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A *Lactococcus lactis* BFE920 feed vaccine expressing a fusion protein composed of the OmpA and FlgD antigens from *Edwardsiella tarda* was significantly better at protecting olive flounder (*Paralichthys olivaceus*) from edwardsiellosis than single antigen vaccines

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ABSTRACT

Edwardsiellosis is a major fish disease that causes a significant economic damage in the aquaculture industry. Here, we assessed vaccine efficacy after feeding oral vaccines to olive flounder (*Paralichthys olivaceus*), either *L. lactis* BFE920 expressing *Edwardsiella tarda* outer membrane protein A (OmpA), flagellar hook protein D (FlgD), or a fusion antigen of the two. Feed vaccination was done twice with a one-week interval. Fish were fed regular feed adsorbed with the vaccines. Feed vaccination was given over the course of one week to maximize the interaction between the feed vaccines and the fish intestine. Flounder fed the vaccine containing the fusion antigen had significantly elevated levels T cell genes (*CD4-1*, *CD4-2*, and *CD8α*), type 1 helper T cell (Th1) subset indicator genes (*T-bet* and *IFN-γ*), and antigen-specific antibodies compared to the groups fed the single antigen-expressing vaccines. Furthermore, the superiority of the fusion vaccine was also observed in survival rates when fish were challenged with *E. tarda*: OmpA-FlgD-expressing vaccine (82.5% survival); FlgD-vaccine (55.0%); OmpA-vaccine (50%); WT *L. lactis* BFE920 (37.5%); Ctrl (10%). In addition, vaccine-fed fish exhibited increased weight gain (~20%) and a decreased feed conversion ratio (~20%) during the four week vaccination period. Flounder fed the FlgD-expressing vaccine, either the single or the fusion form, had significantly increased expression of *TLR5M*, *IL-1β*, and *IL-12p40*, suggesting that the FlgD may be a ligand of olive flounder TLR5M receptor or closely related to the TLR5M pathway. In conclusion, the present study demonstrated that olive flounder fed *L. lactis* BFE920 expressing a fusion antigen composed of *E. tarda* OmpA and FlgD showed a strong protective effect against edwardsiellosis indicating this may be developed as an *E. tarda* feed vaccine.

1. Introduction

Edwardsiella tarda is a motile, rod-shaped, gram-negative, facultatively anaerobic intracellular pathogenic bacterium that causes edwardsiellosis in many types of animals, but is especially important in fish [1,2]. Due to the massive damage caused by *Edwardsiella* infection, antibiotics have been dominantly used for the treatment of edwardsiellosis. However, the emergence of resistant bacteria caused problems in the efficacies of antibiotics and the safeties of human health and environment. Therefore, non-antibiotic means against edwardsiellosis has long been sought by aquaculture researchers [2]. Vaccine development is a fundamental and efficient approach to control infectious diseases. Generating immune memory and reducing usage of antibiotics are the major advantages of utilizing a vaccine strategy. Various types of *E. tarda* vaccines have been developed during the last several decades such as formalin-killed bacterium [3,4], ghost vaccines [5–7], attenuated mutant strains [8–10], recombinant subunit vaccines [11–14], and

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DNA vaccines [12,15–17]. Even though many types of vaccines have been introduced, all have been injection type vaccines and thus have some obvious limitation including the labor-intensive process of injecting massive numbers of cultured fish, secondary skin infections at the injection site, the handling stress caused on the fish [18,19]. Feed vaccination, a simultaneous vaccination in the course of regular feeding, may be an ideal alternative to overcome the disadvantages of injection-type fish vaccines. However, oral vaccinations have sometimes been associated with weaker vaccine-induced protection against the target pathogen.

In this study, we attempted to develop an *E. tarda* fish feed vaccine that can overcome the limitations of both conventional injection-type and oral fish vaccines. Oral vaccination of fish is convenient and less stressful for the fish and induces strong immune protection against the target. *E. tarda* subunits have been intensively studied with the goal of developing *E. tarda* subunit vaccines. Subunit vaccines have many advantages: target specificity, the capacity to introduce further antigen modification for improved efficacy, and safety [11–17]. However, subunit vaccines usually induce only a weak immune response and require strong adjuvants to elicit adequate protection [20,21]. If specific pathogen subunit proteins are used in feed vaccines, oral tolerance is likely to aggravate their inherent weak immunogenicity. Oral tolerance is the suppression of immune responsiveness to antigens encountered orally [22,23]; therefore, oral vaccines require a potent mucosal adjuvant in order to convert oral tolerance into immune activation. To resolve these tasks, we chose *Lactococcus lactis* BFE920 as an antigen delivery vehicle because it has the ability to stimulate IFN-γ production and innate cell activation [24–26]. *L. lactis* BFE920 is a GRAS (generally regarded as safe) grade lactic acid bacteria (LAB) and acts as a probiotic in humans and animals, including fish [24–26]. In addition, several studies have demonstrated that *L. lactis* species including *L. lactis* BFE920 are excellent and safe antigen delivery vehicles for human, mouse, and fish vaccines [27–31]. Recently, we demonstrated that *L. lactis* BFE920 expressing the SiMA antigen of *Streptococcus iniae* showed excellent vaccine efficacy when fed to olive flounder [31].

