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Pathogen recognition receptors in channel catfish: IV. Identification, phylogeny and expression analysis of peptidoglycan recognition proteins

Luyang Sun, Shikai Liu, Ruijia Wang, Chao Li, Jiaren Zhang, Zhanjiang Liu *

The Fish Molecular Genetics and Biotechnology Laboratory, Aquatic Genomics Unit, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, 203 Swingle Hall, Auburn, AL 36849, USA

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ABSTRACT

Peptidoglycan recognition proteins (PGRPs) can recognize bacterial cell wall (peptidoglycan) and activate innate immune system. In addition to its function as pathogen recognition receptors (PRRs), PGRPs are also involved in directly killing bacteria, and regulating multiple signaling pathways. Recently, we have reported catfish PRRs including nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs), retinoic acid inducible gene I (RIG-I) like receptors (RLRs), and Toll-like receptors (TLRs). In this study, we identified and characterized the PGRP gene family in channel catfish which included two members, PGLYRP-5 and PGLYRP-6. Phylogenetic analysis, syntenic analysis and protein structural analysis were conducted to determine their identities and evolutionary relationships. In order to gain insight into the roles of PGRPs in catfish innate immune responses, quantitative real-time PCR was used to investigate the expression profiles in catfish healthy tissues and after bacterial infection. Both PGLYRP-5 and PGLYRP-6 were ubiquitously expressed in all 12 healthy tissues, and most highly expressed in gill and spleen, respectively. Distinct expression patterns were observed for PGRPs after infection with *Edwardsiella ictaluri* and *Flavobacterium columnare*, both Gram-negative bacteria. After infection with *E. ictaluri*, both PGLYRP-5 and PGLYRP-6 were significantly down-regulated at a certain time-point, while both genes were generally up-regulated in the gill after infection with *F. columnare*. Collectively, these findings suggested that PGRPs may play complex roles in the host immune response to bacterial pathogens in catfish.

1. Introduction

Peptidoglycan recognition receptor proteins (PGRPs) are a group of pattern recognition receptors (PRRs) that can recognize bacterial peptidoglycan (PG) and activate innate immune system. PGRPs were first isolated in silkworm (*Bombyx mori*) as a bacterial PG binding protein that activate the prophenoloxidase cascade (Yoshida et al., 1996). Unlike other PRRs such as nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and Toll-like receptors (TLRs), PGRPs can also play roles in directly killing the invading microbes, in addition to their roles as PG receptors (Sukhithasri et al., 2013).

PGRPs are ubiquitous and conserved in most animal species, from insects to mammals (Dziarski and Gupta, 2006a,b; Kang et al., 1998; Sukhithasri et al., 2013). All PGRPs contain at least one conserved PGRP domain, which provides the ability to interact with bacterial PG (Royet et al., 2011). Insects have up to 19 PGRPs, which can be classified into two categories based on the length of their transcripts: short (S) and long (L) (Werner et al., 2000). In mammals, four PGRPs have been identified which are PGLYRP-1, PGLYRP-2, PGLYRP-3 and PGLYRP-4, respectively. Distinct from other three PGRPs in mammals, PGLYRP-2 contains a PGRP domain with amidase activity that can hydrolyze bacterial PG by cleaving the lactyl bond between NAM and stem peptide (Li et al., 2006).

PGRPs have also been identified in several fish species including zebrafish (Li et al., 2007), rockfish (Kim et al., 2010), large yellow croaker (Mao et al., 2010), rainbow trout (Jang et al., 2013), and grass carp (Li et al., 2013, 2014). Up to date, three PGRPs were identified in teleost fish including PGLYRP-2, PGLYRP-5 and PGLYRP-6. Among them, only PGLYRP-2 is homologous to mammal PGLYRP-2, while PGLYRP-5 and PGLYRP-6 are only found in teleost fish. PGLYRP-1, PGLYRP-3 and PGLYRP-4 homologues have not been found from teleost fish.

Numerous studies of PGRPs have been conducted to investigate their roles in innate immune system (Royet et al., 2011). In fruit fly (*Drosophila melanogaster*), PGRPs were reported to control microorganisms in the gut mucosal epithelium by regulating antimicrobial

* Corresponding author. Tel.: +1 334 844 4784; fax: +1 334 844 2937.
E-mail address: liuzhan@auburn.edu (Z. Liu).

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peptide (AMP) and reactive oxygen species (ROS) (Bischoff et al., 2006; Gendrin et al., 2009; Zaidman-Rémy et al., 2006). PGRPs were reported to present a direct bactericidal function and play roles in defense against pathogen adherence in mollusk (Itoh and Takahashi, 2008; Su et al., 2007). Zebrafish PGRPs were induced during embryogenesis in order to protect the embryo from the invasion of bacteria existing in the surrounding water (Li et al., 2007). Suppression of PGLYRP-5 and PGLYRP-6 in zebrafish resulted in reduced resistance to *Salmonella enterica*, *Bacillus subtilis* and *Flavobacterium columnare*, respectively (Chang and Nie, 2008; Li et al., 2007). After *Edwardsiella tarda* infection, PGRPs were induced in rainbow trout (*Oncorhynchus mykiss*) liver cells, suggesting their roles in modulating the innate immune responses (Jang et al., 2013). In grass carp (*Ctenopharyngodon idella*), PGLYRP-5 was found to be involved in anti-stress response and Toll-like receptor signaling pathway (Li et al., 2013), as well as PGLYRP-6 was reported to activate NF-κB activity and induce the expression of NOD2, a PRR gene (Li et al., 2014).

