# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Equipments

Autoclave Model # LS-2D (Rexall industries Co. Ltd., Taiwan)

Automatic micropipettes P10, P100, P200, and P1000 (Gilson Medical Electrical S.A., France

–20 °C Freezer (Whirlpool)

–80 °C Freezer (Revco)

GS Gene Linker™ :UV Chamber (Bio-RAD Laboratories, USA)

Hybridization oven (Hybrid, USA)

Hyperfilm MP (Amersham International, England)

Incubator 37 °C (Memmert)

Larminar flow: Dwyer Mark II Model # 25 (Dwyer instruments, USA)

Microcentrifuge tubes 0.5 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

PCR thermal cycler : Gene Amp PCR System 2400 (Perkin Elmer)

PCR thin wall microcentrifuge tubes 0.2 ml (Perkin Elmer)

PCR workstation Model # P-036 (Scientific Co., USA)

Pipette tips 10, 20, 200, and 1000 µl (Bio-RAD Laboratories, USA)

Power supply: Power PAC 3000 (Bio-RAD Laboratories, USA)

Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Touch mixer Model # 232 (Fisher Scientific, USA)

Transilluminator 2011 Macrovue (LKB)

Vacuum blotter Model # 785 (Bio-RAD Laboratories, USA)

Vacuum pump (Bio-RAD Laboratories, USA)

Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

White/UV transilluminator: UVP ImageStore 7500 (Mitsubishi Electric Corporation, Japan)

### 2.1.2 Chemicals and Reagents

Absolute ethanol, $C_2H_5OH$ (BDH)

Acetic acid glacial, $CH_3COOH$ (BDH)

Agarose (Sekem)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Boric acid, $BH_3O_3$ (Merck)

Bromophenol blue (BDH)

Chloroform, $CHCl_3$ (Merck)

Developer (Eastman Kodak Company, USA)

Diethyl pyrocarbonate (DEPC), $C_6H_{10}O_5$ (Sigma)

100 mM dATP, dCTP, dGTP, and dTTP (Promega)

Dimethyl sulfoxide (DMSO), $C_6H_6SO$ (Amresco)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA), disodium salt dihydrate (Fluka)

Ficoll$^{TM}$ 400 (Amersham)

Fixer (Eastman Kodak Co., USA)

Foetal bovine serum (Gibco BRL)

Formaldehyde (BDH)

Isoamylalcohol (Merck)

Isopropanol (Merck)

Kodak Tri-Xpan400 film

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Phenol crystals, $C_6H_5OH$ (Carlo Erba)

RNase A (Sigma Chemical Co., USA)

Sodium acetate (Merck)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

Tryptic soy broth (Difco)

Trizol reagent (Gibco BRL)

Urea (Fluka, Switzerland)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

$\alpha$-$^{32}$P dATP specific activity 800 Ci/mmol (Amersham International, England)

### 2.1.3 Enzymes

Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, USA)

*Eco* RI (Biolabs)

*Not* I (Biolabs)

*Sal* I (Biolabs)

### 2.1.4 Bacterial strains

*Escherichia coli* strain XL-1 Blue MRF'

*E. coli* strain SOLR

*E. coli* strain DH10B

*E. coli* strain Y1090

*Vibrio harveyi* 1526

## 2.2 Samples

Sub-adult *P. monodon*, (approximately 3 month-old, 20 g of body weight), were purchased from local farms and separated into 2 groups. One was the unchallenged (or normal) shrimp and the other was *P. monodon* experimentally infected with *V. harvei* 1526. Both groups were acclimatized in aquaria at the ambient temperature (28 ± 4 °C) and at the salinity of 15 ppt for at least 1 day before used in the experiments.

**2.3 Preparation of *Vibrio harveyi* infected shrimp (modified from Roque et al., 1998)**

A single colony of *V. harveyi* 1526 (kindly provided by Charoenpokphand Group of Companies) was inoculated in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl at 30 °C for 8 hours. The culture was then diluted 1:100 with a sterile normal saline solution (0.85% NaCl, w/v). The titer of this dilution was monitored by a plate count method in tryptic soy agar (TSA) supplement with 2% NaCl (modified from Austin, 1988). One hundred microlitres of the $10^7$ CFU/ml diluted culture were intramuscularly injected into the $4^{th}$ abdominal segment, whereas the control group (or normal shrimps) was injected with 100 µl of 0.85% NaCl (w/v).

At 48 hours post-injection, haemolymph was collected and shrimps were tested whether the infection was successful by culture the suspensions of hepatopancreas on TSA plates supplemented with 2% NaCl (w/v) and incubated at 30 °C overnight. Colonies of *V. harveyi* 1526 from infected shrimps showed strong luminescence in the dark.

