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

メタデータ	言語: eng
	出版者:
	公開日: 2018-01-04
	キーワード (Ja):
	キーワード (En):
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	所属:
URL	https://oacis.repo.nii.ac.jp/records/1480

Doctoral Dissertation

THE IMMUNE DEFENSE OF SHRIMP GILLS REVEALED BY *Marsupenaeus japonicus* GILL C-TYPE LECTIN (MjGCTL)

September 2017

Graduate School of Marine Science and Technology Tokyo University of Marine Science and Technology Doctoral Course of Applied Marine Biosciences

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Acknowledgements

The completion of this work was a very challenging process, and it will be impossible if not for these very instrumental persons, to whom I pour my utmost gratitude;

To **Prof. Ikuo Hirono**, my academic adviser and mentor, for his guidance, patience and unfailing support, and for showing me what and how is it to do real science.

To **Prof. Hidehiro Kondo**, who recommended my scholarship study in Japan and to this laboratory, and for helping me in my experimental design and trouble shooting.

To **Mrs. Reiko Nozaki**, for her unfailing generosity, and for offering me help and giving me tips and fine-tuning my skills in performing experimental techniques.

To **Prof. Mary Beth Maningas**, for introducing and inspiring me to follow my dream of being a scientist someday, for being the Filipino sempai who introduced me to the Laboratory of Genome Science, and for being always there for me.

To my other Genome Science Lab Filipino sempais **Dr. Cristopher Caipang**, **Dr. Fernand Fagutao**, **Dr. Mudjiekeewis Santos**, **Dr. Sherryl Hipolito**, and **Dr. Benedict Maralit**, for serving as my role models for to look-up to and follow.

To Jade, Moji, Noel, Leo, Ivane, Arianne, Erwin, and Seiji, for helping me in many ways, for bringing the warm feel of the familiar Filipino friendship in Japan.

To the **Genome Science Lab members** whom I shared my three years of fun, learning, and friendship with. To **Koiwai** and **Tip**, to whom I enjoyed discussing, playing, and doing experiments with, and **lang** for sharing with me my journey to Ph.D.

To the Japanese Government MEXT: MONBUKAGAKUSHO Scholarship for funding my study in Japan.

To my **Parents and family**, for supporting me in my studies, for always praying for my well-being, for visiting me in Japan and contacting me, making my time away from home less lonely.

To **Rika Nakamura**, for being my source of happiness, for inspiring and encouraging me to do my best in everything, and for always being there for me helping me in my experiments and my life in Japan.

Finally, to the omnipotent God almighty, the source of all life and love around me, who entrusted Science to man as a way of knowing Him more and for us to be better stewards of all His creations.

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Abstract

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 immunerelated 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 Ca²⁺-dependent, carbohydrate-binding proteins, capable of recognizing PAMPs through carbohydrate moieties, while CTLDs bind to other moieties independent of Ca²⁺. 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 Ca²⁺-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 Ca²⁺. However, upon the removal of Ca²⁺ 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

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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

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penaeidin in gills of MjGCTL-silenced shrimp were found to be down-regulated. Furthermore, microarray analysis of new biodefense genes conducted among immunerelated 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 Ca²⁺- 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.

CHAPTER 1

General Introduction

For Asia-Pacific countries, shrimp has become an indispensable source of revenue. Based on FAO's statistics (2015), among the world's total aquaculture commodity shrimp products accounts for 14% of the total export, which makes it the second most valued aquaculture commodity. The international demand for shrimp is high, where among the total production 86% originates from Asia, however about one-third is exported to European and American countries (FAO, 2015). Shrimp farming has also contributed to the socio-economic development of the many coastal communities in Asia, where in the recent years the average production growth rate is about 10% (Walker and Mohan, 2009). Although the shrimp aquaculture is a fast-growing industry, and an important aquaculture product that contributes to meet the high demand of nutritious sea-food, outbreaks of diseases are hampering the attainment of its sustainability (Subasinghe, 2009).

As witnessed in the recent years, where the global shrimp production was gradually increasing until the year 2012 that it reached its peak followed by the 19% decline in year 2013. This marked the detection of a new shrimp disease which was later identified as acute hepatopancreatic necrosis disease (AHPND) caused by a toxin-carrying strain of *Vibrio parahaemolyticus*. In the history of shrimp diseases, AHPND is the next most devastating disease outbreak that ever hit the industry after white spot syndrome virus (WSSV) that was detected in year 1994 (Flegel, 2012). These two, together with other identified pathogens, both viral and bacterial, continue to plague the shrimp industry up to present (FAO, 2012). The problems posed by the infectious shrimp diseases, together with

other environmental factors, made the sustainable development of shrimp industry a serious concern. For this, management strategies have been proposed to address the pressing concern. Leung and Engle (2008) proposed the research and development in these three major areas for shrimp health management: prevention, detection, and treatment. Prevention involves confirmation of the seed stock quality coming up with a specific pathogen-free (SPF) culture, evaluation of culture environment, and studying the shrimp's biodefense mechanism. A quick and accurate detection of pathogens is also important, which includes the identification of the causative agents of the infection. And finally, as countermeasure, the treatment from infection which entail optimization of delivery and follow-up procedures and monitoring treatment outcomes. Among these three areas, development of detection strategies has shown a significant improvement in the recent years. Unfortunately, it is not enough to detect the presence of pathogens, both treatment or prevention is needed to sustain shrimp production. The development of treatment strategies is made problematic by three major factors: the risk of developing drug-resistant microbes, the shrimp lacking adaptive immune system, and the limited knowledge on the shrimp immune system and how it interacts with pathogens. For this, much attention is now being given to searching for ways to prevent disease outbreaks, particularly by studying the fundamental mechanisms of the biodefense mechanism of shrimp. Understanding how the defense mechanisms of shrimp, both physiological and immunological, works against pathogens will serve as the starting material for the development of preventive strategies.

In the recent years, the number of studies on shrimp immunity and their application to aquaculture have been growing and developing together with the technological advancement in the field of molecular biology. Current approaches include the study of

immune-related genes through high-throughput genome, transcriptome, and proteome sequencing analyses; gene-expression analyses through microarray; and functional studies using gene-knockdown and recombinant protein technologies (Tassanakajon et al., 2013).

Shrimp immune system

Both marine and freshwater habitats are lurking with harmful biotic and abiotic factors. Together with other crustaceans, shrimps are protected by the hard cuticle as a structural barrier. However, against the pathogenic microorganisms that can penetrate the body cavity, a more sophisticated internal immune defense mechanism is required. Several immune mechanisms have been identified in shrimp, together with that of the other closely-related crustaceans (Söderhäll and Cerenius, 1992). Shrimp immune system depends solely on its innate immune responses, comprised of the humoral and cellular responses. These immune responses, just like in any other animal, generally depend on the immune actions of blood cells or hemocytes (Johansson et al., 2000).

On the humoral responses, the primary defense mechanism is blood clotting, which is the rapid sealing of an open wound the to prevent the entry and immobilization of invading pathogen. Among crustaceans, there are two ways of how the clotting system render protection. For crayfishes, blood clotting is the end result of the mechanism (Lee and Söderhäll, 2002). For the horseshoe crabs, and later on found to be the same for shrimps, the clotting system is coupled with the release of antimicrobial peptides (AMPs) in the hemolymph (Maningas, 2008; Iwanaga and Lee, 2005; Iwanaga, 2002). In shrimp, it was discovered that the clotting system proceeds with the Ca²⁺-dependent combination reaction of both clotting protein and transglutaminase protein (Maningas, et al., 2012).

Another major component of the humoral response is the prophenoloxidase (proPO) cascade. It is known in crustaceans that the proPO system can be activated in the presence of microbial component called pathogen-associated molecular patterns (PAMPs) (Fagutao et al, 2009). Upon the detection of PAMPs, the serine protease cascade is initiated where the inactivated enzyme precursor, proPO, is converted to form the phenoloxidase (PO). PO is the catalyst for the oxidation of tyrosine to produce toxic quinone substances and other reaction intermediates which results to the formation of melanin (Tassanakajon et al., 2013). Melanin increases the adhesion of microbes to hemocytes, acting as an adhesive substance which facilitates elimination through nodule formation (Koizumi et al., 1999).

As mentioned above, the clotting system is coupled with the release of AMPs, which are the effectors of the innate immune system that serves as the first line of defense against infectious microorganisms. AMPs are characterized as molecules small in molecular size (150-200 amino acid residues), amphipathic structure with either cationic or anionic properties (Tassanakajon et al., 2013). These AMPs are known to have activity against a wide-array of microorganisms such as bacteria, yeast, fungi, parasites, and viruses (Hancock and Diamond, 2000; Kreptakies et al., 2012). In shrimp, several AMPs have been identified such as crustins, penaeidins, lysozymes, anti-lipopoly-saccharide factors (ALFs), and stylicins (Rolland et al., 2010; Tassanakajon et al., 2010). Most of these AMPs were found to be produced, stored, and released by the hemocytes (Somboonwiwat, et al., 2005). Proteinases and their inhibitors (proteinase inhibitors) have also been reported to be linked to the shrimp immune system (Jiang and Kanost, 2000; Tassanakajon et al., 2013). These molecules are ubiquitous in all living organisms and are involved in several biological and physiological mechanisms. They are linked in biological pathways that lead to the apoptosis and melanization mechanism of shrimp innate immune system. Some families identified in shrimp are the clip domain serine proteinases, Kazal-type serine proteinases, serpins, and alpha-2-macroglobulins (Rimphanitchayakit and Tassanakajon, 2010).

On the other hand, cellular immune reactions of the shrimp immune system are the several actions of the hemocytes, which include phagocytosis, encapsulation, nodulation, and apoptosis (Söderhälll and Cerenius, 1992; Johansson et al., 2000).

The antiviral mechanism in shrimp has also been described, however, is still poorly understood. The evidence of the antiviral mechanism among invertebrates lies in the conserved mechanism of the RNA interference pathway, which is characterized by the sequence-specific gene silencing (Labreuche and Warr, 2013). In shrimp, several novel genes have been characterized through the functional analysis through gene knockdown by RNAi (Maningas et al., 2008; Li and Song, 2010; Shockey et al., 2009; Ponprateep at al., 2012). The RNAi machinery is composed of 3 main features: first the recognition of the dsRNA inducer that is recognized by host receptors and taken up through the endocytic pathway; second, the conserved protein complexes such as the Dicer (Dcr) that cleaves dsRNA into siRNA whose sense strand serve as the guide RNA that forms complex with Argonaute (Ago) molecule to form the RNA induced silencing complex; and degradation of the RNA target in a sequence dependent fashion. This mechanism has been known to be a natural antiviral mechanism of the plant immune system (Shabalina and Kooni, 2008). Later in animals, Fire and Mello (1998) reported their work, where they established the framework for gene silencing in *Caenorahbditis elegans* can be triggered by the introduction of double-stranded RNA (dsRNA). The antiviral mechanism through RNAi machinery was discovered in Drosophila melanogaster when viral dsRNA, either from viral transcription complexes or transcription of DNA viruses, are recognized by Dcr2 and with

the help of a dsRNA binding protein (R2D2) the resulting viral small interfering RNAs (vsiRNAs) are transferred to Ago 2. The protein complex of vsiRNA and Ago2 will then target the viral RNAs for degradation (Sabin et al., 2010). In shrimp, analysis of the transcripts of the molecules involved in the RNAi machinery after challenge test with white spot syndrome virus (WSSV). Transcripts of Dcr2 in *Litopenaeus vannamei* (LvDcr2) and Ago from *Penaeus monodon* (PmAgo) was observed to increase upon WSSV infection (Chen et al., 2011; Unajak, et al., 2006). Also, silencing Dcr1 increased both viral load and mortality in *P. monodon* (Su et al., 2008). Furthermore, it has been demonstrated that the introduction of a non-specific dsRNA is able to produce certain protection against viral infection in *L. vannamei* (Robalino, et al., 2004; Robalino, et al., 2007). These findings suggested that, like vertebrate host cells, invertebrates can detect dsRNA as viral PAMPs that induces the antiviral response through the sequence-independent and sequence-specific RNAi pathway (Sabin and Cherry, 2013).

Indeed, the innate immune system's humoral, cellular and anti-viral responses can perform various defense mechanisms against different types of pathogens. However, a prerequisite to elicit an immune response is the accurate and quick recognition of the pathogens though the PAMPs.

Pattern recognition receptors

Different type of pathogens requires different immune responses and thus are recognized as different types of PAMPs. These PAMPs are recognized by the pattern recognition receptors (PRRs), where the end goal is the rapid humoral and cellular immune responses. These PRRs are germ-line encoded proteins and are therefore different from that of the antibodies produced by vertebrates. Also, rather than recognizing specific parts of the pathogen, the PAMPs that PRRs recognize are molecular structures or patterns found on surface of microbes. Examples of these PAMPs are lipopolysaccharide (LPS) of Gram negative bacteria, peptidoglycan (PGN) or lipotechoic acid (LTA) of Gram-positive bacteria, or glucans components of fungal cells. PAMPs can also be polynucleotides like CpG DNAs of bacteria and viruses, or ssRNA and dsRNA viral stages. The shrimp immune system has PRRs having different ligands and functions eliciting cellular, humoral, or antiviral immune reactions, and are classified to 11 different families: β -1,3-glucanase-related proteins (BGRPs), β -1,3-glucan binding proteins (BGBPs), scavenger receptors (SRs), serine-protease homologs (SPHs), thioester-containing proteins (TEPs), down syndrome cell adhesion molecule (DSCAM), toll-like receptors (TLRs), RNA-binding proteins (TRBPs), fibrinogenrelated proteins (FREPs), galectins, C-type lectins (CTLs) (Wang and Wang, 2013). This section will discuss briefly each of these PRRs except the CTLs, which will be the focus of the next section .

The first group of shrimp PRRs are the β -1,3-glucanase-related proteins (BGRPs), which were also previously called Lipopolysaccharide (LPS) and β -1,3-glycan-binding proteins (LGBPs), or Gram-negative binding protein (GNBP) (Wang et al., 2011). This is with reference to the diverse binding specificity of these proteins which are β -1,3-glucan, and Gram-negative bacteria; although BGRPs were also found to bind to Gram-positive bacteria. Thus, to avoid confusion they were referred to as BGRPs based on the shared domain similar to β -glucanases (glucanase-like domain, GLU) (Pauchet et al, 2009; Wang et al, 2011). In shrimp, the expression of these BGRPs were found to be upregulated by live and inactivated bacteria, as well as PAMPs such as LPS (Cheng et al., 2005; Lin et al., 2008). The localization of shrimp BGRPs are mostly in hemocytes and/or hepatopancreas.

Furthermore, their binding to PAMPs can enhance PO activity in *P. monodon* (Sritunyalucksana et al, 2002).

