Thesis for the degree of Master of Science

Generation of genetically engineered M13 bacteriophage for delivery of heterologous DNA and protein in fish

by

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August, 2023

어류에 외래 DNA 및 단백질 전달을 위한 유전적으로 엔지니어링된 M13 박테리오파지의 제작

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by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in Department of Aquatic Life Medicine, The Graduate School, Pukyong National University

August 2023

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August 18, 2023

Contents

List of Tables

List of Figures

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Abstract

The M13 bacteriophage is a virus that explicitly infects *Escherichia coli* which exhibits an F pilus. It is composed of a simple structure with five different coat proteins surrounding its genome. Since the development of phage display technology that enables the display of foreign peptides/proteins on phage surfaces, a variety of applications ranging from medicine to nanomaterials have attempted to utilize phages. Especially, studies about using phage as a delivery tool for drugs and protective antigens have been getting much attention recently. In this study, a recombinant phage was generated to deliver foreign DNA and proteins via genetic engineering.

The foreign DNA delivered via the phage was CpG-ODN 1668, which was chosen because of its protective efficacy against pathogens

including scuticociliate and *Edwardsiella tarda* reported in olive flounder. The phage was administered by intraperitoneal injection, and the head kidney of the olive flounder was removed 48 h after injection to confirm the activated level of the head kidney phagocytes via chemiluminescence assay. The stimulatory CpG motif harboring phage administered group showed the highest CL intensity compared to other groups.

The delivery of foreign antigens via phage surfaces is based on the phage display technology. With the existing system, the size of the molecule that can be expressed on the major coat protein of the M13 phage is limited to 15aa. By applying the SpyCatcher/SpyTag system, which enables the decoration of virus-like particles with antigens of diverse sizes, we aimed to display foreign antigens that cannot be displayed on the major coat protein with the existing system.

The conjugation reaction between SpyTag displaying phage on the major coat protein and SpyCatcher-fused protein was confirmed by SDS-PAGE and Western blotting, and the protein displaying phage administered group showed a higher antibody titer than other groups in ELISA. Unlike subunit vaccines, which require the addition of an adjuvant to induce a sufficient immune response, phages do not require the addition of an adjuvant as a self-adjuvanting agent. However, to use phages as an antigen delivery tool in vaccine development, it is necessary to confirm the relationship between antibody production and protection efficacy. If so, we can expect the use of phages as versatile delivery tools.

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초록

M13 박테리오파지는 F pili를 갖는 Escherichia coli에 특이적으로 감염되는 바이러스로, 자신의 유전자와 그를 감싸는 5개의 껍질 단백질로 구성된 매우 간단한 구조로 이뤄져 있다. Genetic engineering을 통해 파지 표면에 원하는 물질을 발현시킬 수 있는 phage display 기술이 개발된 이래, 주로 균 감염에 대해 항생제를 대체할 목적으로 파지를 연구해 왔던 과거와는 달리, 현재는 의약학뿐 아니라 나노소재 분야에 걸친 광범위한 분야에서 파지를 이용하고자 하는 시도가 활발히 이루어지고 있다. 특히 파지를 약물, 방어 항원 등에 대한 하나의 전달 도구로써 활용하고자 하는 연구가 최근 많은 관심을 받고 있는데, 본 연구에서는 어류에 외래 물질을 전달하기 위한 도구로써 파지를 이용하고자 genetic engineering을 통해 자기 유전자에 외래 DNA를 갖는 그리고 표면에 외래 단백질을 발현하는 재조합 파지를 제작하였다.

파지를 통해 전달하고자 한 외래 유전자로는 면역 자극 DNA로써

특히 넙치에서 스쿠티카충, *Edwardsiella tarda*와 같은 병원체에 대한 방어 효과가 보고된 바 있는 CpG-ODN 1668를 선정하였다. 파지는 복강주사를 통해 넙치에 전달하였고, 접종 48 hpi에 두신을 절취하여 식세포를 분리 및 선천 면역세포의 활성화 수준을 확인하고자 Chemiluminescence(CL) assay를 진행하였다. 그 결과, 면역 자극 CpG motif를 갖는 파지를 접종한 그룹에서 가장 높은 CL intensity를 나타내는 것을 확인할 수 있었다.

파지 표면에 외래 물질의 발현을 통한 전달은 phage display 기술을 기반으로 하는데, 기존 시스템으로는 파지 표면에 타겟 물질을 많은 수로 발현시키고자 할 때 주로 사용되는 8번 coat protein(p8)에 발현할 수 있는 물질의 크기가 최대 15개 아미노산으로, display 할 수 있는 물질의 크기가 상당히 제한된다. 본 연구에서는 파지와 같이 하나의 전달 도구로써 광범위하게 사용되는 Virus Like Particles(VLPs) 표면을 다양한 크기의 항원으로 장식하는 것을 가능하게 한 SpyCatcher/SpyTag system의 적용을 통해 기존의 파지 디스플레이 시스템으로는 display가 불가능한 크기의 단백질을 p8에 display 하고자 했다. SDS-PAGE 및 Western blot assay에서 conjugation band의 detection을 통해 SpyCatcher/SpyTag 사이의 conjugation reaction이 진행되었음을 확인하였고, 나아가 단백질을 display 하는 파지를 무지개송어에 적용하였을 때 ELISA assay에서 단백질만 접종한 그룹보다 높은 항체가를 나타내는 것을 확인할 수 있었다. 충분한 면역반응 유도를 위해 면역증강제의 첨가가 필수적인 재조합 단백질 백신과 달리, 면역증강제 첨가 없이도 항체가 유도가 가능한 점은 전달 도구로써 파지가 갖는 큰 이점이라고 볼 수 있다. 파지를 항원 전달 도구로써 실제로 이용하기 위해서는 백신 실험을 추가적으로 진행하여 유도된 항체가가 병원체에 대한 방어와도

이어지는지를 확인할 필요가 있지만, 효과가 확인된다면 파지의 다양한 항원에 대한 전달 도구 또는 백신으로써의 활용을 기대할 수 있을 것으로 보인다.

I. Introduction

Bacteriophages, the most abundant organisms on Earth, are viruses that parasitize bacteria as their hosts (Bao et al. 2019). Because of their ability to kill their hosts, they have been used as a treatment against bacterial infection at the beginning of their discovery (Gibb, Hyman, and Schneider 2021). Moreover, the emergence of multidrug-resistant bacteria which has become a major social concern caused by the rise in antibiotic use has drawn more attention to phages for alternatives to antibiotics (Lin, Koskella, and Lin 2017).

