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Short communication

The mucosal expression signatures of g-type lysozyme in turbot (*Scophthalmus maximus*) following bacterial challenge

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ABSTRACT

The mucosal surfaces constitute the first line of host defense against infection, and also serve as the dynamic interfaces that simultaneously mediate a diverse array of critical physiological processes, while in constantly contact with a wide range of pathogens. The lysozymes are considered as key components for innate immune response to pathogen infection with their strong antibacterial activities. But their activities in mucosal immune responses were always overlooked, especially for g-type lysozymes, whose expression patterns in mucosal tissues following bacterial challenge are still limited. Towards to this end, here, we characterized the g-type lysozymes, Lyg1 and Lyg2 in turbot, and determined their expression patterns in mucosal barriers following different bacterial infection. The phylogenetic analysis revealed the turbot g-type lysozyme genes showed the closest relationship to *Cynoglossus semilaevis*. The two lysozyme genes showed different expression patterns following challenge. Lyg2 was significantly up-regulated in mucosal tissues following *Vibrio anguillarum* and *Streptococcus iniae* challenge, while Lyg1 showed a general trend of down-regulation. The significant mucosal expression signatures of g-type lysozyme genes indicated their key roles to prevent pathogen attachment and entry in the first line of host defense system. Further functional studies should be carried out to better characterize the availability of utilization of g-type lysozyme to increase the disease resistance in the mucosal surfaces and facilitate the disease resistant breeding selection.

1. Introduction

The mucosal surfaces constitute the first line of host defense system against infection, and also serve as the dynamic interfaces that simultaneously mediate a diverse array of critical physiological processes, including nutrient adsorption, osmoregulation, waste excretion and oxygen absorption, while in constantly contact with a broad spectrum of pathogens [1]. In particular, as living in the pathogen rich aquatic environment, the mucosal surfaces of fish (skin, gill and intestine) are constantly colonized by a wide range of commensals, opportunistic and primary pathogens [2], and the immune events are happening on these surfaces intensively and continuously to prevent pathogen invading all the time. It has been long hypothesized that the observed differences in disease resistance trait among different fish strains and species are due to the different ability of host to prevent pathogen colonization at the mucosal epithelial sites on fish mucosal surfaces (skin, gill and intestine) [3–5]. Although with the well-recognized importance of fish mucosal surfaces as the first barriers against pathogens, and almost all the most urgent areas of aquaculture research require a comprehensive understanding of fish mucosal immunity, especially for the disease control and prevention measurement, our knowledge of teleost mucosal immunity are still limited [6]. Understanding of the cellular actors and pathways governing mucosal immune responses during pathogen infection is still limited in a handful of teleost species.

Lysozymes have been discovered since 1922 from a bacteria lysis event, and then been identified in all major taxa of living organisms [7]. According to the structural, biochemical and enzymatic

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properties, lysozymes are divided into three major different types, the c-type (chicken or conventional type), the g-type (goose-type) and the i-type (invertebrate type) [8]. The lysozymes are considered as key components for innate immune response to pathogen infection with their strong antibacterial activities. As a group of hydrolytic enzymes, lysozymes are involved in hydrolysation of the bacterial cell walls by cleaving the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan. However, only c-type and g-type lysozymes were identified in teleost, having very different amino acid sequences, different genetic structures, and catalytic mechanisms. The g-type lysozymes were initially identified in egg whites of the Emden goose, and then reported to be dominate lysozyme species in most of birds' eggs [9]. The first report of g-type lysozyme outside of birds was in fish [10], since then, more and more g-type lysozymes have been reported in fish species, including common carp (*Cyprinus carpio* L.) [11], grouper (*Epinephelus coioides*) [12], brill (*Scophthalmus rhombus*) [13], and European sea bass (*Dicentrarchus labrax* L.) [14]. Up to date, many studies have explored their critical roles against bacterial infection in fish species, and found that their activities were dramatically changed during infection. For instance, the g-type lysozyme was induced by LPS, poly I:C, *Edwardsiella tarda* and *Streptococcus iniae* in rock bream (*Oplegnathus fasciatus*) [15]. In half-smooth tongue sole (*Cynoglossus semilaevis*), it was also up-regulated in several tissues following *Vibrio anguillarum* challenge [16]. Collectively, the g-type lysozyme plays vital roles for fish innate immune responses against infection, but its activities in mucosal immune responses were always overlooked. The expression patterns of g-type lysozyme in mucosal tissues following bacterial challenge is still poorly studied.

