Immunoprotection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Lactococcus garvieae* against *Lactococcosis* in tilapia

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In this study, the gene encoding 40 kDa GAPDH of *L. garvieae* was determined and overexpressed by using the *Escherichia coli* expression system. Analysis results indicated that the sequences of GAPDH of *L. garvieae* nucleotide and its amino acid are highly homologous (80.4–100%) to several products of GAPDH from *L. garvieae* and other Streptococcus-related bacteria. According to Western blotting results, rabbit antiserum and tilapia infection serum reacted strongly to the recombinant GAPDH protein. In another experiment, tilapia were immunized intraperitoneally with formalin-killed *L. garvieae* whole cells, recombinant GAPDH (50 µg fish⁻¹) from *L. garvieae* or both. ISA 763A was used as an adjuvant for vaccine and saline was used as a negative control. The fish challenged at 4 weeks after immunization with GAPDH+WC+ISA had the highest survival rate at 100%, followed by fish immunized with WC+ISA or GAPDH+ISA, which had RPS values of 87.5% and 50%, respectively. Additionally, specific antibody responses against *L. garvieae* whole cells and GAPDH were based on enzyme-linked immunosorbent assay. Following 4 weeks of immunization, the specific antibody level of all vaccine groups significantly increased, except for antibody responses against *L. garvieae* GAPDH of those immunized with formalin-killed *L. garvieae* whole cells. Our results further demonstrated that GAPDH from *L. garvieae* protected tilapia from experimental *L. garvieae* infection, implying the potential use of *L. garvieae* GAPDH as a vaccine against *L. garvieae*.

Key Words—glyceraldehyde-3-phosphate dehydrogenase (GAPDH); immunoprotection; *Lactococcus garvieae"

Introduction

*Lacticoccosis* is streptococcosis caused by *Lactococcus garvieae*, which has infected many freshwater fish, marine fish and prawn species worldwide, including giant freshwater prawn (*Macrobrachium rosenbergii*) (Chen et al., 2001), grey mullet (*Mugil cephalus*) (Chen et al., 2002), rainbow trout (*Oncorhynchus mykiss*) (Chang et al., 2002) and tilapia (*Oreochromis* sp.) (unpublished) in Taiwan; yellowtail (*Seriola uestqueradiata*), amberjack (*S. dumerili*) and kingfish (*S. lalandi*) in Japan (Kitao, 1982; Kusuda et al., 1991);
Among the many OMPs, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classical cytosolic glycolytic protein. Notably, GAPDH in several pathogenic bacteria has been characterized as a protein associated with virulence (Brassard et al., 2004; Madureira et al., 2007; Maeda et al., 2004) owing to its ability to either bind to several host proteins (Madureira et al., 2007) or resist reactive oxygen species generated by host phagocytic cells. Group A streptococci (which includes S. pyogenes, S. equisimilis, S. agalactiae, S. suis and S. pneumonia) and Staphylococcus aureus, in which a cell-surface GAPDH is involved in the interaction with host cells, and adhered to host cells and ligands (Brassard et al., 2004; Maeda et al., 2004; Pancholi and Fischetti, 1992). In Edwardsiella tarda, Vibrio anguillarum, Streptococcus iniae, Aeromonas hydrophila and Vibrio harveyi, GADPHs can induce a protective immune response in fish (Liu et al., 2005, 2007; Ra et al., 2009; Zhang et al., 2007; Zhou et al., 2010). According to a recent study, GADPHs of Edwardsiella tarda act as a potential vaccine against E. tarda infection; these GADPHs also protected Vibrio anguillarum in Japanese flounder (Liu et al., 2007). Therefore, GAPDH of bacteria should be viewed as a multi-purpose vaccine candidate against several pathogenic bacteria. However, the feasibility of using GAPDH of L. garvieae to prevent L. garvieae infection in fish has seldom been examined.

