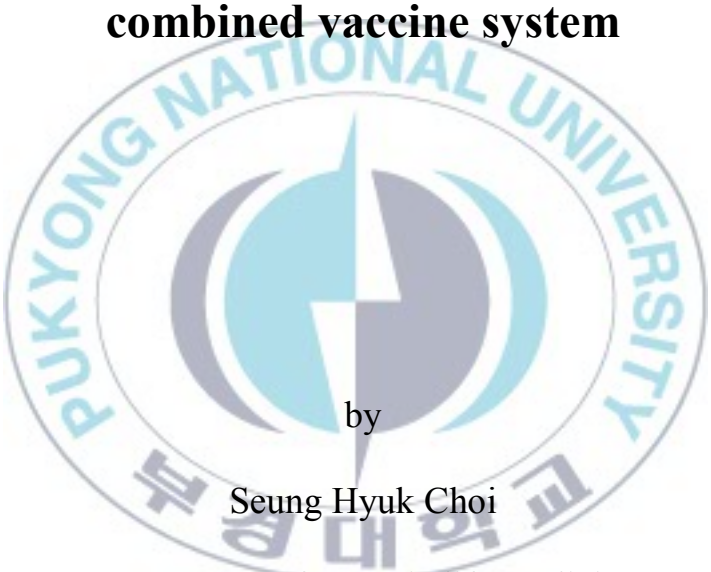
Thesis for the Degree of Doctor of Philosophy

Construction of recombinantly attenuated bacterial vaccines and their use for development of combined vaccine system



by

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재조합 약독화 세균 백신 제작 및 이를 기반으로 하는 복합 백신 시스템 개발

Advisor: Prof. Ki Hong Kim

by

Seung Hyuk Choi

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February 2012

Construction of recombinantly attenuated bacterial vaccines and their use for development of combined vaccine system

A dissertation

by

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ABSTRACT

The aims of the present study were development of live attenuated bacterial vaccines and further assessment of potential of the attenuated bacteria as vehicles for combined vaccines. To achieve the goals, firstly, we generated an auxotrophic *Edwardsiella tarda* mutant (Δ*alr* Δ*asd E. tarda*) by knock-out of two auxotrophic genes that play essential roles in bacterial cell wall biosynthesis - alanine racemase gene (*alr*) and aspartate semialdehyde dehydrogenase gene (*asd*). Virulence of the *E. tarda* mutant in olive flounder (*Paralichthys olivaceus*) was highly decreased, which was demonstrated by approximately 10^6 fold increase of LD_{50} dose compared to wild-type *E. tarda*. Immunization of fish with the auxotrophic *E. tarda* mutant through either intraperitoneal (i.p.) or oral routes induced significantly higher serum agglutination activities and clearly higher survival rates against *E. tarda* challenges. Secondly, to generate auxotrophic *E. tarda* mutant that has no need for supplementation of specific nutrients into culture medium, we newly constructed an *E. tarda* mutant by replacement of *alr* gene promoter with *c*I857-λP_R promoter system plus

another cI857 expression cassette that was driven by a constitutive promoter of *E. tarda* (EtPR C28-1), which allow the mutant bacteria to grow at temperature above 30°C without supplement of D-alanine but to disintegrate below 30°C. In vaccine experiment, olive flounder fingerlings immunized with the temperature-sensitive mutant *E. tarda* showed greatly decreased mortality, and a boost-immunization induced complete protection against *E. tarda* infection. Thirdly, to evaluate the potential of the auxotrophic *E. tarda* mutant as a delivery vehicle for a heterologous antigen, the mutant bacteria was transformed with antibiotic resistant gene-free plasmids harboring cassettes for GFP and *asd* expression, which induced significantly higher ELISA titer against GFP antigen in olive flounder by i.p. immunization. Fourthly, to further evaluate potential of the Δ*alr* Δ*asd E. tarda* as a delivery vehicle for DNA vaccine in fish, olive flounder were immunized with the *E. tarda* mutant harboring plasmids for CMV promoter-driven eGFP, which was successful to express the antigen in the internal organs and to induce humoral adaptive immunity against not only *E. tarda* that was used as a delivery vehicle but also eGFP that was used as the reporter antigen of DNA vaccine. Furthermore, fish immunized with the mutant *E. tarda* harboring plasmids for marine medaka β-actin promoter-driven VHSV G gene showed significantly higher serum neutralization activity and higher survival rates against VHSV challenges. The present results indicate that auxotrophic mutants of fish pathogenic bacteria are effective candidates for fish vaccines and can be used as vehicles for delivery of heterologous antigens or DNA vaccine plasmids.

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GENERAL INTRODUCTION

World production of fish through aquaculture has been rapidly increased to reach almost 50% of the world's food fish (FAO, 2008). Aquaculture in Korea has also continuously grown during the past decades, and provides a significant portion of food fish to people (Park, 2009). However, in accordance with the growing aquaculture production, losses caused by various infectious diseases have been greatly increased, which now become the most threatening factor to aquaculture industry.

Development of vaccines would be an effective way to control infectious diseases. Although various vaccines have been developed and commercialized to prevent mainly bacterial infections in fish, most of the vaccines are prepared through classical ways, such as formalin-killed or heat-inactivated forms (Castro N et al., 2008; Gutierrez MA et al., 1994). Recently, various biotechnological methods are applied to development of improved vaccines, and live vaccines based on recombinantly attenuated pathogenic bacteria have attracted much interest. Advantages of live bacterial vaccines include their mimicry of a natural infection, intrinsic adjuvant properties and their possibility to be administered through mucosal routes. While stimulating antibody and cellular responses, live attenuated vaccines induce both local and systemic immune responses (Clark et al., 2005). Live attenuated vaccines are generally more potent than killed vaccines in activating cellular immunity, and are protective against many wild-type strains encountered in the field (Klesius et al., 1999). This is an advantage over a killed bacterial vaccine that is usually limited in its capacity to provide cross-protection against different strains. Killed vaccines are able to stimulate specific antibody responses (Seder et al., 2000).

Traditionally attenuated bacterial vaccines were produced through selection of nonvirulent

mutant form after multiple passages in culture medium although the attenuation mechanism is not elucidated. In recent years, attenuated bacteria produced by deletion or modification of functionally-known target genes have more potential to improve applicability of the vaccines to more advanced vaccine types. The nature of recombinantly generated attenuated bacteria is deeply related to the function of knock-outed gene(s). Virulence-associated genes can be targets of deletion, however, if the deleted genes are main protective antigens, the vaccine efficacy would be greatly reduced. Furthermore, knock-out of certain virulent genes of pathogenic bacteriae might not guarantee the safety of hosts due to complex mechanisms of bacterial virulence. Compared to the knock-out of virulent genes, deletion or modification of auxotrophic genes responsible for key metabolic processes is more favorable to induce protective immune responses and to guarantee host safety (Oyston PC et al., 2010 ; Roland KL et al., 2010).

The use of attenuated bacteria as a delivery vehicle for heterologous antigens or DNA vaccine vectors is an efficient way to develop combined vaccines that can provide simultaneous protection against more than two pathogens. The auxotrophic mutant bacteriae undergo several limited replication in the host tissues after immunization, and during the course, express heterologous antigens or deliver DNA vaccine vectors. In mammals, expression of heterologous antigens in attenuated bacteria has already been widely agreed as an effective mean to achieve the combined vaccines (Medina et al., 2001; Mollenkopf et al., 2001; Kotton et al., 2004). However despite its importance, little information is available on the attenuated bacteria-based combined vaccines in fish pathogenic bacteria.

The aims of the present study were development of live attenuated bacterial vaccines of *E. tarda*, and further assessment of potential of the attenuated bacteria as vehicles for combined vaccines. To achieve the objectives;

In chapter I, the generation processes of two auxotrophic genes knock-out *Edwardsiella tarda*

(Δ*alr* Δ*asd E. tarda*) are described. Vaccine potential and availability as a delivery vehicle for heterologous antigen were assessed by intra-peritoneal immunization of olive flounder (*Paralichthys olivaceus*) with the auxotrophic *E. tarda* mutant carrying GFP-expressing plasmids. Protective efficacy against *E. tarda* challenge and antibody titers against *E. tarda* and GFP were analyzed. And in protective efficacy of the mutated *E. tarda* that was administered to olive flounder through an oral route was analyzed. To confirm whether both mucosal and systemic protective responses were elicited, the orally immunized fish were challenged through two routes, immersion and intraperitoneal injection.

In chapter II, a temperature-sensitive *E. tarda* mutant was generated by replacement of the promoter region of *alr* gene with λ phage P_R promoter system, which allows the bacteria to grow without supplement of D-alanine but to disintegrate at temperature below 30°C. Vaccine potential of the mutant *E. tarda* was evaluated by immunization of olive flounder.

In chapter III, to evaluate the potential of the mutated *E. tarda* as a delivery vehicle for DNA vaccine in fish, the auxotrophic mutant *ΔalrΔasd E. tarda* transformed with plasmids harboring a cassette for expression of the enhanced green fluorescent protein (eGFP) gene driven by a cytomegalovirus (CMV) promoter was used to immunize olive flounder through either intraperitoneal or oral routes, and the expression of eGFP in the internal organs of fish and generation of antibody against eGFP in fish were analyzed.

In chapter IV, to find more efficient promoter for DNA vaccines in fish, the power of β-actin promoter of marine medaka (*Oryzias dancena*) was compared with that of CMV promoter, in which marine medaka β-actin promoter was clearly stronger than CMV promoter. The availability of attenuated bacteria to deliver DNA vaccine, *ΔalrΔasd E. tarda* was transformed with eukaryotic expression vector harboring the marine medaka β-actin promoter-driven VHSV G gene and immunized to olive flounder. The protective efficacy against VHSV challenge and serum

neutralization titer were analyzed.

Chapter I.

Generation of two auxotrophic genes knock-out *Edwardsiella tarda* and assessment of its potential as a combined vaccine in olive flounder (*Paralichthys olivaceus*)

INTRODUCTION

A Gram-negative bacteria *Edwardsiella tarda*, the causative agent of edwardsiellosis, has been responsible for mass mortality and severe morbidity in a variety of freshwater and marine fish species (Thune et al., 1993; Plumb et al., 1999; Matsuyama et al., 2005). Although chemotherapeutics have been used to control bacterial diseases in cultured fish, several serious problems associated with excessive use of chemotherapeutics, such as antibiotic resistance, water pollution, and harmful effects on human health, have made many countries to reduce use of chemical drugs and to develop immuno prophylactic - measures, such as vaccines. Various types of vaccines against edwardsiellosis have been reported; formalin-killed vaccine (Gutierrez et al., 1994; Castro et al., 2008), ghost bacteria vaccine (Kwon et al., 2005, 2006, 2007; Lee et al., 2008) natural avirulent strain vaccine (Cheng ea al., 2010; Takano et al., 2010), recombinantly attenuated vaccine (Lan et al., 2007), subunit vaccine (Kawai., 2004; Liu et al., 2005; Hou et al., 2009; Jiao et al., 2009, 2010; Sun et al., 2010; Tang et al., 2010; Wang et al., 2010), and genetic vaccine (Jiao et al., 2009; Sun et al., 2010). However, up to now, only a formalin-killed *E. tarda* vaccine is used commercially in Korea.

As live attenuated pathogens possess protective antigens comparable to wild-type pathogens and retain the ability to colonize host tissues through natural infection routes, they can induce adaptive immune responses that are similar to responses induced by wild-type pathogens. Knock-out of genes related to virulence can be used as a strategy to produce attenuated bacterial vaccines, however, loss of a virulent gene(s) may lead to reduction in the effectiveness of adaptive immune responses. Furthermore, knock-out of one or two virulent genes may be not sufficient to guarantee safety. Attenuation of virulent bacteria by knock-out of a certain auxotrophic gene(s) is an

alternative to generate attenuated bacterial vaccines. As the nutrient(s) required for the auxotrophic mutants is not present in vertebrate hosts, the ability of the mutant bacteria to colonize and replicate in the hosts is gradually diminished, which make them suitable for live vaccines with high safety (Oyston et al., 2010; Roland et al., 2010). Several auxotrophic genes have been used for generation of attenuated bacteria that infect cultured fish: the *aroA* gene in *Aeromonas salmonicida* (Vaughan et al., 1993; Marsden et al., 1996), *Edwardsiella ictaluri* (Thune et al., 1999), and *Photobacterium damselae* spp. *piscicida* (Thune et al., 2003); the *purA* gene in *E. ictaluri* (Lawrence et al., 1997); and the *fur* gene in *Pseudomonas fluorescens* (Wang et al., 2009).

Cost and convenience are pivotal requirements for practical use of vaccines in aquaculture farms. The use of combined vaccines, which induce protective immunity against more than two kinds of pathogens, spares the expense related to vaccination processes and reduces the discomfort associated with multiple immunizations. One of the strengths of attenuated bacteria-based vaccines is the usefulness as presenters of heterologous antigens. In mammals, expression of heterologous antigens in attenuated bacteria has already been widely agreed as an effective mean to achieve the combined vaccines (Kotton et al., 2004; Medina et al., 2001; Mollenkopf et al., 2001). However despite its importance, little information is available on the attenuated bacteria-based combined vaccines in fish pathogenic bacteria.

Delivery of vaccines through an oral route would be the most convenient way for immunization of fish, especially for small fishes. Although fish do not have the Peyer's patches as in mammals, many leukocytes are disseminated throughout the intestinal mucosa and the hindgut has a strong antigen-uptake capacity (Rombout et al., 1993; Nelson and Secombes., 1997; Bernard et al., 2006), which might enable oral vaccines to induce adaptive immunity in fish. However, a weak ability of orally delivered antigens to induce protective immunity has been a major

bottleneck for development of effective oral vaccines. Vaccination with auxotrophic mutants of enteric pathogens would be a way to overcome the weak immunogenicity of oral vaccines. Attenuation by mutation of auxotrophic gene(s) would guarantee high safety in fish because of very limited replication of the bacteria in the hosts. Furthermore, as all virulent genes are comparable to those in wild-type bacteria, auxotrophic mutants would undergo the same process of wild-type bacteria in infection and induction of early immune responses, which are important for induction of effective immunity.

In the present study, we have produced two auxotrophic genes - alanine racemase (*alr*) and aspartate semialdehyde dehydrogenase (*asd*) - knock-out *Edwardsiella tarda* (Δ*alr* Δ*asd E. tarda*) for development of a combined vaccine system. Alanine racemase is an enzyme catalytic for change L-alanine to D-alanine that is essential for cell wall synthesis in all bacteria (Wasserman et al., 1984; Hayashi et al., 1990). As D-alanine is not present in vertebrates, *alr* knock-out bacteria are disintegrated after several limited replication in vertebrate hosts. Aspartate semialdehyde dehydrogenase involves in biosynthesis of lysine, threonine, and methionine, as well as diaminopimelic acid and isoleucine. The *asd* mutant bacteria obligatory require diaminopimelic acid (DAP), an essential constituent of bacterial cell wall, and will undergo lysis unless provided with DAP (Pavelka et al., 1996; Viola et al., 2001). The double knock-out of two auxotrophic genes has allowed the mutant *E. tarda* to express plasmid-based foreign protein gene without use of antibiotic resistant gene. In this study, green fluorescent protein (GFP) was used as a model foreign protein, and was produced by transformation of the mutant *E. tarda* with antibiotic resistant gene-free plasmids harboring cassettes for GFP and *asd* gene expression. To assess vaccine potential of the present combined vaccine system, olive flounder (*Paralichthys olivaceus*) were immunized with the GFP expressing mutant *E. tarda,* and analyzed protection efficacy against *E. tarda* challenge and antibody titers against *E. tarda* and GFP.

And the objective of this study was to further investigate the protective efficacy of the mutated *E. tarda* that was administered to olive flounder through an oral route. To confirm whether both mucosal and systemic protective responses were elicited, the orally immunized fish were challenged through two routes, immersion and intraperitoneal injection.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

Edwardsiella tarda NH1, isolated from moribund olive flounder (*Paralichthys olivaceus*) in a natural outbreak of edwardsiellosis from a commercial farm in Korea, was grown in tryptic soy broth (TSB, Difco) containing 1.5% NaCl at 25°C. *Escherichia coli* was cultured at 37°C with Luria-Bertani (LB, Difco) medium. When required, antibiotics (ampicillin) were added to the culture medium at the final concentration of 50 μg/ml. Diaminopimelic acid (DAP, Sigma) was added (50 μg/ml) for the growth of *E. coli* χ7213 (*Δasd*) (Rolands et al., 1999).

2. Fish

Juvenile olive flounder (*Paralichthys olivaceus,* 2-5 g) were obtained from a commercial hatchery in Korea. Fish were acclimated more than 2 weeks prior to initiating experiments, and water temperature was adjusted to 21-22°C throughout experiments.

3. Generation of alanine racemase gene (alr) and aspartate semialdehyde dehydrogenase (asd) gene knock-out E. tarda

The *alr* and *asd* knock-out *E. tarda* NH1 (Δ*alr* Δ*asd E. tarda*) was constructed by allelic exchange mutagenesis using ampicillin-resistant pCVD442 (Addgene plasmid 11074) (Donnenberg et al., 1991), a suicide vector containing *sacB* gene. LB medium containing 1.5% NaCl was used for culture of *E. tarda* used in this experiment. As the first step, the *alr* gene

knock-out *E. tarda* (Δ*alr E. tarda*) was generated by the following procedures. The N-terminal *alr* flanking region (330 bp nucleotides just before *alr* ORF) was PCR-amplified using primers *Alr*f-Fo-*Sac*I and *Alr*f-Re-*Xho*I. A fragment corresponding to 312 bp of C-terminal *alr* ORF was amplified by PCR using primers *Alr*b-Fo-*Nsi*I and *Alr*b-Re-*Xba*I. The amplified PCR products were run on an agarose gel (1.5%) and visualized by ethidium bromide staining. The fragment was purified using a Gel purification kit (Cosmo Genentech, Korea), subcloned into pGEM-T easy vector (Promega), and several clones were sequenced. After digestion of the T-vectors with enzymes corresponding to each fragment, the product was inserted into the plasmid pUC18 (GenScript), in which more restriction enzyme sites (*Sac*I-*Xho*I-*Aat*II-*Spe*I-*Sac*II-*Nsi*I-*Xba*I) were pre-added by insertion of an fragment prepared by annealing of two oligonucleotides (MCS-UP and MCS-DOWN). The pUC18 vector harboring the N-temnal and the C-terminal flanking regions of *alr* ORF was digested with *Sac*I and *Xba*I, and the resulting fragment was ligated into pCVD442 vector, which was predigested with the same enzymes. *E. coli* χ7213 was transformed with the constructed suicide plasmids (pCVD442alr$^-$), and screened on LB agar plates containing 50 μg/ml ampicillin. The wild-type *E. tarda* NH1 was conjugated with *E. coli* χ7213 containing the plasmid pCVD442alr$^-$. Transconjugants carrying *alr*$^-$ by a single crossover of allelic exchange were selected on LB agar supplemented with ampicillin. Secondary recombination of ex-conjugation colonies was performed on LB containing 10% (w/v) sucrose and 50 mM *D*-alanine. The resultant *Δalr E. tarda* was confirmed by PCR with primers *Alr* chro-for and *Alr*b-Re-*Xba*I. As the second step, the *asd* gene of Δ*alr E. tarda* was deleted and generated Δ*alr* Δ*asd E. tarda* by the following procedures. The N-terminal *asd* flanking region (312 bp nucleotides just before *asd* ORF) was PCR-amplified using primers *Asd*f-Fo-*Sac*I and *Asd*f-Re-*Xho*I. A fragment corresponding to 312 bp of C-terminal *asd* ORF was amplified by PCR using primers *Asd*b-Fo-*Nsi*I and *Asd*b-Re-*Xba*I. PCR products were purified using gel purification kit, and subcloned into pGEM T-easy vector. Several clones were sequenced using an automatic sequencer (Applied

biosystems). After digestion of the T-vectors with enzymes corresponding to each fragment, the product was inserted into the plasmid pUC18-MCS. The pUC18 vector harboring the N-temnal flanking region of *asd* ORF and the C-terminal *asd* ORF was digested with *Sac*I and *Xba*I, and the resulting fragment was ligated into pCVD442 vector, which was predigested with the same enzymes. *E. coli* χ7213 was transformed with the constructed suicide plasmids (pCVD442asd$^-$), and screened on LB agar plates containing 50 μg/ml ampicillin. The Δ*alr E. tarda* was conjugated with *E. coli* χ7213 containing the plasmid pCVD442asd$^-$. Transconjugants carrying *asd*$^+$ by a single crossover of allelic exchange were selected on LB agar supplemented with ampicillin and 50 mM *D*-alanine. Secondary recombination of ex-conjugation colonies was performed on LB containing 10% (w/v) sucrose, 50 mM D-alanine, and 50mM DAP. The resultant Δ*alr* Δ*asd E. tarda* was confirmed by PCR with primers *Asd* chro-for and *Asd*b-Re-*Xba*I. Sequences of oligonucleotides used in this study are given in Table 1-1.

4. Construction of antibiotic resistance gene-free and heterologous gene-expressing vector

An antibiotic resistance gene-free vector equipped with the *asd* gene and a heterologous gene (GFP) expressing cassettes was constructed. The *asd* gene expression was driven by a weak constitutive promoter (G02) that was selected from the previously constructed *E. tarda* promoter trap library (Choi et al., 2010). Briefly, the putative promoter region (G02) was PCR-amplified by using a pair of primers (G02 *Aat*II F and G02 *Spe*I R), cloned into pGEM-T vector, and sequenced for confirmation. After *Aat*II and *Spe*I digestion of the vector, the putative promoter region was inserted into T-vector that was predigested with the same enzymes. The rrnBT1 termination sequence, which was made by annealing of two oligonucleotides (rrnBT1-Fo-*Sac*I and rrnBT1-Re-*Nsi*I), was inserted into the *Sac*I/*Nsi*I predigested vector, and named as pG02-rrnBT1. The *asd* ORF of *E. tarda* was amplified from the template *E. tarda* genomic DNA using a pair of PCR

primers, ASD-ORF-Fo and ASD-ORF-Re, of which 5'-ends were designed to possess *Spe*I and *Eco*RI sites, respectively. After cloning and sequence confirmation, the T-vector harboring *asd* ORF was digested with *Spe*I and *Eco*RI enzymes and ligated to the *Spe*I/*Eco*RI-predigested plasmid pG02-rrnBT1, and designated as pG02-ASD. The antibiotic resistance gene (Amp^R) in pG02-ASD was removed by digestion of the plasmid with *Dra*I and *Nar*I, then a sequence containing multiple cloning sites (*Dra*I-*Bgl*II-*Nco*I-*Sca*I-*Nsi*I-*Nar*I) was inserted into the digested plasmid, resulting in pG02-ASD-MCS. The GFP expressing cassette driven by a strong constitutive promoter (EtPR C28-1) of *E. tarda* (Choi et al., 2010) was cut by digestion of plasmid pEtPR-GFP with *Apa*I and *Nsi*I, then, ligated to the pG02-ASD-MCS, and designated as pG02-ASD-EtPR-GFP.

The Δ*alr* Δ*asd E. tarda* was transformed with the pG02-ASD-EtPR-GFP and the expression of *asd* gene was confirmed by reverse transcriptase PCR (RT-PCR). *E. tarda* NH1 and Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-EtPR-GFP were harvested by centrifugation, and total RNA was extracted using RNeasy Mini-Kit (Qiagen) according to the manufacturer's instruction. To synthesize first-strand cDNA, 1 μg of total RNA treated with DNase was incubated with 0.5 μl of random primer (0.5 μg/ml, Promega) at 80°C for 5 min and further incubated at 42°C for 60 min in reaction mixture containing 2 μl of each 10 mM dNTP mix (Takara), 0.5 μl of M-MLV reverse transcriptase (Promega) and 0.25 μl of RNase inhibitor (Promega) in a final reaction volume of 10 μl. PCR was performed with 2×Prime Taq Premix (Genet Bio) and 1 μl of 10^{-1} diluted cDNA template. Thermal cycling conditions were 1 cycle of 3 min at 95°C (initial denaturation) followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. The reaction products were visualized by electrophoresis in a 0.7% agarose gel.

5. *In vitro growth of recombinant E. tarda*

To measure the growth of *alr* and *asd* knock-out *E. tarda* under condition of D-alanine and DAP absence, the mutant bacteria were cultured to an OD_{600} of 0.2-0.3 in D-alanine and DAP supplemented LB and washed with phosphate buffered saline (PBS). The washed bacteria were cultured with LB without supplementation of D-alanine and DAP, or LB supplemented either D-alanine alone or DAP alone, and sampled at various time points to measure cell density at OD_{600}. Growth of the auxotrophic mutant *E. tarda* harboring pG02-ASD-EtPR-GFP was also measured in the presence or absence of D-alanine in culture medium.

6. Determination of LD_{50} of Δalr Δasd E. tarda harboring pG02-ASD-EtPR-GFP

To determine virulence of the Δ*alr* Δ*asd E. tarda* relative to wild-type *E. tarda*, olive flounder (approximately 5 g in body weight) showing no external and behavioral signs of illness were intraperitoneally (i.p.) injected with graded doses (10^2 – 10^8 CFU/ml; 10 fish per each dose) of the mutant *E. tarda* or wild-type *E. tarda*. The fish were observed for 14 days, and any dead fish were removed for confirmation of *E. tarda* presence. The LD_{50} was calculated by the method of Reed and Muench (Reed et al., 1938).

7. In vivo persistence of Δalr Δasd E. tarda harboring pG02-ASD-EtPR-GFP

Experiment 1. (i.p.)

To know the extent of the in vivo persistence of the Δ*alr* Δ*asd E. tarda,* olive flounder were infected with 10^7 CFU of the knock-out *E. tarda* by i.p. injection. At 2, 4, 6, 12, 24, 48, 96 and 168 h post injection, three fish were randomly removed from each tank, and euthanized by transfer to water containing MS-222 (Sigma). From each fish, liver, spleen, and head kidney were

taken aseptically, suspended in 0.5 ml of PBS, weighed, and pulverized. Each tissue lysate was tenfold serially diluted in PBS, and dropped onto *Salmonella Shigella agar* (SS agar, Difco) supplemented with 50 mM D-alanine and 50 mM DAP to count the bacterial colonies. The colonies of Δ*alr* Δ*asd E. tarda* were identified by observation of fluorescence.

Experiment 2. (orally)

The persistence of orally-administered Δ*alr* Δ*asd E. tarda* in internal organs of olive flounder was investigated by analysis of the change in number of colony forming unit (CFU) according to time-lapse. Fish (body weight 2-5 g) were confirmed free-from *E. tarda* before experiments by a routine bacterial examination and a serum agglutination test against formalin-killed *E. tarada* from randomly sampled 10 fish. Fish were intubated with 1 x 10^8 CFU/fish of the *Δalr Δasd E. tarda* that habors pG02-ASD-EtPR-GFP using gastric tubes. At various time points (2, 4, 6, 12, 24, 48, 96 and 168 h) after the intubation, three fish were randomly sampled from each tank, and euthanized by transfer to water containing MS-222 (Sigma). From each fish, liver, spleen, head kidney, and intestine (except stomach) were taken aseptically, suspended in 0.5 ml of phosphate buffered saline (PBS), weighed, and pulverized. Each tissue lysate was tenfold serially diluted in PBS, and dropped onto *Salmonella Shigella agar* (SS agar, Difco) supplemented with 50 mM *D*-alanine and 50 mM diaminopimelic acid (DAP) to count the bacterial colonies.

8 . Vaccination and challenge

Experiment 1. (i.p.)

One hundred forty-four olive flounder fingerlings (2-5 g) were randomly divided into 4 groups with two replicates, and reared in eight 250 L tanks (18 fish/tank) at 20-22°C. Fish were

fed with a commercial pellet and acclimatized for 2 weeks before being immunized, and were anesthetized with MS222 (Sigma) just before injection of the vaccine or blood sampling. Fish in each group were i.p. immunized with 1 x 10^5, 10^6, or 10^7 CFU/fish of Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-EtPR-GFP in 20 μl of PBS. A group that received PBS (20 μl) represented the control. At 3 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, 6 fish in each group were bled to obtain serum for agglutination, ELISA, and Western blot analyses, and the remnant fish were i.p. challenged by 2 x 10^2 CFU/fish of the wild-type *E. tarda* NH1. Deaths were recorded for 21 d post-challenge. Dead fish were collected daily and kidney samples were streaked on SS agar to confirm the presence of *E. tarda*.

