Thesis for the Degree of Master of Science

Improvement of *Vibrio anguillarum* ghost bacteria vaccine and analysis of antigens for subunit vaccine

by

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Vibrio anguillarum Ghost bacteria 백신 개선 및 단위백신을 위한 항원 분석

Advisor: Prof. Ki Hong Kim

by

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Abstract

Vibriosis is the one of the main disease which occurs in cultured fish all over the world including Korea. It also has been reported that this disease is caused by an infection with bacteria to sea fish and freshwater fish.

The study for vaccine to prevent infection of Vibriosis disease has mainly been developed through *Vibrio anguillarum* and it has been using inactivated bacteria by formalin vaccine as antigen until now. Such formalin-killed vaccine can cause a transformation of surface antigenicity while making vaccines, and it results in decrease of protective efficiency. Therefore, in order to get over a defect of the existing killed cell vaccine requires not to transform of surface antigenicity during the process of making vaccine.

As a result, *V. anguillarum* ghost (VAG) bacteria was developed in the past study. Ghost bacteria refers to a bacteria that is empty inside but maintains antigenicity and same shape as the living bacteria with exhaust inner cellular

through the pore after making a small pore between inner and outer membrane of bacteria through expressing Bacteriophage phiX174 E lysis gene. However, VAG produced in the past study showed that the plasmid DNA and genomic DNA still remains because of bacteria that couldn't completely get out of inner cytoplasm in a production of the final vaccine (VAG). Accordingly, this study is focused on the development of new dual vector system that can remove remain of plasmid and genomic DNA of bacteria in a production of the final vaccine to ensure safety of VAG vaccine.

Dual vector is consisted of lysis E gene cassette and including Staphylococcal nuclease A (SNA) gene cassette that dissolves nucleic acid in bacteria, and it is in expressed according to lambda phage P_R-cI system which is controlled by temperature regulatory. After transformation dual vector into *V. anguillarum* using a method of conjugation, the analysis showed that every nucleic acid in bacteria was dissolved and ghost efficiency has been improved compared to the previous VAG while analyzing the results of the production efficiency test of ghost bacteria and the existence of nucleic acid in ghost bacteria.

After making an experiment on Tilapia with vaccine for analyzing differences of immune induction of VAG vaccine and FKC vaccine, result of analysis agglutination activity of each experiment Tilapia serum showed different outcome according to an antigen type (FKC or VAC type) used during the analysis. The fishes immunized against VAG was shown significantly higher agglutination activity to *V. anguillarum* cadaver (VAC) unlike the fishes immunized against FKC antigen type and antibody titer was highly increased by boosting.

In this study, using epitope in outer membrane of *V. anguillarum* for analyzing development possibility of subunit vaccine manufactured

recombinant protein selected Outer mebrane protein U (OmpU) known to influence a thickness of biofilm and bile salt, and flagellin A (FlaA) related to motility and virulence, after then produced to rabbit antiserum each. Using producted rabbit antiserum, as a result of testing bactericidal activity, ELISA and agglutination for *V. anguillarum* and *V. harveyi* it showed highly bactericidal activity, ELISA and agglutination titer in rabbit antiserum for recombination FlaA.

Introduction

Vibriosis is a representative disease in cultured fish world-widely, and causes a haemorrhagic septicaemia to sea fish, brackish water fish and freshwater fish at the period of high water temperature, resulting in great economic losses in aquaculture industry (Austin and Austin, 1993, 1999; Actis *et al.*, 1999). Among the bacteria species causing *Vibriosis*, *Vibrio anguillarum* has been known as the most important pathogen in cultured fish.

Since the use of chemotherapeutics in aquaculture may lead to occurrence of antibiotic resistant bacteria and negatively affect on the environment and on human being, most of the countries have tried to lessen the use of chemotherapeutics and to develop effective vaccines. Although several commercial vaccines produced by formalin inactivated cultures containing mixtures of whole cells of *V. anguillarum* and extracellular products can provide protection against *V. anguillarum* infections in farmed fish, the existence of many O-serotypes in *V. anguillarum* (Sørensen and Larsen, 1986; Rasmussen, 1987; Rasmussen and Larsen, 1987) limited the extensive use of the vaccines across fish species and countries.

By overcoming the previous technical limits, a new vaccine strategy must be developed for better defensive effect in vivo. Until now, studies of vaccine for protecting *Vibriosis* were generally inactive vaccine using formalin killed cell (FKC), and defensive effect showed in various studies differed (Wong *et al.*, 1992; Joosten *et al.*, 1997; Toranzo 1997; Vervarcke1 *et al.*, 2004; Vervarcke *et al.*, 2004).

In fact, Inactivation of pathogenic bacteria by formalin or heating methods inevitably impaired physico-biochemical characteristics of surface antigens,

especially T cell-dependent antigens, and the modification can elicit skewed immune responses in the host. To induce an effective immune response, it is necessary that outer epitopes unchanged before and after physical or chemical treatment of live infective bacteria.

Thus, to make up for the demerits of this variable vaccine, Ghost or SNA vaccine system and Flagellin A or outer membrane protein U - subunit vaccine of V. anguillarum was contrived.

Bacterial ghosts are produced by the controlled expression of bacteriophage PhiX174 lysis gene E, and the E protein leads to the formation of small transmembrane pores through which cytoplasmic contents are expelled (Szostak *et al.*, 1990, 1993, 1996; Szostak and Lubitz, 1991; Witte *et al.*, 1992). Bacterial ghost, is therefore, a recently introduced method for inactivation of gram negative bacteria, may be one of the solutions. If the bacterial surface T cell dependent antigens are not changed by inactivation process, the immune responses induced by the inactivated bacteria may overcome the huddle of O-serotype-limited protection.

The potential usefulness of bacterial ghosts as vaccine candidates have recently been reported in a wide variety of mammalian pathogenic Gram-negative bacteria (Eko *et al.*, 1994; Katinger *et al.*, 1999; Panthel *et al.*, 2002; Marchart *et al.*, 2003) and in a fish pathogenic bacteria, Edwardsiella tarda (Kwon *et al.*, 2005, 2006, 2007).

The other vaccine system suggested *V. anguillarum* SNA (*V. anguillarum* cadaver, VAC) induced degradation intracellular DNA or RNA of V. anguillarum. Because this produce nuclease. This Staphilococcal nuclease exhibits both exo- and endo-5'-phosphodiesterase activities against both DNA and RNA (Heins *et al.*, 1966). For making *V. anguillarum* SNA, *V. anguillarum* conjugated with *SM10λpir* harboring vector including nuclease gene and we were confirmed nuclease activity. And bacterial SNA system

are produced Streptococcal Nuclease A gene same as Ghost expression system of regulatory temperature, and the expressed SNA proteins were degrade genomic DNA, plasmid and RNA of intracellular bacteria.

In the present study, we expect to confirm efficiency and stability of *V. anguillarum* Ghost and SNA vaccine (Jalava *et al.*, 2002), and also check efficiency and stability of integrated vaccine these two components. Furthermore, to avoid the presence of bacterial genomic DNA or RNA and an antibiotic resistance gene in the final VAG product, we have constructed a new vector containing the E-mediated lysis cassette and the staphylococcal nuclease A (SNA) gene- mediated DNA degradation cassette (Haidinger *et al.*, 2003), and have generated safe VAG for use as a fish vaccine. Finally, the potential of the *V. anguillarum* Ghosts or SNA as a vaccine candidate was evaluated by immunization of Tilapia.

Another veccines, we researched into subunit vaccine. The first, characteristic of *V. anguillarum* has motility because of having a flagellum. An earlier study, flagellum has relation to the virulence of *V. anguillarum*. The importance of the flagellum (flagellin A, FlaA) as a potential virulence factor has been proved in other bacteria (Milton *et al.*, 1996). The nonmotile mutants propagated but did not cause the characteristic systemic infection, indicating that motility contributes to the invasive capabilities of this organism (Grant *et al.*, 1993; Ormonde *et al.*, 2000).

For the seconed, the bacterial outer membrane was composed of 50% of the outer-membrane mass and showed phospholipids, LPS and proteins or lipoproteins. Among them, the bacterial major outer membrane protein-U presented. It was used to protect the intracellular contents from destruction of the outside environment or a factor of injury (Koebnik *et al.*, 2000).

The *V. anguillarum* of enterobacterium be faced with bile acid in the route of infection. Therefore, the Outer membrane protein-U (OmpU) of *V.*

anguillarum have influenced on bile salt, motility, virulence and biofilm (Wang *et al.*, 2003; Okuda *et al.*, 2001).

Flagellin-A and Outer membrane protein-U were selected as major antigen of *V. anguillarum*, these two antigens were established since previous studies about a lot of gram negative bacteria proved that these antigens had an important role. The FlaA and OmpU proteins purified and expressed of constructed plasmids.

The purified proteins were obtained antiserum by rabbits, variable measured This flaA or OmpU antiserum analyzed activation using agglutinaton, ELISA and bactericidal activity.

In this study, we expect to know the effects of subunit vaccine on purified flaA and OmpU. We are going to verify utility as vaccine to *Vibrio anguillarum* enhance ghost. Furthermore, we expect to compare with Ghost vaccine and find utility and value as subunit vaccine of *Vibrio anguillarum*.

Materials and Methods

1. Improvement of *Vibrio anguillarum* Ghost bacteria vaccine

1.1. Bacterial strains, media and plasmids

Vibrio anguillarum was provided by National Fisheries Research & Development Institute, Korea, NFRDI, and was routinely cultured at Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar (Difco) at 37C.

Staphylococcus aureus KCCM 11335 for cloning of nuclease gene was purchased from Korean Culture Center of Microorganisms, Korea, and was cultured at Mueller-Hinton Agar with 2% NaCl.