Another family of PRRs are the β -1,3-glucan binding proteins (BGBPs), which show no sequence similarity and are larger molecules (~100 kDA) compared with the previously described BGRPs whose molecular size are usually less than 50kDa. Moreover, BGBPs do not contain GLU domain and are not characterized by any recognizable domains, aside from two short tripeptide motifs RGD and/or RGE. BGBPs forms complex with β -glucans, where the glucan-BGBP complex initiates degranulation of hemocytes and activation of the proPO system (Vargas-Albores and Yepiz-Plascencia, 2000).

A large and structurally diverse family of PRRs are the scavenger receptors (SRs). SRs are transmembrane cell surface glycoproteins that are able to bind to the modified form of low density lipoproteins (LDLs), which are polyanioinic ligands and components of bacterial cell wall. In the innate immune system, SRs are known to recognize PAMPs such as LPS, lipoteichoic acid (LTA), and β -glucans/zymosan of yeasts (Areschoug and Gordon, 2009). SRs facilitate phagocytosis in a non-opsonic fashion, acting as phagocytic receptors. In shrimp, scavenger receptors were identified in *Marsupenaeus japonicus* and are known to promote phagocytosis and clearance to both bacteria and WSSV (Mekata et al., 2011).

Another PRR family are the serine proteases. However, different from their vertebrate counterpart, serine proteases in invertebrates tend to act as more as PRRs specially in crustaceans and are referred to as serine protease homologs (SPHs) (Lee and Söderhall, 2009). Masquerade-like SPH has been identified both in crayfish and shrimp, where these SPH were found to have binding activity with LPS and Gram-negative bacteria, and promote opsonization (Lee and Söderhall, 2009; Jitvaropas et al., 2009).

Thioester-containing proteins (TEPs) are found in both vertebrate and invertebrate innate immune systems. This family of molecules are characterized by the thioester (TE) motif "GCGEQ" and a catalytic histidine residue, where both form covalent bonding with pathogens through a TE bond and facilitate phagocytosis (Levashina et al., 2001). Belonging to this family of TEPs are the protease inhibitor alpha-2-macroglobulin (α 2M), which is comprised of a similar TE motif (GCGEQNM) without the catalytic histidine, a bait region, and a receptor binding domain. These molecule binds to bacterial protease and inhibit the activity of proteases which may decrease the virulence of invading pathogen and promote clearance. In various shrimp species, α 2M were found to be expressed in hemocytes, and its mRNA levels increased upon the administering bacterial component PGN (Gollas-galvan et al., 2003; Rattanachai et al., 2004; Lin et al., 2007). In *Fenneropenaeus chinensis*, however, both bacterial (*Vibrio* sp.) and viral (WSSV) increased α 2M expression in hemocytes and lymphoid organs (Ma et al., 2010).

Invertebrates may not have immunoglobulins similar to the vertebrates' that allow the immune system to recognize in a pathogen-specific manner, however, invertebrates do have those molecules containing the immunoglobulin superfamily (IgSF) domain (Adema et al., 1997). An example of these molecules is the Down syndrome cell adhesion molecule (DSCAM), which was first described in humans and were found to be associated with Down Syndrome where the name was derived from (Yamakawa et al., 1998). The structure of DSCAM is characterized by the presence of immunoglobulin (Ig) and fibronectin (FN) domains, and are expressed in the nervous system and is involved in neural circuit formation (Yamakawa et al., 1998; Hattori et al., 2008). In *Drosophila*, DSCAM has a cytoplasmic tail and a hypervariable extracellular tail that are involved in signal transduction and pathogen recognition, respectively (Watson et al., 2005). Some DSCAM

studies in *Drosophila* appear to condsider invertebrate DSCAM molecules to be serving as the alternative adaptive immunity (Wang and Wang, 2013). DSCAM was also found in crustaceans, and in shrimp particularly in *L. vannamei* LvDSCAM was reported to possibly encode 8,970 isoforms, and the Ig domains might be involved in the response against WSSV infection (Chou et al., 2009).

Among vertebrates, Toll like receptors (TLRs) are PRRs directly recognizing PAMPs. They are characterized as type I transmembrane receptor with the leucine-rich repeats (LRRs), and the intracellular Toll-interleukin 1 receptor (TIR) domain (Kawai and Akira, 2010). In an invertebrate (*Drosophila*) model, however, TLRs were first found to function as indirect PRRs. Early studies found that in the Drosophila Toll pathway, the recognition is done through the spaetzle molecule which is then recognized by TLRs. However, later on some TLRs were found to act as direct PRRs of a virus, similar to that of the vertebrates, inducing antiviral autophagy (Nakamoto et al., 2012). In shrimp, there are three types of TLRs discovered and were found to be expressed in various tissues, and are responsive to bacterial infection rather than viral (WSSV) (Yang et al., 2008; Mekata et al., 2008; Arts et al., 2007; Wang et al., 2012). Thus, TLRs in shrimp appear to act as bacterial PRRs, and their role in the anti-viral immunity remains to be clarified.

The one evident viral PRRs in shrimp are the trans-activation response RNA-binding proteins (TRBPs). Originally referred to as HIV TRBPs, TRBPs were first isolated and characterized from HeLa cells, where it was found to strongly bind to HIV-1 encoded leader RNA (Gatignol et al., 1991). TRBPs are characterized under the double-stranded RNA (dsRNA) – binding proteins, which are characterized by the presence of dsRNA-binding domain (dsRBDs) (Saunders and Barbers, 1991). Among invertebrates, dsRBD-containing proteins were first identified in *C. elegans* which was found to mediate the binding of

dsRNA to Dicer (Dcr), protein kinase R, and PKR-associated activator (Haase et al., 2005; Parker et al., 2008). In shrimp TRBPs were found in *F. chinensis* and *M. japonicus*, both containing three dsRBD and having high sequence similarity between species. Shrimp TRBPs' first dsRBD were found to bind strongly to WSSV dsRNA and other dsRNAs, where the second and third have interactions with eukaryotic initiation factor 6 (eIF6), which is necessary for dsRNA-induced gene silencing (Wang et al., 2009; Wang et al., 2012). Thus, TRBP together with eIF6 are important factors of shrimp anti-viral immunity, where TRBP function as PRR recognizing viral PAMPs.

The last two shrimp PRRs falls under the category of a large carbohydrate-binding group of proteins called the lectins. Lectins act as PRRs by recognizing the carbohydrate components of microorganisms. The collectin family is one representative of the lectins, where one of the early lectin the mannose-binding lectin (MBL) is a member of. MBL is characterized by a short N-terminal region with two or three cysteine residues, a collagen like region, and a conserved carbohydrate recognition domain (CRD) (Holmskov et al., 2003).

Although MBLs are not found in shrimp there are the fibrinogen domain immunolectins or fibrinogen-related related proteins (FREPS) that possess a similar structure as MBLs, however possessing a fibrinogen-like (FBG) domain rather than a CRD (Holmskov et al., 2003). FREPS are representative of mammalian ficolins, which is another family of lectins apart from collectins, only that FREPs are found in both vertebrates and invertebrates (Dong and Dimpoloulos, 2009). The FBG domain has high identity to Cterminal regions of fibrinogen β and γ chains, and on theN-terminal region one or more Nteminal immunoglobulin superfamily (IgSF) domains (Leonard et al., 2001). FREPs can be classified both as a lectin and a member of the immunoglobulin-containing proteins. In *M*.

japonicus, MjFREP1 was found to be upregulated by various bacteria species and WSSV, and displayed a calcium-dependent agglutination activity against Gram-positive bacteria (Chai et al., 2012).

Galactoside-binding lectins or galectins were first identified in vertebrates, where they were found to be lectins that have affinity with β - galactosidases (Barondes et al., 1994). This family of proteins can be characterized by two features: their affinity to β galactosidases, a conserved CRD, and proline and glycine-rich N-terminal region (Hirabayashi et al., 1993). Galectins were previously characterized as S-type lectins because of the association with sulphydril-dependent activities activity of some members of galectins (Yang et al., 2008). These molecules are generally known to be involved in a variety of cellular processes such as cell differentiation, cell proliferation, programmed cell death as well as the innate immune responses (Cooper and Barondes, 1999). Although lacking the conventional signal sequences, galectins are able to interact in the cell surface through its binding with glycoconjugates present in the plasma membrane or extracellular matrix (Hughes, 1999). Among shrimp, galectin was reported in *M. japonicus*, where its mRNA was found to be expressed in the immune related organs such as hemocytes, gills, hepatopancreas, and intestine. This shrimp galectin's expression can be induced by bacterial infection, and its protein activity involves binding with LPS and LTA, which facilitates bacterial clearance (Wang and Wang, 2013).

C – type lectins

C-type lectins (CTLs) are members of the superfamily of metazoan proteins that are characterized by the conserved C-type lectin domain (CTLD), which is a large and diverse group of extracellular proteins referred to as CTLD-containing proteins (CTLDcps) (Zelensky

and Gready, 2005). The growing number of CTLDcps prompted researchers to come up with a way on how to classify the members of the superfamily, where classification of CTLDcps based on the overall domain architecture was introduced (Drickamer, 1999; Drickamer and Fadden, 2002). Generally, CTLDcps were characterized as bona fide calcium-dependent carbohydrate binding lectins, where the name "C-type lectins" were derived from. The binding of CTLs was discovered to be attributed to the C-type CRD or the C-type lectin domain (CTLD), whose structure was present and conserved in all calcium-dependent lectins . Thus, CTLs are therefore defined as a lectin with a CTLD. However, as the number of CTLDcps grew, calcium-independent CTLDcps were also discovered, and they were found to bind not only with carbohydrates but with other moieties such as lipids, proteins, etc.,. Moreover, multiple number of CTLDs in one protein was also discovered, making the classification method problematic. Thus, the term C-type lectin-like domains (CTLDs) was coined to refer to proteins that bind to other moieties in a calcium-independent manner, while sharing the same CTLD/CRD motif with the classical CTLs. The CTL group therefore form a small group under CTLDcps, which is confusing as CTLs are the only members of the superfamily that are actual lectins (Zelensky and Gready, 2005).

Nonetheless, CTLDcps, regardless their dependency on calcium and the ligands they bind to, have been known to form the most number and most diverse group of PRRs. CTLs are known to recognize pathogen through their carbohydrate moieties found on bacterial surface, while others bind to proteins and lipids on the surface of viral pathogen. They have been known to act as effective PRRs that are able to act as opsonins for phagocytic cells. CTLDcps can also facilitate several immune reactions, and even signaling (Cambi et al., 2005). Among vertebrates CTLDcps are known to be highly conserved, however, for the invertebrates a considerable diversity can be observed (Cambi et al., 2005; Pees et al., 2015). In shrimp, CTLDcps have been found to be the group of PRRs that can recognize the most number of PAMPs, which can elicit cellular, humoral, and anti-viral responses. Shrimp CTLDcps show diversity in their domain architecture, ligands, localization, expression patterns, and function in shrimp immune system.

Shrimp gills

Gills of crustaceans are multifunctional tissues performing the key roles both in physiological and immunological responses. While acting as a physical barrier separating internal and external environment, gills are also the site of processes maintaining homeostasis such as respiration, ion-exchange and osmoregulation (Cieluch et al., 2007). Gills also participate in the immune defense, giving mechanical aid to hemocytes, trapping phagocytosed materials and other harmful biotic and abiotic factors, then forming nodules that are extruded during molting (Martin et al., 2000). As gills maintains in-contact with the external environment it is made vulnerable to structural damage and the primary target entry site for pathogens (Rattanarojpong et al., 2007).

Prominent shrimp pathogens, such as White spot syndrome virus, Taura syndrome virus, and Vibrio spp., are known to have the gills as their target organs (Clavero-Salas et al., 2007). Several studies on Vibrio infection on shrimp have established gills as the main infection route (Alday-Sanz et al., 2002). It was also demonstrated that *V. vulnificus* infection on *Penaeus monodon*, regardless of bacterial entry site, infection route converges to a common pathway which leads to the gill tissues (Alday-Sanz et al., 2002). Studies on Vibrio clearance in penaeid shrimp indicated that gills and hepatopancreas were the main site of accumulation of culturable bacteria (Alday-Sanz et al., 2002; Burgents et al., 2005; Martin et al. 1993). But unlike the hepatopancreas, the gills don't possess fixed phagocytic

cells capable of eliminating bacteria (Johnson, 1987). Along with other foreign materials, nodules formed from hemocytes and phagocytosed material increase in size and may impair blood flow and normal gill function such as respiration and ion regulation (Martin et al., 2000). Thus, it is intriguing how the gills manage both penetrating and accumulating bacteria in both internal and internal surface.

There is indeed a lack of knowledge how gills protect the shrimp from invading pathogens from the environment. Thus, there is a need to investigate immune molecules residing in gill tissues to elucidate the types of immune reactions that may be taking place in gills.

Objectives and outline of thesis

Previously, a novel C-type lectin was identified as *Marsupenaeus japonicus* gill ctype lectin (MjGCTL), whose high expression in gill tissues implicated its possible involvement in immune responses in its organ of localization. Therefore, this dissertation aims to characterize MjGCTL, taking into account its nature as a pathogen recognition receptor and its localization in the gill tissues, which consequently investigates the poorly understood defense mechanism of shrimp gills.

Chapter 1 presents the importance of shrimp industry and its current state, where the shrimp industry has grown to be an indispensable economic asset for most Asian countries, however, the sustainable growth is hindered by inflicting diseases. The current situation therefore emphasizes the need to understand the underlying mechanisms of the shrimp's immune defense. Thus, the chapter presented in brief the different immune responses in shrimp and highlighted the prerequisite of all these immune responses, which is pathogen recognition by PRRs. C-type lectins are presented here as an important member of PRRs, where their characterization (as well as the discovery of other defense molecules) is vital knowledge on how shrimp is protected from infections. Also, the lack of knowledge on the existing defense mechanism of gill tissues, where apparently, several biodefense genes are differentially expressed and C-type lectin being one of these genes.

Chapter 2 introduces the gene of interest of this dissertation which is *Marsupenaeus japonicus* gill C-type lectin (MjGCTL). The molecular characterization of MjGCTL is described in this chapter, including its classification among C-type lectin domaincontaining proteins. Also, localization of MjGCTL was confirmed which suggested its involvement in the immune defense of gills.

Chapter 3 describes how MjGCTL functions as a PRR by investigating its function *in vitro*, where its binding activity and specificity to certain ligands were determined. Results of the binding specificity also confirmed its classification from the previous chapter.

Chapter 4 presents the in vivo function of MjGCTL in gills which presents it as an evidence to an existing biochemical barrier present in gill that protects the shrimp from bacterial infection.