Filamentous phages belonging to the *Inoviridae* family have unique properties that are non-lytic; they do not kill their host during replication (Rakonjac 2022). The filamentous phage has a thread-like shape composed of five different coat proteins (pIII, pVI, pVII, pVIII, and pIX) surrounding its genome (ssDNA) (Askora, Abdel-Haliem, and Yamada 2012). Among several inoviral species, the M13 bacteriophage specifically infects *Escherichia coli* strains that exhibit an F pilus (Sattar et al. 2015). The M13 phage has a relatively small genome, which makes it easy to manipulate, and has been applied in various fields, including drug delivery, vaccine development, and even in the nanomaterial field, to provide higher performance as a versatile nanoparticle (Vries et al. 2021; Palma 2023).

Smith et al. developed the phage display technique in which a foreign peptide or protein DNA fragment genetically fused to the phage

gene could be expressed in a fused form with phage coat protein without hindering the phage's replication ability and infectivity in 1985 (Smith 1985). The most frequently utilized coat proteins for displaying foreign peptides or proteins are the major coat protein pVIII, which consists of ~2700 copies per virion, and one of the minor coat proteins pIII, which comprises five copies per phage particle and plays a crucial role in the infection process via the F pilus (Sioud 2019; Samoylova et al. 2017; Palma 2023).

Researchers can choose coat proteins to use depending on the purpose and type of protein to be displayed (Ebrahimizadeh and Rajabibazl 2014). For short peptides and multivalent displays, pVIII is usually preferred and frequently used. For the display of peptides with more than 8 amino acids or proteins, it is appropriate to use pIII (Ebrahimizadeh and Rajabibazl 2014). The type of vector used for phage preparation affects the valency of the molecules displayed on the phage surface (Baek et al. 2002). When directly modifying the phage genome, we can achieve a multivalent display in which all coat proteins are expressed in the fusion form; however, in this case, the size of the fused protein is limited (Ebrahimizadeh and Rajabibazl 2014).

During the assembly of phage particles, all the coat proteins are buried in the inner membrane of the host cell before budding (Bao et al. 2019). Therefore, if the size of the fused molecule exceeds what the host cell can tolerate, it can be a burden for the host and a virion with a deficiency in its replication ability and infectivity could be produced (Loh, Kuhn, and Leptihn 2019).

Alternatively, a plasmid encoding only the recombinant coat protein gene called a phagemid, could be introduced to express larger proteins on the phage surface (Qi et al. 2012). In this system, all the other proteins needed for phage replication are provided by the helper phage, which has a deficient origin of replication; therefore, when phage particles are assembled, phagemids are preferentially encapsulated (Paschke 2006). However, the wild-type coat proteins provided by helper phages account for the majority, and only a small number of recombinant proteins are displayed through the phagemid system (Sidhu 2000).

As an alternative, indirect display can be carried out using phage-displaying specific peptides that can bind to the protein to be displayed (Vries et al. 2021). For indirect display, we applied the SpyTag/SpyCatcher system to the phage display technology. SpyTag is a short peptide of 13 amino acids that forms an immediate isopeptide bond with its protein partner, SpyCather (Hatlem et al. 2019). The reaction between them is specific, rapid, and irreversible; therefore, this powerful protein-ligation tool has been utilized in a variety of applications including vaccine development (Hatlem et al. 2019).

Virus-like particles (VLPs) are attractive tools for delivering immunostimulatory antigens against target pathogens. Initially, it was difficult to display the desired proteins of a certain size or larger on the surface of the VLPs; however, the SpyTag/SpyCatcher system broadened the size limitation by decorating the surface of the VLPs with a spy tag and then simply mixing it with the target protein linked

to the SpyCatcher (Thrane et al. 2016; Brune et al. 2016). Similarly, in the present study, we generated phage particles displaying the target protein on P8 by simply mixing SpyTag-displaying phage on P8 and SpyCatcher-fused protein. To determine the possibility of an efficient antigen delivery tool, the protein-displaying phage was intraperitoneally (IP) injected into rainbow trout, and a specific antibody titer was measured via ELISA.

In addition to their ability to display heterologous antigens on their surface by modifying their coat protein genes, antigens or drugs can also be delivered by inserting an expression cassette of interest into the phage genome or phagemid (Bao et al. 2019). Once phage particles enter the body and their genetic material is exposed to the environment, the antigen or drug encoded in the cassette is expressed in the desired form, similar to the DNA vaccine (Karimi et al. 2016). The delivered DNA is protected by the coat proteins of the phage and is therefore much more stable from degradation (Tada et al. 2017; Farr, Choi, and Lee 2013). Genetic material can also be delivered specifically to the target site by displaying ligands that the target cell can recognize on the phage surface (Farr, Choi, and Lee 2013; Karimi et al. 2016).

CpG-ODN is a short synthetic oligodeoxynucleotide containing an unmethylated CpG motif that stimulates the innate immune response by being recognized by pathogen recognition receptors (PRRs) as a danger signal (Krieg 1999). Between many kinds of developed CpG-ODNs, the protective effect of CpG-ODN 1668 belonging to class

B CpG ODN (CpG-B ODNs) has been demonstrated against fish pathogens including scuticociliate and *Edwardsiella tarda (E.tarda)* in olive flounder (Kang and Kim 2014; Lee et al. 2003). Because CpG-ODNs consist of single-stranded DNA, they are vulnerable to degradation by various nucleases within the body (Qu et al. 2017).

A method to modify their backbones was suggested to enhance the stability of CpG-ODNs. However, it was reported that when backbone-modified CpG-ODNs were administered at a high concentration, toxicity was induced (Givens, Geary, and Salem 2018; Ham et al. 2021). In addition, gene synthesis is costly, regardless of the length of the gene, making it inappropriate to adopt synthesized CpG-ODNs in aquaculture, and it has become necessary to develop a new strategy to deliver CpG-ODNs.

Alternatively, stimulatory CpG motifs can be delivered via plasmid DNA in the inserted form (Cornélie et al. 2004). A large amount of DNA can be obtained at a much lower cost than that of DNA synthesis. Kim et al. reported a higher survival rate in the challenge experiment against fish pathogens mentioned above when plasmids harbored multiple copies of CpG-ODN 1668 (Kang, Kim, and Kim 2014; Kang and Kim 2015). We assumed that immunostimulatory CpG motifs might be delivered much more safely by using a phage as a delivery vehicle.