Escherichia coli DH5a or *SM10λpir* was used as a competent cell in cloning and ligation procedure. Ligation and cloning plasmid were stabilized with *Escherichia coli DH5a* (Invitrogen) cultured in Luria Broth (LB, Difco Laboratories, Detroit, USA) containing 30 ㎍/㎖ ampicillin. After that, *Escherichia coli SM10λpir* used as a donor for conjugation, was cultured in LB containing 30㎍/㎖ kanamycine (Sigma) and used in conjugation of *V. anguillarum*. For sequencing, each constructed cassette was cloned into pGEM-T easy vector (Promega, Madison, WI, USA). A mobilizable vector, pRK415, was used for the transformation into *Escherichia coli SM10λpir a*nd then cultured LB agar with 15 ㎍/㎖ tetracycline. Incubation temperatures for repression and expression of ghost or cadaver gene were 27℃ and 42℃. The Cell lysis and DNA degradation of bacterial cultures were measured by optical density at 600 nm (OD_{600}).

1.2. Construction of pRK-λP_R-c I -Elysis vector

Lysis E gene was amplified by polymerase chain reaction (PCR) using genomic DNA of Bacteriophage PhiX174 (New England BioLabs Inc., Beverly, MA, USA) with oligonucleotide Primers (Forward : 5'-ATGGTACGCTGGACTT TGTG-3' and Reverse: 5'-ACATTACATCACTCCTTCCG-3'). Lambda P_R-c I ^{857} regulatory system which controlled the gene according to conditions of temperature was amplified by PCR from pLDR20 (American Type Culture Cell, ATCC, USA) with oligonucleotide primers (Forward : 5'-CCGCGGCCCTT TAGCTGTCTTGGTTTGC-3' and Reverse : 5'-GGGCCCGACCAGAACACCTTG CCG-3' (Restriction enzyme sites are underlined))which contained *Sac Ⅱ* and *Sma I* restriction sites, respectively. Then PCR products were cloned into pGEM-T easy vector. Lysis E gene fragment was then excised by restriction enzyme digestion with *Sac Ⅱ* and *Apa I*, and ligated to pGEM-T easy vector containing lambda PR-cI system digested with same enzymes, leading lysis E gene expression vector controlled by lambda PR-c I regulatory system (pλ PR-c I -Elysis) (Kwon *et al.*, 2006). This way as Lambda PR-c I regulatory system linked with lysis E gene is named pGhost-V or ghost cassette. The λ PR-c I -Elysis cassette containing *Pst I* and *BamH I* enzyme site was obtained by PCR using pλPR-c I -Elysis with the oligonucleotide primers LC I *Pst*-F and E *BamH*-R (*PstI* and *BamHI* sites (underlined) (Forward : 5'-C TGCAGGACCAGAACACCTTGCCGAT-3' and Reverse : 5'-GGATCCACATTAC ATCACTCCTTCCG-3') PCR amplifications were performed for 1 cycle of 3 min at 95℃, 30 cycles of 30 s at 95℃, 30 s at 55℃, 1 min at 72℃, with a final extension step of 7 min at 72℃. The PCR reactions were performed using the iCycler thermal cycler (Bio-Rad). Each amplified PCR product was visualized on 0.7% agarose gels stained with ethidium bromide, purified with gel extraction kit (Nucleogen, Si Hung, Korea). After *Pst I* and *BamH I*

enzyme digestion of pGhost-V, ghost cassette was inserted into the digested pRK415 plasmid with the same restriction enzymes, and named as pRK-λ P_R-c I -Elysis (Fig. 1).

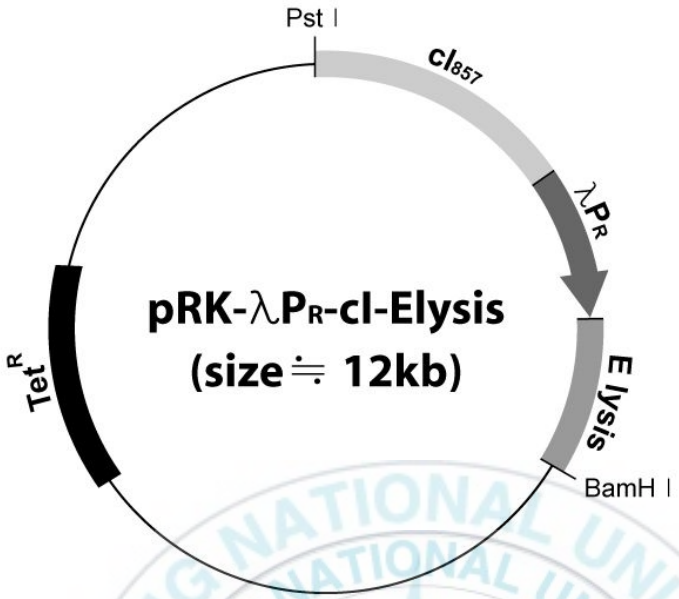


Fig. 1. The pRK-λP_R-cI-Elysis constructed vector map expressed plasmid for E-lysis gene. Tet^R, Tetracycline resistance gene; cI_{857}, gene encoding the temperature sensitive repressor for the lambda P_R promoter; *PstI* or *BamHI*, restrictions sites used for the construction.

1.3. Construction of staphylococcal nuclease A (SNA) expressing vector

The gene fragment encoding *Staphylococcal nuclease A* gene was amplified by the polymerase chain reaction (PCR) using genomic DNA of *Staphylococcus aureus* as a template with oligonucleotide pair primer (*EcoRI* and *PstI* sites (underlined), SNA *EcoRI* Forward : 5'-GAATTCATGGCAACTT CAACTA-3' and SNA *PstI* Reverse : 5'-CTGCAGTTATTGACCTGAATCAGCG -3'), producing primer based on staphylococcal nuclease A gene.

The SNA gene fragment was then excised by restriction enzyme digestion with *EcoR I* and *Pst I*, and ligated to pGEM-T easy vector containing lambda P_R-cI regulatory system digested with same enzymes, leading SNA gene expression vector controlled by lambda P_R-c I regulatory system (pλ P_R-c I -SNA). This λP_R-c I -SNA to ligation production is named SNA cassette. SNA cassette containing *BamH I* and *Pst I* enzyme site was amplified by PCR using the oligonucleotide primers LC I *BamH*-F and SNA *Pst*-R (Forward primer : 5'-GGATCCTCAGCCAAACGTCTCTTCAGG-3' and Reverse primer : 5'-CTGCAGTTATTGACCTGAATCAGCG-3' (Restriction enzyme sites are underlined)). PCR amplifications were performed for 1 cycle of 3 min at 95℃, 30 cycles of 30 s at 95℃, 30 s at 50℃, 1 min at 72℃, with a final extension step of 7 min at 72℃. SNA cassette was inserted into pRK415 vector, leading to pRK-λP_R-c I -SNA (Fig. 2).

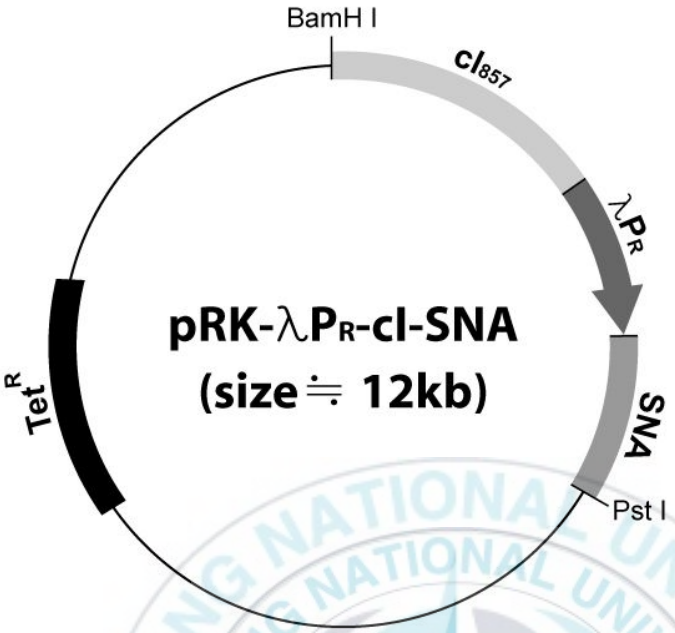


Fig. 2. The pRK-λP_R-cI-SNA constructed vector map expressed plasmid for cadaver (SNA) gene. Tet^R, Tetracycline resistance gene; cI_{857}, gene encoding the temperature sensitive repressor for the lambda P_R promoter; *BamHI* or *PstI*, restrictions sites used for the construction.

1.4 Construction of dual vector expressing PhiX 174 lysis E gene and staphylococcal nuclease A gene

The λP_R promoter in λP_R-c I -Elysis cloned in pGEM-T easy vector (Promega) was exchanged with $\lambda 2_R$ promoter which was λP_R promoter from which cro- gene was removed. Then, for terminating lysis E gene transcription, termination sequence (TS, rrnBT1) was inserted the rear of E lysis gene sequence. The SNA cassette was ligated after termination sequence. This constructed $\lambda 2_R$-c I -Elysis-TS(rrnBT1)-λP_R-SNA (2_R-P_R) gene was obtained by combining these two cassette systems which was named as 2_R-P_R cassette (dual vecter system). 2_R-P_R containing *Xba I* and *sac I* enzyme site was obtained by PCR from p$\lambda 2_R$-c I -Elysis-TS-λP_R-S.nucA using the oligonucleotide primers LC I *Xba I* -F and SNA *sac I* -R (Forward primer : 5'-TCTAGAATGGCAACTT CAACTAAAAATTAC-3' and Reverse primer : 5'-GAGCTCTTATTGACCTGAA TCAGCG-3'). PCR amplifications were performed for 1 cycle of 3 min at 95℃, 30 cycles of 30 s at 95℃, 30 s at 50℃, 2 min at 72℃, with a final extension step of 7 min at 72℃. 2_R-P_R was inserted into the pRK415 vector, leading to pRK-$\lambda 2_R$-c I -E-SNA (Fig. 3). Thus, The pRK-λP_R-cI-E-SNA plasmid, which was used in coexpression of both lysis gene *E* and staphylococcus nuclease A gene.