**2.4 Haemocyte collection and total RNA preparation**

Haemolymph was collected from the ventral sinus of each shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 µl of anti-coagulant (10% sodium citrate, w/v). Haemolymph was immediately centrifuged at 800x*g* for 10 minutes at 4 °C to separate haemocytes from the plasma. The haemocyte pellet was resuspended in 1 ml of Trizol reagent (Gibco BRL) and briefly homogenized. The homogenate was stored at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. After that 200 µl of chloroform was added and vigorously shaken for 15 seconds. The resulting mixture was stored at room temperature for 2 - 5 minutes and centrifuge at 12,000x*g* for 15 minutes at 4 °C. The colorless upper aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube. RNA was precipitated by the addition of 500 µl of isopropanol. The mixture was left

at room temperature for 5–10 minutes and centrifuged at 12,000x*g* for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 500 ml of 75% ethanol. The RNA pellet was kept under 75% ethanol until used. When required, the samples were centrifuged at 12,000x*g* for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was briefly air-dried for 5-10 minutes. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water.

The concentration of total RNA was determined by measuring the OD at 260 nm and estimated in μg/ml using the following equation,

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40^*$$

*A 1 OD unit at 260 nm corresponds to approximately 40 μg/ml of RNA (Sambrook et al., 1989)

## 2.5 Formaldehyde-agarose gel electrophoresis

A 1.0% (w/v) formaldehyde agarose gel was prepared using 1x MOPS buffer (diluted from a 10x MOPS buffer to 0.2mM MOPS, 50mM NaOAc, 10 mM EDTA, pH7.0 final concentration). The gel slurry was boiled until complete solubilization, and allowed to cool to 60 °C. Formaldehyde (0.66M final concentration) and ethidium bromide (0.2 μg) were added to the gel and poured into a chamber set. The comb was then inserted.

Ten to twenty micrograms of total RNA in 3.5 μl of DECP-treated $H_2O$, 5 μl of formamide, 1.5 μl of 10x MOPS and 2 μl of formaldehyde were combined, mixed well and incubated at 65 °C for 15 minutes. The mixture was immediately placed on ice. One-forth volume of the gel-loading buffer (50%, v/v, glycerol; 1mmol/l EDTA, pH 8.0, 0.5%, w/v bromphenol blue) was added to each sample. The sample was loaded to the 1.0 % agarose gel containing formaldehyde. The RNA marker was used as a standard RNA marker. Electrophoresis was carried out in 1x MOPS buffer at 50 volts, until bromphenol blue migrated approximately ¾ of the gel length. The gel was

washed 4-5 times of water before stained with ethidium bromide (0.5 μg/ml) for 1 hour. The EtBr stained gel was visualized total RNA as fluorescent bands by a UV transilluminator (UVP Inc.).

**2.6 Preparation of mRNA**

The mRNA was purified from total RNA using a QuickPrep® Micro mRNA Purification kit (Amersham Pharmacia Biotech). The oligo (dT)-cellulose was gently swirled and 1 ml of the cellulose suspension was immediately pipetted into a 1.5 ml microcentrifuge tube. This tube was centrifuged for 1 minute and the buffer was removed. The solution containing 50 μl of total RNA, 400 μl of the extraction buffer containing quanidinium thiocyanate and N-lauroyl sarcosine) and 750 μl of elution buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA) was added onto the oligo (dT)-cellulose. The tube was inverted gently for 3 minutes to resuspend the oligo (dT)-cellulose. The sample was centrifuged at the top speed for 1 minute. The supernatant was removed. The sample was washed with the high-salt buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 0.5 M NaCl) five times followed by three times with the low-salt buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 0.1 M NaCl). After the last washing step, the resin was resuspended with 300 μl of the low salt buffer and transferred to a microspin column placed in a microcentrifuge tube. The column was added with 500 μl of the low-salt buffer and was centrifuged at the full speed for 1 minute. The effluent in the collection tube was discarded. This step was repeated twice. After that, the column was placed in a sterile 1.5 ml microcentrifuge tube. Two hundred microlitres of prewarmed (65 °C) elution buffer was added to the top of the resin bed. The eluted mRNA was collected by centrifugation at the full speed for 1 minute. The eluted mRNA was kept at -80 °C until used.

## 2.7 Preparation of host bacteria XL 1 blue MRF', SOLR, Y1090 and DH10B

A single colony of a particular bacterial strain was inoculated into 2 ml of LB broth and incubated at 37 °C with shaking overnight. Fifty microlitters of the starter were added into 5 ml of LB broth containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ and incubated at 37 °C for 4 hours. The culture was then transferred into microcentrifuge tubes and centrifuged 800xg for 3 minutes at room temperature. The supernatant was removed and the bacterial pellets were resuspended in 500 µl of 10 mM $MgSO_4$. These bacterial suspension could be kept at 4 °C for 1 week.

