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Transcriptomic analysis of the head kidney of Topmouth culter (*Culter alburnus*) infected with *Flavobacterium columnare* with an emphasis on phagosome pathway

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ABSTRACT

Flavobacterium columnare (FC) has caused worldwide fish columnaris disease with high mortality and great economic losses in cultured fish, including Topmouth culter (*Culter alburnus*). However, the knowledge about the host factors involved in FC infection is little known. In this study, the transcriptomic profiles of the head kidney from Topmouth culter with or without FC infection were obtained using HiSeq™ 2500 (Illumina). Totally 79,641 unigenes with high quality were obtained. Among them, 4037 differently expressed genes, including 1217 up-regulated and 2820 down-regulated genes, were identified and enriched using databases of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The differently expressed genes were mainly associated with pathways such as immune response, carbohydrate metabolism, amino acid metabolism, and lipid metabolism. Since phagocytosis is a central mechanism of innate immune response by host cells to defense against infectious agents, genes related to the phagosome pathway were scrutinized and 9 differently expressed phagosome-related genes were identified including 3 up-regulated and 6 down-regulated genes. Five of them were further validated by quantitative real-time polymerase chain reaction (qRT-PCR). This transcriptomic analysis of host genes in response to FC infection provides data towards understanding the infection mechanisms and will shed a new light on the prevention of columnaris.

1. Introduction

Flavobacterium columnare (FC) is the causative agent of columnaris disease of fish and is a member of the family *Flavobacteriaceae* [1]. The FC infection can result in fin erosion, gill necrosis, and skin lesions, leading to high mortality and severe economical losses in aquaculture industry [2]. Columnaris is a major disease in many wild and cultured fish species, such as channel catfish (*Ictalurus punctatus*), perch (*Perca fluviatilis*), rainbow trout (*Oncorthynchus mykiss*), Topmouth culter (*Culter alburnus*) [3–5]. Topmouth culter, a predatory fish of Cyprinidae, is one of the most popular fish species in Chinese aquatic markets and widely distributed in East Asia [6]. It has now been a national protected fish species due to the sharply declined natural resources [7]. To prevent the columnaris in Topmouth culter culture, identification of host factors involved in FC infection was significantly important. Although the expression profiles of immune-related genes in Mandarin fish, channel catfish, and grass carp upon FC infection have been analyzed using transcriptomic sequencing, real-time PCR, or two-dimensional electrophoresis immunoblotting [8–10], systematic analysis of host factors related to the interactions between FC and Topmouth culter is not available. Genome-wide transcriptomic analysis using

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ultrahigh-throughput sequencing technologies has been widely used to study pathogen infections [6,8,11–14]. Host response upon bacteria infection was associated with several biological processes such as immunity response and phagocytosis. Phagocytosis is a critical process responsible for the elimination of pathogens, such as fungi and bacteria. In the present study, the transcriptomic sequencing of Topmouth culter with and without FC infection was performed, and the differently expressed genes related to phagosome were scrutinized. Moreover, the expression patterns of five differently expressed phagosome-related genes were verified by qRT-PCR. These results will help to reveal the complexity of the FC-Topmouth culter interactions and may provide new insight to counteract the FC infection.

2. Material and methods

2.1. Fish, bacteria and infection

Flavobacterium columnare (FC) was isolated from diseased yellow catfish (*Pelteobagrus fulvidraco*) and kept in our laboratory. All animal experiments were performed using wild Topmouth culter bred in our laboratory and were approved by guidelines of the Committee on the Ethics of Animal Experiments of the Shandong Freshwater Fisheries Research Institute. Fish were maintained at 28–30 °C in a recirculating freshwater system and acclimatized in laboratory for 2 weeks before they were divided into two groups with 10 fish/group (mean body mass 152 ± 13.1 g). Topmouth culter was intraperitoneally infected with FC at the dose of 10^4 c.f.u/g body weights or equal volume of PBS as negative control. Head kidney was collected at 40 h post of infection. The samples were snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA extraction, cDNA library construction and transcriptome sequencing

RNA extraction and quantification of the samples from FC-infected and non-infected groups (ten fish per group) were performed using TRIzol reagent (Invitrogen, USA) and Eppendorf Biophotometer (Hamburg, Germany) respectively. The cDNA was reverse transcribed from these RNA samples and then quantified using several kits according to the manufacturer's instructions as previously reported [8]. The cDNA libraries of each group were mixed by equal volume with normalized 10 nM and then diluted to 4–5 pM for sequencing using HiSeq™ 2500 (Illumina).

