Evaluation of DNA vaccination of spotted sand bass (Paralabrax maculatofasciatus) with two major outer-membrane protein-encoding genes from Aeromonas veronii

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Abstract

Genes encoding two major outer membrane proteins (OMPs) of the bacterial pathogen Aeromonas veronii, Omp38 and Omp48, were used to construct DNA vaccines. The protective effect of such vaccines against motile aeromonad septicaemia was evaluated in spotted sand bass (Paralabrax maculatofasciatus), an endemic species of the Mexican Northwest Pacific coast and a potential resource for the aquaculture industry. Weak protein expression, as determined by immunoblotting, was observed after transfection of eukaryotic cells with the DNA vaccines. Fish immunized with a single intramuscular injection of 20 μg of the omp38 and omp48 DNA vaccines showed slightly, but significantly elevated serum antibody levels 4 and 6 weeks after vaccination, compared to fish vaccinated with the control plasmid pcDNA3.1. Spotted sand bass vaccinated with the omp38 and omp48 DNA vaccines and challenged with A. veronii by intraperitoneal route recorded a relative percent survival (RPS) between 50 and 60%. Histopathological signs of motile aeromonad septicaemia were observed in around 40% of omp38 and omp48-vaccinated fish and 80% of pcDNA3.1-vaccinated control fish. The results indicate that P. maculatofasciatus vaccinated with a single dose of DNA plasmids
encoding the major OMPs from A. veronii shows partial protection against infection and mortality by A. veronii experimental infection.

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1. Introduction

Aeromonas veronii is a Gram-negative bacterium that has been reported as an opportunistic pathogen involved in human gastroenteritis and extraintestinal infections [1]; and together with Aeromonas hydrophila is the causative agent of bacterial hemorrhagic septicaemia (motile aeromonad septicaemia) of numerous cultured fish species [2,3].

The spotted sand bass (Paralabrax maculatofasciatus) is an endemic fish of the Northwest Pacific coast of Mexico and is considered a candidate species for the aquaculture industry. As occurs for other economically important cultured fish species, bacterial diseases mainly caused by Vibrio spp. and Aeromonas spp. (including A. veronii and A. hydrophila) are a major problem in spotted sand bass farming [3]. Although several studies have shown that different vaccine formulations including subunit, whole killed and live attenuated vaccines may provide protection against A. veronii or A. hydrophila infections [4–7], there are currently no vaccines commercially available against these important pathogens. Although attenuated live vaccines stimulate an effective immune response, these vaccines may represent a risk of residual and reverted virulence. On the other hand, while inactivated vaccines are safer, they are usually poorer immunogens. Consequently, current vaccine research is oriented to replace conventional vaccines with new more effective and safe approaches, such as DNA vaccines [8,9].

Starting with the article by Anderson et al. [10], several other reports have demonstrated the effectiveness of DNA vaccination in fish against viral infections, including infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicaemia virus (VHS) [11,12]. Little is known, however, about the feasibility of DNA vaccination against bacterial pathogens in fish.

Outer membrane proteins (OMPs) are highly immunogenic bacterial components due to their exposed epitopes on the cell surface. OMPs from Aeromonas spp. have been identified as suitable targets for vaccine development in fish [5,6,13–15]. We have recently cloned and characterized the genes encoding the Omp38 and Omp48 proteins, which are the major OMPs from A. veronii [16]. In addition, our preliminary studies indicate that these proteins seem to be involved in bacterial adhesion to cultured epithelial cells, which make them suitable candidates for vaccine development [16,17].

Herein, we report the construction of DNA vaccines using omp38 and omp48 genes and its evaluation in eliciting immune response and protection in spotted sand bass against A. veronii experimental infections.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The A. veronii biotype veronii strain A186 used in the present study was obtained from the Microbial Culture Collection of the Hospital of the University of Lund, Sweden, and was kindly provided by Prof. T. Wadström. It was identified by its fatty acid profile (Sherlock System) at Auburn University, AL, USA (Bacterial Strain Identification and Mutant Analysis Service). Escherichia coli DH5α (Stratagene, La Jolla,
CA, USA) was used as a host for DNA manipulations. *E. coli* and *A. veronii* strains were grown at 37 °C in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg ml⁻¹) when needed [18].

