The immune defense of shrimp gills revealed by Marsupenaeus japonicus gill C-type lectin (MjGCTL)

［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

専　攻 Major	APPLIED MARINE BIOSCIENCES	氏　名 Name	ALENTON ROD RUSSEL REYES
論文題目 Title	The immune defense of shrimp gills revealed by *Marsupenaeus japonicus* gill C-type lectin (MjGCTL) (クルマエビのエラ C 型レクチン研究によるエラの生体防御機構の解明)		

Shrimp aquaculture has become an economic asset specially for the Asian countries, however, its growth is threatened by outbreaks of diseases caused by both virus and bacteria. Thus, there is a need for studying shrimp's biodefense mechanisms that will help formulate defense strategies against these pathogens. For this, various studies have been conducted to identify the key factors and mechanisms that protect the shrimp from these pathogens, such as the immune-related organs and biodefense molecules. Organs such as hemocytes, hepatopancreas, lymphoid organ, and gills have shown to be involved in immune response of shrimp. Whereupon in the presence of pathogens these immune-related organs are equipped with immune cells and molecules executing the cellular and humoral responses. Several immune mechanisms have been identified such as blood clotting, melanization, release of antimicrobial peptides, phagocytosis, encapsulation, nodule-formation by hemocytes. The bulk of these immune reactions are performed by the hemocytes in the hemolymph. However, in search for new biodefense molecules among the immune-related organs, our laboratory conducted microarray result analysis which led to the discovery of immune molecules that are highly expressed in the gills rather than hemocytes. One biodefense molecule identified were the C-type lectins (CTLs).

Together with other C-type lectin-like domains (CTLDs), CTLs forms a protein superfamily that is one of the most abundant pathogen recognition receptors (PRRs) that mediate immune responses by the recognition of pathogen associated molecular patterns (PAMPs). The CTLs are Ca^{2+}-dependent, carbohydrate-binding proteins, capable of recognizing PAMPs through carbohydrate moieties, while CTLDs bind to other moieties independent of Ca^{2+}. Previously, a novel CTL was identified to be highly expressed in gills and was named as *Marsupenaeus japonicus* gill CTL (MjGCTL), where its immune function as a CTL localized in gills is still unknown.

Gills of penaeid shrimp act as the gateway between internal and external environment, functioning in gas and ion exchange, filtering out harmful biotic and abiotic factors. To the immune system, gills are known to merely assist mechanically through the removal of trapped foreign materials during molting. Apart from this, the immune role of shrimp gills remains unclear.

This dissertation focuses on the immune role of gills through the characterization of MjGCTL. For this, functional analysis of MjGCTL carried out both *in vitro* and *in vivo*. This study demonstrates that MjGCTL is an evidence that gills, aside from being a physical barrier, serve as a biochemical barrier of biodefense molecules.

Molecular characterization of MjGCTL, based on bioinformatics analysis, revealed that its characteristics are that of a Ca^{2+}-dependent carbohydrate binding CTLs. Phylogenetic analysis revealed that MjGCTL clustered apart from mannose and galactose-binding CTLs, where it may possess a non-canonical binding specificity. The distribution of MjGCTL protein in different tissues revealed that it is secreted on the surface of gills on the gill mucus. Through recombinant protein of (r)MjGCTL expressed in *Drosophila* S2 cells, rMjGCTL's bacterial agglutination on Gram-negative *Vibrio parahaemolyticus*, EGFP-expressing *Escherichia coli*, and Gram-positive *Streptococcus agalactiae* was observed in the presence of Ca^{2+}. However,

upon the removal of Ca^{2+} and the addition of EDTA, the agglutination of rMjGCTL was inhibited. Through bacterial agglutination inhibition, using various carbohydrates and bacterial components peptidoglycan (PGN) and lipopolysaccharide (LPS), the specific ligands of MjGCTL was determined. rMjGCTL was found to have affinity with LPS and PGN, as well as other carbohydrates except mannose and galactose. In an *in vitro* encapsulation assay using shrimp hempcytes, agarose beads coated with rMjGCTL were immediately encapsulated by hemocytes from 0h followed by melanization at 4h post-incubation with hemocytes, as compared to the negative controls where no encapsulation were observed. Corroborating the results of bacterial agglutination inhibition, encapsulation was also inhibited by the carbohydrate ligands of rMjGCTL, confirming its binding specificity.

To investigate the function of gill mucus *in vivo*, total soluble proteins and mucus of gills were extracted. Similar to rMjGCTL, the gill proteins and mucus agglutinated bacteria. To investigate the involvement of MjGCTL in the agglutination ability of gill mucus, anti-MjGCTL rabbit serum antibody was added, where the agglutination activity of mucus was inhibited. Using lactose-agarose beads MjGCTL was partially purified from gills, where the eluted MjGCTL likewise promoted bacterial agglutination. Furthermore, using the eluted MjGCTL, *in vivo* phagocytosis assay was performed using flow cytometry where the addition of MjGCTL increased phagocytosis by hemocytes on PKH67-labelled *Streptococcus agalactiae*. *In vivo* functional analysis of MjGCTL was done by silencing MjGCTL by RNAi, where shrimp were injected with MjGCTL-dsRNA, with GFP-dsRNA and PBS as control groups. Effects of silencing were investigated through agglutination assay, *Vibrio* and total bacteria count, and challenge test by immersion using a virulent strain of *V. parahaemolyticus*. Results showed silencing MjGCTL inhibited bacterial agglutinating capability of gill mucus, as compared with the control groups. Also, increased *Vibrio* and total bacteria count in gills and the hemolymph were observed among MjGCTL-silenced shrimp. Challenge test were conducted twice, results showed MjGCTL-silenced shrimp was more vulnerable to infection reducing survival to 20% and 0% at 7 days post-infection on trial 1 and 2, respectively. In addition, expression of other antimicrobial peptides crustin in penaeidin in gills of MjGCTL-silenced shrimp were found to be down-regulated. Furthermore, microarray analysis of new biodefense genes conducted among immune-related organs showed 27 biodefense genes differentially expressed (> 2-fold higher) in gills as compared to the other organs, where most are specifically expressed in gills.

In summary, as shown by the results of the molecular characterization and *in vitro* functional analysis, MjGCTL is a Ca^{2+}- dependent CTL with a non-canonical carbohydrate binding specificity, where MjGCTL may act as a PRR. This was confirmed in the *in vivo* characterization where it was demonstrated that in the shrimp immune system, MjGCTL functions as a PRR agglutinating bacteria and acting as an opsonin to promote encapsulation and phagocytosis by hemocytes. MjGCTL is secreted on the gill surface giving the gill mucus ability to agglutinate invading bacteria, and tagging the bacteria as opsonins to be recognized by hemocytes. *In vivo* silencing revealed that MjGCTL is involved in maintaining the shrimp microbiota, whereupon silencing MjGCTL caused an increase in bacterial growth. MjGCTL is also important for bacterial infection resistance, as silencing MjGCTL made the shrimp more vulnerable to infection and increased mortality.

This study has some research implications such as the interaction of MjGCTL with other immune molecules as well as with hemocytes, which will be interesting to investigate. The down-regulation of crustin and penaeidin upon silencing of MjGCTL showed that it may be involved in the expression pathway of other antimicrobial peptides however the mechanism is still unclear. Furthermore, as encapsulation by hemocyte was inhibited by carbohydrate ligands, MjGCTL appears to interact hemocyte through a receptor which can be a carbohydrate or protein. These aspects, together with the other gill-specific molecules are potential research areas which will further elucidate the shrimp's immune defense.