This study focuses on identifying and cloning the gene-coding for 40 kDa L. garvieae GAPDH. GAPDH protein in large quantities is then expressed to purify the protein and evaluate the immunoprotection of L. garvieae GAPDH against lactococcosis in tilapia (Oreochromis niloticus).

**Materials and Methods**

**Bacterial strains and growth conditions.** Lactococcus garvieae was cultured on Todd-Hewitt broth (THB) and in Todd-Hewitt broth (THB) at 25°C with moderate shaking at 125 rpm. The isolate was identified as L. garvieae by standard methods (Zlotkin et al., 1998). Bacteria were stored at ̶80°C in THB containing 20% glycerol. Vector pET151D/TOPO used for overexpression of L. garvieae GAPDH was provided by Invitrogen (Carlsbad, CA). E. coli DH5α was utilized as a host for plasmid constructions, and E. coli BL21 (DE3) was used to produce a recombinant protein. Unless otherwise stated, E. coli was grown on Luria-Bertani (LB) medium with 1.5% (w/v) agar at 37°C. Finally, ampicillin was added to the LB medium with a concentration of 50 μg/ml.
DNA preparation. DNA was extracted from *L. garvieae* by using the method described by Chen et al. (2001). Overnight cultures (3 ml) were isolated by phenol/chloroform extraction, followed by precipitation with isopropanol. The DNA was then dissolved in 100 µl sterilized distilled water and stored at −20°C until used. Small scale DNA plasmid was prepared via alkaline lysis and purified using the Plasmid Miniprep kit (Biokit Biotechnology, Inc., Hsinchu, Taiwan).

PCR amplification of the GAPDH gene coding protein. Based on the sequence of the gene-coding of *L. garvieae* GAPDH protein from GenBank (Accession No. FJ524849), primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/). The nucleotides and amino acid sequences were analyzed using CLUSTALW 2.0 (http://www.ebi.ac.uk/Tools/clustalw2/). The gene-coding GAPDH was then amplified by designing the following primers: LacGAPDH-F 5′-CACC ATG GTA GAA TTC ATT AAC G-3′ and LacGAPDH-R 5′-TTA TTT AGC GAT TTT AGC GAA-3′. The forward primer was added to four bases (CACC) at the 5′ end. Overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Polymerase chain reaction (PCR) was performed on a thermal cycler (Bio-Rad, Hercules, CA) with reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM deoxynucleoside triphosphate, 10 µM primer, 2 µl (50 ng) of template DNA, and 0.625 U of Blend Taq<sup>®</sup> DNA polymerase (Toyobo, Osaka, Japan) to a final volume of 50 µl. The following cycling program was run: preheating at 95°C for 5 min and 33 cycles of denaturing at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min 20 s, followed by a final cycle with a 10 min extension step at 72°C. The PCR amplified product was resolved in 1% agarose gel by electrophoresis and analyzed using horizontal electrophoresis chamber Genius SK-24 (SK Bio, Japan).

Cloning of GAPDH gene. PCR amplified product of the GAPDH gene was ligated to the commercial pET 151D/TOPO linearized vector (Invitrogen) at 25°C for 15 min. The ligated product was transformed into chemically competent *E. coli* DH5α cells by heat shock at 42°C for 45 s. The recombinant transformations were then selected using ampicillin (50 µg/ml) on LB agar plates. The recombinant in plasmid colonies containing the GAPDH gene was identified and confirmed by electrophoresis and sequencing.

Expression of recombinant GAPDH proteins. *E. coli* BL21 (DE3) cultures of recombinant clones of GAPDH grown overnight were inoculated into LB broth containing 50 µg/ml ampicillin at 37°C with shaking at 200 rpm. When *E. coli* BL21 (DE3) cultures reached 0.6–0.7 at an optical density of 600 nm (OD<sub>600</sub>), cells were induced with 0.4 mM isopropyl thiogalactoside (IPTG) (Amresco, Solon, OH) for 16 h at 20°C. Whole cell lysates of the bacteria were then prepared and the expression of the GAPDH recombinant protein was identified by running it on 12% SDS-PAGE. An Uninduced recombinant clone and *E. coli* BL21 host cells (with and without IPTG) were used as controls.

