

Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile in response to various immunostimulants and challenge with WSSV

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ABSTRACT

A crustin-like antimicrobial peptide from the haemocytes of giant tiger shrimp, *Penaeus monodon* was partially characterized at the molecular level and phylogenetic analysis was performed. The partial coding sequence of 299 bp and 91 deduced amino acid residues possessed conserved cysteine residues characteristic of the shrimp crustins. Phylogenetic tree and sequence comparison clearly confirmed divergence of this crustin-like AMP from other shrimp crustins. The differential expression of the crustin-like AMP in *P. monodon* in response to the administration of various immunostimulants viz., two marine yeasts (*Candida haemulonii* S27 and *Candida sake* S165) and two β -glucan isolates (extracted from *C. haemulonii* S27 and *C. sake* S165) were noted during the study. Responses to the application of two gram-positive probiotic bacteria (*Bacillus* MCCB101 and *Micrococcus* MCCB104) were also observed. The immune profile was recorded pre- and post-challenge white spot syndrome virus (WSSV) by semi-quantitative RT-PCR. Expressions of seven WSSV genes were also observed for studying the intensity of viral infection in the experimental animals. The crustin-like AMP was found to be constitutively expressed in the animal and a significant down-regulation could be noted post-challenge WSSV. Remarkable down-regulation of the gene was observed in the immunostimulant fed animals pre-challenge followed by a significant up-regulation post-challenge WSSV. Tissue-wise expression of crustin-like AMP on administration of *C. haemulonii* and *Bacillus* showed maximum transcripts in gill and intestine. The marine yeast, *C. haemulonii* and the probiotic bacteria, *Bacillus* were found to enhance the production of crustin-like AMP and confer significant protection to *P. monodon* against WSSV infection.

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Introduction

Antimicrobial peptides (AMPs), widely distributed in the whole living kingdom, play an important role in the immunological defense especially in those organisms which lack adaptive immunity (Boman 1995; Dimarcq et al. 1998; Hancock and Lehrer 1998; Zasloff 2002; Otvos 2002; Bulet et al. 2004). AMPs are promptly synthesized at low metabolic cost, easily stored in large amounts and readily available shortly after an infection, to rapidly kill a broad range of microbes (Hancock 1997, 2001; Prenner et al. 1999a,b). Due to their small size, amphipathic structure and cationic character, AMPs can rapidly diffuse to the point of infection (Brogden 2005). AMPs can kill bacteria in micromolar concentration supporting a non-receptor mediated mechanism as their mode of action. Many antibacterial peptides show a remarkable specificity for prokaryotes and low toxicity for eukaryotic cells; a phenomenon

which has favored their investigation and exploitation as potential new antibiotics (Zasloff 1992).

Crustins, a widely distributed family of AMPs were first isolated from the shore crab, *Carcinus maenas* as an 11.5 kDa peptide by Relf et al. (1999). Crustins are cationic, cysteine-rich AMPs with a molecular weight of 7–14 kDa and isoelectric point of 7.0–8.7 and one whey-acidic protein (WAP) domain at the carboxy terminus (Smith et al. 2008). Several isoforms of crustins have been described in a wide range of penaeid prawns including *Litopenaeus vannamei* (Bartlett et al. 2002), *Litopenaeus setiferus* (Bartlett et al. 2002), *Penaeus monodon* (Supungul et al. 2004; Chen et al. 2004; Amparyup et al. 2008), *Marsupenaeus japonicus* (Rattanachai et al. 2004), *Litopenaeus schmitti* (Rosa et al. 2007), *Fenneropenaeus chinensis* (Zhang et al. 2007), *Farfantepenaeus brasiliensis* (Rosa et al. 2007), *Farfantepenaeus paulensis* (Rosa et al. 2007), *Farfantepenaeus subtilis* (Rosa et al. 2007) and *Fenneropenaeus indicus* (Antony et al. 2010).

White spot syndrome virus (WSSV) is one of the most devastating shrimp pathogens, and it has caused serious damage to the worldwide shrimp culture industry (Takahashi et al. 1994; Wang et

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Table 1
Rearing conditions and water quality parameters.

Tank capacity	500 l
Stocking density	12 nos.
Feeding level	10–15% of body weight
Feeding frequency	Twice daily
Water temperature	24–27 °C
pH	7.5–8
Salinity	15–18‰
Ammonia	0.01–0.02 mg l ⁻¹
Nitrite	0.00–0.01 mg l ⁻¹
Nitrate	Below detectable level
Alkalinity	50–60
Dissolved oxygen	6–7 mg l ⁻¹

al. 1995). WSSV is an enveloped virus with a large, double-stranded, circular DNA genome (~300 kb) containing approximately 180 putative open reading frames (ORFs), most of which have no homology with any known genes or proteins in public databases (Chou et al. 1995; Wongteerasupaya et al. 1995; Wang et al. 1995; Chang et al. 1996; Chen et al. 1997; Yang et al. 2001). Proper husbandry and management of farms with the application of immunostimulants, probiotics and bioremediators can save the industry from the onslaught of diseases to a certain extent.

The selection of suitable compounds with potent immunostimulatory property present a bewildering task. Clearly the over-riding criteria for the selection of suitable immunostimulants are cost, ease of administration, efficacy and low toxicity to the host (Smith et al. 2003). In most cases the initiation of the defense reactions in shrimps is triggered by the presence of pathogen-associated molecular patterns (PAMPs), which include bacterial cell wall components such as lipopolysaccharide (LPS) and peptidoglycan (PG), β -1,3 glucan of fungal cell wall and double-stranded RNA of viruses (Lee and Soderhall 2002). The exact mechanism of action of immunostimulants and the antiviral defense mechanism of crustaceans is poorly understood at the molecular level. AMPs provide a useful way of assessing and studying innate immunity at the biochemical and molecular level.

In the current study, a crustin-like AMP-cDNA from giant tiger shrimp *Penaeus monodon* was cloned and partially characterized at the molecular level. The expression profile of the crustin-like AMP gene in response to various immunostimulants/probiotic bacteria and on challenge with white spot syndrome virus (WSSV) was also analyzed using semi-quantitative RT-PCR. The expression of seven WSSV genes were also analyzed for confirmation of WSSV infection.

Materials and methods

Experimental animals and rearing conditions

Healthy adult *P. monodon* (20–25 g body weight) PCR negative for WSSV were purchased from a local shrimp farm in Vypeen, Kochi. They were transferred to aquarium tanks of 500 l capacity and acclimatized for 1 week under laboratory conditions. Shrimps were fed standard diet (Higashimaru, India) twice daily during acclimatization period. Constant aeration was provided in all tanks during the experiment. Bioreactor was set in all the aquarium tanks for the effective removal of ammonia and nitrate. Physico-chemical parameters such as salinity, pH, alkalinity, ammonia, nitrite, nitrate, dissolved oxygen and temperature were monitored regularly (Table 1).

Immunostimulants/probiotics used

Two marine yeasts, *Candida haemulonii* S27 and *Candida sake* S165 (isolated from the Arabian Sea and maintained in the Microbiology Laboratory of Department of Marine Biology, Microbiology

Table 2
Immunostimulants/probiotics used for studying the expression profile of the crustin gene.

