Research article

Failure to Protect Yellowtail *Seriola quinqueradiata* against Nocardiosis by Vaccination with a Recombinant Protein and pcDNA4 Expression Vector of *Edwardsiella tarda* Glyceraldehyde-3-phosphate Dehydrogenase

Masayuki Imajoh^{1*}, Kazuki Tamura¹, Kazuki Oguro¹, Sumire Kurihara¹, Jin Yamane¹, Masato Shimizu¹, Syun-ichirou Oshima² and Kenji Kawai³

¹Laboratory of Fish Disease, Faculty of Agriculture, Kochi University, Kochi 783-8502, Japan

²Graduate School of Kuroshio Science, Kochi University, Kochi 783-8502, Japan

³The United Graduate School of Agricultural Sciences, Ehime University, Ehime 790-8566, Japan

(Received December 16, 2013)

ABSTRACT—Vaccination with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *Edwardsiella tarda* has been demonstrated to have cross-protection against some other bacterial pathogens. We prepared a recombinant protein and pcDNA4 expression vector of GAPDH of *E. tarda* and injected those to 0-age yellowtail *Seriola quinqueradiata* to evaluate their protective efficacies against *Nocardia seriolae*. The 60-d survival rates ranged from 0% to 6.7% among vaccinated and control groups, suggesting that the protein and expression vector of *E. tarda* GAPDH had no protective effect against nocardiosis. However, the copy numbers of the 16S ribosomal RNA gene of *N. seriolae* were significantly lower in the gill, kidney, and spleen of vaccinated fish 9 and 18 days post-infection compared with those in the control group that was also challenged with *N. seriolae*. Additionally, the *CC chemokine*, *major histocompatibility complex class II α antigen*, *interleukin 1 β* , and *immunoglobulin M* genes were significantly upregulated in the vaccinated groups. Four predicted B-cell epitopes of GAPDH in *E. tarda*, which may play an important role in cross-protection, differed in amino acid sequences from those of GAPDH in *N. seriolae*. This may explain the lack of a protective effect of *E. tarda* GAPDH against *N. seriolae*.

Key words: *Edwardsiella tarda*, *Nocardia seriolae*, GAPDH, vaccine, adjuvant, yellowtail, B-cell epitope

Nocardia are aerobic, gram-positive, non-motile, filamentous actinomycetes. *Nocardia seriolae* belongs to the genus *Nocardia*, and causes nocardiosis in fish (Kudo *et al.*, 1988). In Japan, the disease is a major problem in cultured yellowtail *Seriola quinqueradiata*, amberjack *Seriola dumerili*, striped jack *Pseudocaranx dentex*, and olive flounder *Paralichthys olivaceus* (Takahashi *et al.*, 2011).

There is currently no licensed vaccine for *N. seriolae*. Several studies have attempted to develop formalin-killed, heat-killed, and live attenuated vaccines of *N. seriolae* (Kusuda and Nakagawa, 1978; Kusuda *et al.*, 1989; Shimahara *et al.*, 2005; Itano *et al.*, 2006). The formalin-killed vaccine did not have a protective effect against *N. seriolae* in vaccinated yellowtail, in which anti-*N. seriolae* antibody titer increased (Shimahara *et al.*, 2005). Live attenuated vaccine suc-

cessfully protected yellowtail from lethal challenge by *N. seriolae*, but its long-term safety remains unclear (Itano *et al.*, 2006). Recently, Kato *et al.* (2012) showed that *Mycobacterium bovis* bacillus Calmette-Guérin vaccine had the same protective properties as the live attenuated vaccine.

A 37-kDa outer membrane protein (OMP) of *Edwardsiella tarda* is conserved among the different serotypes, and is a good vaccine candidate against edwardsiellosis in olive flounder (Kawai *et al.*, 2004). The 37-kDa OMP is homologous to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a cytoplasmic enzyme common in organisms (Liu *et al.*, 2005). Our previous study showed that recombinant GAPDH (rGAPDH) protein of *E. tarda* expressed in *Escherichia coli* conferred definitive protection on olive flounder against vibriosis caused by *Vibrio anguillarum* (Liu *et al.*, 2007). Liang *et al.* (2012) reported that immunoglobulin M (IgM) antibodies induced by rGAPDH of *E. tarda* exhibited obvious cross-reactions with *V.*

* Corresponding author
E-mail: m-imajoh@kochi-u.ac.jp

anguillarum, *Vibrio alginolyticus*, *Vibrio harveyi*, and *Aeromonas hydrophila*. We postulated that GAPDH of *E. tarda* may be a multipurpose vaccine candidate against different pathogenic bacteria. Moreover, GAPDH of *E. tarda* can be used as a potential adjuvant for subunit vaccine of red sea bream iridovirus (Shimmoto *et al.*, 2010).

