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# Identification of a type I interferon (IFN) gene from Japanese eel and its expression analysis *in vivo* and *in vitro*

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ABSTRACT

Type I interferon (IFN) is a key cytokine of innate immune response for viral infection in teleost fish. In present study we report the identification of a type I IFN gene (*Aj*IFN) from Japanese eel (*Anguilla japonica*) and provide evidence that *Aj*IFN has been involved in Japanese eel host response against not only the virus but also the bacterial infection. The full-length cDNA of *Aj*IFN (826 bp) has an ORF of 531 bp. The analysis of NCBI CDD showed that the *Aj*IFN protein had the typical conserved domains, including IFabd conserved domain, and two conserved cysteine residues potentially forming disulphide bridges. Based on the phylogenetic analysis, *Aj*IFN was classified into type I IFNs families belonging to the subgroup-a of the 2Cys group. *In vivo*, the *Aj*IFN expressions in liver and kidney were induced following injection with LPS, the viral mimic poly I:C, and *Aeromonas hydrophila* infection. *In vitro*, the *Aj*IFN transcripts of Japanese eel liver cells were enhanced by LPS, poly I:C and CpG-DNA stimulation and no change of the expression level was found post PGN treatment. Following *A. hydrophila* infection, the low concentration of $1 \times 10^6$ cfu/mL failed to induce the expression of *Aj*IFN whereas the concentration of $1 \times 10^7$ cfu/mL and $1 \times 10^8$ cfu/mL successfully induced the expression of *Aj*IFN. These results collectively suggested *Aj*IFN is an inducible gene possibly involved in Japanese eel defense against viral and bacterial infection.

## 1. Introduction

The interferons (IFNs) are a family of multifunctional cytokines as the first line against virus infections and can be induced through different signaling pathways in response to pathogen infection or pathogen associated molecular patterns (PAMPs) stimulation (González-Navajas et al., 2012). In teleost fish, both the type I and II IFN systems are pivotal to antiviral defense in innate and adaptive immunity against viral infection (Zou and Secombes, 2011). Type I IFN genes have been cloned and analyzed in various fish species, such as medaka (*Oryzias latipes*) (Maekawa et al., 2016), turbot (*Scophthalmus maximus*) (Pereiro et al., 2014), rock bream (*Oplegnathus fasciatus*) (Wan et al., 2012), Atlantic salmon (*Salmo salar*) (Sun et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Chang et al., 2009; Zou et al., 2007), zebrafish (*Danio rerio*) (Altmann et al., 2003; Zou et al., 2007), and European seabass (*Dicentrarchus labrax*) (Casani et al., 2009).

Two groups of type I IFNs, namely group I and group II, have been clarified in fish according to their primary protein structures (Zou et al., 2007). The group I IFNs have two cysteine residues and are present in all teleost fish, while the group II IFNs have four cysteine residues which have been found only in salmonids and cyprinids to date (Zhu et al., 2013; Zou et al., 2007). Furthermore, given the phylogeny trees of the mature peptides, teleost type I IFNs are further classified as IFNa, -b, -c, -d, -e, -f and -h subgroups, with IFNa, -d, -e and -h belonging to the group I and IFNb, -c and -f the group II of type I IFNs (Ding et al., 2016; Zou et al., 2014; Zou and Secombes, 2011). Previous studies have been mainly focused on the kinetics of gene expression induced by viral infection and/or dsRNA poly I:C as well as the antiviral activity of recombinant proteins (Chang et al., 2009; Ohta et al., 2011; Pereiro et al., 2014; Svingerud et al., 2012; Wan et al., 2012). Recently, Yeh et al. (Yeh et al., 2013) reported that the activity of CpG-ODNs with the TLR9 and TLR21 in Zebrafish to generate the strong antimicrobial responses. However, the modulation of expression of type I IFNs in the host response to bacterial infection or to its PAMPs such as LPS and PGN is still

*Abbreviations:* cDNA, DNA complementary to RNA; mRNA, messenger RNA; ORF, open reading frame; RACE, rapid amplification of cDNA ends; UTR, untranslated region; IFN, interferon; *Aj*IFN, IFN gene from Japanese eel (*Anguilla japonica*); qRT-PCR, quantitative real-time polymerase chain reaction; PAMPs, pathogen associated molecular patterns; LPS, lipopolysaccharide; poly I:C, polyinosinic-polycytidylic acid; PGN, peptidoglycan; TSB, tryptone soya broth; TLR, Toll-like receptor; RLRs, RIG-I-like receptors

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limited in teleost fish (González-Navajas et al., 2012; Zou and Secombes, 2011).

*Aeromonas hydrophila* is one of major pathogenic bacteria in freshwater farming of eel and other fishes (Guo et al., 2014; Zhang et al., 2014). In present study we report the identification of a type I IFN gene (*Aj*IFN) from Japanese eel (*Anguilla japonica*). In addition, the expression modulation of *Aj*IFN *in vivo* and *in vitro* with bacterial/viral PAMPs stimulation and different concentration of *A. hydrophila* infection was also investigated. We hope that the information presented here will promote better understanding of the function and regulation of fish type I IFNs in response to both virus and bacteria infection.

## 2. Materials and methods

### 2.1. Fish collection and immune challenge

Healthy Japanese eels, weighing 45–50 g, were purchased from an eel farm (Fuqing, China). They were kept in a 1000-L tank at 25 °C with recirculated and aerated water for a week to acclimate to laboratory conditions. Liver, spleen, gills, kidney, intestine, heart, skin, and muscle were harvested and then frozen in liquid nitrogen and then stored at − 80 °C for RNA extraction.