2.2. Construction and preparation of DNA vaccines

The genes coding for Omp38 and Omp48 were amplified from *A. veronii* A186 genomic DNA by the polymerase chain reaction (PCR) with different sets of specific primers (Table 1) and cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Frederick, MD, USA). This vector carries the human cytomegalovirus (CMV) immediate early promoter, the bovine growth hormone (BGH) polyadenylation signal for transcription termination, the ampicillin resistance gene and the ColE1 origin of replication for maintenance in *E. coli*. The forward primers contained a *BamHI* restriction site, and a Kozak sequence (CCACC) just before the start codon to ensure proper translation of prokaryotic genes in eukaryotic cells. The reverse primers contained the C-terminal sequence of Omp38 and Omp48 including the translation stop codon (Table 1). Appropriate primer pair combinations were used to amplify the coding sequence for the primary (including the amino acid leader sequences) and mature forms of Omp38 and Omp48 proteins.

PCR reactions were performed with *Pwo* DNA polymerase (Roche, Indianapolis, IN, USA) and PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer’s protocol. The genes were excised from the pGEM-T vectors by *BamHI*-NotI (omp38 gene) or *BamHI*-EcoRI (omp48 gene) digestion, inserted into pcDNA3.1(+) and transformed into *E. coli* DH5α (Fig. 1). Recombinant clones were selected by ampicillin resistance and confirmed by restriction analysis of plasmids and DNA sequencing. Plasmids were named as follows: pOMP38P (primary Omp38), pOMP38M (mature Omp38), pOMP48P (primary Omp48), and pOMP48M (mature Omp48) (Fig. 1). Plasmids were purified with the EndoFree Plasmid Mega purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, aliquoted at 1 μg ml⁻¹ in sterile endotoxin-free phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and stored at −20 °C until further use.

2.3. In vitro expression of Omp38 and Omp48 in eukaryotic cells

The ability of the plasmids to express the Omp38 and Omp48 proteins in vitro was assessed in transfected HeLa cells. The HeLa cells were grown to confluence at 37 °C (95% humidity and 5% CO₂) in 6-well culture plates in RPMI-1640 culture media supplemented with 10% fetal calf serum, 2 mM L-glutamine, and gentamicin (40 μg ml⁻¹). The cells were transiently transfected with 2 μg of each plasmid construction and pcDNA3.1 as a negative control by the cationic lipid method (Lipofectamine 2000, Invitrogen). Simultaneously, the cells were co-transfected with the reporter plasmid pCMV-SPORT-βgal.

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>38F1</td>
<td>GGATCCACCATGA</td>
</tr>
<tr>
<td>38F2</td>
<td>GGATCCACCATGGCTGTCTTACGACAAGACGG</td>
</tr>
<tr>
<td>38R</td>
<td>TTGAAAGTGGTAAGTGTGGCAAGGCAAC</td>
</tr>
<tr>
<td>48F1</td>
<td>GGATCCACCATGAAAATGAAGCAAAGTGCTTCCC</td>
</tr>
<tr>
<td>48F2</td>
<td>GGATCCACCATGGTGTATTTCCACGGGCTACATCG</td>
</tr>
<tr>
<td>48R</td>
<td>TTACACACAAGCTCCGCTTG</td>
</tr>
</tbody>
</table>

Sequences were taken from accession numbers AF538866 [16] and AF538867.

*BamHI* site and start codon are indicated in boldface and the Kozak sequence is italicised.
(Gibco BRL Life technologies, Frederick, MD, USA) as transfection efficiency control and to normalize the protein expression data. β-Galactosidase activity was determined using chorophenol red-β-D-galactopyranoside (CPRG, Roche) as substrate as previously described [19]. Protein expression was assessed in HeLa cell lysates and supernatants from cultures, 48 h post-transfection, by Western blot as described elsewhere [20], using rabbit polyclonal anti-Omp38 and anti-Omp48 antiserum.Slot blot was also performed with the Bio-Dot SF microfiltration apparatus to concentrate the proteins on the membranes (Bio-Rad, Hercules, CA, USA). Optical density of the blots was measured using the software Scion Image (http://www.scioncorp.com). Protein expression data were expressed as relative optical units, consisting of the ratio of the density of the blots to the β-galactosidase activity.

