Fish & Shellfish Immunology 59 (2016) 298–304

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Temperature-dependent regulation of gene expression in Japanese flounder *Paralichthys olivaceus* kidney after *Edwardsiella tarda* formalin-killed cells

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ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form
28 October 2016
Accepted 30 October 2016
Available online 1 November 2016

Keywords:
Edwardsiella tarda
Japanese flounder
Microarray
Temperature

ABSTRACT

Temperature affects the activities of the immune system and the susceptibility of fish to pathogens. To investigate the modulation of temperature on immune related gene expression in formalin-killed cells (FKC) of *Edwardsiella tarda*-injected Japanese flounder *Paralichthys olivaceus*, fish reared at 15 or 22 °C were injected with FKC of *E. tarda*. The up-regulation of immune related genes was detected in FKC-injected fish at both temperatures by qPCR. The mRNA expression of IFNγ was highly up-regulated at 6 h post injection (hpi) in FKC-injected fish at 15 °C, whereas at 22 °C, strong up-regulation of the gene was detected at 3 hpi The mRNA expression level of IRF1 was detected from 3 hpi to day 14 post injection in fish reared at 15 °C, but the gene was up-regulated from 3 to 6 hpi in fish reared at 22 °C. Comprehensive gene expression profiling showed that immune related genes are differentially expressed between 15 and 22 °C. Genes involved in the IFNγ signaling pathway were up-regulated at 22 °C but not at 15 °C. These results demonstrate that gene(s) involved in IFNγ signaling pathway in Japanese flounder stimulated with FKC of *E. tarda* are regulated by temperature.

1. Introduction

Temperature is a factor that influences the activities of the immune systems in fish. The fish innate immune system, which against invading pathogens, has been considered relatively temperature-independent [1]. For example, sockeye salmon *Oncorhynchus nerka* reared at 8 °C showed similar complement activity to that at 12 °C [2]. However, the complement activities were decreased in tilapia *Oreochromis mossabicus* shifted to 19 and 23 °C from 27 °C, while those shifted to 30 and 33 °C were increased [3]. Similarly, rainbow trout *O. mykiss* reared at 15 and 20 °C showed higher complement activities than at 5 °C. These fish also showed different C5a gene mRNA levels [4]. The innate immune system is now considered to be influenced by the rearing temperature.

In many fish species, adaptive immunity, especially for the induction of specific antibodies is delayed or stopped by decreasing temperature [5] [6] [7], and [8]. In several fish, including carp *Cyprinus carpio* [9], Japanese flounder *Paralichthys olivaceus* [10] and hybrid striped bass *Morone chrysops* x *Morone saxatilis* [11], the increase of specific antibody titers after immunization is delayed. In channel catfish *Ictalurus punctatus*, specific antibody titers were increased by immunization with thymus-independent antigen, but not with thymus-dependent antigen [12], suggesting that the function of T-cells is influenced by temperature.

Comprehensive gene expression profiling in Atlantic cod showed that at 10 °C, certain interferon-regulated genes, such as interferon regulatory factor 1 (IRF1), were up-regulated at 24 h after injection of polyriboinosinic polyribocytidylic acid (polyIC) and formalin-killed *Aeromonas salmonicida* cells, while at 16 °C these genes were induced at 6 h [13] and [14]. Similarly, in Japanese flounder, interferon-regulated genes, such as Mx and STAT1, were strongly up-regulated in the fish at 15 °C at 24 hpi with polyIC, whereas at 25 °C the genes were up-regulated at 3 hpi [15]. The genes involved in type I IFN signaling were also reported to be delayed at lower temperatures in sevenband grouper *Epinephelus septemfasciatus* [16]. At 30 and 35 °C, Mx gene mRNA levels peaked at 3 h post polyIC injection, whereas at 20 and 25 °C they peaked at 24 h.

The expression of immune related genes in fish reared at different temperatures and treated with bacteria has also been studied in rainbow trout [17] and Atlantic cod *Gadus morhua* [14].

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http://dx.doi.org/10.1016/j.fsi.2016.10.048
1050-4648/

Table 1
Primers used for qPCR analyses.

Gene name	Sequence
β-actin-F	5′-TGATGAAGCCCAGAGCAAGA-3′
β-actin-R	5′-CTCCATGTCATCCCAGTTGGT-3′
IL-1β-F	5′-CAGCACATCAGAGCAAGACAACA-3′
IL-1β-R	5′-TGGTAGCACCGGGCATTCT-3′
IFN-γ-F	5′-TGTCAGGTCAGAGGATCACACAT-3′
IFN-γ-R	5′-GCAGGAGGTTCTGGATGGTTT-3′
Hepcidin-F	5′-CGTGCTCGCCTTTGTTTG-3′
Hepcidin-R	5′-TGATGTGCCGCAACTGG-3′
IRF-1-F	5′-CGTTGCCTGACATTGAGGAG-3′
IRF-1-R	5′-TACCGTCTTCCCCTGCTTTG-3′
IRF-10-F	5′-TTTGGTCATCTCATCCCAACC-3′
IRF-10-R	5′-CTTCCAGCACAATTCACAGACAC-3′
CUST_166-F[a]	5′-GCACCTGAAAGAGTGGCTGA-3′
CUST_166-R[a]	5′-ATGGTCCTGTCCTCGTCCTC-3′

[a] The sequences used for the design were deposited into the GEO database.