In this study, we evaluated the protective efficacy of GAPDH of *E. tarda* against nocardiosis in yellowtail.

Materials and Methods

Bacterial strain and culture conditions

N. seriolae strain N-2927 was originally isolated from the spleen of cultured yellowtail in Kochi Prefecture, Japan, in 2007. The strain was picked from glycerol stock, and grown on a tryptic soy broth (TSB; Difco) plate for 5 days at 28°C. A colony from the TSB plate was picked, and pre-cultured in 20 mL of TSB medium in a shaking incubator for 4 days at 28°C. A bacterial suspension (100 µL) was inoculated into 300 mL of TSB medium, and cultured by shaking incubation at 28°C for 4 days.

Construction, expression, and purification of rGAPDH protein

The cold shock expression vector pColdI (TaKaRa) was used to express the rGAPDH protein of *E. tarda* strain EF-1, which was originally isolated in 1979 from cultured Japanese eel *Anguilla japonica* in Shizuoka Prefecture, Japan (Kusuda *et al.*, 1988), in *E. coli*. A 1,006-bp fragment including the full-length GAPDH gene, and the *NdeI* and *EcoRI* sites was amplified by polymerase chain reaction (PCR) with the following primer set: forward primer, AACATATGACTATCAAAGTAGGTATTAACG; and reverse primer, AAGAATTCCTTAGAGATGTGAGCGATTAGG (the restriction enzyme recognition sites are underlined). PCR was performed using *Ex Taq* polymerase (TaKaRa). After digestion with *NdeI* and *EcoRI*, the amplified product was purified from 1.0% agarose gel using a High Pure PCR product purification kit (Roche). The fragment was cloned into the *NdeI/EcoRI* sites of the pColdI vector, which was transformed into *E. coli* strain BL21 (DE3). Overproduction of the soluble rGAPDH protein was induced by adding 100 mM IPTG and incubated overnight at 15°C. The recombinant protein was purified using complete his-tag purification resin (Roche), and dialyzed overnight at 4°C in dialysis buffer consisting of phosphate buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol. Protein concentration was measured using a Quant iT protein assay kit (Invitrogen) on a Qubit fluorometer (Invitrogen).

Construction, extraction, and purification of GAPDH-expression vector

The eukaryotic expression vector pcDNA4/V5-His (Invitrogen) was selected. A 1,026-bp fragment including the full-length GAPDH gene and the *EcoRV* and *XhoI* sites was amplified by PCR with the following primer set: forward primer, TAAGATATCATGGGAGCAACTATAATGACTATCAAAGTAG; and reverse primer, AAACCTCGAGCTTAGAGATGTGAGCGATTAGGTCTAG (the restriction enzyme recognition sites are underlined). The digested, purified PCR product was cloned into the *EcoRV/XhoI* sites of the pcDNA4/V5-His vector, and transformed into *E. coli* strain JM109. The vector was purified on a large scale using a Genopure plasmid maxi kit (Roche). The pelleted pcDNA4-GAPDH (pGAPDH) vector was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and was stored at -20°C until use.

Preparation of formalin-killed vaccine

Formalin-killed *N. seriolae* cells (FKC) were prepared as described previously (Kusuda *et al.*, 1989).

Fish

Zero-year-old yellowtail were caught in the Tosa Bay area, Japan. The fish were held in a 2-kL circular fiberglass reinforced plastic (FRP) tank with a continuous flow of sand-filtered sea-water, and were hand fed a commercial dry pellet diet once daily (Marubeni Nissin Feed) corresponding to approximately 3% of the fish body weight.

Administration of pGAPDH vector and rGAPDH protein

Yellowtail weighing about 26.6 g were divided into the following groups: pGAPDH-administered group, 54 fish were injected intramuscularly with the pGAPDH vector (200 µg fish⁻¹); rGAPDH-administered group, 39 fish were injected intraperitoneally with the rGAPDH protein (340 µg fish⁻¹); FKC-administered group, 39 fish were injected intraperitoneally with FKC (8.0 × 10⁵ colony-forming units (CFU) fish⁻¹); FKC- and pGAPDH-administered group, 33 fish were injected intraperitoneally with FKC (4.0 × 10⁵ CFU fish⁻¹) and intramuscularly with the pGAPDH vector (200 µg fish⁻¹); and FKC- and rGAPDH-administered group, 33 fish were injected intraperitoneally with FKC (4.0 × 10⁵ CFU fish⁻¹) and intraperitoneally with the rGAPDH protein (340 µg fish⁻¹). Sixty-two fish were injected with PBS as non-administered groups.

