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Short communication

Construction and characterization of a DNA vaccine encoding the SagH against *Streptococcus iniae*

Chunsheng Liu[a,b,1], Xiucong Hu[a,b,1], Zhenjie Cao[a,c], Yun Sun[a,b,*], Xiaojuan Chen[a], Zhengshi Zhang[a,c]

[a] State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, PR China
[b] Key Laboratory of Tropical Biological Resources of Ministry of Education, Hainan University, PR China
[c] Hainan Provincial Key Laboratory for Tropical Hydrobiology and Biotechnology, College of Marine Science, Hainan University, PR China

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ABSTRACT

Streptococcus iniae is an important aquaculture pathogen that is associated with disease outbreaks in wild and cultured fish species. Streptolysin S has been identified as an important virulence factor of *S. iniae*. With an aim to develop effective vaccines against *S. iniae* for Japanese flounder (*Paralichthys olivaceus*), in this study, we constructed a DNA vaccine based on the *sagH* gene, which belongs to the streptolysin S-associated gene cluster. In fish vaccinated with pSagH, the transcription of *sagH* was detected in tissues and SagH protein was also detected in the muscles of pSagH-vaccinated fish by immunohistochemistry. The immunoprotective effect of SagH showed that fish vaccinated with pSagH at one and two months exhibited a high relative percent survival (RPS) of 92.62% and 90.58% against *S. iniae* serotype I, respectively. In addition, SagH conferred strong cross protection against *S. iniae* serotype II and resulted in an RPS of 83.01% and 80.65% at one and two months, respectively. Compared to the control group, fish vaccinated with pSagH were able to induce much stronger respiratory burst activity, and higher titer of specific antibodies. The results of quantitative real-time PCR demonstrated that pSagH upregulated the expression of several immune genes that are possibly involved in both innate and adaptive immune responses. These results indicate that pSagH is a candidate DNA vaccine candidate against *S. iniae* serotype I and II infection in Japanese flounder in aquaculture.

1. Introduction

Streptococcus iniae belongs to the *Streptococcaceae* family and is a leading gram-positive pathogen associated with disease outbreaks in wild and cultured fish species worldwide [1,2]. By serological and biochemical analyses, *S. iniae* was divided into serotype I and II. Serotype I is positive for arginine dihydrolase (ADH) and ribose utilization, whereas serotype II is negative for both [3,4].

In recent years, the application of vaccines against streptococcosis has been attempted in aquaculture in Chile, Israel, Russia, and Spain [5–7]. Currently, many studies focus on the use of DNA vaccines in fish populations. DNA vaccines are plasmids that encode antigenic proteins derived from pathogenic microorganisms, specifically viruses and bacteria [8–10]. Many researchers have examined the virulence factors of *S. iniae* in an effort to reveal pathogenic mechanisms [11–13]. To date, several virulence factors, including capsular polysaccharides, surface proteins, and extracellular secreted products, have been identified as key factors that contribute to the virulence of *S. iniae* [13–15]. As evidenced by previous studies, the cytolysin possessed by *S. iniae* is a functional homologue of streptolysin S (SLS) from the group A *streptococcus* (GAS) [11] and can damage the cell membranes of certain cells such as erythrocytes, lymphocytes [16], neutrophils, and platelets [17], as well as certain tissue cultures and tumour cells [18].

The streptolysin S-associated gene cluster, which contains nine genes, has been identified as an important virulence factor of *S. iniae* [11]. In our previous study, we reported *S. iniae* DNA vaccines based on the sagE, F, G, and I genes of the streptolysin S cluster, which is known to be involved in the virulence of *S. iniae* [11,43–45]. The results showed that these DNA vaccines which expressed each of these genes could elicit effective immune response and induce highly protection against *S. iniae*. Thus, we wondered whether other components of the streptolysin S cluster also possess immunoprotective potential. To investigate this question, we constructed a DNA vaccine based on the *sagH* gene, and analysed the protection and immune response induced

* Corresponding author. College of Marine Sciences, Hainan University, 58 Renmin Avenue Haikou, 570228, PR China..
E-mail address: syshui207@126.com (Y. Sun).
[1] These authors contributed equally to this work.