Lastly, Chapter 5 summarizes the results of the three chapters presenting important insights and conclusions on how biodefense molecules such as MjGCTL may function in the gill tissues of *M. japonicus*, and on how this molecule may work with other immune responses in shrimp.

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CHAPTER 2

Molecular characterization of MjGCTL

Abstract

C-type lectins (CTLs) are important PRRs of many animals. In mammalian organisms, numerous CTLs have been identified and classified according to their architectural domain and function. Unlike the mammalian CTLs, invertebrate CTLs have shown more diversity and the number of characterized CTL genes or CTLD-containing proteins (CTLDcps) are still few. It has been proposed that using sequence information of the CTL carbohydrate recognition domain (CRD), identifying molecular characteristics, provide the classification and information of the possible biological function of a CTL. Here I present the molecular characterization of MjGCTL, where its sequence information revealed MjGCTL possess a signal peptide, LDLa domain, a CTLD domain, and 7 potential glycosylation sites. MjGCTL has high identity with CTLDcps genes with QAP motif, the highest (77%) being with Litopenaeus vannamei LvCTLD, a calcium-independent CTLD. Phylogenetic analysis revealed MjGCTL clustered with the QAP-motif containing CTLDcps, separate from the usual EPN and QPD motif-CTLDcps. Having an extra disulfide bridge, MjGCTL possesses a long-form CRD, which is shared among other QAP motif-containing CTLDcps. However, despite the sequence identity between MjGCTL and LvCTLD, protein 3D-structure revealed MjGCTL having the characteristics of a calcium-dependent CTLD while LvCTLD is calciumindependent as previously reported. Recombinant and native protein revealed MjGCTL's size is approx. 43 kDa, which is higher than its predicted (37 kDa) molecular size.

Introduction

Carbohydrate complexes from glycans, holds an immensely complex biological information that are not encoded in the genome, and through them pathogens are recognized by PRRs (Kilpatrick 2002; Ghazarian et al., 2011). This recognition of cell surface carbohydrates can be attributed to the lectins, which are proteins that preferentially bind and recognize carbohydrates. In animals, CTLs are the most abundant and diverse superfamily, and are lectins containing one or more C-type carbohydrate recognition domains (C-type CRDs) or C-type lectin domains (CTLDs) functioning in a Calciumdependent manner (Kilpatrick 2002; Ghazarian et al., 2011). A CRD is a double-loop structure stabilized by 2 disulfide bridges, on near both terminal end of the molecule and the other one in the long-loop region. The long-loop region is composed of four Calciumbinding sites, in the second binding site the two tripeptide motifs that determine the carbohydrate specificity are found. The first of these motifs is EPN (Glu-Pro-Asn) or QPD (Gln-Pro-Asp) and the second is WND (Trp-Asn-Asp) (Tassanakajon et al., 2013; Rabinovich et al., 2012). CRDs with EPN or QPD as the first motif are specific to mannose (Man) or galactose (Gal), respectively. Thus, CTLs have been characterized into two groups, identifying their CRDs as Mannose-binding EPN or Gal-binding QPD (Wang and Wang, 2013; Zelensky and Gready, 2003; Zelensky and Gready, 2005). However, variations of these motifs resulting from a mutation of single amino acid were reported. In shrimp CTLs, variants of these motifs include EPK, EPD, EPQ, EPS, QPN, and QPT (Wang and Wang, 2013). Some of these variants retained the binding-specificity of the original motifs, while others had specificities overlapping with other motifs, making the CTLs difficult to characterize.

As literature on both CTLs increased, ambiguity and confusion over the use of terminologies for their classification arose (Zelensky and Gready, 2005). For instance, C-

type CRDs are also referred to as C-type lectin domains (CTLDs) and C-type lectin-*like* domains are also abbreviated as CTLDs, and are used interchangeably (Pees et al., 2016; Wang et al., 2014; Wang and Wang, 2013). C-type lectin-*like* domains (CTLDs) are proteins that possess the same structural identity with the CRD of CTLs but are not actually lectins as they bind to other moieties rather than carbohydrates and in Ca²⁺-independent manner (Zelensky and Gready, 2005). In addition, the latter CTLDs are also referred to as C-type lectin receptors in some literature (CLRs) (Geijtenbeek and Gringhuis, 2009). Thus, identification and classification of new CTLDcps are important. Proposed by Drickkamer and Fadden (2002), classification of CTLDcps can be done through studying the CRD fold, which will dictate the classification of lectin (calcium-dependency) and its binding specificity (sugar-binding motif). This, together with the information on the accessory domains, the possible biological functions of a lectin can also be predicted.

Recently, another binding motif variant with a QAP (GIn-Ala-Pro) sequence has been reported in several shrimp CTLs. These inlcude CTLDs from *Litopenaeus vannamei* (LvCTLD) (Junkunlo et al., 2011) and *Macrobrachium niponense* (MnCTLDcp1 & 2) (Xiu et al., 2015) and LdIrLec 1 & 2 from *Marsupenaeus japonicus* (Xu et al., 2014). The binding specificity of the QAP motif in LvCTLD, was originally reported to be Gal, suggesting QAP is a mutated QPD motif (Zelensky and Gready, 2003).

In this study, we report a different binding specificity in the QAP motif through MjGCTL. Furthermore, the comparison of MjGCTL to LvCTLD provides a good reference on the function-based characterization to discriminate between CTLs and CTLDs.

Materials and Methods

Experimental Shrimp

Marsupenaeus japonicus shrimp weighing 10 grams were obtained from a farm in Miyazaki prefecture Japan and were acclimatized for at least 3 days before the experiment. Shrimp were kept in tanks with recirculating water maintained at 25 °C and 35 ppt salinity.

Sequence analysis of MjGCTL

MjGCTL nucleotide and amino acid sequence identities were analyzed with BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ExPASy (https://www.expasy.org), respectively. The CTL domains were confirmed by SMART (http://smart.emblheidelbelg.de). Analysis of the putative N-glycosilation sites where done through NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Sequences were aligned with ClustalW(http[1]://www.ebi.ac.uk/Tools/msa/clustalw2/). A consensus tree was constructed using Genious 6.1.8 (Biomatters Ltd., New Zealand) through Neighbor-Joining as the tree building method and Jukes-Cantor as the genetic distance model with 1000 bootstrap replications. The amino acid sequence of MjGCTL and LvCTLD (AEH05998) were also aligned using ClustalW in Genious 6.1.8 (Biomatters Ltd., New Zealand) software.

3D-structure and modeling of MjGCTL CRD

To compare the carbohydrate recognition domain (CRD) 3D-structure of MjGCTL and LvCTLD was constructed by first locating each of their CRDs through SMART (http://smart.embl-heidelberg.de/). Then best template used to generate the 3D-model was identified by MUSTER (http://zhanglab.ccmb.med.umich.edu) as C-type lectin mincle (PDB 4KZVA) from *Bos taurus*. Alignment was then made through ClustalW, and the 3D- model was then generated by Swiss-Model and was then edited and by UCSF Chimera (https://www.cgl.ucsf.edu/chimera/).

Cell culture of Drosophila Schneider 2 (S2) cells

Drosophila Schneider 2 (S2) cells were cultured in Schnieder *Drosophila* medium (SDM) (Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) and antibiotics in a culture flask maintained without CO_2 at 28 °C. S2 cells were passaged several times until reaching the optimum cell growth rate following the manufacturer's protocol.

Production of recombinant MjGCTL (rMjGCTL)

The cDNA sequence of MjGCTL was amplified using rMjGCTL primers (Table 1), where *EcoRI* and *NotI* sites were added to the forward and reverse primers (5' and 3' end), respectively. The rMJGCTL PCR was cloned into pGEM T-easy Vector (Promega, USA), digested with the corresponding restriction enzymes, then cloned into the restriction sites of inducible/secreted protein expression vector pMT:BiP:V5-His C (Invitrogen). Recombinant plasmid pMT:BiP:V5-HisC-MJGCTL was then transfected using Effectene Transfection Reagent (Qiagen, Germany) following manufacturer's instructions. Stable cell lines were selected by passaging the cells several times on SDM with 125 mg/ml of blasticidin according to the manufacturer's instructions. Following the large-scale production of the stable cell-lines, protein expression was then induced by CuSO₄ (600 μM). One day after induction, the cells were centrifuged and rMjGCTL was purified from the supernatant using Ni-NTA agarose (Qiagen) purification column with 500mM imidazole, following the manufacturer's protocol. The concentration of purified protein was quantified using DC protein assay (BIO-RAD, USA) following the manufacturer's protocol.

Table 1. Primer sequences used.

Name	Sequence (5'-3')		
MjGCTL_F	GGCATGGAATAACAGCGAGT		
MjGCTL_R	CATTTTGGATGCTGAGCAGA		
EcoRI_MjGCTL	GAATTCGGAATGCACGGGCGACGAAGTGGC		
Notl_MjGCTL	GCGGCCGCGACAGAGGTGACACAAA		

SDS-PAGE and Western blot analysis

Eluted proteins were subjected to 15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked in 5% BSA in 0.05% Tween 20 in TBS (TBST) for 1 h, incubated for 1 h with mouse anti-V5 monoclonal antibody (Thermo Fisher Scientific, USA) diluted to 1:5000 in blocking solution at room temperature with gentle shaking, washed four times with TBST, incubated for 30 min with anti-mouse IgG, AP conjugate (Promega) diluted to 1:10000 in blocking solution at room temperature with gentle shaking, washed three times with TBST, stained with substrate solution containing 5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium (BCIP/NBT, Sigma, USA) for 5 min and washed with distilled water.

Results

Sequence analysis of MjGCTL

The full cDNA sequence of MjGCTL was previously obtained and was submitted to DDBJ with the accession number LC127418. The total length was 1247 bp and the ORF was comprised of 945 nucleotide bases in length encoding 314 amino acid residues (Fig. 1), with a predicted molecular weight of 35 kDa. Analysis of protein domains revealed MjGCTL is

comprised of a signal peptide, low-density lipoprotein class A receptor (LDLa) domain, and a carbohydrate recognition domain (CRD). The predicted amino acid sequence of the CRD of MjGCTL has a QAP binding motif. The WIGL-like motif was also present as "WLDG" sequence. The MjGCTL amino acid sequence have 5 predicted N-glycosilation sites and 2 O-glycosylation sites. The predicted amino acid sequences of MjGCTL and LvCTLD have the same length and are very similar (Table 1) with a sequence identity of 77%. Blastp results from NCBi show 4 proteins with high identity to MjGCTL that also contain QAP-binding motif. Multiple alignment with these proteins sequences reveals 7 conserved Cys residues indicating a characteristic of a long-form CRD.

In a phylogenetic analysis of the amino acid sequences of shrimp C-type lectins and C-type lectin-like proteins (Fig. 3), MjGCTL clustered with other shrimp CTLs with QAP motifs with a 99.9 % bootstrap value. MjGCTL's CRD fold shares the long-form CRD with other WAP-motif containing CTLDcps.

CTL	Organism	Query cover	Identity %	Tissues	Binding Motif	Ligand/Ca ²⁺ - dependency	Function
LvCTLD	Litopenaeus vannamei	100%	77%	Gills, Nerve	QAP	Galactose, ? YHV proteins Ca ²⁺ -independent	Viral (YHV) recognition, encapsulation, PO activity
MnCTLDcp1	Machrobrachium nipponense	96%	42%	Heart	QAP	?	Bacterial agglutination
MnCTLDcp2	Machrobrachium nipponense	97%	39%	Heart	QAP	?	Bacterial agglutination
LdlrLec2	Marsupenaeus japonicus	94%	32%	Hepato- pancreas, Heart	QAP	?	Interact with WSSV VP28

Table 1. Blastp Resuts from NCBI: CTLDcps with high sequence identity with MjGC	TL.
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cccgcacgtccacgcccatcaccATGAAGGCAATCGTGCTTCTTCTGTGCATCGGCTTTG M K A I V L L L C I G F A CAACGGCCCTGGAATGCACGGGCGACGAAGTGGCGTGTGGGAGCGCCGAGCGATGCGTGC TALECTGDEVACGSAERCVP 241 CTTATCGGTACATCTGCGATTTCGATAGCGACTGCTCTGATGGGTCGGACGAGGATCCTT C D F D S D C S D G S D E D P Y YRYI ACCTCTGCTGGGCATGGAATAACACCGAGTGCGAGAGGGGCTCCGCGCAGTGCTTAACCA l C W A W <u>N N T E</u> C E R G S A Q C L T N 361 ACGGGCGGCAGAGTGCATCCCCATTGAGACTTACTGTCATCGTACTCAGCCTGCGTGCT G R A E C I P I E T Y C H R T Q P А C S CTGGGAGTCTTAACCGCCGAGTCTGCTCTATCATAGAAGACAAGAAGTTGGTGCCCCTCG G S L N R R V C S I I E D K K L V P L A CCTCAATTAAATTTATTCCGGATAATGAACCAGCTGATGCATACAACAGGAGCGTTAGCC SIKFIPDNEPADAY<u>NRSV</u>SL 541 TAGGCGCTGAACTGCGCACTAACCTAAACAACACTCTCAGCCATCCAGACTGTCCTGACT GAELRTNLNNTLSHPD<mark>CPDF</mark> 601 TCTATACCCGGGTTGGCGACCAGTGCCTCTCCGTTTTCTATGTTGGAAGATCGAGCTGGG Y T R V G D Q C L S V F Y V G R S S W G GCGAGGCACGGGCGTTCTGCAAGCACATAGGCGGCGATCTGCTCAGCATCCAAAATGCCA EARAFCKHIGGDLLSIQ<u>NAS</u> 721 GCCATTATATCGATCTTGTCAACCATTTATCGGAGAACCAGATTACCAGCGACTTCTGGC HYIDLVNHLSENQITSDFWL TTGGCGGGAGATACGAACTGGACGACCTGAGTTGGATGTGGCTTGATGGAACACCGATGC G G R Y E L D D L S W M W L D G T P M P CACAAGGCACGCCCTTCTGGAGTCTCAGGCGGTACCACCATTGCGACACCCGGAACGTGA QGTPFWSLRRYHHCDTR<u>NVT</u> 901 CGGTGGCGGGAACCTATCAGGTGCTAGAAGCGAACAACGGCGAGTGCTACCATTACACGC V A G T Y Q V L E A N N G E C Y H Y T Q AGGCGCCCGAAGACCCTCCCAGGGGTTTCTGCGCCGCCATTACCTACGGCAAACACTTC A P E D P P R G F C A A I T Y G K H F Y MSDEDCLADMSPLCVTSV* 1081 taactctgtttttttctctgtaaaacatcagcaattaacatgtgggactaaaaggcttgg $1141 \quad taa aatgtaaccggttttctacgaaggaaggcaatgcgttagtatgtgttgttttcgtctt$ gatgtggagtgaccgatcctatgtttgtctgtgcacgctgtaccttc

Fig. 1. Sequence analysis of MjGCTL. Complete nucleotide cDNA and deduced amino acid sequences of MjGCTL. Signal peptide is bold-faced, Low-density lipoprotein receptor class A (LDLa) domain is enclosed in a dotted box, the carbohydrate recognition domain is highlighted with its WIGL-kike and QAP binding motif designated by a solid box. N-glycosylation sites are underlined and O-glycosylation sites are encircled.

