Analysis of Immune Response in Yellowtail upon Vaccination with a Bivalent Vaccine of *Vibrio anguillarum* and *Lactococcus garvieae*

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ABSTRACT—We inoculated yellowtail *Seriola quinqueradiata* with a commercial bivalent vaccine containing inactivated *Vibrio anguillarum* and *Lactococcus garvieae* to better understand the immunological responses to the vaccination. Serum samples were collected from vaccinated and control (PBS-injected) fish at 1, 3, 5, 7, 10, and 14 days post-vaccination (dpv). Compared to the controls, the serum from the vaccinated fish inhibited the proliferation of *V. anguillarum* from 7 dpv. However, vaccination had no effect on *L. garvieae* proliferation. The antibody titer against *V. anguillarum* obviously increased from 7 dpv, but no such increase was detected for *L. garvieae*. We confirmed that the protection conferred by the *V. anguillarum* vaccine was a result of the antibodies against the pathogen. However, humoral factors related to protection were not observed for *L. garvieae*. The expression of immune-related genes in the head kidney was profiled by microarray analysis. Twenty-two immune-related genes were up-regulated during the experimental period. Up-regulation of cellular immune-related genes, such as tumor necrosis factor, CC chemokine, and macrophage colony-stimulating factor receptor, was mostly observed from 1 to 3 dpv, whereas that of humoral immune-related genes was observed from 3 to 14 dpv. Notably, immunoglobulin-related genes showed continuous up-regulation during the 2-week experimental period.

Key words: *Seriola quinqueradiata*, Yellowtail, Vaccine, Immune response, Gene expression, *Vibrio anguillarum*, *Lactococcus garvieae*

Vaccination is an effective tool for the control of infectious diseases in farmed fish. The first vaccine approved for use against vibriosis in ayu was developed in 1988 and following that, a number of other vaccines have been developed. Currently, 14 vaccines are licensed in Japan.

For a vaccine to be manufactured and sold, pharmaceutical affairs law requires licensing of the vaccine by the Ministry of Agriculture, Forestry and Fisheries. The efficacy of fish vaccine is normally evaluated by a challenge test, the exception of which is the *Vibrio* vaccine, which was evaluated by the agglutination antibody titer test.

The challenge test requires a large number of fish and raises several ethical issues. From the perspec-

tive of animal welfare, the number of animals tested and their suffering must be minimized, but it is difficult to do so as long as the challenge test is used as the official licensing examination. The second issue is the facility used. A number of vaccines have been developed recently and a large number of experimental fish are needed to test each product. Therefore, larger spaces are required for stocking experimental fish. This implies a considerable cost increase. One final complication is reproducibility. The challenge test does not always yield reproducible results due to fish size differences and bacterial pathogenicity. Solving these problems requires the development of an alternative to the challenge test.

To develop an alternative to the challenge test, it is necessary to find a suitable parameter for vaccine evaluation. To this end, a large-scale screening of immune response in yellowtail was conducted in this

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study. Yellowtail stimulated with a bivalent vaccine were used to measure antibacterial activity in serum and skin mucus, determine antibody titer, and profile gene expression by microarray analysis.

In Japan, *Seriola* fish, including *Seriola quinqueradiata*, *Seriola dumerili*, and *Seriola lalandi*, is one of the most important farmed fish. At *Vibrio* vaccine co-relationship between agglutination antibody titer and vaccine efficacy is examined on approval process, and there are reports of the antibody response of *Seriola dumerili* to *V. anguillarum* (Zaccone and Mancuso, 2008) and Red Sea Bream Iridovirus (Kwon *et al.*, 2010). The immune response of yellowtail to different phenotypes of *L. garvieae* (Iida *et al.*, 1982; Yoshida *et al.*, 1996, 1997; Hirono *et al.*, 1999; Ooyama *et al.*, 1999, 2002) was studied and vaccination was evaluated (Iida *et al.*, 1982). However, the underlying mechanism of immune induction following vaccination in *Seriola* fish is still not well understood.

