CHAPTER 2

Materials and Methods

Never say, "I tried it once and it did not work." Lord Ernest Rutherford (1871-1937)

2.1 Sources of biochemical and chemical materials

All chemicals were of analytical grade and purchased from the suppliers or manufacturers indicated in Table 2.1 below.

Table 2.1: Sources of chemicals

Biochemical or chemical	*Supplier/Manufacturer*
acrylamide	BDH
agarose	Promega
amberlite MB-1 resin	Bio-Rad
ampicillin	Boehringer Mannheim
ammonium persulfate	ICN Biomedicals Inc.
bactoagar	Difco
bis-acrylamide	BDH
chloroform	BDH
DIG kit	Boehringer Mannheim
Erase-a-base® kit	Promega
ethanol	BDH
ethylenediamine disodium salt	Ajax Chemicals
gelatin	Ajax Chemicals
GeneClean™ II kit	Bio 101 Inc.
glucose	BDH
glycerol	BDH
hydrochloric acid	BDH
isopropanol	BDH
lambda standard DNA	Promega
lysozyme	Boehringer Mannheim
magnesium acetate	Ajax Chemicals
magnesium chloride	BDH

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magnesium sulfate	BDH
maltose	BDH
polyethyleneglycol 8000	BDH
phenol	BDH
potassium acetate	Ajax Chemicals
Promega T7 sequencing system	Promega
restriction endonucleases	Promega, New England Biolabs, Boehringer Mannheim
RNase A	Sigma
[α-^{35}S]dATP	Dupont
sodium acetate	Ajax Chemicals
sodium chloride	BDH
sodium citrate	BDH
sodium dodecyl sulfate	BDH
sucrose	BDH
Super-Base Sequencing Kit	Bresatec
T4 ligase	Promega
TEMED	Bio-Rad
Tris-base	Boehringer Mannheim
Triton-X100	Sigma
tryptone	Oxoid
Tween 20	ICN Biomedicals Inc.
urea (ultra pure)	ICN Biomedicals Inc.
X-gal	Research Organic Inc. or Norbiochem
yeast extract	Oxoid

2.2 Bacterial strains and media

2.2.1 *Dichelobacter nodosus* strains and media

The *D. nodosus* benign strains 819, 1169, and 2483, and virulent strain 1311, were provided by Dr J. Searson (Regional Veterinary Laboratory, NSW Agriculture, Wagga, New South Wales, Australia). Virulent strains A198, B1006, G1220, H1215, D1172, intermediate strain AC3577 and benign strains C305, H1204, AC390, 1493, 3138, 1469 were provided by Dr J. I. Rood (Department of Microbiology, Monash University, Clayton,

Victoria, Australia). *D. nodosus* strain 1311A was derived from parent strain 1311 during routine laboratory growth in this work. All *D. nodosus* strains were cultured on Blood Eugon agar (45 mg/ml Eugonagar, 2 mg/ml yeast extract, 5% (v/v) defibrinated horseblood) or in Eugonbroth (30 mg/ml Eugonbroth powder, 2 mg/ml yeast extract) (Skerman, 1974) 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.

D. nodosus stocks were obtained from plate cultures. 1 ml of Eugonbroth with 20% glycerol was added to a plate of *D. nodosus* cells and resuspended in the Eugon-glycerol by gently scraping cells from the plate with a sterile glass spreader. 200 µl aliquots of the cell suspension were transferred to a sterile microfuge tube and stored at −80°C.

2.2.2 *Escherichia coli* strains and media

E. coli strain DH5-α [F^-, Φ80d*lacZ*Δ M15Δ, (*lacZYZ-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17*, *supE44*, λ-, *thi-1*, *gyrA96*, *relA1*; Bethseda Research Laboratories] was used as a host for recombinant plasmids in both transformations by electroporation (Dower *et al*, 1988) or by the $CaCl_2$ method (Sambrook *et al*, 1989). DH5-α derivatives were cultured at 37°C overnight on 2 × YTA plates (16 mg/ml tryptone, 5 mg/ml yeast extract, 0.86 M NaCl, 15 mg/ml Bactoagar, 100 µg/ml ampicillin) or in 2 × YT medium (16 mg/ml tryptone, 5 mg/ml yeast extract, 0.86 M NaCl; Sambrook *et al*, 1989) supplemented with 100 µg/ml ampicillin (2 × YTA).

E. coli strain LE392 [F^-, *hsdR574* (r_K^-, m_K^+), *supE44*, *supF58*, *lacY1* or Δ (*lacIZY*)6, *galK2*, *galT22*, *metB1*, *trpR55*] (Murray *et al*, 1977) was used as a host for recombinant lambda bacteriophage. LE392 cells were cultured at 37°C overnight in LB broth (10 mg/ml tryptone, 85.6 mM NaCl, 50 mg/ml yeast extract, 1% (w/v) maltose) supplemented with 1% maltose (Sambrook *et al*, 1989).

Bacterial stocks (500 µl) were stored at –20°C in 20% glycerol, or at –70°C in 7% DMSO. All bacterial growth media were autoclaved at 121°C for 20 minutes prior to use.

2.3 Preparation of DNA

2.3.1 Preparation of *D. nodosus* genomic DNA

D. nodosus genomic DNA was prepared from 150 ml liquid cultures. 1 ml of Eugonbroth was added to a plate culture of *D. nodosus*, and cells were resuspended in the Eugonbroth by gently scraping the bacteria off the solid medium using a sterile glass spreader. Using a sterile pipette, the 1 ml cell resuspension was then transferred into 150 ml of Eugonbroth and grown under anaerobic conditions (Section 2.2.1) at 37°C for 48 to 72 hours, depending upon the strain of *D. nodosus*. *D. nodosus* genomic DNA was prepared using the method described by Anderson *et al* (1984).