Sl. no.	Immunostimulants/probiotics	Diet code
	β -1,3 glucans	
1	<i>Candida haemulonii</i> S27 glucan	CHG
2	<i>Candida sake</i> S165 glucan	CSG
	Marine yeasts	
3	<i>Candida haemulonii</i> S27 whole cell	CHY
4	<i>Candida sake</i> S165 whole cell	CSY
	Gram-positive bacteria	
5	<i>Bacillus</i> MCCB101	B
6	<i>Micrococcus</i> MCCB104	M
7	<i>Bacillus</i> MCCB101 and <i>Micrococcus</i> MCCB104 – combination	BM

and Biochemistry, CUSAT); two glucan preparations from *Candida haemulonii* S27 and *Candida sake* S165; two gram-positive bacterial probionts, *Bacillus* MCCB 101 and *Micrococcus* MCCB 104 and a Combination of these two probionts (*Bacillus* MCCB 101 + *Micrococcus* MCCB 104) (obtained from National Center for Aquatic Animal Health (NCAAH), CUSAT) were tested for its efficacy as immunostimulants by observing the expression profile of the crustin-like gene (Table 2). The two marine yeasts, *C. haemulonii* S27 and *C. sake* S 165 have been proved to be good source of immunostimulants by Prabha (2007) and Sajeevan et al. (2006), respectively. *Bacillus* and *Micrococcus* are commercial probionts used in shrimp culture (Antony and Philip 2008).

Experimental diets used

The experimental feeds were prepared by incorporating the different immunostimulants/probiotics to a standard shrimp diet (Higashimaru, India) which was used as the control feed. Seven different types of experimental diets were prepared: i.e., two glucan diets (CHG and CSG), two yeast diets (CHY and CSY) and three probiont incorporated diets (B, M and BM). The two glucan diets (CHG and CSG) were prepared by incorporating 0.2% glucan (extracted from *C. haemulonii* S27 and *C. sake* S165) with the standard diet based on previous studies (Sajeevan et al. 2006, 2009). For yeast diets (CHY and CSY), the two marine yeast (wet weight) biomass (*C. haemulonii* S27 and *C. sake* S165) were incorporated at a concentration of 10% (w/w) in the standard diet. In the case of probiotic diets, *Micrococcus* (M) and *Bacillus* (B) biomass were prepared and mixed with the standard diet at 10³ cells/g feed. The probiotic combination diet (BM) was prepared by incorporating *Micrococcus* and *Bacillus* at 10³ cells each per gram diet (2 × 10³ cells/g). All feed preparations were kept at –20 °C until used.

Feeding experiment and WSSV challenge

Shrimps were randomly divided into eight groups and were fed the experimental diets for 14 days. Group 1 shrimps fed standard shrimp diet served as the control. Group 2 was fed the experimental diet CHG (0.2% *C. haemulonii* glucan) and group 3, CSG (0.2% *C. sake* S165 glucan). Group 4 was fed experimental diet CHY (10% *C. haemulonii* S27 biomass) and group 5, CSY (10% *C. sake* S165 biomass) (Sajeevan et al. 2006). Group 6, 7, and 8 were fed experimental feeds B (*Bacillus* MCCB101 incorporated diet (50 cells/g animal/day)); M (*Micrococcus* MCCB104 (50 cells/g animal/day)) and BM (*Bacillus* MCCB101 + *Micrococcus* MCCB104 (100 cells/g animal/day)), respectively. The animals were fed twice daily with the experimental diet, except the glucan diets which was given only once in seven days and the control diet on the rest of the days as per the optimized feeding schedule of Sajeevan et al. (2009). Five animals from each group were sampled after 14 days. Only those in

Table 3

Primers used for the study.

ORF/gene	Primer sequence (5'-3')	Annealing temp. (°C)	Amplicon size (bp)	References
Crustin	F – tgttcccacgacttcaagtgtgc R – caaagattcaactaataaacag	60	299	Chen et al. (2004)
β-actin	F – cttgtggtgacaatggctccg R – tgggaaggagtagccacgctc	60	520	Zhang et al. (2007)
18S rRNA	F – ttgtacgaggatcgagtggga R – atgctttcgagtaggtcgt	52	350	Supungul et al. (2004)
Latency related gene	F – cttgtgggaaaagggtcctc R – tcgtcaaggcttacgtgctc	53	647	Liu et al. (2005)
VP28	F – ctgctgtgattgctgtattt R – cagtgccagataggtgac	54	555	Liu et al. (2005)
DNA polymerase	F – tgggaagaagatgcgagag R – ccctccgaacaacatctcag	54	586	Liu et al. (2005)
Endonuclease	F – tgacgaggagattgtaaag R – ttatggttctgtatttgagg	50	408	Liu et al. (2005)
Thymidine kinase	F – gagcagccatcgggtaaac R – gcgagcgtctacctaatac	54	412	Liu et al. (2005)
Protein kinase	F – tggagggtggggaccaacggacaaaac R – caaattgacagtagagaattttgcac	55	512	Liu et al. (2005)
Ribonucleotide reductase	F – atctgctagtcctgcacac R – aaagagggtggaaggcagc	53	408	Liu et al. (2005)

Table 4

Result of BLAST analysis of crustin-like AMP nucleotide (FJ535568).

Closest species	Accession number	E-value	% identity
<i>Farfantepenaeus paulensis</i>	EF182747	1e–31	94%
<i>Litopenaeus vannamei</i>	AF430072	5e–31	94%
<i>Farfantepenaeus subtilis</i>	EF450744	7e–30	93%
<i>Farfantepenaeus brasiliensis</i>	EF601055	7e–30	93%

the intermoult stage were sampled during the study. On the 15th day all the groups were challenged with WSSV by feeding WSSV infected *P. monodon* tissue at the rate of 1 g/animal. The animals were maintained on their respective diets post-challenge WSSV. After 48 h five animals each from all the groups were sampled for the gene expression analysis.

Haemolymph and tissue collection

Haemolymph was collected from the rostral sinus using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase-free, 10% sodium citrate, pH 7.0). Tissues including gill, muscle, heart, hepatopancreas and intestine were collected. Haemolymph and the tissues were suspended in TRI reagent (Sigma) for total RNA isolation.

Total RNA isolation and reverse transcription

Total RNA was extracted from the haemocytes and the target tissues using TRI Reagent (Sigma) following manufacture's protocol. RNA was quantified and the purity was checked by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratio ($A_{260}:A_{280}$) ≥ 1.8 were used for further experiments. First strand cDNA was generated in a 20 μ l reaction volume containing 5 μ g total RNA, 1 \times RT buffer, 2 mM dNTP, 2 μ M oligo d(T₂₀), 20 U of RNase inhibitor and 100 U of M-MLV reverse transcriptase (New England Biolabs, USA). The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min.