Experiment 2. (orally)

Olive flounder fingerlings (body weight 2-5 g) obtained from a local fish farm in Korea were acclimatized in the laboratory tanks for 2 weeks before being used for experiment. One hundred eighty fish were randomly divided into 6 groups of two replicates, and reared in six 500 L tanks (30 fish/tank) at 20-22°C. Fish were deprived of food for 24 h before immunization and blood sampling. Each fish in each experimental group was orally inoculated with 50 μl of 10^8 or 10^9 CFU/fish of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP, and fish in the control groups were orally administered with 50 μl of PBS alone. At 2 weeks post-primary immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, 6 fish in each group were randomly sampled and collected skin mucus, gut mucus, and serum. The remnant of fish in each tank were divided into two subgroups and were challenged either by i.p. injection with 2 x 10^2 CFU/fish of the wild-type *E. tarda* NH1 or by immersion with 10^9 CFU/3 L of the *E. tarda* NH1 for 3 h. Mortality of the fish were monitored for 21 days post-challenge. Dead fish were collected daily and kidney samples

were streaked on SS agar to confirm the presence of *E. tarda*.

To collect skin mucus, each fish was placed in an empty, sterile vinyl-bag, and the secreted mucus was collected. Intestinal mucus was collected by removing the digestive tract except stomach, slitting it lengthways, and then washing with PBS to remove the contents. Then, the mucus was scraped with a scalpel, and transferred to previously weighed microcentrifuge tubes. The skin and intestinal mucus suspensions were centrifuged at 10,000 g for 20 min at 4°C, and supernatant was stored at -70°C. Blood collected from caudal vein was allowed to clot at 4°C for 6 h, and serum was obtained after centrifugation at 5,000 g for 15 min and stored at -70 °C.

9. Agglutination activity of serum against E. tarda

For preparation of formalin-killed *E. tarda* (FKC), wild-type *E. tarda* NH1 was grown for 24 h at 25°C in TSB containing 1.5% NaCl. For FKC preparation, formalin was added to a 24 h culture of the bacterium to make the final concentration 0.5%. After 24h incubation, cells were washed three times with PBS and resuspened in 10 ml PBS. The suspensions were streaked on TSA containing 1.5% NaCl for checking sterility and stored at 4°C until use.

The agglutination test was conducted in 'U'-shaped microtitre plates. The mucus and serum collected from fish in each group were serially diluted two-fold, to which a constant amount of FKC (approximately 4 mg/ml) were added and kept overnight at room temperature. The agglutination activity was determined as the first serum dilution where no agglutination occurred, and expressed as the reciprocal of that dilution.

10. ELISA

Mucus and Serum antibody titers against *E. tarda* FKC and GFP in each group of fish were measured by ELISA analysis. The flat-bottomed 96-well plates (Corning) were coated with 50 µl of recombinant GFP (100 µg/ml) stock and incubated at room temperature for overnight. The plates were then washed thoroughly with PBST (PBS containing 0.1% Tween 20) and blocked with 200 µl of 2% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Subsequently, the plates were washed thoroughly with PBST and incubated with 100 µl of olive flounder serum (1:100) at 37°C for 1 h. After washing 3 times, incubated with 100 µl of rabbit antiserum against olive flounder IgM (1:1000) for 1 h, washed 3 times with PBST, and further incubated with 100 µl of goat anti-rabbit IgG conjugated with alkaline phosphatase (1:1000, Santa Cruz Biotechnology) for 1 h at room temperature. The plates were washed with PBST and developed with the substrate p-nitrophenyl phosphate in substrate buffer at dark room. After 30 min incubation, the optical density was measured at 415 nm using an automated ELISA reader (Bio-Rad).

11 . Western blot

To determine whether the immunized olive flounder produced an antibody against GFP or not, we conducted western blot analysis. Purified recombinant GFP protein were mixed with SDS sample buffer (10% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 0.3M Tris-HCl pH 6.8, 0.05% bromophenol blue, 50% glycerol), boiled for 10 m at 95°C, and resolved by 10% (wt/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membrane with transfer buffer (12 mM Tris-HCl, 96 mM glycine, 20% methanol, pH 8.3) by using a Trans-Blot SD Cell (Bio-Rad) at 25V for 90 min. The membrane was blocked with blocking solution (3% BSA in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with olive flounder serum (1:100) for 2 h at RT and washed. The membrane was incubated with rabbit

antiserum against olive flounder IgM (1:1000) for 2 h at RT. After washing 3 times, the membrane incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2000, Santa Cruz) for 1.5 h at RT. After washing off unbound thirdly antibody, the specific antigen-bound antibody was visualized with nitroblue tetrazolium and 2-bromo-2-chloro-2-indoly phosphate (NBT-BCIP) substrate buffer (Sigma).

12. Statistical analyses

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

Table 1-1. Oligonucleotides used in this study

Primers	Sequence (5'-3')
MCS-UP	CCTCGAG GACGTCACTAGTCCGCGGATGCATT
MCS-DOWN	CTAGAATGCATCCGCGGACTAGTGACGTCCTCGAGGAGCTC
***Alr*f-Fo-*Sac*I**	GAGCTC GACCTTTAACGGTCAGTGGTCG
***Alr*f-Re-*Xho*I**	CTCGAG GCAATAAGGCGACTGTGCG
***Alr*b-Fo-*Nsi*I**	ATGCAT GATACCCGTCTCGGGGTG
***Alr*b-Re-*Xba*I**	TCTAGACTACGCCTCTTCGCCGATATA
***Asd*f-Fo-*Sac*I**	GAGCTC TCCCCCTGCGGTGC
***Asd*f-Re-*Xho*I**	CTCGAG TGCGAGGTTGAAAAAAAGACCG
***Asd*b-Fo-*Nsi*I**	ATGCAT AGGCGATTCCGATCGATGG
***Asd*b-Re-*Xba*I**	TCTAGA CGGCGGCGCCCCACAG
***Alr* chro-for**	ATCATGTTCATCTATCGCGATGAGGTCTAT
***Asd* chro-for**	GCGATCAGTATTGCGCGG
ASD-ORF-Fo	ACTAGT ATGAAAAACGTTGGTTTTATCGGCTGG
ASD-ORF-Re	GAATTC CTAGAGCAGCAGCCTCAGCATACGGC
rrnBT1-Fo-*Sac*I	ATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATAGCT
rrnBT1-Re-*Nsi*I	ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATTGCA
G02 *Aat*II F	GACGTC CGTCCGCGCCGTCGGTAAGCG
G02 *Spe*I R	ACTAGT AGAGAAGAATGCCGGCGGGAAGATC

Underlined nucleotides indicate cutting sites for restriction enzymes

RESULTS

1. Generation of alanine racemase gene (alr) and aspartate semialdehyde dehydrogenase gene (asd) knock-out E. tarda (Δalr Δasd E. tarda)

Using allelic exchange technology, alanine racemase gene (*alr*) and aspartate semialdehyde dehydrogenase gene (*asd*) knock-out *E. tarda* (*Δalr Δasd E. tarda*) was generated. Knockout of the two genes was confirmed by PCR amplification, in which the size of PCR products of mutant *E. tarda* was smaller than that of wild-type *E. tarda* because of partial deletion of the corresponding genes (Fig. 1-1 A). In vitro growth of the mutant *E. tarda* was similar to wild-type *E. tarda* when D-alanine and DAP were supplemented to growth medium. However, without D-alanine and/or DAP supplementation, the mutant showed very limited growth (Fig. 1-1 B).

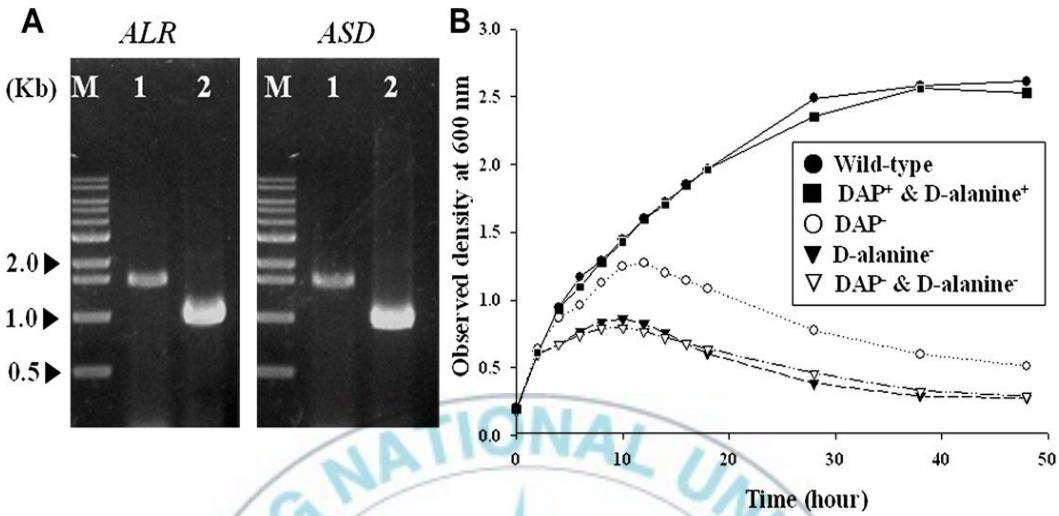


Fig.1-1. Generation of alanine racemase gene (alr) and aspartate semialdehyde dehydrogenase gene (asd) knock-out E. tarda (Δalr Δasd *E. tarda*) (A) Confirmation of *alr* & *asd* knock-out *Edwardsiella tarda* (*Δalr Δasd E. tarda*) by PCR amplification. Lane 1 is the PCR fragment amplified from genomic DNA of the wild-type *E. tarda* NH1. Lane 2 is the PCR fragment amplified from genomic DNA of the *Δalr Δasd E. tarda*. Lane M is a 1 kb ladder (Bioneer, Korea). PCR products of mutant *E. tarda* were smaller than that of wild-type *E. tarda* because of partial deletion of the corresponding genes. (B) The growth of *alr* & *asd* knock-out *Edwardsiella tarda* (*Δalr Δasd E. tarda*) under condition of D-alanine and/or diaminopimelic acid (DAP) absence in culture medium. The bacteria were cultured to an OD_{600} of 0.2-0.3 in D-alanine and DAP supplemented LB (DAP^+ & D-$alanine^+$), and, then, transferred to LB without supplementation of D-alanine and DAP (DAP^- & D-$alanine^-$), or LB supplemented either D-alanine alone (DAP^-) or DAP alone (D-$alanine^-$). Wild-type *E. tarda* was grown in LB. The cultured bacteria were sampled at various time points to measure cell density at OD_{600}.

2. Constuction of plasmid pG02-ASD-EtPR-GFP

Plasmid pG02-ASD-EtPR-GFP, an antibiotic resistance gene-free vector equipped with an *asd* gene and GFP gene expressing cassettes, was constructed (Fig. 1-2 A). The *Δalr Δasd E. tarda* transformed with pG02-ASD-EtPR-GFP showed a similar growth pattern of wild-type *E. tarda* when D-alanine was supplemented in the medium. However, without D-alanine, the growth of mutant *E. tarda* harboring pG02-ASD-EtPR-GFP was severely limited (Fig. 1-2 B). The expression of GFP in the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP could be observed even with naked eyes. The expression of *asd* gene in the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP was analyzed using RT-PCR, and two bands encoding the full ORF of *asd* gene on the pG02-ASD-EtPR-GFP (larger band) and the mutated *asd* gene in the chromosome of the mutant bacteria (smaller band) were amplified (Fig. 1-2 C).

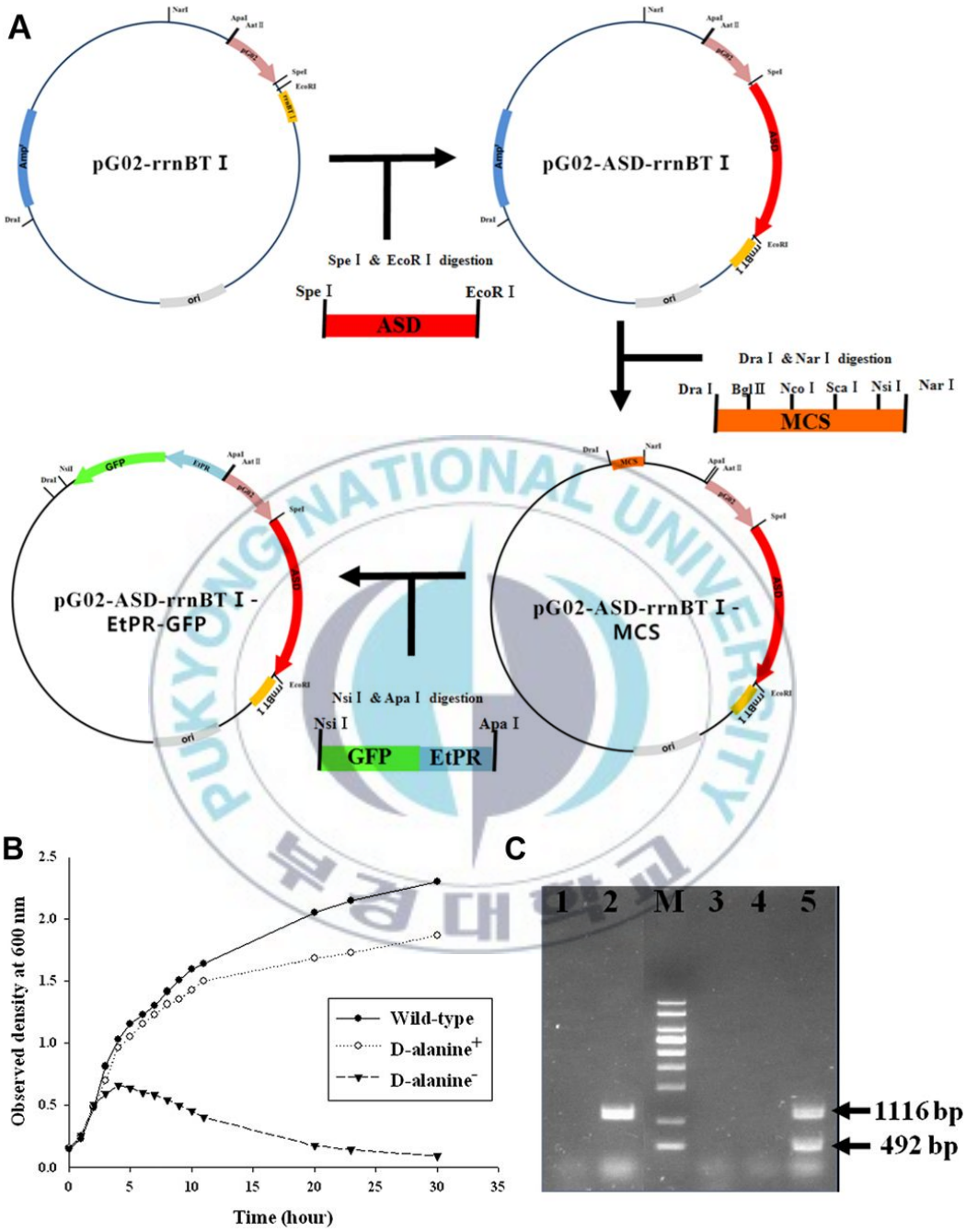


Fig.1-2. Constuction of plasmid pG02-ASD-EtPR-GFP (A) Plasmid pG02-ASD-EtPR-GFP constructed in the present study. Vector pG02-ASD-EtPR-GFP contains two cassettes. One has a

strong constitutive promoter (EtPR) cloned from *Edwardsiella tarda* and the green fluorescent protein (GFP) gene as a model foreign protein. The other cassette consists of a weak constitutive promoter (G02) of *E. tarda*, aspartate semialdehyde dehydrogenase (ASD) gene, and a transcriptional terminator (rrnBT1). The details of the construction are given in section of materials and methods. (B) The growth of *Δalr Δasd E. tarda* harboring plasmid pG02-ASD-EtPR-GFP under condition of presence or absence of D-alanine in culture medium. (C) Confirmation of *asd* gene expression by RT-PCR. Total RNA was isolated from wild-type *Edwardsiella tarda (Lane 1 and 2) or alr* & *asd* knock-out *E. tarda* harboring plasmid pG02-ASD-EtPR-GFP (Lane 3, 4, and 5). Primers used in Lane 1 and 3 were Asd chro-for and ASD-ORF-Re, which were used for confirmation of genomic DNA contamination; in Lane 4 were G02 *Aat*II F and ASD-ORF-Re, which were used for confirmation of plasmid DNA contamination; in Lane 2 and 5 were ASD-ORF-Fo & ASD-ORF-Re, which used for amplification of *asd* gene ORF. Lane M is a 1 kb ladder (Bioneer, Korea).

3. Virulence and persistence of Δalr Δasd E. tarda in olive flounder (Experiment 1)

The LD_{50} of wild-type *E. tarda* NH1 in olive flounder fingerling by i.p. injection was approximately 1 x 10^2, whereas the LD_{50} of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP was 1 x 10^8.

Colony number of the mutant bacteria harboring pG02-ASD-EtPR-GFP isolated from the spleen, kidney, and liver of i.p. infected fish was increased at an early period (from 2 h to 6 h), then, gradually decreased according to time lapse, and no colony was detected at 168 h (Fig. 1-3 A). The mutant bacteria containing the expression vector were easily identified by fluorescence (Fig. 1-3 B).

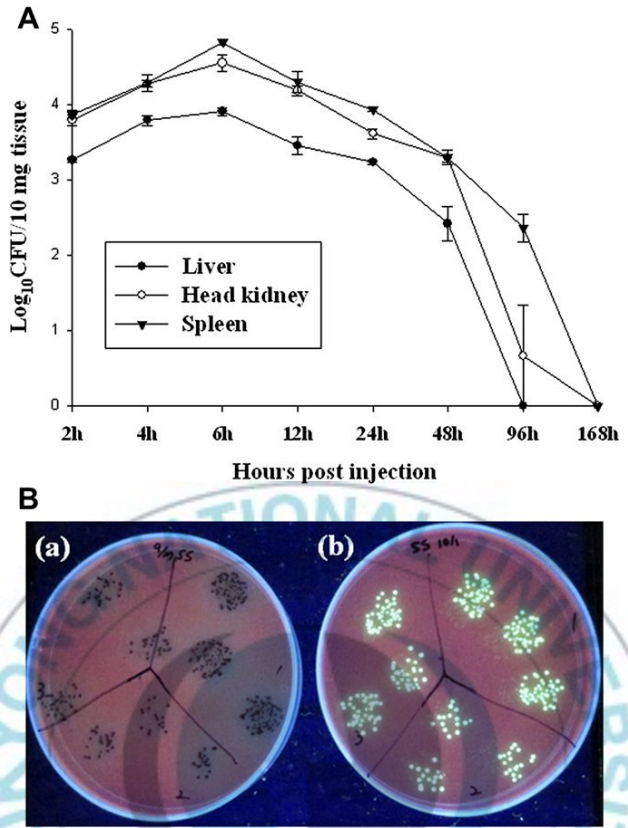


Fig.1-3. Virulence and persistence of Δalr Δasd E. tarda in olive flounder (A) The mutant *Edwardsiella tarda* (*Δalr Δasd E. tarda*) harboring plasmid pG02-ASD-EtPR-GFP recovered from each internal organ (liver, head kidney, and spleen) of olive flounder (*Paralichthys olivaceus*) that were intraperitoneally (i.p.) injected with 10^7 CFU of the bacteria. At 2, 4, 6, 12, 24, 48, 96 and 168 h post injection, the mutant bacteria recovered from each tissue were counted by plating onto *Salmonella Shigella* agar (SS agar) supplemented with 50 mM D-alanine and 50 mM DAP. Each point represents mean ± standard deviation of the bacteria numbers per 10 mg of each tissue. (B) Observation of (a) wild-type *E. tarda* NH1 and (b) *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP on UV light. The colonies of Δ*alr* Δ*asd E. tarda* were easily identified by observation of fluorescence.

4. Vaccine efficacy of Δalr Δasd E. tarda (Experiment 1)

In challenge experiment, groups of fish immunized with 10^7 CFU of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP showed no mortality, which was irrespective to boost immunization (Fig. 1-4). The cumulative mortality rates of fish immunized with 10^6 or 10^5 CFU of the mutant bacteria were lowered by a boost immunization (Fig. 1-4 B). All dead fish were positive for *E. tarda*. On the other hand, no colony was observed from the survived fish that were randomly sampled (5 fish) from the groups immunized with 10^7 CFU of the mutant *E. tarda*.

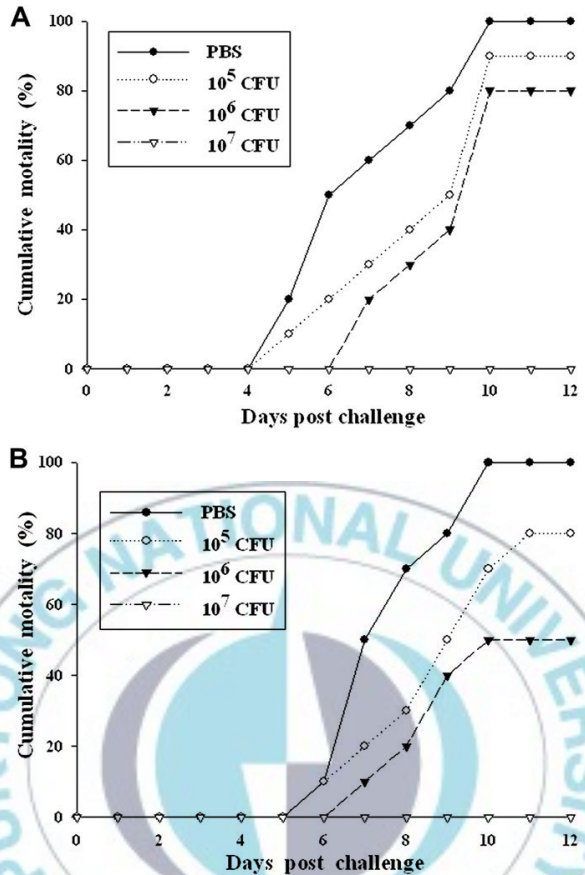


Fig.1-4. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) immunization. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) immunized by intraperitoneal (i.p.) injection of phosphate buffered saline (PBS), or *Δalr Δasd Edwardsiella tarda* harboring pG02-ASD-EtPR-GFP (10^5, 10^6, and 10^7 CFU/fish). At 3 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, fish were i.p. challenged by 2 x 10^2 CFU/fish of the wild-type *E. tarda* NH1. (A) Cumulative mortality of groups of fish administered only a primary immunization and challenged after 6 weeks of the primary immunization. (B) Cumulative mortality of groups of fish administered primary and boost immunizations, and challenged after 3 weeks of the boost immunization.

5. Serum agglutination activity, ELISA, and Western blot (Experiment 1)

Fish immunized with 10^7 of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP showed significantly higher agglutination titer against formalin-killed *E. tarda* than fish immunized with 10^6 or 10^5 of the mutant bacteria (Fig. 1-5 A). The agglutination titer was significantly increased by a boost immunization in fish injected with 10^7 and 10^6 bacteria.

ELISA titers against GFP by a primary immunization was significantly higher only in fish injected with 10^7 of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP than control fish (Fig. 1-5 B). Fish immunized with 10^6 of the mutant bacteria showed significantly higher ELISA titer than control fish by the boost immunization. The presence of serum antibody against GFP in fish immunized with 10^7 of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP was further confirmed by Western blot analysis (Fig. 1-6).

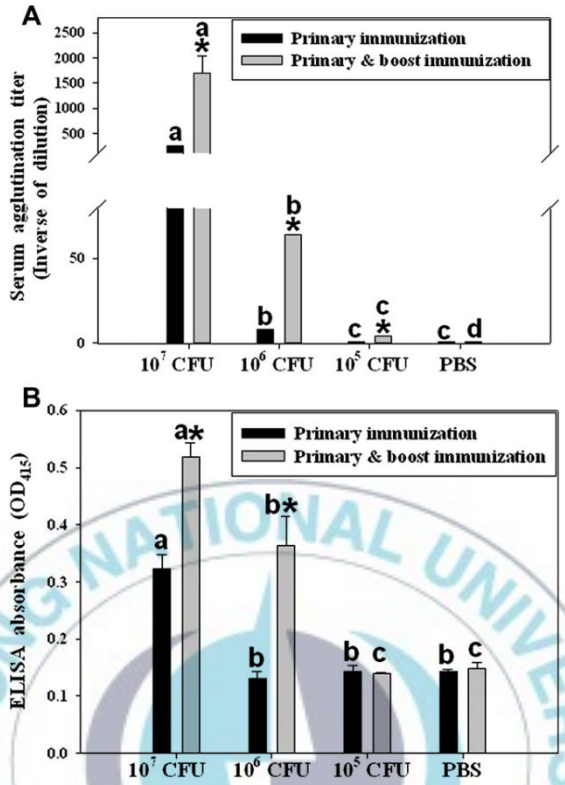


Fig.1-5. Serum agglutination activity, ELISA (Experiment 1). (A) Agglutination activity against formalin-killed *Edwardsiella tarda* NH1, (B) ELISA titer against green fluorescent protein (GFP) in sera of olive flounder (*Paralichthys olivaceus*) immunized by intraperitoneal (i.p.) injection of phosphate buffered saline (PBS), or *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP (10^5, 10^6, and 10^7 CFU/fish). At 3 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, 6 fish in each group were bled to obtain serum for agglutination, ELISA, and Western blot analyses. Values are mean ± standard deviation. Asterisks on the bars represent significantly different at $P < 0.05$ between groups of fish immunized only once and twice with a same dose of bacteria. Different letters on the bars indicate statistically significant differences at $P<0.05$ among primary immunized groups and among boost immunized groups, respectively.

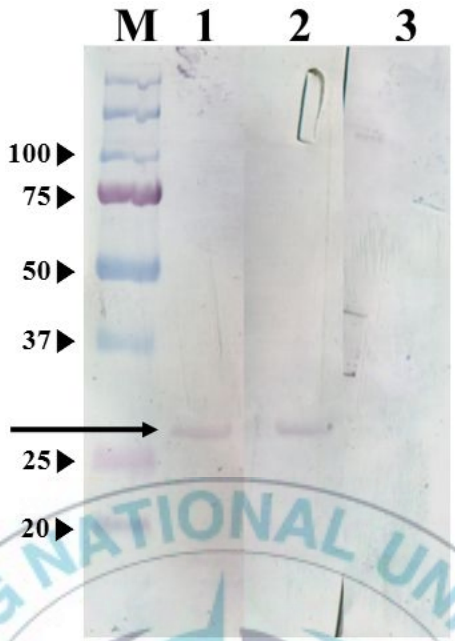


Fig.1-6. Western blot (Experiment 1).Western blot analysis for confirmation of the presence of serum antibody against green fluorescent protein (GFP) in fish immunized with 10^7 of the *Δalr Δasd Edwardsiella tarda* harboring pG02-ASD-EtPR-GFP. M, prestained protein marker (Bio-Rad); Lane 1, serum of twice immunized fish; Lane 2, serum of once immunized fish; Lane 3, Serum of PBS injected fish. The band indicated by an arrow corresponds to the molecular weight of GFP protein.

6. Persistence of orally administered Δalr Δasd E. tarda in olive flounder (Experiment 2)

Invasiveness (or colonization) and persistence of the *Δalr Δasd E. tarda* in internal organs including intestine, spleen, head kidney, and liver were analyzed after a single oral inoculation containing 10^8 CFU of the mutated bacteria. At 4 and 6 h post-administration, the bacteria was found in the intestine in more than 10^4 CFU/10 mg tissue, and then decreased to less than 10^3 CFU at 24 h post-administration (Fig. 1-7). No colonies were found from the spleen, head kidney, and liver to the end of the experiment.