2.3.2 Preparation of Plasmid DNA

(i) Alkaline lysis plasmid preparation

Plasmid DNA for nucleic acid sequencing (Section 2.7) was prepared by using a modified version of the alkaline lysis method described by Birnboim and Doly (1979). A 10 ml overnight culture of DH5-α cells carrying the recombinant plasmid was centrifuged for 10 mins at 1 500 *g* in an MSE benchtop centrifuge. To lyse the cells and remove RNA, the pellet was resuspended in 300 µl of Lysozyme/RNase A (100 mM glucose, 50 mM Tris, pH 8.0 and 20 mM EDTA, 2 mg/ml Lysozyme, 50 µg/ml RNase A) and stored on ice for 30 mins.

To denature chromosomal DNA 600 µl of an alkaline SDS solution (0.2 M NaOH, 1% SDS) was mixed with the suspension, and incubated on ice for 5 mins. To precipitate cell debris, protein and chromosomal DNA, 450 µl of cold 5 M KAc was added, and the

sample left on ice for a further 5 mins.

Cell debris, protein and precipitated chromosomal DNA were removed by centrifuging the suspension and then the supernatant at 12 000 *g* in a microfuge (Clements Orbital 100) for 10 mins. To precipitate the plasmid DNA, 2 × (v/v) of 100% ethanol at room temperature was added, and left to incubate for 5 mins at room temperature. The plasmid DNA was collected *via* centrifugation at 12 000 *g* for 15 mins at 4°C, dried under vacuum, and resuspended in 20 µl of TE buffer (10 mM Tris base, pH 8.0 & 0.25 mM Na_2EDTA, pH 8.0). To remove any remaining contaminants, the 20 µl sample was extracted twice with phenol-chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol alone (Section 2.4.2). For preparation of pBR322 DNA, plasmid DNA was amplified prior to extraction using chloramphenicol (Sambrook, Fritsch & Maniatis, 1989).

(ii) Holmes & Quigley plasmid preparation - large scale

To prepare plasmid DNA for probe preparation (Sections 2.8.2 & 2.8.3), subcloning or screening of transformants (Section 2.6.6), a method modified from that described by Holmes and Quigley (1981) was used.

DH5-α recombinant cells from a 10 ml overnight culture were centrifuged for 10 mins at 1 500 *g* in an MSE benchtop centrifuge. To remove chromosomal DNA, proteins and cellular debris, the pellet was resuspended in 500 µl of STET (50 mM Tris pH 8.0, 50 mM EDTA, 8% sucrose, 5% Triton-X100), after which 40 µl of Lysozyme (10 mg/ml in 50 mM Tris pH 8.0) was added. The sample was then boiled for 90 secs, cooled on ice, and centrifuged (Clements Orbital 100) at 12 000 *g* for 10 mins. The resulting gelatinous precipitate was discarded and the plasmid DNA precipitated by adding 500 µl of isopropanol to the supernatant, which was subsequently held at -20°C. After 20 mins the plasmid DNA was collected by centrifugation at 12 000 *g* for 10 mins, washed with 70% (v/v) ethanol, dried under vacuum, and resuspended in 100 µl of TE buffer.

(iii) Holmes & Quigley plasmid preparation - small scale

The small-scale plasmid preparation was employed to rapidly screen large numbers of possible transformants, and was adapted from the method described by Holmes and Quigley (1981).

Putative transformants were streaked onto 2 × YTA plates and incubated at 37°C overnight. Streaks were then transferred to microfuge tubes and resuspended in 50 µl of STET (50 mM Tris pH 8.0, 50 mM EDTA, 8% sucrose, 5% Triton-X100) and 4 µl of lysozyme (10 mg/ml in 50 mM Tris pH 8.0), boiled for 90 secs, cooled briefly on ice and then centrifuged (Clements Orbital 100) at 12000 *g* for 10 minutes, after which the chromosomal DNA, proteins and cellular debris were removed as a gelatinous precipitate. To precipitate the plasmids, 50 µl of isopropanol was added to the remaining supernatant, which was then held at -20°C for 20 mins, after which the plasmid DNA was collected by centrifuging at 12000 *g* for 20 mins. Plasmid DNA was washed with 70% (v/v) ethanol, dried under vacuum, and resuspended in 30 µl of TE buffer.

2.3.3 Liquid method - lambda DNA preparation

A culture of lambda sensitive *E. coli* strain LE392 (Section 2.2.2) was grown in LB broth overnight from which a 0.5 ml aliquot was transferred to a fresh 50 ml LB broth and incubated at 37°C until the optical density at 600 nm was 0.4. LE392 cells were collected by centrifugation (Beckman J2-21 with JA-20 rotor) at 6000 *g* at 4°C for 5 mins.

Lambda DNA was then titrated (Sambrook *et al.*, 1989) to determine the dilution of lambda stock required for confluent lysis on L & G plates (10 mg/ml tryptone, 5 mg/ml yeast extract, 2 mg/ml glucose, 85.6 mM NaCl, 10.0 mM Tris, 1 mM $MgCl_2$, 1.5% (w/v) bactoagar), which was then used for lambda DNA preparation. DNA from recombinant bacteriophage lambda was prepared by the method modified from a protocol described by Promega (Promega, 1996).

