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Transcriptome analysis of the effect of *Vibrio alginolyticus* infection on the innate immunity-related TLR5-mediated induction of cytokines in *Epinephelus lanceolatus*

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ABSTRACT

Epinephelus lanceolatus, considered to be an aquaculture fish species of high economic value in East Asia, is one of the largest groupers in the *Epinephelus* genus. *Vibrio alginolyticus* is a bacterial species that causes high morbidity in marine fish; infection can cause exophthalmia, ulcers, septicemia, and corneal opaqueness in fish. *Epinephelus lanceolatus* larvae infected with *Vibrio alginolyticus* were subjected to transcriptome analysis to study the immune regulation pathway. Grouper larvae were injected with 2.6×10^4 CFU/fish in 20 μl of *V. alginolyticus* and control larvae were injected with TSB; RNA samples were then collected at 4, 6, 8, 10, 12, 16, 24, and 48 h after infection. Extracted RNA was subjected to reverse transcription, and used to examine the immune gene response of *E. lanceolatus* by Real-time PCR. Samples taken at 6 h were subjected to next-generation sequencing, resulting in a total read value of 28,705,411 and total base number of 2,152,905,850. The unigene number was 100,848, and 5913 unigenes were filtered using FPKM > 0.3, 2FC, $p < 0.05$. Gene Ontology (GO) analysis of the filtered genes revealed a total of 30 GO numbers in the cellular component, and 58 GO numbers for both biological processes and molecular functions. Of the GO group related to immune pathways, 27 unigenes related to biological processes involving the immune response, 31 related to the immune system, 9 related to the inflammatory response, and 43 related to the response to stress were identified. KEGG pathway analysis only detected 1 to 4 genes, and as such, we selected the GO analysis results for further analysis using GeneSpring. This demonstrated that *V. alginolyticus* probably stimulates TLR5 activity via the bacterial flagellum, through an MyD88-dependent pathway; the resulting production of IL-1β and IL-8 through the NFκB pathway induces pro-inflammatory and/or chemotactic effects. Alternatively, serum amyloid A may stimulate neutrophils that induce the secretion of MMP9 from infected tissues, resulting in the cleavage and activation of IL-8. IL-8, in turn, would enhance neutrophil chemotaxis. Infection also induced expression of genes encoding C3, C6, C7, C8, and C9, which induce the complement system and form the membrane attack complex to lyse the bacteria membrane. The qPCR results indicated that TLR5 is significantly increased between 10 and 16 h, IL-1β between 8 and 16 h, IL-8 between 8 and 12 h, and C6 between 4 and 16 h, as compared to levels in the control. One antimicrobial peptide, hepcidin, was also strongly expressed between 4 and 10 h in infected fish. The results indicate that *V. alginolyticus* infection probably induces an immune response via TLR5-mediated regulation of down-stream cytokine gene expression. A second possibility is that the complement system and hepcidin may be involved in the immune response. These results may be applied by examining the immune effects of feeding *E. lanceolatus* larvae on a recombinant protein mixture based on the up-regulated genes.

1. Introduction

Grouper are economically-important cultured fish in East Asia. In Taiwan, the following species are often used in aquaculture:

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orange-spotted grouper (*Epinephelus coioides*), tiger grouper (*Epinephelus fuscoguttatus*), Malabar grouper (*Epinephelus malabaricus*), and giant grouper (*Epinephelus lanceolatus*). *E. lanceolatus* is one of the largest groupers in the world, and, with its fast growth and high price, it swiftly became the favored species for marine aquaculture in Taiwan [1]. However, crowding stress triggers disease in grouper, which causes economic loss. *E. lanceolatus* are vulnerable at the larval stage. Bacterial pathogens of grouper include *Vibrio alginolyticus*, *Vibrio carchariae*, and *Vibrio harveyi*, which cause Vibriosis, a serious disease [2]. *V. alginolyticus* is a halophilic Gram-negative bacteria species, which resides in marine and estuarine environments, and causes exophthalmia, ulcers, septicemias, and corneal opaqueness in fish, as well as mortality of shrimp under stressful conditions [3,4]. Hence, *V. alginolyticus* is a key pathogen of grouper that causes huge economic losses in Taiwan and China, and is therefore of concern to aquaculture.