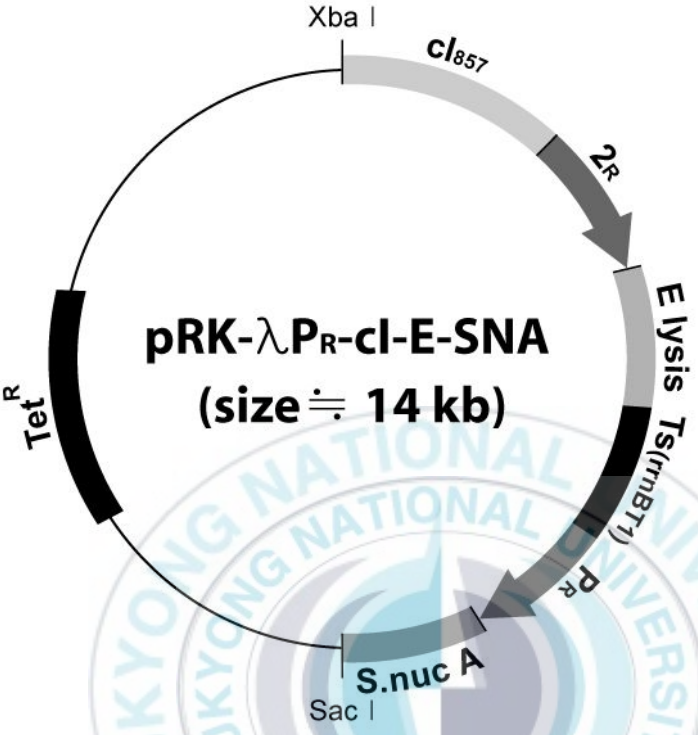


Fig. 3. The pRK-λ2_R-cI-E-SNA constructed vector map expressed plasmid for lysis gene E and cadaver (S. nuc A) gene. Tet^R, Tetracycline resistance gene; cI_{857}, gene encoding the temperature sensitive repressor for the lambda 2_R and P_R promoter; *XbaI* or *SacI*, restrictions sites used for the construction.

1.5. Conjugation of *V. anguillarum*

Escherichia coli SM10λpir harboring each pRK-λP_R-c I -Elysis, pRK-λP_R-c I -SNA or pRK-λ2_R-c I -E-SNA were used as a donor cell for mating of *V. anguillarum* in recipient cell. The suspensions were streaked on tryptic soy agar containing 1.5% NaCl for checking sterility and stored at 4℃ until use. *V. anguillarum* (NFRDI) was plated on tryptic soy agar (TSB, Difco) containing 1.5% NaCl and *E. coli SM10λpir* harboring pRK415 linked each cassette gene was plated on LB agar containing 15 ㎍/㎖ tetracycline. After incubation for 42h at 27℃, the donor and recipient cell were mixed at the ratio of three to one using sterilizing swab, respectively, and spread on LB agar plate. Again after 42 h at 27℃ incubation, transconjugants (Conjugated *V. anguillarum*) were selected by plating on Thiosulphate-Citrate-Bile-Sucrose agar (TCBS, Difco) containing 15 ㎍/㎖ tetracycline (Sigma). Transconjugants were verified by plasmid preparation and PCR.

1.6. Induction of lysis E gene and/or SNA gene

1.6.1 Induction of lysis E gene

Lambda P_R-c I regulatory system controled expression of lysis E gene according to conditions of temperature was not able to operated λP_R promotor because expressed product sticks by operating c I repressor at 27℃. It is the system that the Lysis *E* gene was expressed by operating λP_R promotor due to not operate c I repressor by temperature upshift to 42℃.

The *V. aguillarum* Ghost (VAG) was cultured LB medium 3㎖ containing 15 ㎍/㎖ tetracycline and incubated with vigorous agitation (200 rpm) for overnight at 27℃. Next day, when growing VAG was subcultured to 500㎖ LB medium containing 15 ㎍/㎖ tetracycline. Then lysis E gene was expressed by temperature upshift at 42℃ when reached OD_{600} of 0.2. At the end of lysis, about 2~3hour later by a temperature shift, VAG was collected to centrifuge at 10,000 × g for 10 min at 4℃ and washed three times with PBS (Phosphate Buffered Saline) and then lyophilized.

The efficacy of killing of *V. anguillarum ghost* was estimated by plating samples of appropriate dilutions of lyophilized VAG on LB agar containing 15 ㎍/㎖ tetracycline and results indicated a 100% killing efficiency as no colony-forming units (cfu) were found on plates with lyophilized VAG preparations at any dilution.

1.6.2 Induction of SNA gene

The pRK415 vector inserted with c I repressor-λP_R-SNA cassette was conjugated into *V. anguillarum*. The λP_R promotor was not operated by regulating c I repressor at 27℃ and λP_R promotor was operated due to do not expression c I repressor by temperature upshift to 42℃, as a result the expression of SNA gene was induced. That is to say, P_R promoter was operated by regulator of c I repressor.

The *V. aguillarum* SNA (*Vibrio anguillarum* cadaver, VAC) was cultured with LB medium 3㎖ containing 15 ㎍/㎖ tetracycline and incubated with shaking (200 rpm) for overnight at 27℃. Next day, growing VAC was subcultured and incubated with shaking at 27℃ up to OD_{600} of 0.5, and then the expression of SNA gene was induced by temperature upshift to incubated with shaking at 42℃ and the same time for nuclease activity was added to 1mM $Mgcl_2$ and 10mM $CaCl_2$. The nuclease activity made degradation of intracellular DNA and RNA. And genomic DNA was isolated per 1~2 hour and was analyzed by electrophoresis for checking successful DNA degradation owing to nuclease revelation. Genomic DNA extraction made almost same as the number of cell to measured at OD_{600} and was isolated by the *AccuPrep*® genomic DNA extraction kit according to the manufacturer's instructions (Bioneer, Taejon, Korea). At the end of DNA and RNA degradation, VAC cell was collected to centrifuge at 10,000 × g for 10 min at 4℃ and washed three times with PBS (Phosphate Buffered Saline) and then VAC stocks was stored at -80℃.

The efficacy of killing of *V. anguillarum SNA* was estimated by plating samples of appropriate dilutions of VAC on LB agar containing 15 ㎍/㎖ tetracycline and results indicated a 100% killing efficiency as no colony-forming units (cfu) were found on plates with VAC preparations at any dilution.

1.6.3 Induction of lysis E gene linked with SNA gene

The pRK415 vector inserted with c I repressor-$\lambda 2_R$-E-P_R-SNA (2_R-P_R) gene was conjugated into *V. anguillarum*. The $\lambda 2_R$ and P_R promotor was not operated by operating c I repressor at 27℃ , and $\lambda 2_R$ and P_R promotor was operated due to do not expression c I repressor by temperature upshift to 42℃, the expression of lysis E gene was induced by operating $\lambda 2_R$ promotor and the expression of SNA gene was induced by operating P_R promotor.

The *V. aguillarum* 2_R-P_R (VAGC, *V. aguillarum* Ghost Cadaver) was cultured with LB medium 3㎖ containing 15 ㎍/㎖ tetracycline and incubated with shaking (200 rpm) for overnight at 27℃. Next day, growing VAGC was subcultured and incubated with shaking at 27℃ up to OD_{600} of 0.2, and then induced by temperature upshift to 42℃. After 3 hours by a temperature upshift to 42℃, namely, after 3 hours ghost gene expression, activity of nuclease produced by expression of SNA gene was appeared by add 1mM $Mgcl_2$ and 10mM $CaCl_2$. The nuclease activity made degradation of intracellular DNA and RNA. At this moment, genomic DNA was isolated per 1 hour by the *AccuPrep*® genomic DNA extraction kit according to the manufacturer's instructions (Bioneer, Taejon, Korea) and was monitored by electrophoresis for checked out DNA degradation depend on nuclease revelation. At the end of DNA degradation, VAGC cell was collected and washed three times with PBS (Phosphate Buffered Saline) and then VAGC stocks was stored at -80℃.

The efficacy of killing of *V. anguillarum* (VAGC) was estimated by plating samples of appropriate dilutions of VAGC on LB agar containing 15 ㎍/㎖ tetracycline and results indicated a 100% killing efficiency as no colony-forming units (cfu) were found on plates with VAGC preparations at any dilution.

1.7. Preparation of bacterial genomic DNA and electrophoretic analysis

Cultured bacteria was pelleted by centrifugation at 10,000 × g for 10min. After that, supernatant was discarded and remaining sediment pellet was washed three times with PBS (Phosphate buffered saline).

All of genomic DNA preparation was extracted using *AccuPrep*® genomic DNA extraction kit according to the manufacturer's instructions (Bioneer, Taejon, Korea). The quality and quantity of DNA was determined by ultraviolet (UV) spectrophotometer (*Ultraspec*® 3100 *pro*, Amersham Pharmacia Biotech).

And Electrophoresis was analysed using Mupid®-2plus Electrophoresis machine system (Advance or Optima, Japan). Agarose powder 0.7g and TAE (Tris-Acetate-EDTA) 100ml (0.7% agarose gel) were mixed and heat to completely melt down using Microwave oven and put to room temperature for 20~30min. After cooling up to 60°C, gel was poured into gel plate comb (gel bed) to make hard. Gel bed installed into electrophresis migration tank and then filled with TAE buffer. DNA sample 1㎕ and loading dye 1㎕ for electrophoresis were mixed and put carefully in hole comb of gel. Put 1 kb marker (Bioneer, Korea) was used 1㎕. Dye mixed with a DNA sample was monitored to move from comb starting electrophoresis after put the lid to gel tank. Electrophoresed gel was stained for 10min in x1 DW (Distilled Water) containing 0.5 μg/ml ethidium bromide and then size of DNA sample was monitored by ultraviolet (UV) rays.

1.8. Preparation of formalin-killed *V. anguillarum* (FKC)

V. aguillarum (NFRDI) was cultured in tryptic soy broth (TSB, Difco) medium 3㎖ containing 1.5% NaCl overnight at 27℃. The cultured *V. anguillarum* was added with formalin 1% to final total volume and further cultured 27℃ for 24 hours. Formalin killed *V. anguillarum* (FKC) was obtained pellet by centrifugation at 8000rpm for 10min. After that, supernatant was taken out and remaining sediment pellet was washed three times with PBS (Phosphate buffered saline).

Suspensions were checked sterility on tryptic soy agar (TSA, Difco) plate containing 1.5% NaCl. And pellet was stored at -4℃.

Pelleted FKC was measured a volume using wet weight.

