Vibrio harveyi modulated gene expression in *Penaeus monodon* and *Fenneropenaeus indicus*

A Thesis submitted to Goa University for the Award of the Degree of
DOCTOR OF PHILOSOPHY
in
Marine Sciences

By

Mr. Sagar Nayak, M. Sc.

Goa University,
Taleigao Goa
(2011)

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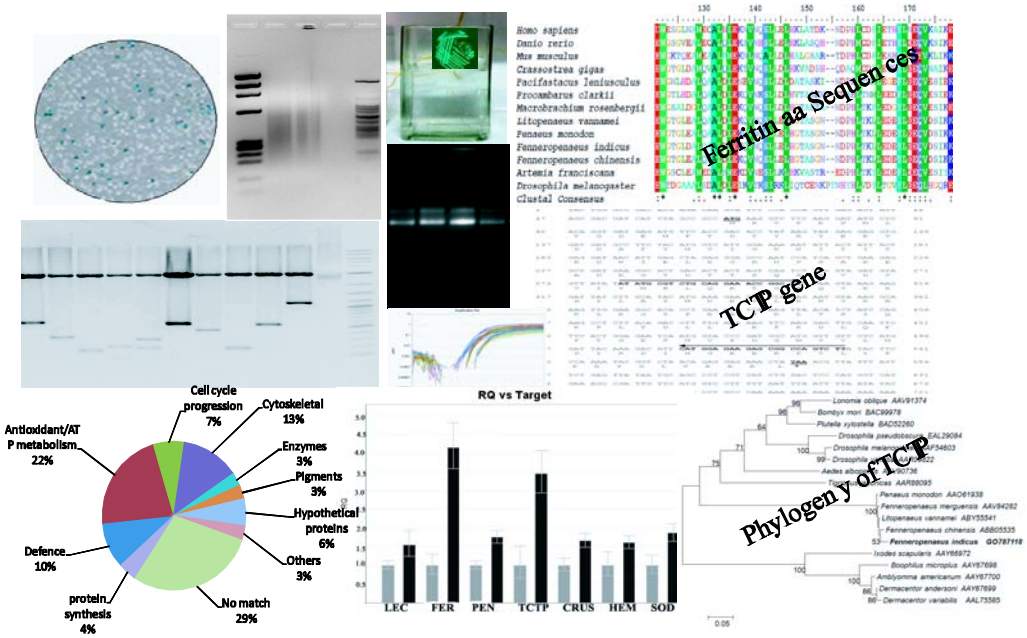

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Sagar Nayak

National Institute of Oceanography
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Under the Guidance

Of

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October 2011

Dedicated To My
Beloved Family

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CERTIFICATE

This is to certify that Mr. Sagar Nayak has duly completed the thesis entitled "*Vibrio harveyi* modulated gene expression in *Penaeus monodon* and *Fenneropenaeus indicus*" under my supervision for the award of the degree of Doctor of Philosophy.

This thesis being submitted to the Goa University, Taleigao Plateau, Goa for the award of the degree of Doctor of Philosophy in Marine Sciences is based on original studies carried out by him.

The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institutions.

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Date: October 4, 2011
Place: Dona Paula

DECLARATION

As required under the University Ordinance 0.19.8 (iv), I hereby declare that the present thesis entitled **"*Vibrio harveyi* modulated gene expression in *Penaeus monodon* and *Fenneropenaeus indicus*"** is my original work carried out in the National Institute of Oceanography, Dona-Paula, Goa and the same has not been submitted in part or in full elsewhere for any other degree or diploma.

The literature related to the problems analyzed and investigated has been appropriately cited. Due acknowledgements has been made wherever facilities and suggestions has been availed of.

Sagar Nayak

ACKNOWLEDGEMENT

I express my deep sense of gratitude and sincere thanks to my research guide Dr. N. Ramaiah, Scientist, National Institute of Oceanography, Goa, for his continuous guidance, useful advices and invaluable support throughout my research work. I thank him in helping me choose my research topic, allowing me to work under his supervision and for all the efforts he has put in reviewing the thesis and all my manuscripts. I appreciate all his contributions of time, discussions, critical comments and innovative ideas of presenting science effectively.

I thank Dr. S. R. Shetye, Director, National Institute of Oceanography for giving me an opportunity to be associated with this institute.

For this thesis I would like to thank my FRC committee members: Dr. S. C. Ghadi, Dept. of Biotechnology, Goa University for his kind consideration to be my co-guide and Dr. Savita Kerkar my VC's nominee, for her valuable comments, support and encouragement.

I thank Professor G. N. Nayak, the former Head of the Department of Marine Sciences, Goa University, and Professor H. B. Menon Head of the Department of Marine Sciences, Goa University, for their constant help and support.

I gratefully acknowledge the funding sources that made my thesis work possible. I thank the DBT, for providing initial financial assistance when I worked in the functional genomics project and CSIR for the senior research fellowship award which enabled me to complete my thesis research work.

I am grateful to Dr. Dileep Kumar for supporting me in his project during my tenure at NIO which helped me to continue with my thesis work.

I express my sincere gratitude to Mr. R. A. Sreepada for allowing me to use the aquaculture facility and for procurement of shrimp post-larvae whenever required for the experiments. I also thank all the lab members from the aquaculture group who have been very friendly and helpful to me all the time.

I would like to acknowledge Dr. M. P. Tapaswi, Librarian, and his staff for their efficient support in obtaining the literature and maintaining good journals.

I am indebted to all my labmates Dr. Jane Paul, Dr. Sanjay Kumar Singh, Dr. Veronica Fernandes, Dr. Sultan Alkawri, Dr. Rakhee Khandeparker, Vera, Geeta, Seema and Areena who have contributed immensely to my personal and professional life and for all the help and support they have rendered to me whenever I needed. Thanks to Mr. Ram Murti Meena for helping me with the sequencing of samples.

I sincerely appreciate Mr. Satyaranjan Sahu, Dr. Anil Pratihary, Dr. Karmabeer Jena, Dusmant, Udhaba and Priyabrata for their friendship, goodwill and support during my research work. My sincere thanks to all my other friends and colleagues whose names I could not include here for want of space.

I also take this opportunity to thank my long time friends Jayant and Shaktiprava who have always been with me in all times and looked out for my wellbeing.

I would like to thank my parents and family members for their love, support and encouragement.

Last but not the least; I thank God for giving me strength and courage to complete my work.

Thank you all…

Sagar Nayak

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Chapter 1

General Introduction

1.1 Introduction

In global trade, fish is also considered as the fastest growing among various agricultural commodities. Fish and fishery products represent one of the most valuable and nutritious food item for humans having most of the essential nutrients for human health. Fisheries sector occupies a very important place in the socio-economic development of the country. As a major source of export and foreign exchange earnings in many developing countries it has been recognized as a powerful income and employment generating entity as it stimulates growth of a number of other subsidiary industries. Most importantly, it is the source of livelihood for a large section of economically backward population of the country. In the recent years, there has been a significant growth in fish production in the country. Fish production during the year 2008-09 was 76.2 lakh tonnes which consisted of 29.8 lakh tonnes of marine fish and 46.4 lakh tonnes of inland fish. Further, there has been a steady growth in the export of fish products. Marine products-export in the country during April 2010 – March 2011 touched the US$ 2.84 billion mark for first time in the history of this industry and, the shrimp continued to be the major export commodity accounting for 44.26 % of the total US $ earnings (MPEDA, 2011). Shrimp exports during the period 2010- 2011 increased by 15.40%, 36.21%, 42.36% in quantity, rupee value and US $ value respectively (MPEDA 2011). This considerable increase in export figures is attributed due to high productivity of *Penaeus monodon* along with large scale production of *Litopenaeus vannamei.* As such, efforts are currently being made to enhance the export potential through diversification of products for export.

On a global scale, aquaculture is noticeably the fastest growing food-producing sector. In global trade, fish and fishery products are among the most sought after of various agricultural commodities. They represent the most valuable and nutritious food items for humans as they have most of the essential nutrients for human health. India's contribution to global aquaculture production is significant and ranks third in world aquaculture production. The aquaculture contribution produce of 3.12 million tones is nearly 45 percent of the total fish production of 7 million tones. The inland

and freshwater aquaculture sectors being the major contributors. Indian shrimp production was 0.4 million tonnes for the year 2009-10 from both the capture and cultured source. The *Penaeus monodon* and the Indian white shrimp *Fenneropenaeus indicus* are the two major commercial species of the penaeid family which have significant economic importance. *F. indicus* in India supports commercial fisheries in both marine and estuarine environments on the east and west coasts. India has been a major supplier of shrimp to countries like Japan, Europe, and USA.

Although the growth in the production of cultured shrimp has increased, there have been considerable, periodic losses due to disease of the farmed shrimps. An estimate for total global loss due to disease over the past 15 years may be in the order of US$15 billion (Flegel et al., 2008). This illustrates the significance of disease intervention/ control required for this industry. In this regard, infectious pathogens have a significant impact on the growth of the shrimp culture industry. The 2001 Global Aquaculture Alliance (GAA) survey with respect to agents responsible for diseases has already revealed that 58% of losses due to diseases were attributable to viruses and about 22% to bacteria (Flegel et al ., 2008). Therefore in the coming future, disease control ought to be a priority. The defense mechanisms of crustaceans are poorly understood, but knowledge of these is a prerequisite for the development of disease prevention strategies. Thus it is very important to obtain a better understanding of the defense system of the major cultured shrimp species, in order to design suitable health management practices. Basic knowledge of the interaction between the pathogens of cultivated shrimp and the defense responses of the animal are very poorly understood, which further complicates the development of intervention strategies. Therefore infectious diseases continue to be major barriers to the development of shrimp aquaculture (Mialhe et al., 1995; Rosenberry, 2001).

1.1.1 Biology of Penaeus monodon and Fenneropenaeus indicus

Crustaceans comprise a large, primitive and diverse animal group that includes many well-known, commercially exploited members, such as shrimp, crab, crayfish and lobster. Crustaceans are primarily marine organisms inhabiting the world oceans, but

they are also present in freshwater, terrestrial and semi-terrestrial habitats. This group of animals is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. *Penaeus monodon* and *Fenneropenaeus indicus* belong to the penaeid family of prawns. Penaeid shrimps belong to the largest phylum in the animal kingdom, the Arthropoda. The following is the taxonomic position of these two species.

1.1.2 Penaeus monodon

Giant tiger prawn, scientific name *Penaeus monodon* (Fabricius, 1798)

Phylum : Arthropoda
Subphylum : Crustacea
Class : Malacostraca
Order : Decapoda
Superfamily : Penaeoidea
Family : Penaeidae
Genus : *Penaeus*
Species : *monodon*

P. monodon can attain a maximum total length upto 336 mm and weigh between 60 to 130 g and exhibits the highest growth rate of all cultured penaeids (Lee and Wickins, 1992). This is one of the major commercial species of the world. It is a marine shrimp, which likes mud or sand bottoms at all, depths from shallows to 110 meters (360 feet). It is widely distributed over a huge range from East and Southeast Africa, through the Red Sea and Arabian Gulf, around the Indian subcontinent, and throughout the Malay Archipelago to Northern Australia and Japan. The species is also one of the major aquaculture shrimp species in India.

1.1.3 Fenneropenaeus indicus

The Indian white prawn, scientific name *Fenneropenaeus indicus* (H. Milne-Edwards, 1837) formerly known as *Penaeus indicus.*

Phylum : Arthropoda
Subphylum : Crustacea

Class : Malacostraca

Order : Decapoda

Suborder : Dendrobrachiata

Family : Penaeidae

Genus : *Fenneropenaeus*

Species : *indicus*

F. indicus in India supports commercial fisheries in both marine and estuarine environments on the east and west coasts. It attains up to 228 mm (nearly 9 inches) in length up to 14-15 g in weight. It is a marine shrimp (with estuarine juveniles), prefers mud or sandy-mud bottom and can be found from 2 to 90 m depth. It can tolerate low water quality, high salinities and high temperatures. It is readily available in the wild, can also attain sexual maturity and spawn in culture ponds. It is one of the most important shrimp caught off the East African coast, and is probably also the most important Indian commercial species, especially for the inshore fisheries and for rice field culture in Kerala. *F. indicus* in India supports commercial fisheries in both marine and estuarine environments on the east and west coasts.

1.1.4 Life cycle of penaeid prawns

The penaeid life cycle has several distinct stages that are found in a variety of habitats (Figure 1.1). Marine shrimps mature and breed only in the marine habitat. The females lay 50,000 to 1 million eggs, which hatch after some time into tiny nauplii. These nauplii feed on yolk reserves within their body and then undergo a metamorphosis into zoeae. This second larval stage feeds in the wild on algae, and after a few days metamorphoses again into the third stage to become mysis. The mysis looks like a tiny shrimp and feed on algae and zooplankton. After another three to four days it metamorphoses finally into post-larva with all the characteristics of adults. This whole process takes about 12 days from the initial hatching.

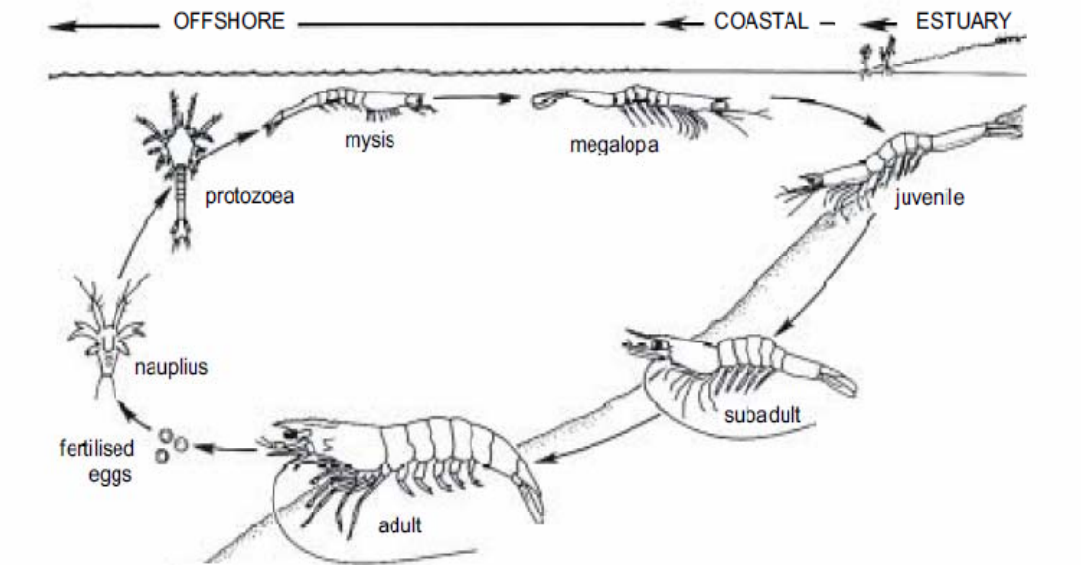

Fig. 1.1 Life cycle of penaeid shrimps. Eggs hatch within 16 hr after fertilization. The larval stages comprise nauplius (6 stages), protozoea (3 stages), and mysis (3 stages). The megalopa and early juvenile are also known as post-larvae (after Motoh, 1984).

Transition from juvenile to sub-adult takes some 135-255 days and subsequently completion of sexual maturity occurs. In the wild, the post-larvae then migrate into estuaries, which are rich in nutrients and low in salinity. There they grow and eventually migrate back into open waters when they mature. Adult shrimp are benthic animals living primarily in the sea.

1.1.5 Luminous bacterial diseases in farmed shrimps

Of the infectious diseases, bacterial and viral infections cause most of the production losses. These pathogens can cause detrimental effects either singly or in combination. Bacteria, though considered opportunistic or facultative pathogens, do cause serious diseases like systemic vibriosis and necrotizing hepatopancreatitis. Bacteria belonging to the genus *Vibrio* have been recognized as common disease causing agents in the farmed shrimps. The *Vibrio* disease is variously known as vibriosis or bacterial disease, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red-leg disease. The luminescent *Vibrio harveyi* is recognized as one of the major agent causing disease and mass mortalities of larval stages in penaeid shrimp hatcheries throughout the world (Lightner et al., 1992; Lavilla-Pitogo et al.,

1998; Chen et al., 2000). Undoubtedly, luminescent vibriosis is a known major constraint of shrimp production throughout the world. Signs of vibriosis in shrimps include lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity and melanization. In many cases, vibrios are opportunists, only causing disease when the host organism is immune suppressed or otherwise physiologically stressed. Most of these bacterial infections are strongly correlated to stressful conditions such as overcrowded ponds, high temperatures, and poor water quality.

1.1.6 Taxonomic position of Vibrio harveyi

Kingdom : Bacteria
Phylum : Proteobacteria
Class : Gammaproteobacteria
Order : Vibrionales
Family : Vibrionaceae
Genus : *Vibrio*
Species : *harveyi*

Vibrio harveyi is a Gram-negative bioluminescent marine bacterium. It is both a free-living, as well as a symbiont with many marine animals. In free-living form, it can be found throughout the water column, in the sediment as well as on the exterior surfaces of marine organisms. It causes systemic infection resulting in mortalities in larvae and post-larvae, which sometimes reaches up to 100% of affected population. The mode of *V. harveyi* infection is similar to many other bacterial diseases. It enters through the mouth and forms plaques then spreads to the guts and the appendages. It also causes loss of limb function, appendage degradation, lethargy and loss of appetite. As the disease progresses, the skin may become discolored, red and necrotic. Contamination can spread all the way to egg and larval tanks, thus causing an even bigger problem for shrimp farmers. Luminous vibriosis has been documented in many other crustaceans all of which glow in the dark when infected.

1.1.7 Defense mechanisms in shrimps against pathogens

Vertebrate immunity is composed of innate and adaptive responses and it is widely accepted that adaptive immunity is restricted to jawed vertebrates whereas invertebrates have only innate defense system to combat infections. This defense system includes the protection by physical barriers together with local (epithelial immunity) and systemic immune responses. Invertebrates including arthropods, lack lymphocytes and antibody-based adaptive immune system and therefore depend only upon the innate immune system to defend themselves against different pathogens (Salzet, 2001). Crustaceans lack the complex and highly specific adaptive immune system of vertebrates, which is based on lymphocytes, immunoglobulins and immunological memory (Soderhall and Cerenius, 1998). There are various strategies for the invertebrates to combat different invading pathogens. These internal immune defense responses are relatively less specific, but are fast and efficient against many types of microbes and intruders.

The innate defense system of crustacean is also known as natural or non-specific defense system which involves both cellular and humoral components (Figure 1.2). Both cellular and humoral mechanisms are activated upon immune challenge to the crustaceans. These two systems work synergistically for detecting as well as eliminating all foreign organisms which are harmful to the host (Jiravanichpaisal et al., 2006). The cellular components involve hemocytes which are responsible for various functions like encapsulation, nodule formation and phagocytosis (Johansson and Söderhäll, 1989; Ratcliff et al., 1985; Theopold et al., 2002). Whereas the humoral components include the secretion, activation and release of various molecules from the plasma stored within hemocytes such as anticoagulant proteins, agglutinins, phenoloxidase enzyme, antimicrobial peptides, protease inhibitors, histones, lysosomal enzymes, lipopolysaccharide, β-1, 3-glucan binding proteins, and recognition molecules etc. (Holmblad and Söderhäll, 1999; Jiravanichpaisal et al., 2006; Lemaitre and Hoffmann, 2007).

Besides the above mentioned processes, two more reactions: coagulation and melanization are activated immediately upon infection or injury and play vital roles in the defense mechanisms against invading pathogens. Melanization is a process in which phenolic intermediates and melanin formation takes place at the site of invasion or wound and the invading pathogen is then immobilized and eliminated from the host system (Lee et al., 2002; Söderhäll and Cerenius, 1998). One significant fact that has come out by studying the invertebrate immune system is that the cellular and humoral responses are very closely related to each other and cannot be separated completely in their immune responses.

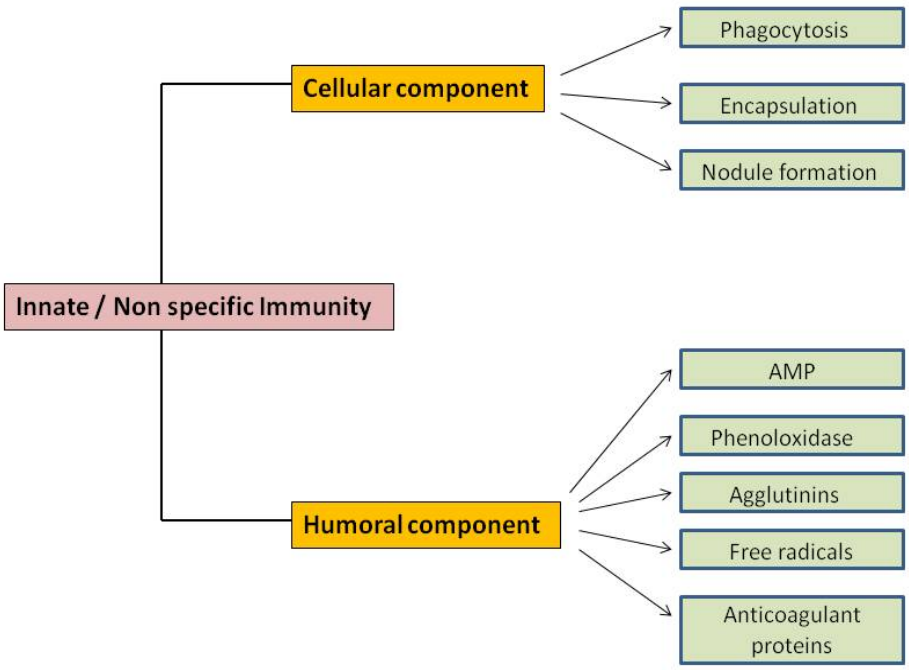

Figure 1.2 Cellular and humoral components of innate immune system in crustaceans (modified from Martinez, 1997)

1.1.8 Elucidation of disease pathology through study of differential gene expression

Alteration in gene expression is generally associated with a large spectrum of biological and pathological processes. The phenotype of an organism is determined by the combined activities of thousands of genes that are coordinated both temporally and spatially. A phenotype results ultimately from the expression of genes and gene products. An understanding of patterns of gene expression observed during changes (in physiological state, or as responses to pathogen challenge) leads to insights on molecular basis of phenotype from the cellular to the whole organism level. Thus, study of differentially expressed genes lead to generate insight into the molecular mechanism underlying disease progression or biological development.

Suppression subtractive hybridization (SSH) is one of the widely followed techniques to study differential gene expression (Diatchenko et al., 1996). This is a method for separating DNA molecules that distinguish between two closely related DNA samples in the absence of sequence information (Fig. 1.3). In subtractive hybridization, mRNA is first isolated from tester and driver populations. A tester is a population having a set of differentially expressed genes and driver is a population which is the control or the reference sample for the experiment. The mRNA is then converted to cDNA and a process of hybridization and selective amplification of differentially expressed genes leads finally to construction of a subtracted cDNA library. Construction of subtracted cDNA library is one the most effective and direct approaches for studying altered gene expression. Subtractive cDNA library allows cloning of genes that are expressed differentially in two different cell types. The representation of inserts derived from functionally expressed mRNAs found in a cDNA library also reflects the functional activities of the cells from which the library was established. The major advantage of subtractive libraries over other libraries lies in the fact that it allows cloning of genes without any previous knowledge of their sequences or functions.

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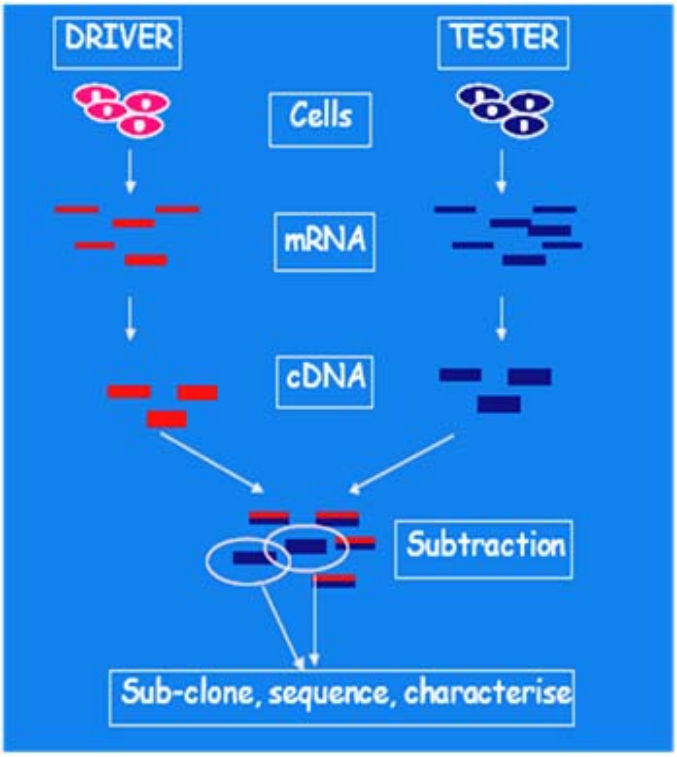

Figure 1.3 Schematic representation of suppression subtractive hybridization (after Diatchenko, 1996)

Disease prevention is always more important than treatment therefore studies towards a better understanding of defense mechanisms in shrimp is a significant problem-solving approach. Knowledge of immune gene expression in response to pathogens is of prime importance to understand the immune capability of shrimps and for further work on genetic selection and also on the establishment of health monitoring. Quantitative assays are useful for analyses of immune effectors and defense molecule encoding genes which thereby help evaluating the immune competence of the animal. In this context, the main aim of my research was to contribute to a better understanding of the defense system in the post-larvae of *P. monodon* and *F. indicus* upon challenge with a virulent strain of *V. harveyi*. Present study involved the use of SSH for construction of subtracted cDNA libraries in order to identify individual genes expressed in the post-larvae as a result of the challenge. Through qPCR

analysis, gene expression profiles of different immune related genes in the post-larvae of *P. monodon* and *F. indicus* were studied. Furthermore, this study also included full length cloning and characterization of two important immune transcripts: ferritin and translationally controlled tumor protein (TCTP) from the Indian shrimp *F. indicus*.

1.2 Objectives

While important progress is in place on key aspects of crustacean immune system and on elucidation of disease processes in farmed shrimps, much more information is needed on the molecular mechanisms of shrimp post-larval stages which are more susceptible to bacterial pathogens than the juvenile or adult populations. Studies on this aspect are limited mainly due to the lack of knowledge of shrimp genomic data and genome organization. Though *F. indicus* is one of the economically important mariculture species in many Asian countries, very few molecular studies in relation to its disease biology exist. Moreover, research reports on Indian prawn *F. indicus* using expressed sequence tag (EST) approach are very rare. Keeping these aspects in the forefront, the following objectives were planned.

- **To identify the genes up-regulated during *Vibrio harveyi* challenge in prawn post-larvae through cDNA library construction**.

  The rationale behind this objective was to identify key genes involved in the innate immune response of the post-larvae of both *P. monodon* and *F. indicus* which are highly susceptible to bacterial pathogens. In fact, there are almost no studies on gene expression changes in post-larvae of Indian shrimp during *Vibrio harveyi* infection.

- **Quantitative analyses of selected transcripts and their relative enhancement due to *Vibrio harveyi* challenge**

  Investigations on changes in immune gene expression in commercially valuable species will yield valuable information on immune mechanisms involved in the defense responses of the crustaceans. Also, quantitative analyses of gene

expression would give a more comprehensive idea on the effect of microbial challenges on individual gene products in the post-larvae.

- **Characterization and cloning of a few up-regulated genes in the shrimp due to *Vibrio harveyi* challenge.**

  Full-length cDNA sequences are superior means for identifying genes thorough homology searches and also in realizing their functions and products. They are important for the correct annotation of genomic sequences. They also facilitate the finding of transcription initiation and termination sites, 5′ and 3′ untranslated regions, promoter regions, and exon-intron splice sites.

Chapter 2

Review of Literature

2.1 Introduction

Shrimps are among the most economically important species in aquaculture due to their high demand world-wide. With a gradual but definitive gaining of experience and sustained effort in the development of production technologies, many cultured shrimp species have become important export products for many countries, in particular along the Indo-Pacific region. Besides, in many economies with rapid growth in the region, the domestic consumption of shrimp has also increased (FAO 2005). Consequently, penaeid shrimp aquaculture is one of the major industries which has rapidly grown during the past three decades in tropical and subtropical areas of the world (FAO 2009). It has become one of the few sources for economic development in coastal areas (Adger et al., 1998).

Shrimp culture has been a booming business since the beginning of the 1990s, reaching a worldwide production of 1 million metric tons in 2002 (Rosenberry, 2002). Marine products export in India during April – March 2011 was 2.84 billion US$. According to reports, farmed shrimps continued to be the major export item accounting 44.26 % of the total US$ earnings (MPEDA 2011). Shrimp aquaculture is thus a major commercial activity in India. The rise of large scale high density shrimp aquaculture industries has led to several problems in the management of shrimp diseases including pathogens (Kautsky et al., 2000). The major current viral diseases are white spot syndrome and yellow head diseases, which are caused by white spot syndrome virus (WSSV) and yellow head virus (YHV), respectively (Flegel et al., 1997). In addition, vibriosis, caused by bacteria in the genus *Vibrio*, is a major bacterial disease (Jiravanichpaisal et al., 1994). Tables (2.1a, b, and c) list the most common diseases that prevail in farmed penaeid shrimps throughout the world.

2.1.1 Outbreaks of vibriosis in reared shrimps

Vibriosis is ubiquitous throughout the world and all marine crustaceans, including shrimps, are susceptible. Epizootics occur in all life stages, but are more common in hatcheries. The outbreaks of these diseases have led to the near or total collapse of the

Table 2.1a List of important viral diseases in farmed penaeid shrimps.

Disease	Abbreviation / Name of disease Agent	Disease/Symptom	Reference
White spot syndrome	WSSV(White spot syndrome virus)	Darkened (red or pink) body surface and appendages, loose shell, heavy surface and gill fouling by external parasites. Shrimp become lethargic, mortality rates of 100% within days. White spots on the carapace and a red hepatopancreas.	Flegel, 1995; Wang et al., 1995; Wongteerasupaya et al., 1995; Lo et al., 1996
Baculoviral midgut gland necrosis	BMN (Baculoviral midgut gland necrosis virus)	Reduced growth, loss of apetitite, hepatopancreas usually turns white can be seen through abdomen in post-larvae, high mortality.	Francki et al., 1991,
Infectious hypodermal and haematopoietic necrosis	IHNV (Infectious hematopoietic necrosis virus)	Stunted growth, deformities, and cellular inclusion bodies. Affects cuticular epidermis, haematocytes, haemopoietic organs/connective tissue, deformed rostrums grow to one side.	Lightner, 1983; Bell and Lightner, 1984; Bonami, 1990;
Hepatopancreatic parovirus disease	HPV (Hepatopancreatic parovirus)	Microscopic lesions and tissue abnormality	Lightner, 1996
Taura syndrome (TS)	TSV (Taura syndrome virus)	Lesions in cuticle, gills, digestive tract and appendages. Multiple irregularly shaped and randomly distributed melanised cuticular lesions, death usually at moulting.	Brock et al., 1990; Lightner et al., 1994; Bonami et al., 1997

Table 2.1b List of important bacterial diseases in farmed penaeid shrimps.

