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Short communication

Comprehensive gene expression profiling in Japanese flounder kidney after injection with two different formalin-killed pathogenic bacteria

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ABSTRACT

Pathogenic bacteria possess some components, that are recognized by the host immune receptors. Different components are recognized by distinct receptors, and thus it is speculated that these components may regulate different gene sets. To investigate the gene expression profiles regulated by different bacterial components in Japanese flounder *Paralichthys olivaceus*, Japanese flounder were intraperitoneally injected with formalin-killed bacterial cells (FKC) of *Edwardsiella tarda* and *Streptococcus iniae*. The numbers of differentially regulated genes were much larger in the fish injected with *E. tarda* than those with *S. iniae*. Comprehensive gene expression profiling showed that almost all of the genes differentially regulated by injections of *E. tarda* FKC were also differentially regulated by injections of *S. iniae* FKC. mRNA levels of inflammatory cytokines, including interleukin (IL)-1β, IL-8, interferon γ and tumor necrosis factor were upregulated in both of the injected groups. Each of these mRNAs except for IL-8 mRNA were also much higher in the *E. tarda* FKC injected group than in the *S. iniae* FKC injected group. Furthermore, mRNA levels of IL-6 were strongly up-regulated in the *E. tarda* FKC injected group. The *E. tarda* FKC might induce higher inflammatory responses than *S. iniae* FKC.

Disease control in aquaculture is essential to improve fish production. Vaccination is one of the measures to prevent mass mortality caused by disease outbreaks. A number of fish vaccines have been developed and used in aquaculture [1,2]. Fish vaccines are generally prepared by inactivate pathogenic organisms by formalin treatment. For vaccines against bacterial pathogen, formalin-killed cells (FKC) induce not only specific adaptive immunity but also innate immune responses. This is because host immune systems recognize molecules associated with pathogenic bacteria. Molecules such as lipopolysaccharide and peptidoglycan are called pathogen-associated molecular patterns (PAMPs) because they are recognized by pattern recognition receptors (PRRs), and induce innate immune responses [3,4]. In mammals, PRRs are responsible for different PAMPs molecules, and thus it is speculated that different pathogens might induce different host innate immune responses [3,4]. Different bacteria have been shown to regulate some genes in different expression patterns [5–7].

A number of bacterial pathogens have been reported in fish aquaculture. For example, bacterial diseases such as Edwardsillosis caused by *Edwardsiella tarda* and Streptococcosis caused by *Streptococcus iniae* are severe problems in aquaculture of Japanese flounder *Paralichthys olivaceus*, one of major aquaculture species in China, Japan and Korea [8,9]. These are Gram-negative and -positive bacteria and they possess distinct PAMPs, lipopolysaccharide (LPS) and peptidoglycan (PG) as a major cell-wall component, respectively. cDNA microarray studies of Japanese flounder have shown that a number of genes are differently regulated by vaccination with *E. tarda* FKC [10] and that *E. tarda* and *S. iniae* FKC induce the expressions of different genes with distinct expression profiles [11]. Here we developed a new Japanese flounder oligo-microarray, which contains more than 13,000 unique probes. In order to evaluate the comprehensive gene expression profiles regulated by different bacteria, the fish were intraperitoneally injected with formalin-killed cells of different bacteria, and the gene expression profiles in the kidney were analyzed using the microarray.

The Agilent 8 × 15 k custom oligo DNA microarray was based on nucleotide sequences deposited in the public databases and transcriptome data obtained from Japanese flounder leukocytes using next generation sequencer [12]. Gene annotation on the microarray was performed using the blast2GO program [13]. All sequence data on the microarray are available on the Gene Expression Omnibus (GEO) database. *E. tarda* and *S. iniae* FKC were prepared according to

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Dumrongphol et al. [11], and colony-forming units were counted. Fish (approximately 8 cm) were reared at 18 °C and intraperitoneally injected with the 1.0×10^7 CFU/fish *E. tarda* and *S. iniae* FKC, and PBS was injected as a control. Tissue samples were collected from the kidney at 6 and 12 h post injection. Total RNA was extracted from the kidney using an RNeasy Mini Kit and used to prepare Cy3-labeled cRNA using a QuickAmp Labeling Kit, one-color (Agilent, USA). The labeled cRNA was hybridized with the microarray for 15 h at 65 °C as described in the Agilent QuickAmp Labeling kit one-color protocol. The microarray was scanned with an Agilent G2565CA scanner and image data were analyzed with Feature Extraction Software v9.5.3.1 (Agilent, USA). The data were normalized and analyzed using GeneSpring GX v11.5.1 Software (Agilent, USA).