Semi-quantitative RT-PCR analysis of gene expression

Expression of the target gene when supplemented with different immunostimulants was determined by semi-quantitative RT-PCR analysis using β -actin and 18S rRNA as the internal control pre and post-challenge WSSV (Marone et al. 2001). PCR amplification of 1 μ l of cDNA was performed in a 25 μ l reaction volume containing 1 \times Standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 3.5 mM MgCl₂, 200 μ M dNTPs, 0.4 μ M each primer and 1 U Taq DNA polymerase (New England Biolabs). Amplification was performed using the target gene primers, Crustin (Forward – 5'-tgttcccacgacttcaagtgtgc-3' and Reverse – 5'-caaagattcaactaataaacag-3') and β -actin (Forward –

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gttcccacgacttcaagtgtgctggcctcgataaagtgttgccttcgacaggtgtttgggagaa
S H D F K C A G L D K C C F D R C L G E
cacgtgtgcaagcctccttcttctacggaaggaatgttaaaggatgatgagaataaaca
H V C K P P S F Y G R N V K G * * E * T
aaagaccaactgacagacaaccgatgatttggaaattaggaccacgaatgttcaatctac
K D Q L T D N R * F G I * D H E C S I Y
tggtatgtcaagtaccaagcaatctgagagtactattatctgtaaaaaataaataaaca
C Y V K Y C A I * E Y Y Y L * K I N K Q
ataataaatgtaacagggaaatgaactactccttctgtttatattagttgaatctttg
I I N V T G N E L L L S V Y L V E S L

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Fig. 1. Nucleotide and amino acid sequences of crustin-like AMP from the haemocyte of the Giant tiger shrimp, *Penaeus monodon* (GenBank accession no. FJ535568). Asterisk indicates stop codon.

5'-cttgggttgacaatggctccg-3', Reverse – 5'-tggtgaaggagtagccacgctc-3') as the internal control. As rRNA is considered as a reliable reference for quantitative RT-PCR (Bustin 2002), 18S rRNA was also included as an internal control (Forward – 5'-ttgtacgagatcgagtgga-3', Reverse – 5'-atgctttcgcagtagtctgt-3'). The thermal profile used was an initial denaturation at 94 °C for 2 min followed by 27 cycles of denaturation at 94 °C for 15 s, extension at 68 °C for 30 s and a final extension at 68 °C for 10 min. for the target genes. Annealing temperature varied for the different genes as given in Table 3. The PCR cycles had been optimized so that the target gene and house-keeping gene amplification were at logarithmic phase. The PCR reaction of each sample was carried out in triplicate. PCR product was analyzed by electrophoresis using 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light. The intensity of the gel bands were measured using Image J analysis software. The relative expression level of tiger shrimp crustin-like AMP mRNA was expressed as the ratio of tiger shrimp crustin mRNA to β -actin mRNA.

Cloning and sequencing

PCR product was cloned into the pGEM-T Easy vector system (Promega). Recombinant clones were identified and plasmid with the insert was isolated and purified using Gen Pure Plasmid isolation kit (Sigma) and was sequenced at Microsynth, Switzerland.

Sequence analysis

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/blast>). Gene translation and prediction of deduced protein were performed with ExPASy (<http://www.au.expasy.org/>). Two multiple sequence alignments were performed with amino acid sequences of known crustin/crustin-like peptides from shrimps and crustaceans separately (NCBI GenBank) using CLUSTALW and GENDOC version 2.7. Phylogenetic and molecular evolutionary analysis were carried out and the consensus trees were compared by bootstrap employing MEGA version 4.0 (Tamura et al. 2007). The neighbour-joining (NJ) tree obtained was validated with the minimum evolution tree. 1000 bootstraps were performed for the NJ tree to check for repeatability of the results. ScanProsite program was used for confirming WAP domain signature.

PCR analysis of WSSV genes

Seven genes viz. DNA polymerase, endonuclease, latency related gene, protein kinase, ribonucleotide reductase, thymidine kinase and VP28 required for WSSV metabolism and infection were selected for the study (Table 3). Expression profiles of these genes when supplemented with different immunostimulants were studied. Shrimps fed standard diet and challenged with WSSV served as the positive control and the unchallenged shrimps as the negative control. PCR amplification of 1 μ l of cDNA was performed in a 25 μ l reaction volume as described above.

Results

Crustin-like AMP gene in *P. monodon*

A partial mRNA transcript of 299 bp, belonging to the crustin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 1). The nucleotide sequence of the tiger shrimp crustin cDNA was submitted to GenBank under the accession number FJ535568. The sequencing was performed in both directions

and sequence was analyzed by homology searches against GenBank data for both nucleotide and amino acid similarity using BLAST program which showed that the crustin-like AMP shared maximum similarity with other crustins of *F. paulensis* (EF182747) (94%), *L. vannamei* (AF430072) (94%), *F. subtilis* (EF450744) (93%) and *F. brasiliensis* (EF601055) (93%) (Table 4). The sequence also showed high similarity to the crustin (No GenBank Submission) isolated from *P. monodon* by Chen and co-workers (Chen et al. 2004).

Multiple alignment of the deduced amino acid sequence of the crustin-like AMP using GenDoc program showed highly significant homology with other crustins isolated from prawns (Fig. 2A). Multiple alignments showed three conserved regions indicating the C₄–C₈ cysteine residues of WAP domain (Fig. 2A). Multiple alignment of the deduced amino acid sequence of the crustin-like AMP with all known crustins showed a characteristic conserved “KCC” region with the C₅ and C₆ conserved cysteine residues, characteristic of the WAP domain of crustins. This region was found to be conserved in all the isolated crustins of prawns, lobsters, crabs and crayfishes (Fig. 3A) (Bartlett et al. 2002).

Phylogenetic analysis of crustin family

All known crustins were retrieved from the GenBank and phylogenetic analysis of both the nucleotide and amino acid sequences of the crustin-like AMPs were performed. The crustins of both shrimps and that of all crustaceans were subjected to phylogenetic analysis using MEGA 4.0 software.

Phylogenetic analysis of crustins isolated from shrimps showed three branches (Fig. 2B). Group I included crustins isolated from the shrimps, *L. vannamei*, *L. setiferus*, *F. paulensis*, *F. subtilis*, *L. schmitti* and crustin-3 of *F. chinensis*. The deduced amino acid sequence of the crustin-like AMP of *P. monodon* in this study belonged to this group at 93% similarity. Group II included the five crustin isoforms isolated and characterized from *M. japonicus* and Group III consisted of crustins from *F. chinensis* (crustin 1 and 2) and *P. monodon* (crustin 1).

Phylogenetic analysis of crustins isolated and characterized from the entire crustacean group showed five major branches (Fig. 3B). Group I included all the crustins from prawns and Group II included all the crustins from lobsters, *H. americanus* and *H. gammarus*. Group III consisted of crustins from the crayfish *P. leniusculus* whereas the crustins from crabs, *Scylla paramamosain* and *Hyas araneus* formed Group IV.

Expression of crustin-like AMP genes in response to various immunostimulants

Crustin gene was found to be up-regulated significantly when *P. monodon* was fed probiotic bacteria, *Bacillus* MCCB101, *Micrococcus* MCCB104 (Fig. 4) and a combination of both *Bacillus* MCCB101 and *Micrococcus* MCCB104 incorporated diets. But the application of yeasts and glucans showed significant down-regulation in the expression profile of crustin-like AMP gene before WSSV challenge.

Expression of crustin-like AMP genes in response to various immunostimulants post-challenge WSSV

All the test diets induced up-regulation of the crustin gene post-challenge WSSV. Marine yeasts viz. *C. haemulonii* S27 (Group 2) and *C. sake* S165 (Group 3) and their cell wall glucans up-regulated the crustin gene significantly. Considerable up-regulation of the gene could be noticed in the case of probiotic fed groups also. In the control group, a down-regulation of the crustin gene could be noticed post-challenge WSSV (Fig. 4).