The up-regulation of cytokine genes, interleukin 1β (IL-1β), IL-10 and interferon γ (IFNγ), were faster and higher in rainbow trout intra-peritoneally injected with a bacterin of *Yersinia ruckeri*, at 25 °C than those at 5 °C [17]. In contrast, in Atlantic cod intra-peritoneally injected with a bacterin of *A. salmonicida*, fewer genes were up- or down-regulated at 10 °C than at 16 °C in comparison with those with polyIC injection [14]. However, temperature-dependent modulation of immune related gene induction by bacterial injection has not yet been well characterized in Japanese flounder. In this study, we investigated the expressions of certain immune related genes in Japanese flounder affected by rearing temperature (15 °C and 22 °C) and by injection of formalin-killed cells (FKC) of *Edwardsiella tarda*. Furthermore, comprehensive gene expression profiling was performed by using oligo-microarray to examine the effect of temperature on the expression of Japanese flounder genes.

2. Materials and methods

2.1. Fish and treatment

Edwardsiella tarda strain 54 [18] was cultured in heart infusion medium (DIFCO) and colony forming unit was counted. The cells were incubated with formalin at a final concentration of 0.3% at 25 °C for 2 days and washed with phosphate-buffered saline (PBS) twice.

Japanese flounder with an average size of 10 cm in total length were acclimated and reared at 15 °C or 22 °C for a week prior to the start of the experiment. For gene expression profiling, fish were intraperitoneally injected with 50 μL of the FKC (3.3×10^8 cells/fish). Spleens were sampled at various times before (day 0) and after injection, soaked in RNA later and stored at −30 °C.

2.2. Gene expression profiling by qRT-PCR

Total RNA was extracted from the spleen of each sample (n = 5)

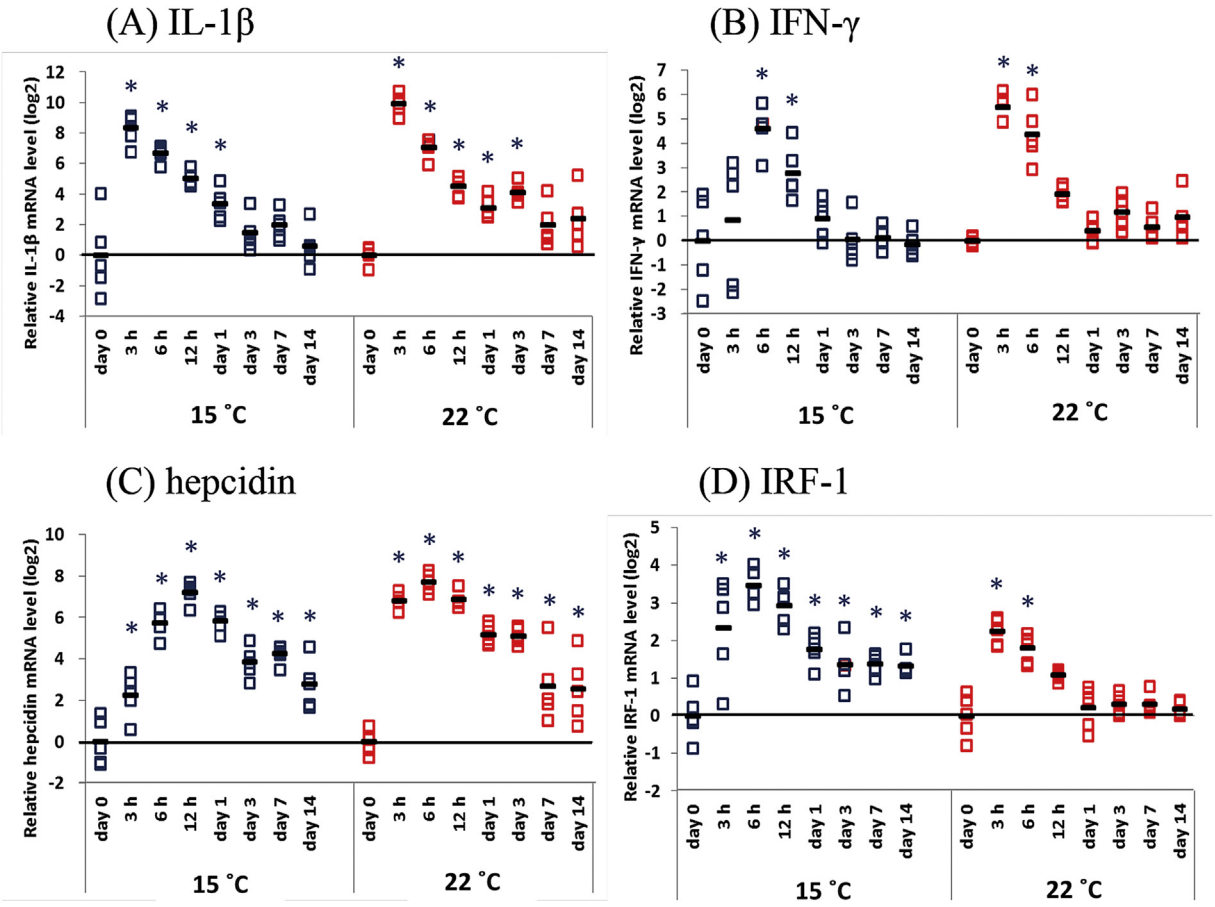

Fig. 1. Relative mRNA expression of immune-related genes. (A) IL-1β, (B) IFN-γ, (C) hepcidin, and (D) IRF1 in the spleen of normal fish and FKC-injected Japanese flounder reared at 15 and 22 °C (n = 5) at the indicated times after injection. Expressions are normalized to the expression of β-actin. The expression values are represented by Log2 values. Asterisks (*) indicate statistical significance by *t*-test ($p < 0.05$).