Challenge experiment

The bacterial suspension was added to 10 L of sea-water at a concentration of 7.5 × 10⁵ CFU mL⁻¹. At 28 days after administration, all administered and 37 non-administered fish were immersed in sea-water for 10 min, and each group was immediately placed into a

separate 200-L tank. Water temperature in the tanks was 27.5°C–29.0°C during the challenge period. The remaining 25 non-administered fish were immersed in 10 L of sea-water mixed with 50 mL of sterile TSB medium. One fish in each group was sampled randomly at 1, 3, 5, 7, 9, 14, 18, 36, and 60 days post-infection (p.i.).

Expression analysis of immune-related genes

Total RNA was extracted from the liver and spleen tissue samples at 1, 3, 5, 7, 14, 36, and 60 days p.i. using Sepasol RNA I super G (Nakalai Tesque). Total RNA (1 mg) was transcribed to complementary DNA using ReverTraAce qPCR RT master mix with gDNA remover (Toyobo).

The expression levels of five immune-related genes were analyzed by quantitative real-time RT-PCR (qRT-PCR) assay. Based on the GenBank sequences of the *CC chemokine* gene (GenBank accession number BB997690), the *major histocompatibility complex (MHC) class II α antigen* gene (GenBank accession number BB997597), the *IgM* gene (GenBank accession number BB9976453), the *interleukin 1 β (IL-1 β)* gene (GenBank accession number BB997494), the *T cell receptor (TCR) α chain* gene (GenBank accession number BB997438), and the *β -actin* gene (GenBank accession number AB179839), primers were designed using Primer Express version 3.0 software (Applied Biosystems). The primer sequences were as follows: forward primer for the *CC chemokine* gene, 5'-GGACAGTCCTGAAGTGCTTTGA-3'; reverse primer for the *CC chemokine* gene, 5'-GGTTTGATGCAATGC-CATTT-3'; forward primer for the *MHC class II α antigen* gene, 5'-CCGGTATTGGACCTGCAGTT-3'; reverse primer for the *MHC class II α antigen* gene, 5'-CTGCACTCGTTCCTTTGATG-3'; forward primer for the *IgM* gene, 5'-TACGGCCGGTTTTGACTACTG-3'; reverse primer for the *IgM* gene, 5'-AGAACTACATGG-CCTCAGAGGAA-3'; forward primer for the *IL-1 β* gene, 5'-TGAAATGCAACTTGAGCGAGAT-3'; reverse primer for the *IL-1 β* gene, 5'-TGTCATTGGATGATGGGAGATC-3'; forward primer for the *TCR α chain* gene, 5'-GTTGAAACCAGGGAGGATTACG-3'; reverse primer for the *TCR α chain* gene, 5'-TGAGTGCGTTGTATCT-GCTGAA-3'; forward primer for the *β -actin* gene, 5'-GCCCCACCAGAGCGTAAATA-3'; and reverse primer for the *β -actin* gene, 5'-CATCGTACTCCTGCTTGCT-GAT-3'. qRT-PCR was performed in triplicate using THUNDERBIRDSYBR qPCR mix (Toyobo) on an ABI7300 real-time PCR instrument (Applied Biosystems). The relative gene expression level was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), normalized to β -actin, and represented as mean n -fold change compared with the level in the non-administered group not challenged with *N. seriolae*.

Quantification of the 16S ribosomal RNA gene of *N. seriolae*

Brain, gill, heart, kidney, liver, and spleen tissue samples at 9 and 18 days p.i. were homogenized in 200 μ L of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Nonidet-40, 1.0% Tween 20, and 0.5% sodium dodecyl sulfate). Proteinase K (2 μ L, 20 mg mL⁻¹; TaKaRa) was added to the lysis buffer, incubated at 50°C overnight, and inactivated at 98°C for 10 min. Lysozyme (5 μ L, 2 mg mL⁻¹; Sigma Aldrich) was added to the lysis buffer, and incubated at 37°C for 30 min. Proteinase K (2 μ L) was added again, and incubated at 55°C for 2 h. Genomic DNA was purified by phenol-chloroform extraction and the ethanol precipitation method. The pelleted genomic DNA was resuspended in 500 μ L of TE buffer.