1.9. Immunization and preparation of serum samples

Various size of Tilapia (Oreochromis niloticus) were randomly obtained from the fish farm in Pukyong National University, Korea. Tilapia were stocked divided 4groups (5 fish / group) in one aquariums, and acclimated for 2 weeks. Water temperature was regulated at 23℃. The FKC, VAG and VAC stocks were suspended with PBS and adjusted to be 2.92×10^7 cells/㎖. The Tilapia in aquarium being 4 group was injected PBS, FKC, VAG and VAC with 2.92×10^7 cells/㎖ intraperitoneally. Each group in aquarium was distinguished by tail fin clip. After first vaccination, second boosting was conducted after 2 weeks and then, 2 weeks later, all of them in each group was collected blood 1㎖ in caudal vein for agglutination tests with challenged by *V. anguillarum*. Collected blood of each group was located for 1 hour at RT and then kept for 1 hour at 4℃, supernatant separated after centrifugation at 8000rpm for 10 min. It was stored at -80℃.

1.10. Agglutination activity of serum

The agglutination activity test was conducted in 96-well 'U'-shaped microplates. The sera were serially diluted two-fold (initial dilution ratio, the original serum), mixed with FKC (4 ㎎/㎖, OD_{600} 0.645)or VAC (adjust OD_{600} 0.645) and incubated overnight at 27℃.

1.11. Challenge test

For attack experiment to Tilapia, *V. anguillarum* was cultured on tryptic soy broth (TSB, Difco) medium containing 1.5% NaCl and incubated for 24h at 27℃. Cultured bacteria was washed three times with PBS and adjusted to 5.7×10^7cells/㎖. And measured efficiency as colony-forming units (cfu) were found on tryptic soy broth (TSB, Difco) agar plates containing 1.5% NaCl. Prepared *V. anguillarum* suspension was injected to fish intraperitoneally with 100㎕ after 2 weeks giving boosting. Dead fish were checked and tissue of kidney and liver was plated on TCBS to confirmed the presence of *V. anguillarum.*

1.12. Statistical analysis

Serum agglutination data were analyzed by the Student's *t*-test, and challenge test data were analyzed by the chi-square test. Significant differences were determined at $P < 0.05$.

2. Analysis of antigens for subunit vaccine

2.1. Bacterial strains, media and plasmids

The pET-28a (Novagen, USA) was used as vector for expression of His-tagged fusion proteins. *Escherichia coli BL21* (DE3) was used as host cell for expression of His-tagged fusion proteins and cultured at 37℃ on Luria-Broth (LB, Difco) agar containing 30 ㎍/㎖ kanamycin and ampicillin.

2.2. Cloning and sequence of Flagellin-A or Outer membrane protein-U

The Flagellin-A (FlaA) or Outer membrane protein-U (OmpU) gene of *Vibrio anguillarum* (NFRDI) were used sequence registered in genbank of NCBI (National Center for Biotechnology). Using this sequence, it was designed FlaA primers ((BamHI and NotI sites underlined) Forward primer ; 5'-GGATCCATGACCATTACAGTAAATACTAACG-3', Reverse primer ; 5'-GCGGCCGCTTACTGCAATAGTGACATTGC-3') or OmpU primers ((BamHI and HindⅢ sites underlined) Forward primer; 5'-GGATCCATGGGTGAGCTATACAACCA-3', Reverse primer; 5'-AAGCTTTTAGAAGTCGTAACGTAGACCTAG-3') . The FlaA or OmpU genes was amplified by each primer pairs using PCR and PCR template was used genomic DNA extract of *V. anguillarum*. PCR amplificated condition were performed for 1 cycle of 3 min at 95℃, 30 cycles of 30 s at 95℃, 30 s at 50℃, 1 min at 72℃, with a final extension step of 7 min at 72℃ (iCycler, Bio-Rad). Each amplified PCR products were purified with Gel Extraction Kit (Nucleogen, Si Hung, Korea), was visualized on 0.7% agarose gels stained with ethidium bromide, and for sequencing, each products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA). The sequenced products were checked with GenBank database through BLAST search in NCBI.

2.3. Construction of recombinant protein *expressing* vector

FlaA or OmpU of the sequenced gene and pET-28a, protein expressing plasmid, were digested by restriction enzymes (BamHI and NotI or BamHI and HindⅢ)) and ligated (pET28a-FlaA, pET28a-OmpU) (Fig. 4). This fusion recombinant plasmid were transferred into *Escherichia coli BL21*(DE3) competent cells.

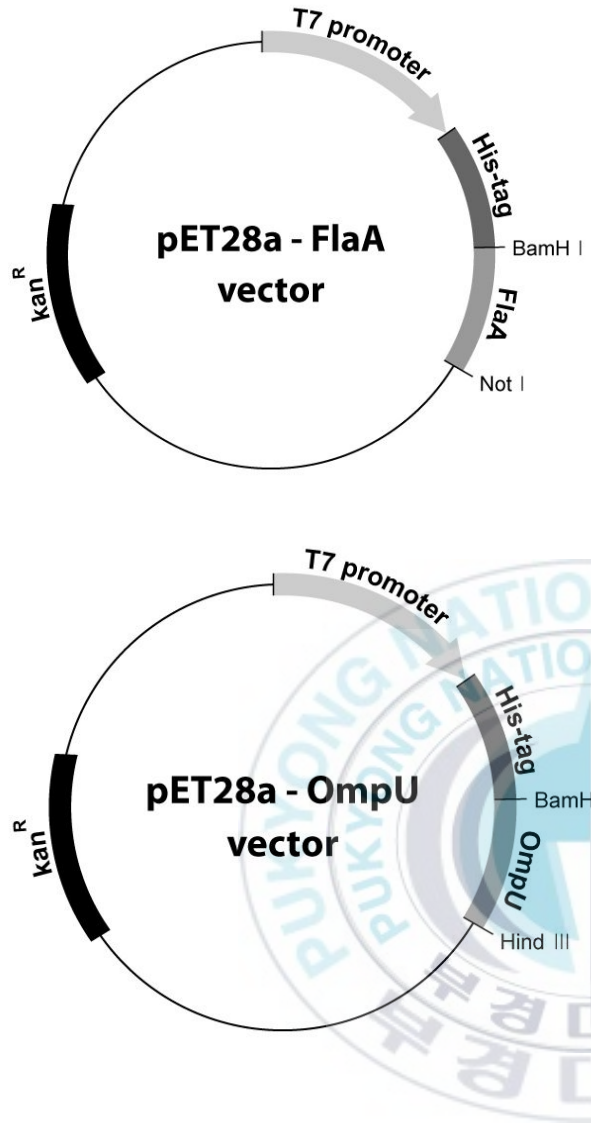


Fig. 4. The pET28a-FlaA or -OmpU constructed vector map expressed plasmid for FlaA and OmpU gene. kan^R, Kanamycin resistance gene; *BamHI and NotI or HindIII,* restrictions sites used for the construction.

2.4. Expression and purification of recombinant proteins

Transformed BL21(DE3) harboring pET28a-FlaA or pET28a-OmpU were grown in Luria Broth (LB, Difco) containing 30 ㎍/㎖ kanamycin (Sigma) at 37°C. For repression and expression of the FlaA or OmpU gene, Isopropyl 1-thio-β-D-galactoside (IPTG) was added to a final total volume of 1 mM when the optical density at 600 nm (OD_{600}) reached about 1.0. Expression BL21 were harvested by centrifugation at 2000 × g at 4℃ for 10 min and then added with binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris/pH7.9) and Lysozyme. The cells were sonicated using Sonic Dismembrator (Fisher Scientific) for 5 min at intervals of 30 sec. Sonicated cells were centrifuged at 12,000rpm at 4℃ for 30 min and the pellets was collected. The collected pellets were melted to bind buffer and UREA adjust 8 mole. And His-tagged fusion proteins were dialyzed and then purified. The His-tagged fusion proteins were purified by chromatography under native conditions on Ni-nitrilotriacetic acid resin (His affinity culumn) according to the manufacturer's protocols (Novagen). The purified His-tagged proteins, quantitative analysis of FlaA and OmpU were measured using Bicinchoninic Acid (BCA) Assay (Sigma, MO, USA).

2.5. SDS-page and Western blot

For measure of FlaA or OmpU was executed by SDS-page (Sodium dodesyl sulfate - Polyacrylamide gel electrophoresis). Stacking gel was used gel (4.0%(v/v)) including 0.5M Tris-HCl, 10% SDS and 30% acrylamide, and separating gel was used gel (10%(v/v)) including 1.5M Tris-HCl, 10% SDS and 30% acrylamide. FlaA or OmpU diluted 40㎍/㎖ (D.W) with sample buffer (1 : 4) and then loaded each 20μL. Marker (Pierce) was loaded 5μL. The electrophoresed gels were monitored bands of FlaA or OmpU after dye at Coomassie blue.

After that, proteins in electrophoresis gel were transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with blocking solution (3% bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT) and then washed with TTBS (0.05% Tween 20 in TBS, pH 7.5). That was incubated with first antibody solution, rabbits anti-FlaA or OmpU sera diluted at 1: 500, for 2 hour at RT and then washed with TTBS again. Membranes incubated with second antibody solution, sera of goat anti rabbit IgG conjugated alkaline phophatase diluted at 1:2000, for 2 h at RT and then washed with TTBS again. Finally, for attaching a substrate, the membranes were developed after adding nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate (NBT-BCIP) substrate buffer (Sigma).

2.6. Production of Antibody (against FlaA or OmpU from rabbits)

Two males of New Zealand White rabbits (Samtako) were used for obtaining the immunized sera. The purified FlaA or OmpU proteins were injected to rabbits intraperitonea with complete Freund's adjuvant (FCA, Sigma) for first injection. After 2 weeks, a same dose of the proteins were injected to rabbits intraperitonea with incomplete Freund's adjuvant (FIA, Sigma) for boosting.

After 2 weeks, post-boost immunization, all rabbits collected blood to obtain serum. Obtained serum were rabbit anti-FlaA or OmpU serum. These sera were stored at -80℃.

