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Review

THE POTENTIAL ROLES OF INTERFERON IN MANAGING VIRAL DISEASES IN CRUSTACEAN

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ABSTRACT

World crustacean aquaculture has developed considerably since the 1980's and is now worth more than USD 10 billion per year. However, the economic growth of this industry has been severely affected by problems related to diseases and environmental degradation. Viral disease outbreaks are particularly concerning and have caused massive economic losses around the world. However, there is still no effective treatment for most viral diseases. Current research on crustacean diseases focuses on the role of innate immune system as the first defence mechanism against viral infections. Of the available antiviral immune responses, interferons (IFNs) are known to have ability interfering effect on viral replication, particularly in vertebrates. This paper reviews the function and molecules involved in the vertebrate interferon system and whether similar molecules and pathways may exist in crustacean immune systems. Therefore, IFN or IFN-like proteins in crustaceans may provide the key to managing viral diseases.

Key words: Crustacea, IFN-like protein; Interferon; Viral diseases

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INTRODUCTION

Global crustacean aquaculture is one of the most economically important sectors that is worth more than $US10 billion per annum (Johnson, *et al.,* 2008). Approximately 75% of the world's production of farmed shrimp comes from Asian countries, particularly from China and Thailand followed by Vietnam, Indonesia, and India (McClennan, 2004) providing income to rural communities and the developing economic sector (Johnson, *et al.*, 2008).

Currently, shrimp production continues to suffer from problems related to environmental devastation and diseases, both infectious and non-infectious (Bachère, 2000) that have caused massive losses to shrimp aquaculture industries throughout its 40-year existence. Farmed shrimp are susceptible to a wide variety of pathogens, including protozoa, fungi, bacteria, and viruses (Johnson, *et al.*, 2008), particularly from the introduction of wild broodstock. The main contributors to these losses were viral diseases (Lotz, 1997). Due to their epizootic spread and economic impact, white spot syndrome virus (WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHNNV) (OIE, 2003), and recently infectious myonecrosis virus (IMNV) (Tang, *et al.,* 2005) have caused substantial losses within aquaculture facilities (**Table 1**).

Over 20 different shrimp viruses has been recorded and many of these have spread through farms and have several host species (McClennan, 2004). Water is the main transfer vector of many of these viruses and high stocking densities are thought to increase the rate of the spread of a virus (NCFE, 1999). Consequently, any virus outbreak also carries the risk of decimating shrimp living in the wild. Viral diseases are also thought to spread by the movement of live and frozen shrimp through import-export activities (Dhar, *et al.,* 2001).

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Table 1. List of crustacean viral diseases that have caused significant losses in aquaculture

Diseases	Gross Signs	Organisms	Reference
WSSV	White circular, inclusions or spots in the cuticle, often accompanied by red coloration in overall body. Stop eating followed by swimming near the surface at pond edges.	*Penaeus monodon* *Litopenaeus. vannamei*	OIE, 2003
YHV	Yellow in cephalothorax and general bleaching of body colour. Moribund shrimp congregated near the surface the edge of ponds.	*P. monodon* *L. vannamei* *L. stylirostrus* *Farfantepenaeus aztecus* *F. duorarum*	OIE, 2003
TSV	Black cuticular lesions and loose shell in *P. Monodon* Expansion of the red chromatophores cause overall pale reddish coloration, tail fan and pleopods distinctly red Having soft shells, an empty gut and often in the late D stages of the molt cycle. Hypoxic on affected shrimp and swim to the pond surface, looking for higher level of DO	*P. monodon* *L. vannamei* *L. stylirostris*	Lightner et al., 1995; OIE, 2003; Limsuwan & Churchid, 2007
IHHNV	In *L. stylirostris* have white coloured spots in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. Juvenile *L. vannamei* with RDS display bent or deformed rostrums, wrinkled antennal flagella, cuticular roughness, and other cuticular deformities.	*P. monodon* *L. vannamei* *L. stylirostris*	OIE, 2003
IMNV	The appearance of whitish, opaque lesions in the skeletal muscle, and infected shrimp eventually become lethargic.	*P. monodon* *L. vannamei* *L. stylirostris*	Tang et al., 2005