In this study, a phagemid harboring 30 copies of CpG 1668 was constructed and the phage particles were amplified. To confirm the

stimulatory effect of the phage containing a specific CpG motif on the innate immunity of olive flounder, the phage was administered via intraperitoneal (IP) injection and a chemiluminescence (CL) assay was performed to analyze the effect of the phage on the innate immune response of the fish.

II. Materials & Methods

1. Bacterial and phage strains

E.coli DH5a strain used for plasmid DNA production and *E.coli* strain TG1 used for phage amplification were cultured at 37℃ in Luria-Bertani (LB, LPS solution) broth and 2x yeast extract tryptone (2xYT, KisanBio) medium, respectively. *Vibrio anguillarum* (*V. anguillarum*) II strain isolated from a moribund olive flounder in a natural outbreak of vibriosis was routinely cultured at 27 ℃ in LB medium. M13KO7 helper phage purchased from New England Biolabs (NEB) was used to amplify phagemid particles.

2. Delivery of immunostimulatory CpG-ODNs via genetically engineered phage

2.1. Construction of immunostimulatory CpG motif harboring phagemid

Phagemid harboring multiple copies of the immune-stimulatory CpG motif was constructed in a previous study. Briefly, 30 copies of the CpG ODN 1668 motif were inserted into the LITMUS 28i vector (NEB) and designated as pLit-CpG30. The sequence of CpG-ODN 1668 is 5'-TCCATGACGTTCCTGATGCT-3'. The phagemid sequence was confirmed by sequencing prior to use.

2.2. Preparation of electro-competent TG1 cell

A single colony of *E. coli* TG1 was picked and cultured in 2xYT broth overnight. The next day, 1/100 volume of overnight culture was inoculated into fresh 2xYT medium and incubated at 37°C, 210rpm until the OD600 value reached 0.4~0.6. Cultures in a baffled Erlenmeyer flask were then transferred to a centrifuge bottle and incubated on ice for 30 min. The cells were pelleted by centrifugation at 3500rpm, 4°C for 20 min. After discarding the supernatant, the bacterial pellet was resuspended in 1mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, Biosolution) and centrifuged under the same condition. After repeating the last two steps, the cells were diluted with 10% glycerol in 1mM HEPES and centrifuged at 4°C, 4000rpm for 20 min. Finally, after removing the supernatant, cells were resuspended in 10% glycerol, aliquoted into microcentrifuge tubes (Axyzen) and stored at -80°C until use.

2.3. Electroporation of the phagemid DNA

For phagemid particle production, phagemids were transformed into *E. coli* TG1 cells via electroporation. 80ul of electro-competent TG1 cells were mixed with 1ul of the phagemid vector, and the mixture was transferred to an ice-cold cuvette. Electroporation was performed at 2500V, 25ms using an Eporator® (Eppendorf), and the cells were immediately resuspended in pre-warmed SOC medium and recovered at 37°C, 210rpm for 1h 30 min. After recovery of the cells, they were spread onto an LB agar plate containing the appropriate antibiotic (100mg/ml of ampicillin). Positive clones were selected by colony PCR and used for further experiments.

2.4. Production of phagemid particles harboring stimulatory CpG ODN within its genome

To obtain phagemid particles harboring pLit-CpG30 or pLITMUS28i, a single colony of *E. coli* TG1 electroporated with each phagemid from an LB agar plate containing ampicillin (100ug/ml) was picked and grown overnight in LB medium containing ampicillin (100ug/ml) at 37°C 220rpm. The overnight culture was diluted 1:100 in fresh LB broth containing ampicillin (100ug/ml) and grown at 37°C with vigorous shaking until the optical density at 600 nm (OD600) reached 0.4-0.6. Cells were then infected with M13KO7 helper phage at > 1 x 10^11 pfu/mL, and kanamycin (50mg/ml) was added. Cultures were incubated for 30 min at room temperature without shaking, incubated for 30 min at 37 °C with vigorous shaking, centrifuged for 10 min at 3000rpm at 4°C, and the pellet was resuspended in 2xYT containing ampicillin (100mg/ml) and kanamycin (50mg/ml) for overnight incubation at 37°C, 220rpm.

After infection with the helper phage, the overnight culture was centrifuged for 10 min at 6000rpm at 4 °C, and the supernatants were transferred to a fresh tube. A 20% PEG 8000/2.5M NaCl solution was gently added to the supernatant at a ratio of 1 to 5 and mixed well by inverting the tubes. After 1 h or overnight incubation at 4 °C, phages were pelleted at 8000rpm, 4 °C for 10 min, and supernatants were discarded. The PEG precipitation step was repeated to increase the

purity of the phagemid particles. After resuspending the pelleted phage in phosphate-buffered saline (PBS) at a ratio of 1/10 volume of the initial culture, the remaining cell debris was removed by centrifugation at 6000rpm for 10 min at 4 °C. Supernatants were filtered with a 0.45um syringe filter (Advantec MFS) and transferred to a fresh tube. 1/5 volume of PEG/NaCl was added and incubated on ice for 1h after inverting the tubes. After incubation, phage particles were pelleted by centrifugation at 8000rpm, 10 min at 4 °C, and supernatants were discarded. Finally, the pelleted phage particles were resuspended in 1/100 volume of PBS and used as a stock solution. The pellet was resuspended in 10% glycerol in PBS for long-term storage and kept at -20℃ until use. The titer of phage particles was determined by counting phage plaques or colonies on the agar plate after infection with the *E. coli* TG1 strain.

2.5 Phage administration

Juvenile olive flounders (average length 14.3 cm, average weight 17.6 g) obtained from a local fish farm in Korea were used for the in vivo experiment. They were confirmed to be pathogen free before the experiment and divided into three groups consisting of three fish per group. Before injection, fish were anesthetized with MS-222 (Sigma) and intraperitoneally (IP) injected with 50ul of PBS, 1x10^10 pfu/50ul of phage harboring stimulatory CpG-ODNs phagemid (pLit-CpG30), or 1 x10^10 pfu/50ul of phage harboring unmodified phagemid (pLitmus28i). At 48h after injection, the head kidney was aseptically removed to conduct the chemiluminescence (CL) assay.

