Fusion of M13 bacteriophage pVIII protein with antigen protein using SpyTag/SpyCatcher system to increase antibody titer in rainbow trout (*Oncorhynchus mykiss*)

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M13 bacteriophage is a virus that specifically infects *Escherichia coli* exhibiting an F pilus. Its structure comprises five different coat proteins surrounding its genome. Since the development of phage display technology, which enables the display of foreign peptides/proteins on phage surfaces, various applications from medicine to nanomaterials have utilized phages. Notably, studies using phages as delivery tools for drugs and protective antigens have garnered significant attention. The delivery of foreign antigens via M13 phage display limits the size of the molecule expressed on the major coat protein (pVIII) to 15 amino acids. In this study, by applying the SpyTag/SpyCatcher system, we displayed a foreign antigen that cannot be displayed on the pVIII with the existing system. The conjugation reaction between SpyTag-displaying phage on the pVIII protein and SpyCatcher-fused protein was confirmed by SDS-PAGE and Western blot. In the immunization experiment in rainbow trout (*Oncorhynchus mykiss*), the group administered the foreign protein displaying phage showed a higher antibody titer than the group administered the foreign protein alone. These results suggest that large antigens can be displayed on the pVIII protein of M13 phage using SpyTag/SpyCatcher system. Furthermore, unlike subunit vaccines requiring adjuvants to induce a sufficient immune response, the phage can act as a self-adjuvanting agent.

Key words: M13 bacteriophage, pVIII display, SpyTag/SpyCatcher system, Antigen delivery, Rainbow trout

Introduction

Filamentous bacteriophages, belonging to the *Inoviridae* family, are non-lytic (Rakonjac, 2024) and have a thread-like shape composed of five different coat proteins (pIII, pVI, pVII, pVIII, and pIX) surrounding their genome (ssDNA) (Askora et al., 2012). Among several inoviral species, the M13 bacteriophage specifically infects *Escherichia coli* strains exhibiting an F pilus (Sattar et al., 2015). The M13

phage has a relatively small genome, making it easy to manipulate, and has been applied in various fields, including drug delivery, vaccine development, and nanomaterials (deVries et al., 2021; Palma, 2023).

Smith et al. (1985) developed the phage display technique, allowing a foreign peptide or protein DNA fragment genetically fused to the phage gene to be expressed in a fused form with the phage coat protein without hindering the phage's replication ability and infectivity. The most frequently utilized coat proteins for displaying foreign peptides or proteins are the minor coat protein pIII, which comprises five copies per phage particle and plays a crucial role in the infection

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process via the F pilus, and the major coat protein pVIII consisting of ~2700 copies per virion (Sioud, 2019; Samoylova et al., 2017; Palma, 2023). The pIII is more suitable for displaying larger antigens that require correct folding, but it is limited by the low number of copies per phage. The pVIII is advantageous for displaying a high number of small peptides, providing a dense array of antigens, but it is limited by the size of antigens it can display. Researchers choose coat proteins based on the purpose and type of protein to be displayed (Ebrahimizadeh and Rajabi-bazl, 2014).

Since the pVIII number on M13 phage is exceedingly higher than pIII number, pVIII fused antigens would be advantageous in inducing adaptive immune responses. However, to use the pVIII as a structural protein for phage display, the size limit for foreign proteins (around 10 amino acids) should be overcome (Iannolo et al., 1995; Malik et al., 1998). In this study, to display a large antigen on the pVIII of the M13 phage, we applied the SpyTag/SpyCatcher system. SpyTag is a short peptide of 13 amino acids that forms an immediate isopeptide bond with its protein partner SpyCatcher (116 amino acids) (Hatlem et al., 2019). The reaction between them is specific, rapid, and irreversible, making this powerful protein-ligation tool useful in various applications, including vaccine development (Hatlem et al., 2019). In fish pathogens, Yang and Kim (2022) reported the application of the SpyTag/SpyCatcher system to display an antigen of *Streptococcus iniae* on the surface of nervous necrosis virus (NNV) virus-like particles (VLPs) by the fusion of the viral capsid protein with SpyTag and bacterial antigen with SpyCatcher, respectively.

The SpyTag/SpyCatcher system broadened the size limitation of the pVIII by decorating the surface of M13 phage with pVIIIs fused to SpyTag and then simply mixing it with the foreign protein linked to the SpyCatcher. In this study, to determine the possibility of an efficient antigen delivery tool, the foreign protein-displaying M13 phage was administered to

rainbow trout (*Oncorhynchus mykiss*), and a specific antibody titer was measured via enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Bacterial and Phage Strains

E. coli DH5α strain used for plasmid DNA production, *E. coli* TG1 strain used for phage amplification, and *E. coli* BL21(DE3) for recombinant protein expression were cultured at 37°C in Luria-Bertani (LB, LPS solution) broth and 2x yeast extract tryptone medium (2xYT, KisanBio), respectively. M13KO7 helper phage, purchased from New England Biolabs (NEB), was used to amplify phagemid particles.

