Fish and Shellfish Immunology 81 (2018) 92–98

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Transcriptome analysis of *Artemia sinica* in response to *Micrococcus lysodeikticus* infection

Yulong Zhang[1], Di Wang[1], Zao Zhang, Zhangping Wang, Daochuan Zhang**, Hong Yin*

The Key Laboratory of Zoological Systematics and Application, College of Life Sciences, Hebei University, 071002, Baoding, PR China

ARTICLE INFO

Keywords:
Artemia sinica
Transcriptome
Immunity genes
Micrococcus lysodeikticus

ABSTRACT

To enhance genomic resources and understand the molecular immune mechanisms underlying the response topathogens, we first performed a comparative gene transcription analysis from *Micrococcus lysodeikticus*-immunized *Artemia sinica* and from a control group through RNA-Seq technology, meanwhile the differentially expressed genes (DEGs) were investigated. In total, 80, 113, 984 clean reads were obtained and then assembled into 71,536 unigenes with an average length of 1115 bp and an N50 of 1783 bp. Unigenes were annotated by comparing against nr, Swiss-Prot\KEGG\ COG\ KOG\ GO and Pfam databases, and 27,689 unigenes (38.7%) were annotated in at least one database. After bacterial challenge, 183 and 298 genes were identified as remarkably up-regulated or down-regulated, respectively, amongst 481 were associated with 168 pathways, including classical immune-related pathways, such as 'Toll-like receptor signaling', 'the complement cascades', 'MAPK signaling pathway' and 'Apoptosis'. Besides, eight genes which were differently expressed immune-related were confirmed by using quantitative real-time PCR. This study characterized a gene expression pattern for normal and *M. lysodeikticus* -immunized *A. sinica* for the first time and sheds new light on the molecular mechanisms thus enabling future efforts on disease control programs in this valuable aquaculture species.

1. Introduction

The brine shrimp, *Artemia sinica*, belongs to Arthropoda, Crustasea, Branchiopoda, Anostacea, *Artemidae* and *Artemia* [1]. *A. sinica* is a high salt-resistant small crustacean distributed widely in saline inland lakes and coastal salterns which commonly known as a main food resource in aquaculture and applied in basic research areas ranging from developmental biology to evolution and ecology [2–7]. However, just as other aquatic organisms, *A. sinica* is suffering from various pathogenic challenge in recent years. One of the most devastating pathogens of *A. sinica* is *Micrococcus lysodeikticus*, a typical Gram-positive bacterium. Several studies have analyzed the immune response to *M. lysodeikticus* in aquatic animals, such as *Sinonovacula constricta*, *Litopenaeus vannamei*, and *A. sinica*, and many immune-related genes have been identified, including galectins, Complement component 3, IRAK-4 [8–10]. But the results of these studies are limited as they were only able to identify one or several immune-related genes and their protein activity.

The rapid advances in next-generation sequencing (NGS) technology have led to a revolution for transcriptomics and genome characterization in recent years. This high-throughput sequencing technology is most frequently used to facilitate functional genomics researches, including global gene expression, novel gene discovery, full-length gene assembly [11,12]. Moreover, differentially expressed genes (DEGs) analysis has provided new insights into immune responses against bacterial infections in various aquaculture animals. By deep sequencing RNA from infected and uninfected samples, it is possible to identify immune-related genes that are differentially expressed in infection status and ultimately lead to a better understanding of molecular mechanisms underlying the organism's response to immunogenic stimuli [13]. There have been several reports of immunological reaction to bacteria using transcriptome analysis in aquatic organisms, such as *Marsupenaeus japonicus*, *Mytilus coruscus* and *Pelteobagrus fulvidraco* [14–16]. With regard to *A. sinica*, transcriptome resources addressing immunity and disease remain scarce.

In the present study, we used Illumina technology for transcriptome profiling analysis of the *A. sinica* with *M. lysodeikticus* infection. These results will provide new valuable information for further research into the immune mechanisms of anti-infection immunity of *A. sinica*.

* Corresponding author.
** Corresponding author.
E-mail addresses: zhangdc@hbu.edu.cn (D. Zhang), yinhong@hbu.edu.cnm (H. Yin).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2018.06.033
Received 5 March 2018; Received in revised form 12 June 2018; Accepted 15 June 2018
Available online 10 July 2018

Table 1
Primers used for qRT-PCR.

Primer name	Forward Primer (5′-3′)	Reverse Primer (5′–3′)	Purpose
CXCL-2	CCAGAGGCAGTTTCTACAG	AGCAGTGCGGATATGTTG	qRT-PCR
ACBP	TATTATCCCGTATGGAGCAG	GGTTGTCCGGCAATCAGT	qRT-PCR
PIK3C	CGAGCATTTGGTAGAAGG	CAACGCTGATAATCACAAGG	qRT-PCR
CACNA2D2	CACTGGTCTGGCTCATCTCC	TTGTCCCCACTCTATGGTTG	qRT-PCR
CREB3	TCTATCCCCTGAGTCGAACA	CATCCTCAAATTCCCTTATCC	qRT-PCR
MEF2C	GCATGAGTCGAGGACTAACAACG	GCCATCGCCATCATCATCAC	qRT-PCR
NFKB1	CAAGATCAAGCCGTAAGTG	AGCAAACGAGTCAACAGAG	qRT-PCR
CALR	GATGGAGATGCTTGGGAACA	ACCGTGCGTCTTGGGATG	qRT-PCR
β-actin	GTGTGACGATGATGTTGCGG	GCTGTCCTTTTGACCCATTCC	qRT-PCR