Purification of protein. Recombinant GAPDH proteins were purified by nickel–nitrilotriacetic acid (Ni–NTA) affinity chromatography (Invitrogen) based on the manufacturer’s protocol. Briefly, 500 ml *E. coli* BL21 (DE3)-expressed recombinant GAPDH protein was centrifuged for 15 min at 8,000 rpm in an R31 rotor of the Himae CD21E centrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan). The pellets were then resuspended in 15 ml of native binding buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.5 mM NaCl; pH 8). In total, 15 mg of lysozyme was added to suspension and then incubated on ice for 30 min. Next, cells were disrupted with a sonicator, the Soni prep 150 (Henderson Biomedical, London, UK) at 400 W for 20 min, with a 10 s interval between each 10 s sonication, while cooling on ice. The disrupted cell extracts were centrifuged at 10,000 rpm for 15 min. The 8 ml supernatant containing released proteins was again mixed with 1.5 ml of Ni-NTA agarose slurry (Invitrogen) and shaken in an ice box for 1 h. Finally, the purified protein was eluted with 6–8 ml native elution buffer (250 mM imidazol, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, and
0.5 M NaCl; pH 8) after being washed 4 times with 8 ml native wash buffer (20 mM imidazol, 50 mM NaH₂PO₄, 0.5 M NaCl; pH 8). Finally, the protein concentration was estimated using a Bio-Rad protein assay kit (Bio-Rad).

**SDS-PAGE and Western blotting.** SDS-PAGE was performed according to the method described by Laemmli (1970). Consisting of 4% (w/v) stacking gel and 12% (w/v) separating gel, the gel was stained with Coomassie brilliant blue (CBB) R-250. The E. coli and 12% (w/v) separating gel, the gel was stained with Laemmli (1970). Consisting of 4% (w/v) stacking gel performed according to the method described by. SDS-PAGE was SDS-PAGE and Western blotting was estimated using a Bio-Rad protein assay kit (Bio-Rad).-infected pellets corresponding to 3 to 5% of total body weight. Before vaccination, the fish were monitored using standard bacteriological and serological methods to confirm the absence of pathogens that could confound the experiments.

Prepared vaccine: The formalin-killed WC vaccine was prepared according to the method of Yoshida et al. (1996) with slight modifications. The L. garvieae vaccine was grown in THB while being shaken at 150 rpm for 8 h at 25°C to obtain a final concentration of 1 × 10⁹ cells ml⁻¹. Bacterial cells were inactivated by adding 0.3% (v/v) formalin and incubated at 25°C for 24 h. Vaccine absorbance (A = 600 nm) was adjusted to 1 (representing a concentration of 1 × 10⁹ cells ml⁻¹) with a sterile saline solution of 0.85%. Sterility was confirmed by spreading the vaccines on blood agar with 5% goat red blood cells. The absence of toxicity was confirmed by injecting the vaccine into 10 tilapia. The aqueous bacteria obtained as described elsewhere were mixed with the nonmineral oil adjuvant Montanide ISA 763 AVG (SEPPIC, Paris, France), at a ratio of 40 : 60 to acquire a stable fluid emulsion, based on the recommendations of SEPPIC.

**Vaccination** : Tilapia (Oreochromis niloticus) were anesthetized using tricaine methanesulfonate (MS222) and divided into four groups, each with 35 fish. Fish were immunized intraperitoneally (i.p.) with 0.2 ml vaccine. With this bacterin, group 1 (WC+ISA) was vaccinated by i.p. injections with the ISA 763 AVG adjuvant (6.5×10⁸ bacteria/fish); group 2 was i.p. injected with bacterin with ISA 763 AVG adjuvant plus purified recombinant GAPDH protein (50 µg/fish) (GAPDH+WC+ISA); group 3 was i.p. injected with purified recombinant GAPDH protein (50 µg/fish) emulsified with ISA 763 AVG adjuvant (GAPDH+ISA); in addition, group 4 was i.p. injected with 0.85% saline (0.2 ml/fish) as a control. The fish were reared in aquaria at 25°C for 4 weeks, followed by bleeding of the 10 fish in each group and use of sera to detect specific antibody titers.