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by SagH. The results reveal that our DNA vaccine is a useful candidate against *S. iniae* (both serotype I and II) and they provide insight into the protective mechanisms of teleost DNA vaccines.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Streptococcus iniae SF1 (serotype I) is a pathogenic bacterium isolated in our lab and *S. iniae* 29177 (serotype II) was purchased from ATCC (American Type Culture Collection) [19]. SF1 and 29177 cells were cultured in Todd-Hewitt broth (THB) at 28 °C. *Escherichia coli* DH5 alpha (Takara, Dalian, PR China) samples were cultured in Luria–Bertani (LB) broth at 37 °C. The cell cultures were maintained in culture medium containing 15% (v/v) glycerol at −80 °C for long-term storage.

2.2. Plasmid and strain construction

The amino acid sequence of SagH was analysed in the BLAST program and SignalP 3.0 was used for signal peptide search. SagH is composed of 375 residues and predicted to be a cytoplasmic membrane protein. It contains a signal peptide (residues 1 to 38), and one predicted major domain (residues 111 to 340), which includes predicted antigenic epitopes (Fig. S1).

To construct DNA vaccine (pSagH), a pair of primers (SagHF1/SagHR1) was used to amplify *sagH* without signal peptide (Table S1) with *S. iniae* SF1 (serotype I) genome DNA as a template. Briefly, the PCR product was ligated into the pEASY®-T1 Simple Cloning Vector (Transgen, Beijing, China); then the recombined plasmid was digested by restriction enzyme *Sma*I and inserted into pCN3 [10] at the *Sma*I site and was transformed to *E. coli* DH5 alpha. After ampicillin-resistance screening and restriction, the clone was checked with PCR primers (CNF1/SagHR1). Thus, pSagH, which expresses a His-tag, was constructed. The plasmids were prepared by the EndoFree plasmid Kit (Tiangen, Dalian, China). The selective plasmid DNA was assessed by agarose gel electrophoresis to exam the integrity, and the purity was measured spectrophotometrically using the $A_{260/280}$ and $A_{260/230}$ absorbances.

To construct pETSagH2, the primers SagHF2/SagHR2 (Table S1) were used to amplify the predicted major domain of *sagH* (residues 111 to 340). The digestion products were ligated into the recombined plasmid, digested with NdeI/XhoI and inserted into pET258 [10]. Subsequently, BL21 (DE3) was transformed with pETSagH2, and recombinant SagH2 (rSagH2) was purified from the transformant using nickel-nitrilotriacetic acid columns according to the manufacturer. The purified protein was checked by SDS-PAGE and visualized after staining with Coomassie brilliant blue R-250.

2.3. Vaccination

Japanese flounder were purchased from a commercial fish farm in Shandong province, China, and acclimated in the laboratory environment for one week. Fish were maintained in 530 L tanks at 20 °C containing sand filtered, activated carbon-absorbed, and aerated seawater that was changed twice daily. The density of the fish was maintained at less than 150 fish/tank. Fish were fed daily with commercial dry pellets (purchased from Shandong Sheng-suo Fish Feed Research Center, Shandong, China) at 0.15 g/day. Before the experiments, 7 fish were randomly selected from each group to confirm that they were in fact free of bacterial pathogen. Plasmids were diluted with PBS to 150 μg/ml. For vaccination with the DNA vaccine, fish (average weight, 13.2 ± 0.7 g) were randomly divided into three groups, designated A, B and C (130 fish/group), and injected intramuscularly (i.m.) above the lateral line on a fish with 100 μl pSagH, pCN3, and PBS control, respectively. The fish bacterial pathogens were cultured at an OD_{600} of approximately 0.8 and then resuspended in PBS to a concentration equivalent to an approximately LD50 dose (SF1: 5.3×10^5 bacteria/fish; 29177: 6.7×10^4 bacteria/fish), respectively. To examine whether SagH could induce cross protection against *S. iniae* serotype I SF1 and serotype II 29177, 25 fish per group were challenged with 100 μl *S. iniae* SF1 (10^7 CFU/ml) via intraperitoneal (i.p.) injection, and 25 fish per group were challenged with 100 μl *S. iniae* 29177 (10^6 CFU/ml) via i.p. injection at one month post-vaccination (p.v.), separately. A second challenge two months p.v. was performed in exactly the same way as the one-month p.v. challenge. For all vaccinations, the mortality rate of the fish was monitored for two weeks. Among the fish that died during the study period, only SF1 or 29177 was isolated from the spleen, kidney, or liver of any fish. All vaccination trials were repeated in one subsequent replication, and RPS values are reported here as the mean mortality results. Relative percent survival (RPS) was determined as follows: RPS = 100*(1 - (% mortality in vaccinated fish/% mortality in control fish)).