2.6. Chemiluminescence (CL) assay

The isolated head kidney was placed in a 100um cell strainer (SPL Life Sciences) and 1 ml of L-15 medium (L15, Sigma) supplemented with 1% anti-anti (Ab, WELGENE) and 10U heparin (Sigma). It was gently ground in a cell strainer by using a sterile syringe piston (SUNGSHIM). The separation of phagocytic cells was performed using SepMate density gradient centrifugation tubes (STEMCELL Technologies) according to the manufacturer's instructions. The cell suspension was carefully loaded into SepMate tubes containing 47% Percoll® (GE Healthcare) and centrifuged at 600 g, 4 ℃ for 30 min with natural breaking. Cells in the top layer were transferred to a fresh 5 ml round bottom tube by pipetting. Phagocytes were washed three times with L15 supplemented with 1% Ab and 10 U heparin by centrifugation at 3000rpm, 4 ℃ for 5 min. The viability of the cells was confirmed to be > 95% using the Trypan blue exclusion test. The number of cells was adjusted to 1 x 10^7 cells/ml. 40ul of cells added to each well of a white 96 well plate (SPL Life Sciences) and incubated with 70ul of luminol (Sigma) at RT for 10 min. Then, 30 ul of zymosan (Sigma) was treated to each well, and CL emission was measured for 60 min using a VICTOR 3 multilabel plate reader (PerkinElmer).

3. Delivery of heterologous antigen using phage display technology

3.1. Construction of SpyTag displaying phagemid on P8

To produce a recombinant phage particle displaying SpyTag on its major coat protein(P8), the pComb8 phagemid vector was purchased from Addgene and used as template. Briefly, 39bp of the SpyTag sequence was inserted between the pelB signal sequence and the p8 protein gene of the pComb8 vector using the Overlap Cloner kit (Elpis). The sequence of the constructed vector was confirmed by sequencing and the final construct was named pComb8-SpyTag. Primers used in this study are listed in Table 1. Primers were designed to have 15~20bp of the overlapped region for the ligation reaction using the Overlap Cloner kit, according to the manufacturer's instructions.

Table 1. Primers used for pComb8-SpyTag phagemid construction

Primers		Nucleotide sequence (5' to 3')
Fragment #1	pComb8_SpyTag_OC_F	ATGGTGGACGCCTACAAGCCGACGAAG GCTGAGGGTGACGATCCCGCAAAAGCG
	AmpR_504_R	CACGCTCGTCGTTTGGTATGGCTTC
Fragment #2	pComb8_SpyTag_OC_R	GTAGGCGTCCACCATCACGATGTGGG CCTGGGCCATGGCTGGTTGGGCAGCGA
	AmpR_514_F	CAAACGACGAGCGTGACACCACGATG

(Underlined sequences indicate overlapped sequences)

3.2. Preparation of the SpyTag displaying phage

To produce SpyTag-displaying phage on its major coat protein(p8-st), pComb8-SpyTag was electro-transformed into electrocompetent TG1 cells, and cells were plated on LB agar containing Ampicillin (100mg/ml). Transformants were selected using colony PCR and used for the recombinant phage particle production.

A single colony of *E. coli* TG1 containing pComb8-SpyTag was picked and grown in LB medium with ampicillin (100mg/ml) overnight at 37℃, 220rpm. The overnight culture was inoculated into fresh LB medium containing ampicillin (100mg/ml) at a dilution ratio of 1:100 and grown until the OD600 value reached 0.4-0.6. Then 1 x 10^11 pfu of M13KO7 helper phage and kanamycin (50mg/ml) were added to the culture and incubated at RT without agitation for 30 min. Further incubation was carried out in a shaking incubator at 37 ℃ for 30 min, and then the culture was centrifuged at 3000rpm, 4 ℃ for 10 min. After discarding the supernatant, the pellet was resuspended in 2xYT medium containing Ampicillin, Kanamycin, and 0.1mM IPTG, then incubated overnight at 27℃, 220rpm.

The overnight culture was centrifuged at 6000rpm at 4 ℃ for 10 min, and supernatants containing phage particles were transferred into fresh conical tubes. To precipitate phage particles, 1/5 volume of 20% PEG 8000/2.5M NaCl was added to the supernatant. The mixture was gently mixed by inversion and incubated on ice for 1h or O/N.

The precipitated phage was pelleted by centrifugation at 8000rpm, 4 ℃ for 10 min. Phage precipitation was performed twice to increase the purity of phage particles. The phage pellet was resuspended in 1/10 volume of PBS from the original culture and centrifuged at 6000rpm at 4 ℃ for 10min. The supernatant was transferred into fresh tubes after filtration using a 0.45um syringe filter, and then 1/5 volume of 20% PEG 8000/2.5M NaCl was added and mixed well by inverting. The mixture was incubated on ice for 1 h and then centrifuged at 8000rpm, 4℃ for 10 min. The supernatant was discarded, and the pellet was resuspended in PBS at 1/100 volume of the initial culture, or sterile glycerol was added for long-term storage at -20 ℃. The Titer of the phage particles was determined using a plaque assay.

3.3. Vector construction for expressing SpyCatcher fused protein

Green fluorescent protein (GFP) was used to confirm the conjugation reaction between p8-st and SpyCatcher-fused proteins. The expression vector for GFP linked to SpyCatcher was constructed in our previous study. Briefly, the cassette of GFP linked to SpyCatcher using a flexible linker was cloned into a pET28a+ vector and designated as pGFP_Sc. The sequence of the vector was confirmed before transformation into the *E. coli* BL21(DE3) strain for protein overexpression.

Based on this construct, other protein genes to be displayed were replaced with GFP. For *V. anguillarum* antigen *FlaB* display, genomic DNA of *V. anguillarum* was extracted using Geneall Exgene Clinic SV mini kit, and the FlaB gene of *V. anguillarum* was amplified by conventional PCR method with the specific primers listed in Table 2. Amplified PCR products were visualized on 0.7% agarose gel, extracted using the FavorPrep TM GEL/PCR Purification kit (Favorgen), and cloned into T easy vector (Promega). The concentration of the purified product was measured using an Eppendorf BioSpectrometer (Eppendorf). The cloned *FlaB* sequence was determined using an ABI 3730xl system (Macrogen Sequencing Service). Primers for the SpyCatcher-fused *FlaB* expression vector were designed based on the sequencing data.