**Challenge** : The remaining fish in each group (n=20) were infected by i.p. injection with the live Lactococcus garvieae (1 × 10⁹ CFU fish⁻¹). Mortality was observed for at least 14 days after the challenge. Additionally, all dead fish were examined to confirm re-isolation of the inoculated strain from internal organs (i.e. brain, spleen and kidney) by streaking directly onto THA plates. The effective protection of vaccines was expressed in terms of relative percent survival (RPS) (Kawai et al., 2004).
**Enzyme-linked immunosorbent assay (ELISA).** By using a standard indirect enzyme-linked immunosorbent assay (ELISA), the specific serum antibodies in fish after immunizations were quantified based on a method described elsewhere (Shimahara et al., 2005; Zhang et al., 2007) with slight modifications. Polystyrene microtiter plates (Jet Biofil®; Guangzhou JET Biofiltration Products, Co., Ltd., Guangzhou, China) were first coated with the antigen of *Lactococcus garvieae* whole cell (2 x 10^6 bacteria/well) and purified recombinant GAPDH (800 ng/well); the plates were then incubated overnight at 4°C. The antigen-coated plates were blocked with 2% skimmed milk to prevent non-specific immune reactions. Antigen-coated plates were then made to subsequently react with antiserum from vaccinated or control fish. The antiserum fish were diluted twofold with phosphate-buffered saline (PBS). Next, antibodies binding to the antigens were detected using the rabbit antiserum against tilapia IgM (1:1,000). Each sample was loaded in duplicate and negative control serum was added to each plate. Negative control serum from tilapia was not immunized. Following the reaction by goat antiserum against rabbit IgG conjugated with peroxidase (1:5,000) for 1 h at 25°C. Wells were washed with PBS containing 0.3% Tween-20 (PBST) 5 times before each reaction. Additionally, a color reaction was carried out with a substrate buffer (0.6 mg/ml 2,2’-amino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt in 0.1 M citric acid buffer; pH 4.0) containing 0.003% H2O2 for 15 min at 25°C. The reaction was stopped with 1% sodium dodecyl sulfate. Each plate was then subjected to a microplate reader (Multiskan® Spectrum Microplate Spectrophotometer; Thermo Scientific, Vantaa, Finland) at 405 nm. Analytical results were considered positive if absorbance was at least twice that of the control sera; in addition, antibody titers were scored as the highest positive dilution. Finally, differences were calculated by using two-way analysis of variance (ANOVA). Significant differences by ANOVA corresponded to p<0.05.

**Results**

**Isolation and identification of GAPDH *L. garvieae* bacteria**

The strain from diseased grey mullet and tilapia had the same characteristics as those described in morphological, physiological and biochemical tests (Chen et al., 2002). Identity of the *L. garvieae* strains was then confirmed by using specific primers to amplify the 1,100 bp fragment of 16S rDNA of *L. garvieae* (Zlotkin et al., 1998). Other DNA bacteria of *Lactococcus lactis* were negative controls in the PCR assay. Experimental results indicated that only DNA from *L. garvieae* was positive with PCR-amplified 1,100 bp fragments (Data not shown).

**Purified plasmid inserted GAPDH and nucleotide sequence analysis**

New LacGAPDH-F and LacGAPDH-R primers were designed to amplify the complete GAPDH gene from the *L. garvieae* isolate. The PCR product size was 1,015 bp. The optimal PCR conditional reaction was run: preheating was conducted at 95°C for 5 min of the initial denaturation, 33 cycles of denaturing at 95°C for 1 min, annealing at 52°C for 1 min, and an extension at 72°C for 1 min 20 s, followed by a final cycle with a 10 min extension step at 72°C (Fig. 1). The PCR products were electrophoresised on 2% gel agarose and purified with the Clean and Gel Extraction Kit (Biokit Biotechnology, Inc.).