2.4. Fish

Spotted sand bass (P. maculatofasciatus) with an average weight of 10 g were held in 500 L tanks supplied with running sand-filtered and UV-treated seawater at 24 °C, salinity 37 ppm, pH 8.2 and 7.25 mg l⁻¹ of

Fig. 1. Schematic diagram for the construction of plasmids for DNA immunization. (a) The synthetic genes encoding for the primary and mature Omp38 and Omp48 proteins were amplified by PCR and cloned in the vector pGEM-T. (b) The genes were inserted in pcDNA3.1, which carries the human cytomegalovirus immediate early promoter (CMV); the bovine growth hormone polyadenylation signal for transcription termination (BGH pA); the ampicillin resistance gene (Amp'); and the origin of replication for maintenance in E. coli (ColE1). (c) The resulting plasmids contained the omp38 and omp48 genes, under the regulatory sequences of pcDNA3.1. Abbreviations: lp: leader peptide, B: BamHI, N: NotI, E: EcoRI.
dissolved oxygen. The fish were daily fed ad libitum with 2–4 mm dry pelleted diet (56% protein, 22% lipid, 9% carbohydrate and 13% ash) during the acclimatization and the experimental periods.

2.5. Fish vaccination

Spotted sand bass were randomly divided into groups of 10 fish and vaccinated as follows: fish were anaesthetized by immersion in a 100 µg ml⁻¹ solution of tricaine methane sulfonate (MS-222) and intramuscularly injected at the base of the dorsal fin with 20 µg of the specified DNA vaccine solution (1 µg µl⁻¹). Three replica groups of 10 fish per treatment were assessed. The treatment groups tested for humoral immune response consisted of fish vaccinated with: (i) pOMP38P; (ii) pOMP48P; and two negative control groups including (iii) pCDNA3.1 and (iv) sterile PBS. For evaluation of protective immunity groups of 30 fish were assessed and an additional treatment group consisting of a mixture of pOMP38P and pOMP48P was assayed.

2.6. Analysis of antibody response

Four and six weeks post-vaccination, five fish from each treatment group were assayed for antibody response against Omp38 and Omp48 by enzyme-linked immunosorbent assay (ELISA). Omp38 and Omp48 proteins were isolated and purified as described previously [16,17]. Fish were sacrificed by an overdose of anaesthetic (MS-222, 500 µg ml⁻¹) and the blood was collected directly from the heart. After coagulation, the blood was centrifuged and the serum was collected and stored at −80 °C. ELISA plates (96-wells; Nunc MaxiSorp, San Diego, CA, USA) were coated overnight at 4 °C with Omp38 and Omp48 proteins (10 µg ml⁻¹) in 100-µl aliquots in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). The plates were washed once with PBS containing 0.05% Tween 20 (PBS-Tween) and then blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 22 °C. After washing with PBS-Tween, serial dilutions of the sera (1:10 and 1:100) obtained from individually vaccinated fish were added to triplicate wells of the plates and incubated for 90 min at 37 °C. The plates were then washed twice and rabbit anti-spotted sand bass immunoglobulins (1:2000) [21] were added to the plates and incubated for 2 h at 37 °C. After washing three times, peroxidase-conjugated goat anti-rabbit IgG (1:2000) (Sigma Chemical Co., St. Louis, MO, USA) was added to the wells and incubated for 2 h at 37 °C. After washing, the reaction was developed by addition of 200 µm of o-phenylenediamine (Sigma) and 0.04% H₂O₂. After 30 min incubation in darkness at 30 °C, the reaction was stopped by the addition of 5% H₂SO₄ and then the plates were read with a microplate reader (Bio-Rad) at 492 nm.