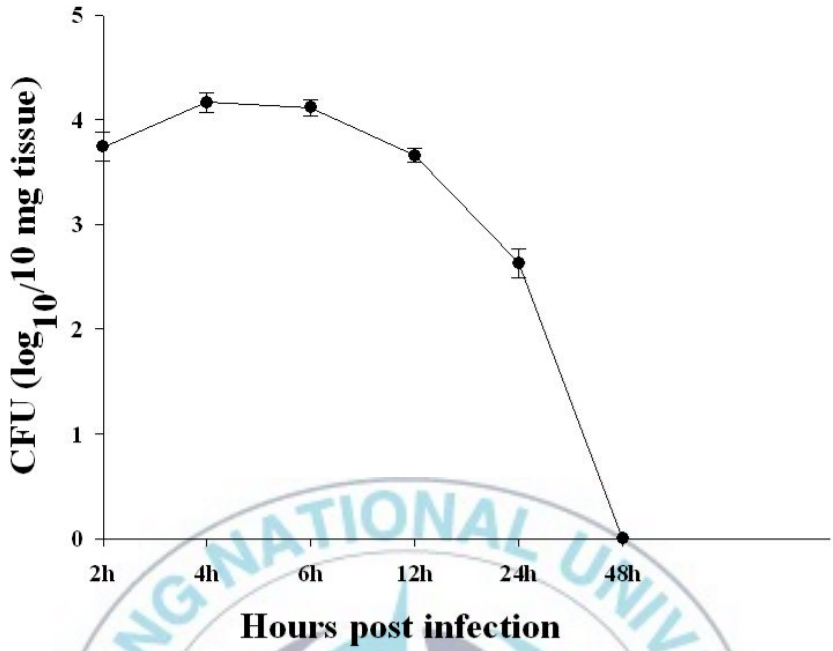


Fig.1-7. Persistence of orally administered Δalr Δasd E. tarda in olive flounder. Recovery of the mutant *Edwardsiella tarda* (*Δalr Δasd E. tarda*) harboring plasmid pG02-ASD-EtPR-GFP from intestine of olive flounder (*Paralichthys olivaceus*) that were orally administered with 10^8 CFU of the bacteria. At 2, 4, 6, 12, 24, 48, 96 and 168 h post-administration, the recovered mutant bacteria were counted by plating onto *Salmonella Shigella* agar (SS agar) supplemented with 50 mM D-alanine and 50 mM diaminopimelic acid (DAP). Each point represents mean ± standard deviation of the bacteria numbers per 10 mg of tissue. The mutant bacteria were not recovered from other internal organs including liver, head kidney, and spleen.

7. Protection of fish by oral immunization with Δalr Δasd E. tarda (Experiment 2)

Fish received a single oral administration containing 10^9 CFU of the *Δalr Δasd E. tarda* showed 100% protection against wild-type *E. tarda* challenges that were conducted through either i.p. injection or immersion (Fig. 1-8). The cumulative mortalities of the fish vaccinated with a single oral 10^8 CFU of the mutated bacteria were 38% when challenged by i.p. injection and 31% when challenged by immersion. The protective efficacy was increased by a boost immunization. The cumulative mortalities of fish immunized twice and challenged via i.p. injection and immersion routes were 0% and 8%, respectively (Fig. 1-8). The cumulative mortalities of fish in the control groups were 82-100%.

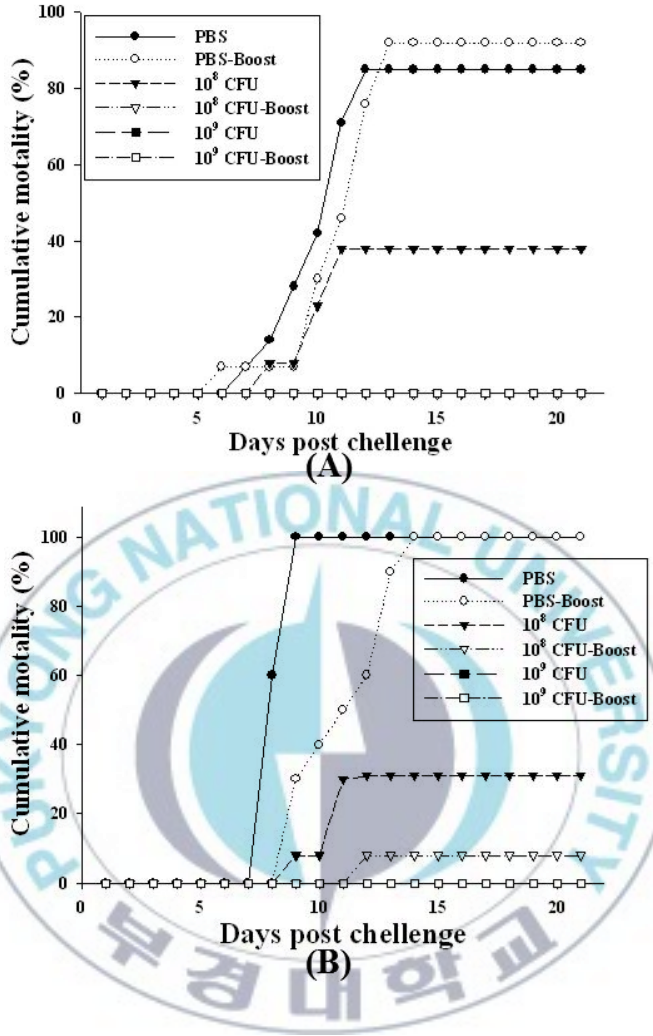


Fig.1-8. Protection of fish by oral immunization with Δalr Δasd E. tarda. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) immunized by oral administration of *Δalr Δasd Edwardsiella tarda* harboring pG02-ASD-EtPR-GFP (10^8 and 10^9 CFU/fish) or phosphate buffered saline (PBS). At 2 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, fish were challenged by (A) intraperitoneal injection (i.p.) of 2 x 10^2 CFU/fish of the wild-type *E. tarda* NH1 or (B) immersion for 3 h in water containing 1 x 10^9 CFU/3 L of *E. tarda* NH1.

8. Agglutination activity, ELISA, and Western blot (Experiment 2)

Skin and intestinal mucus collected from all immunized fish showed no agglutination activity against formalin killed *E. tarda*. A weak agglutination activity of serum was observed in the fish immunized with 10^9 CFU of the bacteria, and the activity was significantly increased by a boost immunization (Fig. 1-9 A). No ELISA titers to *E. tarda* FKC were detected in the skin and intestinal mucus of all immunized fish. However, ELISA titers of sera in immunized fish were dose- and boosting-dependently increased (Fig. 1-9 B).

In Western blot analysis, a band corresponding to GFP was not detected in sera of all immunized fish (Data not shown).

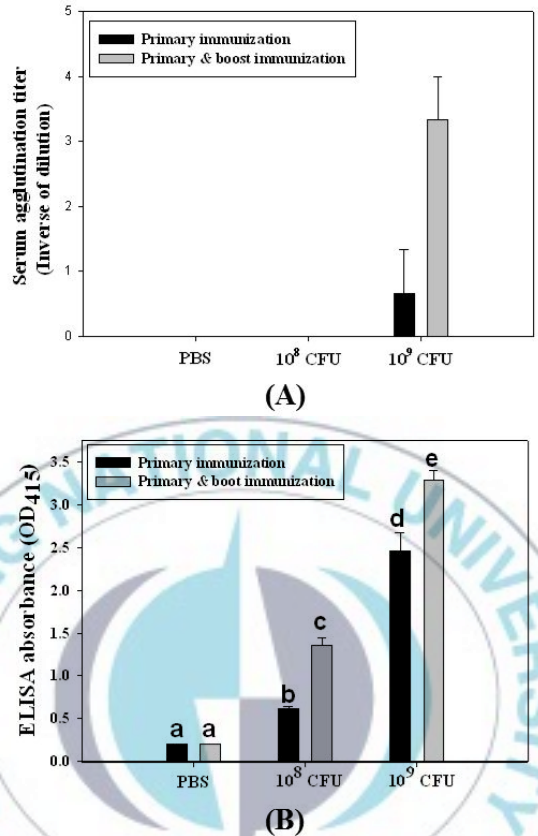


Fig.1-9. Agglutination activity, ELISA (Experiment 2). (A) Agglutination activity and (B) ELISA titer against formalin-killed *Edwardsiella tarda* NH1 in sera of olive flounder (*Paralichthys olivaceus*) immunized by oral administration of *Δalr Δasd Edwardsiella tarda* harboring pG02-ASD-EtPR-GFP (10^8 and 10^9 CFU/fish) or phosphate buffered saline (PBS). At 2 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, 6 fish in each group were bled to obtain serum for agglutination, ELISA, and Western blot analyses. Values are mean ± standard deviation. An asterisk on the bar in (A) and different letters on the bars in (B) represent significantly different at $P < 0.05$ among groups of fish.

DISCUSSION

In the present study, we successfully produced two auxotrophic genes knock-out *E. tarda* (*Δalr Δasd*) that are completely dependent on the presence of D-alanine and DAP for survival. In *Escherichia coli* and *Salmonella typhimurium*, two different alanine racemase genes are present (Wasserman et al., 1983; Walsh et al., 1989). A deletion or mutation of either gene alone does not decrease the bacterial growth in spite of the absence of D-alanine in culture medium, because sufficient amount of D-alanine is produced by the remained, intact alanine racemase gene. Therefore, in *E. coli* and *S. typhimurium*, the two *alr* must be deleted or mutated for generation of bacteria which completely require supplementation of exogenous D-alanine. In several Gram positive bacteria, since D-alanine can be made through catalytic action of not only alanine racemase but also D-amino acid aminotransferase (Pucci et al., 1995; Thompson et al., 1998), inactivation of both genes is indispensable to production of a requirement for exogenous D-alanine. In contrast to these bacteria, *E. tarda* has only one copy of alanine racemase gene (Wang et al., 2009), and knock-out of the gene in this study was sufficient to produce a mutant bacteria, which cannot survive without exogenous D-alanine.

In attenuated bacteria-based combined vaccines, stable expression of a heterologous antigen(s) is essential to induce effective immune responses. A foreign antigen(s) can be expressed by transformation of the attenuated bacteria with an expression vector or by insertion of the foreign antigen gene into the bacterial chromosome. The plasmid-based expression (multicopies) is more efficient than expression by chromosomal insertion (monocopy) because of differences in gene copy number. Although antibiotics and their resistance genes are used for stable maintenance of foreign gene-bearing plasmids, they are not desirable for application to

commercial live bacterial vaccines due to biosafety and environmental concerns. Furthermore, the antibiotic pressure for plasmid maintenance would not work in vivo, which would lead to quick loss of the plasmids. Balanced-lethal host-vector system provides a tool to maintain plasmids in the attenuated bacteria without using antibiotics (Nakayama et al., 1988; Ryan et al., 2000). The plasmid equipped with a cassette for expression of a gene that is essential for survival of the bacteria complements a chromosomal mutation of the vital gene, which ensures plasmid maintenance. In this study, we constructed a plasmid vector expressing *asd* gene under the control of a weak constitutive promoter isolated from *E. tarda,* and successfully complemented the deletion of *asd* gene in the chromosome of *E. tarda*. The expression of *asd* by the plasmids enabled the mutant bacteria to replicate without supplement of DAP and antibiotics.

The in vitro growth of the attenuated *E. tarda* harboring pG02-ASD-EtPR-GFP was similar to that of the wild-type *E. tarda* when D-alanine was supplemented into the culture medium, but was decreased in the absence of D-alanine. The in vivo growth of the attenuated bacteria was also decreased at the similar time of the in vitro experiment and not isolated at 168 h post-infection. These results suggest that the mutant *E. tarda* can replicate several hours and persist for several days in fish in the absence of *D*-alanine, which would strengthen immunogenicity of the vaccine. The virulence of the present auxotrophic mutant *E. tarda* was decreased, which was demonstrated by approximately 10^6 fold increase of LD_{50} dose compared to wild-type *E. tarda*. As the alanine racemase is not related to virulence but involved in cell wall synthesis, the limited replication and persistence of the bacteria by knock-out *alr* might indirectly lead to the decrease of virulence. And in this study, orally administered live *Δalr Δasd E. tarda* showed limited ability to invade into internal organs. The mutated bacteria were recovered only from the intestine till 24 h post-administration, and no colonies were found from the head kidney, spleen, and liver. This result suggests that the invasive capability of the mutant *E. tarda* might be damaged during the course of

arriving to the intestine or too small number of the bacteria might survive to penetrate into other internal organs within their limited survival time. Various encapsulation methods have been reported to prevent destruction of orally delivered antigens in the stomach of fish (Plant and LaPatra, 2011). The effect of encapsulation or mixing with the feed on the persistence and invasiveness of *Δalr Δasd E. tarda* should be further investigated.

In the present study, olive flounder i.p. immunized with 10^7 CFU/fish of the mutant *E. tarda* were completely protected against challenge infection, irrespective of boost immunization. Compared to inactivated vaccines, live attenuated vaccines can more effectively stimulate both humoral and cellular immunities (Marsden et al., 1996), which are most important especially in intracellular pathogens. As the present *alr* & *asd* knock-out *E. tarda* not only possesses all virulent genes corresponding to wild-type *E. tarda* but also has a limited ability to replicate in fish, the immunogenicity would be comparable to live-wild *E. tarda*, and at the same time the possibility of arising attenuation-associated safety problem, such as virulence restoration, would be considerably lowered. Therefore, the present attenuated *E. tarda* can be expected to be a promising vaccine candidate against edwardsiellosis.

It is known in mammals that mucosal vaccinations can effectively induce mucosal immune responses and also can elicit systemic immunity, but injected immunizations are less effective to induce mucosal immunity (Lamm, 1997; Neutra and Kozlowski, 2006). As the majority of pathogenic bacteria of fish infect through mucosal surfaces, mucosal adaptive immunity induced by oral immunization would provide effective defense tools for fish to defeat invading pathogens. In the present vaccine experiment, oral immunization of olive flounder with the auxotrophic mutant *E. tarda* (*Δalr Δasd E. tarda*) induced high protection against wild-type *E. tarda* challenge through either i.p. injection or immersion. This result suggests that protective mucosal and systemic immunities might be induced by oral administration of live attenuated bacteria. Similarly,

Cheng et al. (2010) had assessed the vaccine potential of a natural avirulent *E. tarda* isolate (ATCC 15947) by immunization of olive flounder. They fed the fish with feed containing the bacterial alginate microspheres for 5 days (daily consumption was approximately 10^9 CFU/fish), and challenged with virulent *E. tarda* after 5 weeks of the immunization, in which the cumulative mortalities of the fish challenged by i.p. injection and immersion were 36% and 29% in vaccinated fish, respectively, whereas the cumulative mortalities of fish in control groups were 82%. In the present study, olive flounder orally immunized with 10^9 CFU/fish of *Δalr Δasd E. tarda* (without mixing with feed or entrapment with other materials) were completely protected against challenge through either i.p. injection or immersion, even in fish administered only once, suggesting the present auxotrophic mutant *E. tarda* might has a strong immunogenicity to induce protective immunity in olive flounder. In this study, fish administered twice (primary and boost immunization) with 10^8 CFU of the mutated bacteria (total 2 x 10^8 CFU) showed similar protective efficacy to the fish immunized with 10^9 CFU of the bacteria. As cost is one of pivotal requirements for fish vaccine, multi-administration of a low dose of vaccine would be more favorable than single-administration of a high dose if there were no significant differences in protective efficacy.

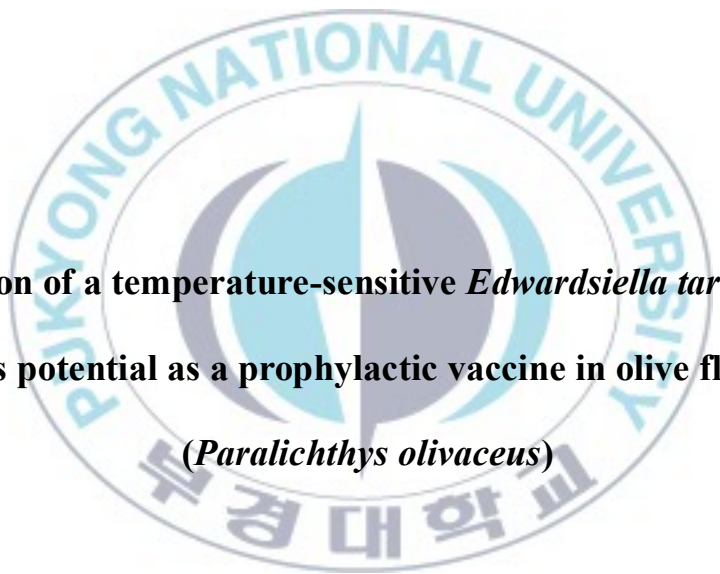
In the present study, in spite of delivering the bacteria through oral route, intestinal and skin mucus showed no agglutination activity and no ELISA titers, whereas serum showed agglutination activity and ELISA titers. These results suggest that antibody responses in the mucus of olive flounder might be hardly induced by oral inoculation of the mutated *E. tarda* or antibodies in mucosal secretions might not be captured by the present analysis procedures. However, the induction of serum antibody responses suggests that antigens of the mutated *E. tarda* might be transmitted to the regions that are engaged in the systemic immune responses, even no live *Δalr Δasd E. tarda* was found from immunologically important organs. Further experiments on the

adaptive cellular immune responses are needed to elucidate the immune mechanism involved in the present protection induced by orally administered bacteria.

In this study, fish immunized with the attenuated *E. tarda* at doses of 10^6-10^7 CFU/fish showed significantly higher serum agglutination activities against FKC than PBS-injected control fish. Furthermore, fish immunized with 10^6-10^7 CFU/fish of the mutant *E. tarda* harboring pG02-ASD-EtPR-GFP showed significantly higher ELISA titer against GFP antigen than fish in other groups. These results indicate that the present double auxotrophic genes knock-out *E. tarda* coupled with a heterologous antigen expression has a great strategic potential to be used as combined vaccines against various fish diseases.

However, in this study, GFP expressed in the cytoplasm of *Δalr Δasd E. tarda* failed to induce antibody responses when administered through an oral route, which might be the results of rapid degradation of GFP protein in the digestive tract or insufficient immunogenicity of the GFP to stimulate intestinal immune responses or further factors that remained to be investigated. New approaches that can strengthen antigenicity of foreign antigens should be conducted to use the present auxotrophic mutant *E. tarda* as an oral combined vaccine.

Chapter II.



Generation of a temperature-sensitive *Edwardsiella tarda* mutant and its potential as a prophylactic vaccine in olive flounder (*Paralichthys olivaceus*)

INTRODUCTION

Edwardsiella tarda is a Gram-negative, rod-shaped bacterium that infects not only fish but also mammals including humans (Janda et al., 1991; Thune et al., 1993). Infection of *E. tarda* in fish is associated with haemorrhagic septicemia, leading to severe losses in farm-reared freshwater and marine fishes (Plumb et al., 1999; Matsuyama et al., 2005; Mohanty et al., 2007). Considering various negative effects caused by excessive use of chemotherapeutics in aquaculture, prophylaxis through development of highly effective vaccines would be an ideal way to control edwardsiellosis. Various vaccine preparations against edwardsiellosis have been reported. As inactivated or subunit vaccines, formalin-killed vaccine (Gutierrez et al., 1994; Castro et al., 2008), ghost bacteria vaccine (Kwon et al., 2005, 2006, 2007; Lee et al., 2008), antigenic protein vaccine (Kawai et al., 2004; Liu et al., 2005; Hou et al., 2009; Jiao et al., 2009. 2010; Sun et al., 2010; Tang et al., 2010; Wang et al., 2010), and genetic vaccine (Jiao et al., 2009; Sun et al., 2011) were described. As live vaccines, natural avirulent strain vaccine (Cheng et al., 2010; Takano et al., 2010) and recombinantly attenuated vaccine (Lan et al., 2007) were reported. Recently, an immersion vaccine made with formalin-killed *E. tarda* was commercialized in Korea (Park, 2009).

Live attenuated bacteria are known as attractive and promising vaccine types for inducing protective immunity. Although the possibility to restore virulence was the bottle-neck for broad utilization of attenuated vaccines, recent genetic engineering technologies including artificial allelic recombination allow developing safe attenuated vaccines. Highly attenuated but almost fully immunogenic property would be the most prerequisite for live vaccines. Mutations of genes related to virulence can be exploited to generate attenuated bacterial vaccines, but may have problems such as weak immunogenicity and/or insufficiency to guarantee safety. Lately, we

developed an attenuated mutant *E. tarda* (Δ*alr* Δ*asd E. tarda*) by knockout of two auxotrophic genes, alanine racemase gene (*alr*) and aspartate semialdehyde dehydrogenase gene (*asd*), that play essential roles in bacterial cell wall biosynthesis (Choi & Kim , 2011). As the molecules, D-alanine and diaminopimelic acid (DAP), produced by enzymatic activity of the two genes are not synthesized by vertebrate hosts, the growth of the mutant *E. tarda* is severely limited, which make the mutant *E. tarda* suitable for live vaccines with high immunogenicity and high safety.

To produce auxotrophic mutant bacterial vaccines, the mutant bacteria should be cultured with a medium supplemented with molecule(s) compensating for knockout of the auxotrophic gene(s). However, requirement of specific nutrient(s), such as D-alanine and DAP for Δ*alr* Δ*asd E. tarda*, is a factor of increasing cost for vaccine production. As cost is an important requirement for vaccines in aquaculture farms, development of ways to produce auxotrophic mutants without use of specific supplement(s) would be favorable. In the present study, we newly generated an auxotrophic mutant *E. tarda* by replacing promoter region of *alr* gene with λ phage P_R promoter system, which allow the bacteria to grow without supplement of D-alanine but to disintegrate at temperature below 30°C. Furthermore, vaccine potential of the temperature-sensitive mutant *E. tarda* was evaluated by immunization of olive flounder (*Paralichthys olivaceus*).

MATERIALS AND METHODS

1. Bacterial strains and culture

Edwardsiella tarda NH1 (Choi & Kim, 2011) was cultured in tryptic soy broth (TSB, Difco) containing 1.5% NaCl at 25°C. *Escherichia coli* was grown in Luria-Bertani (LB, Difco) medium and when required, was supplemented with 50 μg/ml ampicillin. For culture of *E. coli* χ7213 (*Δasd*), 50 μg/ml of diaminopimelic acid (DAP, Sigma) was added into the culture medium.

2. Generation of temperature-sensitive E. tarda mutant by replacement of alr gene promoter with a temperature-sensitive promoter system

Primers used in the present study were listed in Table 2-1. The upstream flaking region (840 bp) of *alr* open reading frame (ORF) of *E. tarda* was amplified using a pair of PCR primers, *PAlr*F-Fo-*Sac*I and *PAlr*F-Re-*Xho*I. A fragment corresponding to 1092 bp of *alr* ORF was amplified by PCR using primers *OAlr*B-Fo-*SpeI* and *OAlr*B -Re-*XbaI*. The fragment of cI_{857} λP_R was amplified from the template plasmid pλPR-cI-Elysis (Kwon et al., 2005) using a pair of primers *C1*-Fo-AatⅡ and λP_R- *RE*- *Spe*I. To express more cI857 protein, a constitutive promoter of *E. tarda* (EtPR C28-1) and *c*I857 ORF were amplified by primers of C28-1 *Aat*II F & EtPR Re *Sal*I and C1 ORF Fo *Sal*I & C1 Re *Xho*I, respectively, using the plasmid pEtRP-InaN-GFP-Ghost (Choi et al., 2010) as a template. Each PCR product was purified using gel purification kit (Cosmo Genentech, Korea), and subcloned into pGEM-T easy vector (Promega). Each fragment in the T vector was digested with each corresponding enzyme, and cloned into pTOP-MCS vector that was made by insertion of multi-cloning sites (MCS; *Sac*I-*Xho*I-*Aat*II-*Spe*I-*Sac*II-*Nsi*I-*Xba*I) into pTOP

vector (Enzynomics, Korea). The pTOP-MCS vector harboring the EtPR C28-1-driven CI857 cassette and *c*I857-λP_R driven *alr* cassette (EtPR-*c*I857+*c*I857-λP_R-*alr*) was digested with *Sac*I and *Xba*I, and the resulting fragment was ligated into a suicide vector pCVD442 (Addgene plasmid 11074) that was pre-digested with the same enzymes, and the resulting plasmid was designated as pCVD442-EtPR-*c*I857+*c*I857-λP_R-*alr*. *E. coli* χ7213 was transformed with the constructed pCVD442 plasmids and screened on LB agar plates containing 50 μg/ml ampicillin. The wild-type *E. tarda* NH1 was conjugated with *E. coli* χ7213 containing the plasmid pCVD442-EtPR-*c*I857+*c*I857-λP_R-*alr*. Transconjugants were selected by growth on LB agar supplemented with ampicillin, and, then, the selected *E. tarda* was plated on LB agar containing 10% (w/v) sucrose and 50 mM *D*-alanine for a second cross-over. Insertion of EtPR-*c*I857+*c*I857-λP_R in front of *alr* ORF of *E. tarda* chromosome (Δ*Palr*:: EtPR-*c*I_{857} λP_R *alr*) was confirmed by severely weakened ability of the strain to grow at 25°C and by PCR with primers *Alr* chlo-for and *Alr*f-Re-*Xho*I. To more tightly control the λP_R activity, the resulting temperature-sensitive *E. tarda* mutant was further transformed with the T vectors harboring the EtPR C28-1 driven *c*I857 cassette (pEtPR-*c*I857) by electroporation (Gene Pulser, Bio-Rad).

3. In vitro growth of temperature-sensitive E. tarda mutant

The temperature-sensitive *E. tarda* mutant grown in LB at 39°C were washed with phosphate buffered saline (PBS), and cultured at 20°C or 25°C. At different time points post-inoculation, the change of optical density at 600 nm (OD_{600}) was monitored until no further increase in OD of wild-type *E. tarda*. Growth of the *E. tarda* mutant harboring pEtPR-*c*I857 was also measured in the same way.

4. Semi-quantitative RT-PCR analysis

To measure the tightness of CI857 in inhibition of λP_R-driven *alr* gene expression at low temperature, the level of *alr* transcription in the mutant *E. tarda* or the mutant harboring pEtPR-*c*I857 was analyzed by semi-quantitative RT-PCR. At 6, 12, 24 and 48 h post- temperature shift (from 39°C to 25°C or 20°C), total RNA from cells was extracted using RNeasy Plus Mini Kit (Qiagen). To synthesize first-strand cDNA, 1 μg of total RNA treated with DNase was incubated with 0.5 μl of random primer (0.5 μg/ml, Promega) at 80°C for 5 min and further incubated at 42°C for 60 min in reaction mixture containing 2 μl of each 10 mM dNTP mix (TaKaRa), 0.5 μl of M-MLV reverse transcriptase (Promega) and 0.25 μl of RNase inhibitor (Promega) in a final reaction volume of 10 μl. PCR was performed with 2×Prime Taq Premix (Genet Bio) and 1 μl of 10^{-1} diluted cDNA template. Oligonucleotide primer pairs for a target (*alr*) and a control (16S ribosomal RNA) genes are provided in Table 1. Thermal cycling conditions were 1 cycle of 3 min at 95°C (initial denaturation) followed by 18 cycles (for 16S rRNA) or 25 cycles (for *alr*) of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the 1% agarose gel, and stained with ethidium bromide (EtBr).

5. In vivo virulence of temperature-sensitive E. tarda mutant

To determine 50% lethal dose (LD_{50}), olive flounder fingerlings (approximately 5 g in body weight) were intraperitoneally (i.p.) injected with 50 μl of graded doses (10^2 – 10^8 CFU/fish; 10 fish per each dose) of wild-type *E. tarda* or the temperature-sensitive *E. tarda* mutant or the mutant harboring pEtPR-*c*I857, and were monitored twice a day for 14 days.

6. in vivo persistence of temperature-sensitive E. tarda mutant

For in vivo persistence analysis, olive flounder fingerlings were i.p. injected with 10^7 CFU/fish of the temperature-sensitive *E. tarda* mutant harboring pEtPR-*c*I857. At 2, 4, 6, 12, 24, 48, 96 h, 7 d, 14 d, 21 d and 28 d after injection, three fish were euthanized by transfer to water containing MS-222 (Sigma), and samples of liver, spleen, and head kidney were taken aseptically, suspended in 0.5 ml of PBS, weighed, and pulverized. The bacterial load of each organ was enumerated by dropping dilutions of the tissue lysate onto *Salmonella Shigella agar* (SS agar, Difco) supplemented with 50 mM D-alanine.