Development of treatments for crustacean viral diseases

Most viral diseases cannot be treated effectively in crustacea since it is common for crustacea, such as shrimp to be infected by viruses without any apparent symptoms (Flegel, *et al.*, 2004). Crustacea do not have an adaptive immune response that enables them to recognise previous exposure to pathogens (Johnson, *et al.*, 2008). Consequently, vaccines are unable to be used as a method of prevention for crustacea. Preventing and controlling disease outbreaks lies in strengthening the innate immune response to viral diseases. However, the molecular mechanisms involved in antiviral immune responses remain unknown for the majority of crustacean species. Of the available antiviral immune responses, interferons (IFNs) are particularly interesting because of their ability to interfere in viral replication (Stark, *et al.*, 1998). Since the recent increase in the commercial production of human interferons, there is the possibility of using human interferons in antiviral therapy for crustacea. It may also be possible to use interferons that are present in the innate immune system of crustacea for treatment of viral infections since all living cells appear to be able to produce interferons (Sanders, 1981). In fact, information on any interferon-based antiviral system in crustacea which resembles or has functions similar to that of vertebrates is poorly understood. This is irrespective of a number of processes of the antiviral system being identified in crustacea, such as the innate immune response from Toll homolog in Chinese shrimp *Fenneropenaeus chinensis* (Yang, *et al.*, 2008) and the activation of the signal transducers and activators of transcription (STAT) in response to WSSV infection (Chen, *et al.*, 2008; Liu, *et al.*, 2009). In light of these studies, this review attempted to build an understanding of antiviral systems in crustacea. Expanding knowledge of any interferon-based antiviral system in crustacea

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may provide the link of antiviral immunity between vertebrates and invertebrates, as well as aid in developing treatments for viral infections in crustacea. This review will discuss the interferon system, the induction of type I IFNs and their pathways (focusing on interferon alpha and interferon beta) during antiviral immune response in vertebrates. Finally, this review will discuss evidence for several parts of interferon-based antiviral system in crustacean that may facilitate the general understanding if IFN or IFN-like proteins do exist in crustacea.

Interferon as an Antiviral Immune Response

Introduction to the interferons system

Interferons (IFNs) are a type of glycoprotein called cytokines that were first described for their interfering effect on viral replication (Issacs and Lindenmann, 1957). Later on, IFNs were shown to possess a wide range of biological activities (Biron and Sen, 2001) such as growth of cells, differentiation, apoptosis and modulation of the immune response (Samuel, 2001).

In the first line of defence against pathogens such as viruses, bacteria or parasites in vertebrates, antigen-presenting cells such as macrophages and dendritic cells detect the invading pathogens via pattern recognition receptors (PRRs) including membrane-associated Toll-like receptors (TLRs) (Kawai and Akira, 2007). These receptors recognise conserved pathogen-associated molecular patterns (PAMPs) that are present in microbial proteins, nucleic acids, lipids and carbohydrates (Smith, *et al.,* 2005). The main signalling of the IFN pathways involves specific receptors binding to the Janus kinases (JAKs), signal transducers and activators of transcription (STATs) followed by inducing expression of many genes that establish the responses (Stark, *et al.,* 1998). IFN-treated cells synthesise IFNs in response to viral infection by conferring an antiviral state to the cells (Samuel, 2001; Stark, *et al.,* 1998).

IFNs are separated into three groups based on affinity and activation of their receptors (Galligan, *et al.,* 2006): type I, type II (Smith, *et al.,* 2005) and type III (Zhou, *et al.,* 2007). Type I IFN consist of 13 α-genes encoding 12 IFN-α subtypes, one β-gene encoding a single IFN-β subtype, a single IFN-ω gene encoding IFN-ω (Mogensen, *et al.,* 2004; Smith, *et al.,* 2005), IFN-ε, IFN-κ and IFN-υ (Pestka, 2007). Type I IFNs are commonly found in mammals and bind to a specific type of cell receptor known as interferon alpha receptor (IFNAR) while IFN γ binds to a specific type of receptor known as interferon gamma receptor (IFNGR). Type II IFN consists of a single gene that codes for the cytokine IFN-γ (Smith, *et al.,* 2005). Type III IFN consists of IFN-λ molecules (IFN-λ1-3) and its isoforms (IL-28A, IL-28B and IL-29) that bind specifically to a heterodimeric receptor consisting of the interleukin 10 receptor (IL10R2) and class II cytokine receptor subunit interferon lambda receptor 1 (IFNLR1) (Lindahl, 2006; Smith, *et al.,* 2005; Vilcek, 2003).

