Doctoral Thesis

Mechanisms of protection induced by atypical *Edwardsiella tarda* vaccine in red sea bream *Pagrys major*

summary

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Edwardsiella tarda infection (edwardsiellosis) is an important bacterial disease of fish in the world. Edwardsiellosis was first reported in farmed channel catfish *Ichtalurus punctatus* and Japanese eel *Anguilla japonica* in the 1970s. The disease has become more serious in farmed marine fish species in Japan, particularly Japanese flounder *Paralichthys olivaceus* and red seabream *Pagrus major* since 1980s, where two phenotypes of *E. tarda* strains were recognized as the causative agent. One type is motile strain, designated as typical type, and the other is non-motile strain, designated as atypical type. Both are different in the pathogenicity and host specificity. A number of virulence factors of *E. tarda* have been reported, and intracellular parasitic nature of *E. tarda* is considered as one of the most important factor that causes difficulty in controlling the disease. The pathogen survives inside phagocytic cells and is inaccessible by chemotherapeutic agents or antibody. Virulence mechanisms of the pathogen are still poorly understood. Several studies have attempted to develop vaccine against edwardsiellosis, but presently there is no licensed vaccine for the atypical *E. tarda* infection of red seabream.

In this thesis, I analyzed the virulence-associated factors (genes) and pathogenicity of typical and atypical *E. tarda* strains (Chapter 1), and I demonstrated the role of fimbriae as an important virulence factor in *E. tarda* infection under marine environments (Chapter 2). I developed an injection vaccine for red seabream edwardsiellosis (Chapter 3) and demonstrated the protection mechanisms conferred by the bacterin (Chapter 4).

Chapter 1. Comparison of virulence-associated factors between two *Edwardsiella tarda* phenotypes

Two phenotypic strains of *E. tarda*, FK1051 (typical) and MEE0309 (atypical), isolated from diseased Japanese flounder and red seabream, respectively, were used in this study. Full genome data of the *E. tarda* strains were analyzed to predict the virulence factors. Pathogenicity of the bacteria was evaluated by intraperitoneal infection in Japanese flounder and red seabream. The bacterial responses to the alternative pathway of complement cascade were evaluated by the serum bactericidal assay.

Total of 116 virulence-related genes were identified. Among them, 86 open reading frames (ORFs) were found in both strains, of which 45 ORFs were identical, based on the deduced amino acid sequence analysis. Infection experiments indicated pathogenicity of both strains to Japanese flounder and red seabream. The typical strain FK1051 was highly pathogenic to Japanese flounder ($LD_{50}=7\times10^2$ CFU/ 100g body weight) but less pathogenic to red seabream. Both strains were similarly pathogenic to red seabream with LD_{50} value of 4×10^6 CFU/100g. Red seabream serum complement system has no ability to inhibit the bacterial growth, indicating that the complement killing does not work well in protection against *E. tarda* infection in red seabream.

Chapter 2. Sodium chloride-enhanced fimbriae expression of *Edwardsiella tarda*

E. tarda is pathogenic to marine fish, but the previous studies revealed that the survival time in seawater is fairly short compared with that in freshwater, and that high concentration of sodium chloride (3% NaCl) in the growth medium induced the hemagglutination and cell adherence activities. In my study, both typical and atypical *E. tarda* strains exhibited faster growth in liquid medium supplemented with 0% to 2% NaCl and slower growth in the 3% NaCl conditions. Hemagglutination activity against guinea pig erythrocytes was detected only in the 2% NaCl and/or 3% NaCl cultures. Electron microscopy revealed two types of fimbriae. The first type was wide (ca. 9 nm) and expressed by the only typical strain in the 0% NaCl culture, designated as thick fimbriae and the second type was thin (ca. 4 nm) and produced by both *E. tarda* strains in the 3% NaCl cultures, designated as thin fimbriae. Flagella of the typical strain were lost in the 3% NaCl culture. Comparative genomic analysis indicated that amino acid sequences of the fimbrial operon (etfA, etfB, etfC, etfD) were homologous between the strains. Expressions of the major fimbrial subunit gene (*etfA*) in both strains were significantly higher in the 3% NaCl cultures than in the 0% NaCl cultures. The atypical *E. tarda* cells from 3% NaCl culture adhered in the intestine of red seabream more abundantly than those from 0% NaCl culture. In conclusion, the high-salt conditions of seawater are highly stressful for the typical and atypical *E. tarda*, causing the decrease in the survival time and growth rate and loss of the flagella. The thin fimbriae structure, induced by the *etf*A gene expression, might be required by the typical and atypical *E. tarda* as the mediator of adherence into host cells to escape from such unfavorable seawater conditions.

Chapter 3. Effectiveness of atypical *Edwardsiella tarda* bacterin in red seabream *Pagrus major*

In this study, the formalin-inactivated atypical *E. tarda* cell bacterin derived from 2% NaCl culture was selected as a vaccine candidate against red seabream edwardsiellosis. Lipopolysaccharides and outer membrane proteins were detected as antigenic substances in the bacterin. A single intraperitoneal (IP)-injection of the bacterin (10^8 cells/fish) to red seabream juveniles induced high antibody production at 3 weeks post-immunization. The bacterin induced no apparent abnormalities in fish conditions even at 10 times higher dose. High protection with relative percentage survival (RPS) values of more than 60% was achieved by the immunization, when the fish were IP-challenged with the homologous strain. The protections were dose-dependent and lasted at least up to 4 months post-immunization. Minimum effective bacterin dose was 10^7 cells/fish. Serum antibodies (agglutinins) were detected at high titers from all immunized fish but not correlated with the protection. The injection bacterin developed in this study will be applicable to red seabream farming facilities to control edwardsiellosis.

Chapter 4. Protective mechanisms in red seabream *Pagrus major* induced by atypical *Edwardsiella tarda* bacterin

To clarify protection mechanisms induced by the bacterin, some *in vivo* and *in vitro* experiments were performed. The numbers of *E. tarda* live cells in the blood, kidney, spleen and liver of the immunized fish at 48-h post bacterial challenge were significantly lower than those of the non-immunized fish, but with no total bacterial clearance. Factors contributing to inhibition of the bacterial growth were then investigated. At the early stage of infection, both classical and alternative pathways of fish serum complement cascades have no *in vitro* bactericidal effects to the atypical *E. tarda*. However, the immune macrophages with aids of the immune sera exhibited higher phagocytic activity and inhibition of intracellular growth of *E. tarda*, though clearance of the bacteria was not completed. Fish conditions at the late stage of infection were investigated by histopathological and immunohistochemical methods. Development of granulomas were detected in the kidney, spleen and liver with most dominant appearance in the kidney. Accumulation of phagocytic cells was detected after 4 days of infection and different types of granulomas were found after 2 weeks of infection. Granulomas in the immunized fish were mainly composed of progressively developing ones in the forms of walled off phagocytic cells aggregates and localized aggregates from surrounding normal tissue with centralized or disappearing bacteria in it, while those in the non-immunized fish were dominated by enlarging granuloma with spreading bacteria. Melanomacrophage centers (MMCs) occupied the area of immunized fish tissue more widely than those of non-immunized fish, and most of them were associated with granulomas. Based on these results, I concluded that protection of red seabream by immunization with the inactivated bacterin was brought by cellular immunity; the activated macrophages with aids of opsonins, granulomas formation and MMCs.