Nécom		10 	20 	30 	40 	50 	60
MJGCIL LUCTED AFH05998	PDFITF	VGCOCLSVF	VUCSSSWCE	ARAFCAHIG	CDLLSIQ-NASI	IYUDI WNHLS	IDNRTT
Mn CTLDcp1 AK022256	POLYTE	IGDOCVSVF	FIGNLTWLES	SOAFCOAIG	GELFVVKSDLSI	VIALLOHFN	IDNOVT
Mn CTLDcp2 ALF45198	PNLYTE	VGDICVSLF	FIGNMTWGE	NOFCEVIG	GKLFTPE-KSEI	LDILGRHMF	EHPIS
Mj_ldlrLec2_AHA83583	PMLYTE	VGGKCLAFF	SLAKVPWPE	ARQFCKSIY	GDLVTFK-NLQI	FGHVIEYL	TLIT
MjGCTL	SDFWLGO	RYELDDLSW	MW-LDGTPMI	PQGTPFWSL	RRYHHCDTRN	-VTVAGTYQV	LEANN
LvCTLD_AEH05998	SDFWLGO	RYEVDDLSW	TW-LDGTPMI	RGTPFWSL	RRYDSCNPRN	-VTLTGTSEV	REANN
Mn CTLDcp1 AKQ22256	KDFWIGO	MYVNSSTGW	QW-TDERSIG	QLGAPIWAV	RHTPVCSTRV	-VTSPSFNV1	REAND
Mn CTLDcp2 ALF45198	SDFWIGG	NFYNNTHGW	RW-TDGRAM	LSTYHWAL	RHDSTCYQRE	-LNWEHRNVF	VDAND
Mj_ldlrLec2_AHA83583	SDFWIGO	RYDMDVNAW	AWAIDDTPM	PLGSPYWAE	RYSSSCVPRSPI	PHTDPFSNPF	SALPE
MjGCTL LvCTLD_AEH05998 Mn_CTLDcpl_AKQ22256 Mn_CTLDcp2_ALF45198 Mj_ldlrLec2_AHA83583	GECYHYI GECYHYI GICYHYR GVCYSYF APCYNYI	QAP-EDPPR QAP-ETPPK QAP-SEPLT QAPWVSNPY QAP-KQRNQ	GFCAAITYGH GFCAAITYDH GNCLAITYEH GSCVAMTYKH GWCSAITYEH	KHFYMSDED KHFYMSDED HYYFASDQE RFYYLSDED HFYFISDDE	CLADMSPLCV CLADMSPLCV CWTRKSPLCV CGQKKSPLCKIV CONSRSPLCLL	/KA	

Fig.2. Amino acid alignment of QAP-motif containing CTLDcps highlighting the conserved Cys residues and QAP bindng motif.



Fig. 3. Phylogenetic analysis of shrimp CTLDcps carbohydrate recognition domains (CRDs) using Neighbhor-joining methods with 10000 replicates. MjGCTL is represented with a (•). Each CTLDcps are labeled with their sugar-binding motifs. *Protein 3D-modeling of CRD fold*

The 3D-structure of MjGCTL CRD corroborates the sequence analysis showing

MjGCTL possess a long-form of CRD (Fig. 4A). Like the usual long-form CRD of CTLs, the

extra 2 Cys (C) residues (C0 & C0') stabilizes an auxiliary anti-parallel β -plated sheet on the 5'-end of the fold. The whole fold is stabilized by C 1-5 forming a disulfide bridge between α 1-helix and β 5-sheet, while the long-loop region is stabilized by C 2-3 at β 2- β 2 sheet. The tripeptide QAP binding motif is found in β 3-sheet co-localized with the predicted calcium-binding site. The comparison of MjGCTL and LvCTLD CRD fold predicted by Swiss Model is shown in Fig. 4B. The z-scores 19.637 and 19.2970, respectively, indicate good matches to the template (bovine mincle). Black arrows indicate the locations of the CRDs and green spheres indicate the locations of the bound calcium atoms predicted by Swiss Model. Note that the CRD of MjGCTL is co-located with the calcium-binding site while the CRD of LvCTLD is not near calcium-binding site (Fig. 4B).



Fig. 4A. Predicted 3D-model of MjGCTL by Swiss Model using template C-type lectin mincle (PDB 4KZVA). Representations of alpha helix (α) beta-plated sheets (β) are colored yellow and blue, respectiviely, and are numbered according order from 5'-3' direction. Cysteine (C) residues forming disulfide (S-S) brideges are designated by the magenta color. QAP motif is represented by the red color and the calcium-binding site by the green sphere.



Fig. 4B. Comparison of predicted 3D-model of the CRD of LvCTLD (AEH05998) and MjGCTL by Swiss Model using template C-type lectin mincle (PDB 4KZVA). Alpha helix (α) betaplated sheets (β) are colored yellow and blue, respectiviely. QAP motif is represented by the red color and the calcium-binding site by the green sphere. Differences in the location of QAP are indicated by an arrow for both CRDs.

MjGCTL protein and tissue distribution

Determined in a previous study, MjGCTL transcripts were expressed only in gill and stomach tissues of *M. japonicus*, with expression being strongest in the gills. The protein expression was similar, MjGCTL transcripts were only detected in gills and stomach not detected in hepatopancreas, lymphoid organ, hemocyte, muscle, heart intestine, nerve and eye (Fig. 5A & B). In addition, MjGCTL was likewise detected in the gill-mucus.

Successfully purified eluted rMjGCTL from S2 cells subjected to SDS-PAGE and western blot analysis, which revealed its actual size at about 50 kDa (Fig. 5B). Subtracting the size of the extra residues and V5 – His epitope, the actual size of rMjGCTL is about 43 kDa, which is the same size as the detected native MjGCTL from the gill tissues.



kDa rMjGCTL Gill tissues

В

Fig. 5. MjGCTL protein detected by anti-MjGCTL rabbit serum antibody showing (A) MjGCTL's tissue distribution by western blot analysis (a) with SDS-PAGE as loading control (b). (B) Western blot analysis comparing recombinant (r) MjGCTL and native MjGCTL.

Discussion

invertebrate CTLDcps are more diverse both in structure and in function as compared to the vertebrate CTLDcps, where it has been speculated that this diversification appears to be a way of compensating for the lack of the adaptive immune mechanisms such as specificity in pathogen recognition (Zelensky and Gready, 2005). To understanding this mechanism of invertebrate immune system, there is a need to investigate of the diversity of the CTLDcps and their potential to mediate immune specificity, however only a few invertebrate CTLDcps have been characterized.

The classification of CTLs can be done by using their sequence information, identifying their CRDs as mannose- or galactose-binding, which is based on the signature EPN motif or QPD motif, respectively. The protein blast revealed four other CTLDcps that are highly identical to MjGCTL (Table 1), and among them LvCTLD (Junkunlo et al., 2011) shares the highest identity (77%) and is the only one whose sugar binding specificity has been identified, which prompted us to use LvCTLD as a reference to predict MjGCTL's possible biological function. As shown in Fig. 1, MjGCTL possessed a QAP motif, and among shrimp CTLDcps LvCTLD (Junkunlo et al., 2011), MjLdlrLec 1 & 2 (Xu et al., 2014), and MnCTLDcp 1 & 2 (Xiu et al., 2015) possessed QAP motif and shares the same protein domains, with 7 conserved cysteine residues with MjGCTL (Fig. 2). In the phylogenetic analysis comparing the shrimp CRDs (Fig. 3), MjGCTL was clustered closely with these other CTLDcps containing a QAP binding motif. The report on LvCTLD claimed it to have an affinity to galactose, suggesting QAP has the same binding-specificity as QPD implying QAP is a mutated variant QPD motif (Junkunlo et al., 2011). However, in this study, the phylogenetic analysis shows the QAP motif cluster appears to be form a different group from CRDs with

QPD and EPN motifs as well as their mutants (QPT, EPS, etc.) suggesting that QAP is possibly an entirely different motif.

In case of the invertebrate CTLDcps, it is not unusual that each CTLDcps have their own unique individual binding-specificity as some has been reported to have overlapping and divergent sugar-binding specificity of some binding motifs of CTLs (Lee et al., 2011). Although most variants of the EPN and QPD binding motifs (e.g. EPD, QPT, etc.) were previously identified and reported to possess a common binding-specificity with the original motifs EPN (mannose) and QPD (galactose), there were others that do not (Lee et al., 2011). Moreover, some of the CTLDcps with and without the variant motifs, have overlapping sugar-binding specificity (Lee et al., 2011). Some even switched their specificity, as in the case of MjLecB's CRD possess a QPT motif believed to have mutated from QPD, however binds to mannose (Song et al., 2010). In this study, the comparison MjGCTL with LvCTLD (Junkunlo et al., 2011) demonstrated even that two highly identical CTLDcps with the same sugar-binding motif can possess a different sugar-binding specificity. Furthermore, despite their high identity, MjGCTL is a C-type lectin (calcium-dependent), while LvCTLD is a C-type lectin-like (calcium-independent and binds to proteins). Through the generated 3D models of MjGCTL and LvCTLD (Fig. 4A & B) suggests an insight to why MjGCTL is calcium-dependent, where unlike LvCTLD, through the co-location of the predicted calcium-binding site and the QAP motif. This is in accord with the structures of classical C-type lectins, in which calcium-binding site is coupled with their sugar-binding motif in the CRD (Zelensky and Gready, 2003). This is another proof of the diversification of CTLDcps among invertebrates as represented by shrimp model, suggesting classification by function shall be more determinative.

All reported CTLDcps with QAP binding motifs are only found in shrimps, including MjGCTL, all containing a low-density lipoprotein receptor class A (LDLa) domain. Lowdensity lipoprotein receptors (LDLRs) are cell surface receptors found in both vertebrates and invertebrates, and are receptors attributed to the binding of calcium and LDLs (Chang et al., 1998). In shrimp, the reported CTLDcps with an LDLa domain interact with viral particles. LdlrLec1 & LdlrLec2 interact with the VP28 envelope protein of WSSV and were upregulated upon WSSV infection (Xiu et al., 2015) and LvCTLD interacts with YHV particles (Junkunlo et al., 2011). In each case, the binding and interaction was attributed to the LDLa domains. However, in a previous study on the mRNA expression of MjGCTL (data not shown), its transcript level remained unchanged after WSSV infection, possibly because the LDLa domain does not interact with WSSV particles. Furthermore, shrimp CTLDs not containing LDLa were found to bind with viral particles or change their mRNA transcripts in respond to viral infection such as the PmAV (Luo et al., 2003; Luo et al., 2007), LvCTL1 (Zhao et al., 2009), FcLec3 (Wang et al., 2009), MjLecA, B, & C (Song et al., 2010), LvCTL-br1 and br2 (Costa et al., 2011) thus suggesting that MjGCTL's LDLa domain does not necessarily imply the molecule's binding or responding to viral particles, which is a characteristic of Ctype lectin-like molecules. Thus, it can be possible that in case of CTLs LDLRs serves as calcium-binding site, as they have been known to sequester and regulate calcium levels (Nykjaer and Willnow, 2002). However, the unchanged mRNA levels also do not mean MjGCTL does not respond to invading pathogens. This is shown as in the case of MjHeCL, a CTL highly expressed in hemocytes, that although it maintained its mRNA expression level even after bacterial challenge knock-down of this gene caused the pathogenic bacteria proliferation in hemolymph (Wang et al., 2014). It is possible that just like MjHeCL, MjGCTL is constantly expressed in high levels as readily available defense molecules of the shrimp immune system.

Diversification of shrimp CTLDcps are manifested in their tissue or organ of localization, domain architecture, and sugar-binding capability (Wang and Wang, 2013). In this study, we identified and characterized a new C-type lectin (CTL) gene from *Marsupenaeus japonicus* whose mRNA expression was previously determined to be localized to the gill and stomach (data not shown). And in this study we confirm this through the protein tissue distribution which detected MjGCTL protein in gill and stomach with the addition of gill mucus (Fig. 5A), which suggest MjGCTL is a secreted protein which may have an immune function on the gill mucus. The localization of MjGCTL to both gills and stomach may suggest MjGCTL reside in the main entry sites and target organs of major shrimp pathogens (Clavero-salas et al., 2007; Chang et al., 1998; Wu at al., 2001; Lotz and Soto, 2002). MjGCTL's localization supports our hypothesis regarding its special function on its tissue of localization, as most invertebrate lectins are highly expressed in hepatopancreas or hemocyte making the CTL systemic in its functional site.

The diversity of shrimp CTLDs as seen in the results of phylogenetic analysis and the presence of numerous motif variants and their affinity to different carbohydrates, points out with these results, together with the variable affinity among CTLDs with other carbohydrates other than mannose or galactose, it is very tempting to make an inference that QAP is an entirely new binding motif, and that each CTLD have their unique binding specificity regardless of the sequence of its binding motif. Here we demonstrate the glycosylation of CTLDs and their proper folding is essential for their affinity to carbohydrates (Pees et al., 2016). This was confirmed by the rMjGCTL from *Drosphila* S2 cells that detected protein size of 50 kDa. However, deducting the added residues (5.4 kDa)

from the restriction sites and tagging on the vector, rMjGCTL is around 45 kDa in size, which was confirmed by the detection of the native MjGCTL protein on the gill tissues which is also aroun 45 kDa. The increase in the detected protein size of MjGCTL from the predicted size based on its sequence can be attributed to the PTMs that may have occurred. Posttranslational modifications are most likely to have occurred as supported by the presence 5 N-glycosylation and 2 O-glycosylation prediction sites in the amino acid sequences predicted in MjGCTL. Interestingly, in the construction of 3D protein model structure of MjGCTL CRD, the found template, searched bv MUSTER (http://zhanglab.ccmb.med.umich.edu) is a bovine C-type lectin mincle that possess a CRD coupled with calcium that binds to one glucose residue of trehalose Gla1-1Glca headgroup in the manner of classical C-type lectin molecules (Feinberg et al., 2013). Although QAP binding motif is not possessed by the template bovine mincle, the binding of MjGCTL to glucose and its similarity to the structural configuration a glucose-binding CTL confirms the results of MjGCTL specificity to glucose molecule, and that the specificity may depend more in the unique folding and glycosylation of each CTL rather than solely the binding motif.