Genomic DNA (200 ng) was subjected to qPCR assay targeting the *16S ribosomal (r) RNA* gene of *N. seriolae*. The qPCR primers were designed based on the GenBank sequence of the *16S rRNA* gene of *N. seriolae* strain DSM 44129 (GenBank accession number NR041861) as follows: forward primer, 5'-ATG-GTTCGGGTGGAAAGATT-3'; and reverse primer, 5'-GCCGTGTCTCAGTCCCAGTG-3'. qPCR was performed as described above. The obtained threshold cycle value was converted to the copy number of the *16S rRNA* gene by a standard curve. To generate the standard curve, serial 10-fold dilutions of standard plasmid constructed by inserting the *16S rRNA* gene fragment using a Mighty TA cloning kit (TaKaRa) were analyzed by qPCR. The copy number of the standard plasmid was calculated from the DNA concentration according to the plasmid size and Avogadro's number.

Sequence analysis of the GAPDH gene of *N. seriolae*

A fragment of 1,105 bp including the *GAPDH* gene of *N. seriolae* was amplified by PCR using *Ex Taq* polymerase. The primers were designed based on the complete genome sequence of *Nocardia farcinica* (GenBank accession number AP006618) as follows: forward primer, 5'-GACCTTCGACACCCTCGTT-3'; and reverse primer, 5'-ATGAATCCGCTTGAAAGCTG-3'. The amplified product was purified and sequenced using a Big Dye Terminator cycle sequencing kit (Life Technologies) on a 3,130 genetic analyzer instrument (Applied Biosystems). The obtained nucleotide sequence was analyzed using BioEdit. The amino acid sequence was deduced and compared to the GenBank sequences of *E. tarda*, *Edwardsiella ictaluri*, *A. hydrophila*, *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *Lactococcus garviae*, *Streptococcus iniae*, *Streptococcus parauberis*, *Nocardia otitidiscaviarum*, *Nocardia brasiliensis*, *Nocardia cyriacigeorgica*, *Nocardia nova*, *N. farcinica*, and *Mycobacterium* sp. (GenBank accession numbers BAE07151, HQ697334, HQ697336, HQ697332, HQ697335, HQ697333, EOT30873, AHY16571,

GAJ61094, WP029924496, WP014986416, WP014351697, WP025350671, WP011210122, and EPQ48177, respectively).

Prediction of B-cell epitopes of GAPDH of *E. tarda*

B-cell epitopes of GAPDH of *E. tarda* and *N. seriolae* were predicted using BCPred online server (EL-Manzalawy *et al.*, 2008a) where criteria were set to have 80% specificity and 12 amino acids in length. VaxiJen v2.0 online server (Doytchinova and Flower, 2007) was used to analyse the antigenicity of epitopes. The predicted epitopes were checked for their conservation and variability with the Immune Epitope Database conservation analysis tool (Bui *et al.*, 2007).

Statistical analysis

Statistical analysis was performed using Dunnett's one-way ANOVA. In all analyses, $P < 0.01$ was taken to indicate statistical significance.

Results and Discussion

The cumulative survival of fish in the administered and non-administered groups after immersion challenge is shown in Fig. 1. No mortality was observed in the non-administered group not challenged with *N. seriolae*. The mortality of FKC-administered fish started at 12 days p.i., and lasted for 10 days. The cumulative survival was 6.7%. These observations were consistent with the results of Shimahara *et al.* (2005). The cumulative survival of pGAPDH-administered fish was 2.2%. All rGAPDH-, FKC- and pGAPDH-, and FKC- and rGAPDH-administered fish were dead at 21, 18, and 28 days p.i., respectively. Colonies of *N. seriolae* were

recovered from all dead and surviving fish (data not shown). These results indicated that GAPDH of *E. tarda* has no protective effect against *N. seriolae* infection in yellowtail.

To detect and quantify *Nocardia* species, other studies used a qPCR assay that targeted the *16S rRNA* gene (Alfaresi and Elkosh, 2006; Helal *et al.*, 2008; Baek *et al.*, 2009). In this study, we developed a novel qPCR assay that targets the *16S rRNA* gene of *N. seriolae*, and determined the copy number in each tissue of fish in the administered groups and the non-administered group challenged with *N. seriolae* at 9 and 18 days p.i. The specificity of the qPCR assay was confirmed by testing the genomic DNA of bacterial pathogens of yellowtail, such as *L. garvieae*, *Photobacterium damsela* subsp. *piscicida*, and *Mycobacterium* sp. (data not shown). The standard curve used to calculate the copy number of the *16S rRNA* gene had a slope

Fig. 1. Cumulative survival of yellowtail in the administered and non-administered groups after immersion challenge with *N. seriolae*.

Fig. 2. Quantitative analysis of the *16S rRNA* gene of *N. seriolae* in the brain, gill, heart, kidney, liver, and spleen tissues of yellowtail in the administered groups and the non-administered group challenged with *N. seriolae*. pGAPDH-administered group (solid grey); rGAPDH-administered group (black); FKC-administered group (white); FKC- and pGAPDH-administered group (light grey); FKC and rGAPDH-administered group (hatched); non-administered group with challenge (dotted). Values and error bars represent the means and standard deviation of triplicate measurements. Asterisks indicate statistically significant differences ($P < 0.01$) compared with values of the non-administered group challenged with *N. seriolae*.