Disease	Abbreviation / Name of disease Agent	Disease/Symptom	Reference
Vibriosis	*Vibrio* spp.	Larvae become weak opaque white. Infected larvae exhibit greenish luminescence when viewed in complete darkness. Infected adults show red to brown gills, atrophy of hepatopancreas with necrosis. Reduced feeding and lethargic swimming. Systemic infections results in mortalities of larvae and post-larvae reaching up to nearly 100%.	Lightner, 1988, 1996; Lightner 1993; Alvarez et al., 1998.
Shell disease	Chitinoclastic bacteria like *Vibrio*, *Aeromonas* and *Pseudomonas* groups	Appearance of brownish to black erosion of the carapace, rostrum, tail, gills and appendages. Blisters filled with cyanotic gelatinous fluid seen on carapace abdominal segment. Affected appendages show a cigarette butt-like appearance.	Sinderman, 1990
Necrotising hepatopancreatitis	NHP bacteria (alpha-proteobacterium)	Septic hepatopancraetic necrosis reduced feed intake, anorexia, soft shell, heavy surface fouling, and pallid hepatopancreas.	Lightner, 1992; Frelier et al., 1993; 1996.
Hepatopancreatic ricketisis	Unclassified rickettsia	Heavily infected shrimp are lethargic, off-feed, and with atrophy and pale coloration of the hepatopancreas.	Brock et al., 1986; Anderson et al., 1987; Lightner, 1996.
Shrimp tuberculosis	*Mycobacterium marinum* and *M. fortutum*	Multiple lesions in the muscle/cuticle, hepatopancreas appear dark due to melanisation, hemocytic nodules may be visible.	Lightner and Redman, 1986; Lightner, 1996

Table 2.1c List of important fungal diseases in farmed penaeid shrimps.

Disease	**Name of disease Agent**	**Disease/Symptom**	**Reference**
Filamentous bacteria fouling	*Leucothrix* spp.	Presence of fine colorless thread like growth on the body surface and gills, filamentous growth promote further fouling, mortality may result due to direct and indirect effects.	Brock and Lightner, 1990
Larval mycosis	*Lagenidium* spp. and *Sirolpidium* spp.	Causes mortalities in the larval or early PL stages. Infections due to *Sirolpidium* spp. are more often seen in late mysis and early PL stages. Infection of individual shrimp is typically lethal and may be accompanied by a vibriosis and a terminal bacteriemia.	Lightner, 1981; Baticados, 1988
Fusariosis	*Fusarium solani*	Lesions in gills appendages and cuticle, mortality associated with heavily infected populations only might be as result of productions of mycotoxins. Fungal hyphae and conidia are visible in tissue wet mounts.	Lightner, 1996

shrimp farming industry throughout the world. Although viral infections typically have more deleterious effects on shrimp farm stocks, vibriosis can also cause mass mortalities of farmed shrimps (Saulnier et al., 2000). Major epizootics of vibriosis have been reported for *P. monodon* from the Indo-Pacific region, *P. japonicus* from Japan, and *P. vannamei* from Ecuador, Peru, Colombia and Central America (Lightner, 1996).

For a long time many bacterial species from the genus *Vibrio* are generally known to act as disease causing agents in fish and shellfish (Roberts, 1978; Kinne 1980). Numerous *Vibrio* species have been reported as the causal agents of diseases in various penaeid shrimp species (Gopal et al., 2005). As Lightner (1993) observes, the outbreaks of vibriosis have been reported and have become a severe barrier for further development of shrimp aquaculture. Vibriosis is a bacterial disease responsible for mortality of cultured shrimps worldwide (Lightner and Lewis, 1975; Lightner et al., 1992; Lavilla-Pitogo et al., 1996; 1998; Chen et al., 2000). As such, *Vibrio* species are widely distributed in culture facilities throughout the world. *Vibrio*-related infections occur frequently in hatcheries, but epizootics and mass mortalities also commonly occur in pond reared shrimp species (Ruangpan and Kitao, 1991).

Outbreaks of vibriosis, caused by gram-negative bacteria in the family Vibrionaceae, may occur when environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within the shrimp hemolymph (Sizemore and Davis, 1985), or by bacterial penetration of host barriers. The exoskeleton provides an effective physical barrier to pathogens trying to penetrate the external surface of crustaceans. Both the foregut and hindgut also are great barriers to the pathogens access to the cytoskeletal system. *Vibrio* spp. are commonly found among the chitinoclastic bacteria associated with shell disease (Cook and Lofton, 1973) and they usually gain entry through wounds in the exoskeleton of the host (Jiravanichpaisal and Miyazaki, 1994; Alday-Sanz et al., 2002). The gills may appear susceptible to bacterial penetration because they are covered by a thin exoskeleton (Taylor and Taylor, 1992), but their surfaces are cleaned by the setobranchs (Bauer,

1998). The midgut composed of the digestive gland and the midgut trunk referred to as the intestine, is not lined by an exoskeleton and thus seems to be a possible site for invasion of pathogens present in the water, food and sediment (Ruby et al., 1980; Jayabalan et al., 1982).

Members of *Vibrio* are a perpetual component of the natural microflora of wild and cultured shrimps (Sinderman, 1990) and become opportunistic pathogens when natural defense mechanisms are suppressed (Brock and Lightner, 1990). Vibriosis is expressed by way of number of syndromes. These include: oral and enteric vibriosis, appendage and cuticular vibriosis, localised wounds, shell disease, systemic vibriosis and septic hepatopancreatitis (Lightner, 1996). They are usually associated with multiple etiological agents. However, some *Vibrio* species, or strains of certain species, have been identified as primary pathogens (Owens and Hall-Mendelin, 1989; Lavilla-Pitogo et al., 1990; Owens et al., 1992; de la Pena et al., 1995). Pathogenic strains of *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus* have caused massive epidemics in Thailand (Nash et al., 1992) and the Philippines (Lavilla-Pitogo et al., 1990). *V. anguillarum*, *V. campbelli*, *V. nereis*, *V. cholerae* and *V. splendidus* have also been reported in association with disease outbreaks in shrimps (Lavilla-Pitogo, 1990; Chen 1992; Esteve and Quijada, 1993; Sahul-Hameed et al., 1996). According to Jayasree et al. (2006) occurrence of five types of diseases: tail necrosis, shell disease, red disease, loose shell syndrome (LSS) and white gut disease (WGD) is by *Vibrio* spp. in *Penaeus monodon* from culture ponds of coastal Andhra Pradesh. Among these, LSS, WGD, and red disease had caused mass mortalities in shrimp culture ponds. Six species of Vibrio—*V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus* and *V. splendidus*—are associated with the diseased shrimp.

2.1.2 Vibrio harveyi as a normal component of natural microflora

Vibrio harveyi is one of the most commonly isolated marine *Vibrio* species, and can easily be found both as free-living organisms or associated with the normal intestinal microflora of marine animals (Alvarez, 1998; Gomez-Gil et al., 2004). The luminous

vibrios are found in coastal, oceanic waters, lagoons, suspended particles, zooplanktons, sediments, sessile, benthic organisms, in the digestive tracts and light glands of leiognathid fishes (Ramaiah, 1989). *Vibrio* species are phenotypically diverse and hence, complicate their proper identification. Species of the genus *Vibrio* are Gram negative, oxidase positive, grow on TCBS agar, and O/F positive. Besides these characteristics, they are amylase and gelatinase positive, indole producing, citrate utilizing and capable of growth at 35 and 40^{o}C (Hoa et al., 2000). The virulence factors implicated in vibriosis are many. They include the production of flagella (and associated motility), haemolysins, proteases and iron-sequestering siderophores, the presence of a hydrophobic surface antigen and the ability to adhere to and invade host epithelial cells (Wang and Leung, 2000). Virulence of *V. harveyi* is also associated with production of a toxic extra cellular proteins, and luminescence (Manefield, 2000). Inspite of the above studies the pathogenicity mechanisms are imprecisely understood.

2.1.3 Vibrio harveyi as a shrimp pathogen

As detailed above, vibriosis is one of the major disease problems in shellfish and finfish aquaculture. The luminescent *V. harveyi* is implicated as the major cause of mass mortality of all larval stages in penaeid shrimp-hatcheries throughout the world (Sunaryanto and Mariam, 1986; Lavilla-Pitogo et al., 1990; Karunasagar et al., 1994; Jiravanichpaisal et al., 1994; Pizzutto and Hirst, 1995). Luminous bacterial strains have been isolated from the hepatopancreas of moribund shrimps and *P. monodon* and identified to be *V. harveyi* (Liu et al., 1996). Post-larval stages of shrimps are particularly susceptible to *V. harveyi* succumbing to what has been termed luminescent bacterial disease (Lavilla-Pitogo et al., 1990). Among the *V. harveyi* isolates, some are virulent and some are not, suggesting a great deal of molecular and genetic variation within this species. Luminescent *V. harveyi* appears to release exotoxins (Liu et al., 1996) and may cause 80-100% mortality in *P. monodon* hatcheries. Nakayama et al. (2005) reported linear relationship between luminescence and toxicity of *Vibrio carchariae* to the shrimp. The pathogenic mechanism has also been recently attributed to bacteriophage (Vinod et al., 2006). Luminous bacterial disease outbreaks have been reported from Indonesia (Sunaryanto and Mariam, 1986),

Philippines (Baticados, 1988; Pitogo, 1988; Lavilla-Pitogo et al., 1990), Thailand (Boonyaratpalin, 1990) as well as from other Asian countries. Larval mortality associated with *Vibrio* spp have been reported in *P. monodon* and *P. merguensis* hatcheries in Indonesia, Thailand, Philippines, Taiwan and, Ecuador (Sulasmi et al., 1994).

Vibrio harveyi infections in *F. indicus* larvae have been reported by Prayitno and Latchford (1995) in *P. monodon* larvae by Lavilla-Pitogo et al. (1990). Similarly, *V. parahaemolyticus* infections in *P. monodon* juveniles and adults (Ruangpan and Kitao, 1991; Nash et al., 1992) and in *P. orientalis* juveniles and adults (Xu et al., 1994). The *V. vulnificus* infections in *P. monodon* juveniles and adults (Song et al., 1990; Ruangpan and Kitao, 1991; Nash et al., 1992; Chanratchakool et al., 1995.); *V. damsel* infections in *P. monodon* juveniles and adults (Nash et al., 1992.); *V. campbellii* infections in *P. orientalis* larvae (Xu et al., 1994) and *V. splendidus* infections in *P. monodon* larvae (Lavilla-Pitogo et al., 1990) are also available.

In general, mortalities due to vibriosis occur when shrimps are stressed by factors such as: poor water quality, crowding, high water temperature, low DO and low water exchange (Lewis, 1973; Brock and Lightner, 1990). High mortalities usually occur in post-larvae and young juvenile shrimps. *P. monodon* larvae suffer mortalities within 48 hr of immersion challenge with strains of *V. harveyi* and *V. splendidus* (Lavilla-Pitogo et al., 1990). Mortalities involving vibriosis have been reported in market sized *P. monodon* (Anderson et al., 1988). Adult shrimps suffering vibriosis may appear hypoxic, show reddening of the body with red to brown gills, reduce feeding and may be observed swimming lethargically at the edges and surface of ponds (Anderson et al., 1988; Nash et al., 1992). *Vibrio* spp. also cause red-leg disease, characterized by red coloration of the pleopods, periopods and gills, in juvenile to adult shrimps, and may cause mortality of up to 95% during the warm season (Chen, 1992). The eyeballs of infected shrimps turn brown and fall away and mortality occurs within a few days (Chen, 1992). Six *Vibrio* species, including *V. harveyi* and *V. splendidus* cause luminescence that is readily visible at night/in

darkened spaces, in infected post-larvae, juveniles and adults (Ruby et al., 1980; Lightner et al., 1992). Infected post-larvae may also exhibit reduced motility, reduced phototaxis and empty guts (Chen, 1992).

2.1.4 Histopathology of shrimps affected by vibriosis

Shrimps suffering vibriosis may display localized lesions of the cuticle typical of bacterial shell disease, localized infections from puncture wounds, loss of limbs/appendages, cloudy musculature, and localized infection of the gut or hepatopancreas and/or general septicemia (Lightner, 1993). Lesions of bacterial shell disease are brown or black and, appear on the body cuticle, appendages or gills (Sinderman, 1990). Affected post-larvae may display cloudy hepatopancreas (Takahashi et al., 1985). Gills often appear brown (Anderson et al., 1988). Septic hepatopancreatitis is characterized by atrophy of the hepatopancreas with multifocal necrosis and haemocytic inflammation containing high loads of bacteria. Epithelial cells detachment is usually not seen in the presence of probiotic, non-pathogenic bacteria (Chen et al., 2000). Pathogens like *Vibrio* spp., which cause detachment of the epithelium in the mid gut trunk, can affect high mortality in shrimp by eliminating two-layers that protect the shrimp from infections: the epithelium and the peritrophic membrane it secretes. In addition, loss of epithelium may affect the regulation of water and ion uptake (Mykles, 1977; Neufeld and Cameron, 1994). Hemocytes from the infected hepatopancreas generally appear poorly vacuolated, as result of decreased lipid and glycogen reserves (Anderson et al., 1988). As observed earlier vibriosis in case of *P. monodon* is usually associated with the formation of "spheroids" in the lymphoid organ (Nash et al., 1992).

2.1.5 Defense mechanisms against bacterial pathogens

Disease progression in eukaryote is reported to be mediated by programs of differential gene expression. Up to 15% of genes in higher eukaryotes are reported to express at only a certain physiological state (Wang and Lu, 1998). So the study of differential gene expression is a crucial factor for monitoring disease progression. Crustaceans have both cellular and humoral defense systems and haemocytes are the

one that play a major role in both these processes. Haemocytes are involved in phagocytosis of external material, confinement of pathogens by clotting coagulation hardening of the cuticle and encapsulation (Noga, 2000). Haemocytes are also associated with humoral systems such as the prophenoloxidase system, bactercidins and lectins (Kopacek et al., 1993) and their activation results in rapid clotting, cellular degranulation, and activation of the proPO system and subsequently the production of sticky molecules (Johansson and Söderhäll, 1992).

Though the specificity of haemocyte types is not well defined they are generally classified into hyaline, semi-granular and granular cells. Generally halinocytes have been associated with initiation of hemolymph coagulation (Aono and Mori, 1996), but are also phagocytic in some species (Bell and Smith, 1993). Being actively associated with phagocytosis in many crustaceans the granulocytes are larger in size and also possess lysosomal enzymes, important in immune defense system of hosts (Lorenzon et al., 2001). The semi-granular cell is an intermediate between the hyaline and the granular cell (Söderhäll and Cerenius, 1992), these cells participate actively in encapsulation of foreign particles and pathogens.

The first and essential internal defense process is the recognition of invading micro-organism(s), which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-lascencia, 2000). The invertebrate immune system presumably recognizes large groups of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (Söderhäll et al., 1996). Several types of recognition proteins have been described and are called pattern recognition proteins (PRPs). The PRPs recognize carbohydrate moieties of cell wall components of micro-organisms, like lipopolysaccharides (LPS) or peptidoglycans (PG) from bacteria and fungi (Söderhäll et al., 1996; Vargas-Albores et al., 1996). Some PRPs are lectins, and can work directly as agglutinins or opsonins (Kopácek et al., 1993; Söderhäll et al., 1996). After binding of the PRP ligand with the microbial component, a second site becomes active for cellular binding. Haemocyte activation is generated after this second binding step (Vargas-

Albores and Yepiz-Plascencia, 2000).

After detection of foreign material, haemocytes migrate chemotactically to the site of invasion resulting in inflammation, which also appears as a relevant event in case of the vertebrates. The open circulatory system demands a rapid and efficient defense, in which the proteoloytic cascades play an important role (Sritunyalucksana and Söderhäll, 2000). Haemocytes are involved in the synthesis, storage and upon activation discharge of proenzymes and substrates of the clotting and proPO cascades (Johansson and Söderhäll, 1992; Söderhäll et al., 1996; Sritunyalucksana and Söderhäll, 2000). The clotting mechanism entraps foreign material and prevents loss of haemolymph. The transglutaminase (TGase)-dependent clotting reaction of crustaceans is best described in the freshwater crayfish *Pacifastacus leniusculus* (Kopácek et al., 1993). The clotting reaction is induced when TGase is released from the haemocytes or tissues. The Ca^{2+} dependent TGase catalyses polymerization of the clotting protein, found in the plasma, to form a gel (Kopácek et al., 1993; Yeh et al., 1998).

The proPO-activating system in crustaceans is understood only for the freshwater crayfish *P. leniusculus* (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). It is elucidated from these studies that proteins of proPO system occupy a very prominent position in non-self recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active phenol oxidase (PO) by prophenoloxidase activating enzyme. The PO enzyme catalyses stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin. (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). Melanin is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. Melanized matter can often be seen as dark spots in or under the cuticle of arthropods (Söderhäll, 1982; Sugumaran and Kanost, 1993).

An important factor that is associated with the proPO system is peroxinectin, which was cloned from *P. monodon* (Sritunyalucksana et al., 2001). Peroxinectin has

two different functions: cell-adhesion and peroxidase activity. Crayfish peroxinectin is synthesized in the haemocytes, stored in the secretory granules in an inactive form and then released in response to stimuli and, activated outside the cells. Transmembrane receptors of the integrin family of the haemocytes play an important role in the cell adhesion function of peroxinectin (Johansson, 1989). The cell-adhesion is involved in attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading micro-organisms (Johansson and Soderhall, 1989; Kobayashi et al., 1990; Thornqvist et al., 1994). Phagocytosis is the internalisation of small foreign particles by individual cells. After ingestion, shrimp haemocytes, like vertebrate blood cells, use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994; Munoz et al., 2000). If large amounts of particles enter the body or if they are too large to be internalized, several haemocytes will cooperate to seal off the pathogens; these phenomena are called nodule formation and encapsulation, respectively (Söderhäll et al., 1996). Enzyme inhibitors, also produced by the hemocytes, are necessary to regulate the proteinase cascades and prevent over-activation and damage to the host tissue (Franssens et al., 2007). Serine proteinase inhibitors from the Kazal and Serpin families have been identified in crustaceans (Kanost, 1999). Also h2-macroglobulin, which serves as a broad spectrum protease-binding protein, is stored in the haemocyte granules (Armstrong and Quigley, 1999). In addition, haemocytes play the role of production and discharge of agglutinins (e.g. lectins) (Kopácek et al., 1993), of antibacterial peptides (Destoumieux et al., 1997; 2000) and of cytotoxic molecules such as lysosomal enzymes (lysozyme, esterases, phosphatases, phospholipases, peroxidases and proteases (Millar and Ratcliffe, 1994). For an efficient immune defense, all different components of the immune system must work together. Studies conducted in shrimps show that PO activity is mainly centered on granulocytes (Sung et al., 1998, Perazzolo and Barracco, 1997). Similarly lectins which are sugar binding proteins identified in the hemolymph of many crustaceans. While some lectins act as opsonins, others might directly protect against diseases (Matsushita et al., 1996). However, the biochemical structures of many lectins have not been studied clearly.

2.1.6 EST and gene expression studies in penaeid prawns

Expressed sequence tags (ESTs) are typically single-read cDNA sequences obtained from libraries that often represent a single tissue or an organism under pre-set experimental conditions. As such, ESTs can be interpreted as providing a snapshot of the physiological status of an organism (Adams et al., 1991). For organisms where genomic information is limited, ESTs also provide a rich resource for gene discovery (Adams et al., 1991). Assuming that EST frequency approximates levels of expression of a gene, one can also venture into comparing different libraries to make statements about changes in gene expression associated with a particular condition, health status, or developmental stage, as has been done for shrimp infected with virus in previous studies (Rojtinnakorn et al., 2002; Leu et al., 2007). However, relative levels of expression inferred from EST frequency should be considered tentative, and independent experimental confirmation must follow in order to rule out technical biases introduced during cDNA cloning and EST generation (Robalino et al., 2009). The discovery of genes with potential immune function and the characterization of their expression patterns provide vital clues to the molecular basis for immune responses in shrimp.

The first paper involving penaeid shrimp EST data was published in 1999 by Lehnert et al. (1999). In this study, three cDNA libraries were constructed from the cephalothorax, eyestalks and pleopods of *P. monodon*. Subsequent to the Lehnert et al. (1999) report, a second shrimp EST study was conducted using two closely related species of penaeid shrimp, the Pacific white shrimp, *L. vannamei*, and the Atlantic white shrimp *L. setiferus* (Gross et al., 2001). Four different cDNA libraries were constructed from two immune-related tissues (hemocytes and hepatopancreas) from each shrimp species. The study by Gross et al. (2001) was designed to identify immune-related genes as well as compare the four libraries Among the immune related ESTs, antimicrobial peptides (AMP) were most prominent (64%; 172/268) although they were restricted to the hemocyte libraries. Lectins (6.7%; 18/268) were the largest group of immune ESTs in the hepatopancreas, and they were identified exclusively in the hepatopancreas libraries only. Other immune-related ESTs encoded

serine protease, protease inhibitors, heat shock proteins, clottable protein and β-1, 3 glucan binding protein. Most of these were present in one tissue only, with only two of them (clottable protein and ferritin) being detected in both hemocytes and hepatopancreas.

As detailed above, in crustaceans, the hemocytes are the major immune effector cells, and they play important roles in both humoral and cellular-mediated immune responses, synthesizing and releasing many immune effector molecules (Sritunyalucksana and Söderhäll, 2000; Iwanaga, 2002; Smith et al., 2003). Supungul et al. (2002) were the first to use an EST approach to analyze gene expression profiles in hemocytes of *P. monodon*. The defense-related proteins identified from their study included components of the clotting and prophenoloxidase systems, antioxidative enzymes, AMPs, serine proteinase inhibitors and heat shock proteins. Three ESTs were found to encode full-length proteins, two of which were AMPs (antilipopolysaccharide factor (ALF), penaeidin), while the third was a heat shock protein (cpn 10). This was the first time that ALF and cpn 10 had been identified in a penaeid shrimp. Two years later, Supungul et al. (2004) published a related study in which another 447 EST clones generated from *V. harveyi* challenged *P. monodon* hemocytes were analyzed together with the above 615 clones from the normal hemocyte library. Penaeidins were the major AMP in the normal library (36.84%) followed by crustins and ALF (26.3% each). Conversely, in the challenged library, ALF out-numbered (50.0%) both crustins (25.0%) and penaeidins (18.8%), suggesting that *V. harveyi* infection induces the expression of ALF. ALF, crustin and penaeidin-3 genes were mainly expressed in hemocytes. In *V. harveyi* challenged shrimp, ALF expression was elevated, whereas expression of crustin and penaeidin-3 was lowered (Supungul et al. 2004).

2.1.7 Discovery of immune genes through suppression subtractive hybridization

The SSH was first developed in Clontech Laboratories (USA) to find out the genes that are differentially expressed between two samples of mRNA in eukaryotes from a testis specific cDNA library (Diatchenko et al., 1996). This technique can enrich for

rare sequences over 1,000-fold in one round of subtractive hybridization (Diatchenko, 1996). SSH is one of the most powerful methods for isolating differentially expressed, low abundance and, tissue-specific transcripts. It can increase the detection sensitivity by 10 to 100 fold and make the identification of quite rare genes possible (Sargent and Dawid, 1983). Differentially expressed genes, which have high, middle, low, or rare-abundance transcripts, can be cloned with equal probability by using the SSH. Additionally, only 0.5-2 μg of mRNA is required, and it is not necessary to physically separate single and double stranded DNA molecules (Sargent and Dawid, 1983; Hedrick et al., 1984; Duguid and Dinauer, 1990). The suppression PCR prevents undesirable amplification while the enrichment of target molecules proceeds. The technique of SSH has been used widely in the study of cell differentiation and development in animals and in cancer diagnosis in humans.

The SSH technique takes advantage of the differences in mRNA complexity (Robalino et al., 2009) between a sample of interest (e.g. cells infected with a pathogen) and a reference or control sample (e.g. cells that are not infected). One of the defining features of innate immune responses is their dependence on temporary mechanisms that are activated very early upon immune stimulation. In that, the functional genomics approaches are especially suited for characterizing early transcriptomic changes. Based on hybridization between cDNAs that exist in similar concentrations in both samples, and selective amplification of cDNAs that escape hybridization with molecules from the reference sample (i.e. differentially abundant in the sample of interest), libraries can be generated that represent pools of mRNAs that are either up or down-regulated in a condition of interest (Diatchenko et al., 1996).

SSH libraries have been exploited at a relatively high throughput analyses to identify genes from shrimps such as *Litopenaeus vannamei* (Reyes et al., 2007; Zhao et al., 2007). The responses of other penaeid shrimps to WSSV have also been addressed using SSH approaches (He et al., 2005; Pan et al., 2005; Wang et al., 2006), as also of their reactions to multiple infections and environmental stressors (He et al., 2004; de Lorgeril et al., 2005; de la Vega et al., 2007). The SSH was applied to study

gene expression in the prawn, *Litopenaeus stylirostris* infected with pathogenic bacterium *Vibrio penaeicida* and in *Penaeus japonicus* challenged with heat inactivated microbes (He and Liu, 2004). Identification of differentially expressed genes from different species of shrimp upon bacterial and virus challenge has been carried out by many workers worldwide. Identification of genes induced in shrimp due to viral challenges has been carried out by He et al ., 2005; de Lorgeril et al., 2005; de la Vega et al., 2007; Leu et al., 2007; Li and Brouwer, 2009; James et al., 2010; Prapavorarat et al., 2010. Collectively, these studies have uncovered a large numbers of candidate genes whose involvement in shrimp immune response will need to be addressed by use of further advanced experimental techniques. Though SSH, a differential expression cloning technique can provide a first approach to describing transcriptomic changes associated with immune responses, ultimately however, gene expression needs to be directly evaluated to confirm regulation of genes under conditions of immune challenge.

2.1.8 Significance of full length gene cloning

It has been observed that only about a third of the tentative unique genes (TUGs) as derived from EST projects could be annotated by the sequence homologs found in the published sequence databases (Leu et al., 2010). Although it is quite possible that some of these unmatched sequences truly represent unknown genes that exist exclusively in crustacean, many of these TUGs are unmatched either because the nucleotide or encoded protein sequences in TUGs are too divergent to be matched to their true homologous genes in other organisms or the available TUGs are so short that only untranslated regions are sequenced (Leu et al., 2010). The only way to solve these problems is to isolate and sequence the full-length cDNA clones. Full length cDNA sequences are a superior tool for identifying genes thorough homology searches and also for the functional study of genes and their products. They are important for the correct annotation of genomic sequences, and they also facilitate the finding of transcription initiation and termination sites, 5’ and 3’ untranslated regions, promoter regions, and exon-intron splice sites (Alexandrov et al., 2006). In the era of functional genomics, once an EST database has been compiled, the next logical step is the

construction and sequencing of a full length cDNA library (Seki et al. 2002; Carninci et al., 2003; Gerhard et al., 2004). The establishment of a full-length cDNA library for penaeid shrimp in the near future will therefore enable to decipher a lot of hidden information regarding the crustacean immune system. In this context, it was aimed in the present study to carry out cloning of full length genes of two important shrimp immune related molecules namely, translationally controlled tumor protein (TCTP) and ferritin. Both of these transcripts were found to be significantly upregulated due to *V. harveyi* challenge in this study.

Nowadays, the shrimp industry is facing considerable losses caused by both viral and bacterial diseases, which have a significant impact on shrimp aquaculture sector throughout the world. Economic losses due to disease have made it necessary to understand the disease response of the shrimps at the molecular level, which will finally lead to identification of major molecular mediators of innate immunity. They also help unravel the mechanisms of pathogenicity, and identify environmental modulators of the immune response. Compared to other crustaceans, only a few such studies are available for commercially important penaeid shrimps. Control of diseases is of major importance and can be achieved in different ways however, until now chemotherapy and management practices are the only methods available to lower the infection pressure in shrimp farming. Therefore, research on quantitative assays to monitor the defense system of penaeid shrimps and hence the health status should be of high priority. In addition, the cloning and characterization of genes encoding for immunity, and subsequent studies dealing with the expression and analyses of immune responsive genes during/following infection will lead to a better understanding of the functioning of the defense system (Gross et al., 2001). The combination of different approaches would unequivocally contribute to generation of improved understanding of shrimp immunology.

Chapter 3

SSH and EST analyses from *Vibrio harveyi* challenged *Penaeus monodon* post-larvae

3.1 Introduction

Shrimp aquaculture continues to face disease threats including those caused by pathogenic bacteria that gain entry into hatchery systems through water supply or food sources. Among reared shrimps, microbial infections are usually associated with high incidence of larval deformities and, mortality rates (Vandenberghe et al., 2003). As already mentioned earlier, vibriosis caused by *Vibrio harveyi* is one such prevalent bacterial disease which causes mass mortalities in *Penaeus monodon* larval culture and production facilities (Lightner, 1993; Saulnier et al., 2000). For sustainable-shrimp farming, systematic approaches to prevent and/or control diseases, in particular, at their early life-stages are therefore of great significance. Thus understanding molecular responses and defense mechanism(s) in shrimps against bacterial pathogens is essential.

Besides the production of antimicrobial defense related biomolecules, the innate immune/defense mechanisms in crustaceans involve general active processes like phagocytosis, pathogen encapsulation, nodule formation, agglutination and prophenoloxidase (proPO) activating system (Lee and Sodderhal, 2002). Although several genes involved in the antibacterial response of shrimps have been isolated and characterized earlier for *P. monodon*, few reports are available on responses at molecular level in the post-larval stages of *P. monodon* which are highly susceptible to *V. harveyi*. Identification of genes induced in shrimp due to microbial challenges has been carried out by many workers worldwide (He et al., 2005; Leu et al., 2007; de la Vega et al., 2007; Li and Brouwer, 2009; James et al., 2010; Prapavorarat et al., 2010). Most of them had used targeted tissue/organ systems from adult shrimp as a starting material for construction of the EST libraries. In this study 10-14 d post- larval stages of *P. monodon* bath challenged with *V. harveyi* strain D3 were examined by immersion method, a more natural way for challenging the shrimps.

In this study, an effort was made to identify the genes induced in *P. monodon* PL10-14 challenged with *V. harveyi* through the application of suppression subtractive hybridization (SSH), which is a widely used method for separating DNA molecules that distinguish two closely related DNA samples in the absence of sequence

information. Subtractive hybridization is a powerful tool for identifying differentially regulated genes involved in disease pathogenesis.