A strain of *A. hydrophila* B11, isolated from the liver of diseased eels with ulcers on the peduncle was inoculated in tryptone soya broth (TSB) and incubated on a shaker at 28 °C for 24 h (Guo et al., 2015). The bacteria were collected and diluted to the concentration of $4 \times 10^4$ cfu/mL in 0.01 mmol/L PBS (pH = 7.4). Fish immune stimulation was performed by intraperitoneal injection of 250-μL LPS (Sigma, 4 mg/mL) in phosphate buffered saline (PBS), 250-μL poly I:C (Sigma, 2 mg/mL) in PBS, and 250-μL $4 \times 10^4$ cfu/mL *A. hydrophila* in PBS, respectively. Fish injected with 250 μL PBS were used as controls. Four fish were sacrificed for the control group and treatment group respectively for each time point. Liver, spleen and kidney of each group were collected at 0, 6, 12, 24, 48, and 72 h after injection and then frozen in liquid nitrogen to store at − 80 °C for quantitative real-time polymerase chain reaction (qRT-PCR).

### 2.2. Cell culture and treatments

For *in vitro* studies, Japanese eel liver cells were cultured as described in our previous study (Feng et al., 2016). Cells were then treated with 30 μg/mL LPS (Sigma), 50 μg/mL poly I:C (Sigma), 30 μg/mL CpG-DNA (Sangon Biotech, Shanghai), 30 μg/mL peptidoglycan (PGN, Sigma), three different concentration of *A. hydrophila* ($1 \times 10^6$ cfu/mL, $1 \times 10^7$ cfu/mL, and $1 \times 10^8$ cfu/mL, respectively), and the untreated cells were served as control. Four parallel samples were included of each group at 0, 3, 6, 12, 24, and 48 h after treatment. Total RNA from cells was isolated using E.Z.N.A.™ Total RNA Kit II (Omega) following manufacturer's instructions.

### 2.3. Cloning of full-length cDNA of AjIFN

Total RNA from tissues was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA from the liver of Japanese eel was used to synthesize the first-strand cDNA for the RACE reaction using the SMART RACE cDNA Amplification Kit (Takara, China) according to the manufacturer's instructions.

The EST of *Aj*IFN was screened from Japanese eel transcriptome database in our lab. Based on the partial *Aj*IFN sequence, the missing parts of the cDNA were obtained by 5'and 3′ rapid amplification of cDNA ends (5′ and 3′ RACE) was performed as described in our previous study (Feng et al., 2016) and the gene-specific primers of *Aj*IFN gene was listed in Table 1.

### 2.4. Bioinformatics analysis

Sequence similarity analysis was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The full-length cDNA sequence of *Aj*IFN was analyzed with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The deduced amino acid sequence was analyzed using the ExPASy molecular biology server (http://www.us.expasy.org/tools/). Multiple sequence alignment was performed using the CLUSTALW program (http://www.ebi.ac.uk/clustaw/). The protein domain features were predicted using NCBI CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html). The presence of signal peptide was analyzed with the SignalP 3.0 server (http://www.cbs.dtu.dk/services/signalp-3.0/). The phylogenetic tree was constructed using MEGA 5 software based on Neighbor-Joining method with the bootstrapping of 1000 repetitions.

### 2.5. Expression analysis of AjIFN by qRT-PCR

First strand cDNA was synthesized from total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China) following the manufacturer's instructions. Then, synthesized cDNA was diluted with nuclease-free water by 10-fold and stored at − 20 °C until use. Primers for *Aj*IFN and β-actin (endogenous control gene) were designed using Primer 5.0 software (Table 1). The qPCR was performed as described in our previous study (Feng et al., 2014). All data were given in terms of relative mRNA expression as arithmetic means ± standard error of the mean (SEM) of four separate individuals, each assayed in triplicate.

### 2.6. Statistical analysis

Data from all experiments were analyzed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Significance of differences was determined by Student's *t*-test. *P* values smaller than 0.05 were considered as statistically significant.

## 3. Results

### 3.1. Molecular characterization of AjIFN

The full-length cDNA sequence of *Aj*IFN (GenBank accession No: KT156977) is composed of 826 bp with a 5′-untranslated region (UTR) of 47 bp, an open reading frame (ORF) of 531 bp encoding a 177 amino acid protein, and a 3′-UTR of 248 bp within five mRNA instability motifs (ATTTA) and an eukaryotic polyadenylation signal (AATAAA) (Fig. 1). Sequence analysis by using NCBI CDD revealed this protein contained IFabd conserved domain, and two conserved cysteine residues potentially forming disulphide bridges were also founded (Fig. 1).

### 3.2. Structural analysis of AjIFN

Multiple alignments and amino acid sequence similarity comparison of Japanese eel *Aj*IFN with type I IFN in Atlantic salmon, rainbow trout, goldfish, common carp, Japanese pufferfish, turbot, medaka, and Human were shown in Fig. 2. Two conserved cysteines potentially forming disulphide bridges and the type I IFN family signature motif were found in all the above teleost fish. As shown in Table 2, the Japanese eel IFN had the highest identity (50.6%) with the Atlantic salmon IFNa1. The sequence identity between Japanese eel IFN and rainbow trout IFN1, goldfish IFN, common carp IFN, channel catfish IFN2, zebrafish IFNphi1, medaka IFNd, stickleback IFN1, Japanese pufferfish IFN1, medaka IFNa, human IFN a1, and turbot IFN 1 was 48.2%, 42.0%, 40.4%, 38.5%, 37.8%, 32.2%, 30.4%, 30.1%, 26.4%, 19.9%, and 17.6%, respectively. The lowest identity (14.8%) was found between Japanese eel IFN and Human IFN b.