Eugon agar	Becton, Dickinson & Co.
Eugon broth	Becton, Dickinson & Co.
Formaldehyde	Ajax
Formamide	Merck
Glass beads - acid washed	Sigma
Glucose -6- phosphate dehydrogenase	Sigma
D-glucose	Ajax
Glycerol	Merck
HEPES	Sigma
HEPES-sodium salt	Sigma
Hexanucleotide mix 10x	Roche
Hexokinase	Roche
Hide – Remazol brilliant blue R (Hide-powder azure)	Fluka
Hydrochloric acid	BioLab
Isoamyl alcohol	Ajax
Isopropanol	Ajax
Kanamycin	Sigma
Klenow enzyme	Roche
Lab lemco powder	Oxoid
Lambda DNA	Promega
Lysozyme	Sigma
L-arginine	Sigma
L-lactic acid dehydrogenase	Sigma
Magnesium acetate	Ajax
Magnesium chloride	Ajax
Magnesium sulfate	Chem-supply
Maleic acid	Sigma
N-laurylsarcosine	Sigma
Phenol	Wako
Phosphoenol pyruvate	Aldrich
Polyethylene glycol 6000	Ajax
Poly adenylic acid (Poly-A)	Sigma
Polynucleotide Phosphorylase from *Bacillus stearothermophilus*	Sigma
Potassium acetate	Ajax
Primers (oligonucleotides)	GeneWorks
Probes (TaqMan probes)	GeneWorks
Pyruvate kinase	Sigma
Restriction endonucleases	Promega
Ribonuclease A	Sigma
RNaseA	Roche

RNA Later	Ambion
RNaseZap-Wipes	Ambion
Sodium acetate	Merck
Sodium chloride	Ajax
Sodium citrate	Merck
Sodium dodecyl sulfate	Merck
Sodium hydroxide	Ajax
Spermidine	Sigma
Sucrose	Ajax
T4-DNA ligase	Promega
Tetracycline	Sigma
Tris-base	Merck
Triton X-100	Sigma
Trypticase peptone	Becton, Dickinson & Co.
Tryptone	Oxoid
Tween20	Sigma
X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)	Sigma
Xylene cyanol FF	Sigma
Yeast extract	Oxoid
Zwittergent 3-14 Detergent	CalBioChem

2.1.1. Commercial kits used

1. Ambion - RiboPure™ – Bacteria (Cat. No. 1925).
2. Pierce – Coomassie Plus - The better Bradford assay (Cat. No. 23238)
3. Promega – Wizard® genomic DNA purification kit (Cat. No. A1125)
4. Promega – Wizard® PCR Preps DNA purification system (Cat. No. A7170)
5. Promega – pGEM™ –T and pGEM –T Easy vector systems (Cat. No. A1360)
6. Promega – Wizard® Plus Maxipreps DNA purification system (Cat. No. A7270)
7. Qiagen – MinElute™. (Cat. No. 2860)
8. Invitrogen – SuperScipt™ One-step RT-PCR with Platinum® (Cat. No. 11732-020)