The procedure for SSH includes six general steps (Fig. 3.1). To begin with first total RNA is isolated from cell/tissue/organism types being compared. The RNA population containing the differentially expressed sequences is termed as "tester", while the "driver" population works as reference in the experiment. From tester and driver mRNA fragments, double-stranded cDNA fragments are synthesized and digested with a four base-cutting restriction enzyme (*Rsa* I) to create blunt ends (step 1). The tester cDNA fragments are then subdivided in two fractions and ligated with two different kinds of adapters (step 2). Then to a small amount of each tester cDNA excess driver cDNA is mixed. In the third step, the samples are heat denatured and then allowed to anneal, generating the following types of molecules i.e. type a (ss-tester), b (tester-tester), c (tester-driver) and d (driver-driver homohybrid and ss-driver). During this first hybridization step the concentration of high and low abundant cDNAs is equalized, because the reannealing process is faster for more abundant molecules. Less abundant cDNAs remain single-stranded. During the second hybridization (step 4), the two primary hybridization samples are mixed together without denaturing. Under this condition, only the remaining subtracted ss tester cDNAs can reassociate and form a new hybrid type (type e, tester1-tester2). These new hybrids contain the Adapter 1 sequence at one 5'- terminus and the Adapter 2 sequence at the other 5'-terminus. A second hybridization is again repeated and the entire population of molecules is then subjected to PCR using adapter primers, to amplify the desired differentially expressed sequences. During the first cycle of the primary PCR, the adapter ends are filled in by DNA polymerase creating primer-binding sites (step 5). As type d (driver-driver and ss-driver) molecules do not contain primer binding sites, they cannot be amplified. Type a (ss-tester) and c (tester-driver) molecules have only one primer annealing site and can only be amplified linearly. Type b (tester-tester) molecules contain long inverted repeats on the ends and form a pan like structure that prevents their exponential amplification (PCR suppression effect). In this way only type e molecules, having two different adapters at both ends,

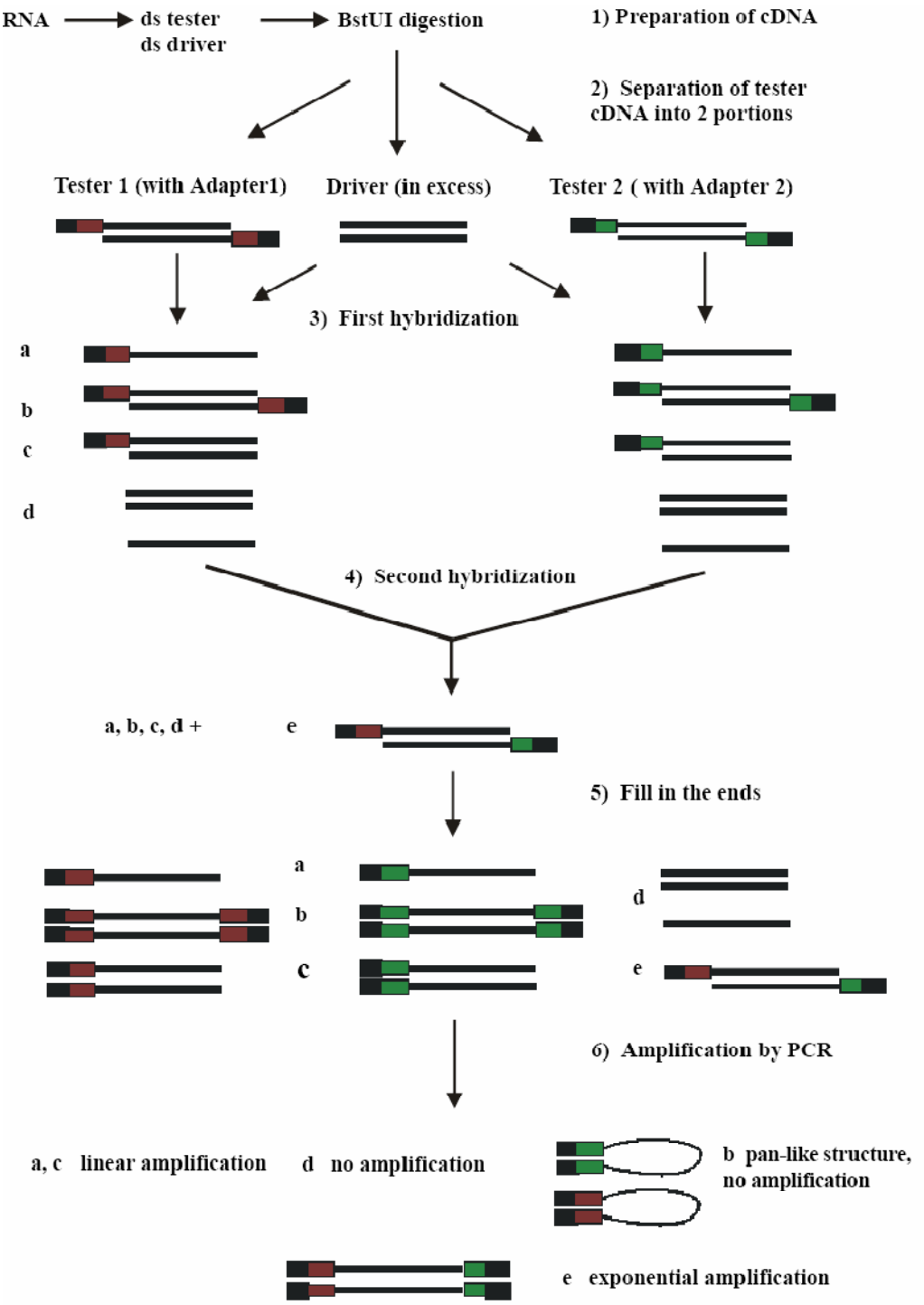

Fig 3.1 Schematic representation of the steps involved in suppression subtractive hybridization (Diatchenko, 1996)

are exponentially amplified. Later a secondary round of PCR amplification is performed using nested primers to further enrich differentially expressed sequences and suppress abundantly expressed genes. The final products can be cloned in vectors thereby enabling construction of subtracted cDNA libraries.

3.2 Materials and Methods

3.2.1 Isolation and biochemical characterization of Vibrio harveyi

Seawater and sediment samples were collected from intertidal regions off Caranzalem and Dona Paula in Goa. These samples were plated on thiosulphate citrate bile salts sucrose (TCBS) and incubated overnight at 37°C. The plates were examined in a darkened room and the luminescent colonies were picked out for further studies. Thirty presumptive isolates of *V. harveyi* were isolated from these sediment and sea water samples. Standard diagnostic biochemical tests were employed for identifying luminescent isolates (Ramaiah and Chandramohan, 1993). In brief, following tests were carried out to characterize *Vibrio harveyi* isolates. They were: indole production, citrate utilization, hydrogen sulphide production, oxidation-fermentation, starch hydrolysis, lipase and, gelatinase production. Five isolates showing bright luminescence and lipase activity were then chosen for further studies. Isolates were streaked on to fresh plates of TCBS agar, subcultured and maintained on TCBS slants until taken up for further studies. In the initial screening for pathogenicity, five strains of *V. harveyi* were examined for their ability to cause any clinical signs of morbidity or mortality in post-larvae (PL10-15). Experiments were set up in duplicate containing minimum of 20 PL per tank. The cells of *V. harveyi* strains grown in seawater nutrient broth were harvested by centrifugation at 5000 rpm for 10 minutes and cell concentrates were added in to experimental tanks to be able to achieve at least 10^4 viable cells ml^{-1}. All the experiment tanks were aerated and the PL were held for 4 days. Mortality, if any, during this period was recorded.

3.2.2 Immune challenge of post-larvae

Ten day old post-larvae (PL10) of *P. monodon* were obtained from a commercial hatchery that maintained them in cisterns (50 cm x 50 cm x 20 cm [height]) with 25-

30L filtered seawater as to reduce bacterial load. After their reaching the laboratory, they were held in a 20 L aquarium tank filled with filtered seawater. The PL were held under continuous aeration. They were fed twice daily with artificial feed and maintained throughout the 4 day experimental period under aeration at 25°C and 28 psu salinity. For the immune challenge replicates of two glass containers of 2-liter capacity were filled with filtered autoclaved seawater. The PL14 of *P. monodon* (50 nos/ container) were acclimatized for a day in these containers under aeration. A strain of *V. harveyi* D3 isolated from sediment samples, identity confirmed through standard biochemical tests and 16S rDNA sequencing (GenBank accession No. HM486479) was used for challenging the PL. This strain had caused noticeable lethargy and >50% mortality in post-larvae of *P. monodon* in trial bath challenge experiments.

Briefly, 24 h old cultures in sea water nutrient broth were used for the experimental challenge. The primary inoculums for bath challenge were prepared by harvesting of cells through centrifugation and then resuspension in sterilized seawater. PL were 'bath' challenged by adding the harvested cells to attain 2.5- 3.2 (X 10^6) cells ml^{-1} in the experimental containers for 48hrs. Cell counts were estimated from the optical density (OD) values at 600nm and the corresponding colony forming units (CFU) were obtained by plating serially diluted broth culture. Two glass containers filled with 2L filtered autoclaved seawater holding ~60 PL without bacterial inoculums cells served as experimental controls.

3.2.3 Isolation of total RNA

Total RNA was isolated from both challenged and control populations of prawn larvae. Animals were first washed in sterile seawater and whole post-larvae were used for RNA extraction using Trizol Reagent (TRI, Sigma, USA) PL were first washed in sterile seawater and, 35 -40 whole PL (weighing ~500mg) were used for RNA extraction. The tissue was ground to fine powder using mortar and pestle by addition of liquid nitrogen. To the homogenized tissue, 10 ml of TRI reagent was added and further homogenized till a uniform suspension was obtained. The homogenate was kept for 10 min at room temperature for complete dissociation of protein complexes.

To the homogenate 1ml of BCP (1-bromo-3-chloropropane) was added. Samples were mixed by vortexing and left to stand at room temperature for 15 minutes and centrifuged at 12000 rpm for 15 min at 4°C. After centrifugation the aqueous phase containing RNA was carefully separated into a separate tube without disturbing the inter-phase. RNA was precipitated from the aqueous phase by mixing with ½ volumes of isopropanol. Samples were stored at room temperature for 10 min and centrifuged at 10,000 rpm for 8 min at 4°C. Supernatant was removed carefully without disturbing the pellet. To the obtained pellet 2.5 volumes of 75% ethanol was added and gently tapped for dissolving. Centrifugation was later carried out at 7,500 rpm for 5 min at 4°C. The supernatant of ethanol wash was carefully removed. RNA pellet was air dried for 3-5 min. Pellet was later dissolved in 50 µl of DEPC Water. The concentration and purity of RNA samples was determined spectrophotometrically by measuring the absorbance at 260nm. An aliquot of RNA sample was also run on 1% formaldehyde denaturing agarose gel for checking structural integrity.

3.2.4 Purification of poly A^+ mRNA from total RNA

The mRNA was isolated using Oligotex mRNA spin columns (Qiagen, USA). Oligotex suspension and elution buffer were heated at 37 °C in a water bath. About 0.25 mg of total RNA was used for mRNA isolation. Total volume of samples was made up to 0.5ml using RNAse free water and Oligotex suspension in a 1.5ml micro centrifuge tube. The suspension was mixed thoroughly by repeated pipetting and, incubated for 3min at 70°C in a water bath. Samples were removed from the water bath and centrifuged at 10,000 rpm for 2 min and supernatant discarded carefully by pipetting. Oligotex mRNA pellet was resuspended in wash buffer and transferred to a new RNAse- free 1.5 ml micro centrifuge tube and then centrifuged for 1min at 10,000rpm. Spin column was then transferred to new RNAse free 1.5 ml micro centrifuge tube and 20µl of hot (preheated at 70°C) elution buffer was added and centrifuged for 1min at 5000 rpm to elute pure mRNA. Structural integrity of mRNA was checked by running a 1µl mRNA sample on 1% agarose gel.

3.2.5 Suppression subtractive hybridization (SSH)

SSH was accomplished using the PCR select cDNA subtraction kit (Clontech, USA).

A forward subtraction was carried out using mRNA from control and challenged post-larvae. The mRNA from normal (control) population was treated as driver and from the challenged population as tester.

Synthesis of double stranded cDNA

Tester and driver mRNA (5 µl each) were taken in two separate 0.5ml RNAse free tubes and 1µl of cDNA synthesis primer (10mM) was added. Tubes were incubated at 70°C for 2min in thermal cycler (Eppendorff, USA). Tubes were later cooled on ice for 2min followed by addition of 2µl of 5X first strand buffer, 1µl of dNTP mix (10mM), 1µl of sterile water and 1µl of AMV Reverse transcriptase ($20U\mu l^{-1}$). Tubes were gently vortexed and incubated at 42°C for 1.5 hours in an incubator. The first strand synthesis reaction was then terminated by placing the tubes on ice. Second strand cDNA synthesis was carried out by adding the reaction mixture to a tube containing 16.0 µl of 5x second strand buffer, 5.0 µl of dNTP Mix (10mM), 4.0 µl of 20X second strand enzyme cocktail and 37.0 µl of sterile water. Reaction mixture was incubated at 16°C for 2 hours in thermal cycler. Later 2µl of T4 DNA polymerase was added and incubated further for 30min at 16°C. Finally 4µl of 20x EDTA was added to terminate second strand synthesis.

RsaI digestion and adapter ligation

Control, tester and driver cDNA populations were digested by RsaI restriction enzyme. The reaction mixtures were incubated at 37°C for 1.5hr. From the digested mixture 5µl was kept aside for analysis of RsaI digestion efficiency. Reaction was terminated by adding 2.5µl of 20X EDTA/ glycogen. Adapters were ligated only to the tester populations. The ligation master mix was prepared by adding 3µl 5X Ligation buffer, 1µl T4 DNA ligase (400units) and 3µl of sterile water. The tester cDNA population was divided into two separate pools which were ligated to two different adaptors (Adaptor 1 & Adaptor 2R). Reaction mixture was incubated overnight at 16°C. To create an unsubtracted tester control population 2µl from each of the adapter ligated cDNA were mixed. Reaction was terminated by addition of 1µl of EDTA /Glycogen mix.

Hybridization of driver and tester cDNA populations

Driver cDNA was added in excess separately to both the pools of cDNA having different adaptors. For the first round of hybridization 1.5µl of RsaI digested driver cDNA was added separately to a tube containing 1.5µl each of both the adapter ligated cDNA pools and 1.0µl of 4X Hybridization buffer separately. Samples were heated to 98°C for 1.5min followed by incubation at 68°C for 8hrs. In order to enrich the differentially expressed transcripts the two samples from first hybridization were mixed with 1µl of fresh denatured driver DNA, 1 µl of 4X Hybridization buffer and 2 µl of sterile water. From this reaction 1 µl of sample was placed in 0.5ml micro centrifuge tube and incubated at 98°C for 1.5 min in a thermal cycler. Freshly denatured driver was mixed simultaneously to the hybridization samples. Mixture was mixed by pipetting, centrifuged briefly incubated at 68°C for overnight. To the reaction mixture 200 µl of dilution buffer was added and heated at 68°C for 7 min in thermal cycler and finally stored at -20°C.

PCR amplification of differentially expressed genes

Differentially expressed cDNA were amplified using two rounds of thermal cycling. The PCR reaction was performed in final volume of 25 µl containing 2.5 of 10X PCR buffer, 0.5 ml of dNTP mix, 1.0µl PCR primer, 0.5µ of 50X advantage cDNA polymerases mix and 1 µl each of subtracted cDNA and corresponding unsubtracted tester control was taken as template. Thermal cycling (PCR) was carried out with an initial denaturation of 94°C for 25 sec followed by 30 cycles at 94°C for 10 sec, 66°C at 30sec and 72°C for 1.5 min. A second round of PCR was carried using 1 µl of the diluted amplified product from the previous reaction, 2.5 µl of 10X PCR reaction buffer, 0.5 µl of dNTP mix, 1.0µl each of Nested PCR primer 1 and Nested PCR primer 2R (provided in the kit) and 0.5µl of 50X advantage cDNA polymerase mix. Thermal cycling (PCR) was carried out with an initial incubation at 75°C for 5 min followed by 15 cycles at 94°C for 10 sec, 66°C for 30sec and 72°C for 1.5 min. From this reaction 8.0µl sample was kept for gel electrophoresis analysis.

3.2.6 Cloning of subtracted product using pGEM-T vector

The PCR products were pooled and purified using a PCR purification kit (Axygen, USA). Ligation reactions were set up in 0.5ml tubes containing 5µl of 2X Rapid Ligation Buffer, 1µl T4 DNA Ligase, 1 µl of pGEM-T Easy Vector (50ng) and 3 µl of PCR product. Reaction mixture was mixed by pipetting and incubated at 4°C overnight for obtaining maximum number of transformants.

3.2.7 Transformation by electroporation and library preparation

Ligation mixture was purified using a PCR purification kit (Axygen USA). Previously prepared *E.coli.*Top10 electrocompetent cells were used for transformation. Aliquot of 50µl competent cells were pipetted into the prechilled cuvettes and 2µl of ligation mixture was added directly into the competent cells and mixed gently with the pipette tip. Transformation was done by giving an electric shock at 2.5kv for 5milli seconds using in an electroporator unit (Bio-Rad gene pulser Xcell system, USA), 950 µl of LB media was added to the transformation mixture and incubated at 37°C for 1hr in a rotary shaker at 150 rpm. Transformation mixture was spread on LB-agar plates containing antibiotic (ampicillin-100 µg ml−1) and selectable markers (X-GAL and IPTG). Plates were incubated at 37°C overnight. After incubation at 37°C overnight, the plates were incubated at 4°C for 2-3 hours to allow for proper color development. Recombinant clones (white in color) were picked and grown overnight in 96 well plates containing LB Amp. All the clones were mixed with 30% glycerol and then stocked at -80°C.

Plasmid isolation by alkaline lysis method

Clones from the subtracted library were grown overnight in 1.5 ml LB with shaking. Cells were centrifuged for 5min and supernatant removed. In order to resuspend the pellet 100 µl of GTE (Glucose Tris EDTA) solution was added to it. After 5 min 200µl of freshly prepared lysis buffer (NaOH and SDS solution) was added and tubes were kept for 5 min. To the above reaction 150µl of 4M potassium acetate solution was added and tubes were placed on ice for 10 min. Tubes were later centrifuged for 1min. Supernatant was transferred to fresh tube & plasmid precipitated with 95% ethanol.

Supernatant was removed and washed with 70% ethanol. Pellet was air dried and 30µl of water was added to dissolve the pellet. Isolated plasmid checked by 1.5% agarose gel electrophoresis.

Restriction digestion of clones

Restriction digestion of the isolated plasmids was carried out with EcoRI in order to check the size of inserts. The reaction was performed in 10 µl reaction volume containing 1µl of EcoRI, 2µl of 10X buffer, 2µl of plasmid DNA and 5 µl sterile water. Reaction mixture was incubated at 37°C for 1hr and the digested mixture was later analyzed on a 1% agarose gel with a long-range ladder.

3.2.8 Sequencing, bioinformatic analyses of expressed sequence tags (ESTs)

All the plasmids were sequenced in single direction using M13F and M13R sequencing primers in an ABI 3130 Genetic Analyzer (ABI, USA). All raw sequences were manually screened for low quality regions and vector sequences using Vecscreen tool available at NCBI. After end trimming, removal of low quality regions and vector sequences, the sequences were analyzed using BLASTX and BLASTN algorithms. Database searches were limited to ESTs > 100 bases in length. The annotated sequences were then grouped into different categories of genes based on their physiological role and function. Blast matching's with E- Value < 10^{-3} were considered as significant and only these sequences were considered for categorization of ESTs into different groups. The generated EST were then submitted to the dbEST in the required format.

3.3 Results

Biochemical characteristics of *V. harveyi* are presented in (Table 3.1). Most strains produced indole, lipase and, gelatinase. They were negative for hydrogen sulphide production. All hydrolyzed starch, were glucose fermentative and capable of citrate utilizing. The 16S rDNA sequencing for the isolate *V. harveyi* D3 used in the bath challenge experiment showed 98 % homology to *V. harveyi* on BLAST analysis. The total RNA isolated from the challenged and control post-larvae population of *P.*

monodon using the TRI reagent had good structural integrity which was evident from the intact 18S and 28S ribosomal bands which could be seen clearly after the gel electrophoresis on a 1.5 % formaldehyde agarose gel (Fig 3.3). From gel electrophoresis the quality and yield of double stranded cDNA synthesis as well as digestion of double stranded cDNA with Rsa1 was confirmed to be successful (Fig. 3.5). After two rounds of hybridization and amplification of differentially expressed genes by PCR the cDNA from the subtracted samples showed enriched bands as compared to the unsubtracted cDNA sample (Fig. 3.8). In order to check discrepancy of reactions at different steps of the procedure a necessary control reactions was also processed simultaneously with the test samples. The control SSH reaction (provided with the kit) performed along with the experimental subtraction reaction showed complete subtraction of skeletal muscle cDNA from the driver ϕx174 /*Hae* III control DNA (Fig 3.7). After completion of the SSH reaction the efficiency of SSH was first evaluated by PCR with a house-keeping gene, β-actin. In this PCR, the depletion of β-actin transcript in the subtracted vis a vis the unsubtracted cDNA was considered as the basis for determining the efficiency of SSH. While β -actin PCR product was detected in the unsubtracted cDNA, they were not detected in the subtracted cDNA under the same PCR conditions. This suggests that a drastic reduction of highly abundant cDNAs was achieved.

From the subtracted library 1152 clones were randomly picked and sequenced. After Blast analysis matchings with E-value $<10^{-3}$ were considered significant and were further used for categorization of genes into different functional categories. After removal of low quality sequences and contaminants, as many as 790 ESTs were available for further analyses. Out of these 298 (37%) did not match to any of the sequences in GenBank. As many as 410 ESTs (51.89 %) were quite similar to sequences in the Database. These ESTs represented 45 different genes (Table 3.3). Apart from these, 20 hypothetical proteins (Table 3.4) were also identified. The remaining 140 ESTs had database matching of E- value $>10^{-3}$.

The annotated ESTs could be grouped into 12 functional categories based on their physiological and functional roles (Fig 3.11). Among these, three genes are likely Table

3.1 Results of biochemical characterization for isolates of luminescent *Vibrio harveyi* from TCBS plates (O, Oxidative, F, fermentative; Y, yellow, R, red). None of the strains produced H_2S in TSI medium

Isolate	Biochemical tests						
	Gelatinase	Indole	Citrate utilization	O/F	Starch hydrolysis	Lipase	TSI slant/butt
A	-	+	+	F	+	+	Y/Y
B	+	+	+	F	+	+	Y/R
C	-	-	+	F	+	+	Y/R
D	+	-	+	F	+	+	Y/Y
E	+	+	+	F	+	+	Y/R
A1	+	+	+	F	+	+	Y/R
B1	+	+	+	F	+	+	Y/Y
C1	+	+	+	F	+	+	Y/Y
D1	+	+	+	F	+	+	Y/Y
E1	+	+	+	F	+	+	Y/Y
F1	+	+	+	F	+	+	Y/Y
G1	-	+	-	F	+	+	Y/Y
H1	+	+	+	F	+	+	Y/Y
A2	+	+	+	F	+	+	Y/Y
B2	-	+	+	F	+	+	Y/R
C2	+	+	+	F	+	+	Y/Y
D2	+	+	+	F	+	+	Y/Y
E2	+	-	+	F	+	+	Y/Y
F2	+	+	+	F	+	+	Y/Y
G2	+	+	+	F	+	+	Y/Y
H2	+	+	+	F	+	+	Y/R
I2	+	+	-	F	+	+	Y/Y
F2	+	-	+	F	+	+	Y/Y
A3	+	+	+	F	+	+	Y/R
B3	+	+	-	F	+	+	Y/Y
C3	+	+	+	F	+	+	Y/Y
D3	+	+	+	F	+	+	Y/Y
G3	-	+	+	F	+	+	Y/Y
H3	+	+	+	F	+	+	Y/Y
H4	-	+	+	F	+	+	Y/Y

Table 3.2 Percent (%) mortality of post-larvae (PL 10) of *Penaeus monodon* bath challenged with five isolates of *Vibrio harveyi.* Twenty individuals were used for testing each of the five strains of luminous bacterial strains.

	V. harveyi strain					
Time (h)	C4	C3	B2	D3	A2	Ctrl
0	0	0	0	0	0	0
24	0	0	5	5	0	0
48	5	5	5	5	5	0
72	15	10	20	25	10	0
96	20	15	30	45	20	0

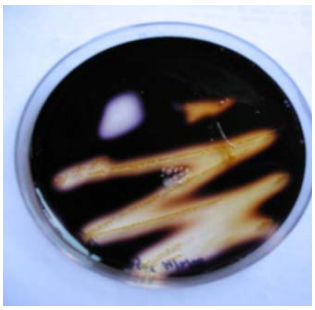

Fig A. Starch hydrolysis indicated by pale yellow and clear areas

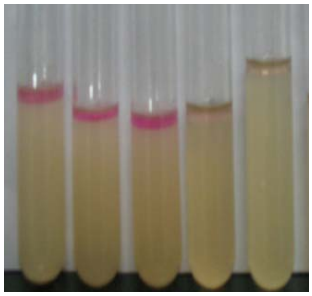

Fig B. Indole positive cultures showing the formation of red colored ring

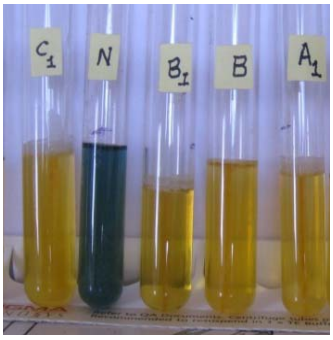

Fig C. Cultures showing positive oxidation and fermentation reaction indicated by the medium turning yellow

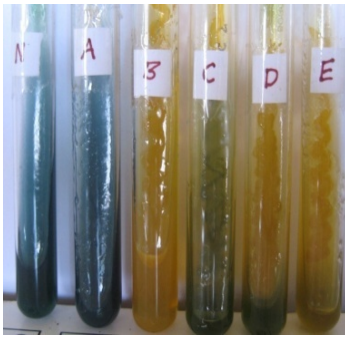

Fig D. Growth of *Vibrio harveyi* on TCBS slant

Fig E. Citrate utilization indicated by Simmon's citrate medium turning blue

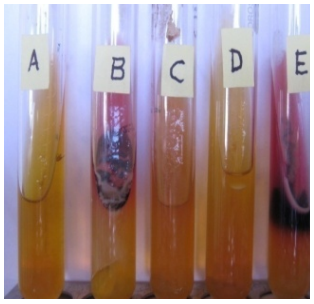

Fig F. Growth of luminescent bacteria on TSI slants

Fig 3.2 Results of various biochemical characterization tests carried out for the bacterial isolates

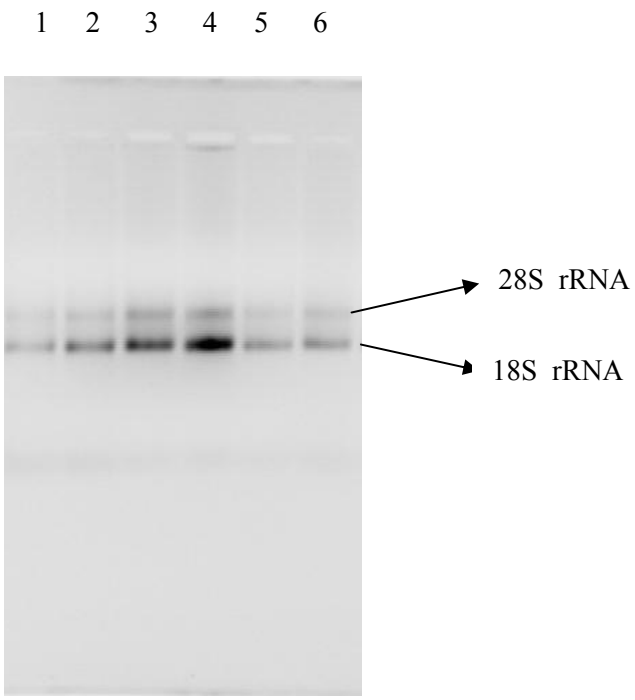

Fig 3.3 Total RNA isolated from post-larvae of *Penaeus monodon* run on a 1% formaldehyde agarose gel. Lanes 3 & 4 - Total RNA from control post-larvae, Lanes 1, 2, 5, 6-Total RNA from *Vibrio harveyi* challenged post-larvae of *Penaeus monodon*

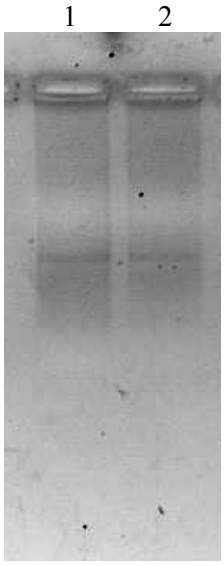

Fig 3.4 Isolated mRNA from control and challenged populations of *Penaeus monodon* postlarvae. Lane 1- mRNA from control post-larvae, Lane 2 - mRNA from *Vibrio harveyi* challenged post-larvae of *Penaeus monodon*

Fig 3.5 cDNA synthesized from mRNA of control and challenged populations of *Penaeus monodon* post-larvae. Lane 1- cDNA after *Rsa*I digestion, Lane 2 – cDNA before *Rsa*I digestion

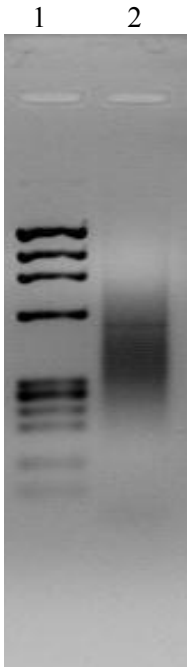

Fig 3.6 unsubtracted cDNA prepared before the subtraction procedure. Lane 1- ϕX174 DNA/Hae III digest marker, Lane 2 – unsubtracted cDNA post larvae of *Penaeus monodon*

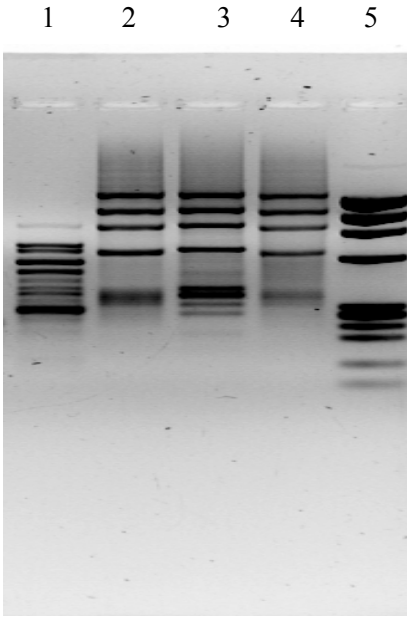

Fig 3.7 cDNA products obtained from the control subtraction reaction run on 1.5 % Agarose Gel. Lane 1- unsubtracted product, Lanes 2 and 3- Primary and secondary PCR products of subtraction, Lane 4- subtracted cDNA from control (human mRNA), Lane 5- ϕX174 DNA/Hae III digest marker

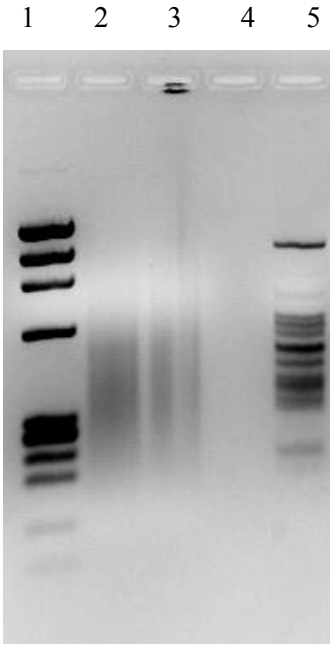

Fig 3.8 cDNA products obtained before and after the SSH reaction run on 1.5 % Agarose Gel. Lane 1- ϕX174 DNA/Hae III digest marker, Lane 2 & 3 - unsubtracted tester, Lane 5- Subtracted cDNA from *Penaeus monodon*

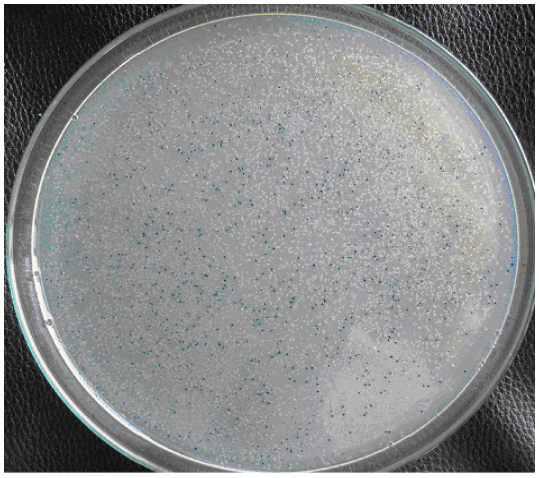

Fig 3.9 Subtracted products cloned using pGEM-T vector and transformed into into *Ecoli.*Top10 electrocompetent cells to generate subtracted cDNA library. Large number of positive transformants (white colonies) can be seen