## 2.8 Construction of cDNA libraries

### 2.8.1 Construction of a haemocyte cDNA library from normal shrimps

2.8.1.1 cDNA synthesis

Five micrograms of mRNA were used to synthesize the cDNA using a Time Saver cDNA Synthesis kit (Amersham Phamacia Biotech). The oligo ($dT_{12-18}$) primer was used to prime at the 3'-end of polyadenylated mRNA for synthesis of the first-standed cDNA, which catalyzed by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. The reaction mixture was incubated at 37 °C for 1 hour. RNA:cDNA hybrid from the 1$^{st}$ step was used to synthesized the second-stranded cDNA by adding Rnase H that nicks the RNA strand. Then, DNA polymerase I replaces RNA with DNA by the nick translation reaction. The cDNA produced was extracted with phenol/chloroform and purified on a spin column previously equilibrated in the ligation buffer (66 mM Tris-HCl ; pH 7.6, 0.1 mM spermidine, 6.6 mM $MgCl_2$, 10 mM dithiothreitol DTT and, 150 mM NaCl). After that, *Eco* RI/*Not* I adapters were ligated to each terminus of the blunt-ended cDNA. Excess and dimerized adapters were removed rapidly using a second spin column.

2.8.1.2 Cloning and packaging λ ZAP II

The *Eco* RI/*Not* I end cDNA was ligated to the *Eco* RI-λ ZAP II arm, catalized by T4 DNA ligase at 16 °C for 30 minutes. After that, the mixture was incubated at 65 °C for 15 minutes to inactivate the ligase activity. Twenty-five microliters of the packaging extract (Epicentre Technologies) were added to the mixture and mixed by pipetting. The reaction was incubated at room temperature for 90 minutes. Five hundred ml of SM buffer (0.1 M NaCl, 0.8 mM $MgSO_4.7H_2O$ and 0.05 Tris-HCl, pH 7.5) and 25 µl of chloroform were added to the reaction and mixed by pipetting. This reaction was kept at 4 °C until used.

2.8.1.3 *In vivo* excision

Ten to the seventh of the lambda phage (packaging phage stock), $10^8$ cfu/ml of XL1-Blue MRF' cell and $10^8$ pfu/ml of ExAssist helper phage were combined in the conical tube and incubated at 37 °C for 15 minutes to allow absorption. Then, 20 ml of LB broth (1% bacto tryptone, 1% NaCl and 0.5% bacto yeast extract) were added. The reaction was incubated with shaking at 37 °C for 2.5–3 hours. The reaction was then heated at 65-70°C for 20 minutes and spun down at 100x*g* for 10 minutes. The supernatant was decanted into a sterile conical tube. This stock contained the excised pBluescript phagemid packaged as filamentous phage particles. This stock was kept at 4 °C before the excised phagemids were plated.

To plate the excised phagemids, 200 µl of freshly grown SOLR cells and 1 µl of the phage supernatant were combined and incubated at 37 °C for 15 minutes. After that, 200 µl of the cell mixture were plated on LB-ampicillin agar plates (50 mg/ml) and incubated overnight at 37 °C.

2.8.1.4 Titering of the phage library

For titering of phage from the normal library, *E. coli* Y1090 was prepared as described in step 2.7. After preparing the bacterial cells, the phage supernatant was serial diluted in the SM buffer. Each phage dilution was added

with the preparing bacteria and incubated at 37°C for 15 minutes. The top agar (1% NaCl, 1% tryptone, 0.5% yeast extract and 0.7% agarose) was added into each serial dilution and the mixture was plated on LB plates (1% NaCl, 1% tryptone, 0.5% yeast extract and 2% agar). These plates were allowed to harden at room temperature for 10 minutes and incubated overnight at 37 °C for 12-16 hours. The number of plaques was counted and the plaque forming units (pfu) per milliliter for each concentration were calculated.

2.8.1.5 Amplification of the cDNA library

The packaged mixture containing about 5 x $10^4$ pfu of bacteriophage and 600 µl of XL1-Blue MRF' cell were combined in the conical tube and incubated for 15 minutes at 37°C. The top agar (6.5 ml) was added and spreaded evenly onto a freshly poured 140-mm NZY agar plate. The plate was incubated at 37°C for 6-8 hours, then overlaid with 8-10 ml of the SM buffer and stored at 4°C overnight. After that, the SM buffer collected into a sterile polypropylene container. The plate was rinsed with 2 ml of the SM buffer and pooled. The pooled SM buffer was added with chloroform to a 5% (v/v) final concentration, mixed well and incubated at room temperature for 15 minutes. The cell debris was removed by centrifuged at 2000x*g* for 10 minutes at room temperature. The supernatant was transferred into a sterile conical tube and chloroform was added to a 0.3% (v/v) final concentration. After 7% (v/v) of DMSO were added, the amplified library was kept at –80 °C.