2.2. Bacterial strains and culture

2.2.1. *Dichelobacter nodosus* strains

D. nodosus strains A198, C305, 1493 and UNE64 (VCS1703A) were provided by Prof. J. I. Rood (Dept. of Microbiology, Monash University, Clayton, Victoria, Australia.). Strains 819 and 2483 were provided by Dr. J. Searson (Regional Veterinary Laboratory, NSW Agriculture, Wagga, NSW, Australia). *D. nodosus* strains were cultured on EYE agar [Eugon agar (45 mg/ml) supplemented with 2 mg/ml yeast extract] and EYEB agar [Eugon agar (45 mg/ml) supplemented with 2 mg/ml yeast extract and 5% of defibrinated horse blood] for 3 to 7 days at 37°C under anaerobic conditions in an atmosphere of 80% (v/v) N_2, 10% (v/v) H_2 and 10% (v/v) CO_2. The pCF5 *pnpA* knockout transformants were cultured with EYETB agar [Eugon agar (45 mg/ml) supplemented with 2 mg/ml yeast extract and 5% of defibrinated horse blood with 1 µg/ml of tetracycline added] and on EYET agar [Eugon agar (45 mg/ml) supplemented with 2 mg/ml yeast extract with 1 µg/ml of tetracycline added] for 3 to 7 days at 37°C under anaerobic conditions. The pSK81 *pnpA* complement transformants were cultured with EYEKB agar [Eugon agar (45 mg/ml) supplemented with 2 mg/ml yeast extract and 5% of defibrinated horse blood with 10 µg/ml of kanamycin added] and on EYEK agar [Eugon agar (45 mg/ml) supplemented with 2 mg/ml yeast extract with 10 µg/ml of kanamycin added] for 3 to 7 days at 37°C under anaerobic conditions.

D. nodosus glycerol stocks were prepared from cells grown on EYEB agar culture plates. EYE broth [Eugon broth (30 mg/ml) supplemented with 2 mg/ml yeast extract] was prepared and 1.5 ml of 50% v/v EYE broth: glycerol was added to plates of *D. nodosus*

cells. The cells were gently scraped with a sterile spreader and 300 µl aliquots of the cell suspension were transferred to sterile microfuge tubes and stored at -80°C.

2.2.2. *Escherichia coli* strains and media

E. coli strain DH5α [F⁻, Φ80d*lacZ*Δ, M15Δ, (*lacZYZ-argF*)*U*169, *deoR*, *recA*1, *ednA*1, *hsdR*17, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1; Bethesda Research Laboratories] was used as a host for recombinant plasmids in heat shock mediated transformations. DH5α derivatives were cultured at 37°C overnight on 2 x YT plates (yeast extract 1% w/v, tryptone 1.6% w/v NaCl 0.5% w/v and bactoagar 1.5% w/v) or YT broth supplemented with one or more antibiotics such as 100 µg/ml ampicillin, 10 µg/ml tetracycline or 20 µg/ml kanamycin depending on the antibiotic markers carried by the particular plasmid.

2.3. Preparation of DNA

2.3.1. Preparation of *D. nodosus* genomic DNA

Genomic DNA was isolated from *D. nodosus* strains using the Wizard® genomic DNA purification kit (Promega). The *D. nodosus* strains were cultured on four EYEB agar plates for 4 days in an anaerobic container. The bacterial culture was scraped from the plates after adding 5 ml of EYE broth and collected in 10 ml sterile tubes, then centrifuged at 1700 *g* for 10 min. The supernatant was discarded and the cell pellet was processed according to the manufacturer's instructions. The isolated DNA was stored at -20°C and was used for Southern blot and PCR experiments.

2.3.2. Preparation of plasmid DNA

(a) Holmes and Quigley method

To prepare plasmid DNA for screening of transformants, sub-cloning (Section 2.4), or probe preparation (Section 2.7.1), a modification of the method described by Holmes and Quigley (1981) was used. A single DH5α recombinant colony was inoculated into 10 ml of YT broth with the appropriate antibiotic and then incubated at 37°C overnight. The culture was collected in a 10 ml sterile tube and centrifuged at 1700 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 500 µl STET (50 mM Tris, pH 8, 50 mM EDTA, 8% sucrose w/v, 5% triton X100 v/v) solution and 40 µl of 10 µg/ml lysozyme was added. The samples were boiled for 90 seconds and transferred to ice for 5 min. After centrifugation at 8800 *g* for 10 min, the supernatant was transferred to a fresh 1.5 ml microfuge tube and 600 µl of iso-propanol was added. The tube was mixed and centrifuged at 4°C at 8800 *g* for 10 min. The pellet was collected and washed with 600 µl of 70% ethanol. The pellet was collected by centrifugation at 4°C at 8800 *g* for 2 min and dried at 37°C for 20 min, and resuspended in 50 µl of sterile TE buffer (10 mM Tris, 1 mM EDTA) or in nuclease-free water.