A 50 ml overnight culture of *E. coli* strain LE392 was centrifuged as before and the pellet was resuspended in 10 ml of 10 mM $MgSO_4$. After lambda titration, three plaques were selected and suspended in 200 µl of λ D.I. (10 mM $MgSO_4$, 10 mM Tris pH 7.5) and incubated at room temperature for 2 hrs, after which 400 µl of resuspended LE392 was added. The 600 µl phage/*E. coli* suspension was incubated for 60 mins at 37°C, added to 45 ml of L & G medium prewarmed to 37°C, and then incubated at 37°C in a shaking water bath overnight.

After lysis was complete, 800 µl of chloroform was added and shaking continued for 30 mins, then the suspension was centrifuged (Beckman J2-21 with JA-20 rotor) at 20 000 *g* at 4°C for 20 mins. The supernatant was transferred to ultracentrifuge tubes and centrifuged (Beckman L8-70M Ultracentrifuge with 60 Ti rotor) for 2 hrs at 50 000 *g*. The pellet was resuspended in 400 µl of SM solution (17 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris pH 7.5, 0.01% (w/v) gelatin), and 10 µl RNase A (1 mg/ml), 10 µl 10% SDS and 50 µl of 2 M Tris /200 mM EDTA, pH 8.0 were added. After 30 mins incubation at 65°C, 50 µl of 5 M KAc was added, and the suspension was incubated on ice for 30 mins, then centrifuged at 12 000 *g* for 10 mins, after which the supernantant was extracted with phenol once, phenol and chloroform/isoamyl alcohol twice, and once with chloroform/isoamyl alcohol before precipitation with ethanol (see Section 2.5.2).

2.4 Preparation of RNA

2.4.1 Preparation of *D. nodosus* total RNA using RNAzol™B

D. nodosus liquid cultures were inoculated with a 1 ml cell resuspension which was subsequently transferred into 150 ml and grown under anaerobic conditions (Section 2.2.1) at 37°C for 48 hours. Cells were collected by centrifugation (Beckman J2-21 with JA-14 rotor) at 15 000 *g* at 4°C for 10 mins. Cells were then washed in 10 ml TE buffer and

centrifuged for 10 mins at 1 500 *g* in an MSE benchtop centrifuge. The 100 mg pellets from each 150 ml culture were resuspended in 2 ml RNAzol™B (Bresatec) and transferred to two pre-chilled, sterile microfuge tubes. 100 µl of chloroform was added to each microfuge tube, vortexed briefly and stored on ice for 5 mins prior to centrifugation (Clements Orbital 100) at 16 000 *g* for 15 mins at 4°C. The upper aqueous phase was transferred to a sterile, pre-chilled microfuge tube, and 500 µl of isopropanol added. The sample was then vortexed for 15 sec and stored on ice at 4°C overnight in order to precipitate the RNA. The RNA was collected by centrifugation at 16 000 *g* for 15 mins at 4°C and washed with 0.8 ml of 75% ethanol at –20°C. Pellets were resuspended in TE buffer by vortexing, and quantified by UV spectroscopy and integrity determined by visualisation using agarose gel electrophoresis under denaturing conditions (Farrell, 1993).

2.5 DNA Purification

2.5.1 GeneClean™

The GeneClean™ Kit II (Bio 101) was utilised to purify DNA fragments from agarose gels in preparation for subcloning. Purification was performed according to the protocol recommended by the manufacturer of the kit. After agarose gel electrophoresis (Section 2.7), DNA fragments were excised from the agarose gel and double-stranded DNA was selectively bound to a silica matrix. Impurities remained in solution and were eliminated by repeated washing.

2.5.2 Phenol chloroform extraction and ethanol precipitation

The procedure for phenol and chloroform extraction and ethanol precipitation of DNA was modified from the method described by Sambrook *et al* (1989), and was utilised to remove contaminating proteins from DNA preparations or digests.

The DNA sample to be extracted was made up to 100 µl with TE buffer, and a half-volume of phenol and a half-volume of chloroform/isoamyl alcohol (24:1 v/v) were added. The sample was vortexed and centrifuged (Clements Orbital 100) at 12 000 *g* for 5 mins. To the supernatant, an equal volume of chloroform/isoamyl alcohol was added, mixed and again subjected to centrifugation at 12 000 *g* for 1 min. The supernatant was collected and a one-tenth volume of 3 M sodium acetate pH 4.5 and 2.5 volumes of 100% ethanol added, and then held at -20°C for 20 mins to precipitate the DNA. The DNA was then collected by centrifugation at 12 000 *g* and 4°C for 10 mins. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in the desired volume of TE buffer.

2.5.3 CTAB (Cetyltrimethylammonium bromide) extraction

CTAB precipitation solution (1% (w/v) CTAB, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0) was used to remove cell wall debris and polysaccharides from *D. nodosus* genomic DNA, using the method of Ausbel, 1989.

2.6 DNA Cloning

The general molecular techniques employed in cloning and analysis of DNA are as described by Sambrook *et al* (1989). Both the DNA fragment to be cloned and the vector were digested with the appropriate restriction enzymes to generate compatible ends for cloning (Section 2.6.1), ligated (Section 2.6.5), and used to transform *E. coli* strain DH5-α (Section 2.6.6), after which possible transformants were screened by agarose gel electrophoresis (Section 2.7) and restriction enzyme digestion.

2.6.1 Restriction enzyme digests

Restriction endonucleases were supplied by Promega, Boehringer Mannheim, and New England Biolabs. The conditions chosen for restriction enzyme digests were dependent upon the amount of DNA in the sample, the optimal salt concentration and temperature for the activity of a given restriction enzyme as indicated by the tables supplied by the manufacturer.

