Fish & Shellfish Immunology 33 (2012) 1303–1310

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Construction and comparative study of monovalent and multivalent DNA vaccines against *Streptococcus iniae*

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ARTICLE INFO

Article history:
Received 28 January 2012
Received in revised form
28 September 2012
Accepted 1 October 2012
Available online 11 October 2012

Keywords:
Streptococcus iniae
Paralichthys olivaceus
DNA vaccine
Cross-serotype protection

ABSTRACT

Streptococcus iniae is an important fish pathogen with a broad host range that includes both marine and freshwater fish species. With an aim to develop effective vaccines against *S. iniae*, we in this study constructed three monovalent DNA vaccines, i.e., pSagF, pSagG, and pSagI, based on *sagF*, *G*, and *I*, which are components of the streptolysin S cluster. The immunoprotective potentials of these vaccines were examined in a model of Japanese flounder (*Paralichthys olivaceus*). The results showed that following intramuscular administration, the vaccine plasmids were transported to spleen, kidney, and liver, where the vaccine-encoding transgenes were expressed. Immunocolloidal gold electron microscopy detected production of the vaccine protein in fish vaccinated with each of the vaccine plasmids. Following lethal-dose *S. iniae* challenge, pSagF-, pSagG-, and pSagI-vaccinated fish exhibited relative percent of survival (RPS) rates of 78%, 65%, and 76% respectively. To examine whether multivalent vaccines composed of different combinations of monovalent vaccines would produce better protections, flounder were vaccinated with FG (pSagF plus pSagG), FI (pSagF plus pSagG), or FGI (pSagF plus pSagG and pSagI). Subsequent challenging study showed that the RPS rates of the fish vaccinated with the divalent and trivalent vaccines were 4%–17% and 13%–26% respectively higher than those of the fish vaccinated with the component monovalent vaccines. Furthermore, FGI exhibited a strong cross protection against both serotype I and serotype II *S. iniae*, apparently due to, as revealed by sequence analysis, the existence of highly conserved SagF, SagG, and SagI homologs in these serotypes. Immunological analysis showed that all vaccines induced (i) specific serum antibody production, (ii) enhanced complement-mediated bactericidal activity, and (iii) significant induction of a wide range of immune genes. However, the levels of gene expression and serum bactericidal activity induced by FGI were in general more potent than those induced by monovalent vaccines. Taken together, these results indicate that the DNA vaccines based on *sagF*, *G*, and *I*, especially when they are formulated as multivalent vaccines, are highly efficacious against *S. iniae* infection.

1. Introduction

Streptococcus iniae is a hemolytic, Gram-positive bacterium that has in the past decade been recognized as a severe pathogen of farmed fish. To date *S. iniae* infection has been documented in at least 27 species of marine and freshwater fish including hybrid striped bass, channel catfish [1], European sea bass [2,3], rainbow trout [4,5], barramundi [6], tilapia [2,7], rabbitfish [8], and Japanese flounder [9–12]. Fish infected by *S. iniae* develop streptococcosis, which in general is characterized by meningoencephalitis, systemic septicemia, and skin lesions [12,13]. Heavy economic losses due to endemic streptococcal outbreaks have been reported in counties such as the United States, Israel, Japan, China, and Singapore [14]. In addition to fish, *S. iniae* is also a zoonotic pathogen and known to cause soft tissue infections and sepsis in humans [15].

Prevention of streptococcosis in farmed fish through vaccination has been tried in Chile, Israel, Russia, and Spain [16,17]. The vaccines are primarily bacterins consisting of inactivated whole bacterial cells. In Israel, large scale application of vaccination with formalin-killed *S. iniae* proved to be successful for a few years until the emergence of new *S. iniae* variants with vaccine-resistant structures [18,19]. In addition to bacterins, molecular vaccines such as subunit vaccines [9,20,21], ghost bacterial vaccine [22], DNA vaccines [23], and attenuated live vaccines [24–26] have been developed in laboratories.

Previous studies have indicated that *S. iniae* possesses a cytolysin that is homologous to the streptolysin S of group A streptococcus

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http://dx.doi.org/10.1016/j.fsi.2012.10.004

and involved in local tissue necrosis and pathogenicity [27,28]. The streptolysin S locus is constituted by 9 genes named *sagA* to *I*, of which, *sagG* and *sagI* encode homologs of ATP-binding cassette (ABC)-type transporters [27]. In this study, we constructed DNA vaccines based on *sagF*, *G*, and *I* and examined in a Japanese flounder (*Paralichthys olivaceus*) model their protective potentials and immune response-inducing capacities as monovalent and multivalent vaccines.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. iniae SF1 (serotype I) is a pathogenic strain that had caused an epidemic in farmed flounder [9]. *S. iniae* 29177 (serotype II) was purchased from ATCC (American Type Culture Collection). Both strains were cultured in Todd-Hewitt broth (THB) at 30 °C. Cell cultures were maintained at 4 °C for short-term storage (1–3 days) or at −80 °C in culture medium containing 15% (v/v) glycerol for long-term storage.

2.2. Plasmid construction and preparation

The primers used in this study are listed in Table 1. To construct pSagF, pSagG, and pSagI, which express Myc-tagged SagF, His-tagged SagG, and His-tagged SagI respectively, *sagF*, *sagG*, and *sagI* were amplified by PCR with the primer pairs SagF-F1/SagF-R1, SagG-F1/SagG-R1, and SagI-F1/SagI-R1 (Table 1) respectively. The PCR products were ligated to the T-A cloning vector pBS-T (Tiangen, Beijing, China); the recombinant plasmids were digested with SmaI, and the *sagF*-, *sagG*, and *sagI*-containing fragments were retrieved and inserted into pCN3 [29] at the SmaI site.