The complete mitochondrial genome of *E. lanceolatus* was previously sequenced to identify new molecular markers (such as expressed sequence tags, ESTs) through population genetics [5]. Next-generation sequencing (NGS) technologies are potentially a large-scale, efficient, and economical means of identifying ESTs. One such technology, 454 pyrosequencing, has been used to analyze the spleen of *E. coioides* infected by Singapore grouper iridovirus, which indicated that mitogen-activated protein kinase (MAPK), toll-like receptor, chemokine, and RIG-I signaling pathways are activated in response to SGIV infection [6,7]. In our previous study, we also utilized NGS technologies to analyze *E. coioides* larvae infected with *V. alginolyticus*, which revealed that the complement system and hepcidin may be induced upon infection [8]. From our work on *E. coioides*, we have already constructed a hypothetical model of the immune response to *V. alginolyticus* infection. The classical pathway involves cleavage of C4 to C3 convertase by C1s, cleavage of C3 to C3b by C3 convertase, combination of C3b with C5, cleavage of the complex to C5b by C5 convertase, and formation of the membrane attack complex (MAC) to lyse *V. alginolyticus*. The lectin pathway involves C-type lectin-mediated cleavage of C4 and C2 by MASP1/2, and cleavage of C3 by C3 convertase. The alternative pathway involves cleavage of C3 by factor B; as in the classical pathway, factor I acts as an inhibitor in the alternative pathway. Hepcidin probably kills the bacterium by disrupting its membrane.

As compared to that of *E. coioides*, less is known of the immune response of *Epinephelus lanceolatus* to bacterial infection at the level of gene expression. Through this study, we hoped to identify novel immune pathways and key immune genes activated in *Epinephelus lanceolatus* larvae in response to *V. alginolyticus* infection. Here, we report immune genes important in grouper, the protein products of which may be suitable for delivery to grouper as fodder in the aquaculture industry.

2. Materials and methods

2.1. Fish and bacterial culture

E. lanceolatus larvae were purchased from the Institute of Biotechnology, National Cheng Kung University Core Facility. Grouper larvae were cultured in a 2 ton FRP tank prior to infection with bacteria. At the time of infection, larvae were 2.03 ± 0.28 cm in length and weighed 0.09 ± 0.01 g, and were 30 days old. *V. alginolyticus* were incubated as previously described [9]. The animal protocol (12-12-447) was approved by the Academia Sinica Institutional Animal Care and Use Committee (IACUC) of the Institute of Cellular and Organismic Biology, Academia Sinica.

2.2. Grouper larvae RNA preparation for next generation sequencing

E. lanceolatus larvae were injected with *V. alginolyticus* (2.6×10^4 CFU/fish in 20 μl), and the fish were sacrificed at 6 h after infection. Three wild-type *E. lanceolatus* larvae were used to construct an EST library for transcriptome analysis. RNA was extracted as described in a previous study [10]. RNA samples for real-time qPCR were extracted from fish sacrificed at 4, 6, 8, 10, 12, 16, 24, and 48 h after infection (n = 6). The NGS analysis was performed as previously described [8].

2.3. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis

GO enrichment analysis and pathway enrichment analysis of KEGG were performed as previously described [8]. The transcriptome was mapped to GO terms from the database (http://www.geneontology.org/). Pathway enrichment was used for KEGG analysis (http://www.genome.jp/kegg/), which uses public databases to examine biological pathways.

2.4. Analysis of gene expression

The real-time qPCR primers designed in the course of this study are described in Supplementary Table 1; real-time qPCR was performed as previously described [9].

3. Results

3.1. Construction of the Epinephelus lanceolatus transcriptome following V. alginolyticus infection by de novo sequencing and sequence assembly

The transcriptomes of grouper larvae (30 days) injected with *V. alginolyticus* were assembled to analyze immune gene regulation pathways. To determine the optimal time after infection to analyze the transcriptome, we analyzed the expression of complement-related genes by qPCR, using primers designed against genes in *E. coioides* (Supplementary Fig. 1). We found that genes were significantly increased in infected grouper as compared to control grouper at 6, 8, and 16 h, and we thus selected the earliest time point (6 h) for transcriptome assembly. The assembled transcriptome possessed a total read value of 28,705,411 and total base value of 2,152,905,850, and its unigene number was 100,848; after filtering with FPKM > 0.3, 2FC, $p < 0.05$, we obtained 5913 unigenes (Table 1). GO analysis identified a total of 30 GO terms related to cellular components, and 58 terms each for biological processes and molecular functions. We selected the group related to the immune pathway for further analysis: 27 unigenes related to biological processes involving the immune response, 31 related to

Table 1
Summary of the assembled transcriptome from *Epinephelus lanceolatus* larvae.

Transcriptome sequences	
Total base	76,854,426
Total contig number	21,867
Unigene number	100,888
Unigene number (FPKM > 0.3, 2FC, $p < 0.05$)	5913
Unigene mean length	761
GO: biological process	58
GO: cellular component	30
GO: molecular function	58
KEGG pathway	47