApoA-IA was expressed at relatively high level in skin, while apoA-IB was expressed at a high level in the intestine. As apoA-I was considered to be responsible for epidermal and mucosal barriers in antimicrobial activities (Concha et al., 2004), two copies in catfish may exert different roles in immune responses within epidermal and mucosal barriers respectively. ApoA-IV was highly expressed in catfish intestine, indicating its role in controlling lipid intake in small intestine (Li et al., 2015a). The catfish apoC-I and apoC-II were also expressed at high levels in liver and intestine. ApoE-A and apoE-B were highly expressed in fish intestine and skin. Nonexchangeable apolipoproteins (apoB-A and apoB-B) were mainly expressed in intestine, followed by liver ([Appendix: Supplemental Figure S12](#)). This expression pattern was consistent with that in humans, in which apoB was mainly expressed in liver and small

intestine (Davidson and Shelness, 2000). Most atypical apolipoproteins (apoD, apoF, apoL and apoO classes) were expressed at relatively high levels in skin ([Appendix: Supplemental Figure S13](#)). Notably, apoM and apo-14kDa showed similar expression profiles, which were expressed at high levels in liver. This is consistent with their expression patterns in *Hemibarbus mylodon* (Kim et al., 2009).

3.4 Expression of apolipoprotein genes after *E. ictaluri* and *F. columnare* infections

After ESC infection, non-exchangeable apolipoprotein genes, apoB-A and apoB-B, were moderately induced in catfish intestine during the pathogenesis of *E. ictaluri*, while three of the seven exchangeable apolipoproteins, apoA-IB, apoC-I and apoE-B, were generally down-regulated starting one day after ESC infection (Fig. 2A). After columnaris infection, three apolipoprotein genes (apoB-B, apoD-B, apoE-A) were suppressed, while four apolipoprotein genes (apoF, apoL-IV, apoO-like, and apo-14kDa) were induced in catfish gill (Fig. 2B). Taken together, a total of 11 apolipoprotein genes were differentially expressed after bacterial infections in catfish, suggesting their potential involvement in bacterial responses. Different apolipoproteins were involved in differentially expressed genes after ESC and columnaris infections. The only overlapping gene was apoB-B that was up-regulated in intestine after ESC infection, but down-regulated in gill after columnaris infection. Such distinct expression pattern of the specific immune related genes was likely caused by very different pathogenesis of these two bacteria in different tissues (Fu et al., 2016; Jiang et al., 2015; Li et al., 2015b). ESC disease starts from intestine and spreads to internal tissues (liver, spleen, and kidney) (Shotts et al., 1986), while columnaris pathogen

mainly infects external mucosal tissues gill and skin (Shoemaker et al., 2008).

Apolipoproteins have been reported to exert functions in modulating inflammatory response to LPS either by directly binding or by regulating LPS-response cells (Berbee et al., 2005). As a component of bacterial outer membrane, LPS is recognized by toll-like receptor 4 (TLR4) and initiate a complex signal-transduction cascade to activate transcription factor nuclear factor- κ B (NF- κ B) and pro-inflammatory cytokines such as tumour-necrosis factor (TNF) (Miller et al., 2005). LPS was reported to be important in the pathogenesis of ESC (Bader et al., 2004; Newton and Triche, 1993) and columnaris (MacLean et al., 2003). After ESC and columnaris infections, many differentially expressed genes with crucial roles in pathogen recognition and inflammatory responses were detected, including pattern recognition receptor (PRR) molecules (e.g. lectins, toll-like receptors) and pro-inflammatory cytokines (e.g. interferons, chemokines) (Li et al., 2012; Sun et al., 2012). The observed differential expression pattern of apolipoprotein genes after bacterial infections suggest their direct involvements in immune responses, likely through the mechanism of LPS-binding as demonstrated in previous studies (Berbée et al., 2010; Ma et al., 2004; Rensen et al., 1997).

ApoA-I, apoC-I, and apoE were demonstrated to bind LPS (Berbée et al., 2010; Ma et al., 2004; Rensen et al., 1997). More specifically, apoA-I could directly bind LPS and neutralize LPS cytotoxicity (Ma et al., 2004). ApoA-I possesses a variety of immune-related properties, including antibacterial activity, antiviral activity and inflammatory responses (Johnston et al., 2008). In teleosts, apoA-I is responsible for both epidermal and mucosal barriers to inhibit the growth of fish bacterial pathogens (Concha et al., 2004). Similarly,

