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Integration of RNAi and RNA-seq uncovers the immune responses of *Epinephelus coioides* to *L321_RS19110* gene of *Pseudomonas plecoglossicida*

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ABSTRACT

Pseudomonas plecoglossicida is a threatening and important pathogen in aquaculture and causes heavy losses. Expression of *L321_RS19110* was found significant up-regulated at 18 °C than at 28 °C, which was confirmed by quantitative real-time PCR. RNAi significantly reduced the content of *L321_RS19110* mRNA of *P. plecoglossicida*, and exhibited the best efficiency of gene silencing with a reduction of 84.9%. Compared with the wild type strain, the infection of *L321_RS19110*-RNAi-1 strain resulted in the onset time delay, and 30% reduction in mortality of *Epinephelus coioides*, as well as alleviates in the symptoms of *E. coioides* spleen. Moreover, compared with wild type strain, the gene silence of *L321_RS19110* in *P. plecoglossicida* resulted in a significant change in transcriptome of infected *E. coioides*. The results of KEGG analysis showed that genes of chemokine signaling pathway and cytokine-cytokine receptor interaction, digestion and absorption of proteins (non-specific immune) and antigen processing and presentation pathways were most affected by *L321_RS19110*-RNAi of *P. plecoglossicida*. In these immune pathways, the most affected immune genes were associated with different number of non-coding RNAs. Among them, most lncRNAs and miRNAs exerted negative regulation on immune genes. The results indicated that *L321_RS19110* was an important virulent gene of *P. plecoglossicida*, the up-regulation of the immune pathways made *E. coioides* more likely to remove *L321_RS19110*-RNAi strain than the wild type strain of *P. plecoglossicida,* the expression of immune genes were regulated by miRNA and lncRNA in a complex way.

1. Introduction

Pseudomonas plecoglossicida, a Gram-negative aerobic and rod-shaped bacterium, posed a serious threat to cultured ayu (*Plecoglossus altivelis*) in Japan, and causing an emerging bacterial disease with bacterial haemorrhagic ascites [1–3]. Recently, *P. plecoglossicida* has been associated with the fulminating infectious disease of several marine fish, such as rainbow trout (*Oncorhynchus mykiss*) and large yellow croaker (*Pseudosciaena crocea*) [4]. The epidemic caused by *P. plecoglossicida* is temperature-dependent and mainly recorded in the seawater temperature range from 15 to 20 °C. In order to reveal the mechanism underlying the pathogenic, the transcriptome of *P. plecoglossicida* incubated under 12, 18 and 28 °C were sequenced, and the data have been deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP107111.

The results of previous comparative transcriptome analysis showed that *L321_RS19110* gene (old_locus_tag: *L321_19357*, a predicted integral component of membrane gene) and *L321_RS24310* gene (old_locus_tag: *L321_*24621, a predicted sequence-specific DNA binding gene) of *P. plecoglossicida* were significantly high expressed under 18 °C [5]. Therefore, *L321_RS19110* and *L321_RS24310* were hypothesized to play roles in the pathogenicity of *P. plecoglossicida.* The interaction of bacterial pathogens with host cells is closely related to the expression of OM proteins [6]. *L321_RS19110* encoded OmpA/MotB domain-containing protein. OmpA is one of the immunodominant antigens and binding of specific anti-OmpA antibodies leads to cell lysis in the presence of complement and can interact with host receptor molecules [7,8]. MotB (and MotA) serve two functions, the MotA/MotB complex attaches to the cell wall via MotB to form the stator of the flagellar motor, and the MotA/MotB complex couples the flow of ions across the cell membrane to movement of the rotor in most of the Gram-negative bacteria. Up to now, the understanding of the function of

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L321_RS19110 is very limited, no research about function of *L321_RS19110* during pathogen infection has been reported.

In view of the great threat of *P. plecoglossicida* to aquaculture and the potential important role of *L321_RS19110* in the virulence of *P. plecoglossicida,* the *L321_RS19110* of *P. plecoglossicida* was knocked down by RNAi, the virulence of wild type strain and *L321_RS19110*-RNAi strain of *P. plecoglossicida* were compared, the spleens of *E. coioides* infected by wild type strain and mutant of *P. plecoglossicida* were subjected to RNA-seq, and the data were compared comprehensive analysed. The aim of this paper is to uncover the function of *L321_RS19110* in the process of *P. plecoglossicida* infection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The pathogenic *P. plecoglossicida* strain (NZBD9) was isolated from a naturally infected large yellow croaker and confirmed as pathogenic by artificial infection [4]. The *P. plecoglossicida* strain was routinely grown in Luria-Bertani (LB) broth or LB agar medium at 18 or 28 °C with shaking at 220 rpm. *Escherichia coli* DH5α was obtained from TransGen Biotech (Beijing, China), which was grown in LB medium (37 °C, 220 rpm).