Antibody titer is the most popular parameter for the determination of vaccine efficacy in mammals. If antibody titer could be used in the licensing of fish vaccines, many of the problems associated with the challenge test would be solved. Except *Vibrio* vaccine, there is little information on the antibody response of *Seriola* fish to vaccination, although the vaccines have a strong protective effect. In other fish, several studies of antibody reaction have been conducted. In Atlantic cod with vibriosis vaccine and the relationship between vaccine efficacy and antibody response has been thoroughly studied but not completely understood (Pilström and Petersson, 1991; Bengtén *et al.*, 1994; Stenvik *et al.*, 2000; Caipang *et al.*, 2008, 2009). Espelide *et al.* (1991) suggested that the efficacy of vibriosis vaccine developed for Atlantic cod is not related to the antibody response of the fish. Atlantic salmon is able to produce a strong antibody response to both *Vibrio salmonicida* and *V. anguillarum* (Steine *et al.*, 2001). Antibody response to lipopolysaccharide (LPS) has been reported in other fish species, including rainbow trout (Hasting and Ellis, 1990), spotted wolfish (Grøntvedt and Sigrun, 2004), and halibut (Schrøder *et al.*, 1992; Ingilæ *et al.*, 2000).

Generally, mucus covering skin surface acts as the first barrier against infection (Ellis, 2001). The important role of mucus in defense against infections is well known and has been studied in several fish species (Hjelmeland *et al.*, 1983; Rombout *et al.*, 1993; Fasta *et al.*, 2002). To compare the antibacterial activities of mucus and serum, a bacterial proliferation inhibition test was performed.

Microarray analysis is an efficient method for large-scale gene expression. It is suitable to search which genes play an important role in vaccine-induced immunity. In this study, we used DNA microarray to examine gene expression profile of yellowtail after vac-

nation, to elucidate their immune response at the molecular level. In yellowtail, vaccines against *V. anguillarum* and *L. garvieae* are the most common. Considering actual usage and history, a bivalent vaccine consisting of inactivated *V. anguillarum* and *L. garvieae* was used in this study.

Materials and Methods

Fish

A total of 48 healthy, artificially hatched yellowtail with weights ranging from 171 to 431 g were used. Six fish each were stocked in 200 L circular tanks at the National Veterinary Assay Laboratory, Japan. Water temperature was kept at 25°C and the salinity of rearing seawater was checked every day. Fish were fed standard commercial diet for yellowtail throughout the experiment. Handling was done according to the National Veterinary Assay Laboratory's animal management guidelines.

Vaccine and vaccination

Commercial inactivated bivalent vaccine containing the antigens of *V. anguillarum* and *L. garvieae* (Kyoritu) was stored at 4°C and injected according to the instructions on the package leaflet. The commercial vaccine has passed the licensing examination by the National Veterinary Assay Laboratory. Vaccine quality was confirmed by National Veterinary Assay Protocol: Antibody titer against *V. anguillarum* should be ≤ 16 in vaccinated fish serum, whereas in control fish serum, it should be ≤ 2 . Mortality resulting from the challenge with *L. garvieae* must show a significant difference ($p < 0.05$) between vaccinated fish and control (phosphate buffered saline (PBS)-injected) fish. Thirty fish were injected intraperitoneally with 0.1 mL of vaccine. Eighteen fish were treated with the same amount of sterile PBS as control.

Blood and skin mucus sampling

Sampling was conducted at 1, 3, 5, 7, 10, and 14 days post vaccination (dpv). Five fish from the vaccinated group and three fish from the control group were anesthetized with FA-100 (Tanabe Seiyaku) and blood was rapidly collected by cardiac puncture using non-heparinized syringes. Blood samples were incubated at room temperature (18 to 23°C) for 1 h and then stored at 4°C for more than 1 h. Serum was obtained by centrifugation at 3,000 rpm for 10 min and stored at -80°C until use. Skin mucus was peeled using a cell scraper according to Staroscik and Nelson (2008). The supernatant of the mucus solution was obtained by centrifugation at 3,000 rpm for 10 min. Serum and skin mucus samples were incubated at 45°C for 20 min before use in tests to deactivate nonspecific factors.

Equal amounts of samples from vaccinated fish col-

lected at the same sampling point were mixed to make pooled samples. Equal amounts of all the control samples were mixed regardless of the sampling point.

Serology

Antigen preparation: A frozen stock of *V. anguillarum* (PT-213 strain) was inoculated onto tryptic soya agar containing 1.5% NaCl (1.5% NaCl-TSA) and incubation was carried out at 25°C for 18 h. Then, a loop of the bacterial preparation was inoculated onto new 1.5% NaCl-TSA. After incubation for 24 h, the collected colonies were washed with PBS and centrifuged three times at 3,000 rpm for 10 min. The bacterial pellet was re-suspended in PBS and then boiled for 1 h. *L. garvieae* (KG9502 strain) was prepared in the same manner except that Todd Hewitt agar (THA) was used as the growth medium.