In summary, this study provides yet another evidence of the complexity and diversity of invertebrate CTLDs through the report of a novel C-type lectin gene MjGCTL, which is a classical C-type lectin that binds to carbohydrates in a calcium-dependent manner, and is capable of bacterial agglutination and opsonization, facilitating encapsulation by the hemocytes. MjGCTL's QAP binding motif was confirmed to have different sugar specificity than that of EPN/QPD-containing C-type CRDs. The finding that MjGCTL is structurally very similar to LvCTLD yet possesses a different function clearly demonstrates that members of the C-type lectin superfamily can be more accurately classified by their function rather than solely by the binding motif they possess. The

divergent binding-motif MjGCTL provides an insight on how invertebrate immune system possess unique and highly specific pathogen recognition receptors through the CTLDcps.

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CHAPTER 3

in vitro characterization of MjGCTL

Abstract

The C-type lectin domain containing proteins (CTLDcps) provide the shrimp immune system with some level of specificity when it comes to pathogen recognition, different CTLDcps recognize different type of pathogen. The recognition of PAMPs by CTLs depend greatly on their ability to bind to different ligands, either carbohydrate or other moieties, depending on its classification. Here, recombinant MjGCTL (rMjGCTL) was used it for the classification of MjGCTL and determination of its function as a PRR in the shrimp immune system. The bacterial agglutination assay demonstrated MjGCTL is a calcium-dependent agglutinating capability against both Gram-negative and Gram-positive bacteria. Through agglutination inhibition using carbohydrates, the affinity of MjGCTL with various carbohydrates was determined, where the MjGCTL binding did not involve either mannose or galactose. In an in vitro encapsulation assay, agarose beads coated with rMjGCTL were immediately encapsulated by hemocytes at 0 h (30 min) and melanization at 4 h postincubation with hemocytes, as compared to the negative controls where no encapsulation were observed. Furthermore, encapsulation of hemocytes were also inhibited by the carbohydrate ligands (glucose and fucose) of rMjGCTL, but not by mannose and galactose. These results confirm that MjGCTL is a classical C-type lectin that can bind to bacteria and hemocytes and act as an opsonin. Furthermore, MjGCTL's CRD QAP binding-motif's carbohydrate-specificity was found to be different from that of mannose and galactosebinding, EPN and QPD CTLDcps, respectively. This suggesting QAP may be a new motif with a different specificity, or that it has lost its affinity to either galactose or mannose.

Introduction

The shrimp immune system, relies solely on the primitive innate immune response that includes humoral and cellular responses for their defense against infectious agents (Maningas et al., 2013; Amparyup et al., 2012; Hanington et al., 2010; Loker et al., 2004; Vasta et al., 2004). Not having the antibody-mediated specificity of adaptive immune system, there is a notion that the immune response of the shrimp is somewhat non-specific. However, there are evidences that the invertebrate immune system can discriminate between pathogen at species and even strain level, which refutes the notion that invertebrate innate immune system is naïve in every infection even with the same pathogen (Pees et al., 2016; Little et al., 2003; Roth et al., 2009; Sadd and Schmid-Hempel, 2006). At present, there is a growing interest in invertebrate immune research in unraveling its secrets on how it generates high levels of specificity without the antibody-, T cell- and B cell-mediated immune response (Pees et al., 2016). The key to understanding such phenomena to investigate the prerequisite of having a pathogen-specific immune responses, which is the diversified array of pathogen recognition receptors (PRRs) recognizing and initiating the necessary immune response through the pathogenassociated molecular patterns (PAMPs) (Cambi et al., 2005; Wang and Wang, 2013). Upon recognition of PAMPs, PRRs will trigger a cascade of immune responses such as agglutination, encapsulation, nodulation, phagocytosis, the release of antimicrobial peptides (AMPs), and the activation of the pro-phenoloxidase (ProPO) system leading to melanization, all of which promote the degradation and clearance of pathogens

(Tassanakajon et al., 2013; Rabinovich et al., 2012; Sun et al., 2008; Wang et al., 2009; Watanabe et al., 2006; Yu and Kanost, 2000; Wang et al., 2014; Schnitger et al., 2009). The shrimp immune system has 11 PRRs (20) and among these are the galectins, β -1,3-glycan-binding proteins (BGBPs), β -1,3-glycanase-related proteins (BGRPs), and C-type lectins (CTLs), specifically bind to cell surface carbohydrates of pathogens (Wang and Wang, 2013a; Wang and Wang, 2013b).

Among the PRRs in shrimp, CTLDcps are the largest and most diversified group binding to a wide array of PAMPs (Wang and Wang, 2013b). It is believed that the diversification of this superfamily among invertebrates is a way for the immune system to compensate the lack of specialized antibodies that can render a highly specific immune recognition and response. Thus, it is important characterize shrimp CTLDcps and identify their specific ligands and the immune reactions they mediate. Here, through the recombinant protein technology we identify MjGCTL's affinity to various carbohydrate ligands and bacteria, and its involvement in some immune reaction.

Materials and Methods

Bacterial agglutination assay

Bacterial agglutination assay was performed following the method of Luo et al. (2006) and Ma et al. (2008) with some modifications. The bacteria used in the assay were two Gram-negative species: *Vibrio parahaemolyticus* and EGFP-expressing *Escherichia coli* and one Gram-positive species, *Streptococcus agalactiae*. Bacteria were re-suspended in TBS^{Ca} (Tris-HCl, pH 7, 100mM NaCl, and 10mM CaCl2) at 1 x 10⁹ cells/ml. Ten microliters of bacteria suspension was then incubated with same amount rMjGCTL (50 mM) at room temperature (25°C) for 1 h. Agglutination were then viewed under light microscope (Nikon)

and fluorescence microscopy for GFP-expressing *E. coli*. The minimum concentration of rMjGCTL causes agglutination was determined with serial dilutions of rMjGCTL in TBS^{Ca} (10mM) and was used in agglutination assay as mentioned above. To test the calcium dependency of rMjGCTL, agglutination was assayed using only TBS (without CaCl₂) and also by adding a chelating agent EDTA with final concentration of 10mM into TBS.

Agglutination inhibition assay of carbohydrates

The sugar specificity of rMjGCTL was expressed as the minimum concentration of a sugar needed to inhibit agglutination. rMjGCTL was incubated with different concentrations of several carbohydrates for 1 h at room temperature prior to the agglutination assay. All assays were done in triplicate.

in vitro encapsulation assay

The ability of MjGCTL to encapsulate and melanize hemocytes was measured in vitro with nickel-NTA agarose beads incubated with shrimp hemocytes (16). The assay was done with both rMjGCTL-coated and non-coated beads. BSA-coated beads and uncoated beads in TNS buffer were used as positive and negative controls, respectively. Prior to adding the hemocytes, the beads were washed three times with 1 ml TNS buffer (10mM Tris-HCl, 140mM NaCl, 20mM CaCl₂, pH 7.9), incubated with 1 ml of His-tagged rMjGCTL or BSA (300 μ M) in TNS overnight at 4 °C with gentle rotation, washed five times with 1 ml TNS and resuspended in 1 ml TNS as a 50% slurry. Hemolymph was collected from *M. japonicus* with a 23-gauge needle and syringe from the second abdominal segment and immediately mixed with 1.5 ml anticoagulant solution Kuruma shrimp PBS (KPBS) (480mM NaCl, 2.7mM KCl, 8.1mM (Na₂HPO₄) 12H₂O, 1.47mM KH₂PO₄, pH 7.6) containing 10 mM EDTA. The

suspension was centrifuged at 600 x g for 10 min at room temperature. The pelleted hemocytes were washed three times with PBS and re-suspended in 1 ml 2x Leibovitz's L-15 medium (Invitrogen, USA). Encapsulation was assayed by adding 10 μ l of the hemocyte suspension to the wells of a 24-well cell culture plate coated with 500 μ l 1% agarose. One μ l of the beads slurry (incubated with rMjGCTL or BSA) was added to each well. Beads suspended in TNS buffer were used as a negative control. Encapsulation by hemocytes was then observed at 0, 2, 4 and 8 h post incubation (hpi) under a light microscope (Nikon).

Hemocyte encapsulation inhibition by carbohydrates

To identify if the opsonization can be attributed to the carbohydrate binding ability of MjGCTL and to confirm its affinity (or lack thereof) to mannose, galactose, glucose, and fucose based on the results of agglutination inhibition assay (Table 2), these carbohydrates (at 100mM final concentration) were incubated with the bound rMjGCTL slurry prior to performing the encapsulation assay as mentioned above. Experimentally added carbohydrates with affinity to MjGCTL will compete with that of the carbohydrate substrates of hemocytes therefore disrupting the encapsulation of the agarose beads. All experiments were done in triplicate and results were then viewed at 8 hpi.

Results

Bacterial agglutination assay

rMjGCTL agglutinated both Gram-negative and Gram-positive bacteria, *Vibrio parahaemolyticus* and *Streptococcus agalactiae*, respectively (Fig. 1). EGFP-expressing *E. coli* was also used for easier visualization, which rMjGCTL also agglutinated in a concentration-dependent manner.



Fig. 1 MjGCTL agglutination activity against *Streptococcus agalactiae* and *Vibrio parahaemolyticus* compared with control BSA and TBS-calcium. Scale bar represents 20 μm.

Calcium-dependent carbohydrate binding and specificity of MjGCTL

rMjGCTL's ability to agglutinate fluorescent (EGFP-expressing) *E. coli* was inhibited by the removal of calcium and by the addition of EDTA to the solution (Fig. 2A), suggesting the calcium-dependency of rMjGCTL's agglutinating activity. Bacterial agglutination inhibition assays using different carbohydrates revealed that mannose and galactose did not inhibit rMjGCTL's ability to agglutinate *V. parahaemolyticus* but that glucose did inhibit the agglutination (Fig. 2B). Agglutination of *V. parahaemolyticus* was also inhibited by the disaccharides lactose and maltose, by N-acetyl-D-glucosamine, and by the bacterial cell surface components lipopolysaccharide and peptidoglycan in varying concentrations (Table 1).

Saccharides	Minimum inhibitory
	concentration
D-Galactose	NI*
D-Glucose	100mM
D-Mannose	NI*
Xylose	62.5mM
Fucose	250mM
N-Acetyl-D-galactosamine	NI*
N-Acetyl-D-glucosamine	250mM
Lactose	250mM
Maltose	125mM
Sucrose	NI*
Lipopolysaccharide	62.5µg/mL
Peptidoglycan	6.5µg/mL
* Not inhibited at 500mM	

Table 1. Carbohydrate specificity and minimum inhibitory agglutination concentration of carbohydrates and bacterial components against MjGCTL.



Fig. 2 Calcium-dependent bacterial agglutination with EGFP-expressing *Escherichia coli* as influenced by the presence (TBS^{Ca}) and absence (TBS⁽⁻⁾) of Ca²⁺ and the addition of a chelating agent EDTA (A). Agglutination inhibition by monosaccharaides mannose, galactose, and glucose (B). Scale bar represents 10 μ m.

rMjGCTL's opsonic effect enhance hemocyte encapsulation

To see if rMjGCTL can act as an opsonin, rMjGCTL-coated agarose beads were incubated with *Marsupenaeus japonicus* hemocytes. Hemocytes bound to the beads immediately after they were added (0 hours post incubation (hpi)) and the binding increased from 2, 4, to 8 hpi (Fig. 3). On the other hand, no binding was observed with BSA-coated beads (protein control) or uncoated beads in TNS buffer (negative control). Melanization (indicated by the brown pigmentation) also increased with incubation time for rMjGCTL-coated beads but not in the protein and negative controls. Results of hemocyte encapsulation inhibition by carbohydrates demonstrated that the opsonic effect that facilitated encapsulation is the CRD or the carbohydrate binding ability of MjGCTL, where glucose and fucose were able to inhibit encapsulation (Fig. 4). Likewise, this assays confirms MjGCTL's preferential binding to glucose and fucose and not to mannose and galactose.



Fig. 3. MjGCTL facilitates *M. japonicus* **hemocytes encapsulation** of Ni-NTA agarose beads. Incubated with rMjGCTL, BSA (protein control), and TNS buffer (negative control) demonstrate encapsulation by hemocyte after 0, 2, 4 and 8 hours post-incubation (hpi).



Fig. 4 Opsonic effect of rMjGCTL for hemocyte encapsulation is attributed to MjGCTL's carbohydrate-binding activity. The effect of incubating mannose, galactose, glucose, and fucose to agarose beads bound to rMjGCTL *from Drosophila* S2 cells after 8 hpi.

Discussion

In this chapter, we demonstrate MjGCTL is a classical C-type lectin with the ability to act as a PRR capable of binding to carbohydrate ligands which promoting bacterial agglutination in calcium-dependent manner, and promoting encapsulation by shrimp hemocytes that lead to melanization. As shown in Fig. 1, MjGCTL is able to agglutinate both Gram-positive and Gram-negative bacteria, and its calcium dependency is shown in Fig. 2A. CTLs are conventionally classified as mannose- or galactose-binding, however, MjGCTL does not bind to either of these sugars as shown in Fig. 2B. The results of the phylogenetic analysis in the previous chapter of MjGCTL's CRD is corroborated with the sugar-bindng specificity where rMjGCTL have no affinity to neither mannose nor galactose, contrary to the previous report on QAP binding-motif's specificity in LvCTLD (Junkunlo et al., 2011). The carbohydrate substrates of MjGCTL listed in Table 1 shows MjGCTL is neither mannose- nor galactose-binding, suggesting a completely different specificity from CTLs with EPN and QPD motif which maybe another evidence of diversification of CTLDcps molecules which displays its potential for generating specificity in pathogen recognition. If we refer to the affinity of QPD and EPN motif based on X-ray crystallographic analysis, QPD motif has an affinity for equatorial/axial 3-OH/4-OH configuration, while EPN equatorial/equatorial 3-OH/4-OH of carbohydrate residues (Kolatkar and Weis, 1996; Ng et al., 1996). The former indicate QPD tends to bind to galactose and N-Acetylgalactosamine, while the EPN to mannose, glucose, N-Acetylglucosamine, and L-fucose (Kolatkar and Weis, 1996; Ng et al., 1996; Lee et al., 2011). As seen in Table 1, among the latter, only mannose does not bind to MjGCTL, suggesting QAP motif has more similar binding specificity to EPN than QPD, corroborating that QAP is a completely different motif from QPD with a different specificity.