### 2.8.2 Construction of a haemocyte cDNA library from the *V. harveyi* infected shrimps

2.8.2.1 cDNA synthesis

Five micrograms of mRNA were used to synthesize cDNA using a Superscript™ lambda system for cDNA synthesis and λ cloning kit (Gibco BRL). The *Not* I-Oligo ($dT_{15}$) primer primed at the 3'-end of polyadenylated mRNA to synthesize the first stranded cDNA by the Superscript II reverse

transcriptase. The second strand synthesis was started, after RNasH was added to nick the RNA strand. *E. coli* DNA polymerase I exhibits nick translation activity and replaced nuclear RNA with DNA. The blunt ended cDNA had the *Not* I restriction sites at the 3'- end. The *Sal* I adapter was ligated to both termini of the synthesized cDNA by T4 DNA ligase. After that, the restriction endonuclease *Not* I was added and cDNA was digested at *Not* I restriction sites , so the final cDNA products had the *Sal* I site at the 5' terminus and the *Not* I site at the 3' terminus.

The adapter-ligated cDNA were sized-functionated through a column chromatography to remove adapters and low molecular weight cDNAs. The column was equilibrated in TEN buffer (10 mM Tris HCl ; pH 7.5, 0.1 mM EDTA, 25 mM NaCl). Aliquots of fractions (about 35 µl per fraction) were collected. Five microlitters of each fraction were analyzed by electrophoresis on a 1% agarose gel. Fractions, having cDNAs with size greater than 500 bp were combined and precipitated with 2 volume of pre-chilled absolute ethanol and immediately centrifuged at room temperature for 20 minutes at 14,000xg. The supernatant was removed. The pellet was washed with pre-chilled 70% ethanol. The reaction was centrifuged for 2 minutes at 14,000xg. The supernatant was removed. The cDNA pellet was dried at 37 °C for 10 minutes.

2.8.2.2 Cloning and packaging of λ ZIP-LOX

Dried cDNA from the cDNA synthesis was resuspended with DEPC-treated water and ligated to the λ ZIP-LOX *Not* I / *Sal* I arms in 5 µl reaction containing 1 µl of 5x ligation buffer, 2 µl of λ ZIP-LOX *Not* I / *Sal* I, 1 µl of DEPC-treated water and 1 µl of T4 DNA ligase (1 U). The reaction was incubated at 4 °C overnight. The ligated reaction was terminated by incubation at 65 °C for 15 minutes. Twenty-five microlitters of the packaging extract (Epicentre technologies) were added to the mixture and mixed by pipetting. The reaction was incubated at room temperature for 90 minutes. Five hundred microlitres of the SM buffer were added to the reaction and 25 µl of

chloroform were added and mixed by pipetting several times. This reaction was kept at 4 °C until used.

2.8.2.3 Titering of phage library

For titering of phage from the *Vibrio*-infected library, *E. coli* strain MRF' were prepared as described in step 2.7. A titering assay was examined as described in step 2.7.1.4

2.8.2.4 *In vivo* excision

To plate the excised phagemids, ten microlitters of the packaged phage solution were combined with 100 µl of DH10B cells and incubated at 37 °C for 15 minutes. After that, 900 µl of SOC medium (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 25. mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) were added and incubated at 37 °C with shacking for 45 minutes. Then, 200 µl of the cell mixture were plated on LB plate containing 100 µg/ml ampicillin and incubated at 37 °C overnight.

2.8.2.5 Amplification of the *Vibrio*-infected cDNA library

One hundred microlittres of the phage supernatant and 100 µl of Y1090 cells were combined in the conical tube and incubated at 37 °C for 15 minutes. The cDNA library was then amplified as described in 2.6.1.4

## 2.9 Plasmid DNA preparation

A recombinant plasmid was inoculated into 2 ml of LB broth containing 50 mg/ml of ampicillin and cultured overnight at 37 °C. The culture was centrifuged at 800x*g* for 3 minutes. The supernatant was removed. The pellets were resuspended in 100 µl of solution I (25 mM Tris – HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose, and 0.5%; w/v lysozyme). The reaction was mixed by vigorous vortexing and placed at room temperature for 5 minutes. Two hundred microlitres of a freshly prepared solution II (0.2 N NaOH and 1% (w/v) SDS) were added and mixed gently for cell lysis and DNA denaturation.