Table 1. Relative expression analysis of five immune-related genes in the liver and spleen of yellowtail in the administered groups and the non-administered group challenged with *N. seriolae*

Group	Days p.i.	Gene name									
		CC chemokine		MHC class II α antigen		IgM		IL-1 β		TCR α chain	
		Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen
pGAPDH administration	1	0.76 [†]	3.15*	-0.38	1.86	N.D.	N.D.	1.18	0.66	-3.92	1.61
	3	-0.17	8.62	-0.74	3.78	0.39	N.D.	N.D.	1.32	-2.31	1.44
	5	-3.16	2.01	-0.36	-0.57	N.D.	-1.99	1.77	2.96	-1.87	-3.31
	7	N.D. [‡]	2.69	1.46	-0.19	1.40	-3.16	3.71	4.97	-0.24	-1.70
	14	-4.05	3.46	-2.21	1.58	-1.88	0.50	-1.75	2.55	-3.18	1.41
	36	2.47	2.46	1.09	1.64	1.97	1.84	1.92	-0.90	-0.25	0.44
	60	0.84	2.00	0.84	1.40	5.28	6.37	1.41	1.07	0.56	2.84
rGAPDH administration	1	1.31	2.65	2.20	2.32	1.19	-1.52	1.71	0.58	0.72	1.57
	3	-2.07	9.34	-1.26	2.78	0.03	N.D.	-0.35	1.37	-2.00	0.63
	5	1.13	4.88	-0.41	3.09	N.D.	1.43	-0.14	2.75	-1.80	1.31
	7	-1.68	3.37	-0.24	1.10	0.14	-1.24	3.45	5.47	-2.53	0.68
	14	-5.31	2.25	-2.69	0.21	-1.77	1.30	-0.32	0.07	-4.04	-0.53
	36	0.36	2.76	1.16	1.02	2.20	1.83	1.88	-0.66	-0.27	-0.18
	60	—	—	—	—	—	—	—	—	—	—
FKC administration	1	-3.37	3.62	-0.95	1.98	-0.13	-0.26	0.51	1.73	-4.02	0.44
	3	N.D.	2.65	0.41	5.31	-0.04	-2.61	1.17	1.13	-1.80	1.77
	5	0.24	2.40	-0.01	2.12	-0.05	0.18	2.28	3.66	-0.51	-0.53
	7	-1.01	3.79	-0.64	2.50	-2.47	0.11	1.95	4.49	-2.31	0.68
	14	0.30	2.57	0.72	0.06	1.08	1.43	4.94	3.70	-0.95	-0.90
	36	2.62	1.56	-1.87	1.01	2.32	2.31	-2.36	1.13	-2.82	1.82
	60	2.43	2.27	1.60	1.49	4.42	4.52	1.29	0.29	2.33	3.03
FKC and pGAPDH administration	1	-1.13	4.96	-0.19	1.82	0.08	-1.83	-0.15	2.89	-2.83	1.61
	3	-2.83	2.38	-0.07	1.13	0.77	-0.14	-1.52	0.63	-3.28	-0.86
	5	-1.46	3.43	-0.45	1.62	-0.80	-1.55	2.50	2.44	-1.73	0.53
	7	-3.29	3.48	-0.13	3.17	-1.40	0.78	1.05	3.83	-1.41	1.54
	14	0.79	3.60	0.38	-1.64	0.89	1.11	3.90	3.98	-2.52	-2.38
	36	0.24	5.13	-1.60	1.64	2.53	1.92	-0.95	1.21	-2.17	1.39
	60	—	—	—	—	—	—	—	—	—	—
FKC and rGAPDH administration	1	2.07	5.43	1.01	2.33	0.07	-0.31	1.54	1.98	-1.19	1.66
	3	-1.83	2.20	0.26	2.26	1.48	-1.41	-0.97	-0.24	-3.34	-1.75
	5	-0.07	2.69	1.23	2.53	0.88	-0.85	3.96	2.47	-0.16	1.41
	7	-2.44	2.30	0.39	2.51	0.16	-0.27	2.33	4.49	-1.81	0.51
	14	0.66	3.93	0.59	-0.54	0.50	0.43	4.25	1.84	-1.34	-1.67
	36	0.61	3.52	0.74	0.83	3.99	4.12	0.60	0.38	-0.72	-0.77
	60	—	—	—	—	—	—	—	—	—	—
non-administration with challenge	1	-4.43	1.04	0.89	0.05	1.46	-0.47	1.28	1.55	-1.31	0.14
	3	N.D.	N.D.	-0.01	1.13	-0.35	N.D.	1.22	1.32	-1.48	1.42
	5	N.D.	N.D.	-0.50	1.71	-0.33	-0.79	0.11	-0.16	-2.06	1.54
	7	-2.73	1.57	-1.13	1.52	-2.58	0.58	0.26	5.42	-3.80	0.76
	14	2.10	1.62	0.20	-1.43	1.30	1.04	6.53	3.13	-1.89	-1.63
	36	—	—	—	—	—	—	—	—	—	—
	60	—	—	—	—	—	—	—	—	—	—