2.7. Experimental A. veronii challenge and evaluation of immunoprotection

Since acute disease caused by A. veronii is strongly stress-associated in nature, it was difficult to achieve the required level of bacterial challenge. In a preliminary assay we determined the median lethal dose (LD₅₀) to be approximately 5 × 10⁶ cfu fish⁻¹. Four weeks post-vaccination fish were anaesthetized and challenged by intraperitoneal inoculation with 50 µl of 1 × 10⁸ cfu ml⁻¹ (5 × 10⁶ cfu fish⁻¹) of A. veronii cell suspension. Bacterial suspension was prepared as follows: A. veronii was grown overnight in LB broth at 37 °C, harvested by centrifugation (1500 × g for 20 min) and washed with PBS buffer three times. Bacteria were suspended in PBS buffer and adjusted to an absorbance value of 1 at 540 nm, which correspond to a cell density of approximately 10⁸ cfu ml⁻¹. To confirm the bacterial concentration, the number of cfu ml⁻¹ were counted by spreading 0.1 ml of 10-fold serial dilutions of bacterial suspension on LB agar plates, which were incubated overnight at 37 °C. Cumulative mortality and clinical signs were recorded daily for three weeks post-challenge and dead fish were autopsied to determine the cause of death.
and for the presence of *A. veronii* in the tissues by bacterial culture in McConkey agar. The relative percent survival (RPS) was calculated according to Amend [22]:

\[ \text{RPS} = 1 - \left( \frac{\% \text{ mortality in vaccinated}}{\% \text{ mortality in control fish}} \right) \times 100 \]

At the end of the experiment, fish that survived the bacterial challenge were euthanized as mentioned above and samples from kidney, intestine, liver, and spleen were dissected for histopathological analysis. Vaccine efficacy was determined by comparing the RPS and the histopathological damage among experimental treatments.

2.8. Statistical analysis

Data from protein expression and antibody response assays were statistically analysed using one-way analysis of variance (ANOVA), and the Student’s *t*-test was conducted where significance was detected at the $P < 0.05$ level. Cumulative percent mortality was analysed by the G-test ($P < 0.05$).

3. Results

3.1. In vitro expression of Omp38 and Omp48

The genes coding for the primary and mature forms of Omp38 and Omp48 were used to construct DNA vaccines against *A. veronii* (Fig. 1). In order to test the ability of the constructed DNA vaccines to express the various forms of Omp38 and Omp48 in a eukaryotic environment, HeLa cells were transfected with pOMP38P, pOMP48P, pOMP38M, pOMP48M, and the negative control pcDNA3.1. After 48 h of incubation, cell extracts and culture supernatants were analysed by Western blot and slot blot with anti-Omp38 and anti-Omp48 antisera. The presence of primary and mature forms of Omp38 and Omp48 could not be detected by Western blots (data not shown). As shown in Fig. 2, the proteins were only detected in the cell lysates by slot blot assay. The expression level was low since high amounts of cell lysate ($50 \mu$g) needed to be loaded and vacuum concentrated on the slots to obtain faint signals, which are not present in the vector controls. The β-galactosidase activity measurements indicated that the transfection was effective and homogenous among the various transfections (data not shown). Albeit Omp38 and Omp48 expression was low, it was significant compared to the pcDNA3.1 control. The expression level of the primary form of Omp48 was higher than the corresponding mature form. Conversely, the expression of mature form of Omp38 was higher than the corresponding primary form (Fig. 2).

3.2. Antibody response to DNA immunization

The humoral immune response of spotted sand bass to immunization with the Omp38 and Omp48 DNA vaccines was assessed by ELISA four and six weeks after vaccination. Fish injected intramuscularly with pOMP38P and pOMP48P produced very low levels of anti-Omp38 and anti-Omp48 antibodies (Fig. 3). Antibody levels against Omp38 and Omp48 were statistically significant at the serum dilution 1:10 in vaccinated fish in relation to PBS buffer or pcDNA3.1 negative controls; however, no statistical differences were observed between the antibody levels at dilutions of 1:100 or higher (data not shown).
3.3. Protection of spotted sand bass from *A. veronii* challenge

Spotted sand bass immunized with the DNA vaccines encoding for OMPs were assessed for protection against *A. veronii* intraperitoneal challenge. As shown in Table 2, mortalities following exposure to the bacteria were lower in fish vaccinated with pOMP38P or pOMP48P, compared to those of the control PBS. Statistical differences were found between treatments with different superscripts ($P < 0.05$).