Another evidence of each CTLDcps having their own unique individual bindingspecificity is the overlapping and divergent sugar-binding specificity of some binding motifs of CTLs (Lee et al., 2011). For instance, variants of the EPN and QPD binding motifs (e.g. EPD, QPT, etc.) were previously identified and reported to possess a common bindingspecificity with the original motifs EPN (mannose) and QPD (galactose), while others do not. Some of these CTLs with variant motifs, even with those with EPN/QPD motifs, have overlapping sugar-binding specificity (Lee et al., 2011). Moreover, some switched their specificity, as in the case of MjLecB's CRD possess a QPT motif believed to have mutated from QPD, however binds to mannose (Song et al., 2010). In this study, our analysis comparing MjGCTL with LvCTLD (Junkunlo et al., 2011) demonstrate that two highly identical CTLDcps with the same sugar-binding motif can possess a different sugar-binding specificity. Furthermore, despite their high identity MjGCTL is a C-type lectin (binds to carbohydrates), while LvCTLD is a C-type lectin-*like* (binds to other moieties).

Establishing the difference between CTLs and CTLDs and the accurate use of terminologies facilitate the proper characterization of these molecules, preventing any overlapping or contrasting in the data on CTLDcps. Among the CTLDcps with QAP motif, apart from MjGCTL, MnCTLDcp 1 and 2 (Xiu et al., 2015) are actually CTLs named as CTLDs,

which may a source of confusion in characterizing. As MjGCTL is a classical C-type lectin, its functions are also that of CTLs binding to carbohydrates and bacterial components, which cause agglutination, all in a calcium-dependent manner. The ability of MjGCTL to bind to lipopolysaccharide and peptidoglycan (Table 1) may explain its ability to agglutinate both Gram-negative and Gram-positive bacteria. MjGCTL can also act as an opsonin, facilitating encapsulation as demonstrated by the encapsulation of agarose beads incubated with rMjGCTL (Fig. 3), which was inhibited when carbohydrate substrates were initially bound to the ligand (Fig. 4). Despite the high identity, LvCTLD only share one function with MjGCTL, which is understandable as CTLDs are non-carbohydrate binding. However, is somewhat odd that LvCTLD have carbohydrate specificity for galactose, as CTLDs are not lectins. Between CTLs and CTLDs, carbohydrate-binding specificity is more of a characteristic of CTLs, as it is more fitting for MjGCTL to have affinity to carbohydrates than LvCTLD. This comparison of MjGCTL and LvCTLD therefore show clearly the demarcation of CTLs and CTLDs, and thus provide a good reference for characterization of the growing number of the diverse superfamily of CTLDcps.

Interestingly, in the construction of 3D protein model structure of MjGCTL CRD, the found template, searched by MUSTER (http://zhanglab.ccmb.med.umich.edu) is a bovine C-type lectin mincle that possess a CRD coupled with Ca²⁺ that binds to one glucose residue of trehalose Gla1-1Glca headgroup in the manner of classical C-type lectin molecules (Feinberg et al., 2013). Although QAP binding motif is not possessed by the template bovine mincle, the binding of MjGCTL to glucose and its similarity to the structural configuration a glucose-binding CTL confirms the results of MjGCTL specificity to glucose molecule, and that the specificity may depend more in the unique folding and glycosylation of each CTL rather than solely the binding motif.

In summary, this study provides yet another evidence of the complexity and diversity of invertebrate CTLDs through the report of a novel C-type lectin gene MjGCTL, which is a classical C-type lectin that binds to carbohydrates in a Ca²⁺-dependent manner, and is capable of bacterial agglutination and opsonization, facilitating encapsulation by the hemocytes. MjGCTL's QAP binding motif was confirmed to have different sugar specificity than that of EPN/QPD-containing C-type CRDs. The finding that MjGCTL is structurally very similar to LvCTLD yet possesses a different function clearly demonstrates that members of the C-type lectin superfamily can be more accurately classified by their function rather than solely by the binding motif they possess. The divergent binding-motif and specificity of MjGCTL provides an insight on how invertebrate immune system possess unique and highly specific pathogen recognition receptors through the CTLDcps.

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CHAPTER 4

in vivo function of *MjGCTL* and the immune defense of shrimp gills

Abstract

Shrimp gill is a semi-permeable organ that maintains contact between internal and external environment, functioning in gas and ion exchange, filtering out harmful biotic and abiotic factors. To the shrimp immune system, gills are known to merely mechanically assist the hemocytes through the removal of trapped foreign materials during molting. Microarray analysis on immune-related organs revealed biodefense genes that are highly expressed and some being specifically localized in gill tissues, where one of these genes is MjGCTL. Here, we demonstrate that gills are also equipped with immune molecules such as MjGCTL. Predicted to be a secreted protein, MjGCTL was hypothesized to be secreted on the gill surface mucous. Supporting this hypothesis are our findings that the gill mucus was also observed to cause bacterial agglutination like the recombinant (r)MjGCTL, and through the detection of MjGCTL by Western blot in gill mucus. Evidence confirming that this agglutination activity is caused by MjGCTL was demonstrated by the inhibition of agglutination ability of gill mucus upon neutralizing MjGCTL with its specific antibody, and the decrease in agglutinating ability upon knockdown of MjGCTLS. Using lactose-agarose beads, MjGCTL was purified from gills, where similar protein functions with rMjGCTL were observed in the purified MjGCTL protein. Phagocytosis assay using flow-cytometry with PKH67-labelled Streptococcus agalactiae revealed that MjGCTL can act as an opsonin, increasing the phagocytic rate of shrimp hemocytes. *In vivo* functional analysis of MjGCTL was done by knockdown of MjGCTL by RNAi followed by challenge test by immersion using a low bacterial concentration. Results showed MjGCTL-knocked down shrimp are more vulnerable to infection reducing survival to 20% and 0% at 7 day post-infection on the first and second challenge test, respectiviely, in contrast to ds. Also, significant increase in bacterial load from 6 to 12 hours post-immersion with *V. parahaemolyticus* was observed in both gills in hemocytes of MjGCTL-silenced shrimp. These evidences suggest that shrimp gill is not only physical, but also a biochemical barriers lined with immune molecules, with MjGCTL as an example.

Introduction

Gills of penaeid shrimp are multifunctional tissues performing key roles both in physiological and immunological processes. While acting as a physical barrier, separating internal and external environment, gills are also in-charge of processes maintaining homeostasis such as respiration, ion-exchange and osmoregulation (Cieluch et al., 2007). Gills likewise participate in the immune defense, giving mechanical aid to hemocytes, trapping phagocytosed materials and other harmful biotic and abiotic factors, then forming nodules that are extruded during molting (Martin et al., 200). As gills maintains in-contact with the external environment it is made vulnerable to structural damage and the primary target entry site for pathogens (Rattanarojpong et al., 2007). Prominent shrimp pathogens, such as White spot syndrome virus, Taura syndrome virus, and *Vibrio* spp., are known to have the gills as their target organs (Clavero-Salas, 2007). Several studies on *Vibrio* infection on shrimp have established gills as the main infection route (Alday-Sanz et al., 2002) demonstrated that *V. vulnificus* infection on *Penaeus monodon*, regardless of bacterial entry site, infection route converges to a common pathway which leads to the gill tissues (Alday-Sanz et al., 2002). Studies on Vibrio clearance in penaeid shrimp indicated that gills and hepatopancreas were the main site of accumulation of culturable bacteria (Alday-Sanz et al., 2002; Burgents et al., 2005a; Burgents et al., 2005b; Martin et al., 1993). Unlike the hepatopancreas, the gills do not have fixed phagocytic cells capable of eliminating bacteria (Johnson, 1987). Together with other foreign materials, nodules formed by hemocytes and phagocytosed material increase in size and may impair blood flow and normal gill function such as respiration and ion regulation (Martin et al., 200). Thus, it is intriguing how the gills manage both penetrating and accumulating bacteria in both internal and internal surface.

Present knowledge on the immune defense of gills suggests that gills are dependent on hemocyte activity, where gills are limited to aiding the hemocytes in a purely mechanical fashion such as the trapping of nodules formed by phagocytosed or degraded materials which is extruded during molting. There are also limited known gill-specific immune molecules expressed by the gills alone to protect itself from pathogens. Even the penaeidin reported to be detected in the cuticular tissue in gills of *Litopenaeus vannamei*, were proven to be synthesized by hemocytes and released to the plasma which infiltrate the gill filaments (Destoumieux, 2000). Nonetheless, phagocytosis and other immune response by hemocytes have long been known to be aided by lectins acting as opsonins labeling foreign materials that may enter the blood circulation (Söderhäll and Cerenius, 1992; Wand and Wang, 2013a; Yu and Kanost, 2004; Yu and Kanost, 2000; Yu et al., 2006). Invertebrate Ctype lectins (CTLs) have a wide array of functions which include recognition, regulation, and elimination of invading pathogen (Rabinovich et al., 2012; Wang and Wang, 2013b; Sun et al., 2008; Schnitger et al., 2009; Watanabe et al., 2006; Wang et al., 2014a). For example, in shrimp *Marsupenaeus japonicus*, a C-type lectin FcLec4/hFcLec4 was found to interact to β -integrin on the surface of hemocytes to promote phagocytosis (Wang et al., 2014b). Previously, we reported *Marsupenaeus japonicus* gill C-type lectin (MjGCTL) as a pathogen recognition receptor capable of acting as an opsonin, binding to both bacteria and hemocytes, rMjGCTL promoted hemocyte encapsulation.

In this study, using native MjGCTL protein from gills and investigating its function *in vivo* through gene-knockdown the role of MjGCTL was investigated, which are evidence that penaeid shrimp gills, aside from being a physical barrier, likewise provides a biochemical barrier aiding the defense of gills from invading bacteria.

Materials and Methods

Experimental shrimp and bacterial strains

Marsupenaeus japonicus shrimp samples were purchased from a farm in Miyazaki prefecture, Japan. A total of 8 shrimp weighing 25 g was used for the protein purification, while 100 were used for the *in vivo* experiments. Shrimp were kept in tanks with recirculating water maintained at 25 $^{\circ}$ C and 30 ppt salinity.

Bacterial strains used in this study were Gram-negative EGFP-expressing *Escherichia coli*, and two strains of *Vibrio parahaemolyticus*, virulent (D6) and non-virulent (N7). Grampositive formalin-killed *Streptococcus agalactiae* were also prepared and labeled with PKH67 Green Flourescent Cell Linker Kit (Sigma, USA) following the manufacturer's protocol.

Microarray analysis

Total RNA was extracted from hemocytes, gills, hepatopancreas, lymphoid organ, and muscles using RNAiso (Takara, Japan) and purified by RNeasy Mini Kit (Qiagen, Germany). Purity of RNA was confirmed with Agilent 2200 TapeStation System (Agilent Technologies, USA). Total RNA (200 ng) was then reverse transcribed and labelled with Cy3 using a Low Input Quick Amp Labeling kit (Agilent technologies, USA) and then hybridized on the microarray following the one-color microarray-based expression analysis protocol. The transcriptome of the five tissues were then analyzed by Agilent Gene Expression oligo microarray. Using an 8x15-k microarray format containing 13, 310 probes, complimentary RNA were hybridized for 17 h at 65 $^\circ\!\mathrm{C}$ and then washed for 1 min with buffers 1 and 2 (Agilent Technologies, USA) at 25 $^{\circ}$ C and 37 $^{\circ}$ C, respectively. Upon drying, slides were scanned using Sure Scan High-Resolution Scanner (Agilent Technologies, USA). Then using the Agilent Feature Extraction Software, data were extracted from scanned slides and imported to GeneSpring GX Software (Agilent Technologies, USA), where data was normalized and filtered for expression, flags and error. Differentially expressed genes in gills (genes that were at least 2-fold higher than normal expression in gills and downregulated in hemocytes, hepatopancreas, lymphoid organ, and muscles) were analyzed, and among those only the immune-related genes were viewed using Heatmap Illustrator (Heml) 1.0 (Deng et al., 2014).

Purification of native MjGCTL from gill tissue

Gills were collected from three *M. japonicus* shrimp weighing 25g and were placed in a glass pestle tissue grinders. Tissues were then homogenized with 2mL PBS⁺(MgCl₂, CaCl₂), then centrifuged at 6000 rpm for 5 min. The supernatant was then collected and mixed

with Lactose-agarose beads in a purification column, then incubated overnight at 4 $^{\circ}$ C with gentle rotation. Lactose-agarose beads were then washed three-times with PBS⁺, then 3 times with 10mM EDTA in PBS. MjGCTL was then eluted with 50mM EDTA followed by dialysis with 1x PBS.

Agglutination assay

Total protein from gill tissues and proteins on the gill surface or gill mucus were collected through washing gill tissues with PBS⁺(with MgCl₂ and CaCl₂) through vortexing in 1.5 mL tube. Gill-mucus in PBS⁺ was then separated from the tissues through filtration. The remaining gill tissues were then washed 3-times, and homogenized in PBS⁺. The homogenate was then centrifuged at 6000 rpm 4 $^\circ\!\mathrm{C}$ the supernatant was then collected as the total gill soluble proteins. Both total protein from gills and gill-mucus were then used for agglutination assay which was carried out using a modification of the method of Luo et al. (2006) and Ma et al. (2008). The bacteria used in the assay were EGFP-expressing *Escherichia coli* and PKH67-labelled *Streptococcus agalactiae*. Bacteria were resuspended in Calcium-rich Tris-buffered saline (TBS^{Ca}) (Tris-HCl, pH 7, 100mM NaCl, and 10mM CaCl2) at 1 x 10⁸ cells/ml. Ten (10) μ l of bacteria suspension was then incubated with same volume of MjGCTL (20 μ g/ml) at room temperature (25 $^{\circ}$ C) for 1 h. Agglutination were then viewed using fluorescence microscopy (Nikon, Japan). Recombinant (r)MjGCTL, was used as a positive control and TBS^{Ca} as a negative control. The eluted native MjGCTL protein from gills was also used in agglutination assay following the protocol mentioned above.

Western Blot Analysis

Tissue samples from gills, hemocyte, hepatopancreas, lymphoid organ, muscle, stomach, heart, intestine, nerve, eye and gill mucus were harvested (approx. 100mg) from three shrimp weighing 25g each and were pooled then homogenized. Equal volume of sample buffer was added to the homogenate prior to incubation at 95 °C for 5 min. Total protein from each tissue were then subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked in 4% skimmed milk in 0.05% Tween 20 in TBS (TBST) for 1 h, incubated for 1 h with rabbit anti-MjGCTL antibody (Eurofins, Japan) diluted to 1:5000 in blocking solution at room temperature with gentle shaking, washed four times with TBST, incubated for 30 min with anti-Rabbit IgG, AP conjugate (Promega, USA) diluted to 1:5000 in blocking solution at room temperature with gentle shaking, washed three times with TBST, stained with substrate solution containing 5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium (BCIP/NBT, Sigma, USA) for 2 min and washed with distilled water. Purified MjGCTL protein described in section 2.5 was likewise confirmed the same protocol.

For the confirmation of MjGCTL silencing, protein samples were prepared separately from 3 shrimp for each treatment group (MjGCTL-dsRNA, GFP-dsRNA and PBS) and was subjected to SDS-PAGE and western blotting using anti-MjGCTL antibody with the conditions mentioned above. As the loading control a commercial antibody for ß-actin (Novus Biologicals, USA) was used.