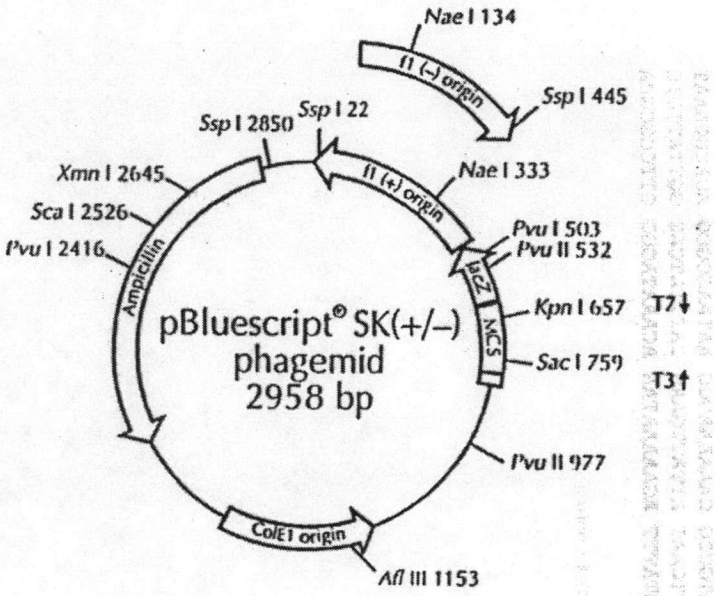
One hundred and fifty microlitres of solution III (3M sodium acetate, pH 4.8) were added, mixed gently and placed on ice for 10 minutes. After centrifugation at 10,000xg for 10 minutes, the supernatant was transferred to a fresh new microcentrifuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed and spun at 10,000x*g* for 10 minutes. The upper aqueous phase was transferred to a new microcentrifuge tube. Pleasmid DNA was precipitated by adding 1/10 volume of sodium acetate and 2 volumes of absolute ethanol. The mixture was kept at – 80 °C for 15 minutes and centrifuged at 10,000x*g* for 10 minutes. The pellet was washed with cold 70% ethanol. The pellet was air dried and dissolved in 50 µl of TE buffer (10 mM Tris –HCl, pH 8.0 and 1 mM EDTA, pH 8.0). Three microlitres of 5 mg/ml of RNase A were added and incubated at 37 °C for 30 minutes. After that, 32 µl of 20 % polyethyleneglycol molecular weight 6,000 (PEG6,000) in 2.5 M NaCl were added. The reaction mixture was mixed gently by pipetting and standed on ice for 1 hour. The mixture was centrifuged at 10,000x*g* for 10 minutes at room temperature and washed with cold 70% ethanol. The pellets were air-dried and resuspended in 50 µl TE buffer.

## 2.10 Determination of insert sizes

After *in vivo* excision, λ-phages containg cDNAs from both libraries were converted to recombinant phagemids (pBluescripSK for the normal library and pZL1 for the infected library). The cDNA insert which size over 500 bp were selected for sequencing, thus sizes of inserts were analyzed by colony PCR or enzymatic digestion if recombinant plasmid.

### 2.10.1 Colony PCR

The pBluescript SK phagemid was 2958 bp in length and was derived from pUC19 (Figure 2.1). This phagemid had 10 unique restriction sites in the multiple cloning region flanked by T3 and T7 RNA promoters, therefore T3 and T7 primers can be used to analyze insert sizes. These primer sets annealed at T3 and T7 RNA promoters and amplified the insert cDNA. Colony PCR was performed in a 25 µl reaction volume containing 1.25 mM of each dNTP




(a)

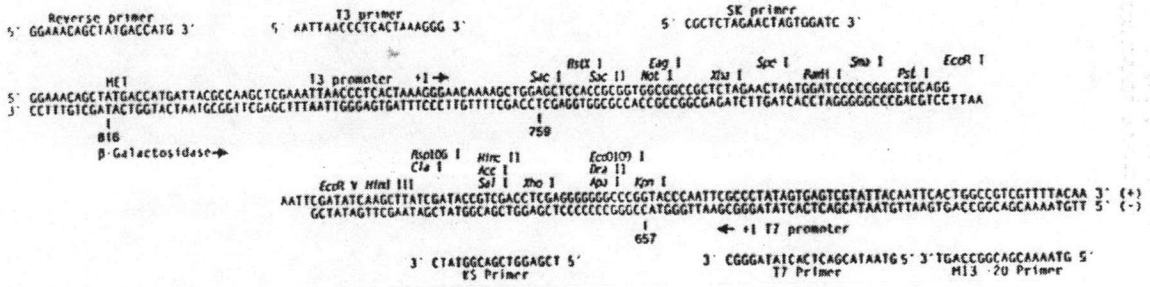



FIGURE 5 Circular map and polylinker sequence of the pBluescript® SK(+/–) phagemid.

(b)

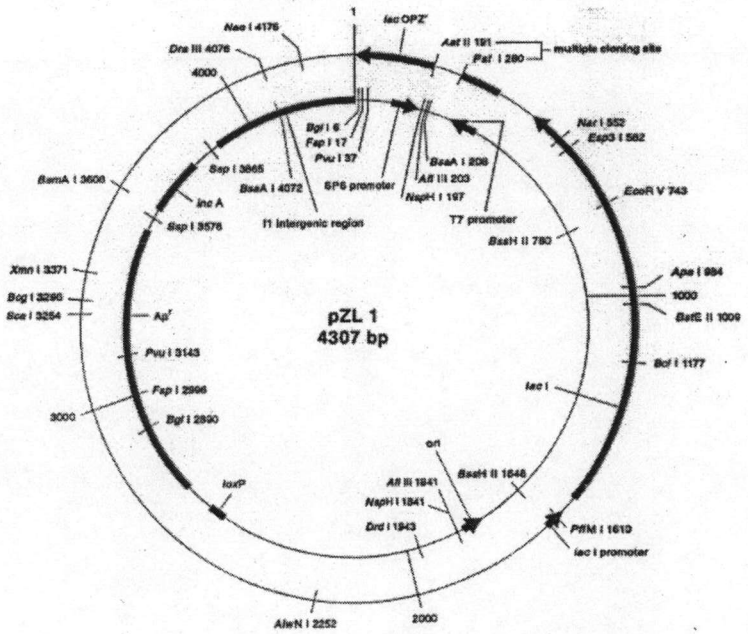
**Figure 2.1** The circular map (a) and polylinker sequence of pBluescript SK (+/-) phagemid.