Turbot (*Scophthalmus maximus*), one of the most important maricultured species in China, suffers from widespread disease outbreaks due to a number of pathogens, including *V. anguillarum*, *S. iniae* and *E. tarda*, resulting in dramatic economic losses to turbot industry. Recently, much efforts have been made to understand the host-bacteria interactions in turbot, many immune related genes and their expression patterns have been characterized during infection, including Stomatin-like protein 2 [17], chemokines [18,19], and MyD88 [20]. But the characterization of mucosal immune cellular actors and their associate immune activities in mucosal tissues against infection is still lacking. The successful infection always starts with the pathogen attachment and entry on the mucosal surfaces, with the strong ability of lysis bacteria cell walls, therefore, lysozyme could be one of the most important players on the mucosal surfaces to clear the attached bacteria. Towards to this end, here, we characterized the g-type lysozyme genes in turbot, and determined their expression patterns in mucosal barriers following bacterial infection. Present study reported the identification, phylogenetic, tissue distribution, and mucosal expression regulation after bacterial infection for the two g-type lysozyme genes in turbot for the first time.

2. Materials and methods

2.1. Sequence identification and analysis

Based on g-type lysozyme sequences from other species, including zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), half smooth togue sole (*C. semilaevis*), and stickleback (*Gasterosteus aculeatus*) and catfish (*Ictalurus punctatus*), we searched turbot transcriptome databases generated by our group using the tBLASTn program with a cutoff E-value of 1e-10 (Li, unpublished data). The retrieved sequences were translated using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted amino acid sequences from ORF predication were further verified by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI non-redundant protein sequence database. The simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/) was used to identify the conserved domain and signal peptides. ExPASy server was used to analyze the theoretical pI, molecular mass and *N*-glycosylation sites [21].

2.2. Phylogenetic analysis

Amino acids sequences of g-type lysozyme from turbot and the other organisms were selected to construct the phylogenetic tree, including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus laevis*), fugu (*Takifugu rubripes*), zebrafish (*D. rerio*), medaka (*O. latipes*), half smooth togue sole (*C. semilaevis*), and stickleback (*G. aculeatus*). Multiple protein sequence alignment was conducted using the ClustalW2 program [22]. Phylogenetic and molecular evolutionary analyses were performed using the neighbor-joining method within the Molecular Evolutionary Genetics Analysis (MEGA 6) package [23]. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 10,000 bootstrapping replications.

2.3. Bacteria challenge and sample collection

In order to characterize the immune roles of g-type lysozyme genes in the host defense against bacterial infection, the Gram-negative bacteria *V. anguillarum* and the Gram-positive bacteria *S. iniae* were selected to conduct the bath challenge. The weight and length of experimental turbot fingerlings averaged 15.6 g and 5.5 cm, respectively. Prior to experimental challenge, turbot fingerlings were obtained from turbot hatchery (Haiyang, Shandong, China), and acclimated in laboratory in a flow-through system for at least one week. The bacterial isolate of *V. anguillarum* and *S. iniae* were provided by the disease lab in Qingdao Agricultural University. After pre-challenge, the bacteria were re-isolated from symptomatic fish and biochemically confirmed before cultured. During challenge, symptomatic fish were confirmed to be infected with *V. anguillarum* and *S. iniae*, respectively. During experiments, the fish were immersed in the bacteria solution for 2 h and then samples were collected at certain time point after treatment under tricaine methanesulfonate (MS-222) at 200 mg/L (buffered with sodium bicarbonate). The water temperature was kept at 27 ± 0.5 °C.

In *V. anguillarum* challenge, bacteria were biochemically confirmed using standard procedure, inoculated in LB broth and incubated in a shaker (180 rpm) at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 10 mL of 10-fold serial dilutions onto LA agar plates. During challenge, the treatment groups were immersed for 2 h at a final concentration of 5×10^7 CFU/mL. Meanwhile, control fish were immersed in sterilized media alone. Aquaria were randomly assigned for 2 h, 6 h, 12 h and 24 h post-treatment sample collection, respectively, and 0 h control with thirty fish in each aquarium. At each time point after treatment, intestine, skin and gill from 15 fish were collected from the appropriate aquaria (three pools of 5 fish per pool).

In *S. iniae* challenge, the bacteria were isolated from a single colony and inoculated in LB medium in a shaker incubator at 28 °C overnight. For the challenge, fish were immersed for 2 h at a final concentration of 5×10^6 CFU/mL, while control fish were immersed in sterilized media alone. At each time point following challenge, namely, 2 h, 4 h, 8 h and 12 h treatment, intestine, skin and gill tissues were collected from 15 fish (three pools of 5 fish per pool) of both the challenge group and the control group, respectively. All