Phagocytosis assay by Flow-cytometry

To assess the role of MjGCTL as an opsonin, purified MjGCTL (20µg/ml) was incubated with EGFP- *E. coli* and PKH67 -labelled *Streptococcus agalactiae* in TBS^{Ca} for 45

min at 25 $^{\circ}$ C. One hundred (100) µl of bacterial solution with MjGCTL was then injected to 5 shrimp, where injection with bacteria solution without MjGCTL was used as control group. Hemolymph (500µl) was then withdrawn 2 hours post-injection mixed with same volume of anticoagulant 1x PBS with 10mM EDTA anticoagulant (480mM NaCl, 2.7mM KCl, 8.1mM (Na₂HPO₄) 12H₂O, 1.47mM KH₂PO₄, pH 7.6 containing 10 mM EDTA). Fluorescence intensity was then evaluated by FACSCalibur (Becton-Dickinson, USA), measuring phagocytosis activity at 530/30 nm (FL-1) as the fluorescence of bacteria engulfed by hemocytes. Ten thousand events were evaluated with Cell Quest Pro software ver. 5.2.1 (Becton-Dickinson, USA). Statistic-analyses were then done using GraphPad Prism 6.0.

in vivo knockdown of MjGCTL by RNA interference

Double-stranded (ds) RNA template was generated by incorporating T7 promoter region to both 5' and 3' end of MjGCTL primers (Table 1), as well as green fluorescent protein (GFP) gene which served as the dsRNA control. dsRNA was generated *in vitro* through T7 Ribo8MAX Express (Promega, USA) and was purified following the manufacturer's instructions. Shrimp samples were then injected twice with 24-hours interval with 5 µg per gram of shrimp dosage of MjGCTL-dsRNA and GFP-dsRNA, while phosphate buffer saline (PBS) was injected as the negative control.

Immune Challenge

Virulent strain of *Vibrio parahaemolyticus* (D6) were cultured in Heart Infusion medium (HI) (3% NaCl) overnight at 30 $^{\circ}$ C. Serial dilutions were then performed on sterile seawater for the bacterial counting performed on HI (3% NaCl) agar plate medium. Three experimental groups (dsMjGCTL, dsGFP, and PBS) were prepared in 20L seawater (30 ppt),

containing 12 shrimp per treatment. At 3-days post treatment (1-day post silencing) all groups were then inoculated with 3.0×10^6 CFU/mL final concentration of *V. parahaemolyticus* (D6 strain). Mortality rate was measured for 7 days and analyzed using Kaplan-Meier survival curve by GraphPad Prism 6.0.

Effect of silencing MjGCTL on bacterial proliferation

Using the same experimental set-up, the effect of silencing MjGCTL on the normal shrimp microflora was assessed in gills and hemolymph of all treatment groups. A total of 90 µg gill tissues, and 500µl of hemolymph suspended in the same volume of anticoagulant (480mM NaCl, 2.7mM KCl, 8.1mM (Na₂HPO₄) 12H₂O, 1.47mM KH₂PO₄, pH 7.6 containing 10 mM EDTA) were collected from 3 shrimp per experimental group at 0 (non-injected) and 3-day post dsRNA-injection (1-day post-silencing). Gill tissues were then suspended in sterile seawater and was mixed through vortexing. Serial dilutions of both gill and hemolymph suspensions were then performed on sterile seawater and plated on TCBS agar medium for the counting of *Vibrio* load and Heart-infusion agar medium with 3% NaCl for total bacterial load.

Simultaneously, to assess the bacterial proliferation after MjGCTL-knockdown, bacterial immersion using the non-pathogenic strain *V. parahaemolyticus* (N7) was conducted as described in section 2.8. Total vibrio count was then done at 0, 6 and 12-hour post-innoculation in gills and hemolymph of all treatment groups (N=3) as described above.

Effect of MjGCTL-knockdown on the expression of other AMPs

Knockdown of MjGCTL transcripts were quantified by qPCR analysis, as well as antimicrobial peptides (AMPs) crustin and penaeidin using THUNDERBIRD SYBR qPCR Mix

(TOYOBO, Japan). The condition was 95 $^{\circ}$ C for 1 minute, 40 cycles of 95 $^{\circ}$ C for 15 seconds and 60 $^{\circ}$ C for 1 minute followed by dissociation analysis step. The log2^{$\Delta\Delta$ Ct} values of target genes were analyzed. The statistical significance (p < 0.05) was analyzed using ANOVA by GraphPad Prism 6.0.

Table 1. Primer sequences used in this study.

Name	Sequence (5'-3')
MjGCTL_F	GGCATGGAATAACAGCGAGT
MjGCTL_R	CATTTTGGATGCTGAGCAGA
MjGCTL_qRT_F	GCGCACTAACCTAAACAACACTCTC
MjGCTL_qRT_R	CATAGAAAACGGAGAGGCACTG
MjGCTL_T7_F	TAATACGACTCACTATAGGGCCTAAACAACACTCTCAGCC
MjGCTL_T7_R	TAATACGACTCACTATAGGGCAAAGGGGACTCATGTCAGC
GFP_T7_F	TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA
GFP_T7_R	TAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA
MjGCRUS_qRT_F	GTGGTTTCGGTGGCTTTCCC
MjGCRUS_qRT_R	CGCAGCAGTACGCTTGGTTC
MjPenaeidin_F	TTAGCCTTACTCTGTCAAGTGTACGCC
MjPenaeidin_R	AACCTGAAGTTCCGTAGGAGCCA
EF-1α_F	ATGGTTGTCAACTTTGCCCC
EF-1α_R	TTGACCTCCTTGATCACACC
EF-1α_qRT_F	ATTGCCACACCGCTCACA
EF-1α_qRT_R	TCGATCTTGGTCAGCAGTTCA

Results

Microarray Analysis

All 13, 310 spotted genes were expressed. There were 101 genes that were at least 2-fold higher than normal expression in gills and down-regulated in hemocytes, hepatopancreas, lymphoid organ, and muscles. Out of these 101 genes 27 were immunerelated, 51 are not immune-related, and 23 were unknown genes (Table 2). The heat-map representation of the 27 immune-related genes are shown in Fig. 1.

Table 2. Sumary of microarray analysis of novel biodefense genes in *Marsupenaeus japonicus* hemocyte, gills, hepatopancreas, lymphoid organ, and muscle.

Total number of spotted genes	13, 310
Total expressed	13, 310
Differentially expressed in gills	101
Immune-related	27
Others	51
Unknown	23



Fig. 1. Microarray analysis of 27 immune-related genes differentially expressed in gills compared to other immune-related organs such as hemocyte, hepatopancreas, lymphoid organ, and muscle. Expression of these genes in gills are at least 2-fold higher than in the other tissues.

MjGCTL in gill-mucus agglutinates bacteria

Gill mucus and total soluble protein extract from gill tissues were found to agglutinate bacteria, similar to the positive control recombinant MjGCTL (Fig. 2). Agglutination was confirmed to be caused by MjGCTL through agglutination inhibition by anti-MjGCTL serum antibody, as compared to the pre-immunized serum as negative control where no agglutination inhibition was observed (Fig. 2).



Fig. 2. MjGCTL secreted from gills agglutinates bacteria. EGFP-Expressing *E. coli* agglutinated by total protein from gills as well as gill-mucus (GM). Agglutination-inhibition by rabbit serum anti-MjGCTL antibody neutralizing GM activity, with pre-immunized serum as negative control. Recombinant (r)MjGCTL was used as positive control and with TBS-Calcium (TBS^{Ca}) as negative control.

Opsonic effect of eluted MjGCTL

Eluted MjGCTL from gill tissues with size of approx. 45 kDa was confirmed by SDS-

PAGE and western blot analysis (Fig. 3A-B). The eluted MjGCTL agglutinated both Gram-

positive PKH67-labelled Streptococcus agalactiae and Gram-negative EGFP-expressing E.

coli (Fig. 3C). Incubating MjGCTL to these two bacteria, increased phagocytic rate in vivo for PKH67-labelled *S. agalactiae* but not for *E. coli* (Fig. 4A). Confirmation of phagocytosis on PKH67-labelled *S. agalactiae* are shown in the histogram of flow cytometric analysis (Fig. 4B), and immunohistochemistry (Fig. 4C).



Fig. 3 Bacterial agglutination activity of eluted native MjGCTL. (A) SDS-PAGE show the elution of native MjGCTL from lactose-agarose beads showing flow-through (1), washing with PBS (2-4), and washing with 10mM EDTA (5-7), and elution with 50 mM EDTA (8-10). (B) Eluted MjGCTL was confirmed using western blot analysis using anti-MjGCTL rabbit serum. (C) Bacterial agglutination activity of eluted MjGCTL against EGFP – *E. coli*, with only TBS^{Ca} as negative control.



Fig. 4. Opsonization activity of eluted native MjGCTL. (A) *in vivo* Phagocytosis assay by Flow cytometry using *Streptococcus agalactiae* labelled with PKH67 and EGFP–*E. coli*. (B) FCM histogram of phagocytic activity of MjGCTL against PKH67- *S. agalactiae*. (C) Fluorescence microscopy images of representative phagocytic activity against PKH67- *S. agalactiae* showing hemocyte nucleus stained with Hoechst (a), PKH67-stained *S. agalactiae* (b), the bright-field image of hemocyte (c), and merged image of a-c demonstrating the phagocytic activity (d).

Knockdown of MjGCTL inhibits bacterial agglutinating activity

Following the dsRNA treatment, lower transcripts of MjGCTL were detected by

semi-quantitative RT-PCR at 1 day post-dsRNA injection, then no transcripts were detected

at 2 and 3 days (Fig. 5A). MjGCTL protein level also decreased after silencing with cognate

dsRNA as compared to the detected MjGCTL in GFP-dsRNA and PBS treated shrimp, and as well as with the loading control β -actin protein (Fig. 5B).

Upon silencing of MjGCTL, gill mucus from MjGCTL knocked-down shrimp showed decrease in agglutinating activity, as compared to GFP-dsRNA and PBS-injected shrimp (Fig. 6A). Further quantification shown by minimum agglutination concentration (MAC), demonstrated that the gill mucus of MjGCTL-knocked down shrimp have significantly higher MAC, similar to the effect of neutralizing MjGCTL with its specific antibody, in comparison with the control groups (Fig. 6B).



Fig. 5. *in vivo* silencing of MjGCTL by RNAi. (A) RT-PCR analysis of MjGCTL transcripts in gills at 1, 2, and 3-day post-injection of MjGCTL-dsRNA, GFP-dsRNA (dsRNA control), and PBS (negative control) using 5µg /gram of shrimp dsRNA dosage. (B) Silencing of MjGCTL protein by cognate dsRNA at day 2 and 3 post-dsRNA injection, using β -actin as loading control. Both RNA and protein samples were collected from three separate samples for each treatment group.



Fig. 6. Effect of MjGCTL-knockdown on gill-mucus bacteria-agglutinating ability. (A) Gillmucus obtained (N=3) at 1, 2, 3-day post-injection (dpi) of MjGCTL-dsRNA, GFP-dsRNA and PBS. (B) Quantification of agglutination-inhibition through minimum agglutination concentration of gill mucus from all treatment groups (obtained 3-dpi) compared with those from non-injected (ni) shrimp, neutralized gill -mucus by anti-MjGCTL antibody serum (ni+MjGCTL-Ab) using pre-immunized serum as negative control.

Silencing MjGCTL increases gill and hemolymph bacterial load.

Total bacteria and vibrio in gills increased upon silencing of MjGCTL, although not significantly (Fig. 7A). In hemolymph, however, total vibrio was only detected in MjGCTLsilenced shrimp; and total bacteria was detected in low count in dsGFP treated shrimp and in significantly higher number in MjGCTL-silenced shrimp (Fig. 7B).

Upon bacterial immersion, vibrio number increased significantly from 6 to 12 hpi among MjGCTL-silenced shrimp, in contrast to dsGFP and PBS treated groups on both hemolymph and gills (Fig. 7C). In the hemolymph, very low vibrio count was detected all treatment groups at 6 hpi, however, increased significantly in MjGCTL-silenced shrimp compared to other groups (Fig. 7D).





(D) hemolymph at 0, 6, and 12 hours post-immersion with *V. parahaemolyticus* (N7-strain) at 3 MjGCTL-silencing, GFP-dsRNA and PBS treatment.

Effect of silencing MjGCTL on shrimp survival

MjGCTL-silenced shrimp survival decreased to 20% after 7 days post-immersion (dpi) with a low concentration of the virulent strain of *V. parahaemolyticus*. On the other hand, control groups treated with GFP-dsRNA sustained a 90% survival at 7 dpi (Fig. 8). Similar results were obtained during the second trial, where dsGFP-treated shrimp also maintained 90% survival, while MjGCTL-silenced shrimp reached 0% survival at 7 dpi. Statistical analysis by Kaplain-meier survival curve indicated a significant difference (p < 0.00) between groups.

Expression of AMPs upon MjGCTL-knockdown

MjGCTL transcripts at 3 dpi with dsMjGCTL were significantly lower than in dsGFP-treated control group (Fig. 9A). Crustin and paneidin, antimicrobial peptides expressed in gills of *M. japonicus*, were downregulated among MjGCTL-silenced shrimp (Fig. 9B).



Fig. 8. Effect of MjGCTL-knockdown on shrimp survival. Bacterial challenge with virulent strain (D6) of *V. parahaemolyticus* after MjGCTL-dsRNA, and GFP-dsRNA treatment recorded up to 7-day post-immersion with *V. parahaemolyticus*. The challenge test was performed twice.



Fig. 9. Expression of antimicrobial peptides after MjGCTL-knockdown. (A) MjGCTL mRNA level at 3 days-post injection of control GFP-dsRNA and MjGCTL-dsRNA. (B) Crustin and penaeidin mRNA levels 3-days post MjGCTL-dsRNA, GFP-dsRNA, and PBS, with untreated shrimp as additional control.

Discussion

The gills of penaeid shrimp has long been considered as an immune organ together with the hemocytes, lypmphoid organ, and hepatopancreas (Tassanakajon et al., 2013; Robalino et al., 2007), however, compared to the others three immune organs, there has been no report on how gills elicit an immune response by itself. Previous studies have mentioned gills to merely give a purely structural and mechanical support to the hemocytes, which are able to perform clotting, melanization, phagocytosis, encapsulation, and release of antimicrobial peptides. The gills serve as the extruding site, where the phagocytic hemocyte directs engulfed materials to be trapped on the gill filaments forming nodules that are lost during molting (Martin et al., 1998). A study by Destoumieux et al. (2000) on penaeidins located on the gill cuticle demonstrated that this chitin-binding antimicrobial peptide is released by the hemocytes to the plasma which then binds to the cuticle. These suggest that the gills greatly depend on the protection provided by hemocytes that readily infiltrate the gill tissues. In this study, however, we argue that gills are also equipped with their own immune molecules which is evidenced by the microarray analysis (Fig. 1). Furthermore, we demonstrate a representative of these molecules which are the C-type lectins (CTLs) that serves as biomolecular barriers, pathogen recognition receptors (PRRs) binding and agglutinating invading bacteria, and tagging them as opsonins.