(b) Wizard® Plus Maxipreps

Plasmid DNA for nucleic acid sequencing, transformation of *D. nodosus* strains (Section 2.5.2) and for RT-qPCR experiments (Section 2.16) was prepared by using the Wizard® Plus Maxipreps DNA purification system (Promega). A single DH5α recombinant colony was inoculated into 10 ml of YT broth with the appropriate antibiotic and incubated overnight at 37°C. A 5 ml aliquot was transferred to 500 ml of YT broth with appropriate antibiotics added and incubated overnight at 37°C, then processed according to the manufacturer's instructions.

(c) Polyethylene glycol 6000 (PEG) purification of the plasmid for sequencing

An equal volume of PEG solution (30% PEG w/v and 1.6 M NaCl) was added to plasmid DNA (Section 2.3.2b) and incubated at 4°C for 30 min. The tube was centrifuged at 8800 *g* for 15 min at 4°C then the supernatant was removed. The pellet was washed by centrifugation at 8800 *g* for 2 min with 1 ml of 70% ethanol and dried at 37°C for 20 min. The DNA pellet was resuspended in 50 µl of sterile water and precipitated by adding 5 µl 3 M sodium acetate and 125 µl of absolute ethanol and then holding at -20°C for 30 min. The DNA was then centrifuged at 4°C at 8800 *g* for 15 min and the supernatant was discarded. The pellet was washed with 600 µl of 70% ethanol, centrifuged at 8800 *g* for 2 min, dried at 37°C for 20 min and finally resuspended in 20 µl of sterile water.

2.4. Construction of plasmids

PCR products which contained restriction enzyme sites suitable for cloning were digested with the appropriate restriction enzymes. The digested DNA was run on an agarose gel and stained with 0.05 µg/µl ethidium bromide. The DNA band of interest was cut out and eluted using a MinElute™ Kit (Qiagen). The plasmid pUC18 was digested with the appropriate restriction enzymes, run on an agarose gel and the appropriate band was cut out and eluted. The concentration of the PCR product (insert) and the pUC18 (vector) were estimated by agarose gel electrophoresis and then added in a molar ratio of 3:1 respectively, together with T4 DNA ligase, according to the manufacturer's instructions. The ligation mixtures were held for 8 – 12 hours at 4°C. Alternatively, PCR fragments were cloned directly into the pGEM-T-easy vector using the pGEM–T and pGEM–T Easy vector systems (Promega) according to the manufacturer's instructions. The ligation

mixes were transformed into competent $CaCl_2$-treated DH5α cells as per section 2.5.1 (Sambrook and Russell, 1989).

2.5. Bacterial transformation

2.5.1. Transformation of *E. coli* strain DH5α

The *E. coli* DH5α competent cells were prepared by treatment with calcium chloride (Sambrook and Russell, 1989). Competent cells were stored at -80°C. The competent cells were subsequently thawed on ice for 20 min, and gently resuspended using a micropipette. Two hundred micro litres of cells were added to 10 µl of ligation mix (Section 2.4) in a sterile microfuge tube. Five micro litre of TE buffer was used as a negative control and 10 ng of uncut pUC18 plasmid DNA as a positive control. The cells were held on ice for 30 min. The bacterial cells were heat shocked at 42°C for 90 seconds and then returned to ice. One ml of 2x YT broth was then added and the cells were incubated for one hour at 37°C. 50 µl of X-gal (20 mg/ml in N-N' - dimethyl-formamide) was spread on 2x YTA (2x YT with 100 µg/ml ampicillin) plates for the selection of blue and white colonies. The bacterial cells were centrifuged at 8800 *g* for 30 seconds. Nine hundred micro litre of the supernatant was removed and cells were resuspended in the remaining 200 µl of 2x YT broth. 50 µl and 100 µl of the culture were plated onto 2x YT plates with the appropriate antibiotics. The plates were incubated overnight at 37°C.