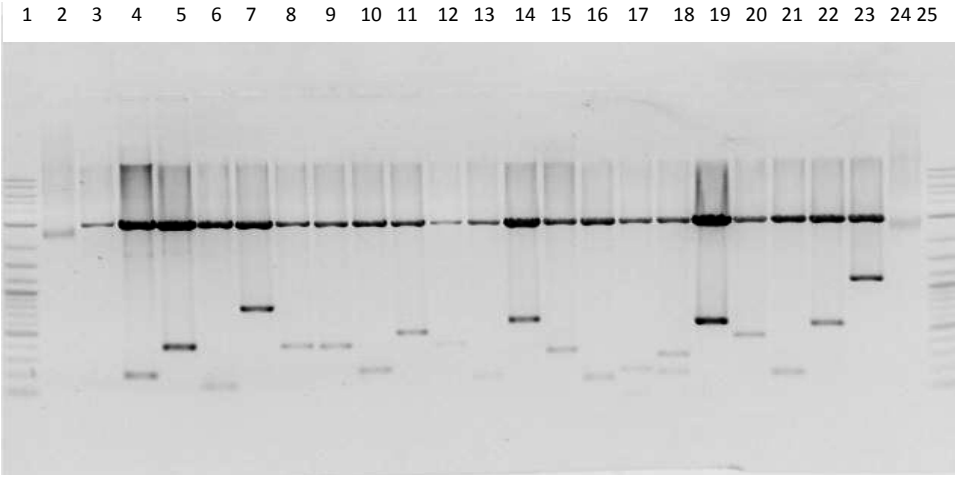

Fig 3.10 Restriction digestion of clones from *Penaeus monodon* SSH library. Randomly selected clones from *Penaeus monodon* library digested using *Eco*RI showing the inserts (ESTs) of various sizes.Lane1- 1Kb ladder, Lane 2 and 24 - uncut plasmid, Lanes 3 to 23- digested plasmids

Table 3.1 Results of biochemical characterization for isolates of luminescent *Vibrio harveyi* from TCBS plates (O, Oxidative, F, fermentative; Y, yellow, R, red). None of the strains produced H_2S in TSI medium

Isolate	Biochemical tests						
	Gelatinase	Indole	Citrate utilization	O/F	Starch hydrolysis	Lipase	TSI slant/butt
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C	-	-	+	F	+	+	Y/R
D	+	-	+	F	+	+	Y/Y
E	+	+	+	F	+	+	Y/R
A1	+	+	+	F	+	+	Y/R
B1	+	+	+	F	+	+	Y/Y
C1	+	+	+	F	+	+	Y/Y
D1	+	+	+	F	+	+	Y/Y
E1	+	+	+	F	+	+	Y/Y
F1	+	+	+	F	+	+	Y/Y
G1	-	+	-	F	+	+	Y/Y
H1	+	+	+	F	+	+	Y/Y
A2	+	+	+	F	+	+	Y/Y
B2	-	+	+	F	+	+	Y/R
C2	+	+	+	F	+	+	Y/Y
D2	+	+	+	F	+	+	Y/Y
E2	+	-	+	F	+	+	Y/Y
F2	+	+	+	F	+	+	Y/Y
G2	+	+	+	F	+	+	Y/Y
H2	+	+	+	F	+	+	Y/R
I2	+	+	-	F	+	+	Y/Y
F2	+	-	+	F	+	+	Y/Y
A3	+	+	+	F	+	+	Y/R
B3	+	+	-	F	+	+	Y/Y
C3	+	+	+	F	+	+	Y/Y
D3	+	+	+	F	+	+	Y/Y
G3	-	+	+	F	+	+	Y/Y
H3	+	+	+	F	+	+	Y/Y
H4	-	+	+	F	+	+	Y/Y

Table 3.3 Genes identified from the cDNA library prepared through suppression subtractive hybridization from *Penaeus monodon* post-larvae bath challenged with the strain *Vibrio harveyi*

GenBank ID	Transcript (no. of clones)	E -value	% ID	Species homology
Defense/Immunity				
GO344439	ferritin (30)	1.00E-65	100%	*Penaeus monodon*
GO349350	manganese superoxide dismutase (3)	1.00E-05	100%	*Taiwanofungus camphoratus*
GO349351	Zinc proteinase Mpc1 (2)	5.00E-27	95%	*Litopenaeus vannamei*
GO349352	zinc proteinase Mpc2 (2)	4.00E-29	96%	*Litopenaeus vannamei*
Antioxidant Metabolism				
GO349353	cyt c oxidase subunit III(13)	1.00E-65	93%	*Penaeus monodon*
GO349354	cyt c oxidase subunit I(3)	7.00E-92	92%	*Fenneropenaeus chinensis*
GO349355	NADH dehydrogenase subunit I(15)	3.00E-60	89%	*Penaeus monodon*
ATP Metabolism				
GO349356	F1F0 ATP synthase subunit G (19)	9.00E-28	59%	*Ixodes scapularis*
GO349357	H+ transporting ATP synthase (10)	2.00E-25	53%	*Bombyx mori*
GO349358	ATP synthase (2)	8.00E-72	84%	*Apis meliferra*
GO349359	ATP synthase Fo subunit6(2)	3.00E-53	84%	*Penaeus monodon*
GO349360	F1-ATP synthase beta subunit (2)	9.00E-75	88%	*Pacifastacus leniusculus*
Cell death/apoptosis				
GO349361	TCTP (15)	3.00E-51	100%	*Penaeus monodon*
Translation				
GO349362	ribosomal protein L10Ae (4)	2.00E-17	85%	*Tribolium castaneum*
GO349364	ribosomal protein L8(1)	5.00E-09	53%	*Litopenaeus vannamei*
Muscle/cytoskeletal				
GO349366	troponin I, fast skeletal muscle (32)	1.00E-14	92%	*Astacus flavuitilis*
GO349367	actin 2 (5)	1.00E-84	98%	*Penaeus monodon*

(Continued)

GO349368	actin 1(5)	4.00E-10	100%	*Penaeus monodon*
GO349369	actin D(1)	9.00E-32	92%	*Litopenaeus vannamei*
GO349371	actin, alpha, cardiac muscle 1 (1)	4.00E-18	100%	*Mus musculus*
GO349370	cardiac muscle actin (3)	8.00E-33	95%	*Homarus americanus*
GO349372	Beta actin(6)	6.00E-17	93%	*Penaeus monodon*
GO349373	Myosin light chain 2(1)	2.00E-16	88%	*Litopenaeus vannamei*
GO349374	myosin heavy chain (13)	6.00E-44	85%	*Farfantapenaeus paulensis*
GO349375	sarcoplasmic ca+ binding protein(9)	4.00E-57	95%	*Penaeus Species.*
GO349376	ATPase (2)	1.00E-14	81%	*Porcelio scaber*
GO349377	arginine kinase (4)	5.00E-100	98%	*Litopenaeus vannamei*
GO349378	allergen Pen m 2 (3)	3.00E-59	98%	*Penaeus monodon*
GO349379	Four and a half LIM domain (15)	2.00E-102	100%	*Macaca mulatta*
Enzymes				
GO349380	reverse transcriptase-like(6)	6.00E-22	40%	*Strongylocentrous purpuratus*
GO349381	catalse(3)	1.00E-09	96%	*Campylobacter jejuni*
GO349382	pyruvate kinase (7)	1.00E-46	97%	*Litopenaeus vannamei*
GO349383	serine carboxypetidase (17)	2.00E-22	42%	*S. purpuratus*
GO349384	trypsin (3)	4.00E-72	88%	*Litopenaeus vannamei*
GO349385	xylose isomerase (3)	1.00E-19	52%	*Bacterium Ellin514*
GO349386	similar to astacin-like protein (3)	2.00E-06	51%	*Strongylocentrotus purpuratus*
GO349387	Phosphoglycerate kinase(1)	4.00E-09	93%	*Branchiostoma belheri*
Eye stalk and vision				
GO349388	rhodopsin (15)	4.00E-09	74%	*Litopenaeus vannamei*

(Continued)

Pigments				
GO349390	crustacyanin-A2 subunit(1)	3.00E-09	70%	*Homarus gammarus*
GO349389	crustacyanin-C1 subunit(5)	8.00E-36	64%	*Homarus gammarus*
Others				
GO349391	Tetraspanin(11)	1.00E-19	49%	*Strongylocentrotus purpuratus*
GO349392	puroinodoline B protein(2)	1.00E-17	63%	*Triticum aestivium*
GO343530	elongation factor 2 (1)	2.00E-48	100%	*Penaeus monodon*
GO448020	unnamed protein product (1)	1.00E-22	47%	*Tetraodon nigroviridis*
GO448021	unnamed protein product (1)	1.00E-71	100%	*Homo sapiens*
GO448024	GK16683 gene product (1)	7.00E-17	58%	*Drosophila willistoni*
GO448025	GE10365 (1)	4.00E-07	50%	*Drosophila yakuba*
GO448026	DKFZP468L0125 protein (1)	8.00E-05	61%	*Pongo abelii*
GO448027	cuticle protein CUT5 (1)	7.00E-14	48%	*Portunus pelagicus*
GO448028	endothelial chloride channel (1)	3.00E-16	53%	*Danio rerio*
GO448029	Suppressor of profilin 2(1)	8.00E-19	51%	*Apis mellifera*

Similarity matches with E-value<10-3 are only included. When multiple significant similarities were found for a single cDNA, only the highest scoring hit is included in the table.

Table 3.4 List of ESTs encoding hypothetical proteins identified from *Penaeus monodon* cDNA library

GenBank ID	**Species homology**	**E- value**	**%ID**
GO448049	*Pongo abelii*	8.00E-16	88%
GO448044	*Trichoplax adhaerens*	7.00E-04	70%
GO448048	*Callithrix jacchus*	7.00E-04	59%
GO448041	*Caenorhabditis elegans*	2.00E-27	71%
GO448043	*Danio rerio*	1.00E-09	33%
GO448040	*Maccaca mulatta*	6.00E-04	59%
GO448042	*Escherichia coli*	3.00E-04	80%
GO448030	*Branchiostoma floridae*	4.00E-23	47%
GO448031	*Thermobia domestica*	4.00E-09	65%
GO448032	*Tetradon nigrovirdis*	8.00E-23	47%
GO448033	*Mus musculus*	1.00E-22	47%
GO448034	*Plasmodium chabaudi*	5.00E-49	97%
GO448035	*Trichoplax adhaerens*	6.00E-04	70%
GO448036	*Candida glabrata*	4.00E-16	75%
GO448037	*Macaca fascicularis*	4.00E-49	59%
GO448038	*Plasmodium chabaudi*	7.00E-36	94%
GO448039	*Plasmodium berghei*	2.00E-27	71%
GO448046	*Mus musculus*	7.00E-04	59%
GO448047	*Homosapiens*	7.00E-04	59%

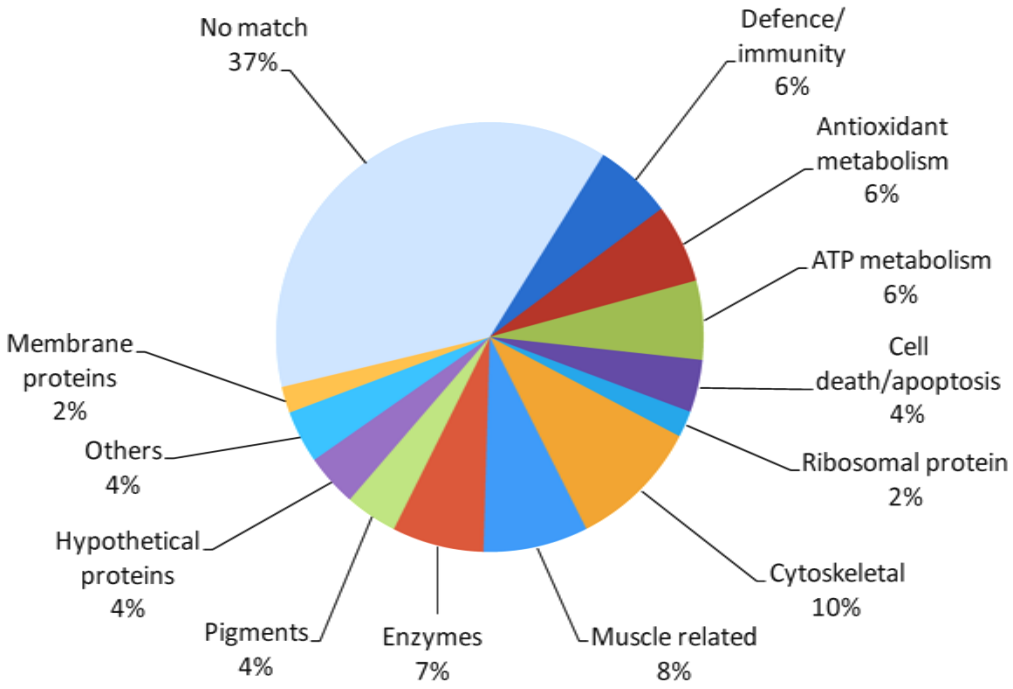

Fig 3.11 Relative proportion (%) of different categories of transcripts identified from the subtracted cDNA library prepared through suppression subtractive hybridization from *Penaeus monodon* post-larvae bath challenged with the strain *Vibrio harveyi* D3

to be immunorelevant and, eight others, related to antioxidant and ATP metabolism. The largest group comprised of the muscle and cytoskeletal related genes which were represented by 12 different transcripts followed by the enzymes which comprised of eight different genes.

The most abundant immune related genes in the subtractive library were ferritin, hemocyanin and translationally controlled tumor protein (TCTP). Besides a large number of sequences encoding antioxidant molecules, such as cytochrome C-oxidase subunit III, cytochrome C-oxidase subunit I and NADH dehydrogenase subunit-I were also identified. This category of genes, known to play significant role in antioxidant metabolism and oxidative phosphorylation pathways, contributed to 6% of the total identified transcripts. ATP metabolism genes such as F1F0-type ATP synthase subunit G and H^+ transporting ATP synthase involved in ATP metabolism were also found in large numbers. Expression of genes related to cytoskeleton motility was at a higher level in the challenged PL. Contributing to 10% of the total, this group of genes was the largest in this study. Furthermore, cDNA fragments encoding for allergens such as arginine kinase, myosin light chain and sarcoplasmic calcium binding protein were also present. Genes encoding for different enzymes such as pyruvate kinase, trypsin, catalase, xylose isomerase and phosphoglycerate kinase were detected, albeit in small numbers. Two genes related to crustacyanin pigment were also identified from the subtracted library.

3.4 Discussion

In this present study SSH method was used to investigate *V. harveyi* responsive genes in the post-larvae of *P. monodon.* Primarily, the frequency of occurrence of immune-related genes is reported to be very high in studies earlier to the present study. This is because the source materials for gene identification targeted were immune organs or tissue systems like hemolymph, hepatopancreas and lymphoid tissue (Somboonwiwat et al., 2006; Tassanakajon et al., 2006). The ESTs from the SSH cDNA library could be grouped into 12 different functional categories with the representing molecules having diverse physiological and functional roles. It is thus inferable from this study that a large number of genes are associated in the post-larval defense of *P. monodon* against *V.*

harveyi. These observations may prove useful for elucidating primary avenues for controlling luminous vibriosis in marine shrimps in their early stages.

3.4.1 Genes related to defense and immunity

In this category, clones of hemocyanin and ferritin were the most frequently encountered ones. Earlier studies (Pan et al., 2005; Zhang et al., 2006) had also found that the level of ferritin gene transcript was up-regulated after challenge with white spot syndrome virus (WSSV). This iron storage protein is crucial in the metabolism of iron as well as in the detoxification of this metal. Its expression is known to play a key role in maintaining iron homeostasis inside the cell (Marcelis, 1980). Binding of free iron is considered bacteriostatic, as bacterial cell growth is inhibited if no iron is available for synthesis of essential iron-containing compounds (Marcelis, 1980). It may also have a role in clotting, as it has been shown earlier in the case of *Drosophila melanogaster* (Karlsson et al., 2004). The high representation of ferritin ESTs in the subtracted cDNA library indicates its possible upregulation due to the *V. harveyi* challenge.

The EST fragments bearing 98 % similarity to the hemocyanin subunit L gene of *Marsupenaeus japonicus* were also numerous in the *P. monodon* library. As the principal component of haemolymph, haemocyanin contributes to 95% of the total amount of serum proteins (Khayat et al., 1995). Earlier studies have shown the evidence of generation of antibacterial and antifungal peptides from the C terminus of haemocyanin peptide from shrimps (Destoumieux-Garzon, 2001). Haemocyanin gene is multifunctional respiratory pigment performing important immune functions in crustaceans. Haemocyanin helps in activation of prophenoloxidase activity by partial proteolysis and also sometimes, can even function by itself as a phenoloxidase under certain conditions (Decker and Rimke, 1998). It is proven to be an important immune factor against WSSV (Zhang et al., 2004) and its diverse peptides are reported from *P. monodon* (Sritunyalucksana et al., 2001). From the occurrence of this gene in the subtracted library of *P. monodon*, its role as a major defense molecule against challenged *V. harveyi* is ascertainable.

ESTs relating to zinc proteinase Mpc1 and Mpc2 subunits were also identified in the library. Zinc proteinase belongs to family of metalloendopeptidase that have many different roles in biological systems like connective tissue remodeling and removal of signal sequences from nascent proteins (Gearing et al., 1994). In general, metal binding proteins such as ferritin and zinc proteinase are involved in animal's immune responses, and some are also involved in activating or degrading signal molecules (Dumermuth et al., 1991). This suggests that metal binding proteins such as ferritn and zinc proteinases may participate in shrimp's humoral response to the bacterial infection.

A single transcript coding for manganese superoxide dismutase (MnSOD), with proven role in antioxidant defenses (Pipe et al., 1993), was recorded. MnSOD plays an important role in crustacean immune defense by eliminating oxidative stress (Bendich, 1993). MnSOD tissue-specific expression in crustaceans may respond to particular types of invaders, as seen in previous studies on *Macrobrachium rosenbergii* challenged with *Lactococcus garvieae* (Cheng et al., 2006). Thus upregulation of MnSOD may be essential in the defense response of *P. monodon*.

3.4.2 Cell death/apoptosis related proteins

Interestingly, the TCTP with 25 clones was one of the most frequently encountered transcripts present in the library. Also known as fortilin, it is believed to protect cells from death under toxic conditions. Fortilin/TCTPs are highly conserved throughout the animal and plant kingdoms, and shrimp fortilin had 64% identity in amino acid composition with human fortilin (Bangrak et al., 2004). Earlier, fortillin gene was detected in WSSV-infected *P. monodon* using SSH. Its expression was reported to decrease during the terminal stage of the infection (Bangrak et al., 2004). TCTP has a proven role in 'histamine release factor' (HRF), triggering histamine release from leukocytes (MacDonald et al., 1995). TCTP also displays more general 'cytokine-like' activities, as it also induces the production of interleukins from basophiles and eosinophiles (Bheekha-Escura et al., 2000) and it is also reported to be induced by certain cytokines (Kang et al., 2001). This study reveals for the first time the upregulation and/or over expression of TCTP due to bacterial challenge. Thus, TCTP may play a critical role

in their response to bacterial infection, through regulation of a cell death pathway (Bangrak et al., 2004).

3.4.3 Muscle and cytoskeleton related proteins

Contributing to 18% of the total ESTs identified, the muscle and cytoskeletal proteins constituted a major category of genes identified in the library. As the cDNA library was prepared out of the whole animal tissue, transcripts of muscle and cytoskeletal specific genes such as actin, myosin, troponin and sarcoplasmic calcium binding protein were observed more frequently. Actins are conserved proteins that participate in muscle contraction, cell motility, cell division, and cytoskeletal structure (Urica et al., 1980). The reorganization of the actin and tubulin cytoskeleton plays a crucial role in many cellular functions such as phagocytosis, encapsulation, and nodule formation as well as cell shape change, cell motility, cell adhesion, and cytokinesis of shrimp haemocytes (Pollard, 1990). The upregulation of these genes result in effective measures to clear away the invading pathogens.

Interestingly, nine transcripts of sarcoplasmic calcium binding protein (SCP) were found in our library. The SCPs are an important Ca^{2+} binding system in the muscles of both vertebrates and invertebrates. (Gao et al., 2006). SCP has been recently described as a novel allergen in shrimp (Kazuo, 2008). Although expressed ubiquitously (Gao et al., 2006), it is usually abundant in muscle. Its increased expression suggests modulation of calcium ion concentration in *P. monodon* due to bacterial challenge.

3.4.4 ATP -metabolism related genes

Contributing ~6% of the total ESTs identified, genes related to ATP-metabolism such as ATP synthase, F1F0 type ATP synthase subunit g and H^+ transporting ATP synthase were quite frequent. ATP synthase (or H^+-ATP synthase) is the terminal complex of the respiratory chain and catalyzes the formation of ATP from ADP and inorganic phosphate (Boyer, 1997). The activation of ATP synthase genes could indicate a high increase in ATP demand which may be due to the increased demand for energy in the bacteria challenged post-larvae.

3.4.5 Antioxidant metabolism

Proteins, particularly those encoded by mitochondrial genes and those involved in oxidative phosphorylation and antioxidant metabolism, contributed to 6% of the identified ESTs, and represented cytochrome C-oxidase and NADH dehydrogenase. The NADH dehydrogenase complex, also called NADH coenzyme Q oxidoreductase, is the first complex of the electron transfer chain which catalyzes the transfer of electrons to the third complex of the chain from NADH to coenzyme Q (Weiss, 1991).As energy production is more efficient with the NADH dehydrogenase pathway the up-regulation of this gene could indicate an increased demand for energy production.

Genes encoding cytochrome c-oxidase were also identified in this group, this molecule is the terminal enzyme in the electron transfer chain responsible for more than 90% of the oxygen consumption by living organism. Similar to the observations from WSSV infection studies (Leu et al., 2007), it is likely that bacterial afflictions also modulate expression of energy metabolism related genes in *P. monodon* post-larvae. Expression of the above mentioned mitochondrial genes related to oxidative phosphorylation, might suggest a functional response to the challenge. Thus the present study is useful to suggest that bacterial infection might also modulate the amount of mitochondrial mRNAs.

3.4.6 Enzymes

The enzymes encountered during this study were either proteolytic such as trypsin, and retinoid inducible serine carboxypeptidase or, metabolic such as xylose isomerase, pyruvate kinase, phosphoglycerate kinase. As many as eight percent of the total ESTs were related to metabolic and proteolytic enzymes. Trypsin, an abundant protease found in the crustaceans, is reported to be responsible for proteolysis in various cellular and metabolic processes (Galgani et al., 1985). Serine carboxy-peptidase has a known serine type endopeptidase activity (Kunugi, 1985). Xylose isomerase and phosphoglycerate kinase identified in the library have also been reported to bear a direct role in glycolysis and carbohydrate metabolism. The reason for high incidence of this category of genes may be due to the fact that the mRNA copy numbers from all these categories was quite

high. In that, the transcripts have been represented in higher numbers as we had macerated the whole post-larvae for extracting the mRNA.

3.4.7 Pigments and other genes

The cDNA clones encoding for crustacyanin and rhodopsin formed a minor group in the subtracted library. Rhodopsin, an eye pigment protein, being derived from the contaminated compound eye in the whole animal macerate was more frequent. Fragments encoding both crustacyanin C1 and crustacyanin A2 subunit were identified in this study. Crustacyanin is a member of lipocalin family of proteins, involved in various physiological functions, such as binding with small hydrophobic molecules (Cianci et al., 2002; Wade et al., 2005) forming complexes with other macromolecules, to ensure numerous physiological processes such as invertebrate cryptic coloration (Riley et al., 1984) and the regulation of cell homoeostasis (Francone et al., 1989; Cancedda et al., 1988). Crustacyanin was first identified in European lobster, *Homarus gammarus* (Wade et al., 2005) and has been shown to bind astaxanthin which gives blue coloration.

Apart from these, transcripts coding for tetraspanin and smooth endoplasmic reticulum calcium, ATPase were identified in this study. These two proteins are integral components of membranes. Tetraspanin is a transmembrane protein and smooth endoplasmic reticulum calcium ATPase aids in calcium ion transport and has ATP binding domain. The increased expression of these genes in the bacteria challenged shrimps indicates that the regulation of calcium flux in the animal is definitely modulated by the bacterial challenge. More studies are needed to elucidate as to how they function and, which is the most likely pathway involved in the response. Astacin, a gene related to cell differentiation and development, was also detected in this study. Some other genes identified were the elongation factor 2, arginine kinase, and calcium ATPase. Besides these, as many as 20 hypothetical proteins were also identified (Table 3.4). Among them, many are being reported for the first time from this shrimp. Many of these might be the products of novel genes with interesting roles in shrimp immune-modulation or in stress management.

Results from this study provide important insights into individual genes and pathways that constitute to the shrimp post-larval response towards the *V. harveyi* challenge. Implicitly, the frequency of occurrence of some genes, in particular those known to be involved in immune modulation, suggests that they are up-regulated due to the bacterial challenge. These observations also demonstrate that application of SSH is very useful for identification and study of *V. harveyi* responsive genes in *P. monodon* post-larvae. In the total of 45 genes that could be identified from the subtracted library, 37% of them had no matches in the databases. These may be novel, interesting molecules for elucidating shrimp defense-mechanism(s) during vibriosis. These confirmation requiring findings are useful to suggest that they quite likely are involved in the host defense mechanisms of pathogen challenged animals. Although the upregulation/increased expression of genes related to immune functions was low, many genes related to various other processes such as ATP/energy metabolism, antioxidant metabolism and apoptosis/cell death were preponderant. Many of the transcripts identified from the subtracted cDNA library might prove to be of great value in adding to our understanding of the shrimp immune system and also in studying the pathogen responsiveness of early developmental stages of shrimps.

4.1 Introduction

Sustainable shrimp farming relies heavily on sound scientific approaches to prevent and/or control diseases particularly at the early stages of development. Culture of penaeid shrimps is an important activity in several parts of the world especially in Asia and Latin America. As the natural stocks are showing the declining trends due to overfishing and ecology related adversities, rearing of commercially vital shrimp species has the potential to sustain high quality seafood supply. However, progress of this industry is severely limited by microbial diseases. Among them, bacterial diseases are common in prawn hatcheries, rearing and production facilities (Lavilla-Pitogo et al., 1990). Members of the Vibrionaceae are involved in several diseases of the farmed shrimps (Lightner et al., 1993). As pointed out already in the previous chapters, vibriosis caused by *Vibrio harveyi* is quite prevalent causing mass mortalities in larval cultures and, affecting shrimp production (Saulnier et al., 2000). Among reared shrimps, microbial infections are usually associated with high incidence of larval deformities and, mortality rates (Vandenberghe et al., 2003).

As documented by FAO, the Indian white shrimp *Fenneropenaeus indicus* was reported to be reared only in India and Vietnam in 1990. But by the year 2000 its global production was 16,444 tonnes which was already twice the value of what it used to be in 1990. This rise in production was mainly due to wide spread culture of this species in countries like Iran and Saudi Arabia. In the year 2005 Saudi Arabia was the largest producer of *F. indicus* producing about 11,300 tonnes. However, in India a fall in the production of *F. indicus* was noticed from the year 2005. This fall in trend was mainly due to occurrence of diseases and other factors like, broodstock shortages, trade barriers, market competition, preference on *P. monodon* etc. So, quite poorer attention was paid to the *F. indicus* although the possibilities of achieving high production rates existed as this species has the potential to tolerate hypersaline conditions and to provide greater ease for hatchery reared seed production. Its increased production can only be expected if the problems related to diseases are treated very seriously.

With a functional genomics approach on the bacteria and shrimp interaction, very important information on the role of individual genes and gene products in disease pathology can be well understood. The EST based gene discovery approaches have taken the lead role with the introduction of high throughput sequencing technologies (Adams et al., 1991). It has always been an economic and efficient approach for gene discovery in those organisms whose genomes have not been sequenced. ESTs not only help in annotating the genome but also are useful in identifying new genes, gene locations and intron-exon boundaries (Hillier et al., 1996; Dias et al., 2000). As ESTs are short fragments of a coding region from a gene they are very helpful in determining the functional status of the cells or tissue from which the library has been created.

In recent years, SSH has been successfully applied as an efficient method to identify differentially expressed genes in shrimps (He et al., 2004; Pan et al., 2005). As such an EST based gene discovery project generates a lot of data which are usually repetitive and not unique. This is mainly due to the fact that there is an over representation of some transcripts which are either in very high copy numbers or, are stably expressed in the organism like the house keeping genes (Lenhert et al., 1999). As a result, the genes which are in low copy numbers or, are rare their mRNA transcripts are lost. In such situations, SSH is one useful tool for identifying differentially expressed genes which are in very low copy numbers and differentially expressed between two samples in comparison (Diatchenko et al., 1996).

Although *F. indicus* is economically important in many Asian countries very few molecular studies in relation to its disease biology exist. In this regard, an understanding of molecular responses and defense mechanisms in shrimps against bacterial pathogens is essential. Unfortunately, very few reports of genomic sequences and ESTs are available for *F. indicus* as compared to other commercially important penaeid species. In this study, the SSH was used to elucidate the interaction between bacteria and shrimp. As detailed in Chapter 3, this study aimed at identifying genes that are induced in the post-larvae of *F. indicus* [akin to that of *P. monodon*] on challenge with *V. harveyi*.

4.2 Materials and Methods

Ten day old post-larvae of *F. indicus* were obtained from a commercial hatchery and were held separately in 500 L tanks filled with seawater. The post-larvae were fed twice daily with an artificial larval feed and maintained throughout the 4 day acclimatization period under continuous aeration at 25°C and 29 psu salinity. As mentioned in Chapter 3, identical protocols of immune challenge, RNA isolation, SSH and constructions of subtracted cDNA library were followed for the post-larvae of *F. indicus* as well.

4.3 Results

Total RNA with good structural integrity was isolated from the challenged and control post-larvae population of *F. indicus* using Trizol reagent. This was evident from the intact 18S and 28S ribosomal bands which could be seen clearly after the gel electrophoresis on a 1.5 % formaldehyde agarose gel (Fig 4.1). In the β-actin efficiency test PCR product was detected in the unsubtracted cDNA, but not detected in the subtracted cDNA under the same PCR conditions. This suggested the reduction of the abundant cDNAs in the subtracted sample.

Identification of genes from the subtracted library

From the subtracted library, 1400 clones were randomly picked and sequenced in single direction using M13F and M13R sequencing primers. After end trimming and removal of low quality sequences (EST length <100bases) as many as 998 ESTs were taken up for further analysis. Sequences with E-value < 10^{-3} from BLASTX analysis were considered as significant and were taken for categorization of genes into different functional categories. Only 809 ESTs showed significant similarities with previously deposited sequences of the database. The remaining ESTs had database matching with E- value >10^{-3}. The analyzed ESTs represented 38 different genes and 8 hypothetical proteins (Table 4.1 and Table 4.2). As many as 29% of the analyzed ESTs had no matches in GenBank. The identified ESTs from the *F. indicus* subtracted library could be broadly grouped into 10 different categories based on their putative physiological and functional role (Fig 4.4). Among the 38 identified ESTs, 8 were immunorelevant genes, 6 were related to antioxidant/ATP metabolism, 9 to muscle/cytoskeletal proteins and, four to cell

cycle progression and protein modification. The other 11 identified ESTs were genes related to enzymes, pigments and other functions.

Although the genes related to ATP/energy metabolism and antioxidant metabolism was preponderant with frequent occurrence, many transcripts having significant roles in the crustacean immunity were identified from this subtracted cDNA library. Defense related genes contributed 10% of the total identified ESTs. Transcripts of metal binding proteins and proteases like carboxypeptidase B (CPB), ferritin, translationally controlled tumor proteins (TCTP), hemocyanin, chitinase and serine carboxypeptidase were prominent ones.

Interestingly, there was higher incidence of genes related to cell cycle progression and protein modification such as S-phase kinase-associated protein (SKP1) and ubiquitin in the cDNA library. The SKP1 in particular is being reported here for the first time in penaeid shrimps from this study. It is of much interest that these transcripts are identified as a result of the bacterial challenge to the post-larvae. Genes coding for retinoid inducible serine carboxypeptidase, and genes associated with cytoskeleton motility seem to be up-regulated after the challenge. Apparently, genes encoding for different enzymes such as pyruvate kinase, trypsin, DNA gyrase and transposase were identified. Most enzymes identified in this study were either DNA related or cellular metabolism associated. The cDNA fragments encoding for allergens such as arginine kinase, myosin light chain and sarcoplasmic calcium binding protein were also present. Besides this genes related to crustacean pigments such as crustacyanin subunit I, subunit II and rhodopsins were also identified from the subtracted library.