7. Determination of protective efficacy of temperature-sensitive E. tarda mutant

Two groups with 2 replicates of olive flounder fingerlings were reared in four 250 L tanks (30 fish/tank) at 20-22°C. Fish were fed with a commercial pellet and acclimatized for 2 weeks before immunization. Fish in a group were immunized by i.p. injection with 1 x 10^7 CFU/fish of the temperature-sensitive *E. tarda* mutant harboring pEtPR-*c*I857 in 50 μl of PBS, and fish in the other group were injected with 50 μl of PBS as controls. At 3 weeks after the primary immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, 6 fish in each group were bled to obtain serum for agglutination assay, and the remnant fish were i.p. challenged with 2 x 10^2 CFU/fish of the wild-type *E. tarda* NH1. Deaths were recorded for 14 d post-challenge. Dead fish were collected daily and kidney samples were streaked on SS agar to confirm the presence of *E. tarda*.

8. Serum agglutination activity

The serum agglutination activity against formalin-killed *E. tarda* was determined according

to the previously described method (Choi & Kim, 2011).

9. Statistical analysis

Data on serum agglutination activity were analyzed by Student's *t*-test. A probability (*P*) value less than 0.05 indicated statistical significance.

Table 2-1. Oligonucleotides used in this study

Name of Oligonucleotides	**Sequence (5`~3`)**
MCS-UP	CCTCGAG GACGTCACTAGTCCGCGGATGCATT
MCS-DOWN	CTAGAATGCATCCGCGGACTAGTGACGTCCTCGAGGAGCTC
*PAlr*F-Fo-*SacI*	GAGCTC GTAAGGATGAGGGACCGAAGAGCATC
*PAlr*F-Re-*XhoI*	CTCGAG TTAAAGGTCAGACGCACCGTCCCGATA
*OAlr*B-Fo-*SpeI*	ACTAGT ATGAAAGCGGCAACCGCCATCATTG
*OAlr*B -Re-*XbaI*	TCTAGA CTACGCCTCTTCGCCGATATATTCC
C1-Fo-*Aat* II	GACGTC AGCCAAACGTCTCTTCAGG
λP_R- *RE- Spe*I	ACTAGT ACAACCTCCTTAGTACATGCAACCAT
C28-1 *AatII* F	GACGTC GGGTAATTGCGCTGC
EtPR Re *SalI*	GTCGAC ATACCTCCTCTTAAAGTTAACATGGTGGCTAT
C1 ORF Fo *SalI*	GTCGAC ATG AGC ACA AAA AAG AAA CCA TTA ACA CA
C1 ORF Re *XhoI*	CTCGAG TCAGCCAAACGTCTCTTCAGGC
Alr chlo-for	ATCATGTTCATCTATCGCGATGAGGTCTAT
*Alr*f-Re-*Xho*I	CTCGAG GCAATAAGGCGACTGTGCG

RESULTS

1. Generation of temperature-sensitive E. tarda mutant

An *E. tarda* mutant designed to grow at temperature above 30°C but to go down at temperature below 30°C was constructed by insertion of nucleotides corresponding to EtPR-*c*I857+*c*I857-λP_R into the just N-terminal end of *alr* ORF using allelic exchange technology. The chromosomal structure of the temperature-sensitive mutant is shown in Fig. 2-1 A, and the insertion of the temperature-sensitive cassettes was verified by PCR analysis using primers that amplify 415 bp of wild-type *E. tarda* nucleotides encoding partial region of promoter to ORF of *alr* gene. In the mutant strain, a band of 2.2 kb that corresponds to the 415 bp of the wild-type plus 1.8 kb of the inserted cassettes was amplified (Fig. 2-1 B). Growth of the mutant strain was not different to that of wild-type *E. tarda* under conditions of culture at 39°C (Fig. 2-2 A) or supplement of D-alanine to growth medium. However, growth of the mutant strain was severely retarded by culturing the bacteria at 25°C or 20°C (Fig. 2-2 B). To further inhibit leakage of λP_R promoter at low temperature, the mutant strain was transformed with a vector harboring a constitutive promoter EtPR C28-1-driven *c*I857 cassette (pEtPR-*c*I857), which resulted in more limited growth compared to the mutant without the plasmids (Fig. 2-2 B).

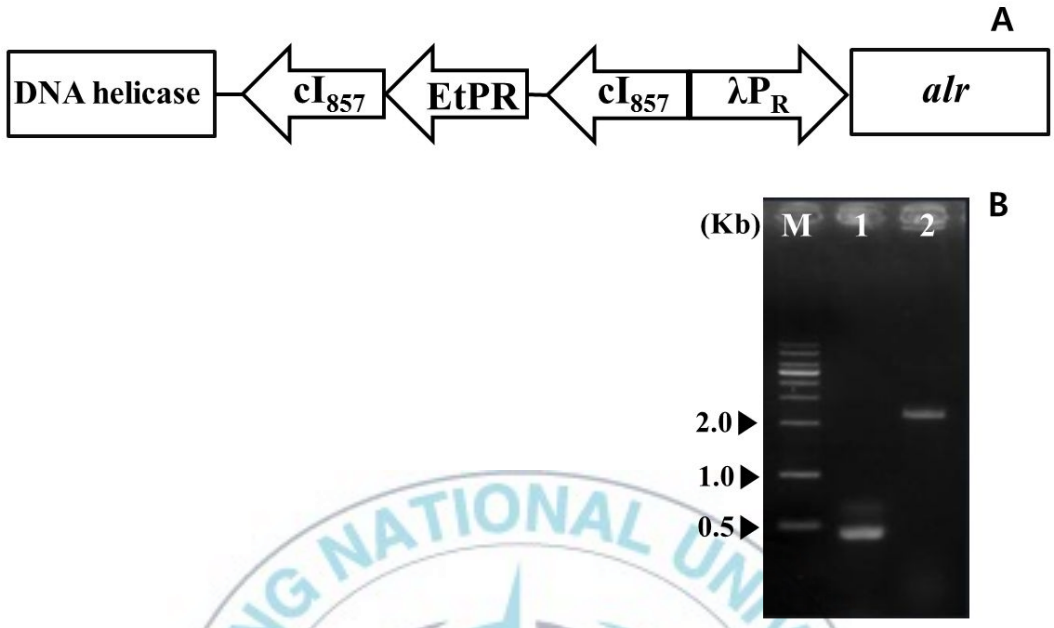


Fig.2-1. Generation of temperature-sensitive E. tarda mutant. (A) Chromosomal arrangement of the present temperature-sensitive cassettes. Nucleotides corresponding to EtPR-*c*I857+*c*I857-λP_R were inserted into the just N-terminal end of *alr* ORF using allelic exchange technology. EtPR is a strong constitutive promoter (EtPR C28-1) cloned from *Edwardsiella tarda*. (B) PCR analysis of the insertion of the temperature sensitive cassettes. Lane 1 is the PCR fragment amplified from genomic DNA of the wild-type *E. tarda* NH1. Lane 2 is the PCR fragment amplified from genomic DNA of the temperature-sensitive *E. tarda* mutant. Lane M is a 1 kb ladder (Bioneer, Korea). PCR product of mutant *E. tarda* was larger than that of wild-type *E. tarda* because of insertion of the nucleotides corresponding to EtPR-*c*I857+*c*I857-λP_R.

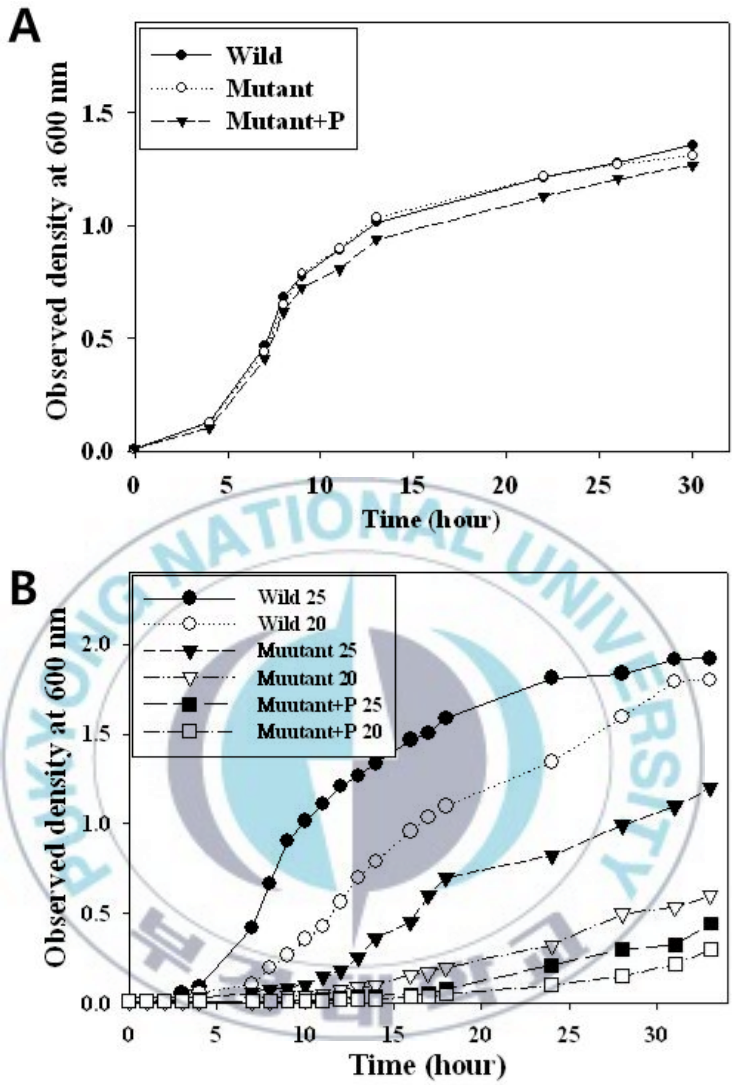


Fig.2-2. Growth conditions. Growth of wild-type *Edwardsiella tarda* NH1 (Wild), temperature-sensitive *E. tarda* mutant without plasmids (Mutant), and the *E. tarda* mutant carrying plasmids pEtPR-*c*I857 (Mutant+P) under conditions of culture (A) at 39°C and (B) at 25°C or 20°C without supplementation of D-alanine. The cultured bacteria were sampled at various time points to measure cell density at OD_{600}.

2. *Semi-quantitative RT-PCR analysis to measure alr* gene transcription

There was no difference in the expression of *alr* gene between wild-type and the mutant *E. tarda* under conditions of culture at 39°C (Fig. 2-3). However, the *alr* gene expression in the mutant *E. tarda* and the mutant harboring pEtPR-*c*I857 was mostly suppressed by culturing the bacteria at 20°C. Under conditions of culture at 25°C, the mutant harboring pEtPR-*c*I857 showed hardly detectable level of *alr* gene expression, but the mutant *E. tarda* without the plasmids expressed more amount of *alr* gene than the mutant with plasmids (Fig. 2-3).

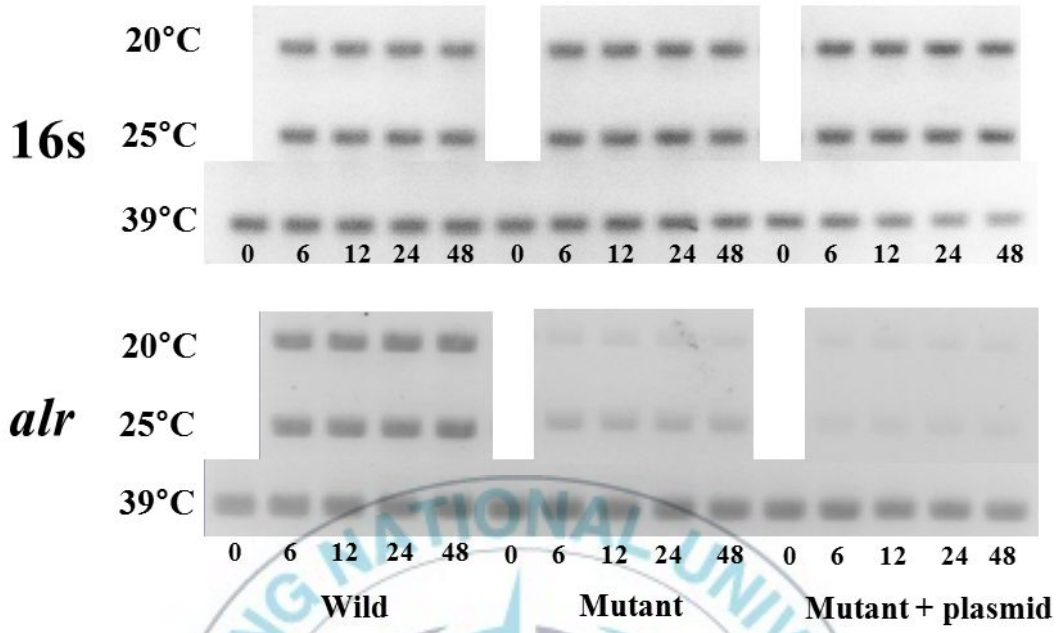


Fig.2-3. Semi-quantitative RT-PCR analysis to measure alr gene transcription. Confirmation of *alr* gene expression in wild-type *Edwardsiella tarda* (Wild), temperature-sensitive *E. tarda* mutant without plasmids (Mutant), and the *E. tarda* mutant carrying plasmids pEtPR-*c*I857 (Mutant+plasmid) using semi-quantitative RT-PCR analysis. The 16S ribosomal RNA (16s) was used as the control.

3. in vivo virulence and persistence of temperature-sensitive E. tarda mutant

No fish were died by an i.p. challenge with 10^7 CFU of the mutant *E. tarda* harboring pEtPR-*c*I857, whereas 20% of fish died by injection with 10^6 CFU of the mutant strain without the plasmids, and 60% of fish died by 10^2 CFU of the wild-type *E. tarda* (Fig. 2-4). Thus the LD_{50} by i.p. injection in olive flounder fingerling was less than 10^6 CFU for the temperature-sensitive *E. tarda* mutant and far less than 10^7 CFU for the mutant strain harboring pEtPR-*c*I857.

The mutant strain harboring pEtPR-*c*I857 was recovered from the head kidney, spleen, and liver in i.p. injected olive flounder fingerling (Fig. 2-5). The colony number in each organ was gradually diminished, and no colony was isolated from all organs at 28 d post-injection.

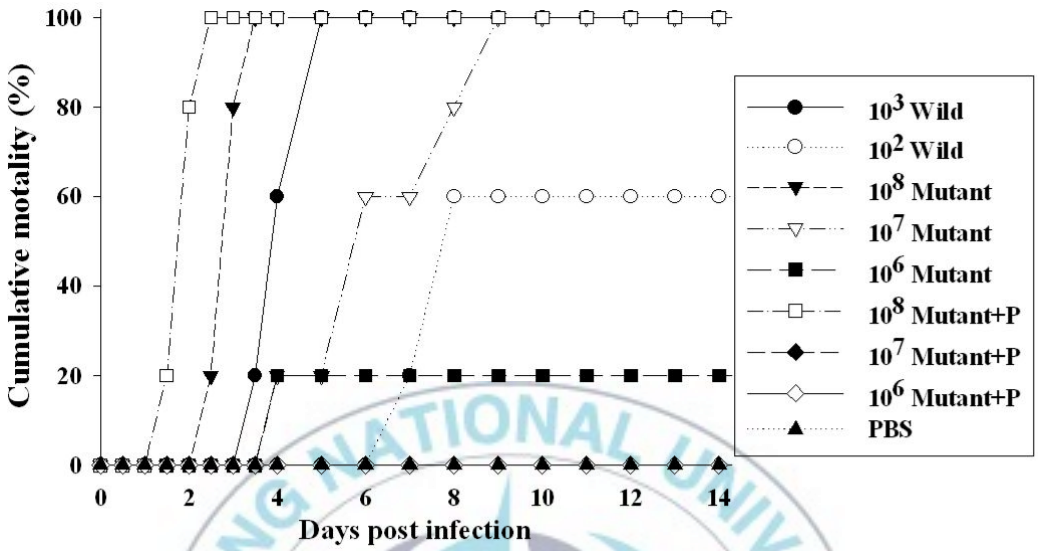


Fig.2-4. In vivo virulence of temperature-sensitive *E. tarda* mutant. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) intraperitoneally (i.p.) injected with phosphate buffered saline (PBS), or various CFUs of wild-type *Edwardsiella tarda* (Wild), temperature-sensitive *E. tarda* mutant without plasmids (Mutant), and the *E. tarda* mutant carrying plasmids pEtPR-*c*I857 (Mutant+P).

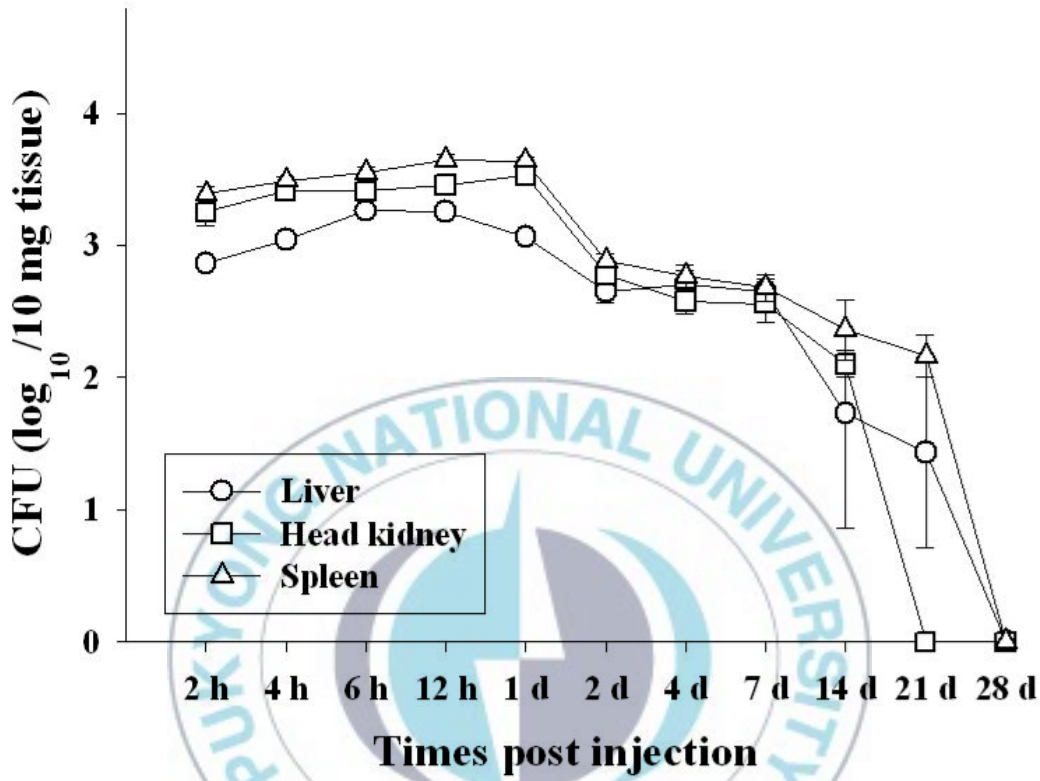


Fig.2-5. In vivo persistence of temperature-sensitive *E. tarda* mutant. Recovery of the temperature-sensitive *E. tarda* mutant carrying plasmids pEtPR-*c*I857 from each internal organ (liver, head kidney, and spleen) of olive flounder (*Paralichthys olivaceus*) that were intraperitoneally (i.p.) injected with 10^7 CFU of the mutant bacteria. At 2, 4, 6, 12, 24, 48, 96 h, 7 d, 14 d, 21 d and 28 d after injection, the mutant bacteria recovered from each tissue were counted by plating onto *Salmonella Shigella* agar (SS agar) supplemented with 50 mM D-alanine. Each point represents mean ± standard deviation of the bacteria numbers per 10 mg of each tissue.

4. Vaccine efficacy of temperature-sensitive E. tarda mutant and agglutination activity of serum

To determine the degree of protection against *E. tarda* by i.p. immunization with 10^7 CFU of the mutant strain harboring pEtPR-*c*I857, olive flounder fingerlings were challenged with 10^2 CFU of the wild-type *E. tarda* at 6 weeks after the primary immunization. As a result, a group of fish immunized with the mutant bacteria showed 27% mortality, and no mortality was recorded in a group of boost-immunized fish, whereas groups of control fish injected with PBS alone showed 100% mortality (Fig. 2-6).

Agglutination activity of serum against formalin-killed *E. tarda* was highly increased by immunization of fish with the temperature-sensitive *E. tarda* mutant harboring pEtPR-*c*I857, and the activity was significantly increased by a boost immunization (Fig. 2-7).

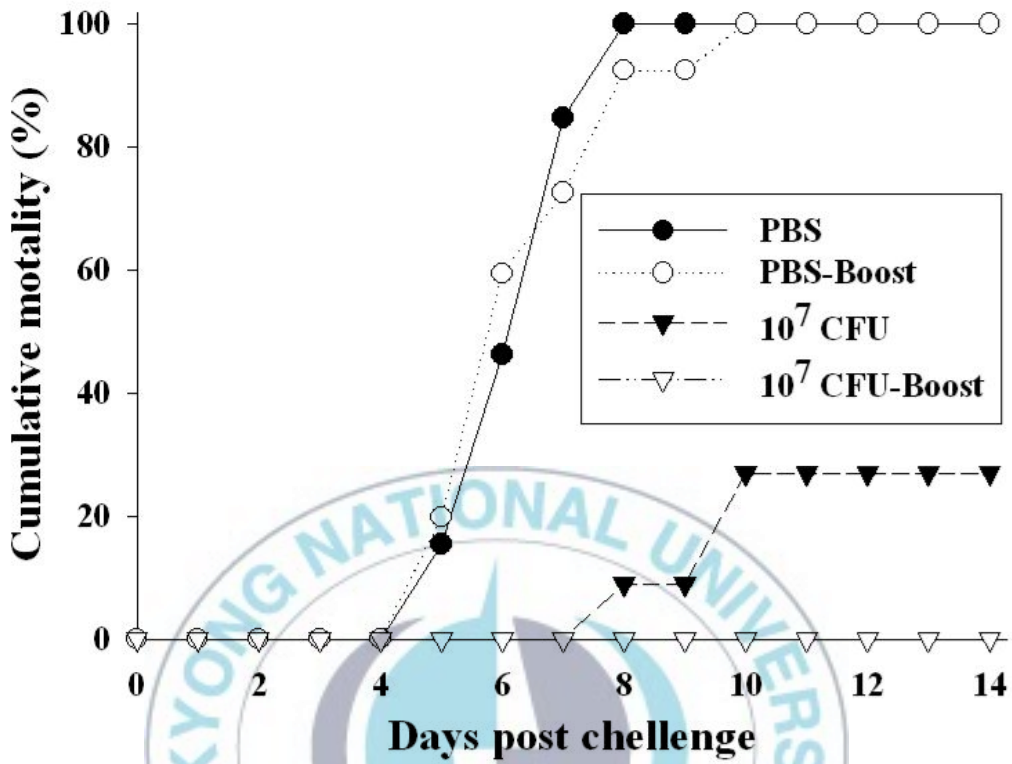


Fig.2-6. Vaccine efficacy of temperature-sensitive *E. tarda* mutant **.**Cumulative mortality of olive flounder (*Paralichthys olivaceus*) immunized by intraperitoneal (i.p.) injection of phosphate buffered saline (PBS), or the temperature-sensitive *E. tarda* mutant carrying plasmids pEtPR-*c*I857 (10^7 CFU/fish). At 3 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, fish were i.p. challenged by 2 x 10^2 CFU/fish of the wild-type *E. tarda* NH1.

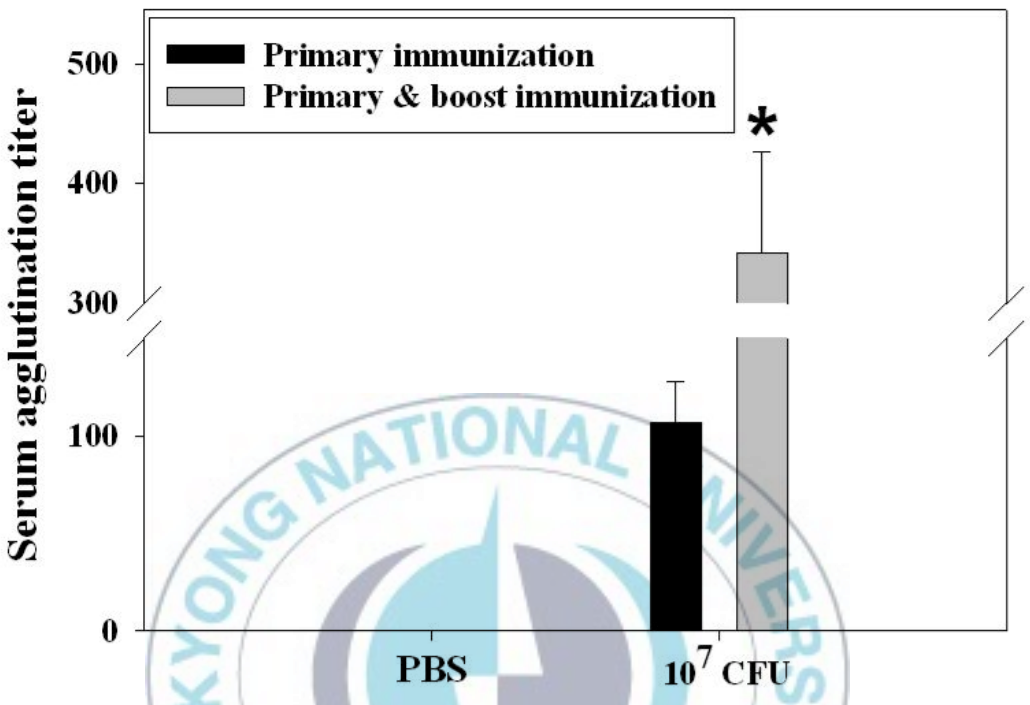


Fig.2-7. Agglutination activity of serum. Agglutination activity against formalin-killed *Edwardsiella tarda* NH1 in sera of olive flounder (*Paralichthys olivaceus*) immunized by intraperitoneal (i.p.) injection of phosphate buffered saline (PBS), or 10^7 CFU/fish of the temperature-sensitive *E. tarda* mutant carrying plasmids pEtPR-*c*I857. At 3 weeks post-immunization, fish in groups of one replicate were boost immunized with the same regime of the primary immunization. At 3 weeks post-boosting, 6 fish in each group were bled to obtain serum for agglutination. Values are mean ± standard deviation. The asterisk on the bar represent significantly different at $P < 0.05$ between groups of fish immunized only once and twice with the mutant bacteria.

DISCUSSION

In the present study, we have generated a mutant *E. tarda* by replacement of *alr* gene promoter with *c*I857-λP_R promoter system plus another CI857 expression cassette that was driven by a constitutive promoter of *E. tarda* (EtPR C28-1) (Choi et al., 2010). In *c*I-λP_R promoter system, the inhibitor protein CI attaches to the operator region of λP_R promoter and suppresses transcription of the λP_R promoter-driven gene. The CI repressor detaches from the operator region by elevation of temperature above 42°C, which allows expression of the λP_R promoter-driven gene. By using a temperature-sensitive mutant of CI repressor, CI857 (Nauta et al., 1997), λP_R promoter-driven gene expression can be induced at temperature above 30°C (but the efficient temperature for inactivation of CI857 repressor ranges between 36-42°C) (Villaverde et al.,1993). Thus the present temperature-sensitive *E. tarda* mutant was expected to grow at temperature higher than 30°C without addition of D-alanine by expression of *alr* gene, but anticipated to be lyzed at temperature below 30°C by inhibition of *alr* gene expression through attachment of CI857 on the operator region. This temperature-controlled gene expression system would be useful because most of cultured fish live at temperature below 30°C at which the temperature-sensitive *E. tarda* mutant would be lyzed.

