

## **Profiling of a few immune responsive genes expressed in postlarvae of *Fenneropenaeus indicus* challenged with *Vibrio harveyi* D3**

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### **Abstract**

We identified 38 genes and 8 hypothetical proteins by sequencing of 1200 clones from a *Vibrio harveyi* challenged *Fenneropenaeus indicus* subtracted cDNA library. Based on physiological roles and functions these genes were categorized into 10 groups with ~29% of the sequences having no matches in the databases. Immune related transcripts in the library were carboxypeptidase B, ferritin, translationally controlled tumor proteins (TCTP), hemocyanin, chitinase and serine carboxy peptidase. Remarkably, qPCR results imply 4.15, 3.45 and 1.86 fold rises in expression of ferritin, TCTP and hemocyanin transcripts respectively. Additionally, minor upregulation of other immune relevant genes lectin, penaeidin, crustin, MnSOD was observed in the challenged postlarvae.

Keywords: *Fenneropenaeus indicus*; *Vibrio harveyi*; SSH; EST; Ferritin; Penaeidin

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## **1. Introduction**

Penaeid shrimp farming is an economically important activity which serves the dual purpose of producing high quality seafood and generation of employment. Bacteria of Vibrionaceae family are involved in several diseases of farmed shrimps (Lightner, 1993) causing mass mortalities in larval cultures, prawn hatcheries, rearing and production facilities (Lavilla- Pitog et al., 1990). Microbial infections are usually associated with high incidence of larval deformities and, mortality rates in cultured shrimps (Vandenberghe et al., 2003). Sustainable shrimp farming relies heavily on sound scientific approaches to prevent and/or control diseases particularly at the early stages of development. In this regard, an understanding of molecular responses and defense mechanisms in shrimps against bacterial pathogens is essential. This study was therefore planned for identifying genes that are induced in the post larvae of *F. indicus* on challenge with *V. harveyi* using suppressive subtractive hybridization (SSH; Ditchenko et al., 1996). Although *F. indicus* is one of the economically important mariculture species in many Asian countries, few molecular studies in relation to its disease biology exist.

## **2. Materials and Methods**

### **2.1 Immune challenge of postlarvae and construction of subtracted cDNA library**

In essence, all the procedures of bath challenging, RNA isolation, reverse-transcription (RT-PCR) and suppressive subtractive hybridization were carried out using standard procedures according to manufacturer's protocol and detailed in our previous work on *Penaeus monodon* (Nayak et al 2010).

### **2.2 Real time PCR quantification of gene expression**

Gene expression analysis using Real Time PCR (qPCR) was carried out for seven different immune related transcripts. Primers used in the qPCR study are listed in Table 1. Shrimp  $\beta$ -actin gene was used as an internal control during all the qPCR run as 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  $C_T$  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 amplifications were carried out in a 96 well plate in a total volume of 25 $\mu$ l using 4  $\mu$ l 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 95°C for 15 min, followed by 40 cycles of 95 °C for 15s and 60°C for 1 min. The relative fold of induction was determined by  $\Delta\Delta$  CT method (Livak and Schmittgen, 2001). The CT values for challenged and control samples for individual target genes obtained from qRT-PCR analysis were subjected to analysis of t-test. A probability (P) value of less than 0.05 was considered significant.

### 3. Results

Sequencing of 1200 clones from the subtracted library resulted in the identification of 38 different genes and 8 hypothetical proteins (Table 2) which could be grouped into 10 categories based on their putative physiological and functional roles (Fig 1). Nearly 29% of ESTs had no matches in the GenBank. Defense and immunity related genes such as ferritin, carboxypeptidase, translationally controlled tumor protein (TCTP), hemocyanin, serine carboxy peptidase, and chitinase accounted for 10% of the identified ESTs. 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. This is the first report of SKP1 from penaeid shrimps. As is evident from the mean CT and  $\Delta\Delta$ CT values for challenged and control samples (Table 3), there is a noteworthy increase in the relative expression of all seven genes examined. With 4.15, 3.45 and 1.86 folds higher expression of ferritin TCTP and hemocyanin transcripts, it is clear that immune relevant genes do get upregulated due to *V. harveyi* challenge (Fig 2).

### 4. Discussion

In recent years, SSH has been successfully applied as an efficient method to identify differentially expressed genes in penaeid shrimps (Pan et al., 2005 and Nayak et al., 2010). Unlike many earlier studies, which have used specialized tissue or organ systems for gene expression studies in adult or juvenile shrimps, we use 10-14 day-old postlarvae for examining molecular response in early life-stages.