(dATP, dCTP, dGTP and dTTP), 1 x PCR buffer, 1.2 mM $MgCl_2$, 2 pmole of each primers and 1 unit of *Taq* DNA polymerase (5unit/μl). A recombinant colony was scraped by the micropipette tip and mixed well in the amplification reaction. The PCR profiles incubated at 96 ° C denaturing step for 2 minutes, followed by 30 cycles of 96 °C for 30 seconds and 70 °C for 2 minutes. The reaction was final extended of at 72 °C for 5 minutes. The resulting PCR products were electrophoretically analyzed in 1 x TBE buffer at 100 volts. A using DNA ladder (100bp) was used as a DNA marker.

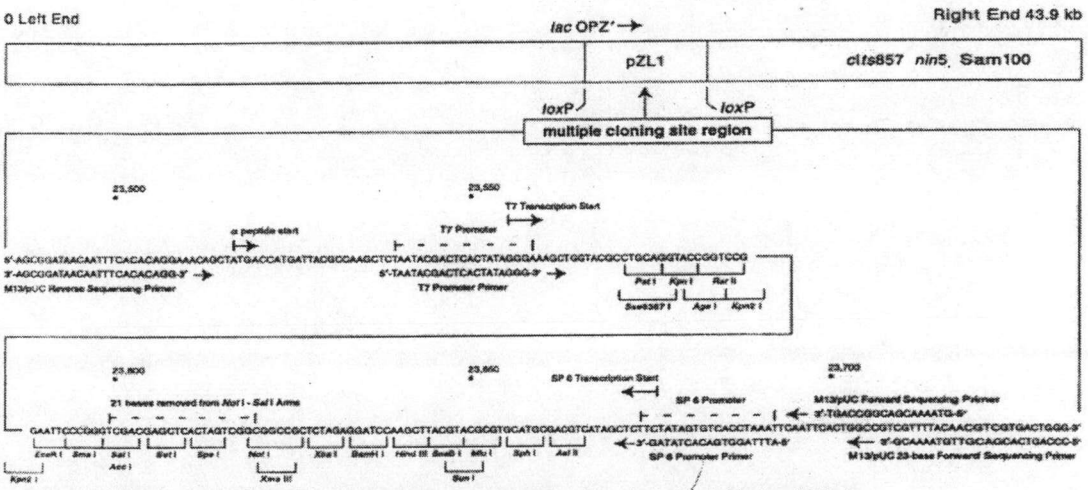
The pZL1 plasmid was 4,307 bp in lenngth and was identical to plasmid pSPORT 1, with the exception that it contained a single loxP site and the incA sequence cloned at the positions indicated in the map (Figure 2.2). This plasmid had 19 unique restriction sites in the multiple cloning region flanked by SP6 and T7 RNA polymerase promoters. Therefore SP6 primer and T7 primer were used for identification of insert sizes. These primer sets annealed SP6 and T7 RNA polymerase promoters and amplified insert cDNAs. The amplification reaction and the PCR profile used were identical to those described for colony PCR of recombinant pBluescriptSK but the primer sets (T3 and T7) were replaced with SP6 and T7 primer sets. The resulting PCR product was analyzed by agarose gel electrophoresis prepared in 1 X TBE buffer at 100 volts. A DNA ladder (100 bp) was used as a DNA marker).

**2.10.2 Restriction enzyme digestion**

Since cDNAs from of haemocytes of normal shrimp contained the *Eco*RI adaptor at the both termini thus, *Eco* RI was used to cut the inserted cDNA from the pBluescript phagemid. Conditions for enzymatic digestion of recombinant plasmid composed of 150 ng of extracted recombinant phagemid, 1x reaction buffer (10mM NaCl, 5 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.9: New England Biolabs), 1 unit of *Eco* RI (New England Biolabs) and 100 μg/ml BSA in a 10 μl reaction volume. The reaction was incubated at 37 °C for 1 hour. After that, 5 μl of loading dye were added to the reaction. The mixture was loaded onto 1.2% agarose gel and electrophoresed




(a)




(b)

**Figure 2.2** The circular map of pZL 1 plasmid (a) and multiple cloning site of the λ ZipLox vector.

in 1 x TBE buffer at 100 volts. Sizes of insert were visualized under a UV transilluminator by comparing with a 100 bp ladder.