245 apoC-I contains structural elements to bind LPS and to enhance the proinflammatory
246 response toward LPS via a LPS-binding protein (LBP)-like mechanism (Berbée et al., 2010).
247 In this LBP-like mechanism, apoC-I directly associates with LPS and presents it toward
248 CD14, and then CD14 ultimately presents LPS to MD2/TLR4 complex to stimulate the
249 TLR4-dependent inflammatory response (Berbée et al., 2010). Likewise, apoE binds to LPS
250 and protects against bacterial lipopolysaccharide-induced lethality (Van Oosten et al., 2001).
251 The down-regulation of apoE-B might be repressed by LPS via inhibition of its promoter
252 activity (Gafencu et al., 2007). The downstream transcription factors (NF- κ B and AP-1)
253 interact directly on the apoE promoter (Gafencu et al., 2007). In addition, apoB class is a
254 crucial component of predominant LPS-binding lipoproteins (LDL and VLDL) (Vreugdenhil
255 et al., 2001), and they were reported to interact with LPS to protect the host from deleterious
256 effects of LPS toxicity (Netea et al., 1998; Van Lenten et al., 1986). Here, we found that two
257 non-exchangeable apolipoproteins (apoB-A and apoB-B) were both mildly up-regulated at 3
258 h and 24 h after infection with *E. ictaluri*, suggesting that they could be similarly involved in
259 LPS binding. After columnaris infection, the repression of apoD might be mediated by
260 transcription factors APRE-3, AP-1 and NF- κ B, which directly bind to the distinct binding
261 sites in the promotor of apoD in response to LPS (Do Carmo et al., 2007). ApoL-IV was
262 mildly up-regulated at 24 h after infection with *F. columnare*. In humans, apoL class could be
263 induced by pro-inflammatory signaling molecules, such as interferons and tumor necrosis
264 factor alpha (TNF-alpha) (Smith and Malik, 2009). As the interferon-inducible inflammatory
265 responses were observed in our datasets (Sun et al., 2012), apoL-IV in catfish gill might be an
266 interferon-inducible gene which was up-regulated via interferon (IFN)-mediated pathways.

The teleost-specific apolipoprotein apo-14kDa was up-regulated with a fold change of 3.19 at 2 d post-infection. As a homologue to apoA-II, apo-14kDa might also contribute significantly to the antimicrobial activity like apoA-II. Consistent with our observations here, apo-14kDa was found to be up-regulated in turbot after injected with viral stimuli, indicating that apo-14kDa may be an immune-related protein (Pereiro et al., 2012). Expression of apoF and apoO-like were both found significantly induced in catfish after infection with *F. columnare*, suggesting their potential involvement in the immune response as well, although there is no report of involvement of these genes in the literature. Further studies are warranted to investigate their roles in immune responses.

In summary, a complete set of 19 apolipoprotein genes were identified and annotated in channel catfish. The expression pattern in healthy tissues indicated that most apolipoprotein genes were mainly expressed in mucosal tissues (skin and intestine). Eleven of the 19 apolipoprotein genes were regulated after infections with *E. ictaluri* and *F. columnare*, suggesting their potential roles in catfish immune response to bacterial infections.

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Figure captions

Fig. 1. Identification and annotation of apolipoprotein genes. (A) Overall phylogenetic analysis of 19 apolipoprotein genes using sequences from catfish, zebrafish, xenopus, chicken, mouse and human. The red and black dots indicate catfish and zebrafish apolipoprotein genes, respectively. Bootstrap (1000 replications) support values appear on the branches. The Latin name and accession numbers were provided in [Appendix: Supplementary File S1](#). (B) Syntenic analysis of apoC-II (apoc2), apo-14kDa (apo14kDa), apoD-A (apoda), apoL-I (apol1) and apoO-like (apool) genes in catfish with human, chicken, zebrafish, tilapia or medaka. The apolipoprotein genes are highlighted by navy color filled boxes. Full gene names were provided in [Appendix: Supplementary S1](#). (C) Copy numbers of apolipoprotein genes in several representative vertebrate genomes. Blue shading indicated the mammal-specific apolipoprotein genes. Grey shading indicated that these genes are absent from teleosts or otherwise as extended into additional taxa. Orange shading indicated that they are teleost-specific genes. Green shading indicated species-specific genes. Species names were abbreviated as in [Appendix: Supplementary S1](#).

Fig. 2. Expression profiles of catfish apolipoprotein genes after two bacterial infections. (A) Expression levels of catfish apolipoprotein genes in intestine after *E. ictaluri* infection at 3 h, 24 h and 72 h. Gene expressions were presented as fold change relative to control samples. Asterisks (*) indicated significant differences (p-value < 0.05, expression fold change > 1.5, read number > 5). Transcripts with total read number less than 5 were not included in the analyses. (B) Expression of catfish apolipoprotein genes in gill after *F. columnare* infection at

473 4 h, 24 h and 48 h. Gene expressions were presented as fold change relative to control
474 samples. Asterisks (*) indicated significant differences (p-value < 0.05, expression fold
475 change > 1.5, read number > 5). Transcripts with total read number less than 5 were not
476 included in the analyses.
477

Figure 1

Figure 2

A)

B)