2.4. Examination of plasmid DNA and SagH expression

At 7 days p.v., the spleen, muscle, and kidney were collected from the vaccinated fish (average weight, 13.2 ± 0.7 g). To assess the plasmid, DNA was extracted from the fish tissues using a Tissue DNA Kit (Promega, Beijing, China). PCR analysis was then performed using the primer pairs SagHF3/CNR1 (Table S1). To detect vaccine gene transcription, the total RNA was extracted from the various tissues with the MiniBEST Universal RNA Extraction Kit (Promega, Beijing, China) using the primers SagHF3/SagHR3 following the manufacturer's instructions (Table S1). The SagH vaccine was detected at the protein level by immunocolloidal gold electron microscopy as reported previously [10]. Briefly, the samples were labeled first with mouse anti-His monoclonal antibody (Tiangen, Beijing, PR China) and then with gold-labeled goat anti-mouse IgG (Bios, Beijing, PR China). After staining with uranyl acetate and lead citrate, the sections were observed with a transmission electron microscope (GEM-1200, GEOL, Japan).

2.5. Respiratory burst (RB) activity

Japanese flounder (average weight, 35.8 ± 1.3 g) were vaccinated with 100 μl pSagH, pCN3 (300 μg/ml), or PBS as described above. Head kidney (HK) macrophages were isolated from vaccinated fish 1-, 2-, 4-, 7-, 14-, 21-, and 28-days p.v. and cultured in L-15 medium (Thermo Scientific HyClone, Beijing, China) as described by Chung and Secombes [20]. To determine the respiratory burst activity of HK macrophages, a nitroblue tetrazolium (NBT, Sigma Aldrich) assay was performed. Briefly, 2×10^5 of macrophages were added to a 96-well microplate with 100 μl of 1 mg/ml nitroblue tetrazolium (Sangon, Shanghai, China). After incubation at 25 °C for 2 h, the reaction was stopped by adding 100% methanol. After washing the plate with 70% methanol, 100 μl of 2 M KOH and 120 μl of DMSO (Sangon, Shanghai, China) were used to solubilize the reduced formazan. The spectrophotometric result was measured at 630 nm, and KOH/DMSO was used as the blank control.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Sera were collected at 1, 2, 3, 4, 5, 6, 7, and 8 weeks p.v. (five fish/time point) from fish (average weight, 13.2 ± 0.7 g) vaccinated with pSagH, pCN3 or PBS and then fish were returned to the tank. To detect antibody titres, ELISA was performed as described previously [21]. Briefly, a 96-well ELISA plate was coated with recombinant SagH2 (rSagH2). Then, the sera were diluted 20-fold in PBST (0.1% Tween-20 in PBS) and added to the wells in triplicate. Next, the plates were incubated at 37 °C for 2 h. Then, rat anti-Japanese flounder IgM antibodies which were prepared in our lab were added after washing the plate with PBST [10]. After incubating and washing as described above,

goat anti-rat IgG HRP (Abcam, England) was added to the plate. The TMB Kit (Solarbio, Beijing, China) was used for colour development, and the plate was read at 450 nm with a microplate reader.

2.7. qRT-PCR

Japanese flounder were vaccinated with pSagH at one month and challenged with SF1 as described above. Spleen was collected from three vaccinated and control fish (average weight, 13.2 ± 0.7 g) at 24 h post-challenge. RNA extraction and cDNA synthesis were performed as described in a previous study [22]. The primers used are listed in Table S1. qRT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) with α-tubulin as an internal reference as described previously [42]. The assay was performed three times. All data are given in terms of relative mRNA, expressed as means plus or minus standard errors of the means (SE).

2.8. Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance analysis was carried out by one-way ANOVA. When the ANOVA identified differences among groups, multiple comparisons were performed by the Tukey HSD test. In all analyses, the significance level was defined as $P < 0.05$.

3. Results

3.1. Expression of SagH in different fish tissues following vaccination

Japanese flounder were vaccinated with pSagH, pCN3, or PBS. The results showed that both plasmid DNA and transcripts of *sagH* were detected in the spleen, muscle, and kidney of pSagH-injected fish 7 days p.v. by PCR and RT-PCR analyses (Fig. S2). In contrast, no *sagH* transcription was detected in the pCN- (Fig. S2) or PBS-vaccinated fish (data not shown). To examine the expression of SagH protein in the vaccinated fish, immunocolloidal gold electron microscopy analysis was performed, which revealed the production of SagH protein in the muscle tissue of pSagH-vaccinated fish but not in that of pCN3-vaccinated fish (Fig. 1) or PBS-vaccinated fish (data not shown).