The purified gel 1,015 bp PCR products were inserted into the pET151D/TOPO cloning vector (Invitrogen), according to the manufacturer’s instructions. The recombinant plasmid pET151D/TOPO-GAPDH was then successfully constructed. The plasmid DNA containing GAPDH of *L. garvieae* was extracted using the Plasmid Miniprep kit (Biokit Biotechnology, Inc.) and was sequenced by Tri-I Biotech Company, Taiwan.

![Fig. 1. Agarose gel 2% showing the polymerase chain reaction (PCR) amplification.](image-url)
The DNA sequences obtained were performed using BLAST (http://www.ncbi.nlm.nih.gov), and DNA Star 5.0 was utilized with neighbor-joining (NJ; Saitou and Nei, 1987) for alignments. Figure 2 shows the full-length GAPDH nucleotide and amino acid sequences. The GAPDH gene of the *L. garvieae* sequence homology percentage identity with the GAPDH gene of other bacteria is shown in Table 1. The GAPDH protein homologies had protein sequences more conservative than the nucleotide sequences in *Streptococcus*-related bacteria.

Expression and purification of recombinant GAPDH

Conditional optimal expression was achieved when bacteria containing pET151D/TOPO-GAPDH were expressed in BL 21(DE3) *E. coli* with 0.4 mM IPTG induction at 20°C for 16 h. Recombinant GAPDH proteins should have molecular masses of 40 kDa. However, owing to fusion with His-tag, the molecular mass was greater than those predicted based on their open reading frames (ORFs) by SDS-PAGE. The size of the recombinant GAPDH protein was approximately 44 kDa, including a 4 kDa His-tag and V5 epitope from the expression vector (Fig. 3A). Under induction by IPTG, the recombinant GAPDH proteins with His-tag were the most abundant proteins in inclusion bodies and a small amount of proteins appeared in soluble form. However, no inserted GAPDH gene or expressed *E. coli* without IPTG displayed the corresponding protein bands (Fig. 3A). The above analytical results suggest the successful expression of recombinant GAPDH proteins. GAPDH was purified using the His-tag system (Invitrogen) via Ni-NTA affinity chromatography. The molecular mass of recombinant GAPDH proteins was estimated at approximately 44 kDa by 12% SDS-PAGE (Fig. 3B).

A protein with 337 amino acid residues and an estimated molecular mass of 40 kDa encoded by ORF of GAPDH had 1,011 nucleotides with C+G contents of 41%. Protein identity was determined with its homolo-

Fig. 2. Full length nucleotide sequence of the *L. garvieae* GAPDH gene and its deduced amino acid sequence.

Annealing site of primers LacGAPDH-F/R are underlined.
Table 1. Homologies of nucleotide and amino acid sequence of *L. garvieae* LG1 GAPDH in Taiwan to those of other bacterial infected fish.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Accession number of GAPDH genes</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nucleotide</td>
</tr>
<tr>
<td><em>Lactococcus garvieae</em></td>
<td>LG2 in this study</td>
<td>99.9</td>
</tr>
<tr>
<td><em>Lactococcus garvieae</em></td>
<td>FJ524849</td>
<td>99.5</td>
</tr>
<tr>
<td><em>Lactococcus lactis subsp. lactis</em></td>
<td>CP001834</td>
<td>90.1</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>AF338416</td>
<td>83.6</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em></td>
<td>AF375662</td>
<td>80.4</td>
</tr>
<tr>
<td><em>Streptococcus iniae</em></td>
<td>AF421902</td>
<td>82.8</td>
</tr>
<tr>
<td><em>Edwardsiella ictaluri</em></td>
<td>CP001600</td>
<td>56.9</td>
</tr>
<tr>
<td><em>Edwardsiella tarda</em></td>
<td>FJ605131</td>
<td>56.3</td>
</tr>
<tr>
<td><em>Vibrio harveyi</em></td>
<td>DQ184650</td>
<td>61.2</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>CP001485</td>
<td>62.1</td>
</tr>
<tr>
<td><em>Vibrio vulnificus</em></td>
<td>AEC16795</td>
<td>61.8</td>
</tr>
</tbody>
</table>