However, unexpectedly, in our preliminary experiments, use of *c*I857-λP_R promoter system instead of *alr* gene promoter did not hinder the growth of *E. tarda* at temperature lower than 30°C. This result suggests that the expressed amount of CI857 was not sufficient to completely stop P_R promoter-driven *alr* gene expression or the CI857-mediated blocking was weakly leaky. It has been reported that the CI is not tight to completely inhibit expression of λP_R promoter-driven gene when λP_L operator region is absent (Révet et al., 1999), and the repressible ability of CI is

influenced by the number of CI molecule (Meyer, 1980; Ptashne et al., 1980). Therefore, in the present study, we have added an additional cassette that constitutively express *c*I857 gene. The mutant *E. tarda* having *c*I857-λP_R promoter system plus another *c*I857 expression cassette instead of *alr* gene promoter showed severely retarded growth at temperature 20 and 25°C compared to wild-type *E. tarda*. To further strengthen the repressible power, we additionally supplied CI857 protein to the mutant *E. tarda* by transformation with plasmids harboring a cassette for constitutively expressing CI857 protein, which resulted in almost interruption of growth and gradual invitalization of the mutant bacteria even at 25°C. The level of *alr* gene transcription in the mutant *E. tarda* and the mutant harboring pEtPR-*c*I857 was well coincided with the result of bacterial growth, suggesting that increased number of CI857 molecule was the major factor to further suppress the λP_R promoter-driven *alr* gene expression at low temperature.

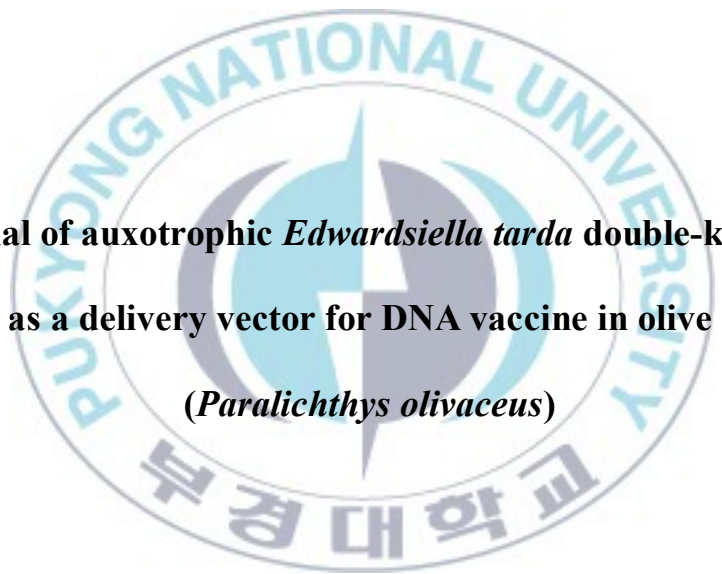
In the present study, the degree of attenuation was estimated by comparison of mortality of olive flounder that were injected with wild-type *E. tarda*, mutant *E. tarda*, or mutant *E. tarda* having the plasmids. Although fish injected with just 10^3 CFU of wild-type *E. tarda* showed 100% mortality within 4 d post-injection, fish injected with 10^6 CFU of mutant *E. tarda* induced 20% mortality, and fish challenged with up to 10^7 mutant *E. tarda* harboring the plasmids showed no mortality. This result suggests that the present mutant *E. tarda* is highly attenuated, and supplementation of CI857 molecule plays a crucial role in attenuation of the present mutant *E. tarda*.

The present result showed that the CFU number of mutant *E. tarda* harboring CI857 expressing plasmids was slightly increased or not changed at early period (12-24 h), then gradually decreased with the lapse of time from the internal organs, such as kidney, spleen, and liver in i.p. injected olive flounder, and no colony was found from fish analyzed at 28 d post-injection. As the ability of in vivo replication and persistence of attenuated bacteria can critically

influence on the induction of efficient adaptive immune responses, the systemic invasion and relatively long persistence of the present *E. tarda* mutant in olive flounder would be promising aspects for a live vaccine against edwardsiellosis.

In the present vaccine experiment, fish immunized with the temperature-sensitive mutant *E. tarda* showed greatly decreased mortality, furthermore, a boost-immunization induced complete protection against *E. tarda* infection. The protection rate of fish was proportional to the serum agglutination titer against *E. tarda*. These results suggest that the present temperature-sensitive mutant *E. tarda* is a good candidate for effective vaccines for prophylaxis of edwardsiellosis in fish. Moreover, the present auxotrophic mutant *E. tarda* can be cultured without supplementation of the corresponding nutrient, and can be attenuated by obstruction of *alr* gene expression at temperature below 30°C, suggesting that vaccines based on the present mutant *E. tarda* would be more economical than previously reported auxotrophic mutant *E. tarda*.

Chapter III.



Potential of auxotrophic *Edwardsiella tarda* double-knockout mutant as a delivery vector for DNA vaccine in olive flounder (*Paralichthys olivaceus*)

INTRODUCTION

Recently, DNA vaccines that are composed of plasmids equipped with an eukaryotic expression cassette have been proved as a promising type of vaccines against viral, bacterial and parasitic pathogens in vertebrates. As the antigen of DNA vaccines is expressed in the cytoplasm of host cell, MHC class I-mediated cytotoxic T cell responses can be elicited, which allows DNA vaccines to defeat intracellular pathogens effectively (Ulcer et al., 1993; Corr et al., 1996; Wang et al., 1998). Furthermore, the antigens expressed in the cytoplasm can be released from the cell and be engulfed by antigen presenting cells (APCs), which can induce MHC class II-mediated helper T cell response that is important to induce humoral adaptive immune responses (Cohen et al., 1998; Wang et al., 1998).

In fish, DNA vaccine-mediated prevention of viral and bacterial diseases has been reported during past decades, such as infectious pancreatic necrosis virus (IPNV) (Mikalsen et al., 2004), infectious salmon anemia virus (ISAV) (Mikalsen et al., 2005), infectious hematopoietic necrosis virus (IHNV) (Anderson et al., 1996; Corbeil et al., 1999; LaPatra et al., 2001), viral hemorrhagic septicaemia virus (VHSV) (Lorenzen et al., 1998, 2001), hirame rhavdovirus (HIRRV) (Takano et al., 2004; Seo et al., 2006), red seabream iridovirus (RSIV) (Caipang et al., 2006), *Aeromonas veroni* (Vazquez-Juareza et al., 2005), *Mycobacterium marinum* (Pasnik and Smith, 2005), *Vibrio anguillarum* (Rajesh Kumar et al., 2007; Yang et al., 2009a), and *Streptococcus iniae* (Sun et al., 2010). Most of DNA vaccines used in fish were injected through intramuscular (i.m.) route in a naked plasmid form. To enhance delivery efficiency and/or convenience of DNA vaccines in fish, other delivery methods have been tried including gene gun bombardment (Corbeil et al., 2000; Lee et al., 2000; Tucker et al., 2001), immersion & ultrasound (Fernandez-Alonso et al., 2001),

and oral administration by encapsulation with chitosan or chitosan nanoparticles (Ramos et al., 2005; Rajesh Kumar et al., 2008). In spite of those researches, need for efficient delivery methods of DNA vaccine is still demanded in aquaculture.

In mammals, genetically attenuated intracellular bacteria such as *Salmonella, Shigella,* and *Listeria* have been used to deliver DNA vaccines to host cells (Sizemore et al., 1995; Darji et al., 1997; Dietrich et al., 1998; Fennelly et al., 1999; Schoen et al., 2004). These attenuated bacteria can penetrate or be engulfed by APCs such as macrophages and dendritic cells, and can do limited replication in host cells because of knockout of genes related to cell wall synthesis, through which DNA vaccine plasmids can enter the cell nucleus. The attenuated bacteria also can be effectively delivered via mucosal sites including an oral route (Thole et al., 2000; Dietrich et al., 2003; Yang et al.,2009b), which is advantageous in vaccination of small animals, such as fish fingerlings. Attenuated bacterial vector for DNA vaccines have been evaluated in numerous animal and human studies, but, to date, there has been no report on the availability of attenuated bacteria as a delivery vehicle for DNA vaccines in fish.

Edwardsiella tarda is an intracellular bacterium of a wide variety of fish (Thune et al., 1993; Plumb, 1999). Recently, we have generated two auxotrophic genes - alanine racemase gene (*alr*) and aspartate semialdehyde dehydrogenase gene (*asd*) - knockout *E. tarda* (*Δalr Δasd E. tarda*), and have shown that intraperitoneal or oral immunization of olive flounder with the auxotrophic mutant induced high protection against challenges with a virulent wild-type *E. tarda* (Choi and Kim, 2011a,b). In this study, to evaluate the potential of the mutated *E. tarda* as a delivery vehicle for DNA vaccine in fish, the auxotrophic mutant *Δalr Δasd E. tarda* transformed with plasmids harboring a cassette for expression of the enhanced green fluorescent protein (eGFP) gene driven by a cytomegalovirus (CMV) promoter was used to immunize olive flounder through either intraperitoneal or oral routes, and the expression of eGFP in the internal organs of

fish and generation of antibody against eGFP in fish were analyzed.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

The recombinant *E. tarda* (Δ*alr* Δ*asd E. tarda*) used in this study was generated in a previous study (Choi and Kim, 2011a). The mutant bacteria were grown in Luria-Bertani (LB, Difco) medium supplemented with 50 mM D-alanine, and 50 mM diaminopimelic acid (DAP, Sigma).

2. Vector construction for DNA vaccine

Previously, an antibiotic resistance gene-free vector equipped with the cassette for *asd* gene expression and the multiple cloning sites (MCS) (pG02-ASD-MCS) was constructed (Choi and Kim, 2011a). In this study, an eukayotic expression cassette that contained CMV immediate-early promoter-driven enhanced GFP (eGFP) gene was inserted into the MCS region of the pG02-ASD-MCS. A fragment containing CMV immediate-early promoter, multiple cloning sites (MCS), and bovine growth hormone polyadenylation (BGHpA) signal was PCR-amplified using a pair of primers, pCMV-F-*Bgl*II (5'-AGATCTCCCGATCCCCTATGGTGCAC-3') and BGHpA-R-*Sal*I (5'-GTCGACCCATAGAGCCCACCGCAT-3'), and the pcDNA 3.1(+) vector (Invitrogen) as a template. The PCR product was purified using gel purification kit (Cosmo Genentech, Korea), and cloned into pGEMT-easy vector (Promega), resulting in pCMV-MCS-BGHpA. A PCR fragment encoding the open reading frame (ORF) of the eGFP gene was amplified using the primers of eGFP-kozak-F-*Hind*III (5'-AAGCTTCGCCACCATGGTGAGCAAGGGCGAGG-3') and eGFP-R-*Xho*I (5'- CTCGAGTTACTTGTACAGCTCGTCCATGC-3'), and plasmid pEGFP-C1

(Clontech) as the template. The amplified fragment was cloned into pGEMT-easy vector, digested with the *Hind*III and *Xho*I restriction enzymes, and inserted into above pCMV-MCS-BGHpA vector, resulting in pCMV-eGFP-BGHpA. The eGFP expressing cassette was cut by digestion of the pCMV-eGFP-BGHpA with *Apa*I and *Nsi*I, then, ligated to the pG02-ASD-MCS, and designated as pG02-ASD-CMV-eGFP (Fig. 3-1).

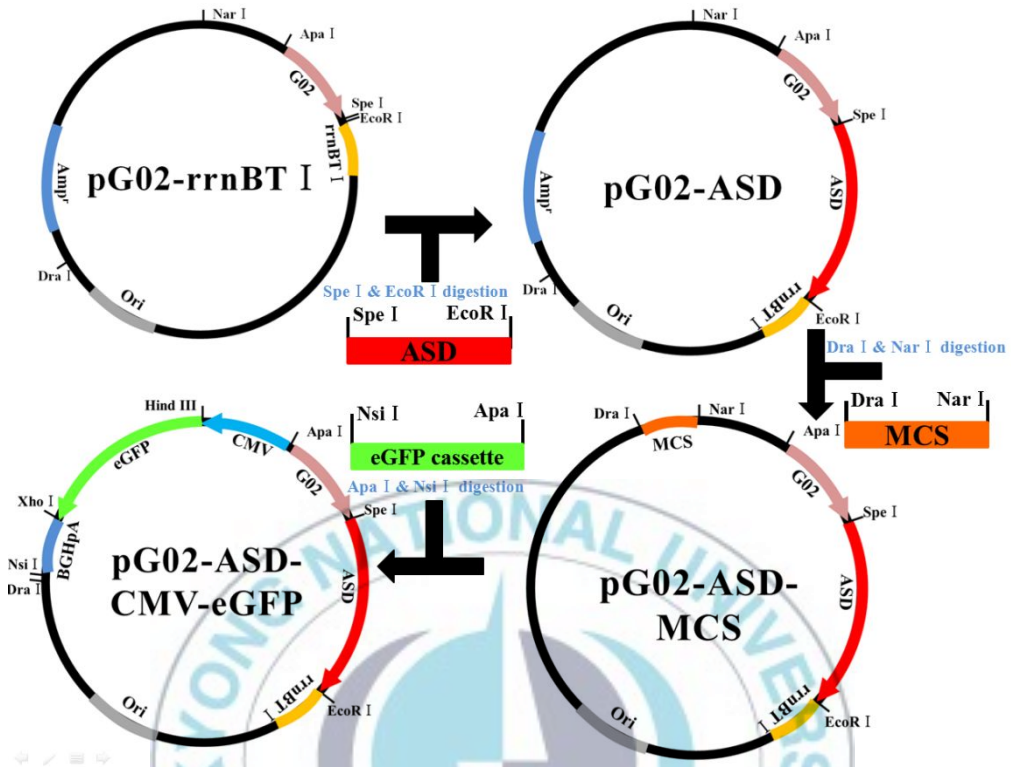


Fig.3-1. Map of the plasmid pG02-ASD-CMV-eGFP. The vector contains two expression cassettes. One is an eukaryotic expression cassette that is composed of the cytomegalovirus immediate-early promoter (CMV)-driven enhanced green fluorescent protein (eGFP) gene and bovine growth hormone polyadenylation (BGHpA) signal. The other is a prokaryotic expression cassette that consists of a weak constitutive promoter (G02) of *E. tarda*, aspartate semialdehyde dehydrogenase (ASD) gene, and a transcriptional terminator (rrnBT1).

3. *In vivo persistence of orally administered Δalr Δasd E. tarda*

In vivo persistence of orally administered *Δalr Δasd E. tarda* was investigated according to the method described in the previous paper (Choi and Kim, 2011b). Twenty-four olive flounder fingerlings (body weight 2-5 g) were orally intubated with 1 x 10^9 CFU/fish of the *Δalr Δasd E. tarda* that habors pG02-ASD-CMV-eGFP, and the colony numbers of Δ*alr* Δ*asd E. tarda* in liver, spleen, head kidney, and intestine were counted at various time points (2, 4, 6, 12, 24, 48, 96 and 168 h) after the intubation from 3 randomly sampled fish at each time point.

4. In vivo administration of Δalr Δasd E. tarda harboring pG02-ASD-CMV-eGFP

For analysis of eGFP expression in internal organs of fish administered *Δalr Δasd E. tarda* harboring pG02-ASD-CMV-eGFP, fifty-four olive flounder fingerlings were randomly divided into 3 groups and reared in three 200 L aquaria (18 fish/aquarium) at 20-22°Cfor 2 weeks prior to the experiment. Fish in each experimental group were intraperitoneally (i.p.) injected with 2 x 10^7 CFU/fish or orally intubated with 1 x 10^9 CFU/fish of Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-CMV-eGFP in 20 μl of PBS. Fish in the control group were i.p. injected with PBS (20 μl) alone. At 1, 3, 7, 14, 21, and 28 days post-administration, three fish were randomly sampled from each group, euthanized by transfer to water containing MS-222 (Sigma) and dissected to excise internal organs including liver, spleen, and head kidney. In the orally administered group, intestine was additionally isolated, and fish sampled at 28 days post-administration were bled to obtain serum before dissection.

5. Western blot analysis to confirm the expression of eGFP in internal organs

Each internal organ sample was homogenized in an extraction buffer (250 mM Tris-Cl, pH 7.8 and 1 mM phenylmethylsulfonylfluoride) using an ultrasonicator at 4 °C, centrifuged at 10,000 × *g* for 10 min, and then the supernatant containing soluble fraction was subjected to eGFP analysis using Western blot. For each sample, total protein content in the supernatant was measured using Protein Assay Kit (Bio-Rad, USA). Each supernatant containing 100 μg of total protein was mixed with SDS sample buffer (10% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 0.3M Tris-HCl pH 6.8, 0.05% bromophenol blue, 50% glycerol), boiled for 10 m at 95°C, and resolved by 10% (wt/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membrane with transfer buffer (12 mM Tris-HCl, 96 mM glycine, 20% methanol, pH 8.3) by using a Trans-Blot SD Cell (Bio-Rad) at 25V for 90 min. The membrane was blocked with blocking solution (3% BSA in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with rabbit polyclonal GFP (1:1000, Santa Cruz) for 2 h at RT and washed. After washing 3 times, the membrane incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2000, Santa Cruz) for 1.5 h at RT. After washing off unbound antibody, the specific antigen-bound antibody was visualized with nitroblue tetrazolium and 2-bromo-2-chloro-2-indoly phosphate (NBT-BCIP) substrate buffer (Sigma).

6. Serum agglutination activity against E. tarda

The serum collected from fish in each group at 28 days post-administration were serially diluted two-fold, to which a constant amount of formalin-killed *E. tarda* (FKC; approximately 4 mg/ml) were added and kept overnight at room temperature. The agglutination activity was determined as the first serum dilution where no agglutination occurred, and expressed as the reciprocal of that dilution. The activities were analyzed by the Student's *t*-test, and significant

differences were determined at $P<0.05$.

7. Western blot analysis to confirm serum antibody against eGFP

SDS-PAGE of purified recombinant GFP protein (50 μg/ml) was conducted as the above method. Trans-blotted membranes were incubated with each of the above olive flounder sera (1:50) for 2 h at RT and washed. The membrane was incubated with rabbit antiserum against olive flounder IgM (1:500) for 2 h at RT. After washing 3 times, the membrane incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2000) for 1.5 h at RT. After washing off unbound thirdly antibody, the specific antigen-bound antibody was visualized with NBT-BCIP substrate buffer.

RESULTS

1. Persistence of orally administered Δalr Δasd E. tarda in olive flounder

Recovery of live *Δalr Δasd E. tarda* harboring pG02-ASD-CMV-eGFP in internal organs including intestine, liver, head kidney, and spleen was analyzed after a single oral intubation of 1 x 10^9 CFU of the mutated bacteria per fish. From 2 to 24 h post-administration, 10^3 -10^5 CFU/10 mg tissue of the bacteria were found in the intestine (Fig. 3-2). Only at 6 h post-intubation, approximately 10^1 CFU/10 mg tissue was found from the spleen. No colonies were found from the head kidney and liver to the end of the experiment.

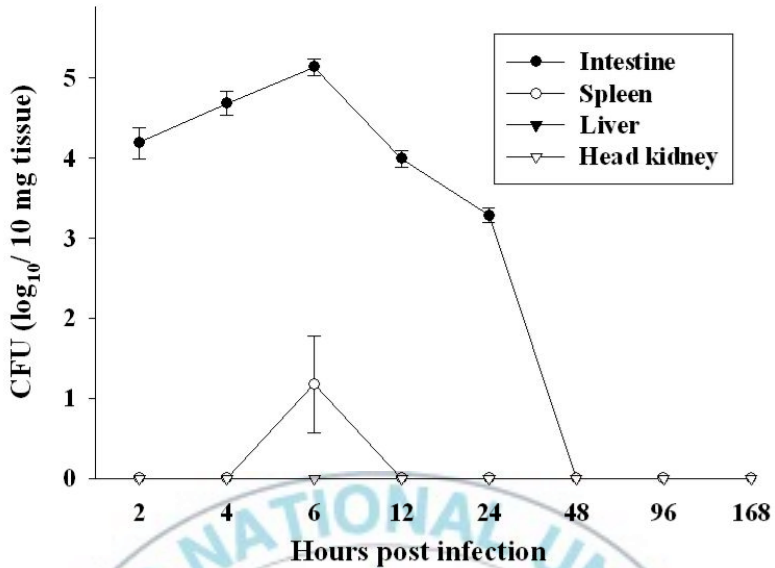


Fig.3-2. Persistence of orally administered Δalr Δasd E. tarda in olive flounder. Recovery of the auxotrophic *Edwardsiella tarda* double-knockout mutant (*Δalr Δasd E. tarda*) harboring plasmid pG02-ASD-CMV-eGFP from each internal organ (head kidney, liver, spleen, and intestine) of olive flounder (*Paralichthys olivaceus*) that were orally intubated with 10^9 CFU of the bacteria. At 2, 4, 6, 12, 24, 48, 96 and 168 h post intubation, the mutant bacteria recovered from each tissue were counted by plating onto *Salmonella Shigella* agar (SS agar) supplemented with 50 mM D-alanine. Each point represents mean ± standard deviation of the bacteria numbers per 10 mg of each tissue sampled from three fish.

2. Expression of EGFP in internal organs

In fish i.p. injected with 2 x 10^7 CFU/fish of Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-CMV-eGFP, expression of eGFP was detected in liver, kidney, and spleen from 1 day to 12-28 days post-injection (Fig. 3-3 A). In fish orally administered with 1 x 10^9 CFU/fish of the bacteria, the eGFP band was detected in liver, kidney, and spleen from 1 day to 7-14 days post-administration, whereas, in intestine, the band was detected only at 1 day post-administration (Fig. 3-3 B).

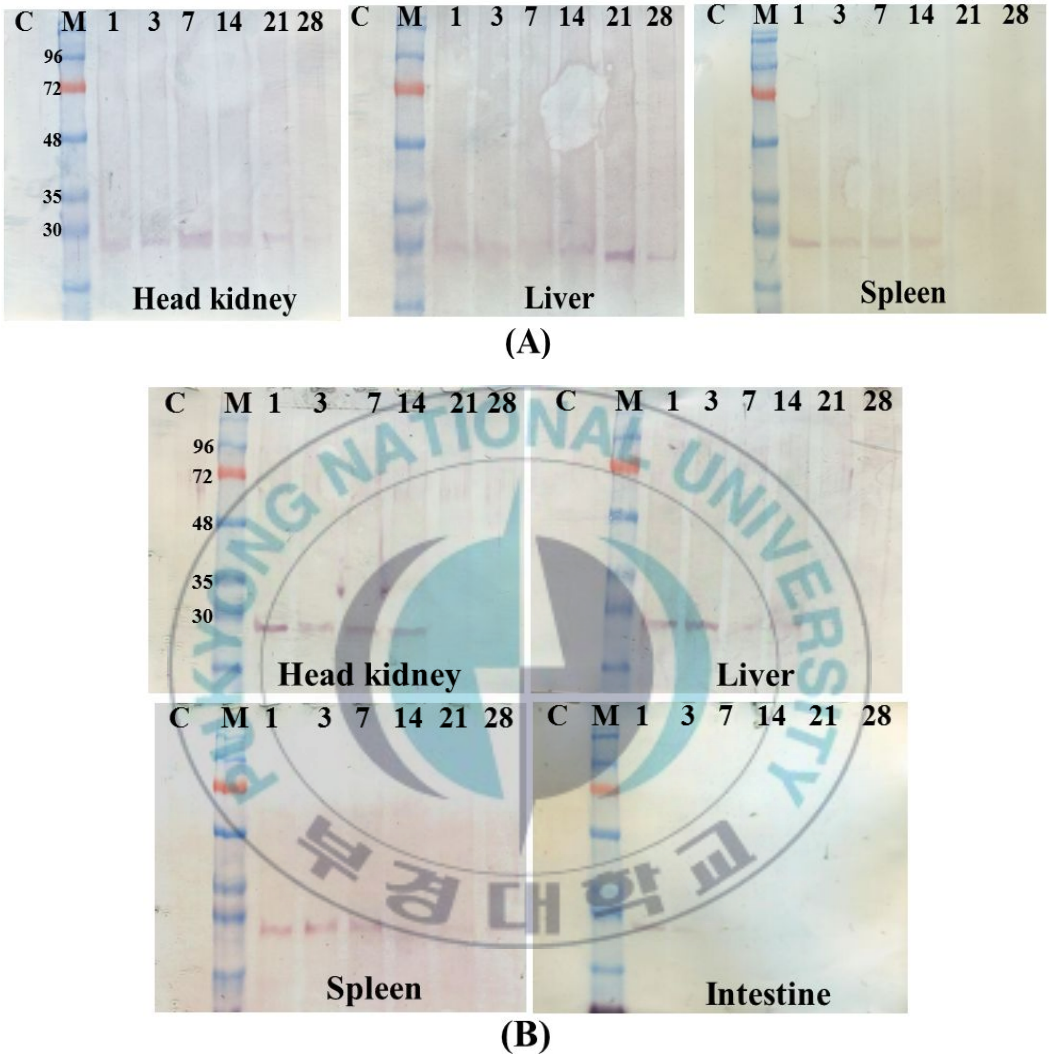


Fig.3-3. Expression of EGFP in internal organs. Western blot analysis to confirm expression of a foreign protein, eGFP, in internal organs of olive flounder (*Paralichthys olivaceus*) that were (A) intraperitoneally (i.p.) injected with 2 x 10^7 CFU/fish or (B) orally intubated with 1 x 10^9 CFU/fish of Δ*alr* Δ*asd Edwardsiella tarda* harboring pG02-ASD-CMV-eGFP in 20 μl of PBS. Fish in the control group were i.p. injected with PBS (20 μl) alone. At 1, 3, 7, 14, 21, and 28 days

post-administration, internal organs were excised from three fish in each tank for western blot analysis. M, prestained protein marker (ELPIS Biotech, Korea).

3. Serum agglutination activity against E. tarda

Serum agglutination activity against *E. tarda* was significantly increased in fish administered Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-CMV-eGFP by either i.p. injection or oral intubation (Fig. 3-4). The activity of i.p. injected fish was distinctly higher than that of orally administered fish.

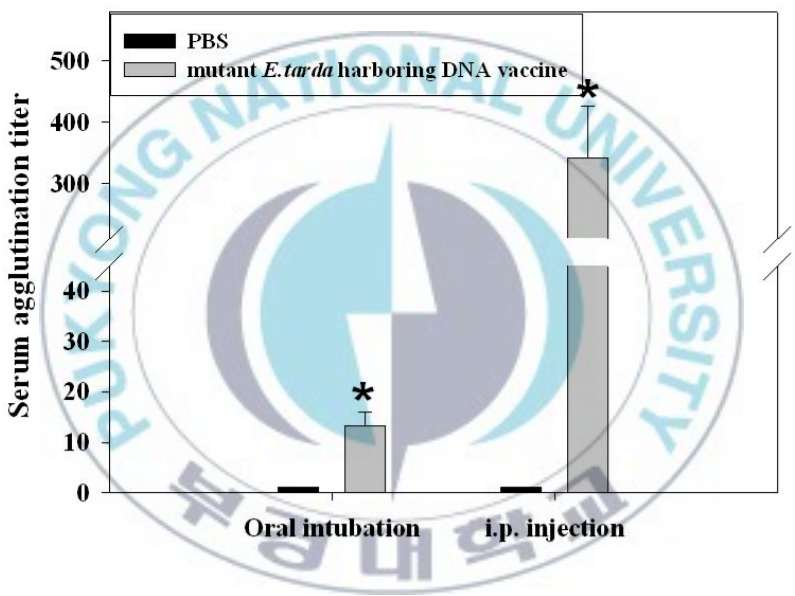


Fig.3-4. Serum agglutination activity against *E. tarda*. Agglutination activity against formalin-killed *Edwardsiella tarda* NH1 in sera of olive flounder (*Paralichthys olivaceus*) that were intraperitoneally (i.p.) injected with 2 x 10^7 CFU/fish or orally intubated with 1 x 10^9 CFU/fish of Δ*alr* Δ*asd Edwardsiella tarda* harboring pG02-ASD-CMV-eGFP. At 4 weeks post-administration, 3 fish in each group were bled to obtain serum for agglutination analysis. Values are mean ± standard deviation. Asterisks on the bars represent significantly different at $P < 0.05$ compared to PBS control group.

4. Generation of serum antibody against eGFP

Production of antibodies against eGFP in fish administered Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-CMV-eGFP by either i.p. injection or oral intubation was confirmed by western blot analysis (Fig. 3-5).