2.5.2. Transformation of *D. nodosus* strains

The *D. nodosus* strains were cultured on EYEB agar plates for 4 days under anaerobic conditions at 37°C. The cells were scraped from plates using 1 ml of EYE broth and

transferred to 1.5 ml microfuge tubes. After 1 min of centrifugation at 8800 *g*, the supernatant was discarded and the cells were resuspended in 1 ml of EYE broth and centrifuged again for 1 min at 8800 *g*. This step was repeated twice and the cells were resuspended into 500 µl of EYE broth and dispensed into 100 µl lots into 10 ml sterile plastic tubes. Between 1 and 25 µg of plasmid DNA was added to the 10 ml tube containing 100 µl of cell suspension. The cell suspensions were incubated under anaerobic conditions for 4 hours at 37°C with the lids open. Then 2 ml of EYE broth was added, and the cells were incubated for 22 hours anaerobically with the lids open. Each transformation mix was dispensed into two 1.5 ml microfuge tubes and centrifuged for 1 min at 8800 *g*. The supernatant was discarded and the cells were resuspended in 100 µl of EYE broth. 50 µl of cell suspension was then plated onto EYETB agar and EYET agar plates and incubated for 5 days at 37°C anaerobically. Individual colonies were plated onto new EYET agar plates (or an appropriate antibiotic resistant plate depending upon the marker carried by the plasmid) and incubated for 4 days anaerobically.

2.6. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out according to Sambrook and Russell (1989), using 1X TEAC (40 mM Tris, 1 mM EDTA, 20 mM acetic acid) buffer. λ DNA cut with *Hind*III was used as a molecular size marker.

2.7. Southern blotting

2.7.1. DNA Probe preparation

One micro gram of plasmid DNA containing the gene of interest was linearised with a restriction enzyme. The digested plasmid was extracted with phenol/chloroform/isoamyl alcohol, then precipitated with ethanol (Sambrook and Russell, 1989) and resuspended in distilled water. The DNA was boiled for 10 min, and then cooled immediately in an ice/water bath. The DNA was then labeled with DIG DNA labeling mix (1X DIG DNA labeling mix, 1x hexanucleotide mix, 2U Klenow enzyme, Roche), according to the manufacturer's instructions.

2.7.2. Southern blot preparation

The genomic DNA was digested with restriction enzymes, then run overnight at low voltage (40 – 55 volts) on a 1% agarose gel, stained with ethidium bromide and photographed. The gel was washed twice for 15 min in 100 ml of 0.25 M HCl and rinsed with 100 ml of distilled water for 2 min. The gel was then washed two times for 15 min with 100 ml of 0.5 M NaOH/1 M NaCl, rinsed with 100 ml distilled water for 2 min, and washed for 15 min in 100 ml of 0.5 M Tris-HCl/1.5 M NaCl, pH 7.4. After rinsing with 100 ml distilled water for 2 min, the DNA from the gel was transferred by the capillary transfer method (Southern, 1975) to a positively charged nylon membrane. After transfer the membrane was baked for 4 hours at 80°C.

The membrane was then prehybridised with 20 ml of hybridization solution [50% formamide, 5X SSC, 2% blocking stock (Blocking stock solution is 10% w/v blocking reagent in Buffer 1: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5), 0.1% sodium N-

laurylsarcosine, 0.02% SDS)] for 4 hours at 37°C. The probe was boiled for 10 min, then kept on ice for 5 min and added to the membrane with 20 ml of fresh hybridization solution and incubated for 6 hours at 37°C.