Haemocyte cDNAs of *V. harveyi* infected shrimps were directional clone to *Not* I and *Sal* I sites therefore *Not* I and *Sal* I were used to cut cDNA inserts from the pZL1 plasmid following conditions described above but *Eco* RI was replaced with *Not* I and *Sal* I. The digested products, after incubation at 37 °C for 1 hour, were electrophoresed in 1.0 % agarose gel prepared with 1x TBE buffer at 100 volts. A DNA ladder (100bp) was used as a DNA marker.

**2.11 Agarose gel electrophoresis (Sambrook et al., 1989)**

One percentage (w/v) of agarose gel was prepared using 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The gel slurry was heated until complete solubilization, then the gel was poured into a chamber set. A comb was inserted. After the gel was solidified, the comb was carefully withdrawn and sufficient 1 x TBE buffer was added to cover the gel for approximately 0.5 cm. Each sample was mixed with ¼ volume of the gel-loading dye (0.25% bromphenol blue, 0.25% xylene cyanol FF and 15% ficoll) and loaded into the well. Lambada-*Hind* III fragments or a DNA ladder (100 bp marker) were used as standard DNA markers. Electrophoresis was carried out in 1 x TBE buffer at 100 volts until the bromophenol blue dye marker migrated about ¾ of the gel length. After electrophoresis, the gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 5 minutes and destained to remove unbound EtBr by submerged in distilled water for 15 minutes. Fractionated DNA was visualized under a UV transilluminator (UVP Inc.)

**2.12 DNA sequencing and Data Analysis**

The dideoxy chain termination-based sequencing method was carried out using a ThermoSequenase Fluorescent Labeled Primer Sequencing kit (Amersham Pharmacia Biotech) with M13 fluorescent dye labeled primers. Three microlitres (0.5-5 μg) of plasmid DNA and two pmol of M13 fluorescent

labeled primer were added into each 4 tubes; 2 μl of A-reagent (Tris-HCl, pH 9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddATP, thermostable pyrophosphatase and ThermoSequence DNA polymerase), 2 μl of C-reagent (Tris-HCl, pH 9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddCTP, thermostable pyrophosphatatase and ThermoSequence DNA polymerase), 2 μl of G-reagent (Tris-HCl, pH 9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddGTP, thermostable pyrophosphatatase and ThermoSequence DNA polymerase) and 2 μl of T-reagent (Tris-HCl pH 9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddTTP, thermostable pyrophosphatatase and ThermoSequence DNA polymerase).

Each reaction was overlaid with light mineral oil and was amplified in a thermal cycling using a 96 °C, 2 minutes initial denaturation followed by a 96 ° C, 30 seconds denaturation, a 60 °C, 30 second annealing temperature, a 72 °C, 1 minute extension for 25 cycles, and a 72 °C, 5 minutes final extension. After amplification was complete, each A, C, G and T-reaction was added with 5 μl of formamide loading dye (formamide, EDTA and funchsin) and 2.5 μl of each reaction were loaded onto 6% denaturing polyacrylamide gel (500 ml of gel mixture prepared from 250 g urea, 75 ml 40% acrylamide stock solution, 4 g N, N methylenebisacrylamide and 187.5 ml deionized water) and were electrophoresed on an automated DNA sequencer (LC4000, LICOR) with 1x TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3) at 50 w for 8-16 hours.

Sequences of cDNAs were edited and compared with sequence in the nucleotide sequence database of the GenBank (the National Center for Biotechnology Information; NCBI) using the BLASTN and BLASTX programs (Altschul et al., 1997). Significant probabilities and numbers of matched nucleotide/proteins were considered when E-values < the $10^{-4}$ and a

match > 100 nucleotides for the BLASTN and a match > 10 amino acid residues for the BLASTX, respectively.

After homology search, matched ESTs were categorized into 6 broad functional categories; 1) gene expression, regulation and protein synthesis 2) internal/external structure and motility 3) metabolism 4) defence and homeostasis 5) signaling and communication and 6) cell division/DNA synthesis, repair and replication, based on significant sequence homology according to the criteria proposed by Adams et al., (1991). EST nucleotide sequences were submitted to the nucleotide sequence database (dbEST) of the GenBank (Altschul et al., 1997).

## 2.13. Semiquantitative Reverse Transcription–Polymerase Chain Reaction

### 2.13.1 Samples

Sub-adult *P. monodon* were infected with $10^7$ cfu/ml of *V. harveyi* 1526 as described in 2.3. Haemolymph was collected from the ventral sinus of each shrimp using sodium citrate as an anti-coagulant at 0, 6, 12, 24 and 48 hours after injection. Haemocytes were prepared by centrifugation of the haemolymph and used to prepare total RNA as described in 2.4.

### 2.13.2 First stranded cDNA synthesis

The 1st standed cDNA was generated using 1 μg of total RNA and 0.5 μ g of oligo ($dT_{18}$) primer with an ImProm-II™ Reverse Transcription system kit (Promega). The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. After that, 4 μl of 5x reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of dNTP Mix (10 mM each), 20 units of Ribonuclease inhibitor and 1 μl of ImProm-II reverse transcriptase were added and gently mixed. The reaction was incubated at 25 °C for 5 minutes and at 42 °C for 60 minutes. Then, the reaction was incubated at 70 °C for 15 minutes to terminate reverse transcriptase actively.