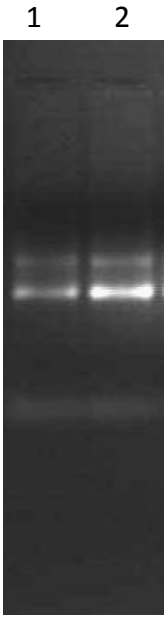

Fig 4.1 Total RNA isolated from post-larvae of *Fenneropenaeus indicus* run on a 1.5% formaldehyde agarose gel. Lanes 1- Total RNA from control post-larvae, Lanes 2 - Total RNA from *Vibrio harveyi* challenged post-larvae

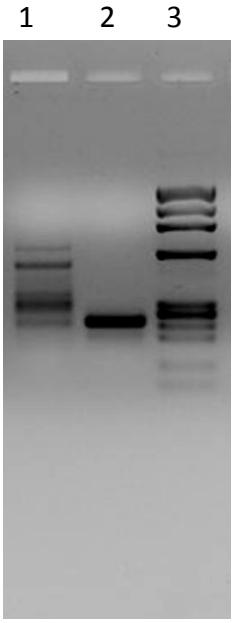

Fig 4.2 Results of experimental subtraction of *Fenneropenaeus indicus*. Lane 1- cDNA after subtraction, Lane 2 – cDNA before subtraction, Lane 3 - ϕX174 DNA/Hae III digest marker

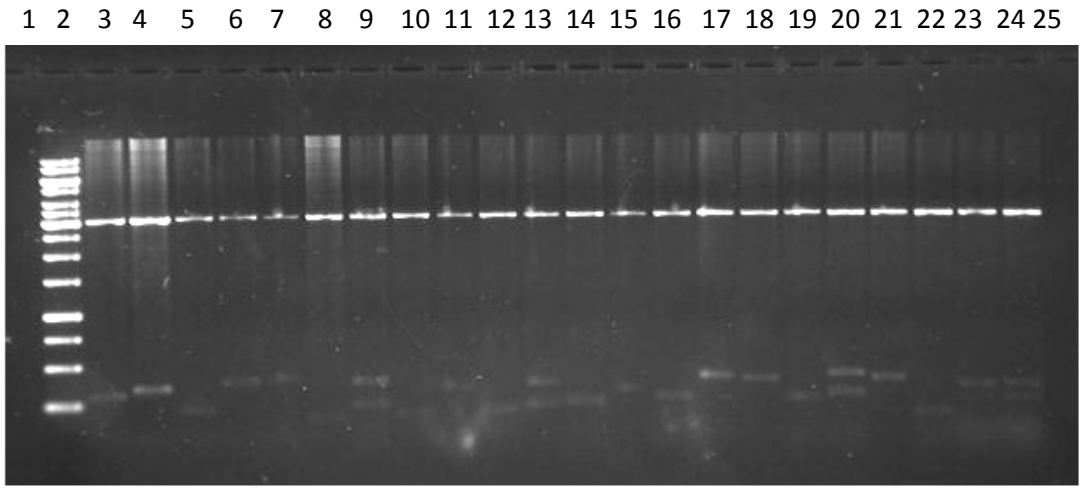

Fig 4.3 Restriction digestion of randomly selected clones from *Fenneropenaeus indicus* subtractive library using *EcoR*I for showing the inserts (ESTs) of various sizes. Lane 1-1kb ladder, Lanes 2 to 24 digested plasmids.

Table 4.1 Genes identified from the cDNA library prepared through suppression subtractive hybridization from *Fenneropenaeus indicus* post-larvae bath challenged with *Vibrio harveyi*

GenBank ID	Transcript (No of clones)	E value	%ID	Homologous species
Muscle/cytoskeletal				
GO787099	alpha actin (5)	9.00E-08	76%	*Oreochromis niloticus*
GO787100	actin (4)	2.00E-05	72%	*Oncorhynchus mykiss*
GO787101	beta-actin (6)	5.00E-28	98%	*Penaeus monodon*
GO787102	cardiac muscle actin (4)	1.00E-33	97%	*Homarus americanus*
GO787103	fast myotomal muscle actin 2 (2)	6.00E-23	97%	*Salmo salar*
GO787104	troponin I (7)	1.00E-14	92%	*Astacus fluviatilis*
GO787105	myosin heavy chain (7)	7.00E-44	84%	*Farfantepenaeus paulensis*
GO787106	four and a half LIM domains 1 (1)	2.00E-88	78%	*Macaca mulatta*
GO787107	sarcoplasmic calcium-binding protein (9)	2.00E-58	96%	*Litopenaeus vannamei*
Cell cycle progression				
GO787108	S-phase kinase- protein 1A (19)	1.00E-62	99%	*Oncorhynchus mykiss*
Antioxidant ATP metabolism				
GO787109	NADH dehydrogenase subunit 4 (17)	4.00E-37	97%	*Homo sapiens*
GO787110	NADH dehydrogenase subunit 1 (32)	6.00E-64	88%	*Penaeus monodon*
GO787111	NADH dehydrogenase subunit 2 (3)	7.00E-62	87%	*Penaeus monodon*
GO787112	NADH dehydrogenase subunit 5 (3)	2.00E-19	89%	*Homo sapiens*
GO787113	cytochrome c oxidase subunit I (3)	5.00E-23	94%	*Homo sapiens*
GO787114	F1F0-type ATP synthase subunit G (9)	9.00E-28	59%	*Ixodes scapularis*
Protein Synthesis and modification				
GO787125	eIF3e (10)	7.00E-44	80%	*Aplysia kurodai*

(Continued)

GO787115	ribosomal protein L26 (6)	6.00E-16	100%	*Penaeus monodon*
GO787116	ubiquitin (10)	2.00E-36	100%	*Salmo salar*
Defense immunity				
GO787117	carboxypeptidase B (4)	3.00E-11	55%	*Litopenaeus vannamei*
GO787118	TCTP(13)	6.00E-51	100%	*Litopenaeus vannamei*
GO787119	ferritin (15)	8.00E-48	100%	*Penaeus monodon*
GO787120	arginine kinase (2)	1.00E-16	95%	*Litopenaeus vannamei*
GO787121	allergen Pen m 2 (6)	5.00E-17	97%	*Penaeus monodon*
GO787122	chitinase (6)	1.00E-15	59%	*Scylla serrata*
GO787123	serine carboxypeptidase (5)	4.00E-22	41%	*Strongylocentros purpuratus*
GO787124	hemocyanin subunit L (14)	4.00E-46	98%	*Marsupenaeus japonicus*
Enzymes				
GO787126	trypsin (4)	1.00E-66	89%	*Litopenaeus vannamei*
GO787127	pyruvate kinase 3 (2)	1.00E-46	97%	*Litopenaeus vannamei*
GO787128	DNA gyrase subunit B (4)	6.00E-51	90%	*Bacterium SD212*
GO787129	transposase IS630 (1)	7.00E-06	100%	*Listonella anguillarum*
Pigments				
GO787130	crustacyanin-C1 subunit (4)	9.00E-36	64%	*Homarus gammarus*
GO787131	rhodopsin (4)	4.00E-09	74%	*Litopenaeus vannamei*
Others				
GO787132	allatotropin neuropeptide precursor (4)	6.00E-06	61%	*Spodoptera frugiperda*
GO787133	reverse transcriptase (1)	8.00E-22	40%	*Strongylocentros purpuratus*
GO78713	putative puroindoline b protein (1)	3.00E-14	78%	*Triticum aestivum*
GO787136	tetraspanin (2)	1.00E-20	41%	*Tribolium castaneum*

Similarity matches with E-value $<10^{-3}$ are only included. When multiple significant similarities were found for a single cDNA, only the highest scoring hit is included in the table.

Table 4.2 Hypothetical genes identified from the cDNA library prepared through suppression subtractive hybridization from *Fenneropenaeus indicus* post-larvae bath challenged with *Vibrio harveyi*

GenBank ID	**Species homology**	**E- value**	**%ID**
GO449031	*Thermobia domestica*	4.00E-09	65%
GO449032	*Tetradon nigrovirdis*	8.00E-23	87%
GO449035	*Trichoplax adhaerens*	6.00E-04	70%
GO449036	*Candida glabrata*	4.00E-16	75%
GO449037	*Plasmodium chabaudi*	4.00E-49	96%
GO449039	*Plasmodium berghei*	2.00E-27	75%
GO449040	*Trichoplax adhaerens*	6.00E-04	70%
GO449041	*Caenorhabditis elegans*	2.00E-27	72%

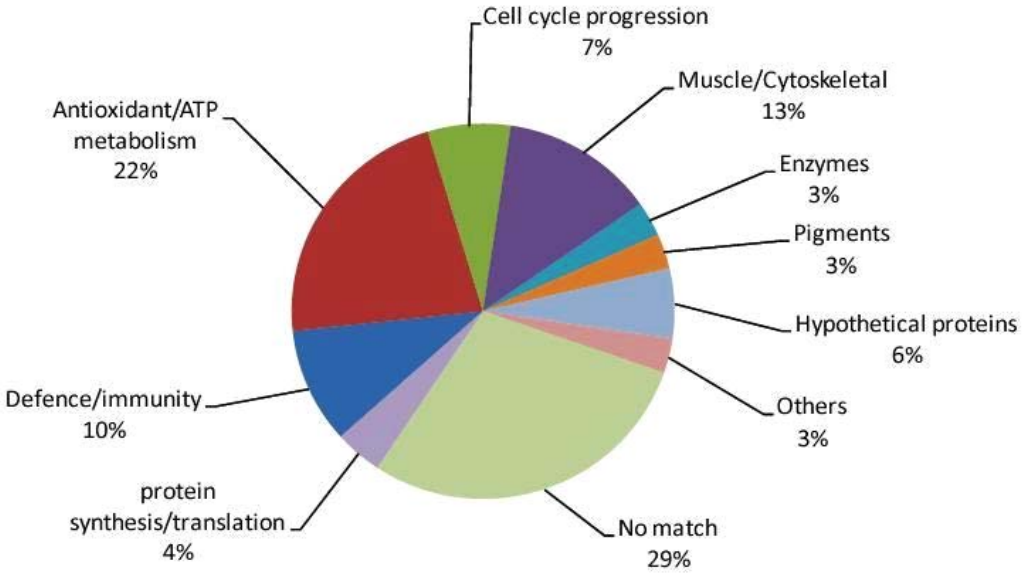

Fig 4.4 Distribution of genes into functional categories. Relative proportion (%) of different categories of transcripts identified from the subtracted cDNA library prepared through suppression subtractive hybridization from *Fenneropenaeus indicus* post-larvae bath challenged with the strain *Vibrio harveyi D3*

4.4 Discussion

As already described in Chapter 3, the SSH method was applied to elucidate *V. harveyi* responsive genes in *F. indicus* post-larvae. As also brought out earlier, this is the first report of ESTs generated from the post-larval stages of the Indian shrimp *F. indicus* challenged with a virulent strain of *V. harveyi*.

During this study, a total of 1400 clones were sequenced from the subtracted cDNA library constructed through SSH. A total of 1150 EST submissions have been made at the GenBank dbEST database. A large number of genes and hypothetical proteins previously not reported for this species were recorded in this study. It is inferable from the analysis of the ESTs that a large number of genes are associated in the post-larval defense of *F. indicus* against *V. harveyi*. These observations may prove useful for elucidating primary avenues for controlling luminous vibriosis in marine shrimps in their early stages.

From the forward subtracted library constructed to identify different *V. harveyi* responsive genes in post-larvae of *F. indicus,* it is apparent that many immune/defense relevance genes are upregulated. As such, the ESTs from the SSH cDNA library could be grouped into 12 different functional categories with the representing molecules having diverse physiological and functional roles. A brief description of the representative transcripts is provided below.

4.4.1 Genes related to defense and immunity

Genes related to defense and immune functions contributed to 10% of the total analyzed sequences with significant matchings. This category included transcripts such as ferrtin, carboxypeptidase, translationally controlled tumor protein (TCTP), hemocyanin, serine carboxy peptidase, and chitinase.

Clones of ferrtin, TCTP and hemocyanin were the most frequently encountered ones as also reported previously for *P. monodon* post-larvae challenged with the same strain of *V. harveyi* in chapter 3. Ferritin is predominantly an iron storage protein which plays crucial role in the cellular metabolism and detoxification of iron. Expression of

ferritin is known to play a key role in maintaining iron homeostasis inside the cell (Marcelis et al., 1980). Previously it was already reported by Marcelis (1980) that binding of free iron has bacteriostatic potential as bacterial cell growth is hampered, in particular of Vibrionaceae, in the absence of iron. Ferritin also has a potential role in clotting, as it was shown in *Drosophila melanogaster* (Karlsson et al., 2004). Earlier studies (Pan et al., 2005 and Zhang et al., 2006) had reported the upregulation of ferritin gene transcripts following the challenge with white spot syndrome virus (WSSV). Recently, it was reported that the purified recombinant ferritin helped in reducing the mortality in shrimps infected with *V. harveyi* (Maiti et al., 2010). The high representation of ferritin transcripts in the library suggests its possible upregulation and involvement in the defense responses against *V. harveyi.* Therefore, it can be suggested that ferritin does play an important and protective role in the post-larvae defense responses against bacterial pathogens and thereby enhancing their survivability.

A large number of clones bearing similarity to translationally controlled tumor protein were observed in the *F. indicus* subtracted cDNA library. Also known as fortilin, TCTP is believed to protect cells under toxic conditions. Fortilins/TCTPs are highly conserved throughout the animal and plant kingdoms. It has an established role as a 'histamine release factor' (HRF), which also initiates the release of histamines from leukocytes (MacDonald et al., 1995). TCTP is also reported to be induced by certain cytokines and acts as a growth factor for B-cells (Kang et al., 2001). TCTP induces the production of interleukins from basophiles and eosinophiles and, is known to display more general 'cytokine-like' activities (Bheekha-Escura et al., 2000). Shrimp fortilin is reported to bear 64% identity with human fortilin in its amino acid composition (Bangrak et al., 2004). An SSH experiment using WSSV-infected *P. monodon* had earlier revealed its role during viral infection in shrimps (Bangrak et al., 2004). Its expression however is reported to decrease during the terminal stage of the infection (Bangrak et al., 2004). As already described in Chapter 3, and reported by Nayak et al. (2010), upregulation of TCTP is also elevated in *P. monodon* post-larvae challenged with *V. harveyi*. In essence TCTP might be critical in defending bacterial infection through regulation of a cell death pathway that is common to shrimps and, humans. Studies on both *P. monodon* and *F.*

indicus post-larvae reveal the upregulation of TCTP due to bacterial challenges. Occurrence of such gene transcript in the subtracted library suggests the relevance of initiation/up-regulation of control-mechanisms on cell death in 10-14 day old post-larvae exposed to *V. harveyi.*

Also identified in this group was the hemocyanin subunit L gene. Hemocyanin a principal component of the hemolymph, contributes to 95% of the total amount of serum proteins (Khayat et al., 1995). In addition to its primary function as oxygen carrier (Holde et al., 1995), hemocyanin has multiple roles and is involved in physiological processes such as osmoregulation, protein storage and enzymatic activities (Paul and Pirow, 1997). Earlier studies have shown the evidence of generation of antibacterial and antifungal peptides from the C-terminus of shrimp hemocyanin (Destoumieux-Garzòn et al., 2001). Its role in immunity is confirmed by the fact that it can be used as a substrate for generating antimicrobial peptides which help in elimination and clearance of microbial pathogens. Hemocyanin gene is multifunctional respiratory pigment which also has important functions in the immune system of crustaceans. It is already proven to be an important immune factor against WSSV infection suggesting its potential role in defense process during virus infection (Zhang et al., 2004). There is diversity of haemocyanin peptides in *P. monodon* as suggested by (Sritunyalucksana et al., 2001). From the incidence of this gene in the subtracted library of *F. indicus*, its role as a major molecule involved in the defense against challenged *V. harveyi* is ascertainable. From these findings it can be suggested that hemocyanin that is readily available in the plasma might augur to shrimp immune system to generate responses against challenged bacteria.

Carboxypeptidase B (CPB) was another significant member of this group that was found in the library. CPB and its homologues have been extensively studied from many species of animals including shrimps (Gates and Travis, 1973). Also it has a proven role in the elimination of intracellular accumulation of amyloidal-beta in the brain and, thereby protecting the neurons from degeneration (Papp et al., 2003). With one zinc atom per molecule, the CPB, a metalloendopeptidase, particularly hydrolyzes arginine and lysine from the carboxyl terminus of proteins. Apparently, CPB and ferritin, both metal

binding proteins identified in this study are involved in animal's immune response, as well as have definite roles in cell signalling pathways (Gearing et al., 1994). The up regulation of this transcript has been reported in WSSV challenge, which was also detected using SSH in *Litopenaeus vannamei* (Zhao et al., 2007). Fragments similar to serine carboxy-peptidase with known serine type endopeptidase activity were also identified from the subtracted library. Occurrence of such transcripts suggests the involvement of various proteolytic enzymes in the innate immunity and defense responses of crustaceans.

4.4.2 Muscle and cytoskeleton related proteins

Muscle and cytoskeleton proteins constituted a major category of genes identified in the subtracted library, contributing ~13% of the total ESTs identified. The obvious reason for high incidence of these genes in the subtracted library is due to the fact that the cDNA library was prepared out of the whole animal tissue in which the copy number of such muscle and cytoskeletal specific genes are quite high. This group consisted of transcripts such as actin, myosin, troponin and sarcoplasmic calcium binding protein. Actins are conserved proteins that participate in functions related to muscle contraction, cell motility, cell division, and cytoskeletal structure (Durica et al., 1990). The reorganization of the actin and tubulin cytoskeleton plays a crucial role in many cellular functions such as phagocytosis, encapsulation, and nodule formation as well as cell motility, and cytokinesis of shrimp hemocytes (Pollard, 1990). The upregulation of these genes result in effective measures to clear away the invading pathogens.

Transcripts encoding for sarcoplasmic calcium binding protein (SCP) were also identified. The SCPs are important Ca^{2+} binding systems in the muscles of both vertebrate and invertebrate species (Gao et al., 2006). SCP has been recently described as a novel allergen in shrimp (Shiomi et al., 2008). Although expressed ubiquitously (Gao et al., 2006), it is usually abundant in the muscle. Its increased level of expression could indicate the modulation of calcium ion concentration in *F. indicus* due to the bacterial challenge.

4.4.3 ATP and Antioxidant metabolism related genes

As much as 22 % of the identified ESTs could be categorized as genes involved in ATP and antioxidant metabolism. NADH dehydrogenase, ATP synthase and cytochrome c oxidase were the major representatives of this group. The NADH dehydrogenase complex is the first complex of the electron transfer chain that catalyzes the transfer of electrons to the third complex of the chain from NADH to coenzyme Q (Weiss et al., 1991). As energy production is more efficient with the NADH dehydrogenase pathway, the up-regulation of this gene could indicate an increased demand for energy as a consequence of microbial challenge.

The activation of ATP synthase genes could indicate a high demand for ATP due to the increased demand for energy in the bacteria challenged post-larvae. Cytochrome c oxidase is the terminal enzyme in the electron transfer chain and is responsible for more than 90% of the oxygen consumption by living organisms. Similar to the observations from WSSV infection studies (Leu et al., 2007), it is likely that bacterial afflictions also modulate expression of energy metabolism related genes in *F. indicus* post-larvae as was also observed in *P. monodon*. The present study is useful to suggest that bacterial infection might also modulate the amount of mitochondrial mRNAs and, expression of mitochondrial genes related to oxidative phosphorylation as a response to challenge by *V. harveyi*.

4.4.4 Genes related to cell cycle progression and protein modification

Very interestingly a transcript similar to S-phase kinase associated protein (Skp1A) with 99% identity to *Onchorhyncus mykiss* fragment was identified. This Skp1A is a highly conserved ubiquitous eukaryotic protein belonging to the SCF complex (Bai et al., 1996; Zheng et al., 2002) and involved in the regulated ubiquitination of specific protein substrates. This Skp1 is reported to target the substrates for degradation by the proteosome (Carrano et al., 1999; Tsvetkov et al., 1999). It is reported that Skp1 is essential as a regulatory protein that controls entry into S phase and said to be clearly important for several cell cycle events (Bai et al., 1996). This is the first report of identification of cell cycle progression related transcripts in *F. indicus*. In this study, a

transcript ubiquitin, 100% identical to *Salmo salar* fragment, was also identified. Ubiquitin is a small and highly-conserved regulatory protein responsible for post-translational modification of proteins. The most prominent function of ubiquitin is labelling of proteins for proteosomal degradation (Voges et al., 1999). Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, and endocytosis (Hershko and Ciechanover, 1998). Further, the ubiquitin system has been implicated in the immune response, development, and programmed cell death (Hershko and Ciechanover, 1998). The identification of ubiquitin and SKP1 together are being reported here for the first time in *F. indicus*. These results are useful to suggest that bacterial challenge may probably lead to elevated levels of such cell cycle regulator-transcripts in the cell cycle machinery.

4.4.5 Enzymes

Various metabolic enzymes such as trypsin, pyruvate kinase and DNA related enzymes such as DNA gyrase subunit B and transposase were identified in the forward subtracted library. Trypsin identified in the library is reported to be an abundant protease in crustaceans. It is also reported to be responsible for proteolysis in various cellular and metabolic processes (Galgani et al., 1985). Pyruvate kinase has a direct role in glycolysis and carbohydrate metabolism. Occurrence of these genes in the subtracted library probably suggests that the expression of such transcripts involved in basic metabolic and physiological functions are elevated during the *V. harveyi* challenge.

4.4.6 Pigments and other genes

The cDNA clones encoding for crustacyanin and rhodopsin formed a minor component in the subtracted library. Fragments encoding both crustacyanin C1 and crustacyanin A2 subunit were detected in the subtracted library. Crustacyanin was first identified from European lobster, *Homarus gammarus* (Cianci et al., 2002) and has been shown to bind astaxanthin which gives blue colouration. It is a member of lipocalin family of proteins, involved in various physiological functions, such as binding with small hydrophobic molecules (Cianci et al., 2002; Wade et al., 2005) forming complexes with other macromolecules to ensure numerous physiological processes such as invertebrate cryptic

coloration (Riley et al., 1984) and the regulation of cell homoeostasis (Francone et al., 1989; Cancedda et al., 1988).

Also, identified in this study were the transcripts related to protein synthesis and translation such as the eukaryotic translation initiation factor and ribosomal protein L26. The upregulation of such transcripts suggests that the bacterial challenges stimulate active division and growth of cells. Apart from this, this study report here for first time the occurrence of reverse transcriptase enzymes for shrimps. In the past, these enzymes were considered important due to their significant role as antiviral defence factors in shrimps mRNA from both RNA and DNA viruses (Flegel et al., 2009). Their occurrence in the *F. indicus* subtracted library probably suggests an interesting antibacterial response. However, further studies are required to analyze their accurate role for antibacterial responses in shrimps.

In the subtracted library two clones encoding for tetraspanin gene were also identified. Tetraspanin are a family of membrane proteins which take significant part in the reorganization and formation of membrane structures during the process of phagocytosis and cytokinensis (Helmer et al., 2001). Very recently, Wang et al., (2010) reported on their active participation in the viral defense responses in the Chinese shrimp *F. chinensis*. It can therefore be hypothesized that this gene might be expressed in bacteria challenged shrimps as a result of phagocytosis processes for elimination of bacterial pathogens.

Besides these, eight different hypothetical proteins were also identified. These might be the products of novel genes with interesting roles in shrimp immune-modulation or in stress management.

4.4.7 Comparative account of upregulated genes in P. monodon and F. indicus

Comparative analysis of the genes identified from both these species in this study reveals that a total of 38 genes and 8 hypothetical proteins were identified from the *F. indicus* whereas 45 genes and 20 hypothetical proteins were identified from *P. monodon.* The

genes observed in both species were: ferritin, TCTP, cytochrome c oxidase subunit I, F1Fo ATP synthase, NADH dehydrogenase, actin, myosin, troponin, sarcoplasmic calcium binding protein, arginine kinase, reverse transcriptase, pyruvate kinase, serine carboxypeptidase, hemocyanin, crustacyanin, tetraspanin, puroindoline b protein, rhodopsin, ribosomal protein, eukaryotic intiation factor (IF3e) and trypsin. Incidence of genes related to muscle and cytoskeletal motility in large numbers was observed likewise in both the libraries. Transcripts encoding genes for ATP synthesis and antioxidant metabolism were higher (22%) in the *F. indicus* library as compared to the *P. monodon* library (12%).

Of the 45 genes detected through the random selection of clones from the P. monodon subtracted library, cuticle protein CUT5, zinc proteinase Mpc1, ATPase, catalse, xylose isomerase, astacin, phosphoglycerate kinase, endothelial chloride channel, suppressor of profilin and manganese superoxide dismutase were not seen in F. indicus library. Similarly four and a half LIM domain protein, allatotropin neuropeptide precursor, ubiquitin, four and a half LIM domain, carboxypeptidase B, chitinase, DNA gyrase subunit B, transposase and S-phase kinase protein 1A were not seen in P. monodon library.

The percentage of ESTs with no matches in the GenBank was higher in *P. monodon* (37%) library than that in *F. indicus* (29%). Defense and immune-related genes contributed 6 % of the total identified ESTs in case of *P. monodon* and 10% in case of *F. indicus*. Similarly, hypothetical proteins in *P. monodon* library accounted for 4 % of total ESTs *vis a vis* 6 % in the *F. indicus* library. Four uncharacterized protein products reported from *Tetradon nigrovirdis, Caenorhabditis elegans, Plasmodium chabaudi* and *Trichoplax adhaerens* were observed in the both the libraries in the form of hypothetical proteins. As can be seen from Tables 3.4 and 4.2 (Chapter 3 and 4) the detection of divergent hypothetical proteins is useful to suggest that there might be different physiological/immunological responses among the post larvae of these two shrimp species.

The interesting feature in *F. indicus* post-larvae is the occurrence of some genes related to cell cycle progression like the ubiquitin and SKP1A proteins which were not detected from the *P. monodon*. Such genes in the *F. indicus* might imply that bacterial challenge elicits elevated levels of some cell cycle regulator-transcripts (Nayak et al., 2011). This observation on the occurrence of these genes as a result of the bacterial challenge is the first time report in *F. indicus*.

In addition to obtaining the first sets of ESTs from *V. harveyi* challenged *F. indicus* post-larvae this study is useful in providing a knowledge-base on the *F. indicus* defense mechanisms especially in its early stages of development. Also, this is the first study depicting the upregulation of many genes in the post-larval stages of *F. indicus* upon bacterial challenge. Many transcripts identified during this study will, certainly prove to be of great value in understanding the shrimp immune system as well as in deciphering the response of early developmental stages of shrimps to bacterial stress.

Chapter 5

Upregulation of certain immune responsive genes in challenged *Penaeus monodon* post-larvae

5.1 Introduction

Vibriosis is an important disease of cultured shrimps across the world which is a major hindrance for growth of this industry. As much as 80% mass mortalities in shrimp post-larvae have been reported due to bacterial diseases (Karunasagar et al., 1994; Robertson et al., 1998; Vandenberghe et al., 1999). Healthy larvae are the key to culture success and thus responsible for sustainable shrimp production. In this regard the understanding of shrimp post-larvae immunity and their response to bacterial pathogens is of major relevance not only for establishment of health monitoring programmes but also for disease prevention strategies. Disease progression in eukaryotes is reported to be mediated by programs of differential gene expression (Huang et al., 2007). About 15% genes in higher eukaryotes are reported to be expressed and/or upregulated in response to a specific physiological state (Wang and Lu, 1998). So the study of gene expression/upregulation is a crucial factor for understanding the gene function(s) and for antinfectious responses during disease processes. Therefore as highlighted by Huang et al., quantification of expression of these genes or their transcripts has always served as an important aid in the research related to gene function and functional genomics.

Susceptibility of shrimp post-larvae to pathogens depends on their immune competence to defend themselves from external pathogens (Jiravanichpaisal et al., 2007). A better understanding of the defense mechanism involved during these stages in the shrimps is thus very significant to combat disease problems as to increase production and for managing the good health of shrimps under intensive farming systems. Shrimps are completely dependent on their innate immune system for their defense against bacterial pathogens and their elimination (Cerenius and Soderhall, 2004). A cascade of genes gets activated as result of entry of an external pathogen or antigen into the host system (Vargas-Albores and Yepiz-Placensia, 2000). Therefore quantification of the expression of such genes will be helpful in understanding the shrimp immune system ontogeny and thereby will be useful in elucidating the differences in combat mechanisms involved in the responses of post-larvae vis a vis their adult counterparts against microbial challenge.

Quantitative reverse transcription PCR (qRT-PCR) is a very popular and sensitive method for gene quantification. It helps revealing differential abundance or copy numbers of genes within different samples analyzed and it is also subjectable to high-throughput analysis (Gentle et al., 2001). Real-time PCR (qPCR) relies on the use of fluorescent reporter dyes in the simultaneous amplification and detection steps which are involved in this type of PCR reaction within a single tube (Fig 5.1). The increase in the signal intensity of fluorescence is proportional to the amount of amplified product during every cycle of the reaction. One distinct feature in this type of reaction is the PCR cycle number at which the intensity of fluorescence passes above the defined threshold or background fluorescence. This PCR cycle value is designated as Ct value. The more the amount of target the less will be its Ct value. This relationship between fluorescence signal and amount of product formed allows the accurate quantification of target molecules during the qPCR reaction (Fig 5.2).

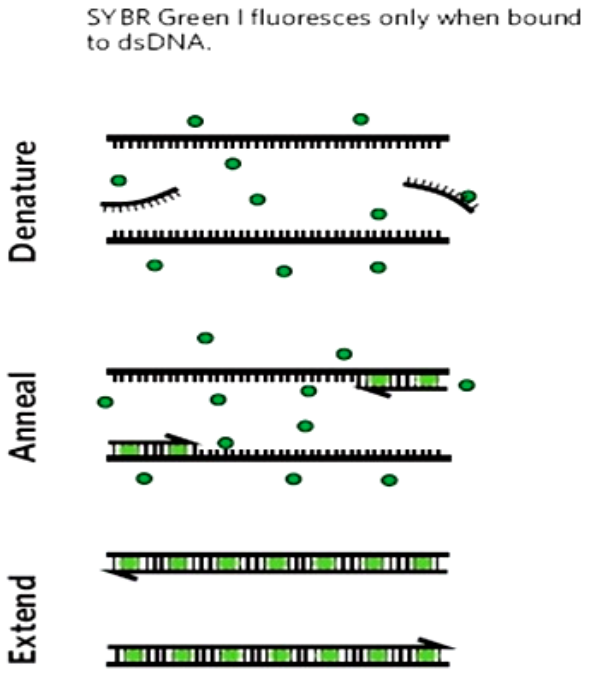

Fig 5.1 SYBR Green chemistry in Real time PCR. SYBR Green dye selectively binds to double stranded DNA. The florescence emitted is proportional to the amount of double stranded products formed in the reaction. (Adapted from Quorani, 2011)

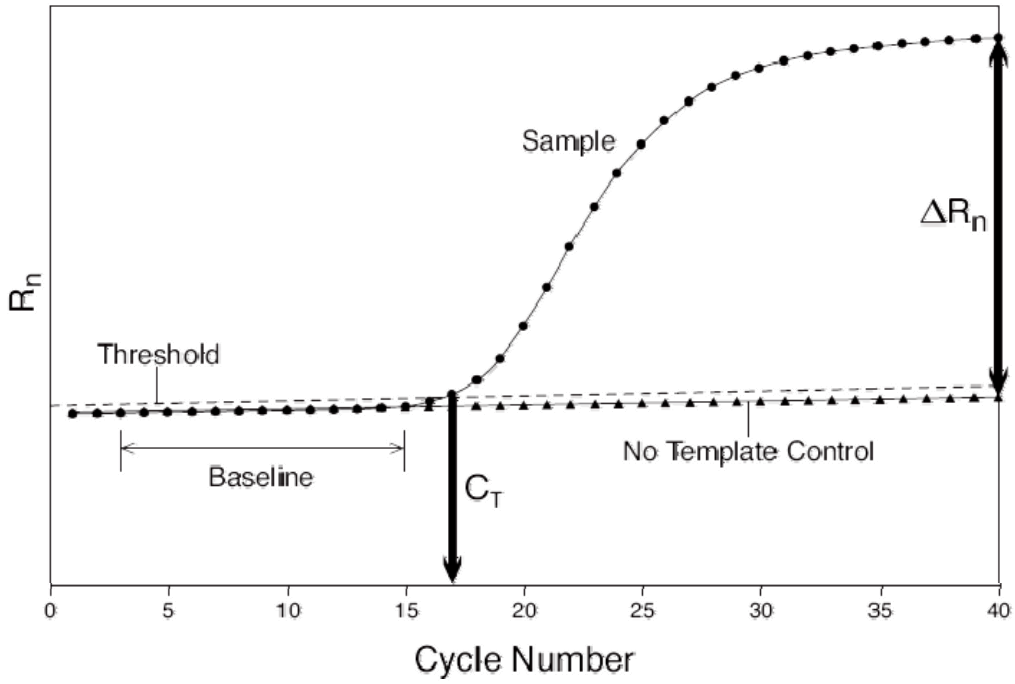

Fig 5.2 Example of a typical Real Time PCR amplification plot.
Threshold – where the threshold and the amplification plot intersect defines C_T, C_T - (cycle threshold) the cycle number where the fluorescence passes the threshold, Rn - Fluorescence at cycle n, ΔR_n- (R_n-baseline), ΔRn is plotted against cycle numbers to produce the amplification curves which gives the C_T value (Adopted from Quorani, 2011).