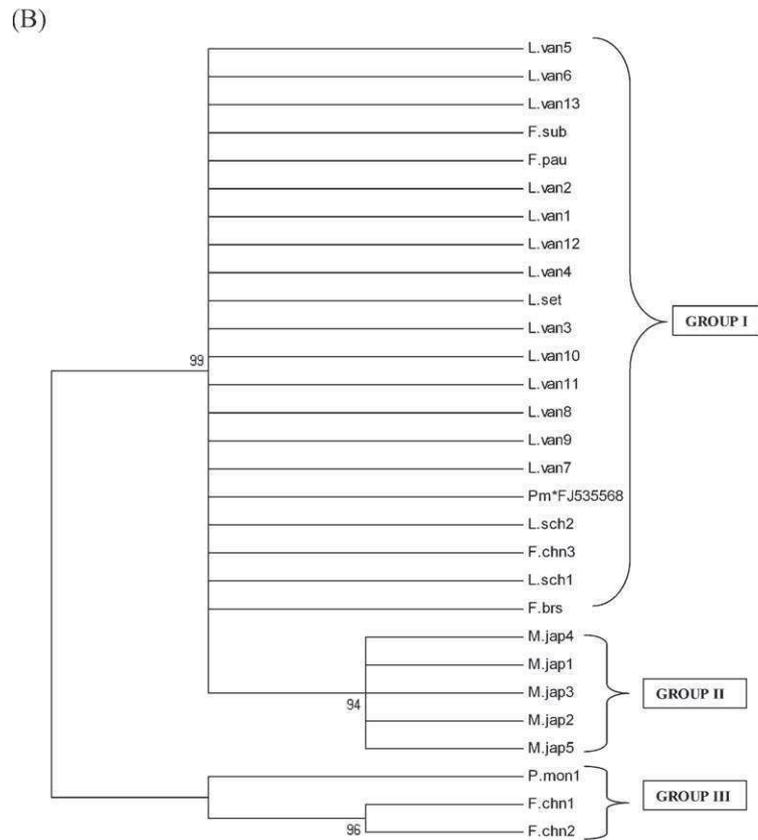
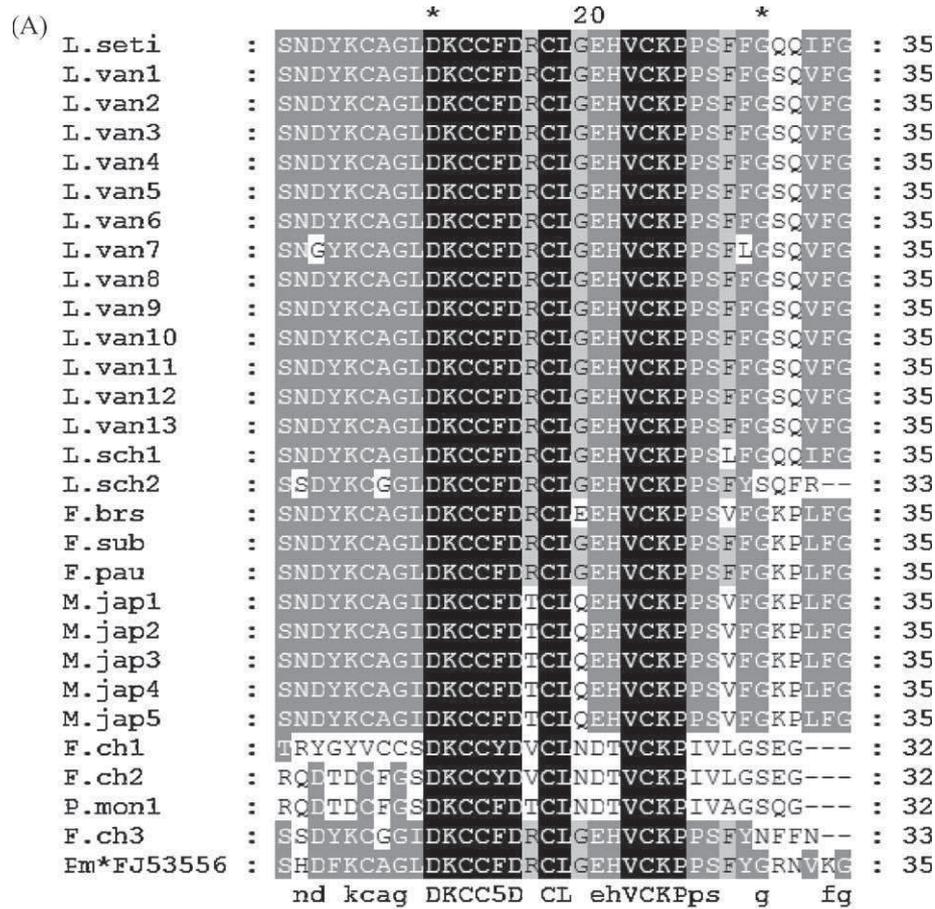


Fig. 2. (A) Multiple alignment of deduced amino acid sequence of the *Peneaus monodon* crustin-like AMP (FJ535568) with other shrimp crustins (L.seti – *Litopenaeus setiferus* AF430078, L.van1 – *Litopenaeus vannamei* 1 AF430075, L.van2 – *Litopenaeus vannamei* 2 AF430073, L.van3 – *Litopenaeus vannamei* 3 AF430072, L.van4 – *Litopenaeus vannamei*

Expression of WSSV genes in the haemocytes of *P. monodon* in response to various immunostimulants

All the seven WSSV related genes (DNA polymerase, endonuclease, latency related gene, protein kinase, ribonucleotide reductase, thymidine kinase and VP28) were found to be expressed in the positive (WSSV challenged) control and glucan treated group of shrimps post-challenge WSSV, confirming WSSV infection in these groups (Fig. 5). Intensity of bands showed that the extent of infection was highest in the control group, followed by *C. sake* glucan and *C. haemulonii* glucan diet fed group of shrimps. Moderate amount of transcripts could be noticed in the case of *C. sake* (yeast) fed group for protein kinase. A non-specific amplification of VP28 could also be noticed for the *Micrococcus* fed group. However no viral gene transcripts could be detected in the case of marine yeast and probiotic fed group.

Expression of crustin-like AMP in various tissues of *P. monodon* in response to the administration of the marine yeast *C. haemulonii* and probiotic bacteria bacillus

Since the performance of *C. haemulonii* and *Bacillus* supplemented diets were found to be the top two post-challenge WSSV, detailed tissue-wise expression was studied for these two treatment groups (Fig. 6). In the control group of animals, crustin gene expression was considerably high in all the tissues pre-challenge and there was a reduction in expression post-challenge. In the case of *C. haemulonii* treated group, the expression was very less pre-challenge and remarkably high post-challenge. However for *Bacillus* treated group, the crustin gene expression was more or less same both pre- and post-challenge. Of the various tissues, the gene expression was found to be maximum in gill and intestine followed by muscle and the least in hepatopancreas.

The expression of WSSV genes were observed only in the control and *C. sake* glucan fed group and no transcripts were noticed in the other immunostimulant/probiotic fed group. Among glucans, the *C. haemulonii* glucan was found to perform better with lesser expression of the WSSV genes.

Post-challenge survival

Generally, marine yeast diet fed groups showed significantly high survival compared to β -glucan diet fed shrimps and the control group. Among the various treatments, *C. haemulonii* S 27 (CHY) fed group showed maximum survival (93%) followed by *C. sake* S165 (CSY) (75%), *Micrococcus* (M) (73%), *Bacillus* + *Micrococcus* (BM) (56%), *C. haemulonii* glucan (CHG) (42%), *C. sake* glucan (CSG) (38%) and *Bacillus* (B) (42%) fed group (Fig. 8).