A typical digest of 1 µg of DNA contained 2 µl of 10 × TA buffer (330 mM Tris, pH 7.9, 660 mM potassium acetate, 100 mM magnesium acetate, 1 mg/ml gelatin), 1 µl of restriction enzyme, the optimal concentration of NaCl and made up to a total volume of 20 µl with MilliQ water. For lambda DNA digests or genomic DNA digests 1 µl spermidine (5 mM) was also added to optimise digestion. The digests were allowed to proceed for 2 hrs for plasmid DNA, 4 hrs for lambda DNA, and 8 to 16 hrs for genomic DNA, at a temperature appropriate for the restriction enzyme employed.

Where multiple restriction enzymes were employed, optimal salt and temperature requirements for each enzyme to be used were considered and where these conditions were not compatible, the enzyme with activity at the lower salt concentration or lower temperature was added to the DNA first and, when the digestion by the first enzyme was complete, the buffer and/or temperature was modified and the second enzyme was then added.

2.6.2 Subcloning - *via* shotgun cloning

Shotgun cloning was employed where no useful 6 bp restriction enzyme sites in appropriate positions were available for subcloning a given insert, or where restriction enzyme sites were unknown. Digests of either a DNA fragment purified using the GeneClean™ II Kit (Section 2.5.1) with 4 bp restriction enzymes generated a number of smaller fragments which were subsequently extracted with phenol, and precipitated with ethanol and then ligated to compatible termini in the pUC18 polylinker. Recombinant

pUC18 was then used to transform *E. coli* strain DH5-α.

2.6.3 Cloning PCR products

(i) Of Taq DNA polymerase amplified products

PCR products amplified using Taq DNA polymerase were purified using the Wizard Magic PCR Product Purification kit (Promega) or gel purified (Section 2.5.1). The purified products were subjected to digestion with the Klenow fragment of *E. coli* DNA polymerase I to remove the dATP 3'-overhangs generated by the template-independent polymerase activity of Taq DNA polymerase; phosphorylated with T4 polynucleotide kinase, and cloned into dephosphorylated *Sma*I-digested pUC18 using the SureClone™ Ligation Kit (Pharmacia P-L Biochemicals) according to the manufacturers instructions.

(ii) Of Deep $Vent_R$ polymerase amplified products

PCR products amplified using Deep $Vent_R$ DNA polymerase (New England Biolabs) were purified using the Wizard Magic PCR Product Purification kit (Promega) or gel purified (Section 2.5.1). Unlike Taq DNA polymerase, Deep $Vent_R$ DNA polymerase generates products that predominantly (95%) have blunt-ends, and thus after purification fragments were cloned directly as described (Section 2.6.5).

2.6.4 Blunt-ending of restriction fragments

T4 DNA polymerase (Promega) was used to fill in 5'-protruding ends with dNTPs or, *via* 3'to 5' exonuclease activity, to convert a 3'-protruding end to a blunt end. For either reaction 0.2-5 µg of a purified restriction fragment was resuspended in T4 DNA polymerase buffer, containing 100 mM of each dNTP, 0.1 mg/ml acetylated BSA and 5 units of T4 DNA polymerase per microgram of DNA. The reaction was incubated at 37°C for 5 mins before the reaction was stopped by heating at 75°C for 15 mins (Promega, 1996). After

phenol extraction, ethanol precipitation, and resuspension in an appropriate volume, the fragments were used for subcloning.

2.6.5 Ligation of vector and insert

Foreign DNA fragments were cloned into a pUC18 plasmid (Yanisch-Perron *et al*, 1985), a small high-copy number *E. coli* plasmid cloning vector. pUC18 carries a 54 bp multiple cloning site polylinker within the *lacZ* gene, which is disrupted by the insertion of foreign DNA fragments; on selective media containing X-gal, disruption of the *lacZ* gene results in the production of white colonies rather than blue colonies. In addition, pUC18 carries an Amp^R marker which was also used for selection of pUC18-containing colonies.

For ligation reactions, a molar ratio for insert to vector of 3:1 was maintained; 1 µl of ligase buffer (Promega) and 1.5 U or 3 U of T4 DNA ligase (Promega) were added for cohesive or blunt-ended DNA, respectively. Ligation mixtures were made up to 10 µl with TE and ligation reactions were incubated at room temperature (~15-25°C) for 4 hrs.

2.6.6 Bacterial Transformation

(i) Transformation of *E. coli* strain DH5-α $CaCl_2$ competent cells

The method outlined by Sambrook *et al* (1989) was modified for transformation of *E. coli* strain DH5-α cells with pUC18 recombinant plasmids. Competent cells were prepared using the $CaCl_2$ method (Sambrook *et al*, 1989), and stored at –70°C.

Five µl of ligation mix was added to 100 µl of competent cells and, after being held on ice for 30 minutes, the competent cells were heat-shocked at 37°C for 2 mins, returned to ice, 1 ml of 2 × YT broth added and then incubated at 37°C. After 60 mins, the cells were collected by centrifugation (Clements Orbital 100) at 3 500 *g* for 1 min. The soft pellet was resuspended in 100 µl of 2 × YT broth, plated on YTA + X-gal (1 mg/20ml) plate and

incubated at 37°C overnight, before screening for transformants.

(ii) Transformation of *E. coli* strain DH5-α by electroporation

The method outlined by Dower *et al* (1988) was modified for transformation of *E. coli* strain DH5-α cells with pUC18 recombinant plasmids. Competent cells were prepared using the method for electroporation competent cells described by Dower *et al* (1989) and stored at −70°C.