Induction of type I Interferon

The important role of infectious agents in initiating the induction of IFN is becoming clear with the knowledge of TLR signalling in humans (Smith, *et al.,* 2005). TLRs are membrane-associated receptors that characterise an initial detection and response to pathogens, such as the nematode *Caenorhabditis elegans* (Fallon, *et al.,* 2001). So far, there are 11 known TLRs and each of them has a number of ligands indicating potential binding sites and act as integrators of signalling with other receptors (Smith, *et al.,* 2005). Some TLRs can change their properties to bind different ligands known as homo or heterodimers (Smith, *et al.,* 2005). Lipid-based structures are recognised by TLR2 (bacterial or spirochetal lipoproteins and peptidoglycan) in combination with TLR1 or TLR6 as heterodimers and TLR4 as a homodimer. Viral and or bacterial nucleic acids are recognised by TLR3 (double stranded RNA (dsRNA)), TLR7 and TLR8 (single stranded RNA (ssRNA)), and TLR9 (CpG motifs in DNA). Protein from pathogens such as flagellin are recognised by TLR5 and profilin are recognised by TLR 11 (but only in mice) (O'Neill, 2006; Smith, *et al.,* 2005; Yarovinsky, *et al.,* 2005).

All TLRs, except TLR3, use the general adaptor protein MyD88 that is responsible for signal transduction (Fitzgerald, *et al.,* 2001) (**Fig. 1**). The TLRs and interleukin 1 receptor (IL-1R) family share conserved cytoplasmic

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regions called the Toll-interleukin receptor (TIR) domains (Smith, *et al.*, 2005). The TIR domain on the cytoplasmic region of the TLRs interacts with the carboxy-terminal TIR of MyD88 allowing a downstream signalling pathway (Imler and Hoffmann, 2003). The serine-threonine kinase IL-1R associated kinase 1 (IRAK1), IRAK2 and IRAK4 are involved in signal transduction between MyD88 and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (**Fig. 1**) (Suzuki, *et al.*, 2002). Serine-threonine activation is linked to the activation of protein kinases such as the mitogen activating protein (MAP) kinase family, leading to activation of TAK1 which regulates p38 and the JNK activation pathways (Lee, *et al.*, 2000) that are linked to programmed cell death AP-1 (**Fig. 1**). TAK1 activation also discharges IκB releasing NF-κB to bind to the promoters of proinflammatory cytokines (**Fig. 1**), such as alpha (TNF-α), IL-6, and IL-12 (Smith, *et al.*, 2005). Although IL-6 alone does not stimulate the expression of Interferon Stimulated Genes (ISG), it improves the cellular response to IFN-α (Weihua. *et al.*, 2000) via a separate pathway.

Besides MyD88, there is an additional adaptor allowing the lipopolysaccharide (LPS) signalling cascade which leads to the activation of the MAP kinase family and NF-κB (Kawai, *et al.*, 1999). TLR2 and 4 have this adaptor molecule in common referred to as, TIR-containing adaptor protein (TIRAP) (**Fig. 1**). Furthermore, identification of a third adaptor protein called Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) (Yamamoto, *et al.*, 2002), also known as Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule 1 (TICAM1) is essential for the TIRAP independent pathway (Oshiumi, *et al.*, 2003a; Oshiumi, *et al.*, 2003b). Over expression of TRIF plays a crucial role in activation of the NF-κB-dependent promoter but unlike MyD88 or TIRAP, TRIF activates IFN-β expressed by the signalling pathway through TLR3 and TLR4 (Yamamoto, *et al.*, 2002). In general, there are two major pathways of initiating IFN induction by TLRs (Smith, *et al.*, 2005).