Discussion

The partial coding sequence, denoted as crustin-like AMP had 299 nucleotides and 91 amino acid residues with conserved cysteine residues characteristic of the WAP domain. The C-terminal segment included a high proportion of cysteine-rich region that

participate in the formation of disulphide bonds. The partial cDNA fragment had the five conserved cysteine residues (C₄–C₈ residues). Multiple polyadenylation consensus sequences (AATAAA) were also present at the C-terminus. The partial cDNA fragment lacked the signal peptide region but possessed the cysteine-rich region characteristic to WAP domain. The putative polyadenylation consensus signal (AATAAA) also confirms the 3' end of the crustin-like AMP. Multiple polyadenylation sites were observed in the fragment.

As predicted by the ScanProsite program, a partial WAP domain signature exists in the C-terminal region and one of the four disulphide core (DSC) domain was found to be located at Cys⁶–Cys²³. Since only partial cDNA fragment and only five of the conserved cysteine regions could be retrieved from the sequence, other locations of the DSC could not be located. Searching against the Prosite database, analysis of the crustin-like AMP revealed the existence of WAP type “DSC” domain signature. The expected WAP type “4DSC core” domain signature is:

$$C_1-(X_n)-C_2-(X_n)-C_3-(X_5)-C_4-(X_5)-C_5-C_6-(X_3-5)-C_7-(X_3-4)-C_8$$

where X is any amino acid residue and X_n is a stretch of *n* residues (Bartlett et al. 2002).

The partial cDNA sequence of the crustin-like AMP showed the presence of C₄–C₈ and it followed the same pattern as expected in the WAP domain except for one extra residue between C₇ and C₈ (C₇–(X₅)–C₈). Similar case was reported for the crustins isolated from *F. chinensis*, GenBank no. DQ097703, DQ097704 (Zhang et al. 2007). The WAP domain signature of the present crustin-like AMP is shown in Fig. 7. Several other consensus sequences also appear in the 4DSC domain, i.e. (1) a conserved aspartate (D) residue between C₃ and C₄. (2) KCC with C₅ and C₆. (3) CXXP with C₈ (Bartlett et al. 2002).

Cysteine residues present in the WAP domain of the crustin were reported to have functions in maintaining the tertiary structure of crustins (Gross et al. 2001). According to the previous reports on the crustin-like proteins, the 4DSC domain played important roles in the biological function (Zhang et al. 2007).

Although much research has been done to investigate various AMP classes and their structure and function, the enhanced production of AMPs using immunostimulants has rarely been evaluated. It is very important to find suitable immunomodulators that could suppress or completely eliminate WSSV by up-regulating the expression of immune genes.

As one of the important AMPs in crustaceans, crustins have gained the attention of many researchers. Until recently, a few sequences of crustins have been described in penaeid shrimps (Gross et al. 2001; Bartlett et al. 2002; Vargas-Albores et al. 2004; Rattanachai et al. 2004; Zhang et al. 2007). As AMPs play an important role in shrimp defense, the expression levels of these molecules are possible indicators of the immune state of shrimps. Haemocytes have been proved to be the site of production and storage of crustins at very high levels (Soderhall and Cerenius 1998; Hauton et al. 2006; Supungul et al. 2007; Amparyup et al. 2008). Hence haemolymph is the best tissue to study the expression of AMPs in relation to various conditions.

4 AF430071, L. van5 – *Litopenaeus vannamei* 5 AF430076, L. van6 – *Litopenaeus vannamei* 6 AF430074, L. van7 – *Litopenaeus vannamei* 7 AY486426, L. van8 – *Litopenaeus vannamei* 8 AY488497, L. van9 – *Litopenaeus vannamei* 9 AY488496, L. van 10 – *Litopenaeus vannamei* 10 AY488495, L. van11 – *Litopenaeus vannamei* 11 AY488494, L. van12 – *Litopenaeus vannamei* 12 AY488493, L. van13 – *Litopenaeus vannamei* 13 AY488492, L. sch1 – *Litopenaeus schmitti* 1 EF182748, L. sch2 – *Litopenaeus schmitti* 2 EF601056, F. brs – *Farfantapeneaus brasiliensis* 1 EF601055, F. sub – *Farfantapeneaus subtilis* 1 EF450744, F. pau – *Farfantapeneaus paulensis* EF182747, M. jap1 – *Marsupeneaus japonicus* 1 AB121740, M. jap 2 – *Marsupeneaus japonicus* 2 AB121744, M. jap3 – *Marsupeneaus japonicus* 3 AB121743, M. jap4 – *Marsupeneaus japonicus* 4 AB121742, M. jap 5 – *Marsupeneaus japonicus* 5 AB121741, F. ch 1 – *Fenneropenaeus chinensis* 1 DQ097704, F. ch2 – *Fenneropenaeus chinensis* 2 DQ097703, F. ch 3 – *Fenneropenaeus chinensis* 3 AY871268, P. mon 1 – *Peneaus monodon* 1 EF654658) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences. (B) A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequences of the *Peneaus monodon* crustin-like AMP (FJ535568) with other shrimp crustins (GenBank accession number details as given above). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