For gene expression studies two standard procedures of qRT-PCR analysis i.e. relative and absolute quantification can be applied depending on the user application and design of the experiment. In relative quantifications, expressions of target gene are compared with one or more reference gene(s). A reference gene is a transcript whose expression is consistent in all the analyzed samples. Generally the housekeeping genes like β-actin, 18S rRNA, elongation factor-1α and GAPDH are frequently used. One easy and fast way of relative quantitation is by the comparative Ct method (Livak and Schmittgen, 2001). In the comparative Ct method the Ct values of the experimental samples are compared with the Ct values of the control (or calibrator) such as a non-

treated sample i.e. mRNA from normal tissue. Before beginning the reaction it is required that the expressions of the control and the samples are normalized with respect to a suitable endogenous housekeeping gene. This can be achieved by observing how the Ct values vary with different dilutions of the template used in the PCR reaction. The values of template dilution and corresponding ΔCT can be plotted. The slope of this plot should be close to zero, which denotes that the amplification efficiencies of the target gene and the internal control genes are similar. This method is also known as the $2^{-\Delta\Delta CT}$ method, where Δ CT = (Target transcript Ct – internal control Ct), ΔΔCT= (Inf ΔCt - Ctrl ΔCt). The value obtained from this $2^{-\Delta\Delta CT}$ method corresponds to the fold change in expression. The validity of this calculation depends on whether the amplification efficiencies of the desired target gene and the internal control have been approximately equalized. If a suitable internal control gene whose efficiency is close to the target gene is not available than the standard curve method for quantitation of target gene should be followed.

Although innate immunity in shrimps have been studied intensively and many molecules have been cloned and characterized, very few reports are available explaining the nature of immune molecules and the defense strategies involved in the post-larval stages against bacterial pathogens. The present study was aimed at investigating the gene expression profiles of six different immune related transcripts in *V. harveyi* challenged *P. monodon* post-larvae. This approach was to realize the individual functional role and level of expressions of these select set of defense related genes.

5.2 Materials and Methods

As mentioned in Chapter 3, identical protocols of immune challenge of *P. monodon* post-larvae and total RNA isolation were carried out. The total RNA isolated from the challenged and control post-larvae were treated with DNaseI to ensure genomic DNA-free RNA preparation. The first strand cDNA was synthesized from 4 µg of DNA-free total RNA with oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, USA). Primers used in the qPCR study for the relative quantification of expression of different genes were designed using Primer express software ver. 3 (ABI, USA).

Detailed list of all the primers their sequence and corresponding amplicon sizes have been mentioned in Table 5.1.Shrimp β-actin gene was used as an internal control during all as the qPCR runs there was hardly any difference in the mean C_T values of *V. harveyi* challenged (C_T= 20.5244) and unchallenged (control; C_T =20.7182) post-larvae. The PCR efficiencies for the individual target genes and the internal control were determined. The CT values <40 were used to calculate the PCR efficiency from the slope determined by the ABI 7500 SDS software ver 2.0.1. All the PCRs had efficiency between 95% and 100%. The PCR efficiencies for the individual target genes and the internal control were determined. The relative fold of induction was calculated using comparative CT method for relative quantification. The amplifications were carried out in a 96 well plate in a total volume of 25µl using 4 µl of cDNA, 200nM of each gene-specific primers pair with 5X qARTA Green qPCR Mix (Axygen, USA) in an ABI 7500 Real-Time PCR system. Cycling parameters were 95ºC for 15 min, followed by 40 cycles of 95 ºC for 15s and 60°C for 1 min.

The specificity of PCR products was confirmed by dissociation curve analysis performed at the end of qPCR by continuously heating from 55 ºC to 95 ºC with an increment of 0.1ºC. Data were analyzed using the ABI 7500 software version 2.0.1. Each sample was run in triplicate for each gene and shrimp β-actin gene was used as an endogenous control. Negative control reactions were included for every primer set used in the reaction by omitting the template DNA in the reaction. The relative fold of induction was determined by ΔΔ CT method (Livak and Schmittgen, 2001). The fold change was calculated using the formula $2^{-\Delta\Delta CT.}$ The CT values for challenged and control samples for individual target genes obtained from qRT-PCR analysis were subjected to analysis of t-test. Statistical analysis was performed with Microsoft Excel program (Microsoft Office 2007). A probability (P) value of less than 0.05 was considered significant.

5.3 Results

The expression of all six genes mentioned above was normalized to β-actin, the internal control. During standardization all the PCR reactions exhibited efficiencies between 95-100%. Amplifications in some NTC (no template control reactions) were obtained but the corresponding Ct values were very high so these were readily ignored. The melt curve analysis performed after the real time cycling reaction ascertains the presence of single peaks denoting the absence of non specific products and primers dimers within the reaction. The amplification plot obtained for analyzed genes were in agreement to the principles of comparative Ct method used in this study for gene expression analysis (Fig 5.3 and Fig 5.4). The shape of the amplification plot was observed and found to be uniform for all the analyzed defense related transcripts. This indicated that the efficiencies of amplification of the desired target genes chosen for the experiment were approximately very similar to each other. The amplification plot also revealed that the fluorescence intensities measured from different target genes were well above the threshold or baseline.

A significant increase in the relative expression of all the genes examined was observed. The hemocyanin transcript showed remarkable upregulation of 11.63 fold in the challenged post-larvae (Fig 5.5). Whereas TCTP gene had 6.47 fold and ferritin a 2.04 fold rise in their expression. Similarly the lysozyme gene had 5.49 fold, penaedin 2.09 fold and MnSOD 3.28 fold rise respectively (Fig 5.6). The values for mean CT and ΔΔCT for challenged and control samples for all the genes analyzed in the *P. monodon* are shown in (Table 5.2).

Table 5.1 List of primers used in the qPCR assay for gene expression analysis in the challenged *Penaeus monodon*

Primer	Sequence	Product size (bp)
β - Actin	F 5’ GACTCGTACGTGGGCGACGAGG 3’	125
	R 3’ AGCAGCGGTGGTCATCTCCTGCC 3’	
Ferritin	F 5’ ATGGGGATCCAAGTCCGCCAG 3’	145
	R 5’ TTAGTGGAATTCCTTATCAAC 3’	
Hemocyanin	F 5’ CATCTCTTCACCAACAGCGA 3’	130
	F 5’ GGAATTCCATATGCCAGGTG 3’	
TCTP	F 5’ CGAGATGGCATGGTTGTTCTCATG 3’	150
	R 5’ CGAAGACATTGATGGAGAAGAGC 3’	
MnSOD	F 5’ GGCCACTTGAACCATACCATCT 3’	119
	R 5’ GGCTCACCACCAGCATCAG 3’	
Penaeidin	F5’ TCCCTGGAGGTCAATTGAGTG 3’	111
	R5’ AGTCGAACATGCAGGCCTATCC 3’	
Lysozyme	F 5’ GGCCTCCGTAAGGAACATTT 3’	125
	R 5’ CTTGCTGTTGTAAGCCACCC 3’	

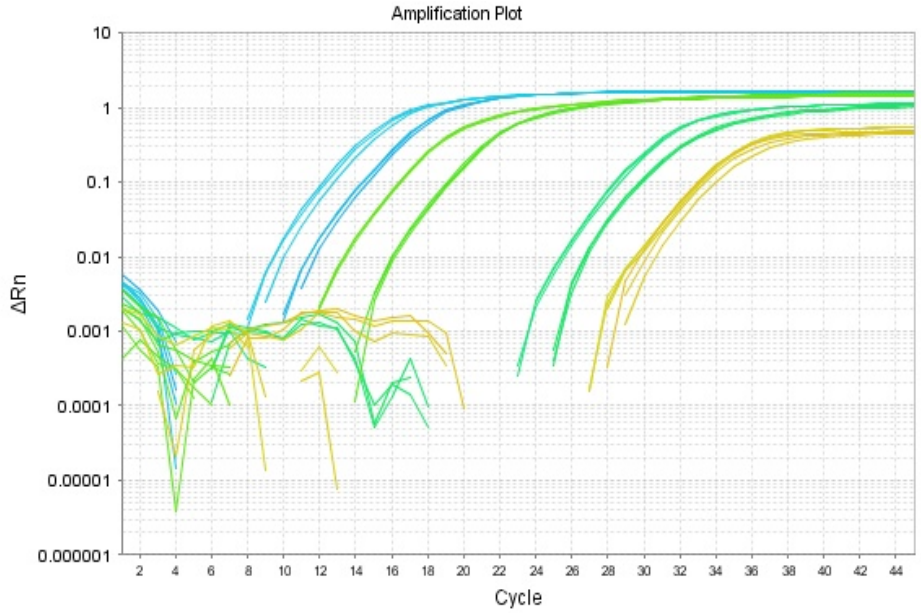

Fig 5.3 Amplification plot for hemocyanin, TCTP and ferritin genes obtained from the qPCR analysis. The expression plot is obtained by plotting the values of ΔRn (Fluorescence intensity) against the PCR cycle number which gives the corresponding Ct values of a particular gene of interest analyzed.

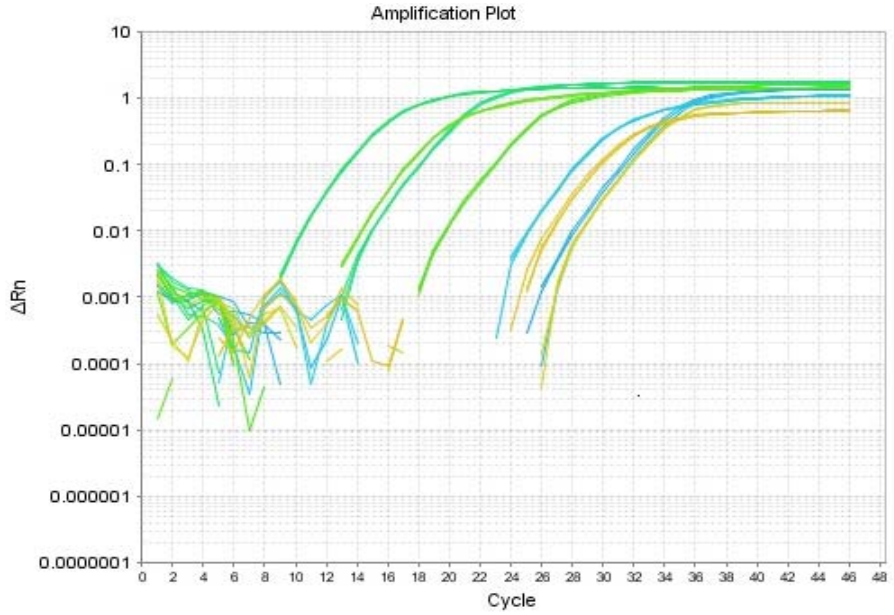

Fig 5.4 Amplification plot for MnSOD, penaeidin and lysozyme genes obtained from the qPCR analysis. The expression plot is obtained by plotting the values of ΔRn (Fluorescence intensity) against the PCR cycle number which gives the corresponding Ct values of a particular gene of interest analyzed.

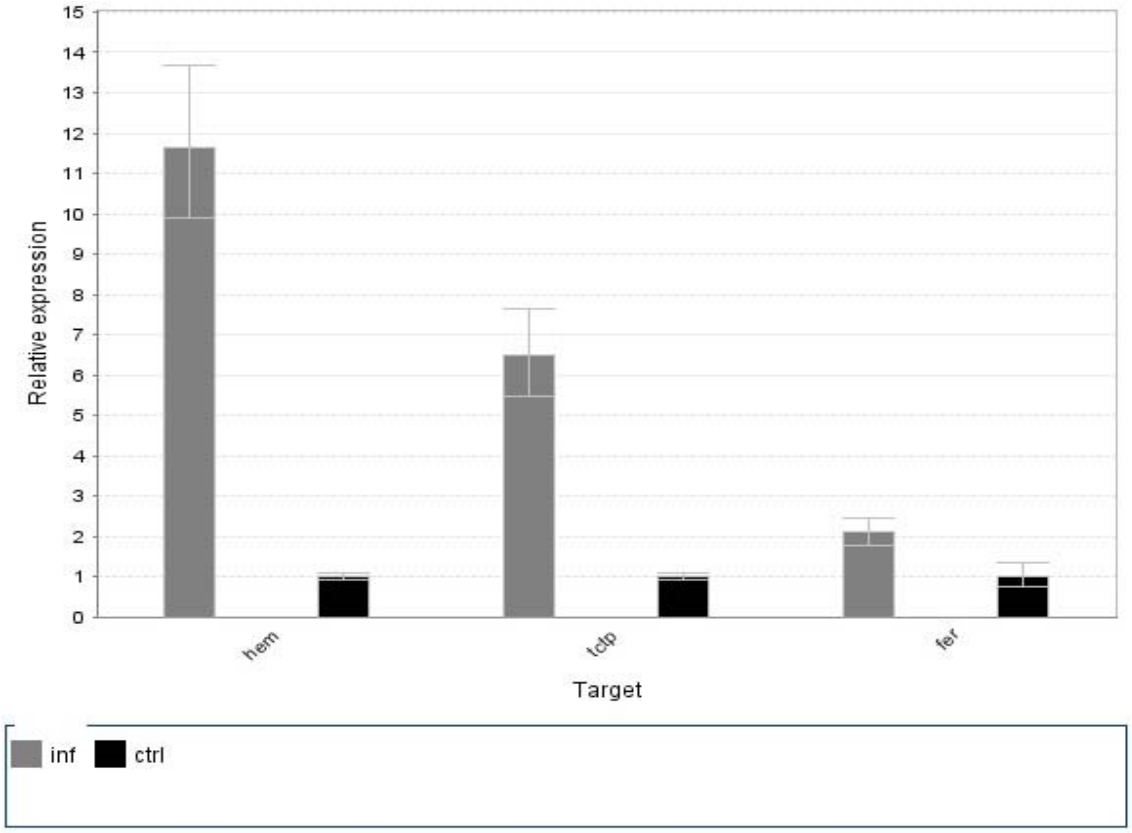

Fig 5.5 Relative expression of immune related genes, hemocyanin, ferritin and TCTP. Expressions normalized with respect to the internal control gene β-Actin. The error bars in the histogram represent the maximum and minimum changes of the relative expression fold changes. B-Actin is compared to itself as a reference and represented here as one fold change in its expression.

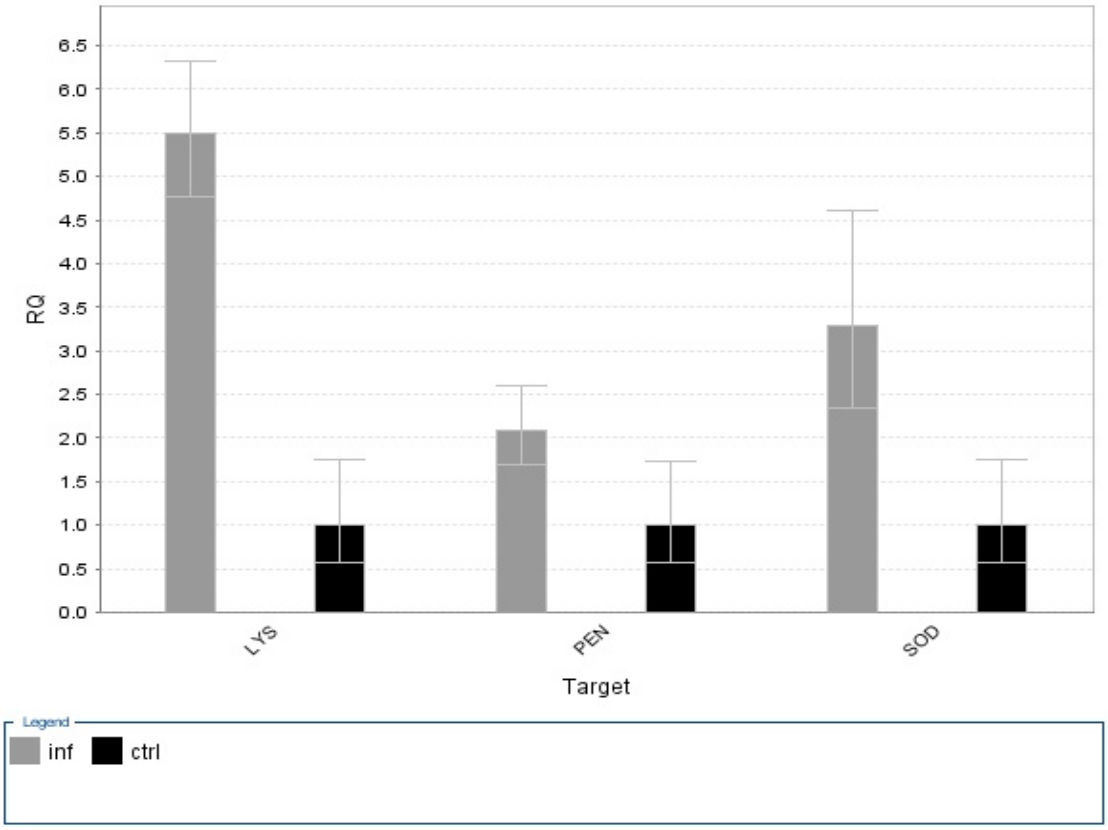

Fig 5.6 Relative expression of immune related genes lysozyme, penaeidin and superoxide dismutase MnSOD. Expressions normalized with respect to the internal control gene β-Actin. The error bars in the histogram represent the maximum and minimum changes of the relative expression fold changes. β-Actin is compared to itself as a reference and represented here as one fold change in its expression

Table 5.2 Quantitative PCR analysis of gene expression in control and *Vibrio harveyi* challenged post-larvae of *Penaeus monodon*

Target Gene	Mean cycle threshold		ΔΔ CT	Fold change in expression ($2^{-\Delta\Delta CT}$*)
	Challenged post-larvae	Unchallenged post-larvae (control)		
Ferritin	28.90	32.00	-1.067	2.09
Hemocyanin	17.92	23.49	-3.540	11.63
TCTP	14.88	19.60	-2.695	6.47
MnSOD	13.99	16.01	-1.714	3.28
Penaeidin	29.49	30.86	-1.068	2.09
Lysozyme	16.61	19.37	-2.45	5.49

Δ CT = (Target transcript CT - β-Actin CT); *ΔΔCT= (Inf ΔCT - Ctrl ΔCT),

5.4 Discussion

The qPCR quantification of expression of the six immune related genes (TCTP, ferritin, hemocyanin, lysozyme, penaedin and MnSOD) with reference to the internal control β-Actin supports the data generated by SSH with respect to the upregulation of defense related genes in particular.

The hemocyanin gene had the highest fold rise in expression of 11.6 folds among all the immune related genes analyzed. Besides their role as oxygen transporters, hemocyanins are well known in the initiation and activation of the prophenoloxidase pathway which is responsible for elimination of bacterial pathogens by phagocytosis and melanization (Nagai and Kawabata, 2000; Nagai et al., 2001). Peptides such as ascidins derived from crustacean hemocyanins have been already reported by research groups (Lee et al., 2004). Hemocyanins have been also reported for production of antifungal peptides from shrimps (Destoumieux et al., 2001). It seems to be very active in the defense against bacterial pathogens. Furthermore antiviral properties of hemocyanin are also well known (Zhang et al., 2004). Therefore, results from this study and earlier studies of crayfish and shrimps (Jiravanichpaisal et al., 2007; Lee et al., 2004) suggests that shrimp hemocyanin definitely plays an important role as major defense factor in the microbial challenged post-larvae of *P. monodon*.

Lysozyme was yet another transcript which had a significant upregulation of 6.47 folds in the challenged post-larvae in this study. It is found in both prokaryotes and eukaryotes and, known to be an integral component in the innate immune system of crustaceans that protects against various types of microbial infections (Ji et al., 2009). It acts as a major molecule involved in the innate immune defense reactions against bacterial pathogens by hydrolyzing their cell walls and eliminating them from the infected host cells (Jolles and Jolles, 1984). In case of penaeid shrimps lysozymes have been very well studied and characterized as they have shown specific activity against a large number of Gram (+) and Gram (-) negative bacterial species, including pathogenic *Vibrio* species (Hikima et al., 2003; de-la Vega et al., 2006; Burge et al., 2007). Also it has been previously reported that the expression of lysozyme gene increased after

challenges with *V. campbellii* and *V. alginolyticus*, in *L. vannamei* and *F. merguiensis* (Burgents et al., 2007; Mai and Hu, 2009). Further, in *vivo* studies of mRNA expression for lysozyme earlier in *L. vannamei* had shown significant increase in its expression in 36 h post challenge with *V. harveyi* (Wang et al., 2010). These findings thus confirm the systemic immunity role of lysozyme against bacterial pathogens and suggest it to be an important component of the shrimp anti-gram negative bacterial defense system. The results from this study are concurrent with the earlier finding from other research groups.

The gene of MnSOD, a well known antioxidant enzyme was found to have 3.28 fold rise in expression in the challenged *P. monodon.* Crustacean defense system usually relies on the production of antimicrobial elements which also includes the reactive oxygen intermediates (ROI) such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radical (Roch, 1999). These ROIs can cause damage to the cellular system. So it is essential that these molecules are continuously removed from the system. These processes require the help of free radical scavenging enzymes like superoxide dismutase (SOD) that scavenges the superoxide anion (Holmblad et a., 1999; Campa-Cordova et al., 2002). Cheng et al. (2006) had cloned MnSOD from the hepatopancreas of *Macrobrachium rosenbergii* and found that the expression of this enzyme had decreased 3h post the bacterial challenge but no significant change in expression was noticed in the hemocytes of challenged prawn at 3- 24 h. In this study increased expression level of this gene even after 48 hrs of challenge suggests the important role of MnSOD as major scavengers of free radicals generated during the process of phagocytosis and elimination of bacterial pathogens.

Penaeidin, an important antimicrobial peptide in crustaceans, was upregulated by 2.09 folds in the challenged *P. monodon* post-larvae. This peptide was first purified and characterized by Relf et al. (1999) from the horseshoe crabs. Later it was reported that antimicrobial peptides like penaeidin are produced and stored in shrimp granulocytes and are released upon microbial challenge (Destoumieus, 2000). Gene expression analysis of PEN3 penaeidin in *L. vannamei* had revealed that PEN3 expression decreased from 6-12 h after microbial challenge, and then gradually increased to normal levels after 48 h post-injection (Munoz et al., 2004). Also recent study by (Fall et al., 2010) on expression of

MjPEN in the *Marsupenaeus japonicus* had shown the upregulation of this transcript in 12 and 48 h after challenge with *Vibrio nigripulchritudo.* Very recently, its expression in the digestive organ of *P. monodon* is believed to have revealed a uniform level of expression throughout the period of microbial challenge (Soonthornchai et al., 2010).

Apart from the above 4 genes, expression of TCTP and ferritin genes from the challenged post-larvae was 6.7 and 2.4 folds respectively. Elevated expression of such genes in the challenged post-larvae is generally considered as first line of defense in the invertebrate immune system. Cloning and characterization of these two transcripts will be dealt separately in Chapter 7 as also to compare them with the same ones from *F. indicus*.

The above observations of upregulation in expressions of these immune related transcripts thereby not only prove the occurrence of these immune related transcripts in the post-larval stages but also testify their significance in the defense mechanisms involved in the elimination of *Vibrio harveyi* from the shrimp post-larvae. Results of gene expression analysis of immune related genes of the six immune related genes TCTP, ferritin, hemocyanin, lysozyme, penaedin and MnSOD with reference to the internal control β-Actin are affirmative enough to suggest that understanding of the innate immune response of shrimp against *V. harveyi* is still in nascent stages. On the basis of these results, it will be very interesting and pragmatic too to determine whether the expression profile of such genes can be used as standards for health monitoring and assessment of immune capability of the post-larvae under the influence of microbial and environmental stressors.

Chapter 6

Profiling of gene expression in challenged *Fenneropenaeus indicus*

6.1 Introduction

There is considerable progress in recent years on the elucidation of invertebrate immunity mostly through the characterization of genes that are related to infection and/or stress responses (De la Vega et al., 2007; Leu et al., 2007; Pongsomboon et al., 2008; Robalino et al., 2009). However, the mechanisms by which commercially important marine invertebrates such as cultured crustaceans can successfully survive an infection remain greatly unexplored. It has been found that resistance to infection is due to genetic variation and it is widespread among insects and other organisms (Sackton et al., 2009). However, the extent to which this variation in resistance is mediated by alteration in infection-induced gene expression is poorly studied.

Knowledge of immune gene expression in response to bacterial pathogens is vital for understanding the immune capability of shrimps for genetic selection as well as for establishing health monitoring protocols (Lorgeril et al., 2000). Shrimp defense system is believed to rely largely on innate immunity. Quantification of expression of defense related genes of the innate immunity will be helpful in understanding the shrimp immune system and thereby prove useful in elucidating the mechanisms involved in the responses of post larvae of shrimps against microbial challenge. Gene expression profiling is a tool that provides vital data about disease mechanisms, regulatory pathways, and gene function. (Shena et al., 1995). As made clear earlier, molecular studies on the effect of microbial stress on the immune response in the *Fenneropenaeus indicus* are limited. In this chapter changes in gene expression profiles of the *F. indicus* post larvae as a result of the *Vibrio harveyi* challenge are described. This study reports for the first time the upregualtion of seven major immune related proteins in *V. harveyi* challenged Indian shrimp *F. indicus* using qPCR.

6.2 Materials and Methods

All methods of immune challenge, RNA isolation, cDNA synthesis and qPCR are the same as detailed in Chapters 3 and 5.

6.3 Results

The expression of all the seven immune related genes mentioned above was normalized to β-actin, the endogenous internal control as was done for screening immune related genes in *P. monodon*. Efficiencies of PCR reaction was determined during the standardization procedures. All reactions exhibited efficiencies between 95- 100%. Amplifications in some NTC (no template control reactions) were ignored because of very high Ct values. The melt curve analysis performed after the real time cycling reaction ascertains the presence of single peaks denoting the absence of non specific products and primer dimers within the reaction. The amplification plot obtained for analyzed genes were in agreement to the principles of comparative Ct method used in this study for gene expression analysis (Fig 6.1). The shape of the amplification plot was observed and found to be uniform for all the analyzed defense related transcripts depicting similar amplification efficiencies between desired target genes chosen for the experiment.

qPCR results for seven immune related genes in *V. harveyi* challenged *F. indicus* post larvae showed significant increase in the relative expression of all the genes examined (Fig 6.2). Major upregulation was observed for ferritin and TCTP (4.15 and 3.45 fold rise in expression respectively). Furthermore, hemocyanin transcript showed 1.86 fold rise in expression. The expression profiles of immune related genes TCTP, ferritin and hemocyanin with reference to the internal control β-Actin supports the data generated by SSH with respect to the upregulation of these genes. Major upregulation in expression of TCTP and ferritin obtained in our study is generally considered as first line of defense in the invertebrate immune system. Furthermore the hemocyanin gene, with an upregulation of 1.8 fold, seems to be active in the defense against bacterial pathogens. Besides this relative quantification of four important antimicrobial defense related genes (MnSOD, penaeidin, crustin and lectin) chosen for this study demonstrated marked upregulation in their expression. The values for mean Ct and ΔΔCT for challenged and control samples for all the genes analyzed in the *F. indicus* are shown in (Table 6.2).

Table 6.1 List of primers used in the real-time PCR assay for relative quantification of genes.

Primer	Sequence	Product size (bp)
β - Actin	F 5' GACTCGTACGTGGGCGACGAGG 3' R 3' AGCAGCGGTGGTCATCTCCTGCTC 3'	125
Ferritin	F 5' TGAAGCCTCCATTAACAAGC 3' R 5' GAGATGAGGATCGTTGTTGC 3'	130
Hemocyanin	F 5' CCTGCC TCGATCTTTGCAA 3' R 5'AAG TGCTCGGAATCTTCGGTAA 3'	123
TCTP	F 5' GAG CCAATCCATCAGCTGAAGA 3' R 5' ACATCAACACCAGACTGACTAGTAGT 3'	145
MnSOD	F 5' GGCCACTTGAACCATACCATCT 3' R 5' GGCTCACCACCAGCATCAG 3'	119
Penaeidin	F 5' ACAGTCGTATTTGTCCCAGCAGGT 3' R 5' AACACCAACCACACACAGACCCAT 3'	111
Crustin	F5' TCCCTGGAGGTCAATTGAGTG 3' R5' AGTCGAACATGCAGGCCTATCC 3'	233
Lectin	F5' AGTGCTGGACGAGTGCTTCTATCT 3' R5' TTGAGAGCATAGACGTTCCTGGGT 3'	170

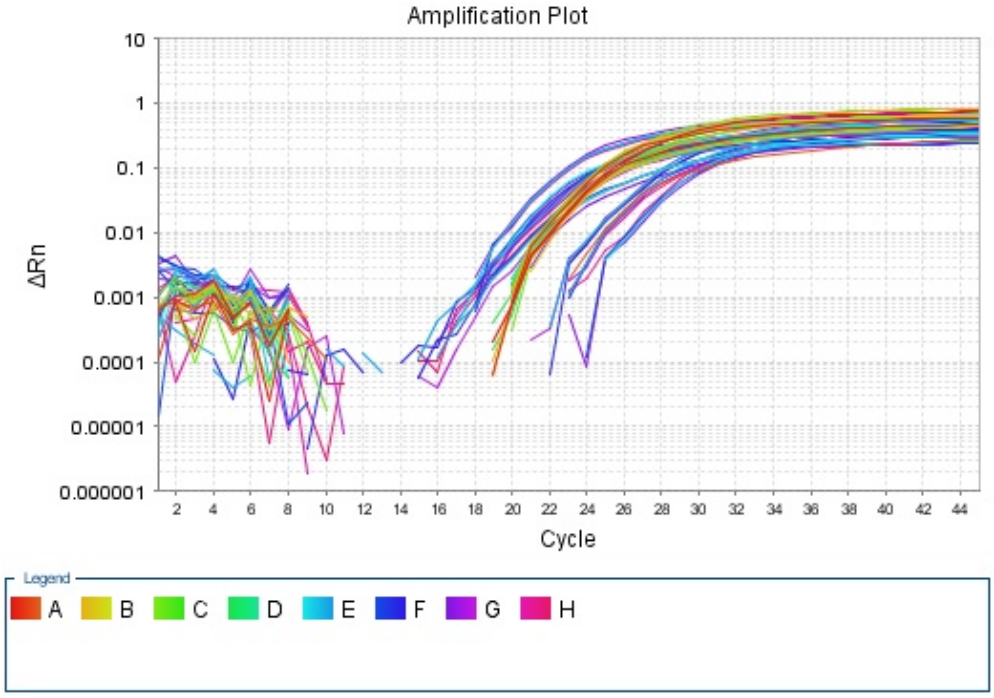

Fig 6.1 Amplification plot for lectin, penaedin, MnSOD, hemocyanin, ferritin, fortillin, and crustin obtained from the qPCR analysis. The expression plot is obtained by plotting the values of ΔRn (Fluorescence intensity) against the PCR cycle number which gives the corresponding Ct values of a particular gene of interest analyzed.