Preparation of electro−competent TG1 Cells

A single colony of *E. coli* TG1 was picked and cultured in $2 \times YT$ broth overnight. The next day, 1/100 volume of the overnight culture was inoculated into fresh $2 \times YT$ medium and incubated at 37°C, 210 rpm, 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 3,500 rpm, 4°C, for 20 min. After discarding the supernatant, the bacterial pellet was resuspended in 1 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, Biosolution) and centrifuged under the same conditions. After repeating the last two steps, the cells were diluted with 10% glycerol in 1 mM HEPES and centrifuged at 4°C, 4,000 rpm, for 20 min. Finally, after removing the supernatant, cells were resuspended in 10% glycerol, aliquoted into microcentrifuge tubes, and stored at -80°C until use.

Electroporation of phagemid DNA

For phagemid particle production, phagemids were transformed into *E. coli* TG1 cells via electroporation. 80 μl of electro-competent TG1 cells were mixed with 1 μl of the phagemid vector, and the mixture was

transferred to an ice-cold cuvette. Electroporation was performed at 2,500 V, 25 ms using an Eporator® (Eppendorf), and the cells were immediately re-suspended in pre-warmed SOC medium and recovered at 37°C, 210 rpm, for 1 h 30 min. After recovery, the cells were spread onto an LB agar plate containing the appropriate antibiotic (100 mg/ml of ampicillin). Positive clones were selected by colony PCR and used for further experiments.

Construction of SpyTag−displaying phagemid on pVIII

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

Preparation of SpyTag−displaying phage and SpyCatcher−fused GFP

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 (100 mg/ml). Transformants were selected using colony PCR and used for recombinant phage particle produc-tion. A single colony of *E. coli* TG1 containing pComb8-SpyTag was picked and grown in LB me-dium with ampicillin (100 mg/ml) overnight at 37°C, 220 rpm. The overnight culture was inoculated into fresh LB medium containing ampicillin (100 mg/ml) at a dilution ratio of 1:100 and grown until the OD600 value reached 0.4-0.6. Then, 1×10^{11} pfu of M13KO7 helper phage and kanamycin (50 mg/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°C for 30 min, and then the culture was centrifuged at 3,000 rpm, 4°C for 10 min. After discarding the supernatant, the pellet was re-suspended in $2 \times$ YT medium containing ampicillin, kanamycin, and 0.1 mM IPTG, then incubated over-night at 27°C, 220 rpm. The overnight culture was centrifuged at 6,000 rpm at 4°C for 10 min, and su-pernatants containing phage particles were transferred into fresh conical tubes. To precipitate phage par-ticles, 1/5 of the volume 20% (W/V) PEG 8000/2.5 M NaCl was added to the supernatant (4/5 of the vol-ume). The mixture was gently mixed by inversion and incubated on ice overnight. The precipitated phage

Table 1. Primers used for vector construction

	Primers	Sequence (5'-3')
Fragment #1	pComb8_SpyTag_OC_F	ATGGTGGACGCCTACAAGCCGACGAAGGCTGA GGGTGACGATCCCGCAAAAGCG
	AmpR_504_R	CACGCTCGTCGTTTGGTATGGCTTC
Fragment #2	pComb8_SpyTag_OC_R	GTAGGCGTCCACCATCACGATGTGGGCCTGGGCCATGGCT GGTTGGGCAGCGA
	AmpR_514_F	CAAACGACGAGCGTGACACCACGATG
	GFP_EagI_F	CGGCCGATGGTGAGCAAGGGCGAGGA
	Spycatcher_HindIII_R	AAGCTTTTAAATATGAGCGTCACCTTTAGTTGCTTT

(Underlined sequences indicate overlapped sequences or restriction enzyme sites)

was pelleted by centrifugation at 8,000 g, 4°C 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 6,000 g, 4°C for 10 min. The supernatant was transferred into fresh tubes after filtration using a 0.45 μm syringe filter, and then 1/5 of the volume 20% PEG 8000/2.5 M NaCl was added and mixed well by inverting. The mixture was incubated on ice for 1 h and then centrifuged at 8,000 g, 4°C 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°C. The titer of the phage particles was determined using a double-layer agar (DLA) plaque assay.

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 (Yang and Kim, 2022). Briefly, the cassette of GFP linked to SpyCatcher using a flexible linker was PCR amplified using primers (GFP EagI F and Spycatcher HindIII R, Table 1). The PCR product was cloned into a pGEM-T easy vector (Promega), and after digestion with EagI and HindIII, the fragment of eGFP-SpyCatcher was ligated with pET28a+ vector digested with the same restriction enzymes. The construct was designated as pGFP-Sc.