3.2. Respiratory burst (RB) activity

Respiratory burst activity showed that macrophages from the pSagH-vaccinated group exhibited significantly enhanced respiratory burst activity ($P < 0.05$) relative to that of macrophages from the pCN3- or PBS-vaccinated group at 4, 7, and 14 days p.v. (Fig. 2).

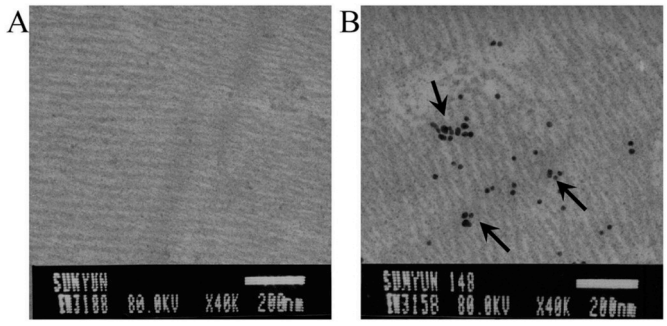

Fig. 1. Production of vaccine proteins in the vaccinated fish. Muscle tissues were taken from Japanese flounder vaccinated with pCN3 (A) and pSagH (B) at 7 days post-vaccination and used for immunohistochemistry with gold-labeled antibodies against His. Arrows indicate gold particles. Bar = 200 nm.

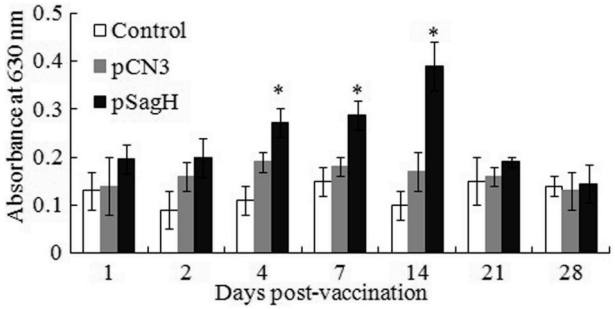

Fig. 2. Effect of pSagH vaccination on respiratory burst activity in head kidney macrophages. Japanese flounder were vaccinated with pSagH, pCN3 or PBS (Control), and head kidney macrophages were collected and used for the analysis of respiratory burst activity. Data are presented as means ± SE ($N = 3$). *$P < 0.05$.

3.3. Serum antibody response induced by pSagH

Recombinant SagH2 (rSagH2) was purified and assessed by SDS-PAGE (Fig. S3). ELISA analysis was performed to evaluate the antibodies. The serum from fish vaccinated with pSagH contained antibodies against the protein. The specific serum antibodies with rSagH2 appeared at 2 weeks p.v. and persisted until at least 8 weeks p.v.; furthermore, the maximum serum titre occurred at 4 weeks p.v. (Fig. 3). No specific serum antibody production was detected in fish vaccinated with pCN3 or PBS.

3.4. Immunoprotective effect of SagH as a DNA vaccine against S. iniae challenge

One or two months p.v., the fish vaccinated with pSagH were challenged with *S. iniae* SF1 or *S. iniae* 29177, and mortality was monitored for 15 days. When challenged with *S. iniae* SF1 at one month p.v., the mean accumulated mortalities of pSagH-, pCN3-, and PBS-vaccinated fish were 6.0%, 76.0%, and 82.0%, respectively (Table 1 and Fig. S4). When challenged with *S. iniae* SF1 at two months p.v., the mean accumulated mortalities of pSagH-, pCN3-, and PBS-vaccinated fish were 8.0%, 80.0%, and 86.0%, respectively (Table 1 and Fig. S4). These correspond to the RPS rates of 92.62% and 90.58% at one and two months p.v. for pSagH-vaccinated fish.