[†] Values indicate mean fold change of gene expression compared with the non-administrated, non-challenged group.[‡] Values not detected are indicated by N.D.* Gray boxes indicate significant upregulation ($P < 0.01$).

of -3.427 and a correlation coefficient R^2 of 0.998 over a range of $2.2 - 2.2 \times 10^9$ copies per reaction (data not shown). As shown in Fig. 2, the copy numbers of the *16S rRNA* gene in the gill, kidney, and spleen of fish in each administered group were significantly lower than those in the non-administered group challenged with *N. seriolae*. The lowest copy number was recorded in FKc-administered group at 9 days p.i among the administered groups. In fact, the sampled fish did not have any internal symptoms, while the kidney and spleen of fish of the other administered groups were enlarged with multiple white nodules, which are typical signs of nocardiosis (data not shown). After 9 days p.i., FKc-administered fish also showed the clinical signs.

Microarray analysis to profile immune-related gene expression was developed recently because of poor understanding of the immune system of yellowtail (Darawiroj *et al.*, 2008). The microarray was applied to examine the immune responses elicited by a commer-

cial bivalent vaccine for *V. anguillarum* and *L. garvieae* (Nakajima *et al.*, 2012). Of the immune-related genes spotted on the microarray, we chose five genes, and determined genes that were upregulated after administrations with pGAPDH and rGAPDH. Significant upregulation of four immune-related genes other than the *TCR α chain* gene was detected in each administered group throughout the 60 days of this study compared with those in the non-administered group not challenged with *N. seriolae* (Table 1). It seemed that these immune responses did not differ significantly among the administered groups. As almost all fish in any administered groups were dead, the expression levels of the *CC chemokine*, *MHC class II α antigen*, and *IL-1 β* genes were possibly insufficient to overcome *N. seriolae* infection. Alternatively, the three genes may play limited roles in protective immunity against *N. seriolae*. The upregulation of the *IgM* gene was likely related to the production of anti-*N. seriolae* antibodies considering

Fig. 3. Comparison of amino acid sequences predicted from the *GAPDH* gene. Conserved amino acid residues are highlighted in bold. Gray boxes represent amino acid residues conserved only in *E. tarda*, *E. ictaluri*, *A. hydrophila*, *V. anguillarum*, *V. alginolyticus*, and *V. harveyi*. Gray boxes with white letters represent amino acid residues conserved only in six *Nocardia* spp. and *Mycobacterium sp.* Predicted B-cell epitopes are underlined.

Kusuda *et al.* (1989) reported that the agglutination antibody titer against *N. seriolae* significantly increased after administration with FK. C.