2.2. Construction of P. plecoglossicida RNAi strain

RNAi strain was constructed according to methods described by Choi and Schweizer [9] and Darsigny et al. [10]. Five short hairpin RNA sequences respectively targeting the *L321_RS19110* and *L321_RS24310* gene were designed and synthetized (Table S1, S2) Invitrogrn Block-iT RNAi Designer (http://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=shrna&pid=7085871032206845) was used to predict shRNA sequences that would lead to silencing of target genes. After linearizing pCM130/tac vectors with the restriction enzymes *Nsi*I (R3127S) and *BsrG*I (R3575S) (New England Biolabs, including TIME-SAVER™ Protocol and Buffer Performance), the oligonucleotides were annealed and ligated to the linearized pCM130/tac vectors using T4 DNA ligase (New England Biolabs) following the manufacturer's recommendations. The recombinant pCM130/tac vectors were transformed into the competent *E. coli* DH5a cells (*Trans5α* Chemically Competent Cell) by heat shock and then were electroporated into *P. plecoglossicida* as described previously [11]. Finally, the expression level of *L321_RS19110* and *L321_RS24310* of each RNAi strain was detected by qRT-PCR.

2.3. Artificial infection and sampling

All animal experiments were conducted under the recommendations in the 'Guide for the Care and Use of Laboratory Animals' set by the National Institutes of Health. The animal protocols were approved by the Animal Ethics Committee of Jimei University (Acceptance NO JMULAC201159).

Healthy *E. coioides* (body length 15 ± 1 cm) were obtained from Zhangzhou (Fujian, China) and acclimatized at 18 °C for one week under specific pathogen-free laboratory conditions. For survival assays, each *E. coioides* was intrapleurally injected with 10^3 cfu/g of *P. plecoglossicida* (wild type strain or RNAi strain). *E. coioides* intrapleurally injected with PBS were used as the negative control. The water temperature during infection was kept at 18 ± 1 °C. The status of the fish was recorded three times a day. For tissue RNA-Seq, the spleens of six *E. coioides* infected with wild type strain *P. plecoglossicida* or RNAi strain were sampled at 24 hpi. Every two spleens were mixed as one sample. For the tissue distribution assays, the spleens, livers, head kidneys, trunk kidneys and blood of three infected *E. coioides* were sampled at 6, 12, 24, 48, 72, 96 and 120 hpi, respectively.

2.4. DNA isolation

DNA purification from spleens, livers, head kidneys and trunk kidneys was accomplished with an EasyPure Marine Animal Genomic DNA Kit (TransGen Biotech, Beijing, China) following the manufacturer's instructions. The EasyPure Blood Genomic DNA Kit (TransGen Biotech) was used for DNA isolation from blood samples.

2.5. RNA isolation and reverse transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, the mixed genomic DNA in total RNA was digested with the Turbo DNA-free DNase (Ambion, Austin, TX, USA). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), while the rRNA in total RNA was removed using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. The quality of total RNA was checked by agarose gel electrophoresis. The cDNA was synthesized by TaKaRa PrimeScript™ RT-PCR Kit (TaKaRa Bio Group, JAPAN) protocol in a system. The synthesized cDNA was used as a new sample template for qRT-PCR and then stored at −20 °C until use.

2.6. qRT-PCR

qRT-PCR was carried out using a QuantStudio 6 Flex (Life Technologies). All primer sequences are provided in Table S3. To further validate the results of RNA-seq, the *P. plecoglossicidais* were cultured under 18 °C and 28 °C. Reaction mixtures (10 μL/UltraFlux®i 8-strip Low Profile 0.1 mL w/individual attached Flat cap) were comprised of 5 μL qMix (Life Technologies), 0.25 μL of the forward primer, 0.25 μL of the reverse primer, 0.5 μL diluted template DNA and 4 μL of nuclease-free water. The copy number of the *gyrB* gene was used to estimate *P. plecoglossicida* abundance [12]. The expression of bacterial genes was normalized using *gyrB*. In *E. coioides*, the expression of mRNA and lncRNA was normalized to *β-actin*, and the miRNA expression was normalized by using *5S rRNA* gene [13]. There were three replicates for each treatment, and the $2^{-\Delta\Delta Ct}$ method [14] was used to calculate the relative level of gene expression.

2.7. Transcriptomic analysis

2.7.1. Library preparation and sequencing

The RNA-seq libraries were prepared using protocols supplied with the TruSeq™ RNA sample preparation Kit (Illumina, San Diego, CA, USA). RNA quality was determined by 2100 Bioanalyzer (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Only high-quality RNA sample (OD260/280 = 1.8–2.2, OD260/230 ≥ 2.0, RIN ≥ 6.5, 28 S:18 S ≥ 1.0, > 10 μg) was used to construct sequencing library. In brief, the rRNA-depleted RNA sample was fragmented in fragmentation buffer, and cDNA synthesis was conducted using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). After end reparation, phosphorylation and poly (A) addition, the cDNA library was amplified using Phusion DNA polymerase (NEB). Small RNA-seq libraries were built using a TruSeqTM Small RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. An Agilent 2100 Bioanalyzer (Agilent Technologies) was used to validate the library quality. Sequencing was performed on the Illumina HiSeq4000 sequencing platforms at Majorbio Biotech Co., Ltd. (Shanghai, China).

2.7.2. Processing and mapping of reads

The trimming and quality control of raw Illumina reads were performed using Sickle (https://github.com/najoshi/sickle) and SeqPrep (https://github.com/jstjohn/SeqPrep) with the default settings [15,16]. For RNA-seq, clean data were mapped to the genome of *P.*