Fig.3-5. Generation of serum antibody against eGFP .Western blot analysis for confirmation of the presence of serum antibody against eGFP in olive flounder (*Paralichthys olivaceus*) that were intraperitoneally (i.p.) injected with 2 x 10^7 CFU/fish or orally intubated with 1 x 10^9 CFU/fish of Δ*alr* Δ*asd Edwardsiella tarda* harboring pG02-ASD-CMV-eGFP. M, prestained protein marker (Bio-Rad); Lane 1, serum of PBS injected fish; Lane 2, serum of orally immunized fish; Lane 3, Serum of i.p. injected fish. The band indicated by an arrow corresponds to the molecular weight of eGFP protein.

DISCUSSION

Use of antibiotic resistance genes in the DNA vaccine vector is one of the problems to be used for commercial vaccines due to bio-safety and environmental concerns (Tonheim et al., 2008). In a previous study, we had demonstrated that a plasmid vector equipped with an *asd* gene expression cassette effectively complemented the knock-down of *asd* gene in *Δalr Δasd E. tarda,* and enabled the mutated *E. tarda* to replicate without supplementation of diaminopimelic acid (DAP) and antibiotics (Choi and Kim, 2011a). In this study, use of DNA vaccine vector harboring both the *asd* expression cassette and the eukaryotic cassette for expression of reporter eGFP gene has allowed the *Δalr Δasd E. tarda* to transport the DNA vaccine plasmids without use of antibiotic resistant gene.

In this study, the *Δalr Δasd E. tarda* harboring pG02-ASD-CMV-eGFP will undergo lysis in the fish because of lack of *D*-alanine. According to the results of our previous studies, the auxotrophic mutant *E. tarda* were recovered untill 96 h post-injection from internal organs (liver, head-kidney, and spleen) of olive flounder that were injected intraperitoneally with 10^7 CFU/fish (Choi and Kim, 2011a). In the results of this study, the expression of reporter protein eGFP in all examined internal organs of fish that were i.p. injected with the mutated bacteria harboring pG02-ASD-CMV-eGFP suggests that the mutated *E. tarda* administered through i.p. route might act as an active transporter for transmission of the DNA vaccine plasmids into host cells of internal organs, which results in the successful expression of the reporter protein, eGFP, in the internal organs of olive flounder. In the present study, although oral dose of the *Δalr Δasd E. tarda* was increased to 10 times higher (1 x 10^9 CFU/fish) than the previous study (oral intubation with 1 x 10^8 CFU/fish) (Choi and Kim, 2011b), the pattern of bacterial recovery was not largely different

from the previous result except more colonies of the mutated *E. tarda* from the intestine and a few colonies in the spleen from one fish at an early period. However, in spite of no or scarce recovery of the mutated *E. tarda* from the liver, head kidney and spleen, expression of the reporter protein eGFP was detected from all the examined internal organs. Although the exact cell types responsible for capturing and expressing DNA vaccines following oral administration with the *Δalr Δasd E. tarda* harboring pG02-ASD-CMV-eGFP are not clear, the present result suggests that the *E. tarda* harboring DNA vaccine plasmids might be transported into the internal organs by phagocytosing APCs such as macrophages.

Although i.m. administration of naked plasmids has been the most frequently used route for DNA vaccine in fish, stress of fish due to injection could be disadvantages. The present attenuated *E. tarda*-based DNA vaccine delivery system induced adaptive humoral immunity in fish against the antigen of DNA vaccine even when administered through oral route, suggesting that this bacteria-vectored DNA vaccine delivery is a promising alternative to i.m. delivery of DNA vaccines in fish.

The present results showed that either oral or i.p. immunization of olive flounder with recombinant *E. tarda* that carried heterologous antigen (eGFP)-expressing eukaryotic plasmids was successful to express the antigen in the internal organs and to induce humoral adaptive immunity against not only *E. tarda* that was used as a delivery vehicle but also eGFP that was used as the reporter antigen of DNA vaccine, suggesting attenuated *E. tarda*-vectored DNA vaccine has a high potential to be used as a combined vaccine against fish infectious diseases.

Chapter IV.

Protection of olive flounder (*Paralichthys olivaceus*) from viral hemorrhagic septicemia virus (VHSV) by immunization with auxotrophic *Edwardsiella tarda* mutant harboring VHSV DNA vaccine

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) causes a fatal disease in a wide range of freshwater and marine fish species worldwide (Schlotfeldt and Ahne, 1988; Schlotfeldt et al., 1991; Mortensen et al., 1999; Isshiki et al., 2003; Skall et al., 2005), resulting in a great reduction of aquaculture productivity. VHSV is an enveloped negative-strand RNA virus belonging to the genus *Novirhabdovirus* of the family Rhabdoviridae (Lenoir and de Kinkelin, 1975; Walker et al., 2000; Tordo et al., 2005). Possession of the nonstructural NV gene in the genome is a distinctive characteristic of *Novirhabdovirus*, and the rest of genes are encoded to a nucleoprotein (N), a polymerase-associated phosphoprotein (P), a matrix protein (M), a glycoprotein (G), and an RNA-dependent RNA polymerase (L) (Schütze et al. 1996, 1999, Trdo et al. 2005). Based on genetic analysis, VHSV have been divided into four genotypes that are correlated with the geographic location, virulence, and host species (Snow et al., 1999, 2005; Einer-Jensen et al., 2004; Brudeseth et al., 2008). Since firstly reported at 2001, epizootics of VHSV genotype IV during low temperature period have severely hampered olive flounder (*Paralichthys olivaceus*) farms in Korea (Kim et al., 2003, 2009). Therefore, effective countermeasures should be urgently established to reduce damages caused by VHSV.

In fish rhabdoviral diseases, DNA vaccines targeting viral G gene have been reported as an effective prophylactic measure against infectious hematopoietic necrosis virus (IHNV) and VHSV in rainbow trout and Atlantic salmon (Traxler et al., 1999; Lorenzen et al., 2001, 2002; Lorenzen and LaPatra, 2005; Chico et al., 2009; Einer-Jensen et al., 2009). However, little information is available on the DNA vaccine against VHSV in olive flounder.

Previously, we have verified that an auxotrophic *Edwardsiella tarda* mutant (*Δalr Δasd E.*

tarda) has a potential to be used as a delivery vehicle for DNA vaccine in olive flounder, in which immunization of fish with the *E. tarda* mutant harboring plasmids equipped with a cytomegalovirus (CMV) promoter-driven heterologous antigen (eGFP)-expressing cassette was successful to induce humoral adaptive immunity against not only *E. tarda* but also eGFP. In the present study, we further evaluated the availability of the auxotrophic *E. tarda* mutant as a delivery vehicle for DNA vaccine against VHSV in olive flounder.

The CMV promoter has been widely used for expression of antigen gene in DNA vaccines in vertebrates including fish. However, it has been reported that the power of CMV promoter is weaker than that of carp β-actin promoter (Ruiz et al. 2008). Thus, in this study, we compared the power of β-actin promoter of marine medaka (*Oryzias dancena*) with that of CMV promoter, in which marine medaka β-actin promoter was clearly stronger than CMV promoter, and used the marine medaka β-actin promoter as the promoter for expression of VHSV G gene in DNA vaccine.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

The recombinant *E. tarda* (Δ*alr* Δ*asd E. tarda*) used in this study was generated in a previous study (Choi and Kim, 2011). The mutant bacteria were grown in Luria-Bertani (LB, Difco) medium supplemented with 50 mM D-alanine, and 50 mM diaminopimelic acid (DAP, Sigma).

2. Oryzias dancena β-actin gene

Full length β-actin gene and cDNA sequence of *Oryzias dancena* was kindly provided by Prof. Yoon Kwon Nam (Department of Aquaculture, Pukyong National University).

3. Cells culture, virus and transfection

Epithelioma papulosum cyprini (EPC) cells were cultured in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS, Gibco).

The viruses used for infection were VHSV KJ2008 (Kim & Kim, 2011). The viruses were propagated in monolayer of EPC cells at 15°C in the presence of 2% FBS and antibiotics. Cultures with extensive cytopathic effect (CPE) were harvested and centrifuged 4000 g for 10 min at 4°C, and the supernatants were stored at −80°C.

EPC cells were cultured in wells of 6-well plates (3×10^5 cells/well) at 28°C in L-15 medium containing 20 mM HEPES and 10% FBS. Cells were grown to about 80% confluence and transfected with above heterologous protein roducing vectors or the control vector (pDsRed2-1) using FuGENE 6 (Roche) according to the manufacturer's instructions.

4. Construction of heterologous expression vector

Expression of reporter constructs driven by *Oryzias dancena* β-actin promoter or hCMV promoter or CAG promoter (CMV enhance + *Oryzias dancena* β-actin promoter) constructed for the *in vitro* transfection experiment using EPC cell lines.

The *Oryzias dancena* β-actin promoter fragment was obtained by PCR isolation using a pair of oligonucleotide primers, OD b-act Fo and OD b-act Re containing *Sal*I and *Age*I restriction sites (underlined), respectively. After TA cloning into pGEM-T easy vector, the β-actin promoter fragment was excised using *Sal*I and *Age*I in order to replace the promoter empty vector (pDsRed2-1 plasmid ; Clontech, USA), and designated as pODβA-RFP.

The hCMV promoter fragment was excised using *Sal*I and *Age*I in order to replace the *Bgl*II-*Age*I *Oryzias dancena* β-actin promoter in pODβA-RFP, which named pCMV-RFP. Next, the *Bgl*II-*Sal*I fragment containing the CMV enhancer fragment, which was obtained from a pCDNA3.1(+) plasmid (Invitrogen), was ligated to the *Bgl*II-*Sal*I site upstream of the *Oryzias dancena* β-actin promoter of pODβA-RFP, and designated as pCAG-RFP. DNA vaccines encoding VHSV G protein gene (pODβA-VG, pCMV-VG) in the expression vector pODβA-RFP, pCMV-RFP were used for this study.

The VHSV G protein gene was obtained by PCR isolation using a pair of oligonucleotide

primers, VG Fo *AgeI* and VG Re *NotI* his containing *AgeI* and *NotI* restriction sites, respectively. After TA cloning into pGEM-T easy vector, the VHSV G fragment was excised using *AgeI* and *NotI* in order to replace the RFP in pODβA-RFP and pCMV-RFP, and designated as pODβA-VG, pCMV-VG.

5. In vitro RFP expression assay

RFP expression level was confirmed by fluorescence microscopic bservation and quantification of fluorescence at 48 after transfection. After microscopic observation, each transfected cell was lysed in 0.1% Triton X-100 buffered with 0.1 M Tris–Cl (pH7.4), centrifuged at 8000g for 10 min, and then the supernatant containing soluble fraction was collected. For each sample, total protein quantity was measured by BCA methods. RFP fluorescence was measured using Polarion fluorescent plate reader (Tecan) using cell lysate of non-transfected cells as back ground value. RFP expression level of each sample was then calculated as fluorescent reading per μg protein.

6. In vivo intramuscular injection RFP expression assay

For the *in vivo* injection experiment olive flounder fingerlings (average body weight =10 ± 2 g) purchased from a local farm were transferred to the laboratory aquarium. Fish (n= 15 per group) were injected intraperitoneally (i.p.) with 100 μg of circular plasmid (pODβA-RFP, pCMV-RFP, pCAG-RFP or pDsRed2-1) resuspended in 50 μL of phosphate buffered saline (PBS; pH 7.6. The control group was 50 μl of PBS as controls. At 48 h after injection, muscle tissue (0.5–1.0 cm^3) around the injection site (1 cm below at the last dorsal fin ray) was sampled

individually. Each muscle sample was homogenized in extraction buffer (250 mM Tris-Cl [pH 7.8], 1 mM phenylmethylsulfonylfluoride) using a motor-driven tissue homogenizer at 4°C, and centrifuged at 2000×g for 10 min. Then, the supernatant containing the soluble fraction was subjected to RFP analysis using a Polarion fluorescent plate reader (Tecan). For each sample, the total protein content in the supernatant was measured using a protein assay kit (Bio-Rad, USA). RFP expression level of each sample was then calculated as fluorescent reading per μg protein.

7. Vaccination and challenge

One hundred twenty olive flounder fingerlings (7-10 g) were randomly divided into 4groups, and reared in eight 500 L tanks (30 fish/tank) at 20-22°C. Fish were fed with a commercial pellet and acclimatized for 2 weeks before being immunized, and were anesthetized with MS222 (Sigma) just before injection of the vaccine or blood sampling. Fish in each group were i.p. immunized with 2 x 10^7CFU/fish of Δ*alr* Δ*asd E. tarda* harboring pODβA-VG, pCMV-VG, or pDsRed2-1 in 50 μl of PBS. A group that received PBS (50 μl) represented the control. At 3 weeks immunization, fish in each group were transferred to tanks equipped with a refrigerating apparatus, and were adapted to 15°C by gradual decrease of water temperature. At 4 weeks immunization, 6 fish in each tank were bled to obtain serum, and the remnant fish were intramuscularly challenged with the pathogenic wild-type VHSV KJ2008 at 10^2, 10^3 PFU/fish. Mortalities in the challenge experiments were recorded daily for 21 d post-challenge.

8. Neutralization test

For neutralization test, the sera were previously heat inactivated at 56°C for 30 min to

inactivate complement. The serially diluted sera (50μl/well) were mixed with an equal volume of wild-type VHSV (10^3 PFU) in U-shaped 96-well plates, and incubated at 15°C for 1h. Then, 100 μl of each mixture was added to triplicate wells containing EPC cells monolayer, and observed everyday CPE. The titer of each serum was the last dilution at which CPE was not observed.

9. Statistical analyses

Serum neutralization data were analyzed by the Student's *t*-test. Significant differences were determined at $P<0.05$.

Table 4-1. Oligonucleotides used in this study

Name of Oligonucleotides	Sequence (5`~3`)
OD b-act Fo	GTCGAC AGCCTGATAGTGACGCTTCAGTTTC
OD b-act Re	ACCGGT GGCTAAACTGGAAAAGAACAAACAAATGAGCC
VG Fo *AgeI*	ACCGGT CGCCACCATGGAATGGAATACTTTT TTCTTGGTG
VG Re *NotI* his	GCGGCCGC TCAATGATGATGATGATGATGGACCATCTGGCTTCTGGAGA
eGFP Fo *AgeI*	ACCGGT CGCCACC ATGGTGAGCAAGGGCGAGGAGCTGT
eGFP Re *NotI*	GCGGCCGC TTACTTGTACAGCTCGTCCATGCCGAG
CMV Fo *SalI*	GTCGAC ATAGTAATCAATTACGGGGTCATTAGTTCATA
CMV Re *AgeI*	ACCGGT GATCTGACGGTTCACTAAACCAGCTCTGC
Enhancer Fo *BglII*	AGATCT ACATAACTTACGGTAAATGGCCCGCCTGGC
Enhencer Re *SalI*	GTCGAC GAT GACTAATACGTAGATGTACTGCCAA

RESULTS

1. Vector Construction of DNA vaccine

The finally constructed vectors are shown in Fig. 4-1

The pCMV-RFP, an eukayotic expression cassette that contained CMV immediate-early promoter-driven enhanced RFP and pODβA-RFP, expression of reporter constructs driven by *Oryzias dancena* β-actin promoter and pCAG-RFP, CAG fragment containing CMV enhance + *Oryzias dancena* β-actin promoter were successfully constructed in this study.

Plasmid pODβA-VG, pCMV-VG, the VHSV G fragment was excised in order to replace the RFP.

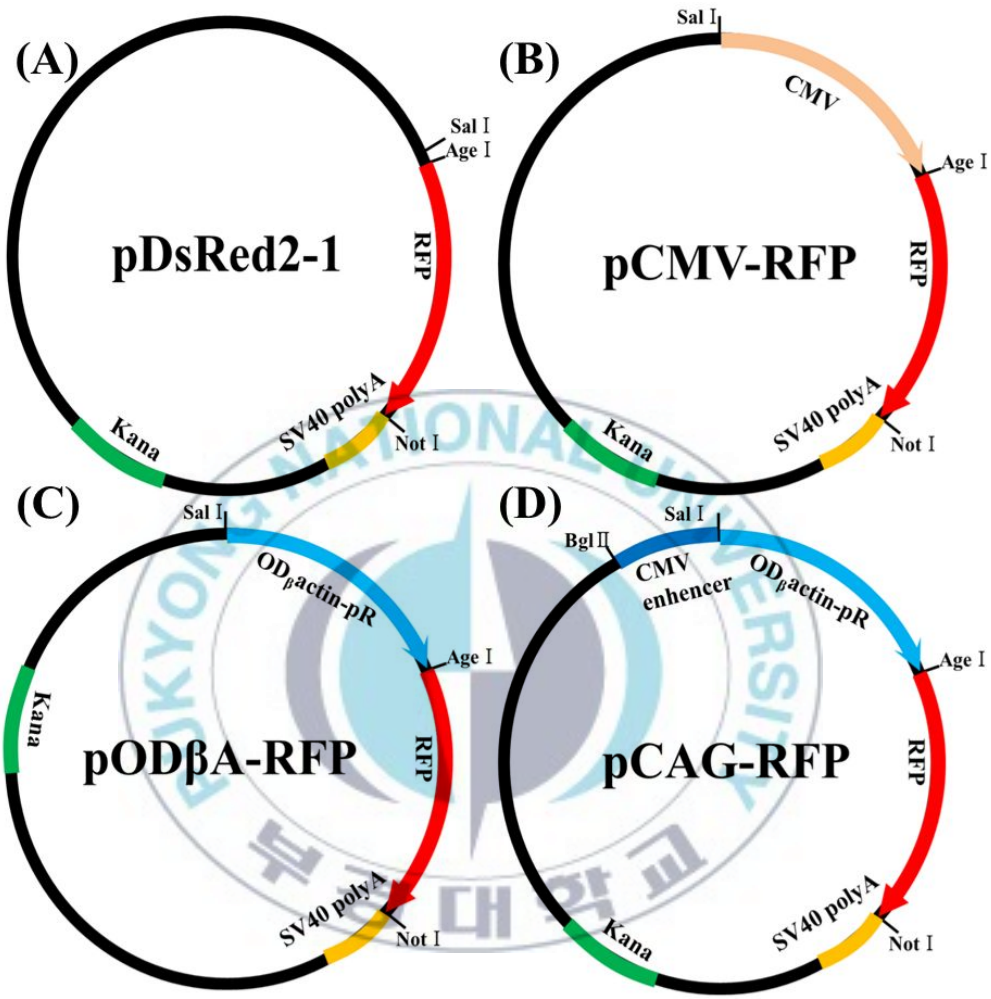


Fig.4-1. Plasmids constructed in the present study.

(A) pDsRed2-1 plasmid :promoter empty vector, (B) pCMV-RFP plasmid : an eukayotic expression cassette that contained CMV immediate-early promoter-driven enhanced RFP, (B) pODβA-RFP plasmid : *Oryzias dancena* β-actin promoter was excised using *Sal*I and *Age*I in order to replace the promoter empty vector, (D) pCAG-RFP plasmid : CMV enhancer fragment was ligated to the upstream of the *Oryzias dancena* β-actin promoter of pODβA-RFP.

2. In vitro & in vitro RFP expression of compared the power promoter

Based on the RFP expression assay was detectable in EPC cells transfected with among pDsRed2-1, pCMV-RFP, pODβA-RFP, pCAG-RFP. The RFP expression were significantly higher in the groups transfected with pODβA-RFP, pCAG-RFP than those with pCMV-RFP (P<0.05 based on student's t-test) in EPC cells.

As a result of the *in vivo* injection experiment, the background fluorescence reading observed from the PBS-injected control was subtracted for the normalization of RFP signals observed in the plasmid construct-injected groups. Among pCMV-RFP, pODβA-RFP and pCAG-RFP induced *de novo* synthesis of RFP protein in injected olive flounder muscle, although significant variation was found in the levels expressed among individuals.

Significantly higher amounts of RFP (more than threefold) were detected in muscles injected with the construct driven by the β-actin promoter (pODβA-RFP), CAG promoter(pCAG-RFP) than in those injected with the hCMV-driven construct (pCMV-RFP).

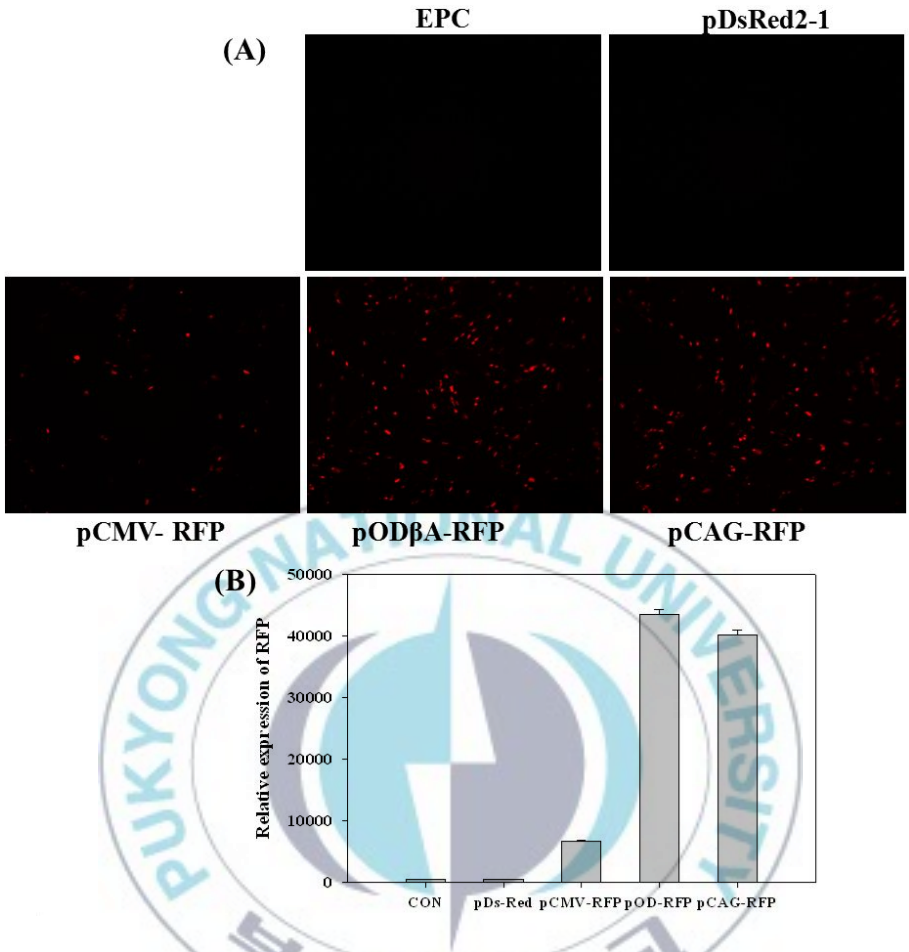


Fig.4-2. In vitro RFP expression of compared the power promoter

Expression of reporter constructs driven by *Oryzias dancena* β-actin promoter or hCMV promoter or CAG promoter in transfected EPC cell lines.(A) Representative fluorescence microscopy observation image of EPC cell lines, transfected with pDsRed2-1 or pCMV-RFP or pODβA-RFP or pCAG-RFP.Expression of reporter constructs driven by *Oryzias dancena* β-actin promoter or hCMV promoter or CAG promoter in transfected EPC cell lines. (B) At 48 h after transfection, RFP expression level was quantified using fluoremeter. RFP expression level was normalized against RFP activity and total protein quantity of each sample. Data was presented as mean of triplicate with standard deviation.

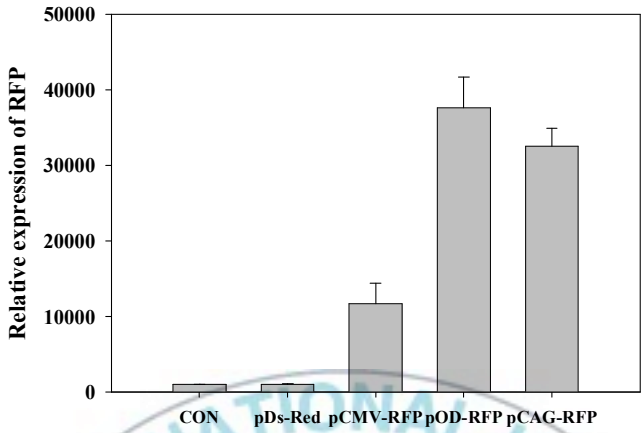


Fig.4-3. In vivo RFP expression of olive flounder intramuscular injection assay

Potency of the *Oryzias dancena* β-actin promoter or hCMV promoter or CAG promoter to drive heterologous expression of the downstream reporter gene in the olive flounder skeletal muscles. Plasmid constructs were introduced into juveniles through a direct intramuscular *in vivo* injection. Relative RFP expression was fluorometrically measured as RFP protein per mg soluble protein). Each value represented mean of 3 independent experiments with standard division.

3. Protective efficacy against VHSV challenges

In the 10^3PFU/fish challenge experiment conducted at 4 weeks after the immunization, the cumulative mortality rates in fish immunized with PBS, Δ*alr* Δ*asd E. tarda*, pCMV-VG/Δ

Δ and pODβA- VG/ΔΔ were 90%, 70%, 70%, and 50%, respectively (Fig. 4-4 A).

In 10^3PFU/fish challenge experiment, the cumulative mortalities of control, Δ*alr* Δ*asd E. tarda*, pCMV-VG/ΔΔ and pODβA- VG/ΔΔ immunized groups were 80%, 60% , 40%, 30% respectively (Fig.4-4 B)

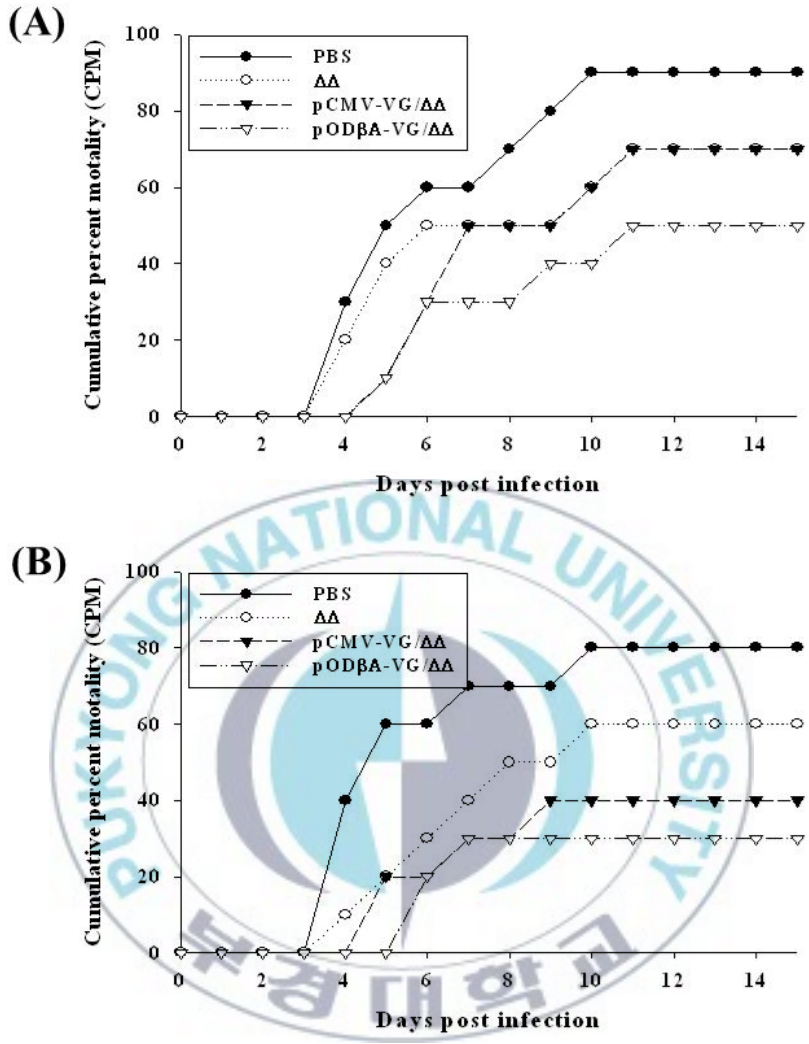


Fig.4-4. Vaccine efficacy of auxotrophic *Edwardsiella tarda* mutant harboring VHSV DNA vaccine. Olive flounder were intraperitoneally (i.p.) immunized with Δ*alr* Δ*asd E. tarda* harboring pODβA-VG, pCMV-VG, Δ*alr* Δ*asd E. tarda* and control PBS. At 4 weeks after the immunization of fish in each experimental group were challenged with (A) 10^3PFU/fish, (B) 10^2PFU/fish of wild-type VHSV KJ 2008. Cumulative mortality rates were recorded daily for 21 d post-challenge.