Sequence analysis revealed that the GAPDH gene of *N. seriolae* has an amino acid sequence identity of more than 90% with those of other five *Nocardia* spp. available in GenBank, while 43% with that of *E. tarda* (Fig. 3). Mapping B-cell epitopes is important for vaccine design, immunodiagnostic tests, and antibody production (EL-Manzalawy *et al.*, 2008b). A total of six epitopes of GAPDH of *E. tarda*, GINGFGRIGRFV (amino acid positions 6 to 17), KYDSTHGRFNLT (amino acid positions 46 to 57), NASCTTNCLAPI (amino acid positions 147 to 158), KDWRGGRGASQN (amino acid positions 192 to 203), KLTGMAFRVPTA (amino acid positions 225 to 236), and GYTEDAVVSQDF (amino acid positions 273 to 284) were predicted using BCPred online server. Antigenic scores were found to be 1.0899 for KYDSTHGRFNLT, 2.2126 for KDWRGGRGASQN, 1.0990 for KLTGMAFRVPTA, and 0.7467 for GYTEDAVVSQDF, respectively, indicating these epitopes as probable antigens. Li *et al.* (2011) showed that rGAPDHs of *E. tarda*, *E. ictaluri*, *A. hydrophila*, *V. anguillarum*, *V. alginolyticus*, and *V. harveyi* shared high levels of identity (over 80%) with each other, and gave remarkable cross-protective immunity against those different pathogenic bacteria. Two epitopes, KYDSTHGRFNLT and KDWRGGRGASQN, are completely conserved in *E. ictaluri* and three *Vibrio* spp., and show approximately 92% conservation in *A. hydrophila*. KLTGMAFRVPTA or GYTEDAVVSQDF epitope shows approximately 92% or 75% conservation in *E. ictaluri*, approximately 83% conservation in *A. hydrophila*, and 100% or approximately 92% conservation in *V. harveyi*. The two epitopes are completely conserved in *V. anguillarum* and *V. alginolyticus*. These findings suggested that four predicted B-cell epitopes play an important role in cross-protection. This may be a reason that GAPDH of *E. tarda* is not a protective antigen of *N. seriolae*, which was supported by the result that the predicted B-cell epitopes of GAPDH of *N. seriolae* differed from those of *E. tarda*. Because GAPDHs of members of the family Streptococcaceae i.e., *L. garviae*, *S. iniae*, and *S. parauberis*, or of members of the suborder Corynebacterineae i.e., *N. seriolae*, *N. otitidiscaviarum*, *N. brasiliensis*, *N. cyriacigeorgica*, *N. nova*, *N. farcinica*, and *Mycobacterium* sp. share a high amino acid sequence identity, there is a possibility that they have potential for cross-protection. GAPDH of *S. iniae* was identified as immunogenic in olive flounder and Nile tilapia *Oreochromis niloticus* (Shin *et al.*, 2007; LaFrentz *et al.*, 2011), and therefore its vaccination is expected to cross-protect against fish streptococcosis caused by *L. garviae* or *S. parauberis*. *Mycobacterium* sp. causes mycobacteriosis in cultured yellowtail in Japan, and no effective treatment for this

disease is yet available in the aquaculture industry (Kusuda *et al.*, 1987; Kato *et al.*, 2011). We must further examine whether GAPDH of *N. seriolae* protect yellowtail against not only nocardiosis but also mycobacteriosis.

Acknowledgments

This research was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

- Alfaresi, M. and A. Elkosh (2006): Rapid identification of clinically relevant *Nocardia* species using real-time PCR with SYBR Green and melting-curve analysis. *J. Med. Microbiol.*, **55**, 1711–1715.
- Baek, K. H., B. D. Yoon, D. H. Cho, B. H. Kim, H. M. Oh and H. S. Kim (2009): Monitoring bacterial population dynamics using real-time PCR during the bioremediation of crude-oil-contaminated soil. *J. Microbiol. Biotechnol.*, **19**, 339–345.
- Bui, H.-H., J. Sidney, W. Li, N. Fusseder and A. Sette (2007): Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. *BMC Bioinform.*, **8**, 361.
- Darawiroj, D., H. Kondo, I. Hirono and T. Aoki (2008): Immune-related gene expression profiling of yellowtail (*Seriola quinqueradiata*) kidney cells stimulated with ConA and LPS using microarray analysis. *Fish Shellfish Immunol.*, **24**, 260–266.
- Doytchinova, I. A. and D. R. Flower (2007): VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinform.*, **8**, 4.
- EL-Manzalawy, Y., D. Dobbs and V. Honavar (2008a): Prediction of linear B-cell epitopes using string kernels. *J. Mol. Recognit.*, **21**, 243–255.
- EL-Manzalawy, Y., D. Dobbs and V. Honavar (2008b): Predicting Protective Linear B-Cell Epitopes Using Evolutionary Information. *IEEE International Conference on Bioinformatics and Biomedicine*, 289–292.
- Helal, Z. H., M. I. Khan, M. S. Ashour and S. A. Eissa (2008): Detection and characterization of *Nocardia* from patients diagnosed as tuberculosis in Egypt. *Int. J. Biomed. Sci.*, **4**, 179–184.
- Itano, T., H. Kawakami, T. Kono and M. Sakai (2006): Live vaccine trials against nocardiosis in yellowtail *Seriola quinqueradiata*. *Aquaculture*, **261**, 1175–1180.
- Kato, G., K. Kato, K. Saito, Y. Pe, H. Kondo, T. Aoki and I. Hirono (2011): Vaccine efficacy of *Mycobacterium bovis* BCG against *Mycobacterium* sp. infection in amberjack *Seriola dumerili*. *Fish Shellfish Immunol.*, **30**, 467–472.
- Kato, G., H. Kondo, T. Aoki and I. Hirono (2012): *Mycobacterium bovis* BCG vaccine induces non-specific immune responses in Japanese flounder against *Nocardia seriolae*. *Fish Shellfish Immunol.*, **33**, 243–250.
- Kawai, K., Y. Liu, K. Ohnishi and S. Oshima (2004): A conserved 37 kDa outer membrane protein of *Edwardsiella tarda* is an effective vaccine candidate. *Vaccine*, **22**, 3411–3418.
- Kudo, K., K. Hatai and A. Seino (1988): *Nocardia seriolae* sp. nov. Causing nocardiosis of cultured fish. *Int. J. Syst. Bacteriol.*, **38**, 173–178.