A single colony of *E. coli* BL21(DE3) transformed with a protein expression vector was picked and grown in LB medium containing kanamycin (50 mg/ml) at 37°C, 220 rpm until the OD600 value reached between 0.4-0.6. Then, cells were induced with 0.1 mM IPTG at 27°C, 220 rpm for 5 h and harvested by centrifugation at 3,000 rpm, 4°C for 10 min. The pellet was resuspended in binding buffer (45 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.9) and the cells were lysed by sonication at 30% amplitude for 30 min. Cell debris was pelleted by centrifugation at 13,000 rpm, 4°C for 10 min, and the supernatant was

filtered through a 0.45 μm syringe filter and transferred to a fresh tube. Protein purification was performed using a Ni-NTA His-Bind Resin® (Novagen). Purified protein in the elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9) was gradually transferred to PBS by ultrafiltration using a 10 kDa Amicon® Ultra-15 Centrifugal Filter. The concentration of the purified protein was determined by the BCA assay.

SDS−PAGE and Western blot

The protein samples were mixed with 5X sample buffer (Elpis) and boiled at 95°C 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 a nitrocellulose membrane (Biorad). After blotting, the membrane was soaked in blocking buffer (3% BSA in TTBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at RT. After three washes with TTBS (0.05% Tween 20 in TTBS, pH 7.5), target proteins were probed using rabbit anti-GFP polyclonal antibody (1:1000, 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 (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 visualize the target protein bands.

Conjugation between SpyTag−displaying phage and SpyCatcher−fused protein

For the conjugation reaction, p8-st at 1×10^{10}-10^{11} pfu/ml and 5 μM of SpyCatcher-fused protein were used. After thoroughly mixing the solutions containing phage and protein by pipetting, the mixture was incubated at RT with rocking for 6 h. Then, 1/6 volume of 30% PEG-8000/3 M NaCl solution was added,

and the mixture was incubated on ice for 1 h to remove unconjugated proteins. After 1 h, the mixture was centrifuged at 13,000 g, 4°C for 20 min, and the phage pellet was resuspended in PBS. The conjugation reaction was confirmed by SDS-PAGE and Western blotting.

Fish immunization

Juvenile rainbow trout (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°C for a week before immunization. Fish were divided into three groups (control, SpyCatcher-fused protein, and conjugated phage), consisting of six fish per group. Before injection, fish were anesthetized with MS-222 and intraperitoneally (IP) injected with 100 µl PBS, 5 µM/100 µl of GFP-Sc, or 100 µl phage-protein conjugate per fish. At three and five weeks post-immunization, fish from each group were anesthetized and bled to collect the serum needed to determine the specific antibody titer against GFP by ELISA.

ELISA

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

Statistical Analysis

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

Results

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 0.1 mM IPTG at 27°C for 5 h. The size of the approximately 45 kDa band was observed after protein purification and dialysis of GFP-Sc on SDS-PAGE and Western blot analysis (Fig. 1).
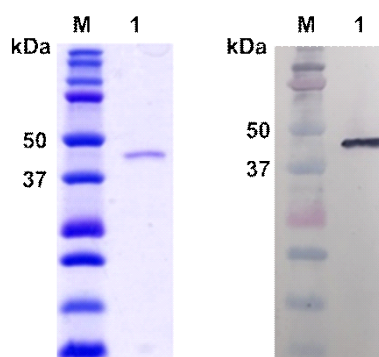

Fig. 1. SDS-PAGE (Left) and Western blot analysis (Right) of the purified GFP-SpyCatcher (GFP-Sc). Rabbit anti-GFP antibody was used as the primary antibody for western blot analysis of purified GFP-Sc. Lane M: protein molecular weight marker, lane 1: purified GFP-Sc.

Conjugation Reaction between p8–st and GFP–Sc

The titer of the finally produced SpyTag-displaying phage was 9.6×10^{13} pfu/ml. SpyTag-displaying phage and GFP fused to SpyCatcher were ligated under different conditions. Based on the Western blot analysis, robust conjugation was observed when 1×10^{10} pfu/ml of phage and 5 µM of protein were incubated at RT for 6 h. The approximate size of the conjugate between p8-st and GFP-Sc was 52 kDa (Fig. 2).