One µl of ligation mix was added to 40 µl of electroporation competent cells and chilled to 4°C. The cells were electroporated in 0.2 cm cuvettes with a 2.5 kV pulse from a 25 µF capacitator with a by-pass resistance of 200 Ω using a BioRad Pulser® *or E. coli* Pulser™ and then transferred to 1 ml of 2 × YT broth. The culture was incubated at 37°C for 60 mins and the cells were collected by centrifugation (Clements Orbital 100) at 3 500 *g* for 1 min. The soft pellet was resuspended in 100 µl of 2 × YT broth, plated on 2 × YTA + X-gal media and incubated at 37°C overnight, before screening for transformants.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was routinely employed to separate DNA fragments ranging from 0.1 kb to 20.0 kb. The appropriate agarose concentrations used to resolve DNA fragments within a given size range are summarised in Table 2.2 (Sambrook *et al*, 1989).

Agarose (%w/v)	Effective range of resolution of linear DNA fragments (kb)
0.5	30 to 1.0
0.8	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

Table 2.2:
Appropriate Agarose Concentrations for Separating DNA Fragments of Various Sizes.

Agarose was made up to the appropriate volume (40 ml, 100 ml, or 200 ml) with Tris acetate gel buffer (40 mM Tris base; 2 mM EDTA pH 7.6), boiled until dissolved, cooled to 55°C, poured into a gel mould, and allowed to set for 30 mins.

DNA samples were prepared by adding 10% (v/v) loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) to the sample; where fragments less than 0.5 kb were to be visualized 1% (v/v) RNase A was also added (0.01% RNase A, 0.01 M Tris pH 7.5, 0.015 M NaCl).

Bio-Rad and Pharmacia Mini-Sub gels (7 cm × 10 cm or 15 cm × 10 cm) were run at a constant 100 V for 1 hr, whilst larger Sub-Cell gels were run at a constant 35 V for 16 hrs. The DNA samples were visualised by staining the agarose gel in an ethidium bromide (1 µg/ml) and TEAC solution for 20 mins, rinsing in water and illuminating under short-wave UV light (302 nm). The gels were photographed with an MP-4 Land camera (Polaroid) using Polaroid 667 film, and the size of sample DNA fragments determined using lambda DNA cut with *HindIII* as standard size markers (yielding fragment sizes: 23.1 kb, 9.42 kb, 6.56 kb, 4.36 kb, 2.32 kb, 2.03 kb, 0.56 kb and 0.12 kb) and migration of the samples through the gel, which is inversely proportional to the logarithm of the size of the DNA fragment (Ausbel *et al*, 1989).

2.8 Southern Blotting

2.8.1 Electrophoresis and membrane transfer

D. nodosus genomic DNA (1-2 µg) was digested with restriction enzyme/s overnight. The digested DNA was electrophoretically size-fractionated on a 1% (15 cm × 20 cm) agarose gel (Bio-Rad) at a constant 35 V for 16 hrs. Pretreatment of the DNA involved partial hydrolysis using 0.25 M HCl for 30 mins, followed by denaturation by soaking in 0.5 M NaOH/1 M NaCl for 30 mins, and neutralisation in 0.5 M Tris-HCl/1.5 M

NaCl for a further 30 mins. The DNA was then transferred to a Hybond nylon membrane (Amersham or Boehringer-Mannheim) or Nitrocellulose membrane (Schleicher & Schuell) *via* capillary action using the method described by Southern (1975). After blotting for 4 hrs, the nylon membrane was baked at 65°C overn ght to fix the DNA to the membrane.

2.8.2 Non-radioactive DIG labelling and detection of DNA probes

Probes used in Southern Blots were prepared using the DIG Kit (Boehringer Mannheim), a non-radioactive labelling and detection kit, and the protocol of the manufacturer. The method involved random-primed DNA synthesis from the linearised probe template with the incorporation of digoxigenin-labelled deoxyuridine-triphosphate (DIG-dUTP). Anti-DIG alkaline phosphatase-conjugated antibodies form complexes with the hybridised DIG-labelled probe which, upon the addition of the chemiluminescent substrate, Lumogen-AMPDD, results in the release of light which is detected on X-ray film.

To prepare DIG-dUTP labelled probes, 1 µg of recombinant plasmid DNA was linearised using the appropriate restriction enzyme (Section 2.6.1), extracted with phenol, precipitated with ethanol (Section 2.5.2) and then resuspended in 17 µl of MilliQ water. The DNA was boiled at 95°C for 10 mins, cooled on ice, then 2 µl hexanucleotide mix, 2 µl dNTP labelling mix and 1 µl Klenow enzyme were added, after which the 22 µl sample was incubated at 37°C for 20 hrs. The reaction was stopped by the addition of 0.25 M EDTA pH 8.0, and the labelled DNA was precipitated by the addition of 9% (v/v) of 4 M LiCl and 60 µl of ethanol at −20°C. The sample was then incubated at −70°C for 30 mins and centrifuged (Clements Orbital 100) at 12 000 *g* for 15 mins to collect the DNA. The pellet was subsequently washed with 70% (v/v) ethanol, dried under vacuum, resuspended in 50 µl of TE buffer and stored at −20°C until use (Section 2.8.4).