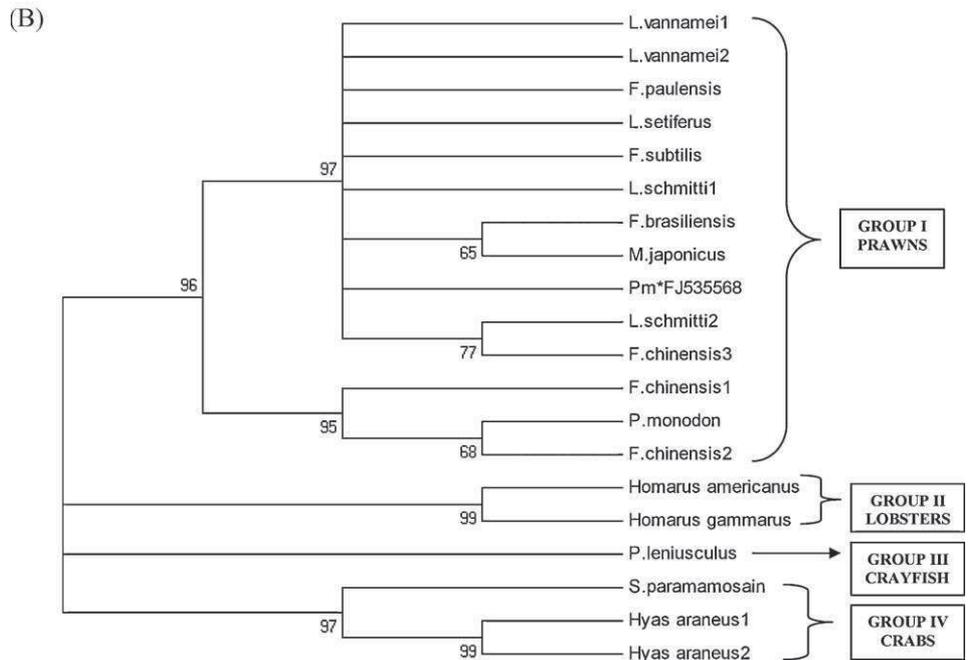
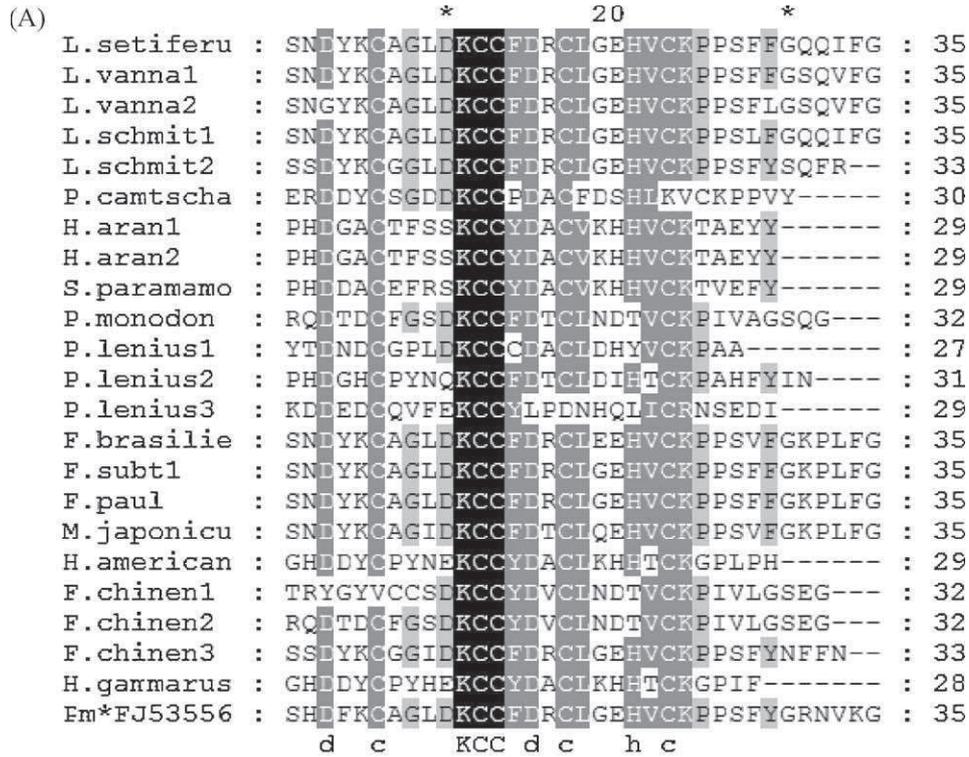


Fig. 3. (A) Multiple alignment of deduced amino acid sequence of the *Peneaus monodon* crustin-like AMP(FJ535568*) with all known crustins (*Litopenaeus setiferus* AF430078, *Litopenaeus vannamei* 1 AF430075, *Litopenaeus vannamei* 2 AY486426, *Litopenaeus schmitti* 1 EF182748, *Litopenaeus schmitti* 2 EF601056, *Paralithodes camtschaticus* EU921643, *Hyas araneus* 1 EU921642, *Hyas araneus* 2 EU921641, *Scylla paramamosain* 1 EU161287, *Penaeus monodon* 1 EF654658, *Pacifastacus leniusculus* 1 EF523614, *Pacifastacus leniusculus* 2 EF523613, *Pacifastacus leniusculus* 3 EF523612, *Farfantapeneaus brasiliensis* 1 EF601055, *Farfantapeneaus subtilis* 1 EF450744, *Farfantapeneaus subtilis* 2 EF182747, *Marsupeneaus japonicus* AB121740, *Homarus americanus* EF193003, *Fenneropenaeus chinensis* 1 DQ097704, *Fenneropenaeus chinensis* 2 DQ097703, *Fenneropenaeus chinensis* 3 AY871268, *Homarus gammarus* AJ786653) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences. (B) A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Peneaus monodon* crustin-like AMP (FJ535568) with all known crustins (GenBank Accession Number details as given above). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

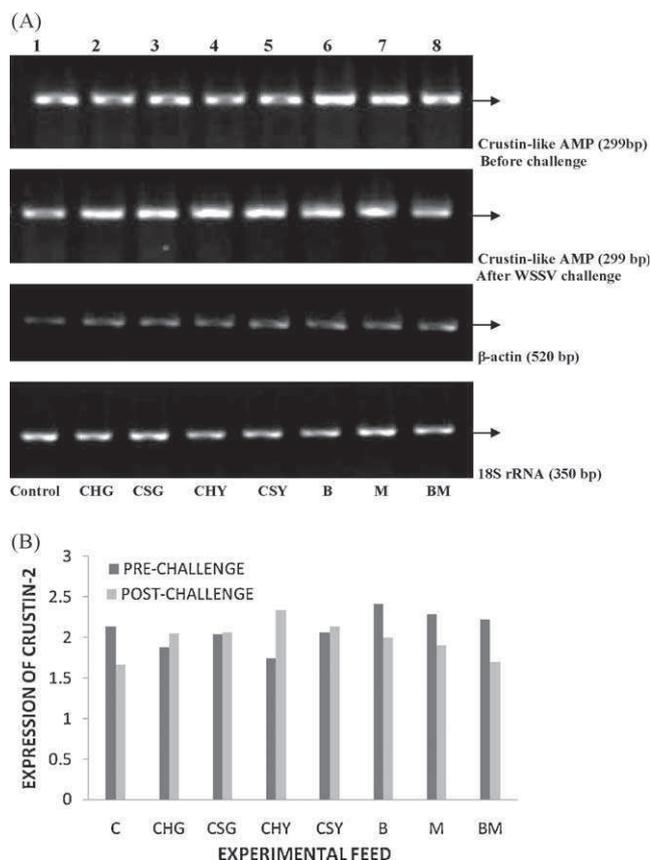


Fig. 4. Expression profile of crustin-like AMP (FJ535568) gene in the haemocytes of *Penaeus monodon* in response to various immunostimulants before and after challenge with WSSV (CHY = *Candida haemulonii* S27 yeast, CSY = *Candida sake* S165 yeast, CHG = *Candida haemulonii* S27 glucan, CSG = *Candida sake* S165 glucan, B = *Bacillus* MCCB101, M = *Micrococcus* MCCB104, BM = combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104). (A) Gel photograph, (B) ratio of target gene/ β -actin (x-axis = immunostimulants used, y-axis = ratio of target gene/ β -actin).

The probiotic gram-positive bacteria incorporated diet up-regulated the crustin gene to significant levels proving its immunostimulatory property. The yeasts and glucans caused a down-regulation of crustin gene expression pre-challenge WSSV. This is in agreement with the earlier work in *L. vannamei*, where no significant variations in the expression of crustins have been reported in β -glucan supplemented diet fed animals (Wang et al. 2007). Significant down-regulation of crustin was noticed with the viral challenge in the control group of organisms. However, all treatment groups showed better crustin expression post-challenge with the best performance displayed by yeast and glucan fed group.

The down-regulation of crustin in the control group 48 h post-challenge showed that the animal had already become weak and succumbed to infection earlier than that of the experimental groups. This observation is in agreement with the earlier work of Sun et al. (2008) where a down-regulation of lectins (another group of AMPs) has been reported 24 h post-challenge WSSV in *F. chinensis*. Vargas-Albores et al. (2004) and Okumura (2007) have also reported a decrease in the number of crustin transcripts when infected with *Vibrio* in *L. vannamei*. Chiou et al. (2005) have reported no up-regulation of AMPs in the haemocytes of tiger shrimps when challenged with *Vibrio*. But contrary observations have been reported in the white shrimps in which AMPs have been up-regulated with bacterial challenge (Jiravanichpaisal et al. 2007; Amparyup et al. 2008).