To examine the cross protection against *S. iniae* 29177, the vaccinated fish were challenged with *S. iniae* 29177 at one month p.v. The results showed that the mean accumulated mortality of pSagH-, pCN3-, and PBS-vaccinated fish were 12.0%, 66.0%, and 70.0%, respectively (Table 1 and Fig. S4). When challenged with *S. iniae* 29177 at two months, the mean accumulated mortalities of pSagH-, pCN3-, and PBS-vaccinated fish were 14.0%, 68.0%, and 72.0%, respectively. Hence,

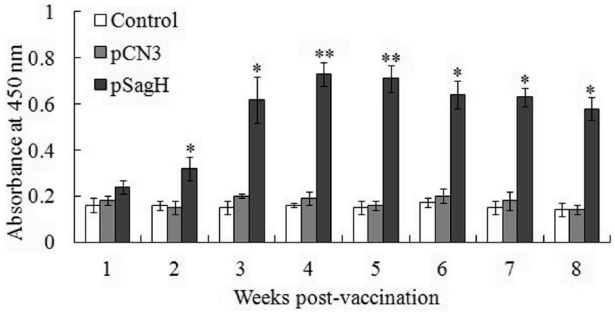

Fig. 3. Serum antibody production in the vaccinated fish. Japanese flounder were vaccinated with pSagH, pCN3 or PBS (Control), respectively. Sera were collected from the fish at 1–8 weeks post-vaccination, and serum antibodies against recombinant SagH2 were determined by ELISA. Data are presented as means ± SE ($N = 3$). *$P < 0.05$; **$P < 0.01$.

Table 1
Summary of the vaccination results.

Vaccine	Challenging strain	Challenging time	Cumulative mortality (%)		RPS[b] (%)
			I[a]	II[a]	
Control	SF1 (serotype I)	1 month	84	80	
pCN3	SF1	1 month	80	72	7.38
pSagH	SF1	1 month	4	8	92.62
Control	SF1	2 months	84	88	
pCN3	SF1	2 months	80	80	6.93
pSagH	SF1	2 months	12	4	90.58
Control	29177 (serotype II)	1 month	68	72	
pCN3	29177	1 month	64	68	5.72
pSagH	29177	1 month	8	16	83.01
Control	29177	2 months	68	76	
pCN3	29177	2 months	64	72	5.57
pSagH	29177	2 months	12	16	80.65

[a] I and II represent the results of two vaccination trials and 25 fish/group.
[b] RPS, relative percentage of survival; in each case, the RPS rate is the mean of two vaccination trials.

the RPS of the pSagH-vaccinated fish against *S. iniae* 29177 was 83.01% and 80.65% relative to the PBS fish at one and two months p.v., respectively (Table 1 and Fig. S4).

3.5. Expression of immune-related genes

To examine the expression of immune-related genes induced by pSagH, qRT-PCR was carried out. Relative to vaccination with pCN3, vaccination with pSagH led to significant ($P < 0.01$ or $P < 0.05$) upregulation of the transcription of the following genes: interleukin 1β (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), tumour necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), interferon-induced Mx protein (Mx), NK cell enhancing factor (NKEF), transforming growth factor β (TGF β), complement C3, immunoglobulin M (IgM), D (IgD) and T (IgT), major histocompatibility complex (MHC) class Iα and class IIα, CD40, and CD8α (Fig. 4).

4. Discussion

Streptococcus iniae infection has been documented in at least 27 marine and freshwater fish species [2,23,24]. There are two serotypes of *S. iniae* [3,4]. Both of them were associated with disease outbreaks. In this study, we constructed a DNA vaccine based on the *sagH* gene, which is one of the genes of the streptolysin S gene cluster in *S. iniae*. And we examined the vaccine potential against both *S. iniae* serotype I and II in Japanese flounder in aquaculture.