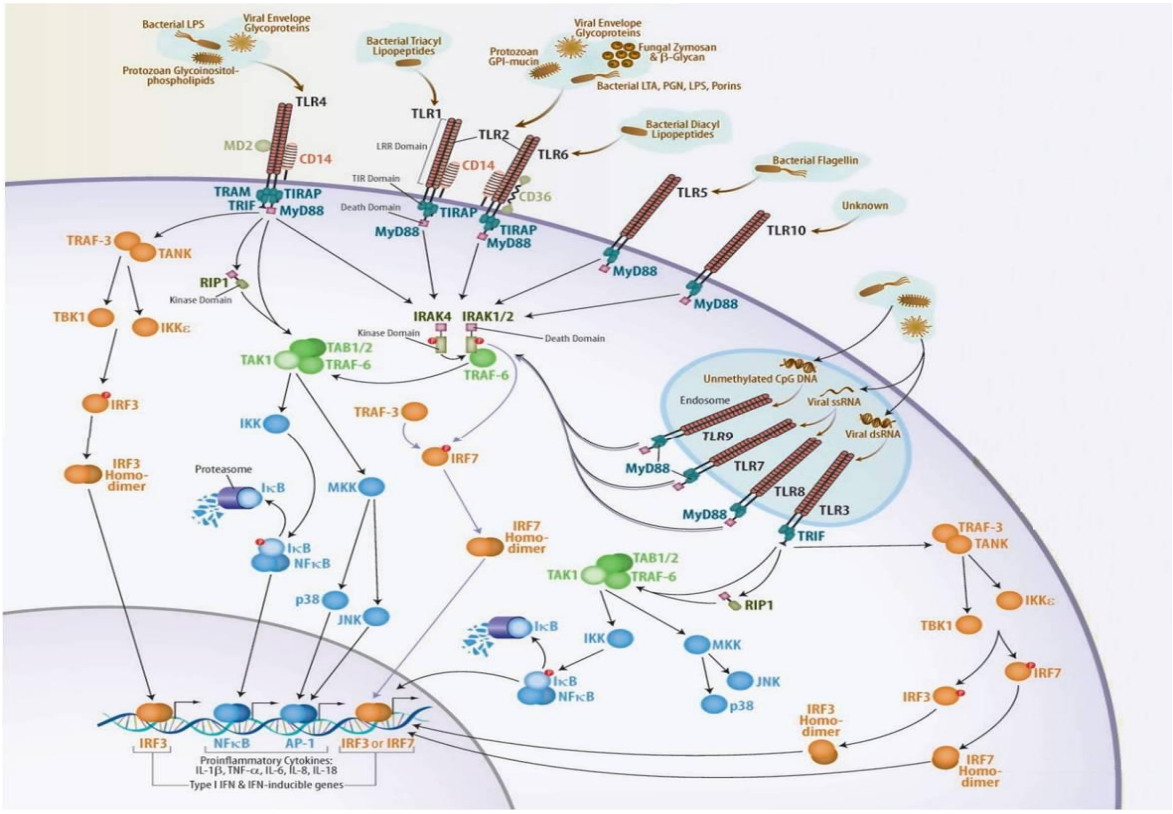

Fig. 1. Illustration of innate immune response involving Pattern Recognition Receptors (PRRs) and membrane-associated Toll-like Receptors (TLRs) (R&DSystems, 2009).

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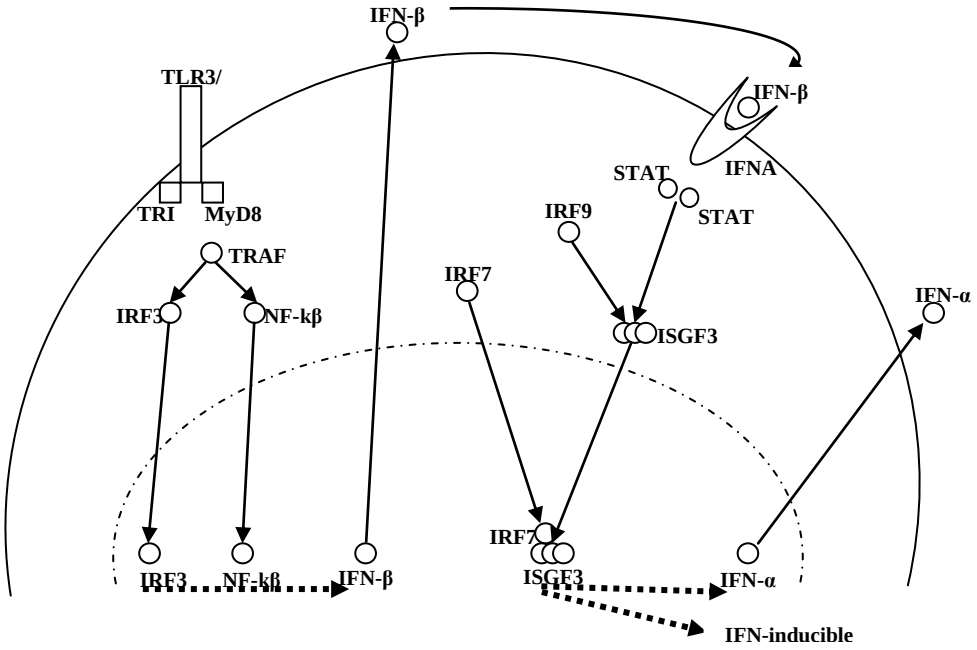