Among the above listed defense and immunity related genes, it can be suggested that ferritin does play defense response role in the postlarvae against bacterial pathogens. Ferritin was first cloned and characterized in crayfish and was shown to possess a unique iron responsive element binding loop in the un-translated region (Huang et al., 1996). It was earlier reported to be upregulated following challenge with WSSV (Pan et al., 2005). Binding of free iron is considered bacteriostatic, as bacterial cell growth, in particular of Vibrionaceae, is inhibited if iron is not available for synthesis of essential iron-

containing compounds (Marcelis et al., 1980). The high representation of ferritin transcripts indicates its upregulation due to *V. harveyi* challenge as was also confirmed by qPCR results.

Also known as fortilin, TCTP is highly conserved throughout the animal and plant kingdoms and believed to protect cells under toxic conditions. In shrimps TCTP was first cloned from *Penaeus monodon* (Bangrak et al., 2004). However, its expression is reported to decrease during the terminal stage of WSSV infection (Bangrak et al., 2004). Up-regulation of TCTP was also observed in *P. monodon* postlarvae challenged with *V. harveyi* (Nayak et al., 2010). In essence, TCTP might be critical in defending bacterial infection through regulation of a cell death pathway that is common to shrimps and humans.

Transcripts of hemocyanin subunit-L gene, a proven immune factor against WSSV infection (Zhang et al., 2004), with 98 % similarity to a *Marsupenaeus japonicus* fragment were numerous in our library, suggesting its potential role in defense process. Earlier studies have shown evidence of generation of antibacterial and antifungal peptides from the C terminus of shrimp hemocyanin (Destoumieux-Garzòn et al., 2001).

Carboxypeptidase B (CPB) identified in our study has been extensively studied from many species of animals including shrimps (Gates and Travis, 1973). Its up-regulation was reported from WSSV challenge, which was also detected using SSH in *Litopenaeus vannamei* (Zhao et al., 2007). Our detection of serine carboxy-peptidase like transcripts suggests involvement of various proteolytic enzymes in innate immunity and defense responses in *F. indicus*.

Accounting for 22 % of ESTs, the mitochondrial genes such as NADH dehydrogenase, ATP synthase and Cytochrome C-oxidase were the major representatives of genes involved in ATP and antioxidant metabolism. As energy production is more efficient with NADH dehydrogenase pathway, its up-regulation could indicate an increased energy demand as a consequence of microbial challenge. Similar would be the case in respect of activation of ATP synthase and Cytochrome C-oxidase like genes. Akin to observations of Leu et al (2007) from WSSV infection studies, it is likely that bacterial afflictions might modulate expression of energy-metabolism related genes in *F. indicus* postlarvae.

Muscle and cytoskeleton proteins constituted ~13% of genes identified from SSH library. Such 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 numbers of muscle and cytoskeletal specific genes are high. Upregulation of these genes results in effective measures to eliminate the invading pathogens. Sarcoplasmic calcium binding protein (SCP) identified in this study is important for Ca<sup>2+</sup> binding (Gao et al., 2006) and has been described as a novel allergen in shrimps (Shiomi et al., 2008). Its increased level of expression could indicate the modulation of calcium ion concentration in *F. indicus* following bacterial challenge.

Besides the 38 genes, eight hypothetical proteins were also identified in this study. These might be the products of novel genes with interesting roles in shrimp immune-modulation or in stress management.

Major upregulation of TCTP and ferritin observed in this study is generally considered as first line of defense in invertebrate immune system. Expression profiles of TCTP, ferritin and hemocyanin with reference to  $\beta$ -Actin the internal control, supports the results of SSH. These observations corroborate with previous studies of differential expression of such genes in various species of shrimps upon microbial stress (Graidist et al., 2006 and Lei et al., 2008). Furthermore, 1.8 fold upregulation of hemocyanin gene seems to imply its active role in the defense against bacterial pathogens through the activation of prophenoloxidase pathway. Also marked upregulation of antimicrobial defense related genes viz., MnSOD, penaeidin, crustin and lectin demonstrates their importance in local immune response following the bath challenge. Recently these genes were found to be expressed as a result of bath challenge in *P. monodon* (Soonthornchai et al., 2010). We can infer that there is differential expression of all these immune related transcripts in the *F. indicus* postlarvae on *V. harveyi* challenge.