2. Materials and methods

2.1. Animal material and bacterial challenge

Cysts of *A. sinica* were collected from the Salt Lake of Yuncheng in Shanxi Province, China, during the summer in 2010 and stored at −20 °C in the dark. The cysts were hatched in saline water under these conditions: a temperature of 28 °C, salinity of 28‰, and light intensity of 1000 lx for 7 days before carrying out experiments [17]. The challenged group was maintained in salt water Gram-positive bacteria *Micrococcus lysodeikticus* with the bacteria concentration of 10^6 cells L^{-1} for 24 h. And the samples in control group were cultured in salt water only. All the samples were rapidly frozen by liquid nitrogen and then stored at −80 °C until use for RNA extraction.

2.2. Library construction and sequencing

Total RNA was extracted from two groups (20 males and 20 females, respectively) with RNAiso Plus (Takara, Dalian, China) according to the user manual in combination with DNase I treatment. The RNA quality was assessed by running the samples on 1% agarose gels and visualizing by staining with ethidium bromide. RNA purity, concentration and integrity were examined by using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) with an absorption at 260 nm.

Sequencing libraries were prepared by using an Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's proposal. After purification, the libraries were sequenced on an Illumina/Hiseq-2500 platform (Illumina, San Diego, CA, USA) by Beijing Novogene Bio-information technology company, with the sequencing read length 100 bp paired-end reads.

2.3. De novo assembly and annotation

The raw reads were filtered by removing the adapters, the primer sequences and low quality sequences. The remaining clean reads were assembled using the Trinity software (v2012_10_05), with the default parameters. The transcripts were assembled and clustered with the Chrysalis clusters software and the longest sequences in each cluster were retained and designated “unigenes” [18]. For functional annotation analysis, all unigenes were searched against following databases: nr (NCBI non-redundant protein sequences), GO (Gene Ontology), KOG (Eukaryotic Orthologous Groups), KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Clusters of Orthologous Groups of proteins) and Pfam database, with the E-value of less than $1.0e^{-5}$ and HMMER of less than $1.0e^{-10}$.

2.4. Differential gene expression analysis

Gene expression levels were estimated by mapping all clean reads to unigene library with the Bowtie method and combined with RSEM (version 1.1.21) for each sample [19,20]. The abundance of all genes was calculated using particularly mapped reads by the FPKM method [21]. In addition, in the process of differential expression analysis, the well-known Benjamin-Hochberg method is adopted to correct the p-value of the original hypothesis test and the FDR (False Discovery Rate) is regarded as a key indicator of differential expression of gene selection. Then, all differentially expressed genes were mapped to terms in GO and KEGG databases for enrichment analysis and the Enrichment factor was used to analyze the enrichment degree of Pathway.

2.5. Experimental validation using RT-qPCR

Eight genes including acyl-CoA-binding protein (ACBP), CXCL2-like chemokine (CXCL2), calcium channel alpha-2/delta-2 (CACNA2D2), adrenergic receptor alpha-1B (ADRA1B), NF-kappa-B p105 subunit (NFKB1), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3C), cyclic AMP-responsive element-binding protein 3 (CREB3) and MADS-box transcription enhancer factor 2C (MEF2C) were selected for confirmation of RNA-seq data by Quantitative real-time PCR (qRT-PCR) using a SYBR Premix Ex Taq kit (Takara, Dalian, China) according to the manufacturer's instructions. The gene-specific primers of qPCR were designed in the Primer Premier 5.0 (Table 1). Real-time qPCR was performed in triplicate for every sample in a parallel design using the LightCycler 96 System (Roche). The qPCR program was 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s, and 72 °C for 30 s β-actin primers (β-actinF, β-actinR, Table 1) were used as a normalization control for target genes expression [22]. The $2-\Delta\Delta Ct$ method was used to calculate the relative quantification (comparative method) [23]. Data obtained from qPCR analysis were analyzed using one-way ANOVA in SPSS 16.0 software. The significance threshold of the means values between the experimental and control groups was set at $P < 0.05$.

3. Results and discussion

3.1. Sequencing and de novo assembly

To identify the genes involved in the response of *A. sinica* to *M. lysodeikticus* infection, two libraries obtained under normal and *M. lysodeikticus* infected conditions, were constructed and sequenced using the high-throughput sequencing. A total of 46, 998, 168 and 40, 103, 202 raw reads were obtained from the control and *M. lysodeikticus* infected libraries respectively. After filtering the low-quality reads, short sequences, and low-complexity sequences, two libraries generated 40, 966, 366 and 39, 147, 618 clean reads left for de novo assembly (Table 2). The values of the Q30% and GC percentage of the clean reads in two DGE libraries were 89.95% and 91.02%, 40.40% and 39.52%, respectively. After aligning all the clean reads to the reference database, 32, 360, 468 (78.99%) and 29, 634, 998 (75.70%) were identified as the unique matches. Reads mapped to an unique sequence were the most critical, as they can explicitly identify a transcript. In this study, the mapped ratio was roughly consistent with the results obtained in other species as *Artemia franciscana*, and *Caligus rogercresseyi* in