In the previous chapter, we established that MjGCTL is a secreted protein having agglutinating ability against some bacteria. Also, we investigate the activity of native MjGCTL protein *in vivo*, where we hypothesized that MjGCTL is secreted on the gill surface binding and agglutinating invading bacteria, and tagging them as opsonins. In chapter 2, we analyzed MjGCTL protein's tissue distribution in gills together with other tissues, where in addition to the different tissues tested, as shrimp gills are known to possess secretory cells, we also collected total protein from the gill surface which we referred to as gill-mucus. The tissue distribution of MjGCTL protein through western blot analysis supported our hypothesis as MjGCTL was detected in gill tissues as well as in gill-mucus. Total protein from gills and gill-mucus were also observed to have agglutinating activity against bacteria (Fig. 2) in the same way as recombinant (r)MjGCTL described in the previous chapter. This then

raised the question whether MjGCTL present in gill-mucus causes the bacterial agglutination. This was confirmed as the agglutination activity of gill-mucus was inhibited by neutralizing MjGCTL through competitive-binding with its own antibody (Fig 2). Corroborating evidence are presented by the results of the *in vivo* RNA interference, where silencing MjGCTL also resulted to the inhibition of bacterial-agglutination activity in gill-mucus (Fig. 6A & B). In fish, CTLs were also discovered in mucus, acting as PRRs capable of binding and recognizing a wide-array of microorganisms (Tsutsui et al., 2015; Tsutsui et al., 2007; Tasumi et al., 2004; Suzuki et al., 2003). In Japanese eel *Anguilla japonica*, two C-type lectins were detected in gill mucus secreted by gill exocrine mucous glands, which also were (Mistry et al., 2001). Interestingly, the expression profile of these lectins was like that of MjGCTL, where strong expression was observed in gill and weak in stomach. Like our assumption as to why these lectins are particularly in these tissues, Mistry, et al. (2001) discussed that these lectins may be serving as barriers to the main entry sites of pathogen, which are through the gills or oral route.

As determined in the previous chapter, lactose is one of the carbohydrate ligands of MjGCTL. Having binding affinity to lactose, we could purify MjGCTL from gills using Lactose-agarose beads (Fig. 3A & B). It was also previously established that rMjGCTL can act as an opsonin promoting bacterial agglutination and encapsulation by hemocytes. Here, we assessed the opsonic activity of the purified MjGCTL through bacterial agglutination and *in vivo* phagocytosis assay, where the PHK67-labelled *Streptococcus agalactiae* incubated with MjGCTL was agglutinated (Fig. 3C) and phagocytosed more than those without MjGCTL (Fig. 5A-C). This corroborates the previous results where rMjGCTL was shown to be capable of agglutinating both *S. agalactiae* and *E. coli*, binding to both bacteria and

hemocytes, which allows MjGCTL to act as an opsonin facilitating encapsulation and phagocytosis.

MjGCTL expressed on the surface of the gills serve as a biomolecule barrier assisting the gills in preventing bacterial penetration. This is demonstrated by the increase in the population of normal microflora in gill as well as in hemolymph upon silencing MjGCTL (Fig. 7A & B). Also, upon immersion with V. parahaemolyticus, Vibrio CFU on gills of MjGCTLsilenced shrimp significantly increased from 6 to 12 h post-immersion (hpi), while those from GFP-dsRNA and PBS control groups showed no significant difference (Fig. 7C & D). Based on this, it appears that without MjGCTL the bacteria that the immune system maintains in low-population can proliferate more, and during infection more bacteria could colonize or adhere more onto the gill surface, as compared to the control groups that appeared to have hindered bacterial proliferation. Vibrio count in the hemolymph allowed us to assess the number of bacteria that could possibly penetrate the shrimp host during immersion. Very few Vibrio CFU were observed at 6 hpi for all groups, which may suggest that 6 hours was not enough for bacteria to penetrate the host. The lower vibrio count in the hemolymph than the gill is expected as the hemolymph, together with the lymphoid organ, unlike the other organ have the ability of clearing bacteria (Smith and Ratcliffe, 1980; van de Braak et al., 2002). Nonetheless, at 12 hpi vibrio count increased significantly in hemolymph of MjGCTL-silenced shrimp (Fig. 7D). Taken together, silencing MjGCTL in gills allowed more bacteria to penetrate, suggesting the presence of bacteriostatic defense on gills where MjGCTL is a key proponent. This is corroborated by the results of the challenge test with a low concentration of V. parahaemolyticus D6-strain, where results show MjGCTL-silenced shrimp became more vulnerable to bacterial infection (Fig. 8). Combined with the results of vibrio counting, decrease in the survival of MjGCTL-silenced shrimp may be due to the increase in number of bacteria caused by the absence of MjGCTL in gills. Contributing to the vulnerability to bacterial infection caused by uncontrolled bacterial proliferation, mRNA expression of antimicrobial peptides in gills crustin and penaeidin were also downregulated upon the silencing of MjGCTL, which suggests a weaker immune system (Fig. 9). Although the connection of MjGCTL to these antimicrobial peptides are still unclear, there have been a report on CTL regulating the expression of antimicrobial peptides such as the MjHeCL of *M. japonicus* (Wang et al., 2014).

In conclusion, MjGCTL localized in gills are secreted to the gill-mucus serving as a biomolecular barrier hamering the growth and penetration of invading bacteria. Shall the bacteria penetrate the gill tissue and enter the blood stream, MjGCTL which acts as a PRR, can bind to the bacteria and cause opsonization, facilitating the phagocytic activity of hemocytes.

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CHAPTER 5

General Conclusion

Among the aquaculture products, aside from being an important commodity, shrimp has become an indispensable source of income for most Asian countries. Shrimp is valued second highest valued commodity, and is highly in demand worldwide. However, achieving sustainability in shrimp production is threatened by numerous outbreaks of diseases caused by both viral and bacterial diseases. As countermeasures, we have to come up with strategies to prevent, detect, and treat diseases. Among these strategies, coming up with ways to prevent diseases is still the most ideal strategy, where prevention is still better than cure. However, for this we have to have a basic knowledge of how the bio defense mechanism of the shrimp work, which is why a lot of studies are now focused on identifying molecules involved in the defense mechanism against infection. Some studies are focused on elucidating immune function of immune-related organs, when hemocytes are the primary subject as immune reactors. Although the hemocytes play a major role in the immune system and are known to possess numerous immune functions identified in both cellular and humoral functions, immune reactions of hemocytes presupposes a complete breach in the internal host system. This is because for most shrimp pathogens, with he exception of fungi, penetrating the hosts defense barrier to enter the internal environment is a prerequisite to infection. In this light, to know how shrimp defends itself from infection, it is fitting to investigate the defense barriers that keep pathogens from entering the host. The shrimp is equipped with the thick and hard cuticle, providing the

physical barrier. However, as soon as the pathogen breaks this physical barrier, the shrimp is left with only the innate immune system to respond to the infection. Thus, it is important to know the different entry sites of different pathogens. Based on the shrimp anatomy and physiology may, the gills is the thin organ that constantly maintains contact with both external and internal environment of the shrimp. This may offer an explanation as to why the gills are the main target organ of major shrimp pathogens such as Vibrio spp., taura syndrome virus/ gill-associated virus, and white spot syndrome virus. It is however unclear as to how the gills protect itself from infection. In the immune mechanisms of shrimp, the gills give mechanical assistance to the hemocytes, trapping and extruding the harmful biotic and abiotic materials which includes the phagocytosis materials. Gills likewise serve as the site for hemocytes nodule formation, which are lost upon molting. There are no reports, however, of the gills having a specific phagocytic cells or molecules providing immune protection. Thus, this study provides a clear evidence of the gills being equipped with immune molecules. This is revealed to us by the microarray analysis of new biodefense genes expression among immune-related organs of Marsupenaeus japonicus. Furthermore, the identification of MjGCTL as one of these novel biodefense genes, its molecular and functional characterization, give a clear example of how these immune molecules may work in the gills.

This study focused on two primary topics which are the functional analysis of a novel biodefense molecule (MjGCTL) expressed in gills, which consequently give us a preview on how the gill defends the shrimp from bacterial infections. Thus, the first two parts (Chapter 2 and 3) on the characterization of MjGCTL as a C-type lectin is the foundation of this study. The molecular characteristics of MjGCTL provided by bioinformatics analyses all pointed out to MjGCTL as a classical C-type lectin. Although

based on the domain structures of MjGCTL, and the existing references on other shrimp Ctype lectin containing proteins (CTLDcps) with QAP motif, is also suggesting that there is a possibility that MjGCTL is also a C-type lectin-like domain protein. That is why the comparison of MjGCTL to the previously reported LvCTLD, that possess high sequence identity, was also vital to the classification of MjGTCL. The comparison of these two molecules provided the idea of how diverse the invertebrate CTLDcps are, where even two molecules with high sequence identity can have an entirely different function from each other. Moreover, this comparison give us an insight that the classification to discriminate between CTL and CTLD can be accurately achieved by investigating the binding capacity and requirements of the molecule to different ligands. Phylogenetic analysis, on the other hand, provided another evidence of the diversity of CTLDcps of shrimp based on their conserved carbohydrate recognition (CRD) fold, where CTLDcps with QAP binding motif are clustered separately from those with EPN and QPD motifs together with their variants. However, based on the phylogenetic information, it is difficult to infer that MjGCTL's binding specificity is different from those CTLDcps with EPN and QPD motif. It is, however, suggestive that the QAP motif is not a variant motif of EPN and QPD, or QAP have an entirely different binding specificity as the other CTLDcps. Moreover, since the phylogenetic analysis includes CRD's of non-carbohydraye binding ligands, it is also difficult to determine MjGCTL's binding specificity. Which is why the use of recombinant protein technology was necessary to clearly investigate MjGCTL's binding specificity to different carbohydrate ligands. The production of recombinant (r)MjGCTL revealed its molecular size which is higher than its predicted size, this was rather expected as MjGCTL's sequence possessed predicted glycosylation sites, and that the molecular functions are also highly dependent on the post-translational glycosylation of the protein.

Utilizing rMjGCTL the binding specificity of MjGCTL as well as its role as a pathogen recognition receptor (PRR) of bacteria. MjGCTL was observed to promote bacterial agglutination for both Gram-negative and Gram-positive bacteria. Using this capability of rMjGCTL to agglutinate bacteria, the dependency of MjGCTL's to calcium was determined as well as various carbohydrates were found to inhibit bacterial agglutination which led to the identification of MjGCTL's affinity to these carbohydrates. The binding affinity of MjGCTL supports the results of the bioinformatics analysis. Firstly, as predicted, MjGCTL binds to carbohydrate ligands in a calcium-dependent manner as that of the classical Ctype lectin. Secondly, the carbohydrate specificity of MjGCTL which showed no affinity to both mannose and galactose, in a way supports the phylogenetic analysis where the MjGCTL is clustered differently from mannose and galactose binding CTLDcps. Apart from carbohydrates, rMjGCTL was also found to bind to bacterial components lipopolysaccharide (LPS) and peptidoglycan (PGN), where this may explain the ability of rMjGCTL to agglutinate both Gram-positive and Gram-negative bacteria. Moreover, rMjGCTL as a PRR not only bind and agglutinate bacteria but also can act as an opsonin for the recognition of hemocytes. This is demonstrated by the increased encapsulation activity of the shrimp hemocytes against the agarose beads with rMjGCTL, which led to melanization, which also suggest that MjGCTL can initiate the phenoloxidase activity that leads to melanization. The inhibition of encapsulation activity hemocytes by the carbohydrate ligands glucose and fucose confirms MjGCTL's specificity. This also implies that MjGCTL is able to bind to shrimp hemocytes through another receptor on the hemocyte surface, which may be a protein or glycans.

From the sequence information, the presence of signal peptide is already suggestive that MjGCTL protein is leaning through the secretory pathway. And this was corroborated
by the tissue distribution of MjGCTL, where aside from being expressed in the gills and stomach, MjGCTL was likewise detected on the gill surface which we designated as gill mucus. Although in this study, I did not provide data supporting the existence of mucussecreting gill cells, other studies have already presented the presence of exocrine cells present in gills of other penaeid shrimp species that help maintain the gills physiological condition while exposed to the water environment. Moreover, I demonstrated that proteins can be collected from the gill surface, which were likewise found to act against bacteria promoting agglutination. Using the serum antibody against MjGCTL to specifically neutralize MjGCTL found in gill mucus demonstrated that MjGCTL is involved in the bacterial agglutinating capability of the mucus. Through MjGCTL's affinity to lactose which is shown in the second chapter, the native MjGCTL was partially purified from the gill tissues. The eluted native MjGCTL was able to perform same functions as rMjGCTL where it also agglutinated bacteria and promotes hemocyte activity through phagocytosis. This finding once more emphasize the ability of MjGCTL to act as a PRR, tagging bacteria for the recognition of hemocytes. The in vivo functional analysis of MjGCTL through RNA interference reveals the importance of MjGCTL in the immune defense of shrimp. It presented MjGCTL as a biodefense gene present on the gill surface, where the consequence of the loss of function of MjGCTL lead to the loss of ability of gill mucus to agglutinate bacteria and increasing the shrimp vulnerability to bacterial infection. Furthermore, the knockdown of MjGCTL suppressed the ability of the shrimp to maintain the normal microflora, which is further aggravated upon the infection of a foreign bacteria increasing the bacterial load in both gill and hemolymph. This increase in bacterial load may be attributed to the impaired ability of gill mucus to agglutinate bacteria and the decreased in expression of antimicrobial peptides upon knockdown of MjGCTL. Although it is not clear

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how MjGCTL directly influences these antimicrobial peptides, other reports exist on CTLs regulating the expression of other antimicrobial peptides. In this study, we have seen MjGCTL to indirectly induce some enzymatic reaction when the encapsulated beads resulted into the production of melanin.

The functional analysis of MjGCTL is just one evidence of the existing defense mechanism of the shrimp gills. As shown in the microarray analysis there are at least 27 other biodefense genes that may also render protection. The functional analysis of these genes would be vital for further understanding the underlying mechanism of the biodefense of shrimp gills. The identification and analyses of these defense genes may serve as the foundation of the formulation of treatments and immune enhancements applicable to the shrimp immune system in the future.