All fragments of the pGFP_Spycatcher, except for the target protein site, were amplified and gel extracted as described above for the ligation reaction. Then, *FlaB* and the rest of the protein expression vector were ligated using the Elpis Overlap Cloner DNA cloning kit following the manufacturer's instructions. After transforming the ligated product into *E. coli* DH5a, the cells were plated on LB agar containing kanamycin (50mg/ml), and colonies harboring cloned vectors were confirmed by colony PCR. The final sequence of the cloned vector was verified by sequencing, and the final construct was named as p*FlaB*-SpyCatcher.

Table 2. Primers used for p*FlaB*-SpyCatcher protein expression vector construction

Primers	Nucleotide sequence (5' to 3')
For the *FlaB* gene of *V. anguillarum* cloning	
VA_*FlaB*_F	AATGAGGTGTACCGACGTGAG
VA_*FlaB*_R	TGGCAGTGAAAACACTGCTTT
For p*FlaB*-SpyCatcher construction	
VA_*FlaB*_pET28a_OC_F	ATGGGTCGCGGATCCGCAATTAATGTAAGACTAACGTGT
VA_*FlaB*_L_R	TCCGCTGCCACCACTCCCACCCAATAGACTAAGAGCTGCAGATG
Linker(GSGGSG)_OC_F	GGGAGTGGTGGCAGCGGA
pET28a_backbone_OC_R	GGATCCGCGACCCATTTGCTGTCCA

(Underlined sequences indicate overlapped sequences)

3.4. Recombinant protein expression and purification

A single colony of *E. coli* BL21(DE3) transformed with a protein expression vector was picked and grown in LB medium containing kanamycin (50mg/ml) at 37℃, 220rpm until the OD600 value reached between 0.4-0.6. Then, cells were induced with 0.1mM IPTG at 27℃, 220rpm for 5 h and harvested by centrifugation at 3000rpm, 4 ℃ for 10 min. The pellet was resuspended in binding buffer (45mM imidazole, 500mM NaCl, 20mM Tris, pH 7.9), and the cells were lysed by sonication at 30% sonication amplitude for 30 min. Cell debris was pelleted by centrifugation at 13000rpm, 4℃ for 10 min, and the supernatant was filtered through a 0.45um syringe filter and transferred to a fresh tube. Protein purification was performed using a Ni-NTA His-Bind Resin® (Novagen, USA). Purified protein in the elution buffer (1 M imidazole, 0.5M NaCl, 20mM Tris, pH7.9) was gradually transferred to PBS by ultrafiltration using an 10kDa Amicon® Ultra-15 Centrifugal Filters. The concentration of the purified protein was determined by the BCA assay.

3.5. SDS-PAGE and Western blotting

The protein samples were mixed with 5X sample buffer (Elpis) and boiled at 95 ℃ for 5 min. The samples were loaded on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and visualized by Coomassie blue staining.

For western blot analysis, proteins separated by SDS-PAGE were transferred onto the nitrocellulose membrane (Biorad). After blotting, the membrane was soaked in blocking buffer (3% BSA in TTBS; 150 mM NaCl, 10 mM Tris-HCl, pH7.5) for 2h at RT or overnight at 4 ℃. After three washes with TTBS (0.05% Tween 20 in TTBS, pH7.5), target proteins were probed using rabbit anti-GFP polyclonal antibody (1:1000, Abcam) or mouse anti 6xHis tag monoclonal antibody (1:500, Abcam) at RT for 2 h with rocking. The membrane was washed three times with TTBS for 10 min again, then incubated with goat anti-rabbit alkaline phosphatase-conjugated IgG or goat anti-mouse alkaline phosphatase-conjugated IgG (1:2000, Abcam). Finally, after washing the membrane with TTBS several times, nitroblue tetrazolium and 2-bromo-2-chloro-2-indoly phosphate (NBT/BCIP, KPL) substrate were applied to the membrane to visualize the target protein bands.

3.6. Conjugation reaction between Spytag displaying phage and Spycatcher fused protein

p8-st between 1 x 10^10-10^11 pfu/ml and 5uM of Spycatcher fused protein was used for the conjugation reaction. After thoroughly mixing the solutions containing phage and protein by pipetting, the mixture was incubated at RT with rocking for 6H. Then, 1/6 volume of 30% PEG-8000/3M NaCl solution was added, and the mixture was incubated on ice for 1 h to remove the unconjugated proteins. After 1 h, the mixture was centrifuged at 13000rpm, 4℃ for 20 min, and the phage pellet was resuspended in PBS. The conjugation reaction was confirmed by SDS-PAGE and western blotting.

3.7. Fish immunization

Juvenile rainbow trout (n= 104, average weight 45.86 g, average length 16.08 cm) obtained from a commercial fish farm in Korea were used for immunization. Fish were acclimated at 16 ℃ for a week before injection and were confirmed to be free from several fish pathogens, including *V. anguillarum*. Fish were divided into three groups (control, Spycatcher-fused protein, and conjugated phage) consisting of three fish per group. Before injection, fish were anesthetized with MS-222 and intraperitoneally (IP) injected with 100 ul PBS, 5uM/100ul of GFP-Sc, or 100 ul phage-protein conjugate. At three- and 5-weeks post-immunization, all fish from each group were anesthetized and bled to collect the serum needed to determine the specific antibody titer against GFP by ELISA.

3.8. ELISA

To quantify the GFP-specific antibody titer in trout serum, 100ul of purified GFP_Sc (5ug/well) in coating buffer (14 mmol/L sodium carbonate, 35mmol/L sodium bicarbonate, pH 9.6) was coated on a 96-well plate at 4℃ for 12h. After washing with 200ul of washing buffer (0.5% Tween 20 in PBS), each well was blocked with 200ul of blocking buffer (1% BSA in PBS) at 37℃ for 1h. After washing three times, 100ul of 1:10 diluted trout serum in dilution buffer (0.1% BSA in PBS) was added to each well and incubated at RT for 1h, followed by three washes. 100ul of anti-rainbow trout IgH monoclonal antibody (1:100, MyBioSource) was added and incubated for 1h at RT. After washing, 100ul of goat anti-mouse alkaline phosphatase IgG (1:1000, Abcam) was added to each well and incubated for 1h at RT. After three washing steps, 200ul of 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP, Sigma) solution was added, and the plate was incubated at RT for 30 min. The reaction was stopped by adding 50ul of stop solution (3M NaOH, then the absorbance was measured at 405nm using a VICTOR X3 Multilabel Plate Reader (Perkin Elmer)

3.9. Statistical Analysis

The Statistical analysis was conducted using GraphPad Prism version 9 software. ESLIA data were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests as a post-hoc test. Statistical significance was accepted at $p < 0.05$.