The membrane was then washed at 65°C twice with washing solution I (2 x SSC, 0.1% SDS) for 5 min and then twice with washing solution II (0.1 x SSC, 0.1% SDS) for 15 min also at 65°C. Then the membrane was washed with washing buffer (Buffer 1 with 0.3% Tween 20) at room temperature. The membrane was washed with Buffer 2 (10% blocking stock solution in Buffer 1) for 30 min at room temperature. The membrane was incubated with anti-DIG buffer (10 µl of anti-DIG-AP conjugate in 100 ml Buffer 2) for 30 min, and then washed twice with washing buffer for 15 min. The membrane was then washed with Buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 – 5 min. A 1% solution of 0.25 mM CSPD (Disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2'-(5'-chloro) tricycle [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate) was made up in Buffer 3 to a volume of 500 µl. After sealing in a plastic bag the diluted CSPD solution was spread over the membrane.

The sealed membrane was kept inside an X-Ray film cassette and incubated for 15 min at 37°C. The membrane was then exposed at various time intervals at room temperature using Fuji X-ray film and the film was subsequently developed. The probe DNA was removed from the membrane with 2 washes of 0.2 M NaOH, 0.1% SDS at 50°C for 20 min and the membrane was then stored in 2 X SSC at room temperature.

2.8. Design of primers for PCR and DNA sequencing

The oligonucleotide primers for PCR were designed using Primer3 software through the Australian Genomic Information Service. The oligonucleotide primers and probes for the real time PCR were designed using the PREMIER Biosoft Beacon Designer^{TM} software (Stratagene). These primers and probes were synthesized by GeneWorks.

2.9. DNA sequencing

DNA samples were sequenced by Newcastle DNA, University of Newcastle, Newcastle, Australia and Macquarie University, Sydney, Australia.

2.10. Polymerase chain reaction (PCR)

The amplification reaction contained 1 µmole of each oligonucleotide primer, 1.8 mM $MgCl_2$, 0.2 mM dNTPS, 1 unit of *Taq* DNA polymerase and approximately 1 – 5 ng of genomic DNA or 1 ng of plasmid DNA and 1X reaction buffer (6.7 mM Tris/HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.2 mg/ml gelatin and 0.45% Triton X-100).

The reaction mixtures were amplified using the PCR conditions shown below. The annealing temperatures were modified for specific PCR reactions.

	Program		
Step	**Temperature in degree Celsius**	**Time (min)**	**Cycles**
1	95	5	1
2	60	1.5	1
3	72	2	1
4	95	1	
5	60	1.5	
6	72	2	
7	Steps 4, 5 and 6 are repeated 34 times	-	-
8	72	10	1
9	4	HOLD	

2.11. Preparation of dialysis tubing

Dialysing tubing was cut into required lengths and boiled for 10 mins in 500 ml of 2% w/v $NaHCO_3$ and 1 mM EDTA, pH 8.0. Then the tubing was rinsed thoroughly in 200 ml of distilled water and boiled in 200 ml of distilled water for 10 min. The tubing was then cooled and stored in TES (20 mM Tris, 1.25 mM EDTA, 2.5 mM NaCl in 200 ml of distilled water and autoclaved) at 4°C.

2.12. PNPase assay

The PNPase assay was modified from that of Fontanella *et al.* (1999). *D. nodosus* cultures were grown on 16 EYEB agar plates for 4 days anaerobically. Cells were scraped into 5 ml EYE broth per plate, collected in 50 ml Sorvall centrifuge tubes and centrifuged at 9000 *g* for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in one ml of 50 mM Tris-HCl, pH 7.5 and transferred to a preweighed 1.5 ml microfuge tube. The cells were then centrifuged at 8800 *g* for 30 sec at 4°C. The supernatant was discarded and the cells were washed twice with 50 mM Tris-HCl, pH 7.5 and centrifuged at 8800 *g* for 30 sec at 4°C. The supernatant was completely removed and the wet weight of the cells was determined. The cells were then resuspended in 500 µl of 50 mM Tris-HCl, pH 7.5 and 100 µl aliquots were placed in fresh microfuge tubes. For each 150 mg of cell pellet, 1 g of acid-washed glass beads (212 – 300 µm, from Sigma) was added. The cells were disrupted by vigorously shaking for 5 x 1 min periods at 4°C, with an idle interval of 1 min in between on ice. The homogenates were incubated with 6 units of bovine pancreas DNase for 10 min at 37°C and centrifuged at 8800 *g* for 20 min at 4°C.