In the present work, glucans and different yeast strains exhibited almost similar efficacy in terms of crustin gene expression.

Whole yeast cells especially *C. haemulonii* were found to perform better under WSSV challenge. It was interesting to note that *C. haemulonii* did not induce the crustin gene pre-challenge, but effected significant up-regulation post-challenge WSSV, proving it to be a good immunostimulant conferring protection to shrimps against WSSV infection.

The response of crustin expression to bacterial challenge is supposed to be enigmatic and often does not follow the pattern expected for immune genes and other AMPs (Gross et al. 2001; Rojtinnakorn et al. 2002; Lorigeril et al. 2005). The present results are consistent with the earlier works where up-regulation of crustin gene has been reported. Application of PG in *M. japonicus* (Rattanachai et al. 2005) resulted in the up-regulation of crustin gene in unchallenged shrimps. In *P. monodon* also a five-fold up-regulation of the crustin transcripts has been reported following challenge with *V. harveyi* (Amparyup et al. 2008). On the contrary a few authors have observed an unexpected down-regulation of crustin transcripts after bacterial challenge with gram-negative bacteria. Administration of LPS in *L. vannamei* (Okumura 2007) resulted in the down-regulation of the same. A down-regulation in the crustin transcripts has also been reported after 24 h of challenge by *Vibrio alginolyticus* in *L. vannamei* (Vargas-Albores et al. 2004; Jiménez-Vega et al. 2004). Also in *P. monodon* crustin transcripts were found to be down-regulated after infection with gram-negative bacteria (Supungul et al. 2007).

The up-regulation by the combination of two strains (*Bacillus* MCCB101 and *Micrococcus* MCCB104) was not up to the level effected individually. Even though a combination of the two strains induced the gene pre-challenge a significant up-regulation was not found under viral challenge. This indicates that these two strains are potent when applied individually and not in combination. *Bacillus* MCCB101 and *Micrococcus* MCCB104 are widely used as probiotics in shrimp culture systems, and the present study proved them to be better immunostimulants when administered individually.

Expression of WSSV related genes post-challenge showed that *C. haemulonii*, *Bacillus* and combination of *Bacillus* and *Micrococcus* treated groups were less infected by WSSV (Fig. 5). This is in agreement with the up-regulation noted for crustin gene except for the combination of *Bacillus* and *Micrococcus*. When the expression of WSSV genes are taken into account, yeasts especially *C. haemulonii* proved to provide better protection to WSSV than glucans. Minor expression of some WSSV genes was noted for *C. sake* yeast and *Micrococcus*. Also *Micrococcus* treated group showed non-specific amplification for VP28. Such non-specific amplification has been reported earlier by Marks et al. (2003). These WSSV gene expressions showed that the extent of protection was lower in *C. sake* whole cell and *Micrococcus* treated group of shrimps compared to *C. haemulonii* and *Bacillus* + *Micrococcus* combination treated groups (Fig. 8).

Tissue-wise expression profile of crustin was in conformity to those found in other arthropods (Iwanaga and Kawabata 1998). Since *C. haemulonii* and probiotic *Bacillus* were found to be the best among the eight experimental groups, tissue-wise analysis of the crustin gene expression was carried out for these two treated group of animals and the control group.

Tissue expression profile of crustins in unchallenged shrimps revealed highest expression in gill followed by intestine, muscle, heart and the lowest in hepatopancreas. Earlier works in *P. monodon* have reported the absence of any crustin transcripts in hepatopancreas (Supungul et al. 2004). In *M. japonicus*, however, crustin mRNA was only detected in haemocyte, not in any other tissue (Rattanachai et al. 2004). Whereas, reports in *L. vannamei* is in agreement with the present result where mRNA transcripts could be observed in all tissues including hepatopancreas (Wang et al. 2007).

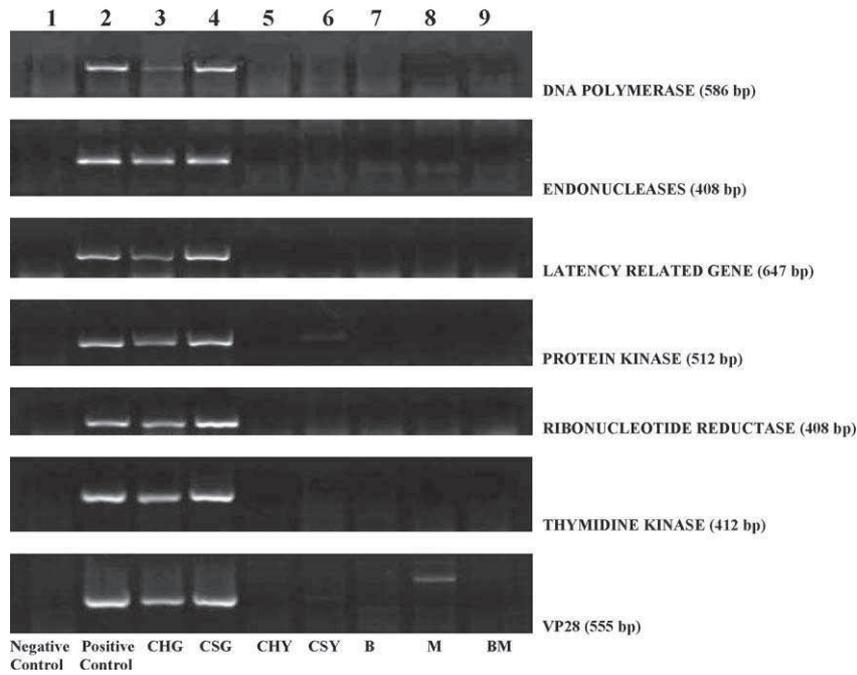


Fig. 5. Expression of WSSV genes in the haemocytes of *P. monodon* in response to the application of various immunostimulants (negative control = pre-challenged control shrimp, positive control = WSSV challenged control, CHY = *Candida haemulonii* S27 yeast, CSY = *Candida sake* S165 yeast, CHG = *Candida haemulonii* S27 glucan, CSG = *Candida sake* S165 glucan, B = *Bacillus* MCCB101, M = *Micrococcus* MCCB104, BM = combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104).