Channel catfish, *Ictalurus punctatus*, is one of the most important aquaculture species in the United States. However, the total catfish value of sales decreased 20 percent from 2011 to 2012 (USDA, 2013). Large scale catfish bacterial disease breakouts were one of the main reasons for the losses of catfish production. Among them, enteric septicemia of catfish (ESC) and columnaris are two of the most severe catfish diseases (Shoemaker et al., 2008; Wagner et al., 2002). To achieve a healthy development of catfish industry, the diseases resistant catfish lines are much needed. Several important genes potentially involved in disease resistance and stress tolerance have been identified and characterized in catfish (Bao et al., 2005, 2006; Baoprasertkul et al., 2007, 2006; Geng et al., 2014; Jiang et al., 2010; Liu et al., 2013; Rajendran et al., 2012a; Wang et al., 2006, 2013; Zhang et al., 2013).

In the previous studies, whereas we have identified three major groups of PRRs in catfish, i.e., NLRs (Rajendran et al., 2012a), RLRs (Rajendran et al., 2012b) and TLRs (Zhang et al., 2013), the knowledge about the breadth and function of the PGRP family in channel catfish is still limited. Therefore, in this study, we sought to characterize PGRP genes and determine their expression patterns after bacterial infection in catfish. Here we report the identification and phylogenies of channel catfish PGRPs, and their expression after infection with the bacterial pathogens *F. columnare* and *Edwardsiella ictaluri*, the causative agent for columnaris and ESC disease, respectively.

2. Materials and methods

2.1. Gene identification and sequence analysis

The catfish PRGPs were identified from the catfish transcriptome database by TBLASTN using human and zebrafish PGRPs as queries, with a cutoff *E-value* of $1e^{-5}$. The transcriptome database was generated by RNA-Seq analysis of a doubled haploid channel catfish (Liu et al., 2012), which has been used as the main resource for identification of full-length gene transcripts in various catfish gene family studies (Liu et al., 2013; Rajendran et al., 2012a, b; Wang et al., 2013, 2014; Zhang et al., 2013). After obtaining the initial pool of PRGP sequences, raw sequences were then aligned by using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) to remove the duplicates and generate a unique set of sequences. The unique set of sequences were used to blast against catfish daft whole genome sequence database (unpublished) with a cutoff *E-value* of $1e^{-10}$ for further confirmation. Open reading frame (ORF) was identified by using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The identities of the predicted ORFs were then verified by BLASTP against NCBI non-redundant (nr) protein database with a cutoff *E-value* of $1e^{-5}$. Also, conserved domains of each obtained sequence were predicted to verify the identities of catfish PGRPs by using NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Putative signal peptides were predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), and the possible N-glycosylation sites were identified by using NetNGlyc 1.0 program (http://www.expasy.ch/tools/protparam.html). The putative isoelectric point and molecular weight were computed using Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The secondary and tertiary structure of catfish PGRP proteins were predicted using Geneious 7.0.6 (http://www.geneious.com/) and 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/), respectively.

2.2. Phylogenetic analysis

The PGRPs from other eight vertebrates, including zebrafish (*Danio rerio*), grass carp, rainbow trout, green-spotted pufferfish (*Tetraodon nifroviridis*), human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*) and cattle (*Bos taurus*), were chosen for phylogenetic analysis with catfish PGRPs. The amino acid sequences of PGRPs from these species were retrieved from the NCBI, Ensembl Genome Browser and UniProt databases. Multiple PGRP protein sequences were aligned using the ClustalW2 program (Larkin et al., 2007). Phylogenetic trees were constructed using MEGA 5.2 with neighbor-joining method (Tamura et al., 2011). Bootstrapping with 1000 replications was conducted to evaluate the phylogenetic tree.

2.3. Syntenic analysis

To identify the flanking genes of catfish PGRP genes in the genome, the catfish PGRP coding sequences were searched against the catfish preliminary genome assembly (unpublished) to obtain the scaffolds containing PGRPs using BLASTN with a cutoff *E-value* of $1e^{-10}$. The flanking genes of PGRPs were predicted using GENSCAN (http://genes.mit.edu/GENSCAN.html). The identities of these genes were confirmed by running BLASTP against NCBI nr database. The syntenic analysis was conducted by comparing with several other fish species with whole genome sequences, such as zebrafish (*D. rerio*), platyfish (*Xiphophorus maculatus*), stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*) and spotted gar (*Lepisosteus oculatus*).

2.4. Bacterial challenge and sample collection

All procedures involved in handling and treatment of fish during this study were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) prior to initiation. All challenge procedures of *E. ictaluri* and *F. columnare* were conducted following previously detailed protocols (Sun et al., 2012; Zhang et al., 2013). Briefly, the bacteria were first cultured from a single colony, inoculated to health catfish, and then re-isolated from asymptomatic fish. Fish for ESC infection were challenged in 40 L aquaria with 4 control and 4 treatment groups. Aquaria with 40 healthy fish each were randomly divided into four sampling timepoints: 4 h, 24 h, 3 d and 7 d post infection for both control and treatment groups. *E. ictaluri* (MS-S97-773) with a concentration of 4×10^8 colony forming unit (CFU)/ml were added into the treatment aquaria for 2 h with the water flow turned off. Control groups were treated with the same routine as treatment groups. At each sampling timepoint, 30 fish were collected from the appropriate control and treatment aquaria and euthanized with MS-222 (300 mg/L) for sample collection. Gill, skin and intestine from 10 fish (3 replicates of 10 fish each) were pooled together