![Fig. 2. Analysis of in vitro expression of Omp38 and Omp48 from DNA constructs in transfected HeLa cells.](image)

![Fig. 3. ELISA detection of anti-Omp38 and anti-Omp48 antibodies in serum from fish immunized with plasmid DNA vaccines and control fish injected with PBS or plasmid control.](image)
groups injected with PBS buffer or pcDNA3.1 alone. The pOMP38P and pOMP48P vaccines protected fish to a similar extent, with a RPS of 53.8% and 60.2%, respectively. The mixture of pOMP38P and pOMP48P vaccines did not elicit an enhanced protective effect with its RPS being similar to that of the individual vaccines (Table 2). Histopathological analyses of vaccinated and non-vaccinated control fish that survived the bacterial challenges revealed signs of tissue lesions resulting from *A. veronii* infection (data not shown). Major lesions were observed in the kidney, liver and spleen, including severe necrosis and hemorrhagic infiltration. The presence of elevated numbers of melanomacrophages was observed in the spleen. Percentages of fish that presented the histological alterations were 80, 83, 41, 37, and 38 for fish vaccinated with PBS buffer, pcDNA3.1, pOMP38P, pOMP48P and the mixture of vaccines, respectively.

### 4. Discussion

Vaccination with OMPs has been shown to be effective against *Aeromonas salmonicida* and *A. hydrophila* infections in fish [5,6,13–15]. However, the need for isolation and purification of the antigens prior to vaccination makes these subunit vaccines costly. DNA vaccines offer a more economic alternative to fish vaccination [10–12], therefore, in this study DNA immunization was employed as an alternative approach to standard immunization protocols. In this work, we investigated the potential for genetic immunization against motile aeromonad septicaemia using the genes encoding for two major outer membrane proteins from *A. veronii*, Omp38 and Omp48.

We show here that immunization of spotted sand bass with these two DNA vaccines induced partial protection against experimental challenge with *A. veronii*. The moderate success of the Omp38 and Omp48 DNA vaccines to elicit a considerable antibody response and efficient protection could be due to: (1) low levels of protein expression in fish tissue; or (2) low levels of antigenicity of the expressed proteins, because of the possibility that the proteins are not expressed in the proper conformation. Although the expression vector used to construct these DNA vaccines was able to drive the expression of Omp38 and Omp48 in HeLa cells in culture (which can resemble the eukaryotic background of fish cells), the levels of expression of these two prokaryotic proteins were very low in whole cell lysates. In addition, as expected for expression vector lacking secretion signal sequence, proteins were absent in the cell culture medium. Inclusion of the sequences coding for the native leader peptides of Omp38 and Omp48 in the constructs did not cause major differences in the levels of expression between the primary and mature forms of the proteins. Thus, the presence or absence of the native leader peptide did not influence the expression level of the proteins in the eukaryotic cells. Since heterologous expression of viral antigenic proteins under the control of the CMV promoter has been achieved in fish cells [10–12], it could be

### Table 2
Cumulative percent mortality and relative percent survival (RPS) of vaccinated spotted sand bass, *P. maculatofasciatus*, following intraperitoneal challenge with *A. veronii*

<table>
<thead>
<tr>
<th>Fish injected with:</th>
<th>Cumulative percent mortalitya (dead fish/total injected fish)</th>
<th>RPSb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS buffer</td>
<td>41.4% (12/29)</td>
<td>–</td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td>43.3% (13/30)</td>
<td>–</td>
</tr>
<tr>
<td>pOMP38P</td>
<td>20% (6/30)</td>
<td>53.8%</td>
</tr>
<tr>
<td>pOMP48P</td>
<td>17.2% (5/29)</td>
<td>60.2%</td>
</tr>
<tr>
<td>pOMP38P + pOMP48P</td>
<td>16.6% (5/30)</td>
<td>61.6%</td>
</tr>
</tbody>
</table>

a Values are cumulated percent of mortality recorded three weeks after challenge.