## **5. Conclusion**

In addition to obtaining the first sets of ESTs from *V. harveyi* challenged *F. indicus* postlarvae this study is useful in providing a knowledge-base on the *F. indicus* defense mechanisms especially in its early stages of development. Further many of the transcripts identified from this subtracted cDNA library will be of great value in understanding the shrimp immune system and the response of early developmental stages of shrimp to bacterial stress. To our knowledge this the first study depicting the upregulation of such genes on bacterial challenge in the postlarval stages of *F. indicus*.

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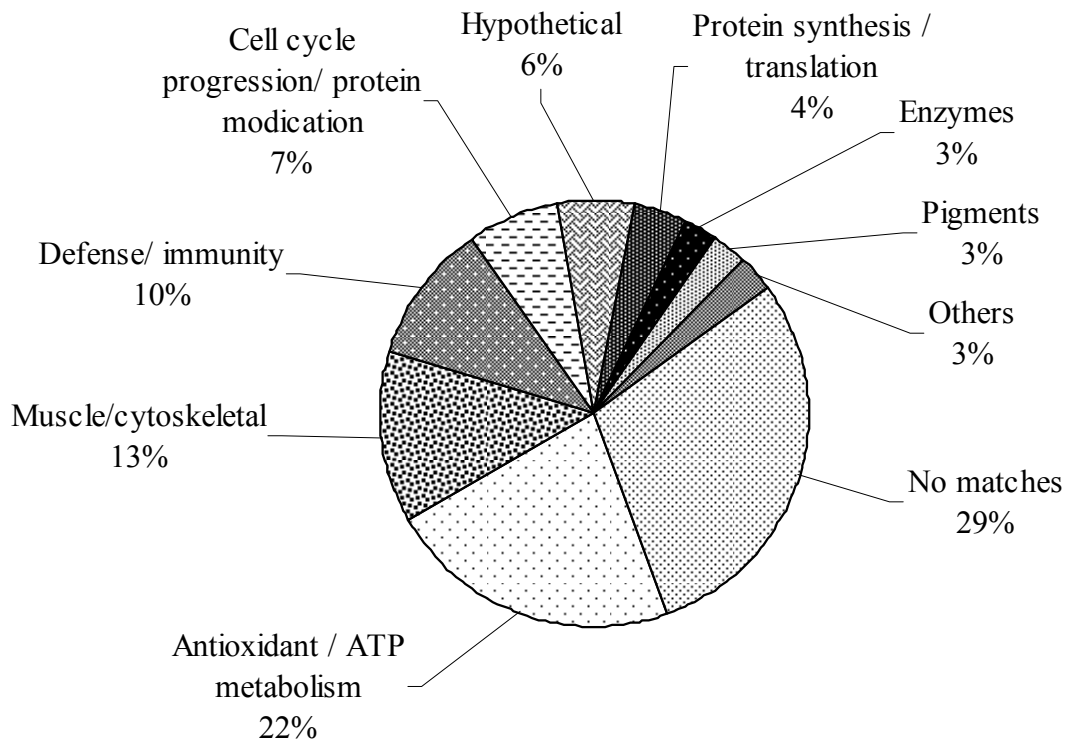
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## FIGURE LEGENDS

Fig 1. 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* postlarvae bath challenged with the strain *Vibrio harveyi*D3