2.8.3 Radioactive ^{32}P-dNTP labelling of DNA probes

Radiolabelled DNA probes were utilised in both Southern blot and northern blot experiments. The Prime-a-gene labelling kit (Promega) was used to label 50 ng of DNA template with 300 Ci/mmol of [α-^{32}P]-dATP or [γ-^{32}P]-dATP in accordance with the manufacturer's instructions. Following the labelling reactions unincorporated nucleotides were removed from the labelled probe using Sephadex G-50 columns. A hole was punched in the bottom of a 0.5 ml microfuge tube, which was then plugged with silanised glass wool (Ausubel *et al*, 1985). The 0.5 ml microfuge tube was then transferred to a 1.5 ml microfuge tube, and 0.6 ml of Sephadex G-50 (2.5% (w/v) Sephadex G-50, 20 mM Spun Column Stop Buffer [10 mM Tris-HCl, 1 mM EDTA, 0.2% SDS]) was added and then centrifuged at 3 500 *g* (Clements Orbital 100) for 2 to 3 seconds. The column was subsequently washed with spun-column stop buffer, and centrifuged at 3 500 g (Clements Orbital 100). The radiolabelled probe mixture was then added to the top of the G-50 column before centrifugation for 2 to 3 seconds. The eluent contains labelled probe molecules whilst unincorporated nucleotides remain in the column, which is discarded. Percent incorporation was determined by comparing the counts per second of the eluent to the column.

2.8.4 Hybridisation and detection of DIG-labelled probes

Membrane-bound DNA was prehybridised with 2.5 ml of hybridisation solution (50% formamide, 5 × SSC diluted from 20 × SSC stock [0.3 M sodium citrate, 3 M sodium chloride], 2% blocking reagent, 0.1% sodium laurylsarcosine, 0.02% SDS) per 10 cm^2 of membrane at 37°C for 8 hrs. The membrane was then hybridised with 10 µl of probe under high stringency conditions and using 1 ml hybridisation solution per 10 cm^2 of filter, and incubated at 37°C for 16-20 hrs, after which the probe/hybridisation solution was decanted and stored for re-use. Membranes were washed with washing buffer 1 (2 × SSC, 0.1% SDS) at 65°C for 10 mins, then with washing buffer 2 (0.1 × SSC, 0.1% SDS) at 65°C for 30 mins and finally with washing buffer 3 (buffer 1 [0.1 M maleic acid, 0.15 M NaCl],

0.3% Tween 20). The membranes were incubated in buffer 2 (blocking solution diluted 10 × with buffer 1) and anti-DIG-AP conjugate (1 µl anti-DIG-AP conjugate per 10 ml buffer 2). After 30 mins, the membranes were washed with buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$) for a further 30 mins.

Lumogen-CSPPD was added to the membrane which was subsequently sealed in a plastic bag, preincubated for 15 mins at 37°C and exposed to X-ray film.

2.8.5 Hybridisation with ^{32}P-dNTP labelled probes

All experiments were performed under high stringency. Membrane-bound DNA was prehybridised with 2.5 ml of hybridisation solution (50% formamide, 4 × SSC diluted from 20 × SSC stock [0.3 M sodium citrate, 3 M sodium chloride], 0.5% blotto from 10% Blotto stock [10% (w/v) Skim milk powder in water, with sodium azide 0.001%], 1% SDS, 0.3 mg/ml sheared salmon sperm DNA) per 10 cm^2 for two to six hours at 37°C. The membrane was then hybridised with 50 µl of ^{32}P-labelled probe was added to 1 ml of hybridisation solution per 10 cm^2 of membrane for 16 hours at 37°C. After hybridisation probes were decanted and stored at −20°C for re-use. The membranes were washed 2 × 10 mins in water and then in 2 × SSC for 30 mins, 2 × SSC/0.1% SDS for 30 mins and 0.1 × SSC/0.1% SDS for 15 mins. All stringency washes were performed at 65°C. Blots were wrapped in cling wrap, and exposed to X-ray film for between 2 hours and 4 days.

2.8.6 Probe removal

(i) of DIG labelled probes

Probe removal was performed using the procedure recommended by the manufacturer of the DIG kit. Membranes were transferred to high stringency washing buffer 2 (0.1% SSC, 0.01% SDS) at 92°C for 10 mins, soaked for 5 mins in 2 × SSC and

washed for 1 min in buffer 1 (0.1 M maleic acid, 0.15 M sodium chloride pH 7.5). The washes and the addition of anti-DIG-AP and CSPD were repeated (Section 2.8.4), after which the stripped membrane was exposed to X-ray film to check that the previous probe had been completely removed.

(ii) of ^{32}P-dNTP labelled probes

250 ml of high stringency wash solution (0.1 × SSC/0.1% SDS) was boiled, and then transferred to a glass dish, to which the membrane was added. The membrane was shaken on an orbital shaker for 30 mins. This procedure was repeated two more times before the membrane was checked for radioactivity with a Geiger counter, then was allowed to air-dry before re-use or storage.

2.9 Northern blotting

2.9.1 Preparation of formaldehyde gel and electrophoresis of RNA samples

RNA was prepared as described in Section 2.4. Prior to electrophoresis, the BioRad mini-gel apparatus or the Owl Buffer Puffer apparatus buffer chamber, combs and gel moulds were submerged in 100 mM NaOH for 30 mins, and then thoroughly rinsed with milliQ water. 10-20 µg of total RNA was denatured by the addition of 1.5 volumes of denaturation mix (50 µl 10× MOPs, 87.5 µl formaldehyde, 250 µl formamide) and heating at 55°C for 15 mins. Loading dye (50% glycerol, 1 mM EDTA, pH 8.0, 0.4% bromophenol blue, 0.4% xylene cyanol) was then added to the RNA samples prior to loading them on a denaturing RNA gel (1.2% agarose, 0.66 M formaldehyde, MOPs [0.04 M 3-[N-morpholino]propane sulfonic acid, 0.01 M sodium acetate, 10 mM EDTA, adjusted to pH 7.2 with NaOH] buffer). Total RNA samples were separated by electrophoresis on a denaturing gel at 50 mA for 2-3 hrs in MOPs running buffer (Farrell,

1993).