Supernatants were then extensively dialyzed against 50 mM Tris-HCl, pH 7.4, dispensed and stored at -20°C. The protein content was assayed using the Coomassie Plus assay (Pierce), using BSA (Bovine serum albumin) as a standard, according to the manufacturer's instructions. The control PNPase assay was performed using commercial PNPase enzyme at various concentrations. The total volume of the assay mixture was 1.5 ml which contained 50 mM Tris-HCl, pH 7.4, 0.1 M KCl, 5 mM $MgCl_2$, 20 µg/ml poly(A), 1.5 mM phospho*enol*pyruvate, 20 mM glucose, 0.5 mM NAD^+, 0.6 U/ml pyruvate kinase, 2 U/ml hexokinase and 4 U/ml glucose-6-phosphate dehydrogenase. 1 mg to 10 mg of crude protein was used in the PNPase assay.

The PNPase assay was carried out at 37°C for 35 min and the absorbance at 340 nm was read in a Cary 50 spectrophotometer using the enzyme kinetics application from Cary WinUV software (Varian). Pnpase, activity was calculated by subtracting A_{340} at 10 min from A_{340} at 40 minutes, divided by 30 (time) and then converted to umol/min/mg of NADH using the molar extinction coefficient of NADH.

2.13. *D. nodosus* protease thermostability assay

The *D. nodosus* strains were grown on EYE agar for two days anaerobically at 37°C. Then 5 ml of EYE broth was added to the culture plates, and they were incubated anaerobically for another two days at 37°C. On the fourth day the liquid was collected from the plates in 10 ml tubes, and centrifuged at 1700 *g* for 10 min. One ml of the supernatant was added to a sterile 1.5 ml microfuge tube and stored at -20°C. The rest of the supernatant was collected in a 10 ml sterile tube and stored at -20°C. A Gram stain was performed on the cell pellet to check for contaminants.

For the protease thermostability assay, the supernatant was thawed in cold water and 0.6 ml volumes of supernatant were dispensed into 1.5 ml microfuge tubes. Tubes were incubated in duplicate at 65°C for 10 min and 20 min. Control tubes were held on ice. After the incubation, the tubes were transferred to ice cold water immediately. Half a ml of the treated supernatant was added to tubes containing 6 mg of hide-powder azure and 0.5 ml protease assay buffer (10 mM HEPES, 2 mM Zwittergent 3-14, 30 mM calcium chloride, pH 8.5). The tubes were then vortexed to mix the supernatant with the hide-powder azure completely and incubated at 37°C in a shaking water bath for 30 min. The tubes were transferred to ice cold water immediately and centrifuged at 4°C at 8800 *g* for 15 min. The supernatants were transferred to fresh 1.5 ml microfuge tubes and kept on ice. A negative control was also performed which contained hide-powder azure, protease assay buffer and EYE broth. The absorbance of the supernatants at 595 nm was estimated using a Cary50 spectrophotometer, and analysed using the simple read application from the Cary WinUV software.

2.14. Twitching motility assay

D. nodosus strains were subcultured from EYE agar plates onto TAS agar plates (1.5% tryptone, 0.5% protease peptone, 0.2% yeast extract, 0.5% lab-lemco powder, 0.5% L-arginine, 0.15% DL serine, 0.2% $MgSO_4.7H_2O$, 1.5% agar), and incubated anaerobically for 4 days. The TAS agar cultures were then used to stab-inoculate fresh TAS agar plates with *D. nodosus* cells using a metal wire. The stab-inoculated cultures were incubated anaerobically for 4 days. Brilliant blue-R dye [0.25% (w/v) brilliant blue R, 40% (v/v) methanol and 7% (v/v) acetic acid)] was then layered over the TAS agar plate to stain the protein. The plates were incubated for 30 min, then treated with destaining solution (10% acetic acid, 40% methanol, 50% water) until the blue background disappeared. Using a

dry tissue, the surface of the plate was gently pressed to remove residual moisture and the zone size was measured and photographed.