2.7. Bactericidal activity of serum

The bactericidal activity about serum was performed according to Yin *et al.* (1996) with some modifications. *V. anguillarum* (NFRDI) was cultured for over night at 27℃, cultured *V. anguillarum* pellet centrifuged at 8,000rpm for 10 min at 4℃ and then washed three times with sterile PBS. The Pellets resuspended with PBS to adjusted optical density at 540 nm (OD_{540}) of 1.0 (1×10^7 cfu / ml). After that, it was diluted 50-fold with PBS and its 25㎕ was mixed with an equal volume of heat-inactivated normal serum or each group antisera. And this diluted of step with PBS. The suspensions were incubated in a 'U'-shaped microtitre plates for 1.5 hour at room temperature. Subsequently, it was dropped on tryptic soy broth (TSB, Difco) agar plates containing 1.5% NaCl and was measured colony forming unit (cfu).

2.8. Agglutination activity of serum

The sera were serially diluted two-fold in 'U'-shaped microtitre plates. And then *Vibrio anguillarum* FKC (4 ㎎/㎖, OD_{600} 0.574) or VAC (adjust to OD_{600} 0.574 with FKC) was added same volume respectively. The plates were incubated for over night at 27℃.

2.9. Enzyme-linked immunosorbent assay (ELISA)

V. anguillarum FKC (4 ㎎/㎖, OD_{600} 0.645) or VAC (adjust OD_{600} 0.645) filled each 50㎕ to 96 micro well plates and incubated for 2 hour at 60℃. It blocked with 200 ㎕ of 2% BSA for 1 hour at 37℃. The test sera were serially diluted two-fold at 1:50 of initial dilution ratio. The plates incubated with 75 ㎕ of rabbit anti- FlaA or OmpU sera (diluted 1:1000) for 30 min at 37℃. And, it was incubated with 75 ㎕ of goat anti-rabbit IgG conjugated with alkaline phosphatase antibody (diluted 1:2000, Santa Cruz Biotechnology, Inc., California, USA) for 30 min at RT. The plates were washed at 3 times with PBST (PBS containing 0.1% Tween 20) at each phase. The washed plates were waited for 30 min with the substrate p-nitropheny phosphate in substrate buffer at darkroom and then it was measured at OD_{415} using an automated ELISA reader (Bio-Rad laboratories, Hercules, CA).

2.10. Statistical analysis

Serum agglutination data were analyzed by the Student's *t*-test, and challenge test data were analyzed by the chi-square test. Significant differences were determined at $P < 0.05$.

Results

1. Improvement of *Vibrio anguillarum* ghost bacteria vaccine

1.1. Generation of *V. anguillarum* ghost bacteria (VAG)

The constructed pRK-λP_R-c I -Elysis was conjugated with *V. anguillarum*. VAG was measured of Optical Density at 600 nm per 1h by a temperature upshift from 27℃ to 42℃. When this system induced by temperature upshift, it check up that was to be ghost because of decreasing value of the Optical Density 600nm when *V. anguillarum* envelope was formed of a transmembrane tunnel structure.

1.2. Generation of *V. anguillarum* cadaver bacteria (VAC)

SNA cassette induced nuclease has brought the promoter regulatory system used in ghost cassette. The constructed pRK-λP_R-c I -S.nucA was conjugated with *V. anguillarum*. VAC was added Ca^{2+}, Mg^{2+} for activity of nuclease as a temperature upshift from 27℃ to 42℃ and monitored by measuring the optical density at 600 nm (OD_{600}) per 1 hour and then performed electrophoresis by DNA extract (Fig. 5).

In addition, the nuclease of *Staphylococcus aureus* is fully dependent on Ca^{2+} to act as a phosphodiesterase (Anfinsen *et al.*, 1971) which cleaves either single- or double-stranded DNA or RNA (Heins *et al.*, 1967). to produce 3-phosphomononucleotides, dinucleotides, and 3,5-nucleoside diphosphates (Alexander *et al.*, 1961; Anfinsen *et al.*, 1971). And, Mg^{2+} was shown a stimulatory effect on the DNase activity of the enzyme (Cuatrecasas *et al.*, 1967).

When this system induced by temperature upshift, genomic DNA of cultured VAC was extracted per 1 hour and checked a degradation of DNA and RNA in VAC using electrophoresis. It was monitored to maintain regularity (Fig. 6).

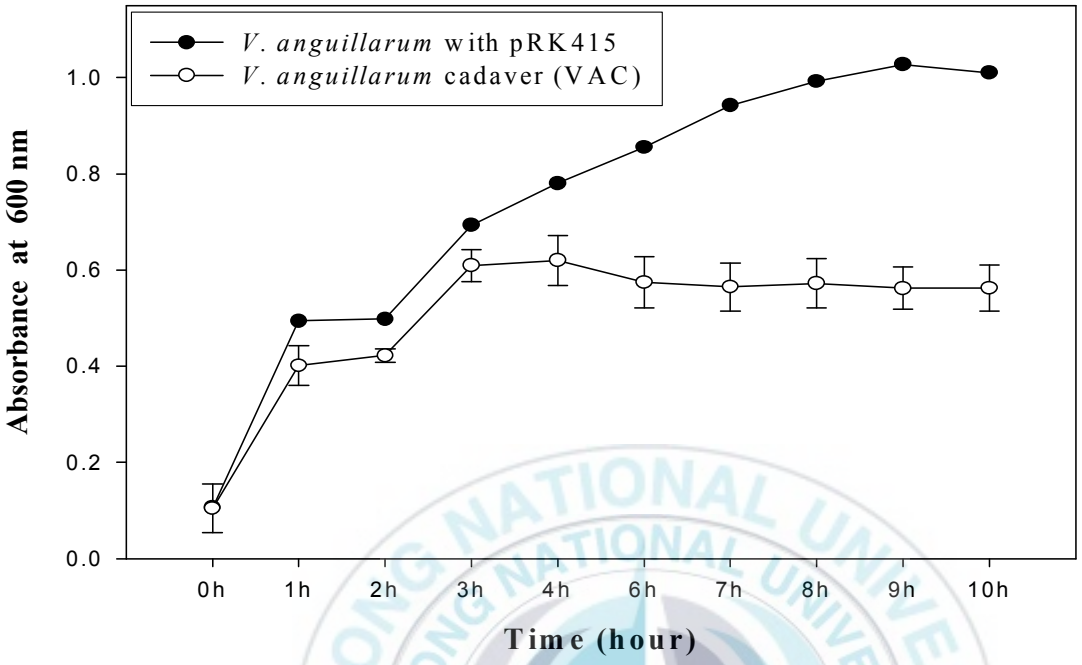


Fig. 5. Spectrophotometric observation for growth of V. anguillarum harboring plasmid pRK415-SNA by temperature induction. Samples were generated by upshift of the incubation temperature from 27℃ to 42℃ and addition of $CaCl_2/MgCl_2$ at 3 hour time. Growth and stop absorvance of *Vibrio anguillarum*, with control bacteria and cadaver bacteria. V. anguillarum with pRK415, *Vibrio anguillarum* harboring pRK415; VAC, *Vibrio anguillarum* harboring pRK-λP_R-c I -S.nucA. Each line graph represents the mean based using 3 VAC samples and T-bars indicate standard errors.

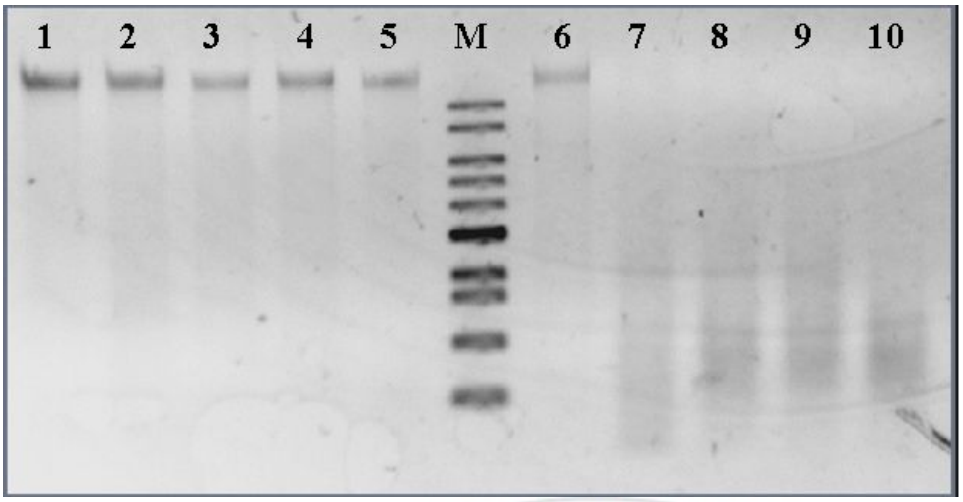


Fig. 6. Degradation of *V. anguillarum* genomic DNA was observed by electrophretic separation with 0.7% agarose gels and extracted genomic DNA extraction kit. Samples were checked every 2 hours by upshift of the incubation temperature from 27℃ to 42℃ (time point: 2, 4, 7, 9, 11 hour). lines 1, 2, 3, 4, 5 - *V. anguillarum* harboring pRK415 (control); line M - 1kb ladder (Bioneer); lines 6, 7, 8, 9, 10 - *V. anguillarum* harboring pRK-λP_R-c I -S.nucA.

1.3. Generation of safety enhanced VAG

The constructed pRK-λ2_R-c1-ghost-P_R-S.nucA (2_Rghost-P_RSNA) was conjugated into *V. anguillarum*. Namely, the system for the efficient expression of ghost, it proved that λ2_R promoter ghost gene expression is better efficient than λP_R promoter ghost gene at same condition because 2_R ghost be caused by decreasing more rapidly the OD_{600} value than P_R ghost. The 2_R ghost removed cro-gene from c I repressor-P_R.

For removing of remaining DNA and RNA in the problem of VAG vaccine, intracellular remaining DNA and RNA were showed degradation inducing Staphylococcal nuclease A gene expression. Thus, 2_Rghost-P_RSNA of Dual-Vector cassette was constructed gene by adding to two systems (Ghoat and SNA). It is expected to make efficient *Vibrio anguillarum* vaccine system as a safe and stable vaccine which is made degradation all of DNA and RNA by nuclease A and bored holes through membrane of bacteria by lysis E. VAGC was monitored value of OD_{600} per 1 hour by a temperature upshift from 27℃ to 42℃ and genomic DNA was extracted per 1 hour. And VAGC was added Ca^{2+} and Mg^{2+} for activity of SNA after 3 hour and induced by a temperature upshift to 42℃.