- Kusuda, R. and A. Nakagawa (1978): Nocardia infection of cultured yellowtail. *Fish Pathol.*, **13**, 25–31. (in Japanese with English abstract)
- Kusuda, R., K. Kawakami and K. Kawai (1987): A fish-pathogenic *Mycobacterium* sp. isolated from an epizootic of cultured yellowtail. *Nippon Suisan Gakkaishi*, **53**, 1797–1804. (in Japanese with English abstract)
- Kusuda, R., K. Ono and F. Salati (1988): Changes in agglutinating antibody titers and serum proteins of immunized eels. *Dis. Aquat. Organ.*, **5**, 87–92.
- Kusuda, R., Y. Kimura and M. Hamaguchi (1989): Changes in peripheral and peritoneal leucocytes in yellowtail *Seriola quinqueradiata* immunized with *Nocardia kampachi*. *Nippon Suisan Gakkaishi*, **55**, 1183–1188. (in Japanese with English abstract)
- LaFrentz, B. R., C. A. Shoemaker and P. H. Klesius (2011): Immunoproteomic analysis of the antibody response obtained in Nile tilapia following vaccination with a *Streptococcus iniae* vaccine. *Vet. Microbiol.*, **152**, 346–352.
- Li, X., H. Wu, M. Zhang, S. Liang, J. Xiao, Q. Wang, Q. Liu and Y. Zhang (2011): Secreted glyceraldehyde-3-phosphate dehydrogenase as a broad spectrum vaccine candidate against microbial infection in aquaculture. *Lett. Appl. Microbiol.*, **54**, 1–9.
- Liang, S., H. Wu, B. Liu, J. Xiao, Q. Wang and Y. Zhang (2012): Immune response of turbot (*Scophthalmus maximus* L.) to a broad spectrum vaccine candidate, recombinant glyceraldehyde-3-phosphate dehydrogenase of *Edwardsiella tarda*. *Vet. Immunol. Immunopathol.*, **150**, 198–205.
- Liu, Y., S. Oshima, K. Kurohara, K. Ohnishi and K. Kawai (2005): Vaccine efficacy of recombinant GAPDH of *Edwardsiella tarda* against Edwardsiellosis. *Microbiol. Immunol.*, **49**, 605–612.
- Liu, Y., S. Oshima and K. Kawai (2007): Glyceraldehyde-3-phosphate dehydrogenase of *Edwardsiella tarda* has protective antigenicity against *Vibrio anguillarum* in Japanese flounder. *Dis. Aquat. Organ.*, **75**, 217–220.
- Livak, K. J. and T. D. Schmittgen (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402–408.
- Nakajima, N., M. Kijima, M. Kawanishi, G. Katou, K. Futami, T. Katagiri, M. Endo and M. Maita (2012): Analysis of immune response in yellowtail upon vaccination with a bivalent vaccine of *Vibrio anguillarum* and *Lactococcus garvieae*. *Fish Pathol.*, **47**, 12–19.
- Shimahara, Y., H. Yasuda, A. Nakamura, T. Itami and Y. Yoshida (2005): Detection of antibody response against *Nocardia seriolae* by enzyme-linked immunosorbent assay (ELISA) and a preliminary vaccine trial in yellowtail *Seriola quinqueradiata*. *Bull. Eur. Ass. Fish Pathol.*, **25**, 270–275.
- Shimmoto, H., K. Kawai, T. Ikawa and S. Oshima (2010): Protection of red sea bream *Pagrus major* against red sea bream iridovirus infection by vaccination with a recombinant viral protein. *Microbiol. Immunol.*, **54**, 135–142.
- Shin, G. W., K. J. Palaksha, Y. R. Kim, S. W. Nho, S. Kim, G. J. Heo, S. C. Park and T. S. Jung (2007): Application of immunoproteomics in developing a *Streptococcus iniae* vaccine for olive flounder (*Paralichthys olivaceus*). *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.*, **849**, 315–322.
- Takahashi, Y., K. Fukuda, M. Kondo, S. Yasumoto, I. Hirono and T. Aoki (2011): Bacterial diseases of marine fish and development of vaccine in Japan. *J. Nat. Fish. Univ.*, **60**, 51–56. (in Japanese with English abstract)