4. Serum neutralization activity

The serum neutralization titer of fish immunized with pCMV-VG/ΔΔ and pODβA- VG/ΔΔ was significantly higher than that of fish immunized with Δ*alr* Δ*asd E. tarda* (Fig.4-5). The serum of fish in the control group showed no neutralization activity.

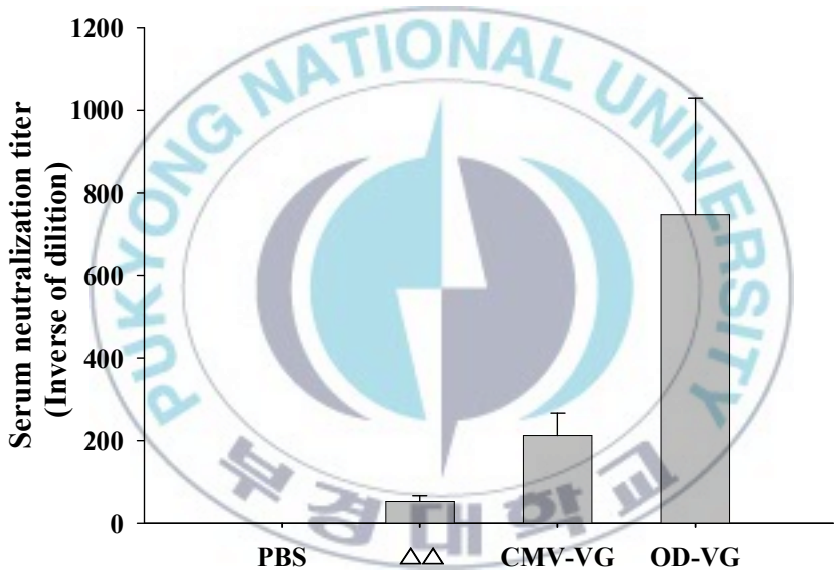


Fig.4-5. Serum neutralization activity. Neutralization activity against wild-type VHSV KJ 2008 in sera of olive flounder (*Paralichthys olivaceus*) immunized by intraperitoneally (i.p.) injection of PBS (Control), or Δ*alr* Δ*asd E. tarda* harboring pODβA-VG, pCMV-VG, Δ*alr* Δ*asd E. tarda*. At 4 weeks post-immunization, 6 fish in each group were bled to obtain serum for neutralization. Values are mean ± standard deviation. Different letters on the bars represent significantly different at $P < 0.05$.

DISCUSSION

The level of antigen expression in host cells transfected with DNA vaccine vector is one of the critical factors for inducing effective adaptive immune responses. Thus, use of an appropriate promoter to express antigen gene is a prerequisite for DNA vaccines. In the present study, the marine medaka β-actin promoter was stronger than CMV promoter. Beta-actin is one of the most abundantly expressed proteins in eukaryotic cells, and the promoter of β-actin has been characterized in mammals and birds (Kawamoto et al., 1988; Quitschke et al., 1989; Danilition et al., 1991), and used to express exogenous genes (Miyazaki et al., 1989; Williams et al., 1991; Zhang et al., 1994). In fish, β-actin promoter has been reported from common carp (*Cyprinus carpio*) (Liu et al., 1990), zebrafish (*Danio rerio*) (Higashijima et al., 1997), tilapia (*Oreochromis niloticus*) (Hwang et al., 2003) and two cyprinid species (Noh et al., 2003; Feng et al., 2006). Recently, Ruiz et al. (2008) reported that transcription activity of carp β-actin promoter in *Epithelial papulosum cyprini* (EPC) cells was about two times stronger than that of CMV promoter. Similarly, in the present study, marine medaka β-actin promoter was stronger more than two times than CMV promoter when the vectors were injected into the muscle of olive flounder, suggesting that certain piscine β-actin promoters would be more appropriate as promoters for DNA vaccine in fish species than CMV promoter. Furthermore, as cytomegalovirus is a virus that can infects human, use of fish β-actin promoter instead of CMV promoter in fish DNA vaccines would be a way to alleviate concerns on the safety of fish DNA vaccines.

To increase immune responses by driving high expression of antigen in DNA vaccines, vectors containing a hybrid CMV enhancer coupled to a modified chicken β-actin promoter (CAG) have been used, and shown to improve immune responses in accordance with increased

antigen expression in mammals (Sawicki et al., 1998; Garg et al., 2004). In the present study, we constructed another kind of CAG promoter that was made by fusion of CMV early enhancer element and marine medaka β-actin promoter. However, the new CAG promoter was not stronger than the marine medaka β-actin promoter alone, suggesting that fusion of CMV early enhancer element to fish β-actin promoter might have no beneficial effects on increasing antigen expression in fish cells.

The present results showed that immunization of olive flounder with recombinant *E. tarda* that carried DNA vaccine construct using either the marine medaka β-actin promoter or CMV promoter induced significantly higher serum neutralization titer against VHSV than immunization with the recombinant *E. tarda* alone or PBS. This result suggests that DNA vaccine plasmids in the recombinant bacteria are transported to the host cell's nucleus, and the expressed VHSV G protein might be exposed to the intercellular region, which allows producing antibodies against VHSV G protein. Interestingly, DNA vaccine vector with marine medaka β-actin promoter induced greater neutralization titer than the vaccine driven by CMV promoter, suggesting that the expressed amount of the antigen might be an important factor for inducing effective adaptive immune responses.

In the present challenge experiments, fish immunized with the *Δalr Δasd E. tarda* harboring DNA vaccine vector driven by the marine medaka β-actin promoter showed higher survival rates against challenge with two different PFUs of VHSV than fish immunized with the bacteria carrying DNA vaccine vector driven by CMV promoter, which are well coincide with the results of serum neutralization titer against VHSV. These results indicate that auxotrophic *E. tarda* mutant harboring marine medaka β-actin promoter-driven DNA vaccine vectors would be a potential system for prophylactics of fish viral diseases. Further studies on the effectiveness of this system by administration through oral or mucosal routes should be conducted for convenient use

of this system in commercial fish farms.

Summary

The aims of the present study were development of live attenuated bacterial vaccines and further assessment of potential of the attenuated bacteria as vehicles for combined vaccines.

In chapter I, Two auxotrophic genes that play essential roles in bacterial cell wall biosynthesis - alanine racemase gene (*alr*) and aspartate semialdehyde dehydrogenase gene (*asd*) - knock-out *Edwardsiella tarda* (Δ*alr* Δ*asd E. tarda*) was generated by the allelic exchange method to develop a combined vaccine system. Green fluorescent protein (GFP) was used as a model foreign protein, and was expressed by transformation of the mutant *E. tarda* with antibiotic resistant gene-free plasmids harboring cassettes for GFP and *asd* expression (pG02-ASD-EtPR-GFP). In vitro growth of the mutant *E. tarda* was similar to wild-type *E. tarda* when D-alanine and diaminopimelic acid (DAP) were supplemented to growth medium. However, without D-alanine and/or DAP supplementation, the mutant showed very limited growth. The *Δalr Δasd E. tarda* transformed with pG02-ASD-EtPR-GFP showed a similar growth pattern of wild-type *E. tarda* when D-alanine was supplemented in the medium, and the expression of GFP could be observed even with naked eyes. The virulence of the auxotrophic mutant *E. tarda* was decreased, which was demonstrated by approximately 10^6 fold increase of LD_{50} dose compared to wild-type *E. tarda*. To assess vaccine potential of the present combined vaccine system, olive flounder (*Paralichthys olivaceus*) were immunized with the GFP expressing mutant *E. tarda,* and analyzed protection efficacy against *E. tarda* challenge and antibody titers against *E. tarda* and GFP. Groups of fish immunized with 10^7 CFU of the *Δalr Δasd E. tarda* harboring pG02-ASD-EtPR-GFP showed no mortality, which was irrespective to boost immunization. The cumulative mortality rates of fish immunized with 10^6 or 10^5 CFU of the mutant bacteria were lowered by a

boost immunization. Fish immunized with the mutant *E. tarda* at doses of 10^6-10^7 CFU/fish showed significantly higher serum agglutination activities against formalin-killed *E. tarda* than PBS-injected control fish. Furthermore, fish immunized with 10^6-10^7 CFU/fish of the mutant *E. tarda* showed significantly higher ELISA titer against GFP antigen than fish in other groups. These results indicate that the present double auxotrophic genes knock-out *E. tarda* coupled with a heterologous antigen expression has a great strategic potential to be used as combined vaccines against various fish diseases.

And a study was undertaken to evaluate the efficacy of oral vaccination of olive flounder (*Paralichthys olivaceus*) with two auxotrophic genes-mutated *Edwardsiella tarda* (*Δalr Δasd E. tarda*) that habors antibiotic resistant gene-free plasmids (pG02-ASD-EtPR-GFP) equipped with expression cassettes for green fluorescent protein (GFP) and aspartate semialdehyde dehydrogenase (*asd*) genes. In a persistence and invasiveness experiment, orally administered live *Δalr Δasd E. tarda* were recovered only from the intestine till 24 h post-administration, and no colonies were found from the head kidney, spleen, and liver. In challenge experiments, fish orally immunized with 10^9 CFU/fish of *Δalr Δasd E. tarda* were 100% protected against challenge through either i.p. injection or immersion, even in fish administered only once. Two doses (primary and boost) of 10^8 CFU of the mutated bacteria given orally led to similar protection rate to administration of 10^9 CFU of the bacteria. In the immunized fish, intestinal and skin mucus showed no agglutination activity and no ELISA titers, whereas serum showed agglutination activity and ELISA titers. GFP expressed in the cytoplasm of *Δalr Δasd E. tarda* failed to induce antibody responses. The present results suggest that oral immunization of olive flounder with the auxotrophic mutant *E. tarda* can induce protective immunity against virulent *E. tarda* challenge through injection and immersion routes. To use the present auxotrophic mutant *E. tarda* as an oral combined vaccine, new approaches that can strengthen antigenicity of foreign antigens should be

conducted.

In chapter II, temperature-sensitive *Edwardsiella tarda* mutant was generated by replacement of *alr* gene promoter with *c*I857-λP_R promoter system plus another CI857 expression cassette that was driven by a constitutive promoter of *E. tarda* (EtPR C28-1), which allow the mutant bacteria to grow without supplement of D-alanine at temperature above 30°C but to disintegrate below 30°C. Growth of the mutant strain was not different to that of wild-type *E. tarda* under conditions of culture at 39°C. However, growth of the mutant strain was severely retarded by culturing at 25°C or 20°C. To further inhibit leakage of λP_R promoter at low temperature, the mutant strain was transformed with a vector harboring a constitutive promoter EtPR C28-1-driven *c*I857 cassette (pEtPR-*c*I857), which resulted in more limited growth compared to the mutant without the plasmids. The level of *alr* gene transcription in the mutant *E. tarda* and the mutant harboring pEtPR-*c*I857 was well coincided with the result of bacterial growth, suggesting that increased number of CI857 molecule was the major factor to further suppress the λP_R promoter-driven *alr* gene expression at low temperature. In vaccine experiment, olive flounder (*Paralichthys olivaceus*) fingerlings immunized with the temperature-sensitive mutant *E. tarda* showed greatly decreased mortality, and a boost-immunization induced complete protection against *E. tarda* infection. The protection rate of fish was proportional to the serum agglutination titer against *E. tarda*. These results suggest that the present temperature-sensitive *E. tarda* mutant is a good candidate for effective vaccines for prophylaxis of edwardsiellosis in fish. Moreover, as the present auxotrophic *E. tarda* mutant can be cultured without supplementation of the corresponding nutrient and can be simply attenuated by shifting temperature below 30°C, vaccines based on the present mutant *E. tarda* would be more economical than previously reported auxotrophic mutant *E. tarda*.

In chapter III, To evaluate potential of an auxotrophic *Edwardsiella tarda* mutant (*Δalr Δasd*

E. tarda) as a delivery vehicle for DNA vaccine in fish, olive flounder (*Paralichthys olivaceus*) were immunized with the *E. tarda* mutant harboring plasmids (pG02-ASD-CMV-eGFP) for eukaryotic expression of the enhanced green fluorescent protein (eGFP) gene through either intraperitoneal (i.p.) or oral route, and the expression of eGFP in the internal organs and generation of antibody against eGFP in fish were analyzed. In fish i.p. injected with 2 x 10^7 CFU/fish of Δ*alr* Δ*asd E. tarda* harboring pG02-ASD-CMV-eGFP, expression of eGFP was detected in liver, kidney, and spleen from 1 day to 28 days post-injection. In fish orally administered with 1 x 10^9 CFU/fish of the bacteria, the eGFP band was detected in liver, kidney, and spleen from 1 day to 14 days post-administration, whereas, in intestine, the band was detected only at 1 day post-administration. Either oral or i.p. immunization of olive flounder with recombinant *E. tarda* that carried eGFP-expressing eukaryotic plasmids was successful to induce humoral adaptive immunity against not only *E. tarda* that was used as a delivery vehicle but also eGFP that was used as the reporter protein of DNA vaccine, suggesting attenuated *E. tarda*-vectored DNA vaccine has a potential to be used as a combined vaccine against infectious diseases in fish.

In chapter IV, the present results showed that immunization of olive flounder with recombinant *E. tarda* that carried DNA vaccine construct using either the marine medaka β-actin promoter or CMV promoter induced significantly higher serum neutralization titer against VHSV than immunization with the recombinant *E. tarda* alone or PBS. This result suggests that DNA vaccine plasmids in the recombinant bacteria are transported to the host cell's nucleus, and the expressed VHSV G protein might be exposed to the intercellular region, which allows producing antibodies against VHSV G protein. Interestingly, DNA vaccine vector with marine medaka β-actin promoter induced greater neutralization titer than the vaccine driven by CMV promoter, suggesting that the expressed amount of the antigen might be an important factor for inducing

effective adaptive immune responses.

In the present challenge experiments, fish immunized with the *Δalr Δasd E. tarda* harboring DNA vaccine vector driven by the marine medaka β-actin promoter showed higher survival rates against challenge with two different PFUs of VHSV than fish immunized with the bacteria carrying DNA vaccine vector driven by CMV promoter, which are well coincide with the results of serum neutralization titer against VHSV. These results indicate that auxotrophic *E. tarda* mutant harboring marine medaka β-actin promoter-driven DNA vaccine vectors would be a potential system for prophylactics of fish viral diseases. Further studies on the effectiveness of this system by administration through oral or mucosal routes should be conducted for convenient use of this system in commercial fish farms.

재조합 약독화 세균 백신 제작 및 이를 기반으로 하는 복합 백신 시스템 개발

최 승 혁

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요 약

그램 음성 세균 (*E. tarda*)은 담수 및 해수 어류 모두에 질병을 유발함으로써 우리나라뿐 아니라, 전세계 양식 산업에 막대한 피해를 주고 있는 것으로 알려져 있다. 양식 어류에서 화학약제를 이용한 질병제어는 내성병원체 출현, 수질 오염 및 인간의 건강에 유해한 영향등의 문제점이 있기 때문에 많은 나라에서는 화학약제의 사용을 줄이는 대신 백신과 같은 예방법 개발이 진행중이다. 여러 백신 방법 중 약독화백신 (attenuated vaccine)은 높은 면역원성 및 방어능력을 유도하기 때문에 고효율 백신으로서의 가치가 높으나, 병원성 회복에 의한 안전성이 가장 큰 단점이라 할 수 있다. 이러한 점에서 볼 때 척추동물에는 존재하지 않는 영양소이면서 세균 생존에는 필수적인 영양소를 생산하는 유전자를 knockout 시킨 영양요구성 돌연변이 균주는 높은 안전성과 함께 약독화백신으로 적합할 것으로 여겨진다. 따라서, 본 연구에서는 homologous recombination 기술을 사용함으로써 live attenuated 재조합 세균를 생산하였고, 이들을 복합 백신의 매개체로 사용하기 위해 그 가능성을 확인하였다.

1. 두 가지 영양요구성 유전자가 knockout 된 *Edwardsiella tarda* 생산 및 vaccine 효과 확인

본 연구에서는 세균의 세포벽 합성에 필수적인 역할을 하는 alanine racemase (*alr*) 유전자와 aspartate semialdehyde dehydrogenase (*asd*) 유전자를 knockout 시킨 영양요구성 돌연변이 *E. tarda* (*Δ alrΔ asd E. tarda*)를 제작하였다. 영양요구성 mutant *E. tarda* 를 wild-type *E. tarda* 와 비교한 결과 넙치에서 약 10^6 정도 감소된 virulence 를 확인하였고, Mutant *E. tarda* 를 olive flounder (*Paralichthys olivaceus*)에 복강주사 및 경구투여하였을 때, *E. tarda* 에 대한 높은 생존율과 serum agglutination activity 가 나타났다.

2. 온도에 민감한 mutant *E. tarda* 제작 및 vaccine 효과 확인

필수 영양소 공급이 필요 없는 auxotrophic *E. tarda* mutant 를 만들기 위해, alr gene 프로모터 대신에 *c*I857-λP_R promoter system 을 적용하였다. 항시고발현 promoter 와 *c*I857 을 추가적으로 삽입하여, 30° C 이상일 때는 D-alanine 공급 없이 정상적으로 성장을 하지만, 30° C 이하 일때는 성장이 저해되는 *E. tarda* mutant 를 제작하였다. 온도에 민감한 mutant *E. tarda* 를 Olive flounder (*Paralichthys olivaceus*)에 면역화 하였을 때, wild type *E. tarda* 에 대해 높은 방어력을 보이는 것으로 나타났다.

3. Heterologous Ag 전달 매개체로서 mutant *E. tarda* 의 가능성 확인

2 개의 유전자를 knockout 시킨 영양요구성 돌연변이 *E. tarda* (*Δ alrΔ asd E. tarda*)를 Heterologous Ag 전달 매개체로 사용하였다.

항생제 내성유전자 사용없이 외래 단백질인 green fluorescent protein (GFP)를 발현할 수 있는 *Δ alrΔ asd E. tard* 를 넙치에 경구 및 복강주사를 통해 면역화한 후 *E. tarda* 감염에 대한 방어효과 및 GFP 에 대한 항체생성능을 분석함으로써 복합백신으로서의 사용가능성을 확인하였다.

4. DNA vaccine 전달 매개체로서 mutant *E. tarda* 의 가능성 확인

Plasmid 의 전달 매개체로 Mutant *E. tarda* 를 사용 가능성을 어류에서 확인하였다. CMV 프로모터와 eGFP 를 결합시킨 플라스미드를 가지고 있는 mutant *E. tarda* 를 넙치에 면역화 하였을때, 내부 장기에서 eGFP 가 발현되는 것을 확인하였다. 또한, *E. tarda* 에 대한 항체가 생겼을 뿐만 아니라 eGFP 에 대한 항체가 만들어진 것을 확인하였다. 더나아가, marin medaka β-actin promoter 와 VHSV G gene 을 결합시킨 vector 를 가진 mutant *E. tarda* 를 넙치에 면역화 하였을 때, VHSV 에 대한 neutralization activity 가 높은 것을 확인하였고, VHSV 에 대한 생존률이 높은 것을 확인 하였다.

이러한 결과는 영양요구성 mutant 가 어류 백신으로 효율적일 뿐만 아니라, heterologous antigen 의 매개체로써, 또한 DNA vaccine 플라스미드의 전달 매개체로 적용이 가능할 것이다.

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먼저, 참으로 부족한 저를 학문의 길로 불러 들여 오늘의 제가 있게 해 주시고, 평생의 스승으로 든든한 버팀목이 되어주신 김기홍 지도교수님께 진심으로 고개숙여 감사드립니다. 선생님 감사합니다. 저는 꼭 약속을 지키겠습니다..

연구와 강의로 바쁘신 가운데도 논문이 완성될 수 있도록 심사해주시고, 아낌없는 관심과 격려를 하여주신, 남윤권 교수님, 김도형 교수님, 정준범 교수님, 권세련 교수님, 수산생명의학이라는 학문을 통해 지식과 정보의 폭을 넓힐 수 있도록 도와주신 박수일 교수님, 정현도 교수님, 정준기 교수님, 허민도 교수님, 강주찬 교수님께도 감사드립니다. 항상 관심을 가져주신 김성구 교수님, 김종명 교수님께도 감사드리며, 대학원 생활 동안 지도하여 주셨던 모든 교수님께 머리 숙여 감사의 말씀을 드립니다.

2002 년 학부 2 학년 때 어패류기생충학 연구실을 들어온 것을 저에게 커다른 행운이였습니다. 어패류기생충학 연구실 선, 후배님들과의 생활은 항상 웃음과 즐거움으로 든든한 버팀목이 되어왔습니다. 항상 아낌없는 조언과 힘이 되어주신 많은 선배님들이 계십니다. 인생에 많은 가르침과 조언을 아끼지 않으셨던 장명덕 선배님, 홍주원 선배님, 황윤정 선배님, 안경진 선배님, 이선정 선배님, 조재범 선배님, 김천수 선배님, 이찬휘 선배님, 정재혁 선배님, 권세련 선배님, 이은혜 선배님, 김성미 선배님, 김형준 선배님, 코스케 형께 감사드립니다. 이 분들의 은혜는 평생 잊지 못할 것같습니다.

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REFERENCES

Anderson E.D., Mourich D.V., Fahrenkrug S.C., LaPatra S., Shepherd J., Leong J.A., (1996). Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol. Mar. Biol. Biotechnol., 5 , 112-122.

Bernard D., Six A., Rigottier-Gois L., Messiaen S., Chilmonczyk S., Quillet E., Boudinot P., Benmansour A., (2006). Phenotypic and fuctional similarity of gut intraepithelial and systemic T cells in a teleost fish. J. Immunol., 176 , 3942-3949.

Brudeseth B.E., Skall H.F., and Evensen Ø., (2008). Differences in virulence of marine and freshwater isolates of viral hemorrhagic septicemia virus in vivo correlate with in vitro ability to infect gill epithelial cells and macrophages of rainbow trout (Oncorhynchus mykiss). J Virol., 82 , 10359-10365.

Caipang C.M., Takano T., Hirono I., Aoki T., (2006). Genetic vaccines protect red seabream, Pagrus major, upon challenge with red seabream iridovirus (RSIV). Fish Shellfish Immunol., 12 , 130-138.

Cárdenas L., Clements J.D., (1992). Oral immunization using live attenuated Salmonella spp. as carriers of foreign antigens. Clin. Microbiol. Rev., 5 , 328-342.

Castro N., Toranzo A.E., Núñez S., Magariños B., (2008). Development of an effective Edwardsiella tarda vaccine for cultured turbot (Scophthalmus maximus). Fish Shellfish Immunol., 25 , 208-212.

Cheng S., Hu YH., Zhang M., Sun L., (2010). Analysis of the vaccine potential of a natural avirulent Edwardsiella tarda isolate. Vaccine., 28 , 2716-2721.

Chico V., Ortega-Villaizan M., Falco A., Tafalla C., Perez L., Coll J., and Estepa A., (2009). The immunogenicity of viral haemorragic septicaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences. Vaccine., 27, 1948-1958.

Choi SH., Nam YK., Kim KH., (2010). Novel expression system for combined vaccine production in Edwardsiella tarda ghost and cadaver cells. Mol Biotechnol., 46 , 127-133.

Choi S.H., Kim K.H., (2011a). Generation of two auxotrophic genes knock-out Edwardsiella tarda and assessment of its potential as a combined vaccine in olive flounder (Paralichthys olivaceus). Fish Shellfish Immunol., 31 , 58-65.

Choi S.H., Kim K.H., (2011b). Protection of olive flounder (Paralichthys olivaceus) against Edwardsiella tarda infection by oral administration of auxotrophic mutant E. tarda (Δalr Δasd E. tarda). Aquaculture., 317 , 48-52.

Clark T.R., and D Cassidy-Hanely., (2005). Recombinant subunit vaccines, potentials and constrains. Progress in Fish Vaccinology. Development in Biological Standardization., 121 , 152-163.

Cohen A.D., Boyer J.D., Weiner D.B., (1998). Modulating the immune response to genetic immunization. FASEB J., 12 , 1611-1626.

Corbeil S., Kurath G., LaPatra S.E., (2000). Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routes of immunization. Fish Shellfish Immunol., 10 , 711-723.

Corbeil S., LaPatra S.E., Anderson E.D., Jones J., Vincent B., Hsu Y.L., (1999). Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout Oncorhynchus mykiss using DNA vaccines. Dis. Aquat. Org., 39 , 29-36.

Corr M., Lee D.J., Carson D., Tighe H., (1996). Gene vaccination with naked plasmid DNA: mechanism for CTL priming. J. Exp. Med., 184 , 1552-1560.

D.J. Lee., S.R. Kwon., K. Zenke., E.H. Lee., Y.K. Nam and S.K. Kim, et al., (2008). Generation of safety enhanced Edwardsiella tarda ghost vaccine. Dis Aquat Organ., 81 , 249–254.

Danilition SL., Frederickson RM., Taylor CY., Miyamoto NG., (1991). Transcription factor binding and spacing constraints in the human beta-actin proximal promoter. Nucl Acids Res., 19 , 6912-6922.

Darji A., Guzman C.A., Gerstel B., Wachholz P., Timmis K.N., Wehland J., Chakraborty T., Weiss S., (1997). Oral somatic transgene vaccination using attenuated S. typhimurium. Cell., 91 , 762-775.

Dietrich G., Bubert A., Gentschev I., Sokolovic Z., Gentschev I., et al., (1998). Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide Listeria monocytogenes. Nat. Biotechnol., 16 , 181-185.

Dietrich G., Spreng S., Favre D., Viret J.F., Guzman C.A., (2003). Live attenuated bacteria as vectors to deliver plasmid DNA vaccines. Curr. Opin. Mol. Ther., 5 , 10-19.

Donnenberg MS., Kaper JB., (1991). Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. Infect Immun., 59 , 4310-4317.

Einer-Jensen K., Ahrens P., Forsberg R., and Lorenzen N., (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. J Gen Virol., 85, 1167-1179.

Einer-Jensen K., Delgado L., Lorenzen E., Bovo G., Evensen Ø., LaPatra S., and Lorenzen N., (2009). Dual DNA vaccination of rainbow trout (*Oncorhynchus mykiss*) against two different rhabdoviruses, VHSV and IHNV, induces specific divalent protection. Vaccine., 27, 1248-1253.

FAO, 2008. ©2005–2010. Fisheries Topics:Governance: Fish health management in aquaculture.Text by Rohana Subasinghe. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 27 May 2005 [cited 22 September 2010].

Feng H., Cheng J., Luo J., Liu SJ., Liu Y., (2006). Cloning of black carp beta-actin gene and primarily detecting the function of its promoter region. Acta Genetica Sinica., 33 , 132-140.

Fennelly G.J., Khan S.A., Abadi M.A., Wild T.F., Bloom B.R., (1999). Mucosal DNA vaccine immunization against measles with a highly attenuated Shigella flexneri vector. J. Immunol., 162 , 1602-1610.

Fernandez-Alonso M., Rocha A., Coll J.M., (2001). DNA vaccination by immersion and ultrasound to trout viral haemmorrhagic septicaemia virus. Vaccine., 19 , 3067-3975.

Franklin F. C., and W. A. Venables., (1976). Biochemical, genetic, and regulatory studies of alanine catabolism in *Escherichia coli* K12. Mol. Gen. Genet., 149 , 229–237.

Garg S., Oran AE., Hon H., Jacob J., (2004). The hybrid cytomegalovirus enhancer/chicken β-actin promoter along with woodchuck hepatitis virus posttranscriptional regulatory element enhances the protective efficacy of DNA vaccines. J Immunol., 173 , 550-558.

Garmory H.S., Brownb K.A., Titball R.W., (2002). Salmonella vaccines for use in humans: present and future perspectives. FEMS Microbiol. Rev., 26 , 339-353.

Garmory H.S., Leary S.E.C., Griffin K.F., Williamson E.D., Brown K.A., Titball R.W., (2003). The use of live attenuated bacteria as a delivery system for heterologous antigens. J. Drug Target., 11 , 471- 479.