If samples were to be transferred in a Northern blot experiment the samples were not stained with ethidium bromide, since this is known to reduce transfer efficiency. However, duplicate samples, and a 3 µg aliquot of stock RNA markers (Promega, 0.36-9.46 kb) were run on the same gel, and later stained in order to check integrity and quantity of the RNA samples, and to allow the size of transcripts to be determined.

2.9.2 Transfer of RNA samples

Following electrophoresis the RNA gel was rinsed in milliQ water three times and then soaked in 10 × SSPE (20 × stock: 3 M NaCl, 200 mM $NaH_2PO_4.2H_2O$, 20 mM EDTA, pH 7.4) for 45 mins, and then washed in 2 × SSPE for 5 mins. The RNA was then transferred to Hybond Nylon N^+ membrane (Amersham) by capillary action as described (Southern, 1975). After blotting for 16 hours, the nylon membrane was rinsed in 2 × SSPE and then baked at 65°C for four hours or overnight to fix the RNA to the membrane (Farrell, 1993).

An alkaline transfer method (Reed, 1987) was also utilised for some experiments in order to ensure that during transfer the RNA sample remained denatured when it bound to the membrane. The alkaline transfer method was the same as that described above except that after electrophoresis there was no equilibration in a high salt solution and RNA transfer was mediated by capillary transfer in 50 mM NaOH for only 2.5 to 5 hours.

2.9.3 Prehybridisation and hybridisation of northern blots

For Northern blots both Denhart's and Blotto hybridisation solutions were used. For Denhardt's (50% deionised formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS,

100 µg/ml denatured salmon sperm) hybridisations the prehybridisation solution and hybridisation solutions were the same. Membranes were prehybridised for 2-4 hours and then hybridised with the probe overnight at 37°C. The membrane was then washed once at 42°C for 10 mins and then once at 65°C for 10 mins.

Membrane-bound RNA was prehybridised with 2.5 ml of Blotto prehybridisation solution (50% deionised formamide, 4 × SSPE, 1% SDS, 0.5% Blotto, 0.3 mg/ml denatured salmon sperm DNA) per 10 cm^2 of membrane at 37°C for 4-8 hours. The membrane was then hybridised with the probe, in 1 ml of hybridisation solution (47% deionised formamide, 10% dextran sulphate, 3 × SSPE, 1% SDS, 0.5% blotto) per 10 cm^2 of filter for 16 hours. The membranes were subsequently washed twice in 2 × SSPE/0.1% SDS for 10 mins at room temperature, and then once in 0.2 × SSC/0.1% SDS for 15 mins. The membrane was then sealed in cling film and exposed to X-ray film for between four and twenty one days before being developed.

2.10 Screening a genomic library in bacteriophage λGEM-12

The titre (7.0 x 10^4 pfu/ml) of the λGEM-12 *D. nodosus* strain A198 genomic library (origin) stock was determined by serial dilution of phage stock using an L + G top agar overlay method (Sambrook *et al.*, 1989). Plaque lifts were done in duplicate on nitrocellulose membranes (Biotrace NT - Gelman Sciences; or Amersham Hybond C) from cooled plates with approximately 200 plaques (Sambrook *et al.*, 1989). After the plaques had been lifted, filters were soaked in 0.5 M NaOH/1.0 M NaCl for 2 min and then in 0.5 M Tris, pH 7.4/1.5 M NaCl for 2 mins, and air-dried before the recombinant phage DNA was fixed to the filter by baking at 65°C for 4 hours or overnight. Plaque lifts were hybridised with DIG-labelled probes under high-stringency conditions as described above (Section 2.8.4). Those lambda plaques that hybridised to the probe were isolated from plates (Sambrook *et al.*, 1989), retitrated and rescreened twice to ensure clone uniformity,

after which lambda DNA was prepared from positive lambda clones (Section 2.3.3). For long-term storage, bacteriophage stocks were stored both at 4°C in SM containing 0.3% chloroform and at –70°C in SM containing 7% DMSO (Sambrook *et al.*, 1989).

2.11 DNA Sequencing

Most of the sequencing data generated in this work was done using a manual sequencing method, though some automated sequencing was done also.

2.11.1 Manual Sequencing reactions

Sequencing reactions were based on the dideoxy-chain termination method of Sanger *et al* (1977), using 2-4 µg of alkaline lysis DNA (Section 2.3.2.*i*) and either the Bresatec Super-Base sequencing kit or the Pharmacia Biotech ^{T7}Sequencing Kit, according to the manufacturer's instructions.

Deaza bases were substituted for normal bases where compressions were to be resolved. Double-stranded sequencing was carried out in both forward and reverse directions using a reverse (Promega) and universal primer (Bresatec) which annealed to either side of the polylinker cloning site in recombinant pUC18 plasmids. Sequencing reactions were labelled with [α-^{35}S]dATP. On some occasions oligonucleotide primers were substituted for pUC18 primers (Section 2.12.1). For manual DNA sequencing, 100 ng of primer was used for each sequencing reaction.

2.11.2 Polyacrylamide sequencing gel

Prior to preparation of sequencing gels the bonded IPC plate of the Bio-Rad SEQUI-GEN sequencing apparatus was treated with a siliconising agent, Rain-X^{TM} to ensure that the polyacrylamide sequencing gel remained on the non-bonded plate during disassembly.