In the aquaculture field, vaccination is generally effective as a preventive tool in the heath management of aquatic organisms and as a tool to control the diffusion of infectious diseases in aquaculture habitats [25]. The stability and distribution of DNA vaccines following vaccination have been studied by many research groups in various animal models [26,27]. In this study, SagH proteins and mRNA could be detected in multiple tissues 7 days after vaccinating with pSagH of the flounder, indicating the successful expression of the DNA plasmids. After a one-month vaccination period, the fish were infected with *S. iniae* SF1 to examine the protective effects of the DNA vaccine. Relative to fish in the PBS group, fish vaccinated with pSagH exhibited an RPS of 92.62%, suggesting that SagH induced protective immunity against *S. iniae* SF1. At two months p.v., RPS was 90.58%, comparable to the one-month level, which suggested that the protective effects of SagH persisted without significant decline for two months. We next focused our investigation on the cross-serotype protection of DNA vaccines against *S. iniae* 29177. The results showed that pSagH had good cross-serotype protection against *S. iniae* 29177, with RPS rates of 83.01% and 80.65% at one and two months p.v., respectively. This phenomenon is likely attributable to the presence of common antigens that were largely conserved (99.2% identity) in *S. iniae* SF1 and *S. iniae* 29177 throughout the vaccination process. As evidenced in previous studies, the SagG, SagH, and SagI proteins of *S. iniae* are predicted to encode ATP binding cassette-type transport systems, and SagE is predicted to be a cytoplasmic membrane protein [11,28,29]. In our previous study, we found SagE was a transmembrane protein and with one major extracellular region named ECR. The results showed that ECR was main immunoprotective domain of SagE [45]. Furthermore, the relationships between haemolytic activity and *sag* genes have been explored in many studies [11,14,30]. These findings are in line with our results and confirm that the *sag* genes play a critical role in *S. iniae* infection.

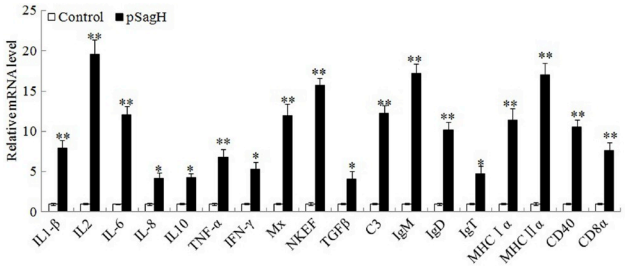

Fig. 4. Expression of immune genes in the vaccinated fish. Japanese flounder were vaccinated with pSagH, or pCN3 (Control). Spleen was taken from the fish at 24 h post-challenge. Total RNA was extracted from the spleen and used for quantitative RT-PCR. For each gene, the mRNA level of the control fish was set as 1. Data are presented as means ± SE ($N = 3$). *$P < 0.05$; **$P < 0.01$.

Previous studies have shown that DNA vaccines are able to induce both innate and adaptive immunity, lending them distinct advantage over other types of vaccines [26,31]. In our study, compared to control fish, fish vaccinated with pSagH showed the strongest respiratory burst activity in HK macrophages occurred at 14 days p.v., suggesting that pSagH can induce the activation of macrophages. In line with this result, Yi et al. also found that a subunit vaccine for Streptococcus agalactiae could induced higher respiratory burst than control at three weeks p.v. and declined at four weeks p.v. [46]. In addition to activating the innate immune response, DNA vaccines have been found to provoke both humoural and cellular immunity in mammals and fish [32–34]. In our study, qRT-PCR analysis showed more than 4-fold increases in the expressions of the examined genes encoding for IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-r, Mx, NKEF, TGF β, C3, IgM, IgD, IgT, MHCIα, MHCIIα, CD40, and CD8α. Previous studies showed that some anti-inflammatory cytokines, such as IL-2, IL-10, IFN-r, and TGF β, co-stimulate T-cells and B cells, which are involved in opsonization, inflammation and the production of immune stimulating factors [35–37]. Furthermore, adaptive immunoregulators influence anti-inflammatory cytokine production, which in turn may influence the activation of antigen-specific immune responses [38,39]. To assess the elevated expression of IgM, an ELISA assay was performed to detect the specific serum antibodies induced by pSagH. ELISA revealed that SagH antibodies were present at 2 weeks and persisted as long as 8 weeks p.v. Many previous studies have shown that antibody-mediated immune responses play an important role in protection against *S. iniae* infection [40,41]. The adaptive immunity usually has a late response and long duration, and the specific serum antibodies last long time [47]. Similar to our results, Pasnik et al. (2005) found that the antibodie titer of Mycobacterium marinum Ag85A were maintained increased level after 70 days p.v. [48]. In our experiment, SagH fulfilled this role. All of these data showed a dynamic interaction between innate and adaptive immunity, suggesting that SagH expression in vivo stimulated both innate and adaptive immunity in fish.

In conclusion, the results of this study demonstrate that pSagH, a DNA vaccine expressing one gene of the streptolysin S gene cluster, can induce the protection in Japanese flounder against both *S. iniae*

serotype I and II infections, which is possibly because of its ability to elicit both innate and adaptive immunity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.045.

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