Endotoxin-free plasmid DNA was prepared using EndoFree plasmid Kit (Tiangen, Beijing, China). The purity of the purified DNA was analyzed spectrophotometrically by measuring absorbance at $A_{260/280}$ and $A_{260/230}$. The integrity of the plasmid DNA was assessed by agarose gel electrophoresis.

2.3. Fish

Japanese flounder (*P. olivaceus*) (average 11.4 g) were purchased from a local fish farm (Haiyang, Qingdao, China) and acclimatized in the laboratory for two weeks before experimental manipulation. Fish were fed daily with commercial dry pellets and maintained at 22 °C in aerated seawater that was changed twice daily. Before experiments, fish (5% of each stock) were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen. No bacteria were detected from any of the examined tissues of the sampled fish. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) as described previously [30].

Table 1
Primers used in this study.

Primer	Sequences (5′ → 3′)[a]	PCR size (bp)
CNF1	CTTGCGTTTCTGATAGGCACCTA	
CNR1	TGCGGGCCTCTTCGCTATT	
SagF-F1	CCCGGGACCACCATGAAAATTATATTAGGACTGC (SmaI)	709
SagF-R1	CAGACCCGGGATGTTGTTCTTTAAAACTAATCAA (SmaI)	
SagF-F2	TATAGACCTGAAAAGCCAAAGC	183
SagF-R2	TAACCGATGCGACCAAACT	
SagG-F1	CCCGGGACCACCATGAGTTTTATCGAGATTCAAA (SmaI)	941
SagG-R1	GTCCCGGGATCTCTTAACTTCTTACCAGT (SmaI)	
SagG-F2	CTCTTTGGCTCACTTTATGGTC	130
SagG-R2	ACGCCTTTTCATTCCTCCC	
SagG-F3	CCCGGGATGGGCAATATTTATGGCTTAT (SmaI)	645
SagG-R3	GCTCCCGGGACCATGATCCATGATAAAA (SmaI)	
SagI-F1	CCCGGGACCACCATGAAAATTGGTGTATTAGATAAGGA (SmaI)	1008
SagI-R1	CGTCCCGGGTACTCTAGAAGGCTTTAATAACA (SmaI)	
SagI-F2	AGTTGCCTTTGCCATCCA	226
SagI-R2	CCATAAACAACCTGCTAACATCG	

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

2.4. Vaccination

To examine the vaccine potential of pSagF, pSagG, and pSagI, the vaccine plasmids were diluted in PBS to 150 μg/ml. Japanese flounder described above were divided randomly into five groups named A–E (40 fish/group) and injected intramuscularly (i.m.) with 100 μl of pSagF, pSagG, pSagI, pCN3, or PBS. At one month post-vaccination, 25 fish were removed from each group and challenged via intraperitoneal injection with 100 μl of *S. iniae* SF1 that had been cultured in THB to mid-logarithmic and resuspended in PBS to 1×10^7 CFU/ml. To examine the vaccine potential of FG, FI, and FGI, the vaccines were formulated as follows. To prepare FG and FI, pSagF, pSagG, and pSagI were diluted in PBS to 300 μg/ml; FG was formulated by mixing pSagF and pSagG at an equal volume; FI was similarly prepared by mixing pSagF and pSagI. To prepare FGI, pSagF, pSagG, and pSagI were diluted in PBS to 450 μg/ml, and FGI was obtained by mixing equal volumes of pSagF, pSagG, and pSagI. Flounder were divided randomly into five groups and injected i.m. with 100 μl of FGI, FG, FI, pCN3, and PBS respectively. The group size was 80 fish except that vaccinated with FGI, which was 120. At one and two months post-vaccination, 25 fish were removed from each group and challenged via intraperitoneal injection with 100 μl of *S. iniae* SF1 as described above. In addition, to examine the cross-serotype protection of FGI, 25 fish were removed from the FGI-vaccinated group and challenged with *S. iniae* 29177 that had been cultured in THB to mid-logarithmic and resuspended in PBS to 2×10^8 CFU/ml. For all vaccination trials, mortality was monitored over a period of 20 days after the challenge, and dying fish were randomly selected for the examination of bacterial recovery from liver, blood, kidney, and spleen as described previously [31]. Relative percent of survival (RPS) was calculated according to the following formula: RPS = {1 − (% mortality in vaccinated fish/% mortality in control fish)} × 100 [32]. All vaccination trials were repeated once, and the mean RPS rates were given in the results.

2.5. Detection of plasmid DNA in fish tissues

Muscle, kidney, liver, and spleen were taken from vaccinated fish at 7 days post-vaccination. DNA was extracted from the tissues with the TIANamp DNA Kit (Tiangen, Beijing, China) and used for PCR analysis with primers CNF1/CNR1 (Table 1), which are specific to the common backbone sequence of pSagF, pSagG, pSagI, and pCN3.

2.6. Detection of vaccine expression by reverse transcriptase-PCR (RT-PCR)

Muscle, spleen, liver, and kidney were taken from vaccinated fish at 7 days post-vaccination and used for total RNA extraction with RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out as described previously using α-tubulin RNA as an internal control [29,33]. The primer pairs used for RT-PCR analysis of *sagF*, *sagG*, and *sagI* were, respectively, SagF-F2/SagF-R2, SagG-F2/SagG-R2, and SagI-F2/SagI-R2 (Table 1).