When this system induced by temperature upshift, it showed degradation of DNA and RNA into *V. anguillarum* (VAGC) by electrophoresis genomic DNA extract (Fig. 7), and monitored that OD_{600} value of VAGC was nearly stopped by activity of SNA after decreasing during 3 hour expressed ghost (Fig. 8).

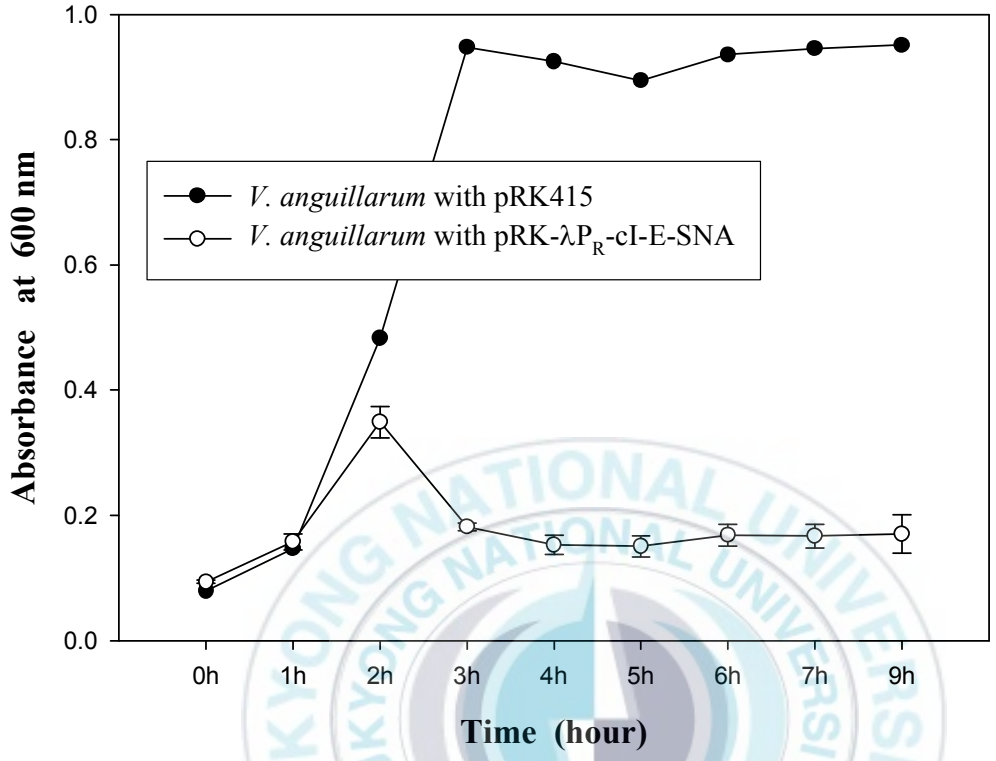


Fig. 7. Spectrophotometric observation for growth of V. anguillarum harboring plasmid pRK415-E-SNA by temperature induction. Samples were generated by upshift of the incubation temperature from 27℃ to 42℃ after 2 hour time and then added of $CaCl_2$ / $MgCl_2$ after 3 hour later. Growth, stop and decrease absorvance of *Vibrio anguillarum*, with each samples. V-pRK415, *Vibrio anguillarum* harboring pRK415; VAGC, *Vibrio anguillarum* harboring pRK-λP$_R$-c I -E-S.nucA. Each line graph represents the mean based using 2 VAGC samples and T-bars indicate standard errors.

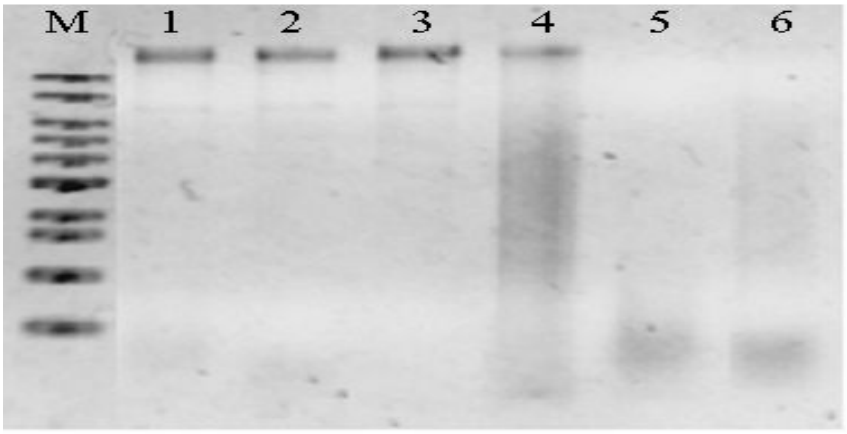


Fig. 8. Degradation of *V. anguillarum* genomic DNA was observed by electrophretic separation with 0.7% agarose gels and genomic DNA extraction kit. Samples were cheked per 2 hour by upshift of the incubation temperature from 27℃ to 42℃ (time point: 4, 6, 8 hour). lines M -1kb ladder (Bioneer); lines 1, 2, 3 -*V. anguillarum* harboring pRK415 (conrol); llines 4, 5, 6 - *V. anguillarum* harboring pRK-λP_R-c I -E-S.nucA.

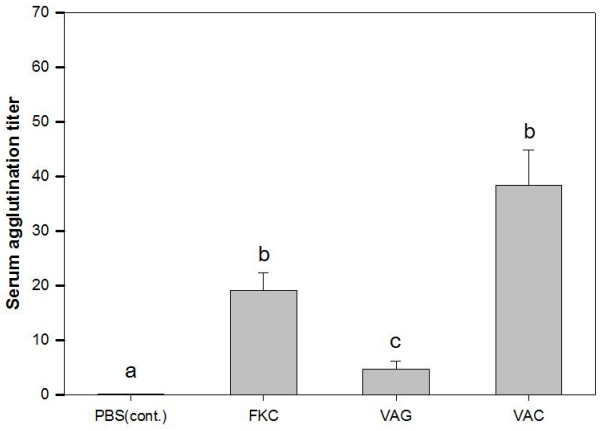
1.4. Protective efficacy of *V. anguillarum* ghosts (VAG) vaccine

After 1st and 2nd vaccination, 2 weeks later, samples performed challenge and was monitored that one fish a day died until 3day respectively in PBS group. The cumulative mortality of the PBS group became 60% same as dying to 3 of the 5 fishes. The rest of groups were not checked dead.

1.5. Agglutination activity of serum

Agglutination antigen was used *V. anguillarum* FKC or VAC respectively. Tilapia immunized with FKC, VAG or VAC showed significantly higher agglutination titer than control injected with PBS. And in two diagram of using the antigen as FKC or SNA, fish immunized with VAC showed significantly higher agglutination titer than fish immunized with VAG or FKC (Fig. 9).

(1)



(2)

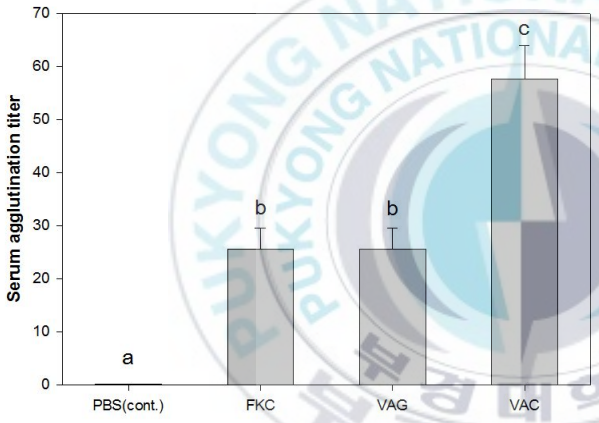


Fig. 9. Serum agglutination titer of tilapia immunized intraperitoneally with formalin-killed *V. anguillarum* (FKC), *Vibiro anguillarum* ghosts (VAG) and *Vibiro anguillarum* cadaver (VAC) or PBS alone (CON) at 2 weeks post boost immunization. (1), using *V. anguillarum* FKC antigen ; (2), using VAC antigen ; Values are mean and T-bars indicate standard error. Different letters on the bars indicate statistically differences at $P < 0.05$.

2 Analysis of antigens for subunit vaccine

2.1. Cloning and sequenceing of Flagellin-A or Outer membrane protein-U

FlaA or OmpU gene of *V. anguillarum* was amplified using PCR and cloned pGEM-T easy vector. After that, it was checked coding sequence flaA or OmpU.

The coding sequence of Fla A comprised 1,140 nucleotides and OmpU comprised 993 nucleotides. But expected cutting site of OmpU was decided using program of SignalP 3.0 Server -prediction results (Technical University of Denmark) on Center for biological sequence analysis(CBS). Cutting site was a lost part sites when it through a nuclear membrane and showed an unnecessary part sites as gene construction. Therefore this size of cutting site was from 5′- start codon (ATG) to 63 nucleotides. Cut OmpU size was 930 nucleotides and after attaching to start codon (ATG), total size was to be 933 nucleotides. And flaA (380 aa, amino acid) and OmpU (311 aa) molecular weight was estimated to approximately 43,655 Da and 37,098 Da.

2.2. Recombinant protein purification and Western blot analysis

FlaA or OmpU gene was amplified from genomic DNA of *Vibrio anguillarum* using PCR. And it was inserted into pET-28a vector. The recombinant plasmid, FlaA or OmpU was transferred into *E. coli BL21*(DE3) and was successfully expressed. Expressed His-tagged fusion FlaA or OmpU protein were measured by SDS-page and Western blot analysis (Fig. 10). And the His-tag fusion FlaA protein was appeared the band of approximately 43 KDa and His-tag fusion OmpU protein was appeared band of approximately 37 KDa. The purified recombinant His-tag fusion FlaA or OmpU proteins were immunized against rabbits to get antisera (rabbit anti FlaA or OmpU sera).