**Fig. 3.** Coomassie brilliant blue-stained SDS-PAGE gel of *E. coli* pellet. (A) The expression of the GAPDH protein. Lane 1: uninduced *E. coli* carrying expression vector; Lane 2: crude extract from induced *E. coli* with 0.4 mM IPTG; Lane 3: soluble fractions from induced *E. coli* with 0.4 mM IPTG; Lane 4: pellets from induced *E. coli* with 0.4 mM IPTG. (B) Ni-NTA purification of *(His)₆* GAPDH produced in *E. coli* BL21. Lane 1: uninduced *E. coli* carrying expression vector; Lane 2: crude extract from induced *E. coli* with 0.4 mM IPTG; Lanes 3–4: eluted fraction of soluble *(His)₆* GAPDH protein. Proteins were resolved by SDS-PAGE on 12% polyacrylamide gel and stained with Coomassie brilliant blue. Lane M: protein maker.

logue from the same species mentioned above (Fig. 4). The GAPDH protein homologies had a protein sequence that appeared to be more conservative than the nucleotide sequence in *Streptococcus*-related bacteria (Table 1).

**Western blot analysis**

Whether recombinant GAPDH protein contains the antigenic epitopes was tested by raising and testing the rabbit antiserum against recombinant GAPDH protein and *L. garvieae*-infected tilapia serum for an immunological reaction with the GAPDH protein. Western blotting results (Fig. 5A) indicated that rabbit antiserum and tilapia infection serum reacted strongly to the 44 kDa recombinant GAPDH protein.

**Specific antibody titers of serum**

A specific antibody titer against *L. garvieae* whole cells and GAPDH were detected in all three vaccinated tilapia sera by ELISA. The specific antibody titer against *L. garvieae* whole cells of fish immunized with WC+ISA, GAPDH+ISA and GAPDH+WC+ISA at
4 weeks was higher than that of control fish, while the specific antibody titer of fish in the group immunized with GAPDH+ISA and GAPDH+WC+ISA was significantly lower than that of WC+ISA (Fig. 6A). However, fish immunized with GAPDH+ISA and GAPDH+WC+ISA at 4 weeks had significantly higher serum antibody titers against GAPDH than those immunized with WC+ISA and control fish (Fig. 6B).

**Effective vaccine protection against *L. garvieae* infection**

The immunoprotective effect of purified recombinant GAPDH protein was investigated by immunizing tilapia separately with WC+ISA, GAPDH+WC+ISA, GAPDH+ISA or Saline. The fish were challenged with the pathogenic *L. garvieae*. Experimental results indicated that fish began to die 1 day after the challenge; the percentage of cumulative mortality of the unimmunized group increased rapidly at 1–4 days and fish stopped dying on day 5 (Fig. 7). The fish immunized with GAPDH+ISA and GAPDH+WC+ISA at 4 weeks had significantly higher serum antibody titers against GAPDH than those immunized with WC+ISA and control fish (Fig. 6B).