Ⅲ. Results

1. Delivery of immunostimulatory CpG-ODNs via genetically engineered phage

1.1. Chemiluminescence (CL) assay

To examine the effect of the immunostimulant delivered by phage on the innate immunity of the olive flounder, a phage harboring stimulatory CpG-ODNs or control phage was injected intraperitoneally, and the CL assay was conducted at 48h post-administration. The CpG-ODNs harboring phage-administered group showed a slightly higher respiratory burst activity than the other groups.

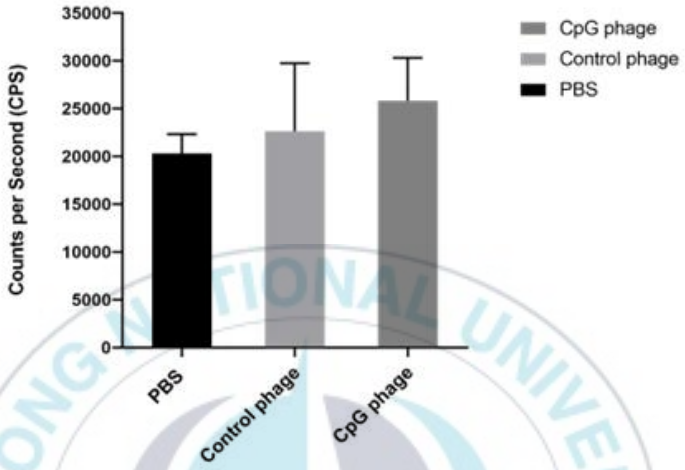

Figure 1. Effect of phage harboring immunostimulatory CpG-ODNs on the respiratory burst activity of olive flounder head kidney phagocytes in chemiluminesence (CL) assay.

2. Delivery of heterologous antigen using phage display technology

2.1. Expression and purification of GFP-SpyCatcher (GFP-Sc)

To produce the GFP-SpyCatcher (GFP-Sc) recombinant protein, *E. coli* BL21(DE3) transformed with pGFP-Sc was induced by 0.1mM IPTG at 27℃ for 5h. The size of approximately 45kDa band was successfully observed after protein purification and dialysis of GFP-Sc on SDS-PAGE and western blot analysis.

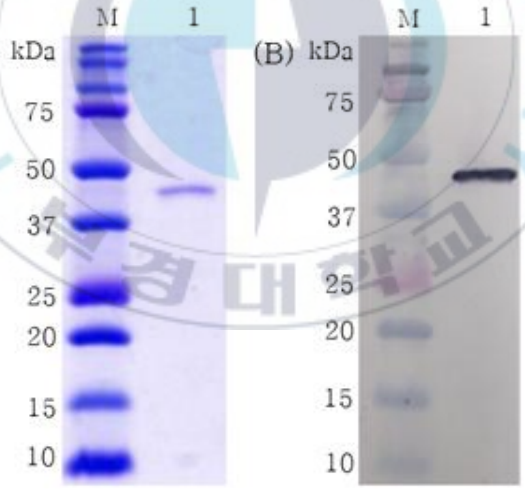

Figure 2. SDS-PAGE (A) and Western blot analysis (B) of the purified GFP-SpyCatcher (GFP-Sc). Rabbit anti-GFP antibody was used as the primary antibody for western blot analysis of purified GFP-Sc. Lane M: protein molecular weight marker, lane 1: purified GFP-Sc.

2.2. Conjugation reaction between p8-st and GFP-Sc

SpyTag-displaying phage and GFP fused to SpyCatcher were ligated under various conditions. The most robust conjugation efficiency was observed when 1x10^10 pfu/ml ≤ of phage and 5uM of protein were incubated under RT, 6H condition by western blot analysis. The approximate size of the conjugate between p8-st and GFP-Sc is 52kDa.

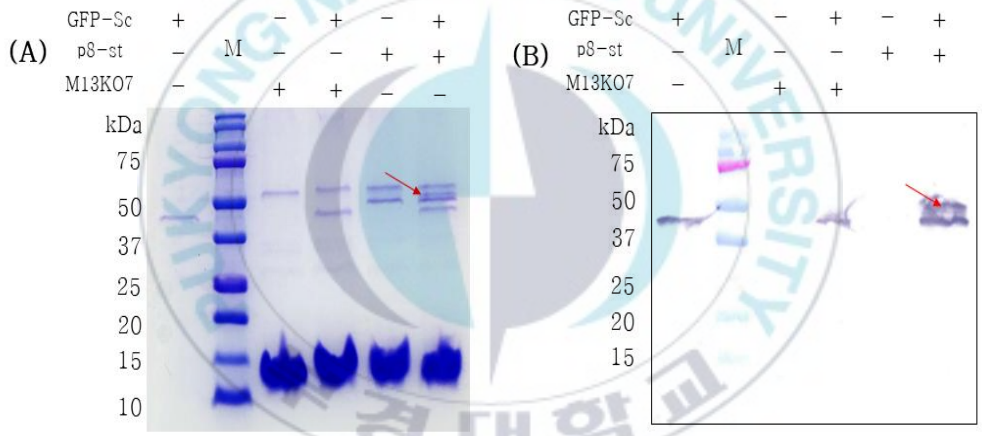

Figure 3. SDS-PAGE (A) and Western blot analysis of conjugation reaction between the p8-st and GFP-Sc. Rabbit anti-GFP polyclonal antibody was used as the primary antibody for the western blot analysis. The red arrow indicates the band corresponding to the ligated product between p8-st and GFP-Sc.

2.3. Specific serum antibody titer against GFP

There was no significant difference in serum antibody titer between GFP and phage-displaying GFP (phage-GFP) group at 3 weeks post-injection. However, at 5 weeks post-injection, the GFP and phage-GFP groups showed significant differences in serum antibody titers against GFP protein.