Fig.2. The two related pathways of induction of type I IFN (IFN-α and IFN-β). Initially, the induction of IFN-β begins with the activation of IRF3 and NF-κB stimulated by MyD88 and TRIF adaptors via TLR3 and TLR4. Secondly, IFN-β autocrinely stimulates the IFNAR resulting in the activation of the JAK-STAT pathway forming ISGF3 and IRF9 to promote the transcription of the IRF7 gene. IRF9, IRF7 and ISGF3 induce the production of IFN-α and prolongs the IFN response as well as encoding IFN-induced proteins, such as PKR, OAS and Mx proteins. Modified from Smith, *et al.,* (2005).

The JAK-STAT pathway

The JAK-STAT pathway of IFN-α and IFN-β requires two receptor subunits (IFNAR1 and IFNAR2), two Janus kinase (JAK) family of tyrosine kinases (Tyk2 and JAK1), two signal transducers, two transcription activators (STAT1 and STAT2), and the IRF family transcription factor p48 (IRF9) (Stark, *et al.*, 1998). Both IFN-α and IFN-β interact with Tyk2 and JAK1, leading to the phosphorylation of interferon alpha-receptor (IFNARs) which allows STAT2 to bind to it. This stimulates the phosphorylation of STAT2 generating a binding site for STAT1, resulting in the formation of STAT1-STAT2 heterodimer after dissociation from the receptor (Leung, *et al.*, 1995). This dimer migrates to the nucleus where it binds to the promoter region of the IFN genes (IRF9/p48) and forms a transcription complex, referred to as interferon-stimulated gene factor 3 (ISGF3) (Leonard and Sen, 1997). This facilitates transcription of the interferon-stimulated genes (ISGs) by binding to the interferon-simulated response element (ISRE) (Fu, *et al.*, 1990). The ISRE is a response element for genes stimulated by IFN-α (Fu, *et al.*, 1990). Type I IFN regulate pleiotropic effects as a result of infectious pathogens; typically the ability to confer an antiviral state (Stark, *et al.*, 1998). An antiviral state established in an infected cell inhibits protein synthesis and stimulates apoptosis reducing the capability of the invading virus to replicate. Hence, the rate of spread of infections also reduced (Smith, *et al.*, 2005).

Interferon-induced proteins

There are many antiviral responses regulated by IFNs through the transcription of several hundred genes (Levy and García-Sastre, 2001). The family of IFN-inducible genes is capable of encoding proteins, such as RNA-dependent protein kinase (PKR), oligoadenylate synthetase (OAS) and Mx proteins. PKR has the capability of inhibiting transcription by preventing viral replication via phosphorylation of the initiation factor eIF. This is different to the Mx proteins that interfere with viral replication by inhibiting viral polymerases (Stark, *et al.*, 1998).

The Oligoadenylate Synthetase (OAS) pathway consists of several enzymes encoded by multiple genes (Levy and García-Sastre, 2001). The role of the OAS pathway in the IFN-induced antiviral responses is supported

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through genetic manipulation of RNase L where its over-expression has been documented to inhibit vaccinia virus and HIV-1 replication (Stark, *et al.,* 1998). Besides facilitating an antiviral role, OAS also plays an important role in controlling cell growth and is effective in the regulation of several apoptotic pathways *(*Rebouillat and Hovanessian, *1999;* Stark, *et al.,* 1998).

Myxovirus-resistance (Mx) proteins are members of IFN-inducible genes of the dynamin-like GTPases (Samuel, 2001). They have the capability to block the replication cycle of a wide range of viruses in the early stage of infection (Haller, *et al.,* 2007). In addition, the range of antiviral activities of the Mx proteins is dependent on the specific Mx protein, its cellular site of localisation, and the type of virus (Samuel, 2001). Not all of these proteins have antiviral activity, but some do provide a resistant state to several RNA viruses when expressed in cells (Levy and García-Sastre, 2001). Specifically, IFN-α or IFN-β appear to be the most important regulators of the antiviral mechanism by Mx proteins (Simon, *et al.,* 1991). Mx proteins are a very sensitive marker for IFN actions (Gresser, *et al.,* 1988; Haller, *et al.,* 2007).