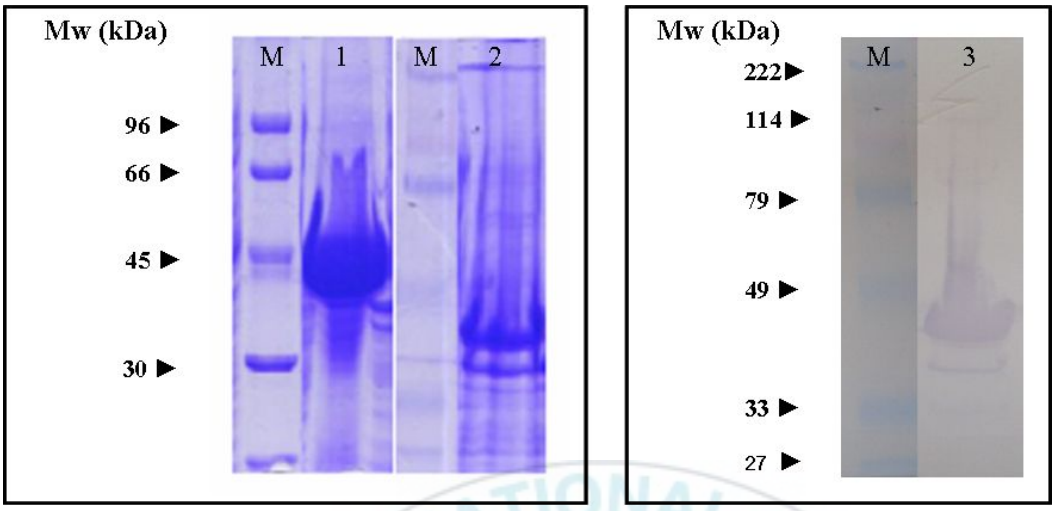


Fig. 10. SDS-PAGE and Western blot analysis FlaA and OmpU. (1) Coomassie blue stained SDS-PAGE gel of *E. coli BL21*(DE3) harboring pET28a-His-tag fusion FlaA or OmpU. (2) Western blot analysis for *E. coli BL21*(DE3) harboring pET28a-OmpU. lane M - prestained protein marker (Pierce); Lane 1 - Recombinant FlaA protein purified by a Ni-NTA His-Bind® Resin (Novagen) open column; Lane 2, 3 - Recombinant OmpU protein purified by a Ni-NTA His-Bind® Resin (Novagen) open column.

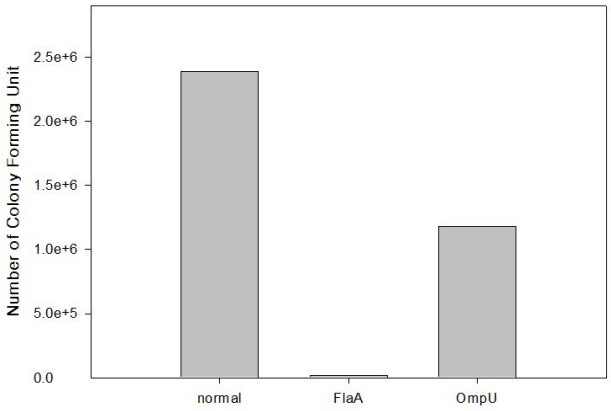
2.3. Rabbit anti FlaA or OmpU serum analysis

2.3.1. Bactericidal activity

The His-tagged fusion FlaA or OmpU antisera was obtained from rabbits.

The result of bactericidal activity test against antisera, colony forming unit (cfu) of rabbit antiserum against PBS (control) was appeared 2.39×10^6 cfu/㎖ and that rabbit antiserum against FlaA was measured 2.06×10^4 cfu/㎖ and that rabbit antiserum against OmpU was 1.18×10^6 cfu/㎖. And colony forming unit (cfu) of *V. haveyi* to control serum was appeared 2.53×10^6 cfu/㎖ and that rabbit antiserum against FlaA was measured 7.6×10^4 cfu/㎖ and that rabbit antiserum against OmpU was 2.01×10^5 cfu/㎖ (Fig. 11). Rabbit antiserum against FlaA or OmpU showed significantly higher bactericidal activities than sera obtained from rabbits injected with PBS.

(1)



(2)

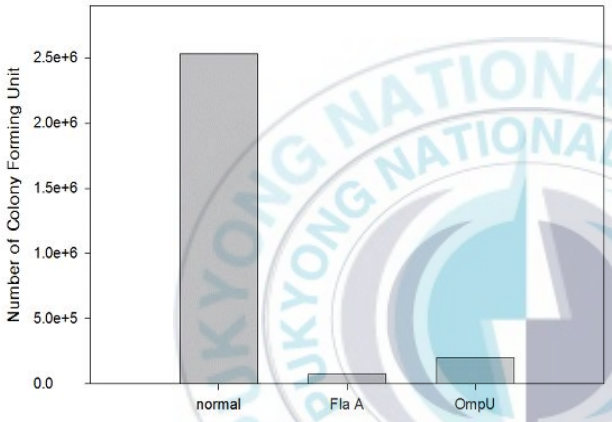


Fig. 11. The bactericidal activity of the sera were expressed colony forming unit (cfu)/ml as the number of *Vibrio anguillarum* (1) and *Vibrio harveyi* (2).

2.3.2. Antibody agglutination titers of serum

Antigen of agglutination titers was used *Vibrio anguillarum* FKC or VAC. The result of agglutination test in rabbit antiserum against FlaA or OmpU, agglutination titer wasn't checked using FKC antigen. But agglutination titer was monitored to all of their sera when using VAC antigen. Agglutination titers using SNA antigen was measured 2^{-5} on FlaA antiserum and 2^{-6} on OmpU antiserum. Rabbit antiserum against OmpU was higher than rabbit antiserum against FlaA. Maybe these differences doesn't have meaning (Fig. 12).

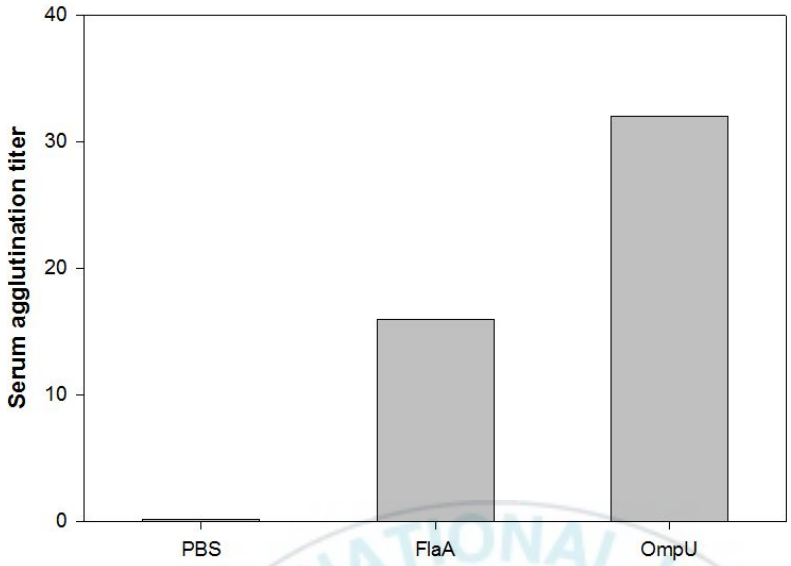
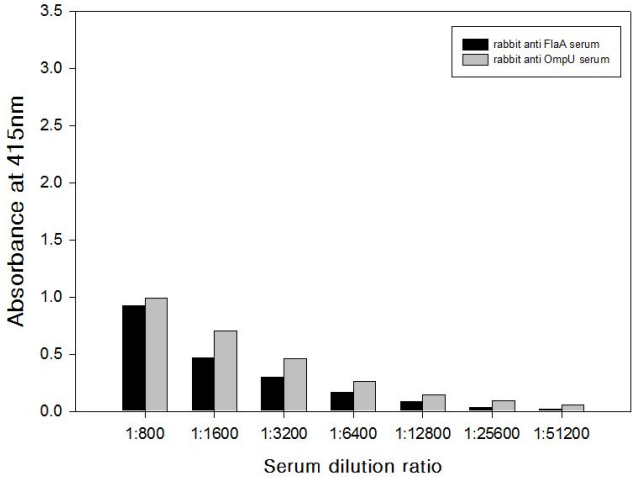


Fig. 12. Sera agglutination titer of rabbits immunized intraperitoneally with purified FlaA, OmpU protein or with PBS (control) after 2 weeks of the boost immunization.

2.3.3. ELISA (Enzyme-Linked Immunosorbent Assay)

Rabbit antiserum against FlaA or OmpU titers were measured by *V. anguillarum* FKC or VAC antigen. On the average ELISA titer using SNA antigen was significantly higher than ELISA titer using FKC antigen. In the titer using FKC antigen, a gap of rabbit antiserum against FlaA and rabbit antiserum against OmpU were insignificant showed, on the average, similar titer. In the titer using SNA antigen, rabbit antiserum against OmpU was measured double higher titers than rabbit antiserum against FlaA (Fig. 13).

(1)



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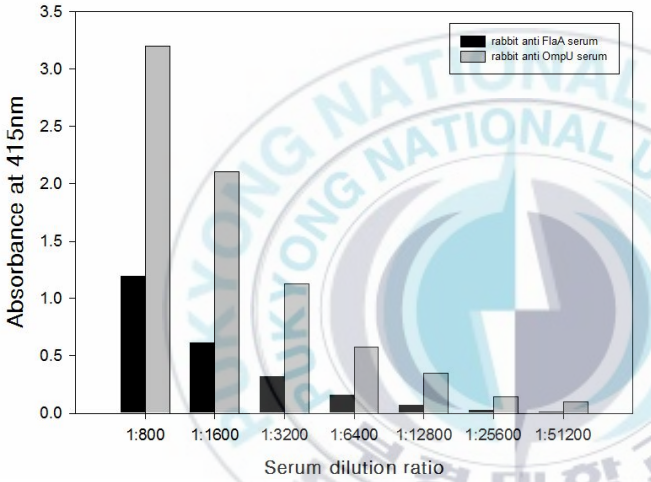

Fig. 13. ELISA antibody titer in the serum from the rabbits immunized with FlaA or OmpU. (1) using *V. anguillarum* FKC antigen ; (2) using VAC antigen.