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**Discussion**

Tilapia culture is widely practiced in many tropical and subtropical regions worldwide. More than 22 tilapia species are cultured worldwide (El-Sayed, 1999). However, streptococcal infection has created tilapia losses in most regions worldwide where this fish is cultured (Eldar et al., 1994; Vendrell et al., 2006). The incidence of streptococcal disease, caused by *Streptococcus* and *Lactococcus* spp., has increased in Taiwan (Chang and Plumb, 1996; Chen et al., 2001, 2002; Tung et al., 1985). Antibiotic treatment is occasionally ineffective under field conditions, necessitating the development of a vaccine to control streptococcal disease (Vendrell et al., 2006). Despite the availability of several injectable vaccines to prevent streptococcosis, many of them differ in their formulation. Previous studies attempted to protect rainbow trout after intraperitoneal (i.p.) vaccination with a for-
malin-killed *Streptococcus* and *Lactococcus* spp. vaccine (Evans et al., 2004; Klesius et al., 2000; Ooyama et al., 2002; Vendrell et al., 2007). However, a subunit vaccine for *L. garvieae* has seldom been investigated. Shin et al. compared three strains of *L. garvieae*. Of those, KG9408 possesses a highly developed capsule and fimbriae-like components; MS93003 has a microcapsule with fimbriae-like components projecting from its cell surface; and NSS9310 has no capsule or fimbria-like structure on its cell surface. Notably, those studies used antisera from olive flounder (*Paralichthys olivaceus*) experimentally infected with either the KG9408 or NSS9310 strain. Proteomic analysis based on 2-DE revealed that the protein profiles of the *L. garvieae* strains differ from each other. Shin et al. noted a similar phenomenon when using rabbit sera (Shin et al., 2007). Those studies also observed GAPDH and other common antigens found in *L. garvieae* stains (Shin et al., 2007, 2009). Moreover, despite studies citing GAPDH as a promising vaccine to protect against Edwardsiella tarda, Vibrio anguillarum and *S. pneumonia* (Ling et al., 2004; Liu et al., 2005, 2007), efficacy of the GAPDH vaccine from *L. garvieae* has not been investigated.

In this study, *L. garvieae* GAPDH was successfully cloned and sequenced. The nucleotide sequence for GAPDH was then determined. According to alignment of the nucleotide and amino acid sequence homologies, GAPDH resembles published GAPDH sequences of other *Streptococcus*-related bacteria. The nucleotide and amino acid sequence of *L. garvieae* GAPDH closely resembles that of GAPDH *Streptococcus*-related bacteria (80.4–100% identity). In particular, nucleotide...
and amino acid sequences had a high homology to the fish pathogenic Streptococcus spp. GAPDH sequence (83.6%) and amino acid sequence (84.9%). A similar study indicated that S. suis GAPDH is highly homologous among species of Streptococcus sp. (Brassard et al., 2004). In analyzing the immunogenicity of this protein, Western blotting of rabbit serum and L. garvieae-infected tilapia sera against GAPDH, respectively, revealed that both sera reacted strongly with purified recombinant GAPDH proteins. A similar observation was made when carried out using rabbit and olive flounder sera based on 2-DE (Shin et al., 2007, 2009).

Humoral immunity plays an important role in protecting yellowtail from L. garvieae infection (Ooyama et al., 1999). This study also analyzed the fish-specific antibody titer against L. garvieae whole cells and GAPDH by an ELISA assay at 4 weeks post-vaccination. According to those results, the specific antibody titer against L. garvieae whole cells of fish immunized with WC+ISA was significantly higher than that of fish immunized with GAPDH+WC+ISA or GAPDH+ISA. Moreover, the specific antibody titer against GAPDH of fish immunized with GAPDH+ISA was significantly higher than that of fish immunized with GAPDH+WC+ISA or WC+ISA. However, the survival of fish immunized with GAPDH combined with WC+ISA was higher (RPS = 100%) than that of fish immunized with WC+ISA (RPS = 87.5%) or GAPDH+ISA (RPS = 50%) alone.