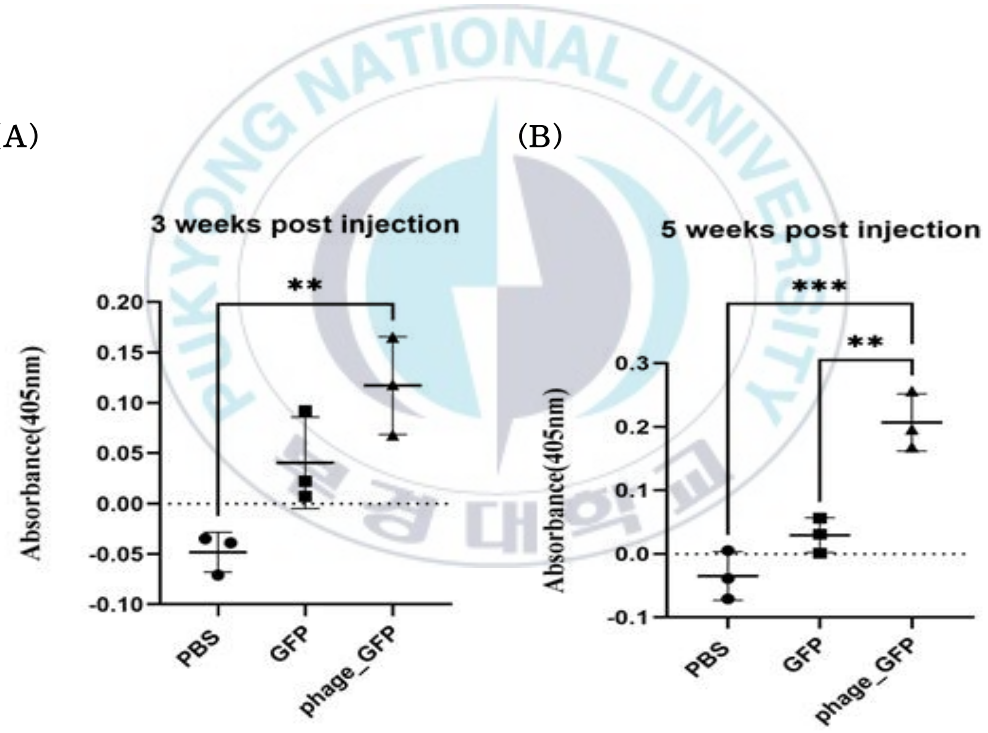

Figure 4. Juvenile rainbow trout were immunized with GFP_Sc (GFP) or phage-displaying GFP (phage-GFP). The control group was injected with only PBS. Asterisks represent statistical significance at $p<0.05$.

2.4. Expression and purification of *FlaB*-SpyCatcher (*FlaB*_Sc)

To express the recombinant *FlaB*-SpyCatcher (*FlaB*-Sc), *E. coli* BL21(DE3) transformed with p*FlaB*-Sc was induced by 0.1mM IPTG at 27℃ for 5h. The size of approximately 57kDa band was successfully observed after protein purification and dialysis of *FlaB*-Sc on SDS-PAGE and western blot analysis.

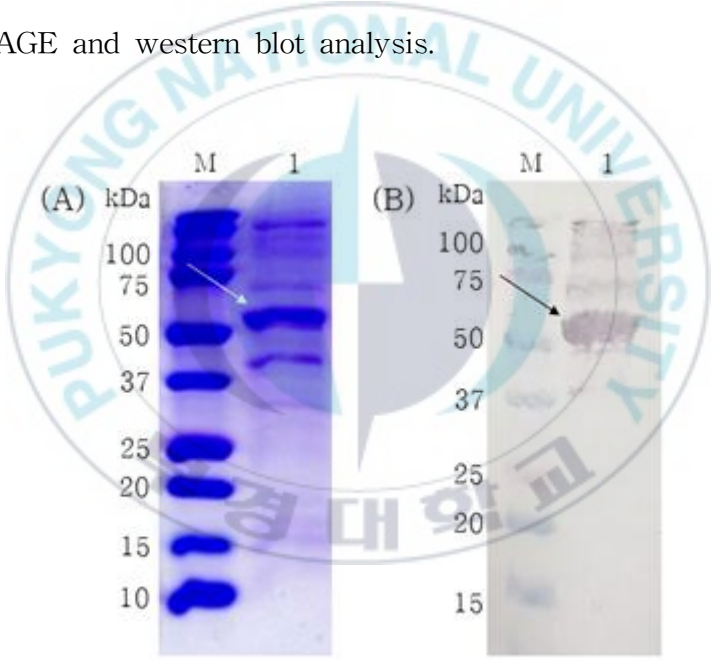

Figure 5. SDS-PAGE (A) and Western blot analysis (B) of the *FlaB*-SpyCatcher (*FlaB*-Sc). Mouse anti-6xHis tag antibody was used as the primary antibody for western blot analysis of purified *FlaB*-Sc. Lane M: protein molecular weight marker, lane 1: purified *FlaB*-Sc.

2.5. Conjugation reaction between p8-st and FlaB-Sc

SpyTag-displaying phage and SpyCatcher-fused FlaB were also ligated under various conditions, and the most robust conjugation efficiency was observed when 1 x 10^10 pfu/ml ≤ of phage and 5uM of protein were incubated under RT and 6H conditions on western blot analysis, similar to the p8-st and GFP-Sc conjugation reaction. The approximate size of the conjugate of p8-st and *FlaB*-Sc is 64kDa.

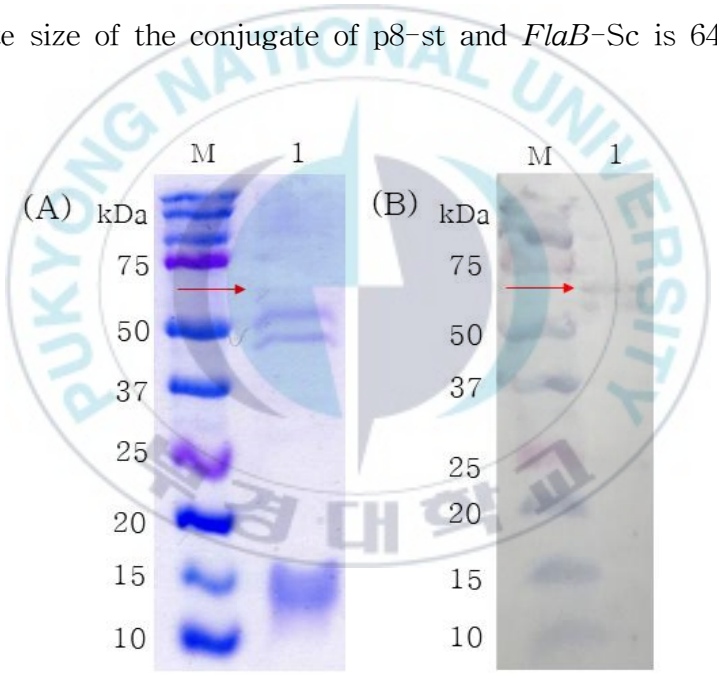

Figure 6. SDS-PAGE (A) and Western blot analysis (B) of the p8-st and *FlaB*-Sc conjugate. Mouse anti-6xHis tag monoclonal antibody was used as the primary antibody for western blot analysis. The red arrow indicates the band corresponding to the conjugated product between p8-st and *FlaB*-Sc.