Evidence of Interferon-based Antiviral System in the Crustacea

Information concerning immune mechanisms directed against virus infections in invertebrates is limited. In fact, there are a lack of genes homologous to IFNs or to the main effectors that induce IFN responses, such as RNA-dependent protein kinase (PKR) (Robalino, *et al.,* 2004) in complete genome sequences of *Drosophila melanogaster* (Adams, *et al.,* 2000) and *Anopheles gambiae* (Holt, *et al.,* 2002). This is interesting, since invertebrates have several parts of interferon-based antiviral system described to be analogous to those present in vertebrates.

A number of other antiviral immune responses also have been described in crustacea. Antiviral activity that inhibits a variety of DNA and RNA viruses such as Sindbis virus, vaccine virus, vesicular stomatitis virus, mengo virus, banzi virus and poliomyelitis has been found in tissue extracts of the blue crab, shrimp and crayfish (Pan, *et al.,* 2000). Other studies proposed a number of immune responsive proteins/genes involved in WSSV pathogenesis in shrimp and crayfish (Dhar, *et al.,* 2003; He, *et al.,* 2005; Liu, *et al.,* 2006; Pan, *et al.,* 2005; Rojtinnakorn, *et al.,* 2002; Roux, *et al.,* 2002). Antibodies against WSSV envelope proteins, such as VP28 (Witteveldt, *et al.,* 2004a; Witteveldt, *et al.,* 2004b, VP68, VP281 and VP466 (Wu, *et al.,* 2005) prolong the survival of the shrimp after WSSV challenge (Liu, *et al.,* 2009). More studies are needed to determine the capability of these antibodies against the other viral proteins. A few other immune stimulants, such as bacterial lipopolysaccharide (Takahashi, *et al.,* 2000), glucans derived from fungi (Chang, *et al.,* 2003; Huang and Song, 1999), dsRNA (Robalino, *et al.,* 2007; Robalino, *et al.,* 2005; Robalino, *et al.,* 2004), and inactivated viruses (Bright, *et al.,* 2005; Melena, *et al.,* 2006) have also been described to elicit an antiviral immune response in crustacea.

There is some evidence for the role of an interferon-based antiviral system in crustacea. Firstly, double-stranded RNA (dsRNA) was found to actively induce an antiviral responses against two unrelated viruses, TSV and WSSV under controlled experimental conditions in *Litopenaeus vannamei* (Robalino, *et al.,* 2004). Similarly, interferon responses in vertebrates are also triggered by dsRNA. Secondly, IFN systems in vertebrates have TLRs as receptors that recognise microbes during infection (Akira, *et al.,* 2006). A Toll homologous to vertebrate TLRs was described from the Chinese shrimp *Fenneropenaeus chinensis* (FcToll) following bacterial and viral stimulation (Yang, *et al.,* 2008). The role of FcToll in the innate immune response may possess a similar role to dToll in *Drosophila*, although dToll does not act as the Pattern Recognition Receptor (PRR) in the host defence system (Hu, *et al.,* 2004). Recently, PmToll and lToll from *Penaeus monodon* and *L. vannamei* respectively have been described (Arts, *et al.,* 2007; Yang, *et al.,* 2007). However, their function in shrimp innate immunity against pathogens remains unclear.

IRF family members which are major regulators of a hosts defence in vertebrates (by the induction of IFN) (Tamura, *et al.,* 2008) are also present in all principal metazoan groups. One IRF gene has also been described from the Chinese mitten crab (*Eriocheir sinensis*) (Nehyba, *et al.,* 2009). Furthermore, the

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JAK/STAT system that is involved in signal transduction following cell activation by IFNs, has a crucial role in blocking viral infection in *Drosophila* (Lemaitre and Hoffmann, 2007) and mosquitoes (Lin, *et al.*, 2004). In shrimp, STAT is activated in the response to WSSV infection and the activated STAT switches on the promoter gene allowing viral transcription in the nucleus (Chen, *et al.*, 2008; Liu, *et al.*, 2009).

Overall evidence suggest that interferon-based antiviral system, analogous to those present in vertebrates, may exist in shrimp and that innate antiviral immunity in vertebrates is possibly linked with invertebrate innate immunity (Robalino, *et al.*, 2004). However, conclusive evidence of the existence of IFNs in crustacea remains elusive. A few studies (He, *et al.*, 2005; Mai, *et al.*, 2009) describe a recombinant interferon-like protein from the virus-resistant shrimp (*Marsupenaeus japonicus*). However, Rosa and Barracco (2008) dispelled these claims and argued that the molecule was only a portion of the mitochondrial F0-ATP synthase rather than an interferon.