Discussion

Vibriosis has been known as a killing disease to various aqua cultured and wide fish, and spread abroad. But, despite of the importance of that, perfectly treated technology was still not established. Nowadays, fishery occurred bacterial disease attempts to use a variety antibiotics but remedial value is not enough. Especially recently, considering several points as outstanding outbreak of bacteria with antibiotic resistance, environmental pollution, and human health risk of residual antibiotics, development of effective vaccine is the best way that can obviate a disease instead of treatment using antibiotics after an attack of a disease.

In the latter half of the 20th century, new developed research technology was get accomplished a lot. Among them, the vaccine has developed more and more, by growth gene-recombination technology. Thus, ghost bacteria is a new and advenced vaccine manufacture that can use a strong and safe vaccine to protect the various infectious diseases (Szostak *et al.*, 1996; Lubitz *et al.*, 1999).

The result, first, Using clones of *V. anguillarum* harboring the constructed plasmids (pRK-Ghost), we have successfully generated *Vibrio anguillarum* ghosts (VAG) by temperature-controlled expression of the phiX174 lysis E gene. We organized into a technology of the micro kernel that release cytoplasm outside, induce micro puncture to bacterial wall that programed a lysis gene (PhiX174 lysis gene E) using vector of expression. The bacteria were completely inactivated by induction of E gene expression.

However, intracellular genomic DNA and plasmid DNA were a little detected from the produced VAG, suggesting the presence of nonlysed or partially

lysed within the bacterial ghost. The presence of genomic DNA containing and an antibiotic resistance gene in the VAG may be problematic to use as a vaccine.

Second, technique of SNA bacteria were transformed in V. anguillarum using vector of expression, and expression of Staphylococcal nuclease A (SNA) was program. As a result, transformation of *V. anguillarum* with Ghost and SNA different kinds of plasmids was difficult, thus, we constructed a new dual vector. It is one plasmid that contained both lysis E gene and SNA gene expressed by each lambda P_R promoter, and successfully generated VAG deficient of genomic and plasmid DNA. This is named VAGC.

VAGC produced by this system were completely removed a living and division possibility, but as structure of bacteria wall was preserved nearly perfectly, it can be offered the surface epitope same as live bacteria. In particular, it was reported that the effect can be obtained a safe and convenient manufacture because of not performed a low temperature or not inducing degeneration of protein that contrary to customary production technology of vaccine. Therefore, this VAG or VAC vaccine system can offer the method that can overcome in the face as reduction of vaccine efficiency and a problem of inaccurate immune response caused by degeneration of antigenic protein frequently accompanying in the middle of manufactured process like a problem of customary inactivated vaccine used prevention about bacterial disease.

In the present study, we first tested the degree of protection and immune response induced after immunization of Tilapia with VAG or VAC vaccine. Although the mortality rate in the challenge experiments was low, the fish immunized with VAG, VAC or FKC showed higher survival rates than fish immunized with PBS.

In the present 2nd in vivo, significantly higher agglutination titers in Tilapia

antiserum against VAG, VAC or FKC than control. However, the agglutination titers of Tilapia immunized with VAG or VAC were greatly affected according to the antigen types (FKC and VAC) used in the analysis of serum agglutination activity, while fish immunized with FKC showed slight differences according to the used antigen types. Thus, the surface of VAG or VAC were expected to has the same antigenic characteristics of live V. anguillarum.

The VAG or VAC vaccine system, the efficiency need to be checked and applies to not only various fish diseases besides *Vibrio anguillarum* but also a germ infected to mammalian. And to make multi-pathogenic vaccine, gene encoding antigen which was reported to induce effective immune response in host was put into a ghost plasmid. Then two immunity about this is obtained by displaying oneself ghost bacteria and epitope expressed of gene about antigen kind of another pathogen. As a method like this, one vaccine will has be capable to defend various pathogens. Therefore, it is expected to need additional study about a multi antigen displayed an apitope of another disease on the surface of VAG and VAC using this study.

The other vaccine system, using subunit vaccine of Flagellin-A (FlaA) or Outer membrane protein-U (OmpU) and the serum from the rabbits immunized with FlaA or OmpU, it was measured the efficiency of the ELISA, agglutination test and bactericidal activity.

At first, we could confirm the result of bactericidal activity to *V. anguillarum*, rabbit antiserum against FlaA showed activity significantly 100 times higher than rabbit antiserum against PBS (control) and rabbit antiserum against OmpU showed activity 2 times higher. Also, V. harveyi showed result of bactericidal activity similar with V anguillarum. Thus, V. harveyi was expected similarity construction of FlaA and OmpU. And, antiserum against FlaA or OmpU might show significant bactericidal activity across *Vibrio* species.

In agglutination test, antigen for agglutination was used *V. anguillarum* FKC or VAC. Then it was not monitored in using FKC-antiogen and, in using SNA-antigen, titers was measured 2^{-6} on rabbit antiserum against FlaA and 2^{-7} on rabbit antiserum against OmpU. Especially rabbit antiserum against FlaA was showed effective bactericidal activity.

The ELISA was used antigen on *V. anguillarum* FKC or VAC, on the average, titers using VAC-antigen showed higher than titers using FKC-antigen.

Therefore, we were expected that epitope of outer membrane in *V. anguillarum* FKC was changed and, on the contrary, that epitope of outer membrane in *V. anguillarum* SNA was not changed. And FlaA seemed to be eligible for the candidate as subunit vaccine. In this study, we will be trying to find a way to make multi vaccine about that included subunit vaccine with vaccine of the whole surface cell like VAG and/or VAC.

Vibrio anguillarum Ghost bacteria 백신 개선 및 단위백신을 위한 항원 분석

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요약

양식 어류의 vibriosis는 우리나라를 비롯한 세계 각지의 양식 어류에 발생하는 주요 세균성 질병의 하나로 다양한 해산어, 담수어에 걸쳐 감염되는 것으로 보고되어 왔다. 어류의 Vibriosis를 예방하기 위한 백신에 대한 연구는 *Vibrio anguillarum*을 중심으로 이루어져 왔으며, 현재까지 주로 포르말린을 이용하여 세균을 불활화시킨것을 항원으로 사용하고 있다. 이러한 포르말린 사균 백신은 백신 제작과정에서 표면항원성의 변형을 야기 시킬 수 있으며, 이로 인한 백신의 방어효율 저하를 초래하게 된다. 따라서 이러한 기존 사균 백신의 단점을 극복하기 위해서는 백신제작과정에서 표면 항원성을 변화시키지 않는 것이 필요하며, 이러한 방법의 일환으로 기존 연구에서 *V. anguillarum* ghost (VAG) 세균이 개발된 바있다. Ghost 세균은 Bacteriophage phiX174 E lysis gene의 발현을 통해 세균의 내막과 외막사이에 작은 pore를 만들고, 그 pore 사이로 세균의 내부 세포질을 배출시킴으로써 세균의 외부는 살아있는 세균과 똑같은 형태 및 항원성을 유지하지만 속은 비어있는 상태의 세균을 말한다. 그러나 기존의 연구에서 제작된 VAG는 최종 백신 산물내에 완전히 내부 세포질이 밖으로 빠져 나가지 못한 일부 세균들로 인해 plasmid DNA와 genomic DNA가 잔존하는 것으로 나타났다. 따라서 본 연구에서는 VAG 백신의 어류 백신으로서의 안전성을 증진시키기 위해 최종 VAG 백신 산물내에 세균의 genomic DNA와 plasmid의 잔존을 없앨 수 있는 새로운 dual vector system을 개

발하였다. 이 dual vector는 lysis E gene cassette와 세균내 핵산을 용해시키는 Staphylococcal nuclease A (SNA)를 포함하는 cassette로 구성되었으며, 각각은 온도에 의해 조절되는 lambda phage Pr-cI system에 의해 그 발현을 유도하였다. Conjugation 법을 이용하여 *V. anguillarum*을 새로 개발한 dual vector로 형질전환한 후 ghost 세균 제작 효율 및 세균내 핵산의 잔존여부를 분석한 결과 기존의 VAG에 비해 ghost 효율이 증가되었으며, 또한 세균내에 핵산이 모두 분해되는 것을 확인할 수 있었다. VAG 백신과 FKC 백신의 면역유도능 차이를 분석하기 위해 틸라피아에 백신 실험을 한 후 각 실험구의 응집항체가를 분석한 결과에 있어서는 응집항체가 분석에 사용한 항원 type의 종류에 따라 상이한 결과가 나타났다. VAG로 면역화한 어류는 FKC로 면역화한 어류와 달리 *V. anguillarum* cadaver (VAC) 항원 type에 대해 유의적으로 높은 응집항체가를 보였으며 또한 boosting에 의해 항체가가 매우 높게 증가되는 현상을 보였다.

본 연구에서는 또한 단위백신의 개발 가능성을 분석하기 위해 *V. anguillarum*의 외막에 존재하는 epitope로, 독력과 운동성에 관련되어 있는 flagellin A (FlaA)와 담즙산과 생체막의 두께에 영향을 준다고 알려진 Outer mebrane protein U (OmpU)를 선택하여 이들의 재조합단백질을 제조한 후, 각각에 대한 토끼 항혈청을 제작하였다. 제작된 토끼 항혈청을 이용하여 *V. anguillarum* 및 *V. harveyi*등에 대한 bactericidal activity, ELISA 및 agglutination test를 실시한 결과 재조합 FlaA에 대한 토끼 항혈청이 유의적으로 높은 bactericidal activity, ELISA 및 agglutination titer를 나타내었다.

감사의 글

부경대학교에서 수산생명 의학도로서 마냥 새로운 맘으로 수업을 듣고 연구했던 일들이 엊그제 같은데 벌써 졸업을 앞두고 있다는 것이 믿어지지가 않습니다. 석사 2년이라는 시간은 제게 정신없이 앞만 보고 달려와 놓친 것, 잃은 것도 많은 시간이었지만, 그 만큼 제 인생 전체를 볼 때는 가장 큰 발전과 발돋움의 기간이었다고 생각됩니다. 이렇게 변화할 수 있게 도와주신 제 주위에 모든 분들께 감사의 말씀을 드립니다.

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