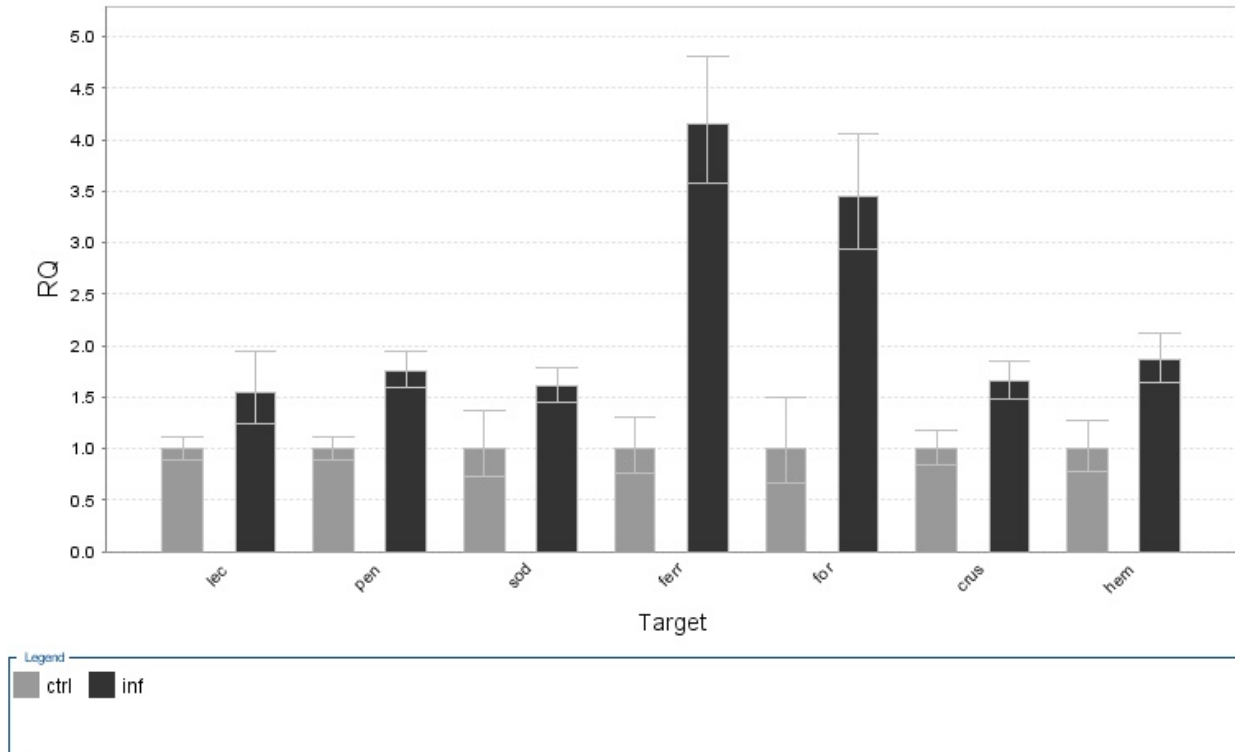
Fig 2. Gene expression plot of analyzed transcripts. Relative expression of immune related genes Lectin, penaeidin, MnSOD, hemocyanin, ferritin, fortillin, and crustin in postlarvae of *Fenneropenaeus indicus* . Expressions normalized with respect to the internal control gene  $\beta$ -Actin. The error bars in the histogram represent the maximum and minimum fold changes of relative expression.  $\beta$ -Actin is compared to itself as a reference and represented here as one fold change in its expression.





**FIGURE 1**

Fig 1. 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* postlarvae bath challenged with the strain *Vibrio harveyi*D3



**FIGURE 2**

Fig 2. Gene expression plot of analyzed transcripts. Relative expression of immune related genes Lectin, penaeidin, MnSOD, hemocyanin, ferritin, fortillin, and crustin in postlarvae of *Fenneropenaeus indicus*. Expressions normalized with respect to the internal control gene  $\beta$ -Actin. The error bars in the histogram represent the maximum and minimum fold changes of relative expression.  $\beta$ -Actin is compared to itself as a reference and represented here as one fold change in its expression.

Table 1: List of primers used in the real time PCR assay for relative quantification of genes

Primer	Sequence	Product size (bp)
$\beta$ - 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' GGCCACTTGAACCATAACCATCT 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' TTGAGAGCATAGACGTTCTGGGT 3'	170

Table 2: Genes identified from the cDNA library prepared through suppression subtractive hybridization from *Fenneropenaeus indicus* postlarvae bath challenged with *Vibrio harveyi*