### 2.13.3 Primers

Expression levels of 8 immune related genes; anti-LPS factor, crustin, penaeidin, HSP 90, HSP 70, lysozyme, serine protease inhibitor and prophenol oxidase were determined and β-actin gene was used as an internal control. Primers were designed from nucleotide sequences of EST clones obtained from both cDNA libraries using the GeneFisher program (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). Sequences of designed primers were shown in Table 2.1.

### 2.13.4 Determination of PCR conditions

Amplification was performed in a 25 μl reaction volume containing 1 μl of cDNA template, 1.25 mM of each dNTPs, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% Tritonx-100), 1 U of *Taq* DNA polymerase, 2 pmole of the interested primers, 2 pmole of β-actin primer and optical concentration of $MgCl_2$ using a Gene Amp PCR System 2400 (Perkin Elmer). Other PCR parameters were optimized and described below.

#### 2.13.4.1 $MgCl_2$ concentration

The optimal $MgCl_2$ concentration between 1-5 mM $MgCl_2$ was examined using the standard PCR conditions. The concentration that gave the highest yield and specificity was chosen.

#### 2.13.4.2 Cycle numbers

The PCR amplifications were carried out at different cycle numbers including 24, 27, 30, 33 and 36. After amplifications, PCR products were run on an agarose gel. The number of cycles that amplified the PCR product in the exponential rage and did not reach a plateau level was chosen.

#### 2.13.4.3 Control for competition between primer sets

To determine the competition between primer sets, cDNAs were amplified at 3 conditions; 1) amplification with the control primer set, 2) amplification with the interesting gene primer set, and 3) amplification with

**Table 2.1** Primers used for semi-quantitation of immune-related gene expression and internal control (β–actin)

| Target transcripts | Primer | Annealing temperature (°C) | Product Size (bp) |
|---|---|---|---|
| ALF | 5'CGCCAGCAAGATCGTAGGGTTG3' (F) | 60 | 250 |
| | 5'AGGCCTATGAGCTGAGCCACTG3' (R) | | |
| SPI | 5'TGGCGTGAGTGTCACTTTCCA3' (F) | 54 | 254 |
| | 5'AAGTCTTGCCATCACTGCCAC3' (R) | | |
| crustin | 5'TCCCTGGAGGTCAATTCGAGTG3' (F) | 53 | 216 |
| | 5'AGTCGAACATGCAGGCCTATCC3' (R) | | |
| penaeidin | 5'AGGATATCATCCAGTTCCTG3' (F) | 50 | 220 |
| | 5'ACCTACATCCTTTCCACAAG3' (R) | | |
| proPO | 5'GAGGATATATTTGGCTCCGAAG3' (F) | 53 | 419 |
| | 5'GGTCGAACGGGAAGCCCATC3' (R) | | |
| HSP90 | 5'TCGTCAATACCCAGGCCAA3' (F) | 50 | 184 |
| | 5'CGACCACAGCATCATCGAAAC3' (R) | | |
| HSP70 | 5'GCACCTGCTGTCGGTATTGATC3' (F) | 50 | 170 |
| | 5'TACAGTGTTGTTGGGGTTCATC3' (R) | | |
| lysozyme | 5'TGGCAGCGATTATGGCAAG3' (F) | 53 | 217 |
| | 5'GGAACCACGAGACCAGCACTC3' (R) | | |
| β–actin (internal control) | 5'GCTTGCTGATCCACATCTGCT3' (F) | 55 | 337 |
| | 5'ACTACCATCGGCAACGAGA3' (R) | | |

both primer sets. PCR products were run on the same agarose gel. The band intensity of each primer set was compared. Decreasing of band intensity indicated competition between two primer sets. When competition was detected, different reaction conditions were tested (e.g. adjusting the concentration of $MgCl_2$).

2.13.4.4 Gel electrophoresis and quantitative analysis

The ratio between the interesting gene and the internal control products was determined by electrophoresis on 1.4% agarose gels prepared as described in section 2.9. Six microlitres of PCR products were combined with ¼ volume of the gel-loading dye before loaded to the agarose gel. A DNA ladder (100 bp marker) was used as a standard DNA marker. After electrophoresis at 100 volts, the gel was stained with 2.5 μg/ml EtBr for 5 minutes and destained in distilled water for 15 minutes. The intensity analysis was performed with Gel Documentation System (GeneCam FLEX1, SynGene) and further quantified using the Genetools analysis software.

2.13.4.5 Data analysis

The expression level of each transcript at a particular time was normalized with that of the control treatment (0 hour) after divided by that of the internal control (actin). Significantly different expression levels were treated using One Way Analysis of Variance (ANOVA) following by a post hoc test (Duncan's new multiple range test)