Antibody titers against *V. anguillarum* and *L. garvieae* were measured by the agglutination method. Serum samples heated to 45°C for 20 min were diluted with a PBS dilution series from 2¹ to 2¹¹ on a 96-well plate. The same volume of antigen solution was added to the plate and incubation was carried out at 25°C for 1 h. After that, the plate was incubated at 4°C for 18 h. Agglutination antibody titer was determined by the highest dilution of a serum sample that caused complete clumping of each antigen.

Inhibition of bacterial proliferation test

A frozen stock of *V. anguillarum* PT-213 strain (serotype J-O-3) (National Veterinary Assay Laboratory, Tokyo, Japan) kept at -80°C was thawed promptly and inoculated into tryptic soya broth containing 1.5% NaCl (1.5% NaCl-TSB), and the whole was incubated at 25°C for 18 h. After incubation, 0.1 mL of the medium was inoculated into 10 mL of new 1.5% NaCl-TSB. *L. garvieae* KG9502 strain (National Veterinary Assay Laboratory, Tokyo, Japan) (Kijima *et al.*, 2007) was prepared with the same procedure as that for *V. anguillarum* except that Todd Hewitt broth (THB) was used as the growth medium.

After the second passage, an overnight culture of *V. anguillarum* or *L. garvieae* with known count was adjusted to approximately 10³ CFU/mL with PBS. One hundred µL of bacterial suspension was transferred to 0.5 mL micro-tubes and an equal volume of undiluted fish serum was added. The bacterial suspension with PBS added served as the control. The solution was mixed with a vortex mixer and kept at 25°C. After incubation for 24 h, bacterial count was determined by inoculating solutions prepared (10¹ to 10⁷) onto 1.5% NaCl-TSA for *V. anguillarum* and THA for *L. garvieae*. The reduction rate of bacteria was calculated with the following formula: reduction rate (%) = 100 - (log₁₀ VS / log₁₀ CS) × 100). VS is the bacterial count in the tested sample obtained from vaccinated fish, and CS is

the bacterial count in the tested sample obtained from control (PBS-injected) fish.

Kidney samples and total RNA isolation for microarray analysis

Kidney samples were excised from fish after blood and skin mucus sampling. Part of the kidney was immediately immersed in an RNA protecting solution, RNAlater (QIAGEN). Total RNA samples were isolated from stabilized kidney using an RNeasy-mini kit (QIAGEN) following the manufacturer's protocol and stored at -80°C. Then, the total RNA sample (30 µg) prepared from each kidney at different time points was used for cDNA synthesis with a LabelStar Array kit, cDNA labeling module (QIAGEN). cDNAs were first labeled with aminoallyl-dUTP and purified with QIAquick spin columns (QIAquick PCR purification kit, QIAGEN). The purified cDNAs were subsequently labeled with Cy3 or Cy5 mono-functional reactive dye (GE Healthcare); RNA samples from control (PBS-injected) fish were labeled with Cy3, whereas samples from vaccinated fish were labeled with Cy5 according to the manufacturer's instructions. This was followed by purification with MinElute spin columns (LabelStar Array kit, cDNA cleanup module, QIAGEN) according to the manufacturer's instructions. Hybridization and detection of fluorescence were done following a previously reported protocol (Yasuike *et al.*, 2007).

Microarray analysis

Microarray chips developed by Daraewiroj *et al.* (2008) were used to survey immune-related gene expression profiles in vaccinated fish. Microarray analysis was carried out according to the protocol reported by Daraewiroj *et al.* (2008). GenePix Pro 6.0 analysis software from Axon Instruments, Inc. (www.axon.com) was used to generate hybridization signals from images obtained by scanning. Feature ratio was calculated from the stimulated sample signal intensity divided by the control signal intensity. The minimum signal intensity was set at 2000. Signals lower than this level were discarded. Only the genes that exhibited feature ratios higher than 2.0 were considered as up-regulated genes (Kurobe *et al.*, 2005).

The data were further analyzed by Cluster Program 3.0 (Clustering Library Version 1.27) using the average linkage hierarchical clustering algorithm with Euclidian distance as the similarity metric (M. De Hoon, S. Imoto, J. Nolan and S. Miyano, Open source clustering software, Bioinf Appl Note 20 (2004), pp. 1453–1454.). Cluster analysis results were visualized with TreeView (version 1.60) (A. J. Saldanha, Java Treeview extensible visualization of microarray data, Bioinformatics 20 (2004), pp. 3246–3248.).