2.3. Raw data cleaning, De novo assembly and gene annotation

The clean reads of FC-infected and non-infected samples were acquired from the raw data by removing the adaptor sequences and low quality sequence using the FastQC program, and then assembling into contigs using the Trinity software. *De novo* assembling of the cleaned reads were performed using sequence data from both the FC-infected and the non-infected samples. The transcripts were subjected to BLASTX for similarity searching against NCBI non-redundant protein database (NR) to identify the unigenes [13]. For functional annotation analysis, all unigenes were searched against the SwissProt, Eukaryotic of Orthologous Groups (KOG), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) [15–17].

2.4. Identification of differently expressed genes and cluster analysis

For differential gene expression analysis, gene expression level was normalized based on Poisson model as previously described [18]. Statistical comparison between FC-infected and non-infected groups was conducted using a web tool DESeq. Genes with *p*-value < 0.05 and fold change >2 were considered differently expressed. Differently expressed genes were enriched and analyzed with GO and KEGG.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

To validate the HiSeq™ 2500 sequencing data, five differently expressed phagosome-related genes between FC-infected and non-infected groups, including syntaxin 12 (STX12), neutrophil cytosolic factor 2 (NCF2), transferrin receptor 1a (TFR1a), ATPase, complement component c3a (C3a) were selected for qRT-PCR assay using the same RNA samples used for the transcriptomic sequencing. Primers were designed and shown in Table 1. The cDNA was synthesized from 1 μg of RNA using M-MuLV reverse transcriptase (Qiagen) and then used as templates for qRT-PCR. The qRT-PCR reaction was performed using similar reaction mixture and condition as previously described [8]. All qRT-PCR reactions were done in triplicate and target specificity was determined by the dissociation curve analysis. The relative expression ratio of the target genes versus 18S rRNA gene was calculated using $2^{-\Delta\Delta CT}$ method and all data were given in terms of relative mRNA expression [19].

3. Results

3.1. Sequencing and de novo assembly

To identify the genes involved in the response of Topmouth culter to FC infection, two cDNA libraries were created from mRNAs extracted from the head kidney of FC-infected and non-infected groups and then sequenced using Illumina HiSeq™ 2500 system. Totally 73,355,532 and 75,508,492 raw reads were identified from FC-infected and non-infected samples respectively (Table 2). After removing of the adaptors and low-quality sequences, 71,624,922 and 73,725,384 cleaned reads were acquired respectively (Table 2). The GC content of the clean reads from both groups is close to 50% (Table 2). The length distribution of the unigenes was organized. It showed that the most abundant unigenes were clustered in a group with 301–500 bp in length (Fig. 1).

3.2. Functional annotation

In order to annotate the unigenes, unigene sequences were subjected to blast search in the NCBI non-redundant (NR) database. A total of 31,678 unigenes, accounting for 39.78% of total unigenes, were annotated using NR database. When the unigenes were further aligned to different databases, the numbers of annotated unigenes were varied (Table 3): Swiss-Prot (25,333, 31.81%), KOG (36,179, 45.43%), GO (17,783, 22.33%), and KEGG (9,711, 12.19%). To better understand the functional distribution of the unigenes, KOG function classification were performed. The unigenes from KOG

Table 1
Primers used for qRT-PCR verification of differently expressed genes.

Gene name[a]	Forward primer (5′-3′)	Backward primer (5′-3′)
C3a	TTGAATACTGGCCGACAAGA	CCTTGATTGTTGCTGAACTTA
ATPase	GCTCAACTCCGCCTCAATA	AGCACCTGACCAATTACC
STX12	GCACTCCTGAGACTCATT	GATCAGTGCTATTGAGCTACAT
NCF2	CTTCACAGACACATGCAGG	GTGCACCAATATGCATCAATTC
TFR1a	CACTCATGTGCTATAATTCGTC	TGGGTTATTTGCCATGATGTC
18S rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

[a] C3a: Complement component C3a; ATPase: vacuolar ATPase; STX12: Syntaxin 12; NCF2: Neutrophil cytosolic factor 2; TFR1a: Transferrin receptor 1a.