b Relative to pcDNA3.1 control.
speculated that the low protein expression obtained in this work is due to the intrinsic features of the *omp38* and *omp48* genes, such as preference codon usage or mRNA stability, instead of regulatory sequences found in the expression vector pcDNA3.1. A number of studies have demonstrated that there is a good correlation between the codon bias of genes encoding for viral, bacterial, and parasitic antigens and its level of expression [23–29]. The antibody titres in the serum of spotted sand bass against Omp38 and Omp48 could be considered a weak immune response since antibodies were detected only at the low serum dilution of 1:10 at four and six weeks post-vaccination. The low antibody response in genetic immunization has been associated with low levels of antigen expression in the host tissue. Several studies have found that codon usage optimization has an enhancing effect on expression levels and immunogenicity of DNA candidate vaccines [23–29].

It should be taken into consideration that a single immunization dose was applied without further booster vaccinations. Although it has been argued that booster vaccinations may not be needed in the case of DNA vaccines, primed-boosted vaccinations may be necessary to enhance the antigenicity of bacterial antigens such as Omp38 and Omp48 [30,31]. It is also possible that lack of secretion of the antigens may have influenced the magnitude of the humoral immune response. After intramuscular injection, plasmid DNA enters the myocytes by an unknown mechanism. Once the antigen is expressed, it is released from the myocytes either as a secreted protein or due to cell damage and/or death and then it is internalised by antigen presenting cells [32]. Although considerable humoral immune response can be obtained against OMP antigens that lack secretion signals [20,33], it has been recently reported that the antibody response is dramatically increased when antigens are expressed as extracellular proteins from plasmid expression vectors carrying the appropriate signal sequence [34–36]. Plasmid DNA vaccines tested in this work did not carry a signal sequence to express the antigen as extracellular protein and it should be considered for further vaccine development based on *omp38* and *omp48* genes.

It is noteworthy that despite the failure of the Omp38 and Omp48 DNA vaccines to induce a significant antibody immune response, these DNA vaccines were able to confer moderate levels of protection to experimental challenge with *A. veronii*, including a reduction in the number of fish with histopathological alterations. These protection levels are similar to those obtained in Atlantic salmon (*Salmo salar* L.) immunized with iron-regulated outer membrane proteins (IROMPs) [13,15], rainbow trout (*Oncorhynchus mykiss*) vaccinated with a 28 kDa OMP (porin) from *A. salmonicida* [14], or mice genetically immunized with the genes coding OMPs from chlamydial pathogens [33,37]. It has been stated that specific antibodies might play a role in the prevention of bacteria from adhering and penetrating the epithelium of the fish gills, skin and fins during the first stages of infection, and afterwards in the opsonization of the bacteria for elimination by phagocytosis [38]. However, there are several conflicting reports regarding the correlation between protection of fish against *Aeromonas* spp. infections and the level of serum specific antibody [7,15,39–42]. The low level of antibody response elicited by the Omp38 and Omp48 DNA vaccines suggest that cellular immunity might be involved in the protection of *P. maculatofasciatus*.

It should be noted that the 60% minimum mortality rate required to calculate the RPS according to Amend [22] was not achieved in the controls, reaching only 41.4 and 43.3% with PBS and pcDNA3.1, respectively, which could be leading to an overestimate of the RPS data. Thus, the moderate RPS values obtained in the present study should be taken cautiously. The assessment of vaccination efficiency against motile aeromonads in terms of protection has not been satisfactory because effective and reproducible challenge in experimental laboratory or field trials has been a difficult task [43].

In conclusion, the results presented here demonstrate that DNA vaccination of spotted sand bass, *P. maculatofasciatus*, with the major OMP-encoding genes from *A. veronii*, failed to induce a significant humoral immune response but conferred partial protection against *A. veronii* experimental infection. Further vaccine designs and vaccination trials should focus on improving the levels of expression and antigenicity of these DNA vaccines. Two main factors are recommended to take into consideration: (1) codon usage optimization of the genes for efficient translation and stability of the mRNA; and (2)
inclusion of a strong heterologous signal sequence to enhance antigen secretion. To the best of our knowledge this study represents the first report of fish DNA vaccination with genes encoding for bacterial OMPs.

Acknowledgements

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