Fig. 1. Equal amounts of samples obtained on the same day and within the same group were mixed to make pooled samples. Equal amounts of all the control samples were mixed regardless of sampling point. Antibody titer (expressed as log₂) against *V. anguillarum* (A) and *L. garvieae* (C) from vaccinated yellowtail serum or skin mucus at each sampling point ($N = 5$). Detection limit is < 2 (2^1). Reduction rate (%) of *V. anguillarum* (B) and *L. garvieae* (D) upon incubation with serum and skin mucus from vaccinated yellowtail at each sampling point ($N = 5$). Reduction rate was calculated with the following formula: reduction rate (%) = $100 - (\log_{10} \text{VS} / \log_{10} \text{CS}) \times 100$. VS: Bacterial count in tested sample obtained from vaccinated fish. CS: Bacterial count in tested sample obtained from control (PBS-injected) fish.

Results

Effects of vaccination against *V. anguillarum*

Serum agglutinating antibody titer in vaccinated fish showed an obvious increase at 7 and 14 dpv (Fig. 1A). A significant correlation ($p = 0.019$) between reduction rate and serum agglutinating antibody titer was observed and the Pearson product-moment correlation coefficient was 0.887 (Fig. 2). In contrast, skin mucus had low antibody titer (2^1 – 2^4) that did not show any change with the number of days post-vaccination.

The mean bacterial count on incubation with serum obtained from control fish was 4.0×10^9 CFU/mL, and that with mucus was 1.4×10^{10} CFU/mL. The reduction rates of *V. anguillarum* due to incubation with serum from 10 and 14 dpv fish were higher than those due to incubation with serum from 1, 3, 5, and 7 dpv fish (Fig. 1B).

Skin mucus agglutinating antibody titer in vaccinated fish was low (2^1 – 2^4).

Effects of vaccination against *L. garvieae*

The antibody titer against *L. garvieae* was below

the detection limit (< 2 (2^1)) in either serum or skin mucus during the experimental period (Fig. 1C).

The reduction rate of *L. garvieae* after incubation with serum and skin mucus obtained from vaccinated fish at each sampling point is shown in Fig. 1D. There was no marked difference in the reduction rate between vaccinated samples and control samples.

Fig. 2. Correlation between serum agglutinating antibody titer against *V. anguillarum* and reduction rate.

Table 1. Microarray analysis of immune-related gene expression stimulated by bivalent vaccine of inactivated *V. anguillarum* and *L. garvieae* in yellowtail

Group	Gene	Accession number	1 dpv	3 dpv	5 dpv	7 dpv	10 dpv	14 dpv
1*3	CC chemokine	BB997690	5.2* ¹	2.9	N.D	0.1	0.6	N.D
	tumor necrosis factor receptor associated factor 2	BB997410	N.D* ²	2.8	2.0	0.1	1.1	0.8
	macrophage colony-stimulating factor receptor	BB997591	1.2	2.1	1.9	1.3	2.0	0.4
	LBP (LPS binding protein)/BP	BB997649	1.7	2.1	0.9	0.6	0.8	0.4
	colony stimulating factor 1 receptor 2	BB997675	N.D	4.5	0.8	0.5	1.0	N.D
	vertebrate interferon regulatory factor family	BB996976	0.8	2.6	N.D	1.3	N.D	1.7
	T cell receptor gamma chain V-J-C1	DC609801	0.9	1.5	3.0	1.4	N.D	N.D
2*4	immunoglobulin light chain	BB997721	1.3	2.7	3.2	3.3	1.9	N.D
	immunoglobulin light chain precursor	BB997682	1.4	2.4	2.1	2.2	1.9	1.3
	immunoglobulin heavy chain	BB997491	1.0	2.5	1.8	2.5	2.0	1.5
	HSP70	BB997674	1.3	2.1	1.6	1.4	2.0	1.6
	MHC class II alpha antigen	BB997597	0.9	2.0	2.3	1.7	2.5	4.5
	neurotoxin/C59/Ly-6-like protein	BB997714	N.D	3.4	2.4	1.0	1.7	5.7
3*5	immunoglobulin M	BB997653	1.1	1.4	1.2	2.1	1.5	0.8
	tumor necrosis factor receptor superfamily. member a	BB997493	N.D	N.D	N.D	2.5	3.7	1.9
	heat shock cognate 70	BB997492	1.2	1.8	1.6	1.4	2.4	1.9
	phospholipase A2-activating protein	BB997002	1.3	1.4	1.7	1.4	2.8	2.8
	natural killer cell enhancement factor	BB997455	0.6	1.6	0.6	1.0	1.4	2.3
	ferritin middle subunit; ferritin M	BB997379	0.6	0.7	0.6	0.8	1.0	5.6
	MHC class II invariant chain-like protein 2	BB997699	0.5	1.1	1.0	1.1	1.2	2.8
	interleukin-18 receptor 1 precursor	BB997065	0.9	1.2	1.3	1.2	2.0	2.4
	Sequestosome 1	BB997245	N.D	1.4	0.9	1.1	0.9	2.2