Gutierrez MA., Miyazaki T., (1994). Responses of Japanese eels to oral challenge with Edwardsiella tarda after vaccination with formalin-killed cells or lipopolysaccharide of the bacterium. J Aquat Anim Health., 6 , 110-117.

Hayashi H., Wada H., Yoshimura T., Esaki N., Soda K., (1990). Recent topics in pyridoxal 5′-phosphate enzyme studies. Annu Rev Biochem., 59 , 87-110.

Higashijima S., Okamoto H., Ueno N., Hotta Y., Eguchi G., (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev Biol., 192 , 289-299.

Hols P., C. Defrenne., T. Ferain., S. Derzelle., B. Delplace., and J. Delcour., (1997). The alanine racemase gene is essential for growth of *Lactobacillus plantarum*. J. Bacteriol., 179 , 3804–3807.

Hou J.H., Zhang W.W., Sun L., (2009). Immunoprotective analysis of two Edwardsiella tarda antigens. J. Gen. Appl. Microbiol., 55 , 57-61.

Hwang GL., Azizur Rahman M., Abdul Razak S., Sohm F., Farahmand H., Smith A., Brooks C., Maclean N., (2003). Isolation and characterisation of tilapia beta-actin promoter and comparison of its activity with carp beta-actin promoter. Biochim Biophys Acta., 1625 , 11-18.

Isshiki T., Nagano T., and Miyazaki T., (2003). Susceptibility of various marine fish species to viral hemorrhagic septicemia virus isolated from Japanese flounder. J Fish Pathol., 38, 112-115.

Janda., Michael J., Sharon L. Abbot., Susan Kroske-Bystrom., Wendy K. Cheung., Catherine Powers., Robert P. Kokka., and K. Tamura.,(1991). Pathogenic Properties of Edwardsiella Species." Journal of Clinical Microbiology 9th ser., 29 , 1997-2001.

Jiao XD., Dang W., Hu YH., Sun L., (2009). Identification and immunoprotective analysis of an in vivo-induced Edwardsiella tarda antigen. Fish Shellfish Immunol., 27 , 632-638.

Jiao XD., Zhang M., Cheng S., Sun L., (2010). Analysis of Edwardsiella tarda DegP, a serine protease and a protective immunogen. Fish Shellfish Immunol., 28 , 672-677.

Jiao XD., Zhang M., Hu YH., Sun L., (2009). Construction and evaluation of DNA vaccines encoding Edwardsiella tarda antigens. Vaccine., 27 , 5192-5202.

Kawai et al., K. Kawai, Y. Liu, K. Ohnishi and S. Oshima., (2004). A conserved 37 kDa outer membrane protein of Edwardsiella tarda is an effective vaccine candidate. Vaccine, 22 , 3411–3418.

Kawamoto T., Makino K., Niwa H., Sugiyama H., Kimura S., Amemura M., Nakata A., Kakunaga T., (1988). Identification of the human beta-actin enhancer and its binding factor. Mol Cell Biol., 8 , 267-272.

Kim M.S., and Kim K.H., (2011). Protection of olive flounder, *Paralichthys olivaceus*, against viral hemorrhagic septicemia virus (VHSV) by immunization with NV gene-knockout recombinant VHSV. Aquaculture., 314, 39-43.

Kim S.M., Lee J.I., Hong M.J., Park H.S., and Park S.I., (2003). Genetic relationship of the VHSV(viral hemorrhagic septicemia virus) isolated from cultured olive flounder, *Paralichthys olivaceus* in Korea. J Fish Pathol., 16, 1-12 (In Korean with English abstract).

Kim W.S., Kim S.R., Kim D., Kim J.O., Park M.A., Kitamura S.I., Kim H.Y., Kim D.H., Han H.J., Jung S.J., and Oh M.J., (2009). An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed olive flounder *Paralichthys olivaceus* in Korea. Aquaculture., 296, 162-168.

Klesius P.H. and C.A. Shoemaker., (1999). Development and use of modified live Edwardsiella ictaluri vaccine against enteric septicemia of catfish. Pages 522-537 in R.D. Schultz, editor Advances in veterinary medicine, 41, Academic Press San Diego, CA USA.

Kotton CN., Hohmann EL., (2004). Enteric pathogens as vaccine vectors for foreign antigen delivery. Infect Immun., 72 , 5532-5547.

Kumar SR., Parameswaran V., Ahmed VP., Musthaq SS., Hameed AS., (2007). Protective efficiency of DNA vaccination in Asian seabass (Lates calcarifer) against Vibrio anguillarum. Fish Shellfish Immunol., 23(2) , 316–326.

Kwon SR., Lee EH., Nam YK., Kim SK., Kim KH., (2007). Efficacy of oral immunization with Edwardsiella tarda ghosts against edwardsiellosis in olive flounder (Paralichthys olivaceus). Aquaculture., 69 , 82-88.

Kwon SR., Nam YK., Kim SK., Kim DS., Kim KH., (2005). Generation of Edwardsiella tarda ghosts by bacteriophage PhiX174 lysis gene E. Aquaculture., 250 , 16-21.

Kwon SR., Nam YK., Kim SK., Kim KH., (2006). Protection of tilapia (Oreochromis mosambicus) from edwardsiellosis by vaccination with Edwardsiella tarda ghosts. Fish Shellfish Immunol., 20 , 621-626.

Lamm, M.E., (1997). Interactions of antigens and antibodies at mucosal surfaces. Annu. Rev. Microbiol., 51 , 311-340.

Lan MZ., Peng X., Xiang MY., Xia ZY., Bo W., Jie L., Li XY., (2007). Jun ZP. Construction and characterization of a live, attenuated esrB mutant of Edwardsiella tarda and its potential as a vaccine against the haemorrhagic septicaemia in turbot, Scophthamus maximus (L.). Fish Shellfish Immunol., 23 , 521-530.

LaPatra S.E., Corbeil S., Jones G.R., Shewmaker W.D., Lorenzen N., Anderson E.D., Kurath G., (2001). Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine., 19 , 4011-4019.

Lawrence M,L,, Cooper R.K., Thune R.L., (1997). Attenuation, persistence, and vaccine potential of an Edwardsiella ictaluri purA mutant. Infect. Immun., 65 , 4642-4651.

Lee DJ., Kwon SR., Zenke K., Lee EH., Nam YK., Kim SK., Kim KH., (2008). Generation of safety enhanced Edwardsiella tarda ghost vaccine. Dis Aquat Org., 81 , 249-254.

Lee J., Hirono I., Aoki T., (2000). Stable expression of a foreign gene, delivered by gene gun, in the muscle tissue of rainbow trout Oncorhynchus mykiss. Mar. Biotechnol., 2 , 252-258.

Lenoir G., and de Kinkelin P., (1975). Fish rhabdoviruses: comparative study of protein structure. J Virol. 16, 259-262.

Ling Li., Xiangyu Mou., and David R. Nelson., (2011). HlyU Is a Positive Regulator of Hemolysin Expression in *Vibrio anguillarum* . journal of bacteriology., 193, 4779–4789.

Liu., Y. Liu., S. Oshima., K. Kurohare., K. Ohnishi and K. Kawai., (2005). Vaccine efficacy of recombinant GAPDH of Edwardsiella tarda against Edwardsiellosis. Microbiol Immunol., 49 , 605–612.

Liu ZJ., Zhu ZY., Roberg K., Faras A., Guise K., Kapuscinski AR., Hackett PB., (1990). Isolation and characterization of beta-actin gene of carp (Cyprinus carpio), DNA Seq., 1 , 122-136.

Lorenzen N., Lorenzen E.., Einer-Jensen K., and LaPatra S.E., (2002). DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. Fish Shellfish Immunol., 12, 439-453.

Lorenzen N., Lorenzen E., Einer-Jensen K., and LaPatra S.E., (2002). DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. Fish Shellfish Immunol., 12 , 439-453.

Lorenzen N., and LaPatra S.E., (2005). DNA vaccines for aquacultured fish. Rev. Sci. Tech., 24, 201-213.

Lorenzen N., Lorenzen E., and Einer-Jensen K., (2001). Immunity to viral haemorrhagic septicaemia (VHS) following DNA vaccination of rainbowtrout at an early life-stage. Fish Shellfish Immunol., 11, 582-591.

Lorenzen N., Lorenzen E., Einer-Jensen K., Heppell J., Wu T., Davis H., (1998). Protective immunity of VHS in rainbow trout (Oncorhynchus mykiss, Walbaum) following DNA vaccination. Fish Shellfish Immunol., 8 , 261-270.

Marsden MJ., Vaughan LM., Foster TJ., Secombes CJ., (1996). A live (delta aroA) Aeromonas salmonicida vaccine for furunculosis preferentially stimulates T-cell responses relative to B-cell responses in rainbow trout (Oncorhynchus mykiss). Infect Immun., 64 , 3862-3869.

Matsuyama T., Kamaishi T., Ooseko N., Kurohara K., Iida T., (2005). Pathogenicity of motile and non-motile Edwardsiella tarda to some marine fish. Fish Pathol., 40, 132-135.

Medina E., and Guzman C.A., (2001). Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. Vaccine., 19 , 1572-1580.

Meyer BJ., Maurer R., Ptashne M.. Gene regulation at the right operator (*O*R) of bacteriophage λ. II.*O*R1, *O*R2, and *O*R3: their roles in mediating the effects of repressor and cro. J Mol Biol 1980;139:162-94.

Mikalsen A.B., Sindre H., Torgersen J., Rimstad E., (2005). Protective effects of a DNA vaccine expressing the infectious salmon anemia virus hemagglutinin-esterase in Atlantic salmon. Vaccine., 23 , 4892-4905.

Mikalsen A.B., Torgersen J., Alestrõm P., Hellemann A.-L., Koppang E.-O., Rimstad E., (2004). Protection of Atlantic salmon Salmo salar against infectious pancreatic necrosis after DNA vaccination. Dis. Aquat. Org., 60 , 11-20.

Miyazaki J., Takaki S., Araki K., Tashiro F., Tominaga A., Takatsu K., Yamamura K., (1989). Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. Gene., 79 , 269-277.

Mohanty BR., Sahoo PK. Edwardsiellosis in fish: A brief review. J Biosci 2007;32:1331-44.

Mollenkopf H., Dietrich G., Kaufmann SH., (2001). Intracellular bacteria as targets and carriers for vaccination. Biol Chem., 382 , 521-532.

Mortensen H.F., Heuer O.E., Lorenzen N., Otte L., and Olsen N.J., (1999). Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea, Kattegat, Skagerrak and the North Sea. Virus Res., 63, 92-106.

N. Castro., A.E. Toranzo., S. Nunez and B. Magarinos, (2008). Development of an effective Edwardsiella tarda vaccine for cultured turbot (Scophthalmus maximus). Fish Shellfish Immunol., 25 , 208–212.

Naka,H., Dias,G.M., Thompson,C.C., Dubay,C., Thompson,F.L. and Crosa,J.H., (2011). Complete genome sequence of the marine fish pathogen Vibrio anguillarum harboring the pJM1 virulence plasmid and genomic comparison with other virulent strains of V. anguillarum and V. ordalii. Infect. Immun., 79 , 2889.

Nakayama K., Kelley SM., Curtiss III R., (1988). Construction of an Asd1 expression-cloning vector: stable maintenance and high level expression of cloned genes in a Salmonella vaccine strain. Bio/Technology., 6 , 692-697.

Nauta A., van den Burg B., Karsens H., Venema G., Kok J. Design of thermolabile bacteriophage repressor mutants by comparative molecular modeling. Nat Biotechnol 1997; 15:980-3.

Nelson MD., Secombes, C., (1997). Isolation of rainbow trout (Oncorhynchus Mykiss) intestinal intraepithelial lymphocytes (IEL) and measurement of the cytotoxic activity. Fish Shellfish Immunol., 7 , 527-541.

Neutra M.R., Kozlowski P.A., (2006). Mucosal vaccines: the promise and the challenge. Nat. Rev. Immunol., 6 , 148-158.

Noh JK., Cho KN., Han EH., Kim A., Lee JS., Kim DS., Kim CG., (2003). Genomic cloning of mud loach Misgurnus mizolepis (Cypriniformes, Cobitidae) beta-actin gene and usefulness of its promoter region for fish transgenesis. Mar Biotechnol., 5 , 242-252.

Oyston PC., Mellado-Sanchez G., Pasetti MF., Nataro JP., Titball RW., Atkins HS., (2010). Yersinia pestis guaBA mutant is attenuated in virulence and provides protection against plague in a mouse model of infection. Microb Pathog ., 48 , 191-195.

Palumbo E., C. F. Favier, M. Deghorain, P. S. Cocconcelli, C. Grangette, A. Mercenier, E. E. Vaughan, and P. Hols., (2004). Knockout of the alanine racemase gene in *Lactobacillus plantarum* results in septation defects and cell wall perforation. FEMS Microbiol. Lett., 233 , 131–138.

Park S.I., (2009). Disease control in Korean aquaculture. Fish Pathol., 44 , 19-23.

Pasnik D.J., Smith S.A., (2005). Immunogenic and protective effects of a DNA vaccine for Mycobacterium marinum in fish. Vet. Immunol. Immunopathol., 103 , 192-206.

Pavelka MS Jr., Jacobs WR Jr., (1996). Biosynthesis of diaminopimelate, the precursor of lysine and a component of peptidoglycan, is an essential function of Mycobacterium smegmatis. J Bacteriol., 178 , 6496-6507.

Plant K.P., LaPatra S.E., (2011). Advances in fish vaccine delivery. Dev. Comp. Immunol., doi:10.1016/j.dci.2011.03.007.

Plumb JA. Edwardsiella septicaemias. In: Woo PTK and Bruno EW editors. Fish diseases and disorders, vol. 3: Viral, bacterial and fungal infections. Wallingford, CABI: 1999. p.479-521.

Ptashne M., Jeffrey A., Johnson AD., Maurer R., Meyer BJ, Pabo CO, Roberts TM, Sauer RT. How the lambda repressor and cro work. Cell 1980;19:1-11.

Pucci MJ., Thanassi JA., Ho HT., Falk PJ., Dougherty TJ., (1995). Staphylococcus haemolyticus contains two D-glutamic acid biosynthetic activities, a glutamate racemase and a D-amino acid transaminase. J Bacteriol., 177 , 336-342.

Quitschke WW., Lin ZY., DePonti-Zilli L., Paterson BM., (1989). The beta actin promoter. High levels of transcription depend upon a CCAAT binding factor. J Biol Chem., 264 , 9539-9546.

R. Thune., L. Stanley and R. Cooper., (1993) . Pathogenesis of Gram-negative bacterial infections in warmwater fish. Annu Rev Fish Dis., 3 , 37–68.

Rajesh Kumar, S., Ishaq Ahmed, V.P., Parameswaran, V., Sudhakaran, R., Sarath Babu, V., Sahul Hameed, A.S., (2008). Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (Lates calcarifer) to protect from Vibrio (Listonella) anguillarum. Fish Shellfish Immunol., 25 , 47-56.

Rajesh Kumar S., Parameswaran V., Ishaq Ahmed V.P., Syed Musthaq S., Sahul Hameed A.S., (2007). Protective efficiency of DNA vaccination in Asian seabass (Lates calcarifer) against Vibrio anguillarum. Fish Shellfish Immunol., 23 , 316-326.

Ramos E.A., Relucio J.L.V., Torres-Villanueva C.A.T., (2005). Gene expression in tilapia following oral delivery of chitosan-encapsulated plasmid DNA incorporated into fish feeds. Marine Biotechnol., 7 , 89-94.

Reed LJ., Muench H., (1938). A simple method of estimating fifty percent end points. Am J Hyg., 27, 492-497.

Révet B., von Wilcken-Bergmann B., Bessert H., Barker A., Müller-Hill B. Four dimers of λ repressor bound to two suitably spaced pairs of λ operators form octamers and DNA loops over large distances. Curr Biol 1999;9:151-4.

Roland KL., Tinge SA., Kochi SK., Thomas LJ., Killeen KP., (2010). Reactogenicity and immunogenicity of live attenuated Salmonella enterica serovar Paratyphi A enteric fever vaccine candidates. Vaccine., 28 , 3679-3687.

Rolands K., Curtiss III R., Sizemore D., (1999). Construction and evaluation of a Δcya Δcrp Salmonella typhimurium strain expressing avian pathogenic Escherichia coli O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis., 43 , 429-441.

Rombout J.H., Taverne-Thiele A.J., Villena M.I., (1993). The gut-associated lymphoid tissue (GALT) of carp (Cyprinus carpio L.): an immunocytochemical analysis. Dev. Comp. Immunol., 17 , 52-66.

Ruiz S., Tafalla C., Cuesta A., Estepa A., Coll JM., (2008). In vitro search for alternative promoters to the human immediate early cytomegalovirus (IE-CMV) to express the G gene of viral haemorrhagic septicemia virus (VHSV) in fish epithelial cells. Vaccine., 26 , 6620-6629.

Ryan ET., Crean TI., Kochi SK., John M., Luciano AA., Killeen KP., Klose KE., Calderwood SB., (2000). Development of a ΔglnA balanced lethal plasmid system for expression of heterologous antigens by attenuated vaccine vector strains of Vibrio cholera. Infect Immun., 68 , 221-226.

Sawicki J. A., R. J. Morris, B. Monks K. Sakai, and J. Miyazaki., (1998). A composite CMV-IE enhancer/β-actin promoter is ubiquitously expressed in mouse cutaneous epithelium. Exp. Cell Res., 244 , 367-369.

Schlotfeldt H.J., Ahne W., Vestergard-Jorgensen P.E., and Glende W.,(1991). Occurrence of viral haemorrhagic septicaemia in turbot (*Scophthalmus maximus*) – a natural outbreak. EAFP Bull., 11, 102-107.

Schlotfeldt H.J., and Ahne W., (1988). Epizootics in brown trout (*Salmo trutta fario*) caused by VHSV-F1. App Ichthyol., 4, 147-148.

Schoen C., Stritzker, J., Goebel W., Pilgrim S., (2004). Bacteria as DNA vaccine carriers for genetic immunization. Int. J. Med. Microbiol., 294 , 319-335.

Schütze H., Enzmann P.J., Mundt E., and Mettenleiter T.C., (1996). Identification of the non-virion (NV) protein of fish rhabdoviruses viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. J Gen Virol., 77, 1259-1263.

Schütze H., Mundt E., and Mettenleiter T.C., (1999). Complete genomic sequence of viral haemorrhagic septicemia virus, a fish rhabdovirus. Virus Genes., 19, 59-65.

Schütze H., Mundt E., and Mettenleiter T.C., (1999). Complete genomic sequence of viral haemorrhagic septicemia virus, a fish rhabdovirus. Virus Genes., 19, 59-65.

Seder R.A., and A.V.S. Hill., (2000). Vaccines against intracellular infectious requiring cellular immunity. Nature., 406 , 792-797.

Seo J.Y., Kim K.H., Kim S.G., Oh M.J., Nam S.W., Kim Y.T., Choi T.J., (2006). Protection of flounder against hirame rhabdovirus (HIRRV) with a DNA vaccine containing the glycoprotein gene. Vaccine, 24 , 1009-1015.

Sizemore D.R., Branstrom A.A., Sadoff J.C., (1995). Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. Science., 270 , 299-302.

Skall H.F., Olesen N.J., and Mellergaard S., (2005). Viral hemorrhagic septicemia virus in marine fish and implications for fish farming – a review. J Fish Dis., 28, 509-529.

Snow M., Cunningham C.O., Melvin W.T., and Kurath G., (1999). Analysis of the nucleoprotein gene identifies distinct lineages of viral haemorrhagic septicaemia virus within the European marine environment. Virus Res., 63, 32-44.

Snow M., King J.A., Garden A., Shanks A.M., and Raynard R.S., (2005). Comparative susceptibility of turbot *Scophthalmus maximus* to different genotypes of viral haemorrhagic septicaemia virus. Dis Aquat Org., 67, 31-38.

Steen A., E. Palumbo M. Deghorain, P. S. Cocconcelli, J. Delcour, O. P. Kuipers, J. Kok, G. Buist, and P. Hols. 2005. Autolysis of *Lactococcus lactis* is increased upon D-alanine depletion of peptidoglycan and lipoteichoic acids. J. Bacteriol., 187 , 114–124.

Strych U., H. C. Huang, K. L. Krause, and M. J. Benedik., (2000). Characterization of the alanine racemases from *Pseudomonas aeruginosa* PAO1. Curr. Microbiol., 41 , 290–294.

Strych U., M. Davlieva J. P. Longtin E. L. Murphy H. Im, M. J. Benedik, and K. L. Krause., (2007). Purification and preliminary crystallization of alanine racemase from *Streptococcus pneumoniae*. BMC Microbiol., 7 , 40.

Strych U., R. 89L. Penland M. Jimenez, K. L. Krause, and M. J. Benedik., (2001). Characterization of the alanine racemases from two mycobacteria. FEMS Microbiol. Lett., 196 , 93–98.

Sun Y., Liu C., Sun L., (2010). Construction and analysis of the immune effect of an Edwardsiella tarda DNA vaccine encoding a D12-like surface antigen. Fish Shellfish Immunol., 30 , 272-279.

Sun Y., Hu Y.-h., Liu C.-s., Sun L., (2010). Construction and analysis of an experimental Streptococcus iniae DNA vaccine. Vaccine., 28 , 3902-3912.

Takano T., Matsuyama T., Oseko N., Sakai T., Kamaishi T., Nakayasu C., Sano M., Iida T., (2010). The efficacy of five avirulent Edwardsiella tarda strains in a live vaccine against Edwardsiellosis in Japanese flounder, Paralichthys olivaceus. Fish Shellfish Immunol., 29 , 687-693.

Takano T., Akiko I., Hirono I., Aoki T., (2004). Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after vaccination. Fish Shellfish Immunol., 17 , 367-374.

Tang X., Zhan W., Sheng X., Chi H., (2010). Immune response of Japanese flounder Paralichthys olivaceus to outer membrane protein of Edwardsiella tarda. Fish Shellfish Immunol., 28 , 332-343.

Thole J.E., van Dalen P.J., Havenith C.E., Pouwels P.H., Seegers J.F., Tielen F.D., van der Zee M.D., Zegers N.D., Shaw M., (2000). Live bacterial delivery systems for development of mucosal vaccines. Curr. Opin. Mol. Ther., 2 , 92-99.

Thompson RJ., Bouwer HGA., Portnoy DA., Frankel FR., (1998). Pathogenicity and immunogenicity of a Listeria monocytogenes strain that requires D-alanine for growth. Infect Immun., 66 , 3552-3561.

Thune RL., Fernandez DH., Batista J., (1999). An aroA mutant of Edwardsiella ictaluri is safe and efficacious as live, attenuated vaccine. J Aquat Anim Health., 11 , 358-372.

Thune RL., Fernandez DH., Hawke JP., Miller R., (2003). Construction of a safe, stable, efficacious vaccine against Photobacterium damselae ssp. piscicida. Dis Aquat Org., 57 , 51-58.

Thune RL., Stanley LA., Cooper RK., (1993). Pathogenesis of gram negative bacterial infections in warm water fish. Annu Rev Fish Dis., 3 , 37-68.

Tonheim T.C., Bogwald J., Dalmo R.A., (2008). What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol., 25 , 1-18.

Tordo N., Benmansour A., Calisher C., Dietzgen R.G., Fang R.X., Jackson A.O., Kurath G., Nadin-Davis S., Tesh R.B., and Walker P.J., (2005). Family Rhabdoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus Taxonomy. Eight Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London., pp. 622-653.

Traxler G.S., Anderson E., LaPatra S.E., Richard J., Shewmaker B., and Kurath G., (1999). Naked DNA vaccination of Atlantic salmon *Salmo salar* against IHNV. Dis. Aquat. Org., 38, 182-190.

Tucker C., Endo M., Hirono I., Aoki T., (2001). Assessment of DNA vaccine potential for juvenile Japanese flounder Paralichthys olivaceus, through the introduction of reporter genes by particle bombardment and histopathology. Vaccine., 19 , 801-809.

Ulcer J.B., Donnelly J.J., Parker S.E., Rhodes G.H., Felgner P.L., et al., (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. Science., 259 , 1742-1749.

Vaughan LM., Smith PR., Foster TJ., (1993). An aromatic-dependent mutant of the fish pathogen Aeromonas salmonicida is attenuated in fish and is effective as a live vaccine against the salmonid disease furunculosis. Infect Immun., 61 , 2172-2181.

Vazquez-Juareza, R.C., Gomez-Chiarrib, M., Barrera-Saldaña, H., Hernandez-Saavedraa, N., Dumasd, S., Ascencio, F., (2005). Evaluation of DNA vaccination of spotted sand bass (Paralabrax maculatofasciatus) with two major outer-membrane protein-encoding genes from Aeromonas veronii. Fish Shellfish Immunol., 19 , 152-163.

Villaverde A., Benito A., Viaplana E., Cubarsi R. Fine regulation of cI857-controlled gene expression in continuous culture of recombinant *Escherichia coli* by temperature. Appl Environ Microbiol 1993;59:3482-7.

Viola RE., (2001). The central enzymes of the aspartate family of amino acid biosynthesis. Acc Chem Res, 34 , 339-349.

Walker P.J., Benmansour A., Dietzgen R., Fang R.X., Jackson A.O., Kurath G., Leong J.C., Nadin-Davies S., Tesh R.B., and Tordo N., (2000) Family Rhabdoviridae. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds) Virus Taxonomy. Classification and Nomenclature of Viruses. Academic Press, San Diego, CA., pp 562-583.

Walsh CT., (1989). Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J Biol Chem., 264 , 2392-2396.

Wang B., Mo ZL., Xiao P., Li J., Zou YX., Hao B., Li GY., (2010). EseD, a putative T3SS translocon component of Edwardsiella tarda, contributes to virulence in fish and is a candidate for vaccine development. Mar Biotechnol., 12 , 678-685.

Wang H., Hu Y., Zhang W., Sun L., (2009). Construction of an attenuated Pseudomonas fluorescens strain and evaluation of its potential as a cross-protective vaccine. Vaccine., 27 , 4047-4055.

Wang Q., Yang M., Xiao J., Wu H., Wang X., Lv Y., Xu L., Zheng H., Wang S., Zhao G., Liu Q., Zhang Y., (2009). Genome sequence of the versatile fish pathogen Edwardsiella tarda provides insights into its adaptation to broad host ranges and intracellular niches. PLoS One., 4 , e7646.

Wang B., Godillot A.P., Madaio M.P., Weiner, D.B., Williams W.V., (1998). Vaccination against pathogenic cells by DNA inoculation. Curr. Top. Microbiol. Immunol., 226 , 21-35.

Wasserman SA., Daub E, Grisafi P., Botstein D., Walsh CT., (1984). Catabolic alanine racemase from Salmonella typhimurium: DNA sequence, enzyme purification, and characterization. Biochemistry., 23 , 5182-5187.

Wasserman S. A., C. T. Walsh, and D. Botstein., (1983). Two alanine racemase genes in *Salmonella typhimurium* that differ in structure and function. J. Bacteriol., 153 , 1439–1450.

Wijsman H. J., (1972). The characterization of an alanine racemase mutant of *Escherichia coli*. Genet. Res., 20 , 269–277.

Williams RS., Johnston SA., Riedy M., DeVit MJ., McElligott SG., Sanford JC., (1991). Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci USA., 88 , 2726-2730.

Y.H. Zhu., Y. Ma., M.F. Shao., P.B. Wang and Y.X. Zhang., (2004). Screening and iron supply optimization of plasmid pEIB1 cured strain of Vibrio anguillarum MVAV6201. High Technol Lett., 14 , 64–67.

Yang H., Cao S., Huang X., Liu J., Tang Y., Wen X., (2009b). Intragastric administration of attenuated Salmonella typhimurium harbouring transmissible gastroenteritis virus (TGEV) DNA vaccine induced specific antibody production. Vaccine., 27 , 5032-5040.

Yang H., Chen J., Yang G., Zhang X.-H., Liu R., Xue X., (2009a). Protection of Japanese flounder (Paralichthys olivaceus) against Vibrio anguillarum with a DNA vaccine containing the mutated zinc-metalloprotease gene. Vaccine., 27 , 2150-2155.

Zhang M., Kim HJ., Marshall H., Gendron-Maguire M., Lucas DA., Baron A., Gudas LJ., Gridley T., Krumlauf R., Grippo JF., (1994). Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. Development ., 120 , 2431-2442.