Mechanism Model of Interferon-based Antiviral System in Crustacea

Based on current evidence for interferon-based antiviral system in crustacea, a model for the mechanism of interferon has been proposed (**Fig. 3**).

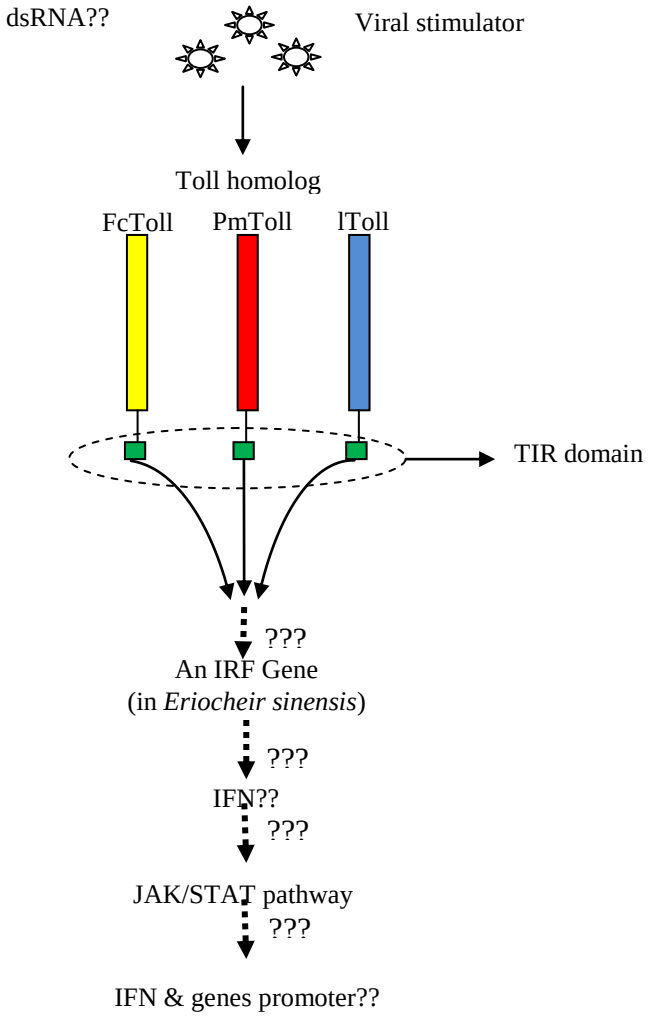

Fig. 3. A hypothetical model for the mechanism of IFN in crustacea. Innate immune response in crustacea may present after viral stimulation that is recognised by Toll homolog receptors, such as FcToll from *Fenneropenaeus chinensis*, PmToll from *Penaeus monodon* and lToll from *Litopenaeus vannamei*. The next pathways remain unknown until an IRF gene was found as a major regulator of host defences through the induction of IFN

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This model simplifies pathway of interferon-based antiviral induction in crustacea including several molecules that responsible to induce interferon. Firstly, invasion by a virus activates innate antiviral responses by Toll homolog receptors containing TIR domains (Yang, *et al.*, 2008; Yang, *et al.*, 2007). The activated IRF gene then encodes transcription factors (possibly by the induction of interferon) that are important in immune defences (Nehyba. *et al.*, 2009). Finally, the JAK/STAT pathway activates the promoter genes allowing viral transcription.

Conclusion and Future Studies

Innate immune responses against microbes such as bacteria, fungi, and viruses in crustacea have been identified in crayfish, crabs, lobster and shrimp (Liu, *et al.*, 2009). Among other innate immune responses, interferons are increasingly becoming the focus in investigations of the antiviral system in crustacea. Although interferons homologous to vertebrates have not yet been demonstrated in any crustacea, parts of an interferon-based antiviral system resembling the functions of interferons in vertebrates have been described. These observations are consistent with the presence of interferons or interferon-like proteins in crustacea. Therefore, instead of the IFN homology issue, focusing on the analogous functions of IFNs could be the key to understanding the evolution of antiviral immunity at the molecular level. Further evidence of similar molecules and pathways of interferons, would allow the development of novel strategies and therapies to control viral diseases in crustacean aquaculture.

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