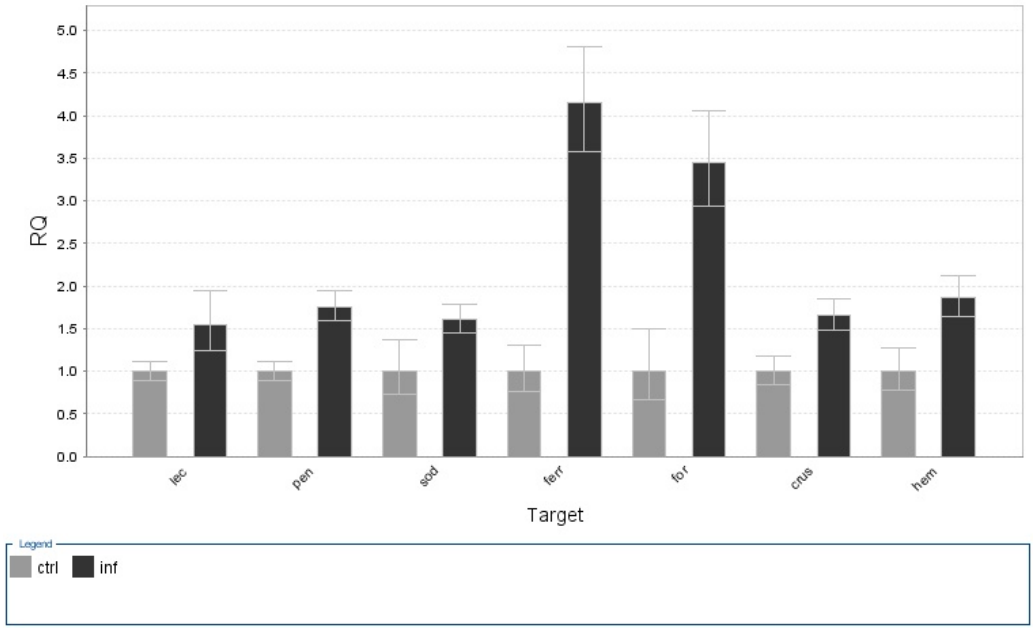

Fig 6.2 Gene expression plot of analyzed transcripts. Relative expression of immune related genes lectin, penaedin, MnSOD, hemocyanin, ferritin, fortillin, and crustin in postlarvae of *Fenneropenaeus indicus*. Expressions normalized with respect to the internal control gene β-Actin. The error bars in the histogram represent the maximum and minimum fold changes of relative expression. β-Actin is compared to itself as a reference and represented here as one fold change in its expression.

Table 6.2 Real time PCR analysis of gene expression in control and *Vibrio harveyi* challenged postlarvae of *Fenneropenaeus indicus*

	Mean cycle threshold			
	V. harveyi challenged postlarvae	Unchallenged postlarvae (control)	ΔΔ CT	Fold change in expression ($2^{-\Delta\Delta CT}$*)
B-actin	20.72	20.54	NA	NA
Ferritin	21.25	23.13	-2.05	4.15
Hemocyanin	22.07	21.35	-0.9	1.86
TCTP	27.01	28.98	-1.78	3.45
MnSOD	23.78	24.56	-0.68	1.60
Penaeidin	25.51	26.02	-0.81	1.76
Crustin	26.24	26.79	-0.72	1.65
Lectin	24.02	24.98	-0.63	1.54

Δ CT = (Target transcript CT - β-Actin CT); *ΔΔCT= (Inf ΔCT - Ctrl ΔCT),

6.4 Discussion

The expression of ferritin, TCTP and hemocyanin genes was multi-folds in *V. harveyi* challenged *F. indicus* post-larvae. The upregulation of these three genes is generally considered as a first line of defense in crustacean immunity (REF). Further, penaeidin and MnSOD are important antimicrobial peptide genes which had also significant upregulation in the challenged *F. indicus* post-larvae. The potential roles and important functions related to these proteins have been already discussed in Chapter 4.

The qPCR analysis of immune related genes in *F. indicus* revealed the upregulation of lectin protein post-*V. harveyi* challenge. Lectin is a well known antimicrobial peptide in the invertebrate immune system. It was found to have 1.54 times rise in expression in the challenged post larvae of *F. indicus.* The innate defense system relies on a group of pattern recognition proteins (PRPs) which distinguish and bind to different molecules present on the surface of invading pathogens as a defense strategy (Janeway et al., 2002). Similarly, lectin is a pathogen-recognition protein in the invertebrates with the property of binding to terminal sugars on glycoproteins and glycolipids present in almost all living organisms (Dodd et al., 2001).

As specific antimicrobial peptides, lectins tend to play a central role in the crustacean innate immunity and help in non self recognition and clearance of the invading pathogens in the host (Vasta et al., 2004). Lectins from some invertebrates have also been reported to be involved in innate defense mechanisms like opsonization, nodule formation and phagocytosis (Mercy et al., 1994; Koizumi et al., 1999; Sierra et al., 2005). They have been also linked with functions of activation of the prophenoloxidase system (Chen et al., 1995; Yu et al., 1999). Animal C-type lectins have been reported to be important in pathogen recognition and cellular interactions (Weis et al., 1998). More specifically, they have been implicated in direct first-line defense against pathogens, cell trafficking, immune regulation and prevention of autoimmunity (Kilpatrick, 2002). Few lectins have been characterized and studies for penaeid shrimps (Cominetti et al., 2002; Luo et al., 2007). Their expression is reported to increase *vis a vis* microbial challenge in *L. vannamei* and *P. monodon* (Liu et al., 2007; Ji et al., 2009).

Recently, an oral challenge of *P. monodon* with *V. harveyi* was shown to have led to its increased expression (Soonthchai et al., 2010). The 1.54 folds increase in its expression observed in this study might be implicit of the fact that the effect of bacterial pathogens on the transcription rates of antimicrobial genes. Also, such genes may get positively modulated due to the challenge. This is the first report of expression of pattern recognition proteins (PRPs) like lectin from post larval stages of any shrimp species.

Crustin was another important antimicrobial peptide which had significant upregulation in *V. harveyi* challenged *F. indicus* post-larvae. It is a hydrophobic molecule with a cysteine-rich region (12 cysteine residues) and a whey-acidic protein (WAP), responsible for forming a four disulfide core with eight cysteine residues (Bartlett et al., 2002). Crustins are a family of anti microbial peptides present in numerous crustacean species (Gross et al., 2001; Nayak et al., 2011). Crustin peptides have been characterized into Type I, II and III based on the composition of amino acid regions (Smith et al., 2008). This protein was first isolated from the shore crab *Carcinus maenas*, and reported to have general antimicrobial activity against Gram-positive marine bacteria (Schnapp et al., 1996). Though crustin has been cloned and characterized recently from *F. indicus* (Swapna et al., 2010), the present study reports for the first time its expression in challenged post larvae of the Indian white prawn. Using the EST approach this gene was also identified in two species of shrimps, *Litopenaeus vannamei* and *L. setiferus* (Gross et al., 2001). After that, crustins are reported in various crustacean species like, *Penaeus monodon* (Supungul et al., 2004), *Marsupenaeus japonicus* (Rattanachai et al., 2004), *Feneropenaeus chinensis* (Zhang et al., 2007), *Homarus gammarus* (Hauton et al., 2006), *Pacifastacus leniusculus* (Jiravanichpaisa et al., 2007), etc.

Earlier studies related to expression of crustin in *P. monodon* and cray fish *P. leniusculus* had revealed minor upregulation of this transcript (Jiravanichpaisal et al., 2007; Soonthornchai et al., 2010). However, some previous studies have reported decreased levels of Type II crustin following the challenge with Gram-negative bacteria in *P. monodon* (Supungul et al., 2008) and in *L. vannamei* (Vargas-Albores et al., 2004). In this study, the observed 1.65 folds rise in its expression in the post-larvae of *F. indicus*

following challenge with *V. harveyi* would indicate the importance of such antimicrobial peptides in the innate immune system. It is therefore hypothesized that the expression of this gene in the post-larvae of *F. indicus* is probably one of the major immune defense mechanisms in the shrimps to eliminate the invading bacterial pathogens and thereby prolonging their survival under stresses brought about by antigens.

From this study it can be summarized that *V. harveyi* infection in *F. indicus* modulates the expressions of various kinds of genes involved in the defense responses of crustacean innate immunity. The qPCR expression profiles of some major defense related transcripts including genes coding for antimicrobial peptides suggests the importance of such quantitative methods as health monitoring tools in aquaculture as well as to realize the linkage between gene expression levels and survival modes of animals exposed to bacterial infections/adversities. As has been emphasized previously, the gene expression data generated in this study will be useful for further investigations dealing with the establishment of disease control in aquacultured penaeid shrimps. It may also be mentioned here that results from this first study on the little-investigated, commercially vital Indian white prawn, *F. indicus* be prominently noted as useful in demonstrating the effectiveness of gene expression-analysis in elucidating and understanding the mechanisms of shrimp responses to bacterial stress at the molecular level.

Chapter 7

Full-length cloning of ferritin and TCTP gene from *Fenneropenaeus indicus*

7.1 Introduction

The invertebrate immune system which lacks immunologic memory is dependent on specific innate molecules for its defense against external pathogens (Lee and Söderhäll, 2002). As mentioned in the earlier Chapters the translationally controlled tumor protein (TCTP) and ferritin genes were the two most important immune related molecules expressed in the shrimp postlarvae as a result of *V. harveyi* challenge (Nayak et al., 2011). In Chapter 6, results of upregulation of these genes in *V. harveyi* challenged *F. indicus* through qPCR analysis are also described.

As the expression levels of TCTP are reported to be significantly upregulated in response to a large number of extracellular stimuli, there have been a large number of studies on this aspect (see Bommer et al., 2004). This protein has been designated with a large array of functions and diverse biochemical activities. It has a significant role in important cellular processes, such as cell growth, cell cycle progression, and malignant cell transformation (Gachet et al., 1999; Liu et al., 2005; Yang et al., 2005). TCTP, known also as p23 or fortillin, is a highly conserved and widely expressed protein throughout the animal and plant kingdoms (Mulenga et al., 2005; Venugopal, 2005). It is reported to protect cells under toxic conditions and from apoptosis (Graidist et al., 2006; Hsu et al., 2007). Previously, it was known as tumor-related protein as observed in mouse erythroleukemic cells (Yenofsky et al., 1983; Chitpatima et al., 1988). It is reported to function as an extracellular cytokine having considerable medical relevance (Chitpatima et al., 1988).

Ferritin has been reported from a wide range of organisms starting from prokaryotes to eukaryotes (Harrison and Arosio, 1996). Ferritins are basically iron storage proteins found throughout the animal Kingdom and belong to a large family of ferritin-like diiron-carboxylate proteins (Theil, 1987). Ferritin is related to innate immune responses as its synthesis is regulated by cytokines responsible for inflammatory responses at transcriptional and translational levels (Konijn et al. 1989; Rogers et al. 1990). Being an iron storage protein, it is very crucial in the metabolism as well as in the detoxification of iron. Its expression has been reported to be associated with the role of

maintaining iron homeostasis inside the cell (Marcelis et al., 1980). It acts effectively against bacterial pathogens by failing the pathogens the chances of binding to iron in the host tissue (Ong et al., 2005). Earlier studies of this transcript in shrimps (Pan et al., 2005; Zhang et al., 2006) had revealed upregulation following challenge with white spot syndrome virus (WSSV).

Suppression subtractive hybridization (SSH) experiment between healthy and *V. harveyi* challenged *F. indicus* postlarvae had revealed high representation of both these transcripts in the subtracted library (Chapter 4). The qPCR analysis of these proteins had also shown upregulation in both *P. monodon* and *F. indicus* postlarvae challenged with *V. harveyi* (Nayak et al., 2010; 2011). Therefore, full length cloning of TCTP and ferritin genes was carried out in order to describe their functions in greater details by comparing their molecular differences, if any with the previously reported observations. For cloning of these transcripts, RACE (rapid amplification of cDNA) technique of Maruyama and Sugano (1994) was followed. RACE is a popular method of PCR amplification to generate full length cDNA sequences when only a small portion of the sequence is known.

In this study, a Gene Racer Kit (Invitrogen, USA) was used to generate the full length gene. RACE-PCR protocol is based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) methods (Fig 7.1), which results in the selective ligation of an RNA oligonucleotide to the 5′ ends of decapped mRNA using T4 RNA ligase (Maruyama and Sugano, 1994; Volloch et al., 1994; Schaefer, 1995). After ligation of the RNA oligo to the mRNA it is reverse transcribed to cDNA.

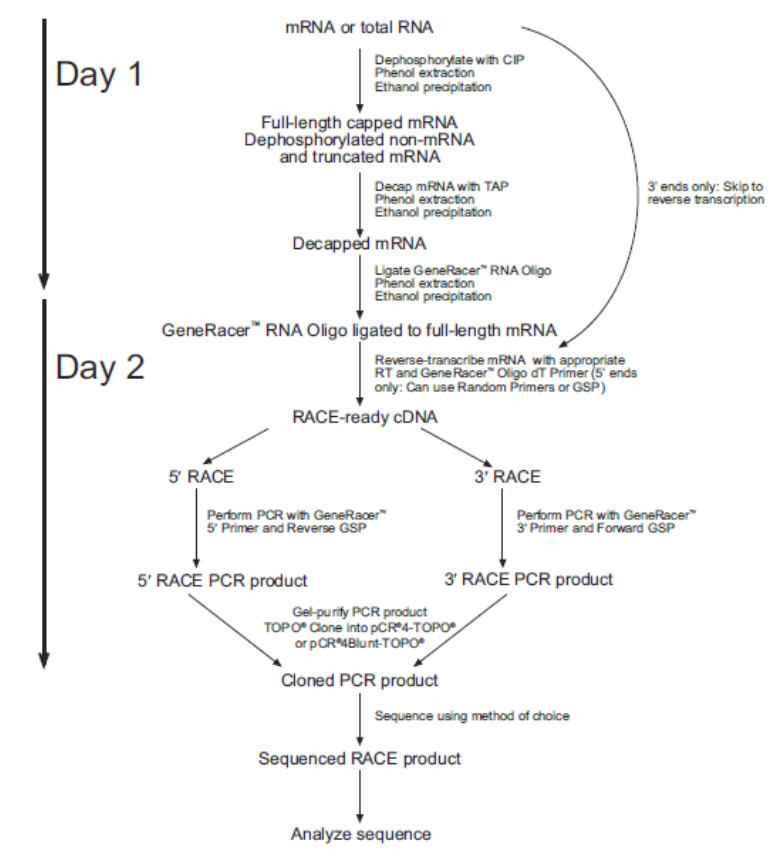

Fig 7.1 Flow chart for the Gene Racer protocol showing the steps involved in generation of the 5' and 3' end cDNA products. The protocol describe a two day long protocol, starting with Day 1 where the mRNA is decapped and RNA oligo is ligated, on the day 2 the mRNA is reverse transcribed into cDNA and 5' and 3 ends are later generated and finally they are sequenced directly or cloned into vector (Invitrogen handbook)

After cDNA synthesis the 5' ends of the cDNA are amplified using a reverse gene specific primer and the GeneRacer 5' primer (Fig 7.2).

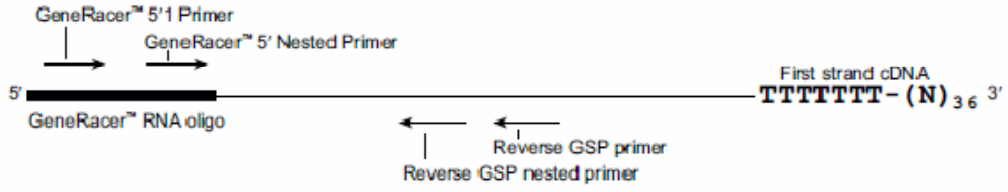

Fig 7.2 The 5'ends of the cDNA are amplified using a reverse gene specific primer and the GeneRacer 5' primer. In this amplfication only the mRNA which have GeneRacer RNA oligo attached to its 5' and which is completely transcribed is amplified. (Invitrogen handbook)

Similarily the 3' ends of the cDNA are amplified using a forward gene specific primer and the GeneRacer 3' primer (Fig 7.3).

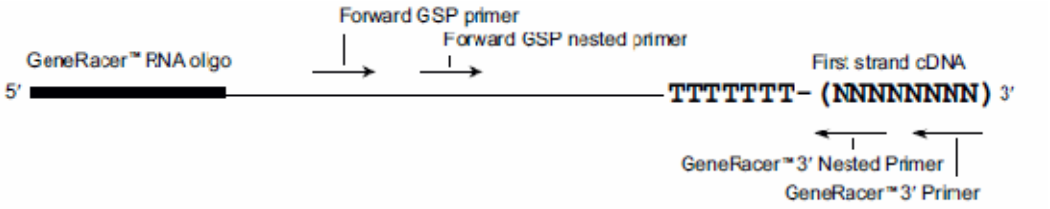

Fig 7.3 The 3'ends of the cDNA are amplified using a forward gene specific primer and the GeneRacer 3' primer. In this amplfication only the the mRNA which have poly A tail and which is completely reverse transcribed is amplified (Invitrogen handbook)

7.2 Materials and Methods

7.2.1 Total RNA isolation and RACE-PCR

Total RNA was isolated from the hepatopancreas of freshly dissected *F. indicus* using Trizol reagent (Sigma, USA). Protocols for the isolation of RNA are same as detailed in chapter 3. To clone the full length cDNA of TCTP and ferritin gene 3' RACE and 5' RACE was carried out using a Gene racer Kit (Invitrogen, USA) following the instructions of the manufacturer. cDNA synthesis was carried out using reagents provided with the Gene racer kit (Invitrogen, USA). Separate reactions for amplifications of 5' and 3' fragments were carried out using gene specific primers and Gene racer primers provide by the manufacturer. The gene specific primers used to amplify the ferritin and TCTP cDNA were designed from the corresponding EST fragments obtained from the *F. indicus* subtracted cDNA library. All gene specific primers used in this study were designed using the Primer express software (ABI, USA) (Table 7.1). The 3' RACE and 5' RACE PCR products obtained were cloned separately into TOPO vector (Invitrogen, USA). The ligation products were transformed into *E. coli* Top10 cells by electroporation. From each cloned library, 10- 12 positive clones were picked out randomly and checked for inserts by carrying out PCR with gene specific primers. Plasmid isolation from the clones was done using a Pure Link plasmid DNA purification

kit (Invitrogen, USA). The 5' and 3' RACE products were later sequenced bi-directionally using Gene specific primers.

7.2.2 Sequence analysis

All raw sequences were manually screened for vector sequences using Vecscreen tool available at NCBI. After removal of vector sequences the sequences were analyzed using blastx and blastn algorithms. The complete nucleotide sequence of the gene was obtained after aligning the 5'and 3' end cDNA sequences. The open reading frame for the obtained nucleotide sequence was determined by using the ORF Finder tool available at NCBI. The full length sequences were submitted at GenBank. Multiple sequence alignment of the deduced protein sequence of TCTP and ferritin along with other homologous sequences from representative species was carried out using Clustal X software following the profile method (Thompson et al., 1997). Sequence editing was carried out using the Bioedit software. Protein sequences used for the phylogenetic analysis of TCTP and ferritin were retrieved from the GenBank database (Table 1a and 1b). Separate phylogenetic trees for the both the proteins were constructed using Neighbour joining method using Mega-4 by default settings of the program. To get the most probable tree, the sequences were boot-strapped 1000 times. Branches with <50% bootstrap support were collapsed in the final tree.

7.3 Results

7.3.1 Full length cloning of TCTP gene and sequence analysis

The 302 bp length TCTP EST fragment identified from *F. indicus* was used as template to design primers for full length amplification of this gene. On Blastx analysis, the identified *F. indicus* complete gene (Accession No gb|JG772274|) was identical to a previous *F. indicus* TCTP sequence (gb|ACR58988.1|). The identified fragment had 98% similarity to *Litopenaeus vannamei* fragment (Accession No. gb|ABY55541.1|) and *Fenneropenaeus merguiensis* (Accession No.gb|AAV84282.1|) also it was found to have 97 % homology to Penaeus monodon TCTP fragment (Accession No. gb|AAO61938.1|).

The complete TCTP nucleotide sequence obtained after RACE PCR was 727 bp in length. Upon analysis of the complete RACE PCR product, it was found that the ORF of this gene consisted of 507 bp coding for 168 amino acids (Fig 7.7). The calculated iso-electric point (pI) was 4.3 and molecular weight was 19.23kDa. Highly conserved nature of TCTP was evident from the amino acid sequence alignment of TCTP sequences from different arthropod representatives (Fig. 7.5). When compared within the penaeid shrimp species particular amino acid substitutions were observed at positions 37, 141 and 155 in the sequence alignment (Fig 7.5). At position 37 a valine to isoleucine substitution was found and at position 141, aspartic acid to valine substitution was observed. A third substitution was observed at position 155 where aspartic acid was substituted by histidine (Fig 7.5).

7.3.2 Phylogenetic analyses of the TCTP gene

The phylogenetic tree constructed in this study clearly shows that the crustacean members distributed in four different groups (Fig 7.6). The *F. indicus* TCTP along with other penaeid family representatives clustered to a separate clade and the protein sequences, taken for the tree construction, clustered with 100% bootstrap value. The homology of different arthropod TCTP sequences as compared to the identified *F. indicus* TCTP sequence in terms of percent identity are presented in (Table 7.2). The phylogenetic tree clearly shows the high homology between TCTP sequences from *F. indicus* and *F. chinensis* which have clustered in the same node (Fig. 7.6). From the phylogenetic tree it can be clearly seen that *Aedes albopictus* and *Tigriopus japonicus* did not fall into any of the four groups. A high similarity shared by TCTP from *F. indicus* with other penaeid species of shrimps suggests the conserved nature of TCTP within the penaeid family.

7.3.3 Full length cloning of ferritin gene

After 3′ and 5′ ends RACE PCR, the full-length cDNA fragment of *F. indicus* ferritin gene was found to be 1221 bp in length (Accession No gb|JN651914|). The full-length nucleotide sequence and the deduced amino acid sequence are shown in (Fig 7.7). Upon Blastx analysis of the obtained ferritin cDNA fragment had 98% identity at

the amino acid level with the *F. chinensis* fragment (ABB05537.1) and *L. vannamei* (AAX55641.1) fragment. The identified fragment also shared 96% similarity with previously deposited *P. monodon* (gb|ABP68819.1) sequence in the Genbank. Upon analysis of the complete nucleotide sequence of ferritin it was found that that the open reading frame (ORF) of this gene was 510 bp in length. The 5′ untranslated region (5′-UTR) was found to be of 130 bp and 3′ untranslated region (3′-UTR) was 568 bp. The ORF encoded 170 amino acids with a predicted molecular weight (MW) about 19385.81 Da and a theoretical isoelectric point (PI) of 5.03. The deduced amino acids sequence also shared high similarity with those of *Pacifastacus leniusculus* (CAA62186), *Macrobrachium rosenbergii* (ABY75225.1), *Scylla paramamosain* (ADM26622.1) and *Procambarus clarkii* (AEB54659.1). Amino acid sequence alignment of the identified *F. indicus* ferritin gene with other representative species however showed no significant amino acid substitutions (Fig 7.8). An iron responsive element sequence (IRE) which is specific features of the ferritin protein was found to be located at positions 5 to 30 bases towards the 5'UTR region (Fig 7.7).

7.3.4 Phylogenetic analyses of ferritin gene

The phylogenetic tree constructed in this study clearly shows that the *F. indicus* ferritin along with other shrimp species representatives clustered to a separate clade (Fig 7.9). The homology of different representative ferritin sequences deposited in GenBank as compared to the identified *F. indicus* ferritin sequence in terms of percent identity is presented in (Table 7.3). Upon analysis of the phylogenetic tree it was seen that there was high homology between ferritin sequences from *F. indicus* and *F. chinensis* which have clustered in the same node (Fig 7.9). A high similarity shared by ferritin from *F. indicus* with other penaeid species suggests the conserved nature of ferritin within the penaeid family. In this phylogenetic tree the *D. melanogaster* ferritin sequence was found to be an outlier.

Table 7.1 Gene specific primers used in the RACE PCR reaction for cloning the full length cDNA of ferritin and TCTP gene from *Fenneropenaeus indicus*.

Primer	Sequence	Product size (bp)
TCTP	F 5' ATGCGTCTGCAGGAAACCGGCTT 3' R 5' AAGACTGGCCGCTCTTCTCCATG 3'	302
Ferritin	F 5' TACCAGAACAAGCGAGGTGGC 3' R 5' ATCTTCAAGAAGCTTAGTGA 3'	216

Table 7.2 Accession numbers of arthropod translationally controlled tumor protein (TCTP) sequences retrieved from GenBank and used for the present study

Accesssion No.	**Arthropod representative**	**No. of aminoacids**	**% ID**
ABY55541	*Litopenaeus vannamei*	168	98.2
ABB05535	*Fenneropenaeus chinensis*	168	97.6
AAO61938	*Penaeus monodon*	168	97.6
AAV84282	*Fenneropenaeus merguiensis*	168	97.6
AAR88095	*Tigriopus japonicus*	172	52.9
AAV90736	*Aedes albopictus*	171	50.2
AAV91374	*Lonomia oblique*	172	48.2
BAC99978	*Bombyx mori*	172	50
BAD52260	*Plutella xylostella*	172	51
AAF54603	*Drosophila melanogaster*	172	55.8
AAR09822	*Drosophila yakuba*	172	55.8
EAL29084	*Drosophila pseudoobscura*	172	54
AAY66972	*Ixodes scapularis*	173	44
AAY67698	*Boophilus microplus*	173	41.9
AAY67699	*Dermacentor andersoni*	173	43.1
AAY67700	*Amblyomma americanum*	173	43.1
AAL75585	*Dermacentor variabilis*	173	43

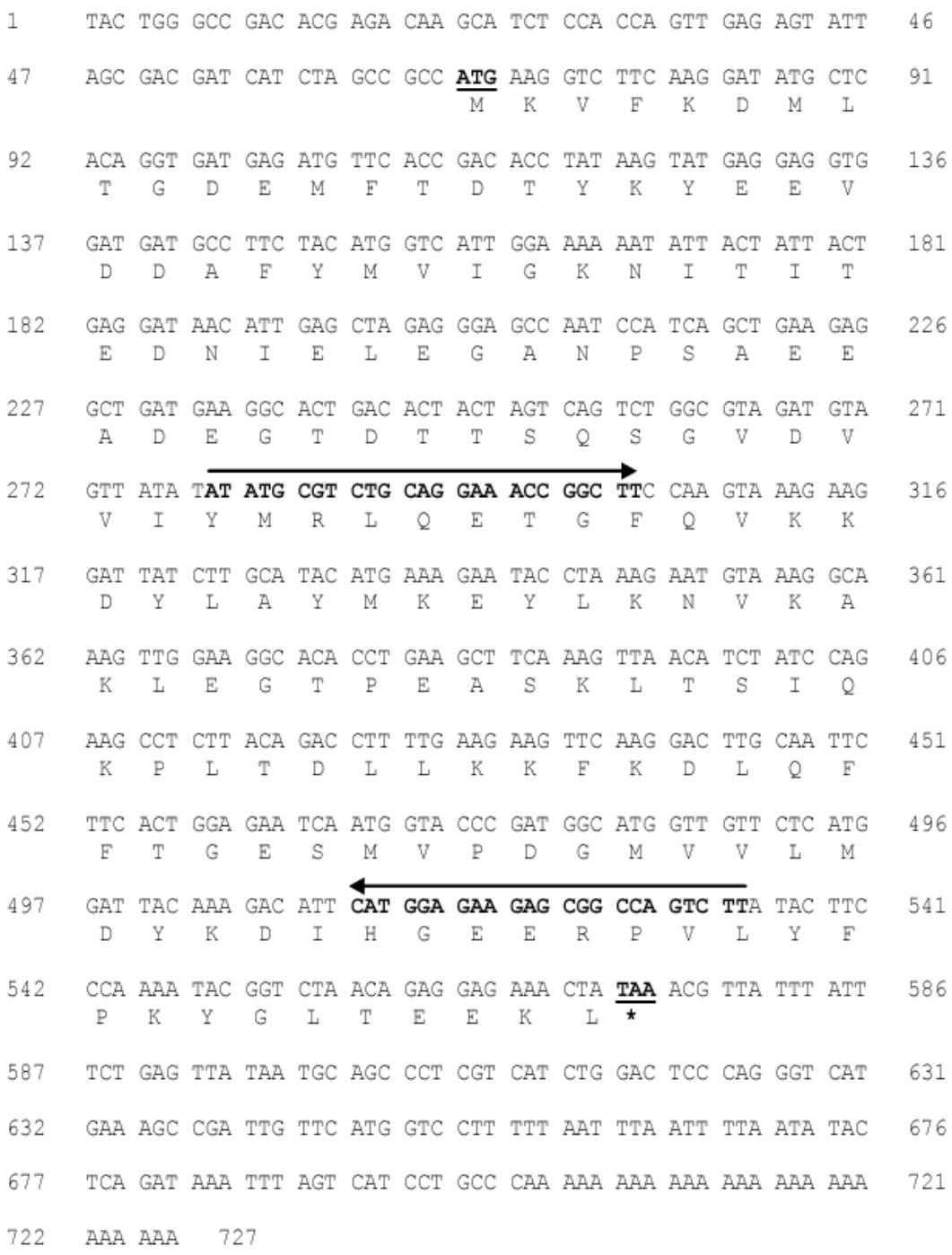

Fig 7.4 Complete nucleotide and deduced amino acid sequence of translationally controlled tumor protein gene from *Fenneropenaeus indiucs* (Accession No gb|JG772274|) obtained in this study using RACE-PCR. Letters in bold and underlined indicate the start (ATG) and stop codons (TAA), arrows indicate the annealing sites of gene specific primers used in the RACE-PCR.