To explain this experimental observation, immunization with the whole cells elicited humoral responses for antibody production associated with several cellular factors and arrays of surface proteins that were recognized by the host immune system. However, as an outer membrane protein, GAPDH represents only small portions of the bacterial constituents, which reduced the contents of a specific anti-GADPH antibody from sera in fish immunized with WC+ISA, as analyzed by ELISA. Additionally, more than an antigenic capable of inducing a humoral response, GAPDH is also expected to function as an adjuvant in the immune response. Therefore, higher specific antibody titers against GAPDH and whole cells of fish immunized with GAPDH+ISA have been observed. Furthermore, Harvey et al. (1992) and Arduino et al. (1994a, b) found that the complement system played a major role in the killing of enterococci in human polymorphonuclear phagocytic cells (Arduino et al., 1994a, b; Harvey et al., 1992). Their results further demonstrated that a slight amount of specific antibodies promoted greater killing by phagocytes than that which occurred in an active complement (Ooyama et al., 1999). The immunostimulatory effects of peptidoglycan have been recognized from laboratory evidence of influences on non-specific immune responses such as phagocytic cell activity, lysozyme levels and complement levels (Itami et al., 1996; Matsu and Miyazano, 1993; Zhou et al., 2006). Thus a fraction of the formalin-killed WC such as the peptide glycan layer or some particular sugar chains can evoke strong antibody responses and exhibit the higher RPS seen in the challenge experiment in this study.

In a previous study, striped bass vaccinated with the Bacillus abortus strain RB51 vaccine expressing a mammalian Mycobacterium sp. 85A antigen revealed antibody and lymphocyte responses that were specific and dose-dependent. Blood and tissue samples from these fish demonstrated significant specific humoral and cell-mediated immune responses towards the 85A antigen in a dose-dependent manner (Pasnik et al., 2003). Further, Bootland et al. (1993) found that immersion vaccination of rainbow trout with infectious pancreatic necrosis virus (IPNV) fusion proteins resulted in protection against waterborne challenge with IPNV that was dependent upon the number and size of the viral proteins expressed. Therefore, in ongoing work, the dose-dependent manner of GAPDH needs
to be determined.

GAPDH is a potential vaccine for protecting against *Edwardsiella tarda* and *Streptococcus pneumoniae* (Ling et al., 2004; Liu et al., 2005). Protective in several ways, GAPDH may protect antigenicity against other pathogenic bacteria in fish (Liu et al., 2007). Shin et al. (2007, 2009) found that *L. garvieae* GAPDH, with its high antigenicity for inducing an immunity response, is highly promising for use in developing an effective vaccine against *L. garvieae* in fish (Shin et al., 2006, 2007, 2009). Our previous study noted a similar finding, indicating that GAPDH can protect fish against *L. garvieae*. In this study, tilapia immunized with recombinant GAPDH combined with WC+ISA had a higher survival rate than that of fish immunized with WC+ISA only; in addition, fish immunized with GAPDH+ISA alone also had a rate of 50% RPS. Previous studies using recombinant GAPDH vaccine, including *Streptococcus iniae*, *Aeromonas hydrophila* and *E. tarda*, revealed that combining recombinant GAPDH protein with WC or recombinant protein vaccines provides better protection than only a subunit vaccine or WC vaccine (Ra et al., 2009; Shimmoto et al., 2010; Zhou et al., 2010).

Moreover, according to analysis of nucleotide and amino acid sequences, *L. garvieae* GAPDH has a high homology among streptococcal pathogens. Compared with other Gram-negative bacteria, high homologies were also observed between *E. tarda* and *V. cholera* (Liu et al., 2005). Additionally, Liu et al. indicated that GAPDH prepared from *E. tarda* protects Japanese flounder effectively in a challenge of *V. anguillarum* (Liu et al., 2007). According to those results, high homology GAPDH from different bacterial species can function in a cross-protective role. Results of this study demonstrate that the 44 kDa recombinant GAPDH of *L. garvieae* can protect tilapia effectively in a challenge of *L. garvieae*. However, the cross-protection GAPDH of *L. garvieae* and other Gram-positive cocci and *Streptococcus* pathogens requires further study.

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**References**


Streptococcus
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Streptococcus
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