GenBank ID	Transcript (No of clones)	E value	%ID	Homologous species
<b>Muscle/cytoskeletal</b>				
GO787099	alpha actin (5)	9.00E-08	76%	<i>Oreochromis niloticus</i>
GO787100	actin (4)	2.00E-05	72%	<i>Oncorhynchus mykiss</i>
GO787101	beta-actin (6)	5.00E-28	98%	<i>Penaeus monodon</i>
GO787102	cardiac muscle actin (4)	1.00E-33	97%	<i>Homarus americanus</i>
GO787103	fast myotomal muscle actin 2 (2)	6.00E-23	97%	<i>Salmo salar</i>
GO787104	troponin I (7)	1.00E-14	92%	<i>Astacus fluviatilis</i>
GO787105	myosin heavy chain (7)	7.00E-44	84%	<i>Farfantepenaeus paulensis</i>
GO787106	four and a half LIM domains 1 (1)	2.00E-88	78%	<i>Macaca mulatta</i>
GO787107	Sarcoplasmic calcium-binding protein (9)	2.00E-58	96%	<i>Litopenaeus vannamei</i>
<b>Cell cle progression</b>				
GO787108	S-phase kinase- protein 1A (19)	1.00E-62	99%	<i>Oncorhynchus mykiss</i>
<b>Antioxidant ATP metabolism</b>				
GO787109	NADH dehydrogenase subunit 4 (17)	4.00E-37	97%	<i>Homo sapiens</i>
GO787110	NADH dehydrogenase subunit 1 (32)	6.00E-64	88%	<i>Penaeus monodon</i>
GO787111	N ADH dehydrogenase subunit 2 (3)	7.00E-62	87%	<i>Penaeus monodon</i>
GO787112	NADH dehydrogenase subunit 5 (3)	2.00E-19	89%	<i>Homo sapiens</i>
GO787113	cytochrome c oxidase subunit I (3)	5.00E-23	94%	<i>Homo sapiens</i>
GO787114	F1F0-type ATP synthase subunit G (9)	9.00E-28	59%	<i>Ixodes scapularis</i>
<b>Protein Synthesis and modification</b>				
GO787125	eIF3e (10)	7.00E-44	80%	<i>Aplysia kurodai</i>
GO787115	ribosomal protein L26 (6)	6.00E-16	100%	<i>Penaeus monodon</i>
GO787116	ubiquitin (10)	2.00E-36	100%	<i>Salmo salar</i>

**Defense immunity**

GO787117	carboxypeptidase B (4)	3.00E-11	55%	<i>Litopenaeus vannamei</i>
GO787118	translationally controlled tumor protein(13)	6.00E-51	100%	<i>Litopenaeus vannamei</i>
GO787119	ferritin (15)	8.00E-48	100%	<i>Penaeus monodon</i>
GO787120	arginine kinase (2)	1.00E-16	95%	<i>Litopenaeus vannamei</i>
GO787121	allergen Pen m 2 (6)	5.00E-17	97%	<i>Penaeus monodon</i>
GO787122	chitinase (6)	1.00E-15	59%	<i>Scylla serrata</i>
GO787123	serine carboxypeptidase (5)	4.00E-22	41%	<i>Strongylocentros purpuratus</i>
GO787124	hemocyanin subunit L (14)	4.00E-46	98%	<i>Marsupenaeus japonicus</i>

**Enzymes**

GO787126	trypsin (4)	1.00E-66	89%	<i>Litopenaeus vannamei</i>
GO787127	pyruvate kinase 3 (2)	1.00E-46	97%	<i>Litopenaeus vannamei</i>
GO787128	DNA gyrase subunit B (4)	6.00E-51	90%	<i>Bacterium SD212</i>
GO787129	transposase IS630 (1)	7.00E-06	100%	<i>Listonella anguillarum</i>

**Pigments**

GO787130	crustacyanin-C1 subunit (4)	9.00E-36	64%	<i>Homarus gammarus</i>
GO787131	rhodopsin (4)	4.00E-09	74%	<i>Litopenaeus vannamei</i>

**Others**

GO787132	allatotropin neuropeptide precursor (4)	6.00E-06	61%	<i>Spodoptera frugiperda</i>
GO787133	reverse transcriptase (1)	8.00E-22	40%	<i>Strongylocentros purpuratus</i>
GO78713	putative puoindoline b protein (1)	3.00E-14	78%	<i>Triticum aestivum</i>
GO787136	Tetraspanin (2)	1.00E-20	41%	<i>Tribolium castaneum</i>
GO787136	hemagglutinin esterase	5.00E-04	80%	<i>Salmon anemia virus</i>

Similarity matches with E-value<10<sup>-3</sup> 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: Quantitative PCR analysis of gene expression in control and *V. harveyi* challenged postlarvae of *Fenneropenaeus indicus*

Target Gene	Mean cycle threshold		$\Delta\Delta$ CT	Fold change in expression ( $2^{-\Delta\Delta$ CT*})
	<i>V. harveyi</i> challenged postlarvae	Unchallenged postlarvae (control)		
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

$\Delta$  CT = (Target transcript CT -  $\beta$ -Actin CT); \* $\Delta\Delta$ CT= ( Inf  $\Delta$ CT - Ctrl  $\Delta$ CT ),