学 位 論 文 の 要 旨

論文題目　Mechanisms of protection induced by atypical *Edwardsiella tarda* vaccine in red sea bream *Pagrus major*

(養殖マダイのエドワジエラ症に対するワクチンの感染防御機構)

広島大学大学院生物圏科学研究科
Bioresource science　専攻
学生番号 D126247
氏　　名 Indah Istiqomah

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 sea bream *Pagrus major* since 1980s, where two phenotypes of *E. tarda* strains were recognized as the causative agents. 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 the phagocytic cells and is unaccessible by chemotherapeutic agents or antibody. However, virulence mechanisms of the pathogen are still poorly understood. Several study have attempted to develop vaccine to induce fish immunity against edwardsiellosis, but there is no licensed vaccine for the atypical *E. tarda* infection for red sea bream in the world.

In this thesis, I analysed the virulence-associated factors (genes) and pathogenicity between typical and atypical *E. tarda* strains (Chapter 1), and I demonstrated the role of fimbriae as an important virulence factor in atypical *E. tarda* infection under marine environments (Chapter 2). In Chapter 3, I developed an inactivated injection bacterin for red sea bream edwardsiellosis 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 *Edwardsiella tarda*, FK1051 (typical) and MEE0309 (atypical), isolated from diseased Japanese flounder and red sea bream, 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 were evaluated by intraperitoneal

infection in Japanese flounder and red sea bream. The bacterial responses to the alternative pathway of red sea bream and other marine fish complement cascade were evaluated by the serum bactericidal assay.

Total of 116 virulence factor genes were identified. Among them, 86 genes were found in both strains, of which 45 genes were identical. Infection experiments indicated pathogenicity of both strains to Japanese flounder and red sea bream. The typical strain FK1051 was highly pathogenic to Japanese flounder (LD_{50}=7×10^2 CFU/ 100g body weight). Both strains were similarly pathogenic to red sea bream with LD_{50} value of 4×10^6 CFU/100g. Red sea bream 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 sea bream. The *E. tarda* strains were also resistant to the serum complement activity of several marine fishes examined.

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

Edwardsiella 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 appeared only in the 0% NaCl culture of typical strain, and the second type was thin (ca. 4 nm) and appeared in the 3% NaCl cultures of both *E. tarda* strains. 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 highly 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 sea bream 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 in decreases 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 sea bream *Pagrus major*

Inactivated atypical *E. tarda* cells (bacterin) derived from 2% NaCl culture was selected as a bacterin candidate. Lipopolysaccharides and outer membrane proteins were detected as antigenic substances in the bacterin. A single IP-injection of the bacterin (10^8

cells/fish) to red sea bream 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 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 antibody (agglutinins) were detected at high titers from all immunized fish but no correlation between the antibody titers and the protection. The inactivated injection bacterin developed in this study will be applicable to red sea bream farming facilities to control edwardsiellosis.

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

To clarify protection mechanisms induced by the inactivated 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 type of granulomas were found after 2 weeks of infection. Tissue ratio of granulomas in the immunized fish were higher than in the non-immunized fish. Granulomas in the immunized fish mainly composed of progressively developing granulomas 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 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 sea bream by immunization with the inactivated bacterin was brought by cellular immunity; in the forms of activated macrophages with aids of opsonins and granuloma formation composed by macrophages and other unidentified cells.