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

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ABSTRACT

Two auxotrophic genes that play essential roles in bacterial cell wall biosynthesis – alanine racemase (*alr*) gene and aspartate semialdehyde dehydrogenase (*asd*) gene – knock-out *Edwardsiella tarda* (*Δalr Δasd 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.

1. 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 [1–3]. 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 [4,5], ghost bacteria vaccine [6–9], natural avirulent strain vaccine [10,11], recombinantly attenuated vaccine [12], subunit vaccine [13–20], and genetic vaccine [21,22]. 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

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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 [23,24]. Several auxotrophic genes have been used for generation of attenuated bacteria that infect cultured fish: the *aroA* gene in *Aeromonas salmonicida* [25,26], *Edwardsiella ictaluri* [27], and *Photobacterium damselae* spp. *piscicida* [28]; the *purA* gene in *E. ictaluri* [29]; and the *fur* gene in *Pseudomonas fluorescens* [30].

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 [31–33]. However despite its importance, little information is available on the attenuated bacteria-based combined vaccines in fish pathogenic bacteria.

In the present study, we have produced two auxotrophic genes – alanine racemase (*alr*) and aspartate semialdehyde dehydrogenase (*asd*) – knock-out *E. tarda* (*Δalr Δasd 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 [34,35]. 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 [36,37]. 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.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. tarda NH1, isolated from moribund olive flounder (*P. 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*) [38].

2.2. Fish

Juvenile olive flounder (*P. olivaceus*, 4–5 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.

2.3. 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 (*Δalr Δasd E. tarda*) was constructed by allelic exchange mutagenesis using ampicillin-resistant pCVD442 (Addgene plasmid 11074) [39], 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* (*Δalr 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 a fragment prepared by annealing of two oligonucleotides (MCS-UP and MCS-DOWN). The pUC18 vector harboring the N-terminal 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 *Δalr E. tarda* was deleted and generated *Δalr Δasd 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-terminal 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 *Δalr 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