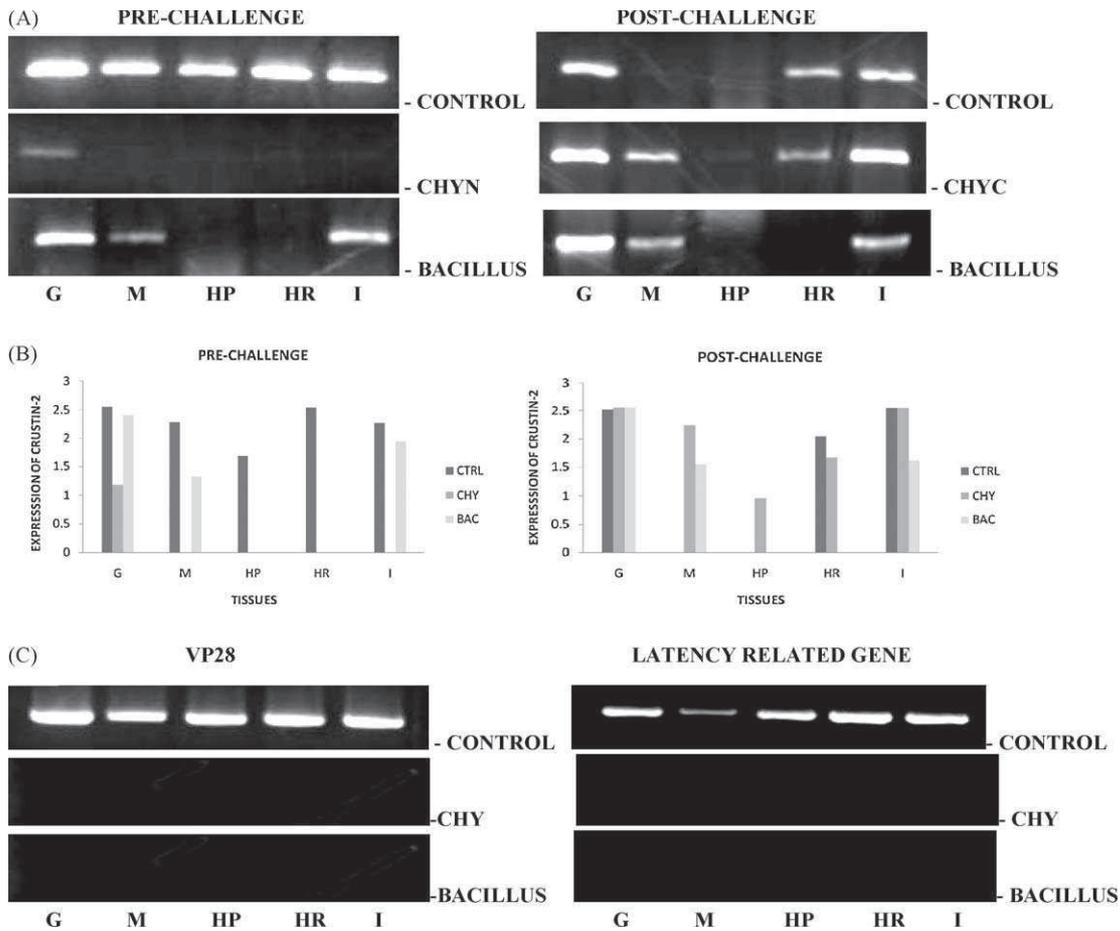


Fig. 6. Expression of crustin in various tissues of tiger shrimp *Penaeus monodon* in response to the application of immunostimulant/probiotic before and after WSSV challenge. G: gill, M: muscle, HP: hepatopancreas, HR: heart, and I: intestine. (A) Gel photograph. (B) Expression of crustin gene in target tissues. (C) Expression of WSSV related gene in target tissues.

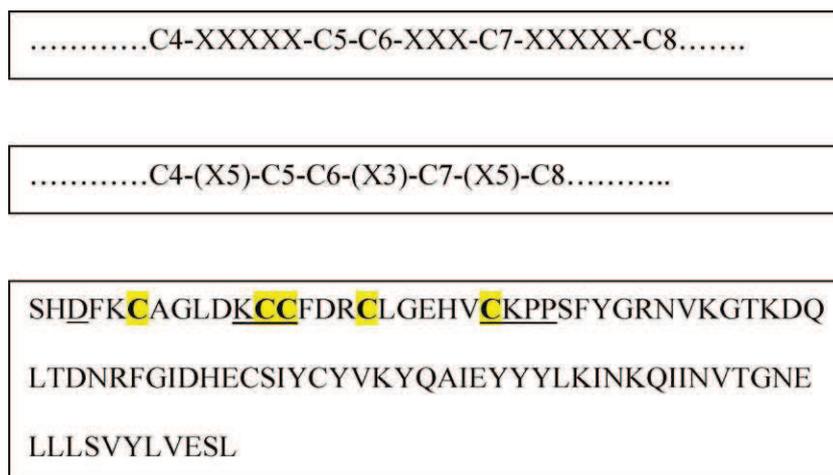


Fig. 7. The WAP domain signature of the crustin-like AMP (FJ535568). Consensus sequences that appear in the 4DSC domain are underlined and the C₄–C₈ cysteine residues are being highlighted.

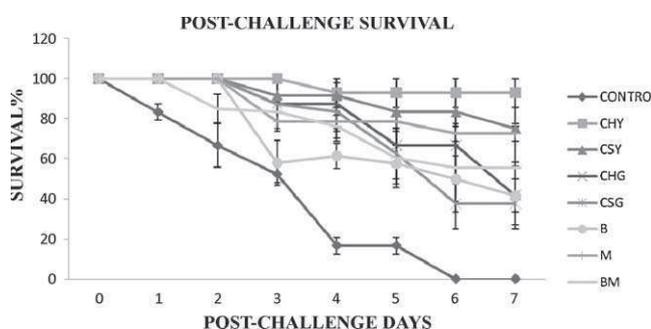


Fig. 8. Post-challenge survival of *Penaeus monodon* fed with different immunostimulant/probiotic bacteria incorporated diets and challenged with WSSV (CHY – *Candida haemulonii* yeast fed group, CSY – *Candida sake* yeast fed group, CHG – *Candida haemulonii* glucan fed group, CSG – *Candida sake* glucan fed group, B – *Bacillus* fed group, M – *Micrococcus* fed group, and BM – *Bacillus* + *Micrococcus* fed group).

Shrimps possess an open circulatory system that allows haemocytes to infiltrate and adhere to many tissues. All gene expressions are from the infiltrating haemocytes and the relative expression levels of these genes would reflect the amount of haemocytes infiltrating or fixed in tissues. Since haemocyte is the site of synthesis of AMPs, there is no point in comparing haemolymph with other tissues. Variation in the expression of crustin genes in various tissues might have been contributed by the differential infiltration of haemocytes into various tissues which in turn is due to the varying levels of virions in the tissues. This tissue-wise variation in crustin expression point to the tissue specificity of viruses for its multiplication.

Secondary expression sites such as intestine or gonads have been reported for AMPs in insects (Hoffmann and Reichart 1997; Manetti et al. 1998). Abundance of mRNA transcripts of AMPs in shrimp intestine suggests intestine to be a possible expression site, besides the haemocytes.

Yeast, *C. haemulonii* and probiotic bacteria, *Bacillus* treated group of animals did not show the presence of any WSSV gene transcripts in any of the tissues tested confirming the absence of WSSV infection.

Yeast, *C. haemulonii* was proved to be the best in terms of AMP gene expression. The constitutive production of these AMPs ensures that animals are able to protect themselves from low-level assaults by pathogens present in the environment. As these molecules play an important role in the shrimp immune system, the expression levels of these AMPs are possible indicators of the

immune state of shrimps. Generally, expression of crustin in the haemocytes was higher on WSSV challenge, suggesting their role in antiviral defense. The up-regulation of the crustin gene by yeasts, glucans and gram-positive bacteria proved that these compounds have potent immunostimulating activity against WSSV infection. Further investigations on the range of activity as well as the regulation of the gene expression of the tiger shrimp crustin during various stages of growth and development would shed light in developing strategies to protect tiger shrimp from infection by WSSV.

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