Specific Serum Antibody Titer against GFP

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

Discussion

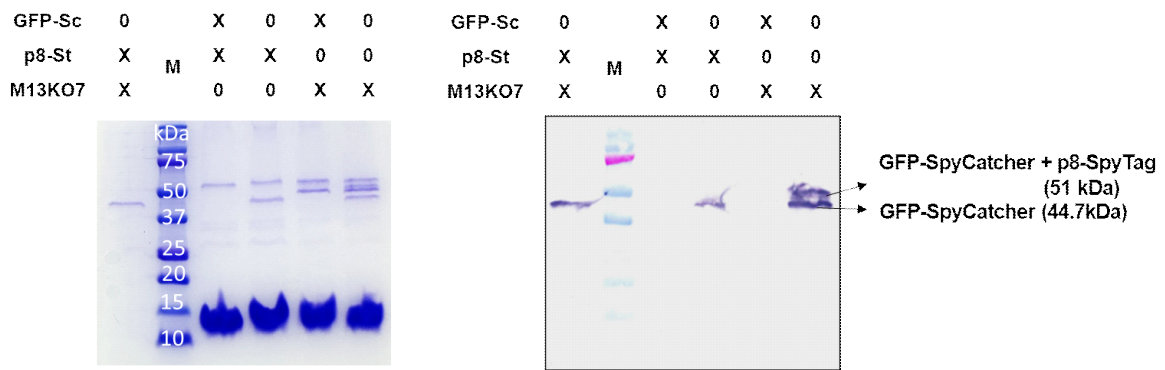In vaccine development, phages are gaining more

Fig. 2. SDS-PAGE and Western blot analysis of conjugation reaction between the p8-SpyTag (p8-st) and GFP-SpyCatcher (GFP-Sc). Rabbit anti-GFP polyclonal antibody was used as the primary antibody for the western blot analysis.
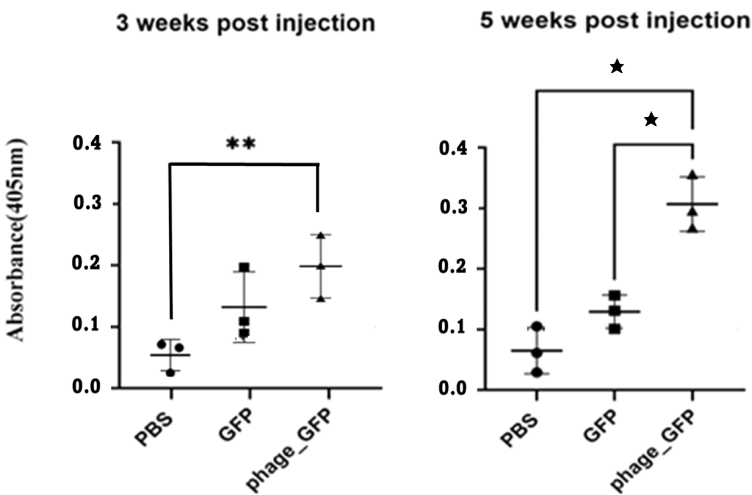

Fig. 3. Juvenile rainbow trout were immunized with GFP-Sc (GFP) or phage-displaying GFP (phage-GFP). The control group was injected with only PBS. Asterisks represent statistical significance at $p<0.05$.

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 (deVries et al., 2021; Bao et al., 2019; Palma, 2023). Furthermore, the structural features of phage particles provide a display of the fused protein in a highly ordered and organized manner, 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 (deVries et al., 2021; González-Mora et al., 2020; Palma, 2023). These attributes make phages more attractive candidates for antigen delivery.

In phage display, it is challenging to display polypeptides longer than 11 amino acids on pVIII of the M13 phage with the existing system (Ebrahimizadeh and Rajabibazl, 2014; Loh et al., 2019). However, indirect display using the SpyTag/SpyCatcher system allows for the display of longer peptides or proteins on pVIII (deVries et al., 2021). Lima et al. (2022) demonstrated that a 200 kDa protein could be displayed on P3 (5 copies/virion) and P8 (~100 copies/virion) using the SpyTag/SpyCatcher conjugation method. Similarly, we confirmed the surface display of the protein, approximately 47 kDa on pVIII. Through the preliminary experiments, the incubation of 1×10^{10} pfu/ml of SpyTag-displaying phage and 5 μM of GFP fused to SpyCatcher for 6 h at RT was determined as the optimum condition.

Intrinsic adjuvanticity is a well-known feature of phages recognized by immune cells that exhibit PRRs as a danger signal (Palma, 2023). Therefore, protective antigens delivered via phage particles do not require the addition of other materials to induce sufficient antibody production, unlike subunit vaccines which require adding an adjuvant to increase vaccine

efficacy. 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 (deVries 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 pVIII 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, suggesting the phage's ability as a self-adjuvant agent.

Although further studies are needed to confirm the potential of phage preparation as an effective delivery vehicle for foreign antigens in fish vaccination, phage-based vaccines may provide new solutions as versatile platforms that are safe and easy to manipulate.

Acknowledgments

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