2.15. RNA isolation and RNA gels

2.15.1. RNA isolation and analysis

D. nodosus strains were grown on four EYE agar plates for four days anaerobically. The bacterial cells were scraped from each plate into 1.5 ml of EYE broth and transferred to sterile nuclease free 1.5 ml microfuge tubes and centrifuged for 1 min at 8800 *g* at room temperature. The supernatant was discarded and the cells were resuspended into 1 ml of EYE broth, pooled in one tube and centrifuged for 30 sec at 8800 *g*. Then the RNA was isolated according to the manufacturer's instructions using the RiboPure^{TM} – Bacteria kit (Ambion). The isolated RNA was dispensed into nuclease free 1.5 ml microfuge tubes and stored at -80°C. Volumes of 3 and 6 µl of the RNA were analysed using an RNA gel (Section 2.15.2). The concentration of the RNA was determined spectrophotometrically using 2 µl of the RNA on the Nanodrop 1000 (NanoDrop Technologies). The RNA was then diluted with nuclease free water as required, and real time PCR (Section 2.16) was then used to estimate the messenger RNA level of the gene of interest.

2.15.2. RNA gels

A 1% agarose gel with 0.1 M sodium ortho phosphate (1% agarose, 0.01 M Na_2PO_4 pH 7.0, 20% formaldehyde) was prepared. 0.01 M Na_2PO_4, pH 7.0 running buffer was used. The samples were prepared by adding two volumes of denaturation mix (60% formaldehyde in 0.02 M Na_2PO_4 pH 7.0,) to one volume of RNA, denatured by heating at

65°C for 10 min and placed on ice. Gel loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 50% glycerol and 0.05 µg/µl ethidium bromide) was prepared. 1:5 v/v gel loading dye was added to the sample and loaded onto the gel. The gel was run at 50 mA for 3 hours, then observed under UV-light and photographed.

2.16. Real time PCR

2.16.1. Construction of standard plasmids and calculation of plasmid copy number

The standard plasmids for the real time PCR were constructed (Section 2.4) by inserting the gene of interest into the pGEM®-T easy vector or pUC18. The plasmids were linearised at different dilutions and the concentration of the plasmid was estimated visually by comparing the brightness of the plasmid band with the lambda *Hind*III standard bands on a 1% agarose gel. The numbers of plasmid molecules were calculated by applying the following standard formula:

$$\text{Molecules of DNA} = \frac{\text{Mass (grams)} \times \text{Avogadro's number}}{\text{Average mol. weight of base pair} \times \text{template length}}$$

The standard curve for quantitative PCR was performed by diluting the plasmid copy number from 10^6 to 10^{-1} molecules per reaction in a ten fold dilution series.

2.16.2. Real time PCR conditions

The 2X reaction mix and SuperScript III RT/ Platinum Taq Mix were supplied from Invitrogen (SuperScipt™ One-step RT-PCR with Platinum®).

Components	Concentration
2X Reaction Mix	1X
Forward Primer	10 µM
Reverse Primer	10 µM
Flurogenic Probe	10 µM
SuperScript III RT/ Platinum Taq Mix	0.5 µl
Template (RNA)	1 pg to 1 ng
Total of 25 µl of reaction made up with milli-Que water	

The absence of DNA in RNA samples was confirmed by substituting SuperScript III RT/ Platinum Taq Mix with the required concentration of Taq DNA polymerase (Promega).

The reaction was performed under the conditions given below in a Cobertt 3000 rotorgene (Corbett Life Science).

	Program		
Step	**Temperature (°C)**	**Time (min)**	**Cycles**
1	50	15	1
2	95	2	1
3	95	15(sec)	45
4	60	30(sec)	45
5	25	5	1
	Step 3 and 4 alternate for 45 cycles		

The results were analysed using the Excel algorithm provided by Cobertt Life Science.