*¹ Values indicate fold change of gene expression at 1, 3, 5, 7, 10, and 14 days post vaccination (dpv) with *V. anguillarum* and *L. garvieae*, compared with non-treated fish.

*² Values not detected are indicated by N.D.

*³ Group 1 genes were up-regulated between 1 to 5 dpv.

*⁴ Group 2 genes were up-regulated from 1 to 14 dpv.

*⁵ Group 3 genes were up-regulated from 7 to 14 dpv.

Changes of gene expression induced by vaccination

Of a total of 88 immune-related genes spotted on the microarray, 22 were up-regulated (feature ratio above 2.0) after inoculating yellowtail with bivalent vaccine (Table 1). The up-regulated bio-defense genes were categorized into three groups according to their expression patterns. Group 1 genes were up-regulated between 1 to 5 dpv; Group 2 genes, from 1 to 14 dpv continuously; and Group 3 genes, from 7 to 14 dpv (Table 1). The six up-regulated genes in Group 1 were related to non-specific cellular immunity, such as tumor necrosis factor receptor associated factor, macrophage colony-stimulating factor receptor, LBP/BP, colony stimulating factor 1 receptor 2, and CC chemokine. Some genes belonging to Groups 2 and 3 were related to humoral immunity, including immunoglobulin heavy chain, immunoglobulin light chain, and immunoglobulin M, whereas others were related to MHC class II. The up-regulated genes of immune-related proteins, such as heat shock cognate 70, phospholipase A2-activating protein, and sequestosome, were mostly observed in Group 3.

Discussion

The immune system of yellowtail is not well understood. The objective of this study was to elucidate the immune mechanism by focusing on vaccine-induced immunity. For this purpose, the immune reactions of yellowtail following vaccination were studied.

We have shown that the serum agglutinating antibody titer against *V. anguillarum* from fish inoculated with commercial bivalent vaccine increased from 7 dpv (> 128 (2^7)) and remained significantly high at 14 dpv ($> 2,048$ (2^{11})). In addition, serum obtained from vaccinated fish showed inhibitory effects on *V. anguillarum* proliferation, and a significant correlation between reduction rate and serum agglutinating antibody titer was observed ($p < 0.05$, $R = 0.887$). *V. anguillarum* bacterial solution incubated with serum from vaccinated fish agglutinated. High-titer serum (antibody titer of 2,048 (2^{11})) more markedly agglutinated *V. anguillarum* than middle-titer serum (antibody titer of 256 (2^8)). In the control serum group, the agglutination of *V. anguillarum* was not observed. From these results, it can be said that antibody plays an important role in vaccine-induced

immunity. Our findings corroborate those of other studies (Thelma and White, 1972; Thuvaner *et al.*, 1987) in which antibody production is induced by vaccination.

L. garvieae is serologically divided into two types, KG⁻ and KG⁺ phenotypes. However, the biochemical characteristics of the two phenotypes are identical and it has been reported that rabbit anti KG⁻ serum agglutinates both KG⁺ and KG⁻ serotypes (Kitao, 1982; Hirono *et al.*, 1999; Ooyama *et al.*, 1999). KG⁻ serotype isolates are more virulent than KG⁺ serotype isolates due to the presence of a capsule (Ooyama *et al.*, 2002) that makes them resistant to opsonophagocytosis and host serum killing (Yoshida *et al.*, 1996, 1997). All vaccine strains have the KG⁻ phenotype and the KG⁻ strain (KG 9502) is used as the standard strains in the challenge test. Yoshida *et al.* (1996) reported that yellowtail vaccinated with the KG⁻ phenotype could not produce high agglutination titer against KG⁻, but could promote phagocytosis of KG⁻ phenotype. Complement may play an important role in immunity against *L. garvieae*. Our results corroborate the above findings: we did not observe any increase of the antibody titer against KG⁻ phenotype *L. garvieae* or any inhibition of pathogen induced by complement-inactivated serum from vaccinated fish. These results suggest that the prophylactic effects of commercial vaccine on *L. garvieae* are not induced by KG⁻ antibody production. Therefore, it is difficult to evaluate vaccine efficacy against lactococcosis by measuring KG⁻ antibody titer and a more suitable marker for evaluating vaccine efficacy is desired.