Ⅳ. Discussion

In vaccine development, phages are gaining more attention as promising delivery tools because they are easy to manipulate, can be produced in large amounts at a relatively low cost compared to other vaccine platforms, and are known to have inherent immunogenicity ((Vries et al. 2021; Bao et al. 2019; Palma 2023). Furthermore, the structural features of the phage particles provide a display of the fused protein in a highly ordered and organized manner, which is known to trigger a strong and long-lasting immune response by enhancing the cross-linkage between B cell receptors (Hashiguchi et al. 2010). While there are unsolved issues regarding how phages are recognized by immune cells, phages are known to stimulate both arms of the immune system, unlike subunit vaccines, which mainly activate humoral immunity (Vries et al. 2021; González-Mora et al. 2020; Palma 2023). These attributes make phages a more attractive candidate for antigen delivery.

In phage display, it is frustrating to display polypeptides longer than 11 amino acids or protein on P8 of the M13 phage with the existing system (Ebrahimizadeh and Rajabibazl 2014; Loh, Kuhn, and Leptihn 2019). However, indirect display, which combines another method such as the SpyTag/SpyCatcher system, allows the display of longer peptides or proteins on P8 (Vries et al. 2021). Guilherme et al. demonstrated that a 200kDa kDa protein can be displayed on P3 (5

copies/virion) and P8 (~100 copies/virion) using the SpyTag/SpyCatcher conjugation method (Lima et al. 2022). Similarly, we confirmed the surface display of the protein, which was approximately 47kDa and 58kDa on P8, respectively.

Regarding the conjugation condition, incubating for 6H at RT showed the highest intensity in the western blot analysis. The titer of the phage and protein concentration also affected the conjugation efficiency between SpyTag and SpyCatcher. To observe the conjugate band via western blotting, the titer of the phage should be more than 1x10^10 pfu/100ul. Concerning the protein concentration, we observed a conjugate band at 700nM. However, the highest efficiency was detected when more than 5 μM protein was added.

Intrinsic adjuvanticity is a well-known feature of phages, recognized by immune cells that exhibit PRRs as a danger signal (Palma 2023). In particular, CpG motifs within the genome trigger immune priming by stimulating the Tall-like receptor 9 (TLR9) cascade (Hashiguchi et al. 2010). Therefore, protective antigens delivered via phage particles do not require the addition of other materials to induce sufficient antibody production, unlike subunit vaccines, which are crucial for adding an adjuvant to increase vaccine efficacy (Skwarczynski and Toth 2017). Many researchers have shown that when whole phage particles displaying an epitope or carrying the genetic information of the target antigen were administered, the epitope-specific antibody was detected in the serum of the phage-injected group (Vries et al. 2021; Bao et al. 2019; Hashiguchi et al. 2010; Samoylova et al. 2017; Palma

2023). To observe the antibody production of the phage-displayed protein, we displayed GFP on P8 of the M13 phage and administered it via IP injection to rainbow trout. As a result, phage-displayed GFP showed higher antibody production than the GFP alone injected group at 5wpi, presenting the ability of the phage as a self-adjuvanting agent.

Based on this result, *FlaB*, one of the flagellin proteins of *V. anguillarum*, the causative agent of vibriosis in rainbow trout, was fused to SpyCatcher instead of GFP and displayed on the major coat protein of the M13 phage. Approximately 61kDa of A conjugation band was observed in the western blot analysis. However, further studies are needed to confirm the potential of phage preparation as an effective delivery vehicle for foreign antigens. In addition, the exact number of SpyTags displayed on P8 should be measured for detailed optimization of the conjugation conditions.

Phages can also be used to deliver foreign genetic materials. In the delivery of the immunostimulant via the phage genome, although the CL intensity between the groups was not statistically significant, the stimulatory CpG motif-harboring phage-administered group showed a slightly higher respiratory burst activity than the other groups, suggesting the potential use of phages as a delivery tool for genetic materials. This may have been caused by outliers in the group, preventing accurate statistical analysis of the acquired data. The slightly increased CPS value of the control phage-treated group compared with that of the control group might be due to the natural CpG motif in the phage genome.

CpG-ODNs are classified into three classes, and the induced immune responses differ slightly depending on the class to which the specific CpG-ODNs belong (Vollmer et al. 2004). CpG-ODN 1668, used in the present study, belongs to class B ODNs, which are known to strongly activate B cells, plasmacytoid dendritic cells(pDCs), and cytokine production (Tada et al. 2017). Although the phage already contained CpG motifs within its genome, we generated a phage harboring CpG-1668 motifs to induce specific immune responses induced by a particular CpG sequence. Moreover, Kim et al. reported that along with several types of CpG-B, CpG 1668 showed promising efficacy against several infectious diseases in olive flounder (Kang and Kim 2012, 2014). We also assumed that applying phage particles might supplement the short half-life of CpG-ODNs or CpG motif-bearing plasmids and alleviate side effects caused by treatment with high doses of CpG-ODN to gain sufficient immunomodulatory effects. Further studies should include not only a comparison between CpG-ODNs and phage-containing stimulatory CpG motifs but also the evaluation of several infectious diseases in fish. Factors that affect enhanced phagocytosis after phage injection and the impact of phage injection on the immune system of fish should also be investigated.

Finally, we can also generate and test a hybrid vaccine by combining the expression of the foreign antigen on the phage surface and incorporating the target gene within the phage genome (Palma 2023; Bao et al. 2019). However, the fish farming industry in South Korea relies mainly on conventional vaccines to combat various fish

diseases (Hwang et al. 2020). Alternatively, phage-based vaccines may provide new solutions as versatile platforms that are safe and easy to manipulate.

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