The microarray data were deposited in the GEO database under accession number GSE47556. The mRNA levels of over 2000 spots differed by at least 2-fold from the PBS injected groups, over 600 spots differed by at least 4 fold and over 200 spots differed by at least 8-fold (Table 1). Interestingly, the numbers of spots showing different mRNA levels by *E. tarda* FKC group were much larger than those by *S. iniae* group at all time points. The heat-map of the expression profiles showed that the differently regulated genes were commonly observed in *E. tarda*- and *S. iniae* groups, but some genes were more strongly regulated in the former group (Fig. 1). Dumrongphol et al. [11] identified some genes that were induced by *S. iniae* FKC but not by *E. tarda* FKC. However, in the present study, we did not observe any genes that were up-regulated only by *S. iniae* FKC. This discrepancy may be partly due to differences in the methods or differences in sampling time points used in the two studies. It should be noted that our microarray detected much number of genes that are differently regulated by the FKC than the oligomicroarray of Dumrongphol et al. Our preliminary microarray data acquired at 6 h after FKC injection (acc. No. GSE29219) also showed more genes were differently regulated by *E. tarda* FKC treatment than by *S. iniae* treatment.

The genes whose mRNA levels changed by more than 4-fold are grouped in their GO functional classes in Fig. 2. Almost all of the genes are up-regulated by *E. tarda* FKC, while their mRNA levels are also slightly changed by *S. iniae* FKC. These genes are involved in many cellular processes besides immunity. A number of genes are differently regulated during innate immune responses [10,11]. Some of the genes might be initially regulated by the PAMPs, and the others may be induced/suppressed by various signal molecules such as cytokines.

Among the cytokines, inflammatory cytokines, including interleukin(IL)-1β, IL-6, IL-8, interferon(IFN)γ and tumor necrosis factor (TNF), have important roles in the regulation of innate immune responses [14]. These cytokines are important in the regulation of gene expression of many genes, and used as markers of innate immune responses [14]. The mRNA levels of IL-1 mRNA levels of up-regulated in both the *E. tarda* and *S. iniae* groups, although they were higher in the former (Fig. 3). The mRNA levels of IL-8 and TNF were also up-regulated in the *E. tarda* and *S. iniae* groups, while IL-6 mRNA levels were up-regulated only in the *E. tarda* groups (Fig. 3). IL-6 induces the production of IgM [15,16]. It also enhances

Table 1
The numbers of spots showing differential gene expression levels after FKC treatments.

	E. tarda 6 h		*E. tarda* 12 h		*S. iniae* 6 h		*S. iniae* 12 h		Total
	Up	Down	Up	Down	Up	Down	Up	Down	
2-fold	760	551	773	885	230	185	305	291	2325
4-fold	272	55	294	187	50	7	79	20	611
8-fold	119	5	139	42	14	0	35	0	222

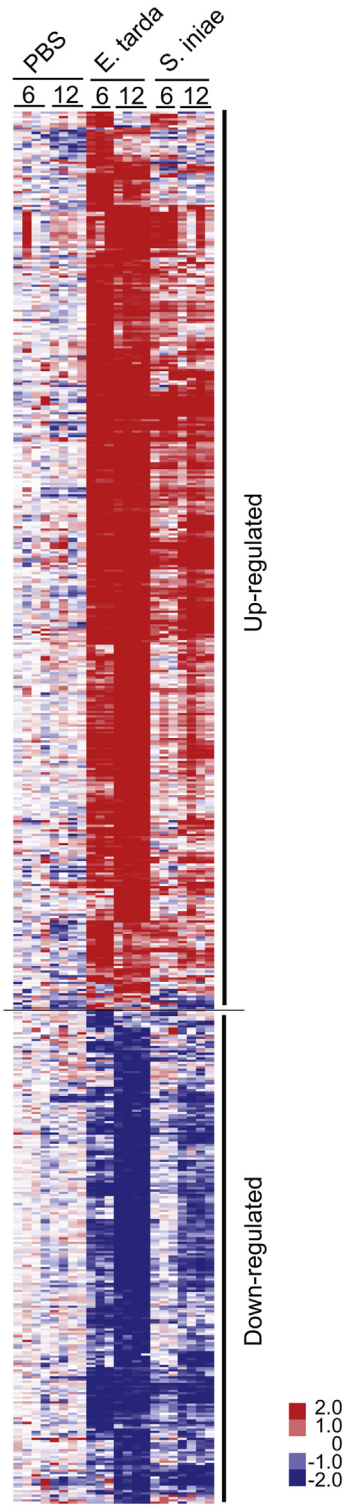

Fig. 1. Heat-map of gene expression profiles at 6 and 12 h after the FKC injections. The genes showing more than 4-fold differently regulated by *Edwardsiella tarda* and *Streptococcus iniae* FKC injections. Rows and lines represent samples and genes, respectively. Red and Blue color indicate up- and down-regulation in Log2 transformed values, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)