To determine other immune responses induced by vaccination, we conducted microarray analysis to profile immune-related gene expression. Only immune-related genes that were up-regulated by vaccination were analyzed in this study. Microarray analysis revealed that 22 immune-related genes showed up-regulation after vaccination. Group 1 consists of innate immune-related genes that are related to macrophage stimulation and inflammation. Immunoglobulin heavy chain (IgH), which is thought to be the major transmembrane receptor on the surface of B cells in all teleost species (Wilson *et al.*, 1990), and its related genes were categorized in Group 2 in this study. Two immunoglobulin light chain related genes were also included in Group 2. The up-regulation of MHC class II alpha antigen (Group 2) and MHC class II invariant chain-like protein 2 (Group 3) possibly indicates that CD4⁺ T cells respond to vaccine antigen. Those results suggest that innate immunity is first stimulated by the vaccination and then humoral immunity is stimulated. The vaccine used in this study contained both Gram-negative *V. anguillarum* and Gram-positive *L. garvieae*. LPS is one of the major antigens of Gram-negative bacteria and known to induce T-cell-independent type I immune response. T-cell-independ-

ent antigens can activate B cells directly through the Toll-like receptor (Fagarasan and Honjo, 2000). The current study shows that T-cell-independent type II antigens, such as polysaccharides, can induce the production of memory B cells (Obukhanych and Nussenzweig, 2006). These findings suggest that T cell is not always needed for the production of specific antibodies for some antigens. The present experiment demonstrated the up-regulation of T cell receptor gamma chain V-J-C1 (Group 1), MHC class II alpha antigen (Group 2), and MHC class II invariant chain-like protein 2 (Group 3). All these are indicative of T cell activation, but are not sufficient to determine which antigen induced the T-cell-dependent immune response.

Skin mucus had low antibody titer against *V. anguillarum*. As the first defense against infection, mucus contains several antibacterial factors (Alexander and Ingram, 1992; Rombout *et al.*, 1993; Aranishi and Nakane, 1997). IgM is one of those. Our results are not sufficient to determine whether an antibody is a specific one or a natural one. Nevertheless, they suggest that mucus contains some antibodies against pathogens.

The HSP 70 related genes were categorized in Groups 2 and 3. HSP 70 is a kind of stress protein present in all cells. It serves as a molecular chaperone in the cell death mechanism (Garrido *et al.*, 2001) and is an extracellular protein with regulatory effects on human monocytes, playing a dual role as a chaperone and a cytokine (Asea *et al.*, 2000). HSP 70 may be up-regulated by vaccination as it induced by stress and inflammation. Other proteins related to cell defense were also up-regulated, such as neurotoxin/C59/Ly-6-like protein (Group 2), phospholipase A2-activating protein (Group 3), and sequestosome 1: P62 protein (Group 3). These also show immune stimulation by vaccine. The IL-18 receptor 1 precursor related gene, which is a kind of cytokine that stimulates both Th1 and Th2 responses, was categorized in Group 3 (Nakanishi *et al.*, 2001) and its expression is increased by LPS (Abu *et al.*, 2008). It is suggested that the up-regulation of this gene is likely induced by vaccination. Microarray analysis suggested that both innate immunity and humoral immunity are stimulated by vaccination. However, the results are due to the use of the bivalent vaccine, and further studies of genes up-regulated by vaccination are needed.

In conclusion, this is the first report of a large-scale immune-related gene expression profiling in vaccinated yellowtail using microarray analysis. Vaccination elicited immune response in yellowtail, activating both non-specific cellular immunity and humoral immunity. We also conducted RT-PCR of some genes to confirm the microarray results (data not shown). The signal intensity scores from the microarray analysis corresponded to the RT-PCR results, suggesting that the gene expres-

sion pattern on the microarray was reliable. Immune response to both antigens, *V. anguillarum* and *L. garvieae*, was observed in this study. Yellowtail may use a different type of immune system for different pathogens. Our results suggest that a specific antibody plays an important role in vibriosis. On the other hand, there was no evidence of acquired immunity to lactococcosis. To determine how yellowtail acquires immunity to *L. garvieae*, further studies of the immune response induced by lactococcosis vaccination are needed.

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