In order to develop an *E. tarda*-specific feed vaccine in this study, two *E. tarda* antigens were selected: an outer membrane protein (Omp) [11,13] and the flagellar hook protein D (FlgD) [14,16]. Immunogenicities were then evaluated between individual antigens and a fusion antigen containing both proteins after feeding the vaccines to olive flounder. The fish fed *L. lactis* BFE920 expressing the fusion protein of *E. tarda* OmpA and FlgD showed a significantly greater T cell response and survival rate when challenged with the pathogen, as compared fish fed single antigen-expressing *L. lactis* BFE920, WT *L. lactis* BFE920, or control. In addition, flounder fed any *E. tarda* vaccine exhibited significantly enhanced growth performance.

2. Materials and methods

2.1. Construction of recombinant vaccine

The DNA containing *OmpA* or *FlgD* antigen genes was prepared by PCR amplification from the genomic DNA of *E. tarda* HFTC0081 isolated from diseased olive flounder sampled in Pohang, Korea. The fusion DNA of *OmpA* and *FlgD* was constructed by linking the two antigen genes by a G/S linker [32] as shown in Fig. 1A. The linked DNA sequences were adjusted to *L. lactis* codon usage to increase its translation efficiency [33]. PCR was conducted as depicted in Fig. 1B with the primers listed in Table 1. To create a fused DNA insert of the two antigens, overlap extension PCR was conducted. Briefly, the PCR products of 5′–*OmpA*-G/S linker–3′ and 5′–G/S linker-*FlgD*–3′ were mixed at a 1:1 M ratio. Approximately 80 ng DNA template DNA concentration was used in the 50 μL PCR reaction using EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan). Initially, the linker-containing template mixture was denatured at 95 °C for 5 min without primers, then the overlap annealing step and the extension step were completed at 70 °C for 10 min. Following the addition of *OmpA*-F and *FlgD*-R primers to the annealing solution, PCR amplification was further processed as shown in Fig. 1B. Each of the PCR products was digested with the *Nsi*I and *Xho*I restriction enzymes and ligated into pCYT using T4 DNA ligase (NEB Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. The ligated plasmid was transformed into *E. coli* DH5α by electroporation using a Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). Positive transformants were selected on Luria-Bertani (LB, Difco Laboratories, Detroit, MI, USA) agar plates containing 25 μg/ml of chloramphenicol and further confirmed employing a PCR conducted using pCYT vector specific primers (Table 1). Plasmids were extracted from the positive transformants and transformed into *L. lactis* BFE920, the vaccine delivery vehicle. Positive L. *lactis* BFE920 transformants were screened on de Man Rogosa Sharpe (MRS, Difco Laboratories, Detroit, MI, USA) agar plates containing 25 μg/ml of chloramphenicol.

Antigen expression from the positive clones was confirmed via western blot analysis. Briefly, recombinant *L. lactis* BFE920 harboring pCYT:*OmpA*, pCYT:*FlgD*, or pCYT:Fusion clones were inoculated (1% v/v) with the fresh overnight culture and further incubated overnight at 30 °C with 200 rpm agitation in MRS broth supplemented with 25 μg/ml of chloramphenicol. For induction of antigen expression, 10 ng/ml of nisin (Sigma–Aldrich, St. Louis, MO, USA) was added to the overnight culture diluted to an OD of 0.5 and incubated for 2 more hours at 30 °C with 200 rpm agitation. The diluted overnight culture was also cultivated without nisin under the same conditions as the control. Bacterial pellets were collected by centrifugation at 4500 rpm for 10 min at 4 °C and washed 2 times with 1× PBS (1.47 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.3). Cell pellets were resuspended in urea denaturation buffer (8 M urea dissolved 1× PBS) maintaining 20-fold concentrated volume and bead-beaten using silica zirconia beads. Supernatants collected by centrifugation at 13,000 rpm for 10 min at 4 °C were used for western blot analysis as described elsewhere [31]. Briefly, the primary antibody used for western blot analysis was 1:1000-diluted mouse anti-6×His Tag antibody (Biolegend, San Diego, CA, USA) and the secondary antibody was 1:3000-diluted HRP-conjugated goat anti-mouse secondary antibody (Bioss Inc, Woburn, MA, USA). Membrane imaging was performed using SuperSignal Femto Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and an Omega LumG imager (Aplegen, San Francisco, CA, USA) utilizing five calculative imaging 15-s intervals.

2.2. Experimental animals and feed vaccination

Healthy olive flounder (*Paralichthys olivaceus*) weighing 86.36 ± 4.31 g purchased from Woo-Jung Hatchery & Fish Farm, Pohang, Korea were acclimated at 20–21 °C in 2000 L cylindrical aquariums for 7 days. Fifty fish per experimental group, in duplicate, were placed in 300 L closed-circulation aquariums. The fish were then fed either a commercial diet (control) or the feed vaccines: the control diet adsorbed with WT *L. lactis* BFE920, OmpA-expressing *L. lactis* BFE920, FlgD-expressing *L. lactis* BFE920, or OmpA-FlgD fusion protein-expressing *L. lactis* BFE920. Feed vaccination was done as described elsewhere [27]. Briefly, overnight-cultured vaccines (~1.4 × 10^9 CFU/ml) were adsorbed to a commercial extruded pellet feed (Flounder Bluechip; Woosung Co., Ltd., Daejeon, Korea) by mixing just before regular feeding (final vaccine