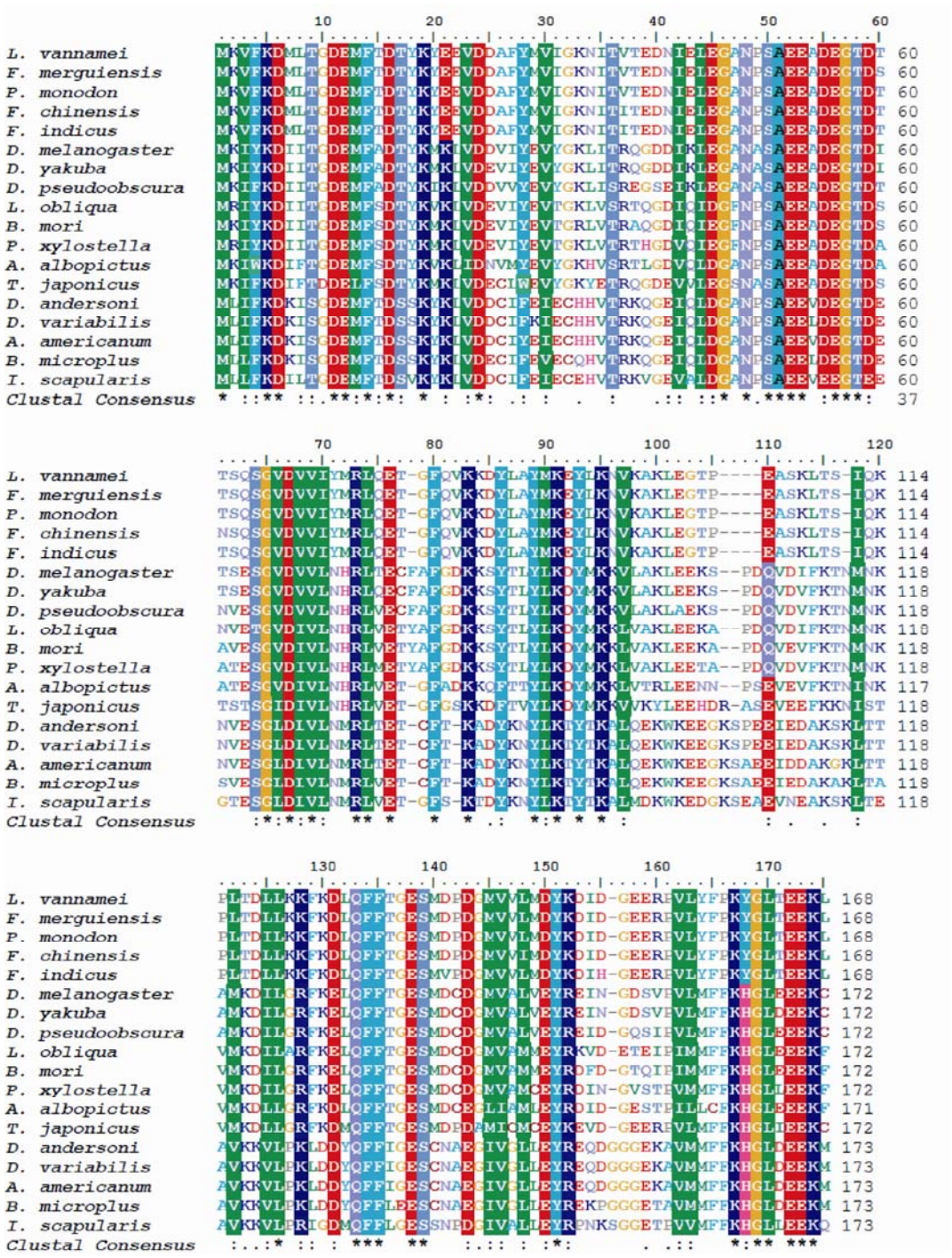

Fig 7.5 Multiple sequence alignment of *Fenerropenaeus indicus* TCTP sequence obtained in this study (Accession No gb|JG772274|) with other representative arthropod TCTP sequences. Conserved aminoacid residues are shaded in grey. Amino acid substitutions for *Fenneropenaeus indicus* are shown in grey square boxes.

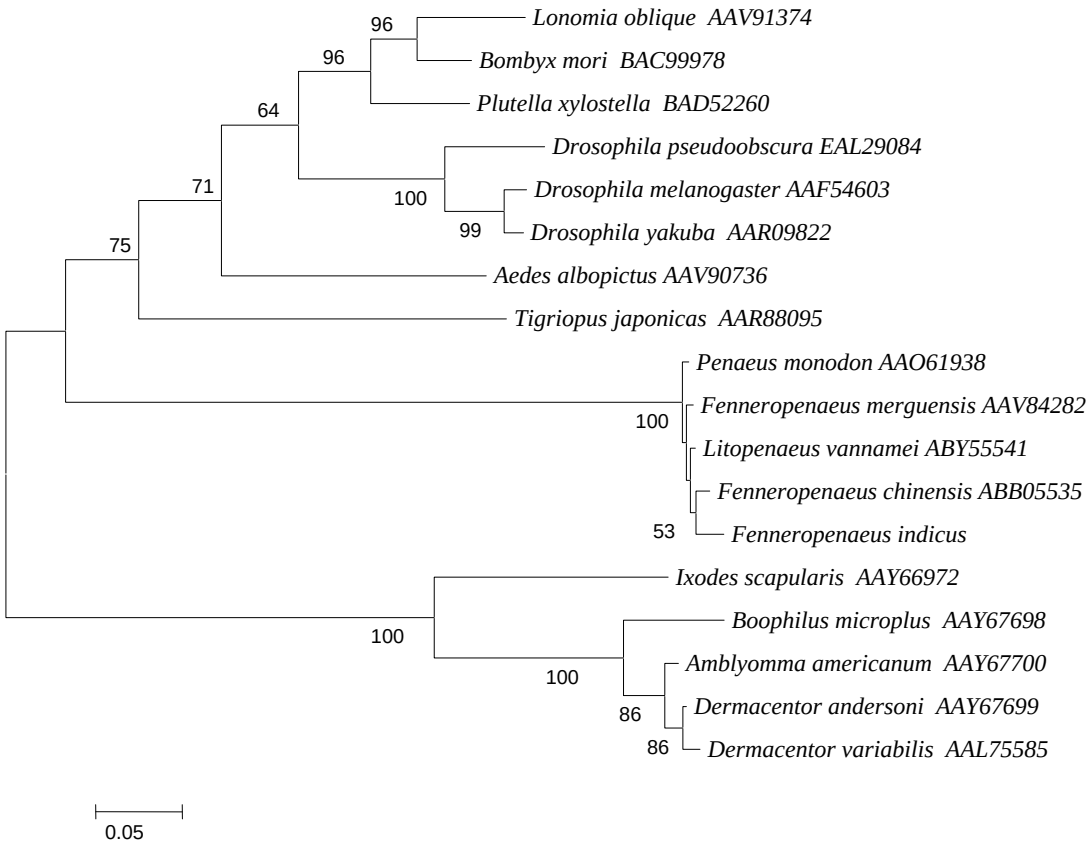

Fig 7.6 Phylogenetic tree of translationally controlled tumor protein (TCTP) gene in arthropods. The tree was constructed using Neighbour joining method using Mega-4. The percentages of boot strap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.

Table 7.3 Accession numbers of ferritin amino acid sequences retrieved from GenBank and used for the present study

Accession No.	Representative organism	No. of amino acids	% ID
AAA35832	*Homo sapiens*	183	55
NP_571660	*Danio reiro*	177	57
AAA37614	*Mus musculus*	183	44
CAD91440	*Crassostrea gigas*	171	60
CAA62186	*Pacifastacus leniusculus*	181	68
AEB54659	*Procambarus clarkii*	170	77
ABY75225	*Macrobrachium rosenbergii*	171	78
ABB05537	*Fenneropenaeus chinensis*	170	98
AAX55641	*Litopenaeus vannamei*	170	98
ABP68819	*Penaeus monodon*	170	95
AAL55398	*Artemia franciscana*	171	60
CAA75724	*Drosophila melanogaster*	205	31

Fig 7.7 Complete nucleotide and deduced amino acid sequence of ferritin gene obtained from *Fenneropenaeus indicus* (Accession no.gb|JN651914|). The start codon (ATG) and the stop codon (TAA) are indicated by underlined letters. The annealing sites of gene specific primers used in the RACE-PCR are shown by arrows. The IRE site is shown in rectangular box in the 5' UTR.

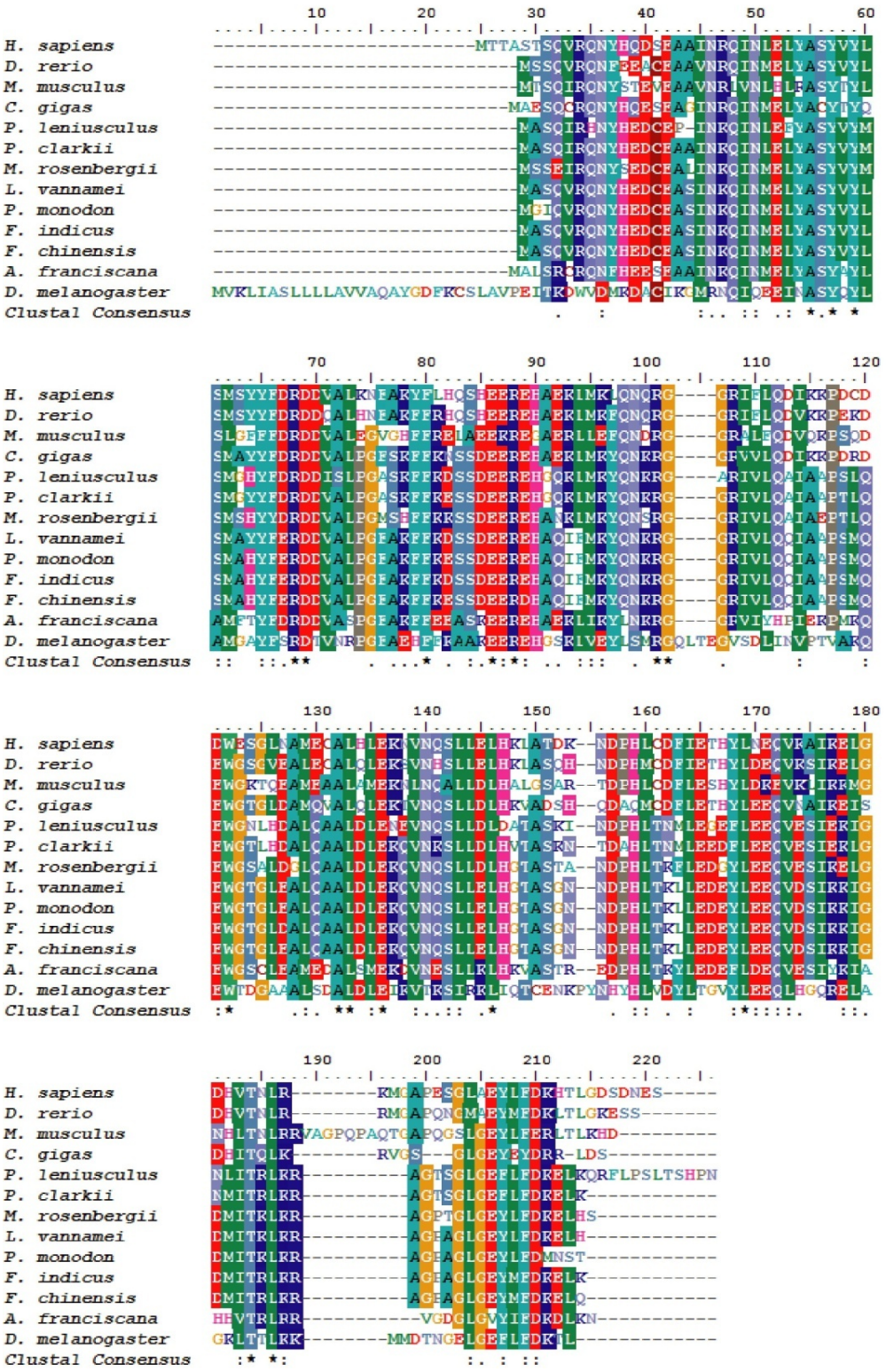

Fig 7.8 Multiple sequence alignment of *Fenerropenaeus indicus* ferritin amino acid sequence obtained in this study (Accession No gb|JN651914|) with other representative ferritin sequences. Identities are marked in asterisk below and similarities are shaded in colored codes.

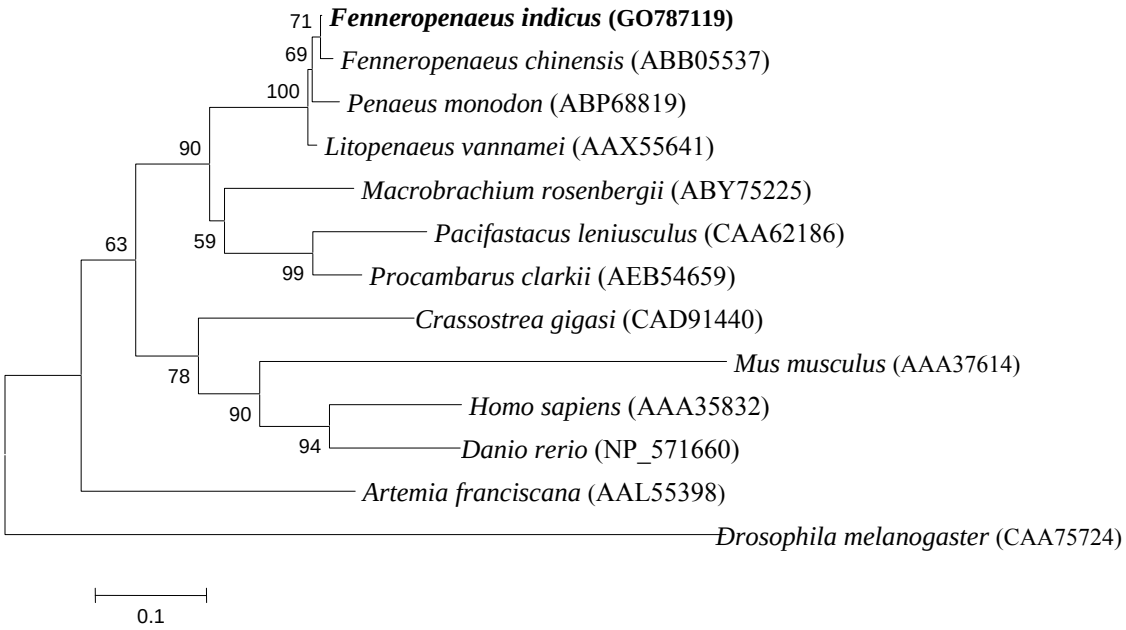

Fig 7.9 Phylogenetic tree of ferritin gene from *Fenneropenaeus indicus*. Phylogenetic tree was constructed from the deduced amino-acid sequences of ferritin along with other several representative organisms. The tree was constructed using Neighbour joining method using Mega-4. The percentages of bootstrap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.

7.4 Discussion

In this study, the full length genes of TCTP and ferritin from *F. indicus* were cloned individually using RACE PCR technique. Since the full length cDNAs are important in characterizing and correct annotation of genomic sequences, this technique has great significance in elucidating the functional profiles of any given protein molecule (Satoh et al., 2007).

Upon amino acid sequence alignment, the *F. indicus* TCTP showed three significant substitutions which were specific for this species. Amino acid substitutions were observed at positions 37, 141 and 155 in the sequence alignment (Fig 7.5). A valine to isoleucine substitution at position 37, aspartic acid to valine substitution at position 141 and aspartic acid to histidine substitution at position 155 are unique to the TCTP gene in *F. indicus* (Fig. 7.5) as compared to other penaeid shrimps. Substitution of histidine might be very significant as histidines are known to impart antinflamatory and antioxidative properties to proteins (Chen et al., 1998; Seo et al., 2010). In this regard observations by Mohankumar and Ramasamy (2006) and, Mathew et al., (2007) are useful to infer that when shrimps undergo severe oxidative stress under viral and/or microbial challenges, such substitutions might prove effective in dealing with the pathogen. Furthermore, the antimicrobial activity of histidine containing peptides against pathogenic bacteria is also well known (Mason et al., 2006). These substitutions might impart TCTP a definite role in defense against bacterial pathogens. Thus expression of TCTP in *F. indicus* postlarvae might protect them from bacterial challenges and usher in their survival due to or, during microbe-induced stress.

Recent studies using SSH, have recognized the protective role of TCTP as an important defense molecule in *V. harveyi* challenged *P. monodon* (Bangrak et al., 2004; Nayak et al., 2010). Recently elevated levels of TCTP expression has been reported in WSSV challenged *F. indicus* (Rajesh et al., 2010). Though, TCTP expression is sometimes reported to decrease during the terminal stage of the infection as in a case of WSSV challenge (Graidist et al., 2006), its manifold expression in challenged *F. indicus* postlarvae might be of greater relevance to the affected hemocytes during the processes

of apoptosis. Along with antioxidant and innate immune responses, the increased expression of TCTP signifies it importance as an integral part of host response against bacterial challenges in cell death/apoptosis mechanisms.

From the complete nucleotide sequence of the *F. indicus* TCTP, it is clear that the structural conservation and uniqueness of this molecule might play significant roles in many biochemical and signaling pathways. Although few but significant amino acid substitutions were observed in this study, these substitutions might play crucial roles in the defense of microbial challenged shrimps and thereby have relevance with the protective functions associated with this molecule. While direct evidences on its anti-apoptotic properties are essential, results from this study do imply its prominent role in innate defense mechanisms of shrimps. This study is useful to recognize the importance of up-regulation of anti-apoptotic mechanisms in shrimp as a result of bacterial pathogens. From this present study, it is clear that TCTP plays a critical role in the survival of the *F. indicus* against *V. harveyi* infection/challenge.

While extensive studies have been carried out on ferritins in vertebrates, very little is known about their sequence, distribution and expression in crustaceans and fishes. Many studies earlier have shown that ferritin synthesis of ferritins is directly affected by inflammatory conditions including those caused by bacterial infections (Orino and Watanabe, 2008). The 1221 bp RACE PCR product obtained in this study had very high similarity to *F. chinensis* ferritin sequence (ABB05537.1). Detailed structure of ferritin gene shows the presence of iron-responsive element (IRE) region which is an important factor in assigning functional importance as an iron binding protein in the invertebrates. This region of ferritin gene codes for a very unique iron regulatory protein (IRPs) that can help in translational regulation of ferritin gene by binding to iron responsive elements (IREs) located in the 5′ untranslated regions (5′-UTR) of the gene (Durand et al., 2004). The IRE was found to be located at positions 5 to 30 bases towards the 5'UTR region. The IRE regions are very conserved and found in almost all major vertebrates and invertebrates. Higher levels of ferritin have been already shown in our previous study in *F. indicus* (Nayak et al., 2011). From this it can be hypothesized that higher expression

levels of ferritin gene signifies its defense mechanism against the invading bacteria as has been shown earlier by Marcelis (1980). In essence, binding of free iron is considered bacteriostatic and bacterial cell growth is inhibited if no iron is available for synthesis of essential iron-containing compounds.

The importance of ferritin is due to its multifaceted roles in diverse number of biological processes like detoxification (Munro and Linder, 1978), inflammation (Roeser et al., 1980) development regulation (Levenson and Fitch, 2000) and neuronal differentiation (Van Landingham and Levenson, 2003). Its immunogenic properties can also be contributed to its unique sequence structure which permits it to act as a iron sequestering machinery (Ong et al., 2006). Their multimeric structure consists of an assembly of 24 individual subunit proteins and each of the subunit has a definite functional role (Harrison and Arosio, 1996). The core formed by this protein is able to bind upto 4500 iron atoms (Harrison and Arosio, 1996). Rise in the levels of ferritin expression has also been reported as a signal or marker for the commencement of inflammatory processes and neoplasms (Ozen et al., 1995; Moroz et al. 1997). Accordingly, ferritin is also well known to play a unique role in the innate immunity processes against viral infection (Zhang, et al., 2006). Although the perfect function of this molecule as an antibacterial agent in shrimps is still to be proved, this study has brought to light its relevance in *F. indicus* as a major defense protein against bacterial agents like *Vibrio harveyi.*

Results from this study demonstrate the structural uniqueness of TCTP and ferritin genes and bring forth their important functions in *F. indicus* innate immunity. Both these two proteins share exclusive sequence structure conservation among their related species implying a strong evolutionary relationship of these genes within different shrimp-species. Although few but significant amino acid substitutions were observed in *F indicus* TCTP gene, they might be vital in the defense of larval stages of *F. indicus*. From the total absence of shrimp-species specific substitution in the ferritin gene, it is ascribable that its protective roles are highly similar to the ones reported from other shrimps. Although direct evidences on anti-apoptotic properties of *F indicus* TCTP or

that of ferritin in multifaceted protective functions would expand our understanding, results from this study in general imply their prominent role in innate defense mechanisms of shrimps.

Chapter 8

Summary

The major denominator in the success of aquaculture industry is diseases. Among the plethora of possible ailments in maricultured shrimps, bacterial diseases are a serious concern due to high mortality of post-larvae in hatcheries and grow-out ponds. Most shrimp producing countries face problems of sustainability and development of this sector. Besides being a true shrimp-pathogen, *Vibrio harveyi* is also an opportunistic pathogen in secondary infections. For long-term production and economic sustainability of this industry, prevention and control of diseases are to be top priorities. Understanding shrimp innate immune system and its molecular mechanisms is therefore essential to prevent as well as control the frequent occurrence of microbial diseases which usually are responsible for huge economic losses.

More recently, the high throughput molecular characterization of genes and gene products has become an important strategy for understanding the shrimp immune system. In this direction, functional genomics approaches like SSH especially allow the easy and effective identification of genes without any previous knowledge on the molecular bases for immune responses. Also gene expression techniques like the qPCR are efficient for describing transcriptomic changes associated with immune responses. Quantitative assays are significant for the evaluation and monitoring of the health status of shrimps which thereby help in designing and, development of efficient disease intervention strategies.

Modulation of gene expression in post-larval stages of two economically important penaeid shrimps *Penaeus monodon* and *Fenneropenaeus indicus* upon bath-challenge with a virulent strain of *Vibrio harveyi* was investigated in this study.

In this extensive and first-time investigation on molecular immune responses in the post-larvae of these species, a number of genes responding to *V. harveyi* challenge were identified using SSH technique. Gene expression analysis of some selected immune related genes carried out using qPCR assays, revealed their significant upregulation due to the *V. harveyi* challenge. Full length cloning and characterization of two important upregulated defense related genes are useful in appreciating the importance of their molecular structure. Thus, an emphasis on the need for gene sequencing to note antimicrobial properties of immune relevant genes could be made.

Following are some of the major observations from this work

- The initial bath challenge experiments with five luminescent strains selected on the basis of their biochemical similarity with some reportedly virulent strains were helpful in identifying the strain *Vibrio harveyi* D3 as a virulent strain as was also confirmed by mortality of the post-larvae.
- After the SSH and cloning procedures, the sequencing of 1152 clones from the subtracted library of *V. harveyi* challenged *P. monodon* resulted in an identification of 45 different genes. Approximately 37% ESTs did not match to any of the sequences in GenBank. This observation be taken to note that these may be novel genes and/or gene products having crucial roles in the defense responses of *P. monodon* against *V. harveyi* challenge. Apart from these, 20 hypothetical proteins were also identified.
- Based on their physiological and functional roles, the annotated ESTs from *P. monodon* library could be grouped into 10 functional categories. Among these, three genes are immune-relevant and, eight others, related to antioxidant and ATP metabolism. The largest group comprised of the muscle and cytoskeletal related genes which were represented by 12 different transcripts followed by the enzymes which comprised of eight different genes.
- Defense and immunity related genes contributed to 6% of the total identified ESTs. The most abundant immune related genes in the subtractive library were ferritin, hemocyanin and translationally controlled tumor protein (TCTP).
- Accounting to 6% of the total identified transcripts, a large number of sequences encoding antioxidant molecules, such as cytochrome c oxidase subunit III, cytochrome c oxidase subunit I and NADH dehydrogenase subunit-I were also identified. ATP metabolism genes such as F1F0-type ATP synthase subunit G and H^+ transporting ATP synthase involved in ATP metabolism were also found in large numbers.
- Occurrence of genes related to cytoskeleton motility was very high in the subtracted cDNA library and, the genes encoding for allergens such as arginine kinase, myosin light chain and sarcoplasmic calcium binding protein were also

present. Genes encoding for different enzymes such as pyruvate kinase, trypsin, catalase, xylose isomerase and phosphoglycerate kinase were detected in small numbers. Two genes related to crustacyanin pigment were also identified from the subtracted library.

- Sequencing of 1400 clones from *V. harveyi* challenged *F. indicus* library, on the other hand, resulted in identification of 38 different genes and 8 hypothetical proteins. Only 809 ESTs showed significant similarities with previously deposited sequences of the database; leaving as many as 29% of the ESTs with no match with any sequence in GenBank. Apparently, all these non-matchable ESTs are new additions to the existing GenBank, in particular of the least investigated *F. indicus* database.
- Based on their putative physiological and functional role as many as 10 broad categories of ESTs were identified from *F. Indicus* library. Among the 38 identified ESTs, 8 were immune-relevant genes, six related to antioxidant/ATP metabolism, nine to muscle/cytoskeletal proteins and, four to cell cycle progression and protein modification. Remaining 11 ESTs were of genes related to enzymes, pigments and/or other functions.
- Although the numbers of genes related to ATP/energy metabolism and antioxidant metabolism was preponderant and occurred frequently, many transcripts having significant roles in crustacean immunity were identified from this subtracted cDNA library. These defense-related genes contributed 10% of the total identified ESTs. Transcripts of metal binding proteins and proteases such as carboxypeptidase B (CPB), ferritin, translationally controlled tumour proteins (TCTP), hemocyanin, chitinase and serine carboxy peptidase were the prominent ones.
- Interestingly, there was also a higher incidence of genes related to cell cycle progression and protein modification such as S-phase kinase-associated protein (SKP1) and ubiquitin The SKP1 in particular is observed and reported for the first time in penaeid shrimps. It is of much interest that these transcripts are identified as a result of bacterial challenge to post-larvae. Genes coding for retinoid-

inducible serine carboxypeptidase, as well as genes associated with cytoskeleton motility seem to have been up-regulated after the challenge.

- Genes encoding for different enzymes such as pyruvate kinase, trypsin, DNA gyrase and transposase were also identified. Most enzymes identified in this study were either DNA related or cellular metabolism-associated. The cDNA fragments encoding for allergens such as arginine kinase, myosin light-chain and sarcoplasmic calcium binding protein were also present. In addition, genes related to crustacean pigments such as crustacyanin subunit I, subunit II and rhodopsins were identified.
- The qPCR analysis of the cDNA from the *V. harveyi* challenged *P. monodon* post-larvae revealed a significant increase in the relative expression of all six examined genes. Hemocyanin gene had the highest fold rise in expression of 11.6 folds whereas TCTP, 6.47 fold and ferritin, 2.04 fold rises. Similarly, the lysozyme gene had 5.49 fold, penaedin 2.09 fold and MnSOD 3.28 fold rises.
- Akin to observations from *P. monodon* post-larvae, the qPCR results for the seven immune related genes in *V. harveyi* challenged *F. indicus* post-larvae showed significant increase in their relative expression. Major upregulation was observed for ferritin (4.15 folds) and TCTP (3.45 folds). Hemocyanin transcript showed 1.86 fold rise in expression. Clearly, importance of hemocyanin is significant in processes such as activation of prophenoloxidase pathway for elimination of pathogens. Besides, the quantification of other four important antimicrobial defense-related genes (MnSOD, penaeidin, crustin and lectin) suggested marked up-regulation in their expressions.
- Full length cDNA of the TCTP and ferritin transcripts from the Indian white prawn, *Fenneropenaeus indicus* were cloned using RACE-PCR.
- Detailed analysis of TCTP RACE-PCR product revealed the open reading frame of the gene to be of 507 bp coding for 168 amino acids. The calculated iso-electric point (pI) was 4.3 and molecular weight was 19.23kDa.
- Highly conserved nature of TCTP was evident from the alignment of TCTP amino acid sequences from different arthropod representatives. Only three amino acid substitutions were found at positions 37, 141, 155 specific to the Indian white

prawn. A valine to isoleucine substitution at position 37, aspartic acid to valine at 141 and, aspartic acid to histidine at 155 are unique to the TCTP gene in *F. indicus.* These substitutions might impart TCTP a definite role in defense against bacterial pathogens. Thus upregulation of TCTP in post-larvae might protect them from bacterial challenges and help their survival due to microbial stress(es).

- Phylogenetic analysis suggested quite close relatedness of *F. indicus* TCTP to *F. chinensis*, *L. vannamei* and *F. merguensis.* Observed high similarity suggests its conserved nature within the penaeid family.
- After 3′ and 5′ ends RACE PCR, the full-length cDNA fragment of *F. indicus* ferritin gene was found to be 1210 bp (Accession No gb|JN651914|). Upon blastx analysis the obtained ferritin fragment had 98% identity at the amino acid level with *F. chinensis* (ABB05537.1) and *L. vannamei* (AAX55641.1). The complete nucleotide sequence of ferritin has a 510 bp open reading frame (ORF). The 5′ untranslated region (5′-UTR) was found to be of 130 bp and the 3′ untranslated region (3′-UTR) was 568 bp.
- The ORF encoded 170 amino acids with a predicted molecular weight (MW) about 19385.81 Da and theoretical isoelectric point (PI) of 4.73. The deduced amino acids sequence also shared high similarity with those of *Pacifastacus leniusculus* (CAA62186), *Artemia franciscana* (AAL55398), and *Crassostrea gigas* (AAP83793).
- An iron responsive element sequence (IRE) which is a region coding for an iron regulatory protein (IRP) that regulates translation of ferritin gene was found to be located at positions 5 to 30 bases towards the 5'UTR.
- High expression levels of ferritin are thus indicative of a defense mechanism against the invading bacteria, as binding of free iron is considered bacteriostatic, as bacterial cell growth is inhibited if no iron is available for synthesis of essential iron-containing compounds. Thus, the immunogenic properties of ferritin can thus be contributed to its unique sequence structure. Although the perfect function of this molecule as an antibacterial agent is still to be proved, this study has brought out its relevance as a major defense protein against bacterial agents like *V. harveyi* for *F. indicus* post-larvae.

The gene expression data generated in this study will be useful for further investigations dealing with the establishment of disease control in aquacultured penaeid shrimps. Notwithstanding the many speculative propositions on the possible functions of the genes identified, it is to be noted that results from this first study on Indian white prawn, *F. indicus*, which is commercially vital but least- investigated, are useful in demonstrating the effectiveness of gene expression-analysis for elucidating and understanding its immune response mechanisms to bacterial stress at the molecular level.

Future prospects

From the foregoing, it is evident that this study was an extensive first time effort in India on identification and expression profiling of genes responding to *V. harveyi* challenge in post-larval stages of two important aquaculture candidate shrimp species. To date, a large number of ESTs identified from this study have no identifiable homologues existing in the database, but whose expression is/was modified in response to exposure to a virulent bacterial strain. Those sequences with no matches might –in all probabilities– be novel proteins encoding genes responsible for dealing with bacterial infection(s). Further characterization of these genes would prove useful in understanding the survivability of shrimps under bacterial stress.

The data generated from this present study provides reliable grounds for further future investigations to target immune related genes which are key in combating bacterial infections. Further work on the *in vivo* effects of these upregulated genes in shrimp using SiRNA or Gene silencing technique will be very valuable in explaining the exact mechanism of action of these important immune genes. Results from *in vivo* biological activity related experiments of individual target genes on the shrimp will also be very helpful in characterization as well as better interpretation of many results obtained from this study.

Even though functional genomics studies are only beginning in shrimps, they already have provided important insights into invertebrate defense system. While the

entire nature of shrimp immune system still remains largely unknown, there is no question that functional genomics tools and approaches have made important contributions to our understanding of shrimp and pathogen interactions. The most significant achievement in shrimp immunology from the perspective of genomics has been the generation and collection of huge amount of ESTs using the high throughput differential expression studies. However, in the absence of a full-genome sequencing effort, other tools such as preparation of bacterial artificial chromosome (BAC) libraries and maps of the shrimp genome need to be developed in order to understand the immune mechanisms in greater details. In the longer term, BACs will provide useful information and thereby help in development of high density shrimp genome maps. Indian shrimp culture industry need to gear up to amass information on immunology of economically vital shrimp species.

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- S. Nayak, S. Kulkarni, R.A Sreepada, N. Ramaiah, *Vibrio harveyi* induced disease response and gene expression in *Penaeus monodon*: A functional genomics approach." Poster presented at the 7th Asia Pacific Marine Biotechnology Conference held at Kochi November 2006.