An 8% polyacrylamide gel solution ([7.72% (w/v) acrylamide, 0.27% (w/v) bis-acrylamide, 18.68% (w/v) urea], TBE [10 mM Tris, 0.2 mM Na_2EDTA, 8.6 mM boric acid] and 23% urea) was prepared, to which 1.26 x 10^{-3} M ammonium persulfate and 3.77 x 10^{-3} M TEMED (Bio-Rad) were added for polymerisation. It was then poured into a SEQUI-GEN sequencing apparatus (Bio-Rad) and allowed to set overnight.

Prior to loading sequencing reactions, gels were covered in 1 × TBE and heated to 50°C. All sequencing reactions were heated to 70°C for 3 mins before loading 2.5 µl onto the gel. The polyacrylamide gel was run at 2500 V, current of 250 mA and at constant power of 110 W, and the gel temperature maintained at 50°C for 4 hrs, before a second loading of samples for a further 3 hours. The 0.4 mm thick gel was transferred to 3 MM Whatman filter paper, dried on a Bio-Rad 583 gel dryer at 80°C on the sequencing cycle for 2 hrs, and exposed to X-ray film (Fuji Medical X-ray film) for 2-14 days.

2.11.3 Autoradiography

Autoradiographs were developed for 3 mins in developer (Kodak), rinsed for 1 min in running water, fixed for 5 mins, before soaking in water for 30 mins.

2.11.4 Automated DNA sequencing

Plasmid DNA for automated sequencing was prepared using the Wizard™ *Plus* Minipreps DNA Purification System (Promega), using the method recommended by the manufacturer for 10 ml bacterial cultures. Sixteen µl plasmid DNA at a concentration of 200 ng/µl was sent to the Supermac DNA Sequencing facility (Eveleigh, New South Wales)where the reactions were performed and sequencing data was generated. The data generated was forwarded by e-mail back to the sender for analysis.

2.11.5 Computer-aided sequencing analysis

The DNA Rodent Computer Package (BIOTEL) was used to read sequencing gels, and the SEQAID II programme (Rhoads & Roufa, Molecular Genetics Laboratory, Kansas State University) was used to analyse DNA sequences. Identity searches with protein and DNA sequences were carried out using software provided by the Australian Genomic Information Service (ANGIS).

2.12 Polymerase Chain Reaction (PCR)

2.12.1 Oligonucleotide synthesis and design

Oligonucleotides were designed for both sequencing and PCR experiments. To optimise the design of these oligonucleotide primers for sequencing, complementarity was tested by using the JROligos program (Rozas, 1991). These oligonucleotides were synthesised using a Pharmacia LKB Gene Assembler and using chemicals and computer programs supplied by Pharmacia. Oligonucleotide synthesis and purification was performed by the Institute of Biotechnology, UNE. More recently, primers have been synthesised by Bresatec.

2.12.2 Using Taq DNA polymerase

Each PCR reaction contained 1.8 mM $MgCl_2$, 1 × reaction buffer (6.7 mM Tris/HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.2 mg gelatin ml^{-1}, 0.45% Triton X-100, from Bresatec), 0.2 mM dNTPs, 1 µM of each primer, 1 unit of Taq DNA polymerase, 1-10 ng of genomic DNA or 1 ng of plasmid DNA in a final reaction volume of 25 µl. The reaction mixtures were amplified for 30 cycles (each cycle consisting of 90 s at 94°C, 60 s at 60°C and 120-300 s at 72°C) in a Corbett FTS-320 thermal cycler (Corbett Research). Oligonucleotide primers used in these experiments and their approximate positions are shown in Appendices 1, 2 and 3.

2.12.3 Using Deep Vent$_R$ DNA polymerase

The 25 µl amplification reactions contained 1 µM oligonucleotide primer, 200 µM dNTPs, 1 × Thermopol™ reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.01% Triton X-100, from New England BioLabs), 0.25 units of Deep Vent$_R$ Polymerase (New England BioLabs) and 10 ng of template DNA. The reaction mixtures were amplified for 34 cycles (each cycle consisting of 90 s at 94°C, 60 s at 60-63°C and 120-240 s at 72°C) in a Corbett FTS-320 thermal cycler (Corbett Research). Oligonucleotide primers used in these experiments and their approximate positions are indicated in Appendices 1 and 3.

2.13 Miscellaneous methods

2.13.1 *D. nodosus* Protease thermostability assays

The supernatant from 10 ml liquid cultures of *D. nodosus* was assayed for proteolytic activity by measuring the release of a soluble blue dye from hide powder azure in protease assay buffer (0.2 M HEPES, pH 8.5, 0.02 M $CaCl_2$) (Green, 1985). Spectrophotometry was used to determine the proteolytic activity present before and after heat pretreatment of the supernatant at 60°C for 10 mins.

2.13.2 Growth, viable count and cell counts of *D. nodosus*

For each of four *D. nodosus* strains studied the cells from 12 plates were resuspended in a total volume of 12 ml of Eugonbroth which was pooled, prior to inoculation into twelve 150 ml Eugonbroths. For each strain two bottles were placed in each of six anaerobic jars. For each of six days, one jar was opened and *(i)* the optical density at 600 nm determined; *(ii)* a cell count was performed using a haematocytometer; and *(iii)* serial dilutions (10^{-1} to 10^{-5}) of broth cultures were plated for a viable count after a further 5 days growth. The data were then plotted and analysed.

2.13.3 Plasmid Incompatibility Assays

Native *D. nodosus* plasmid pDN1 was tested for incompatibility to related IncQ plasmid pKT240 (Bagdasarian *et al.*, 1983), a derivative of RSF1010 (Scholz *et al.*, 1989) using a method described previously (Couturier *et al.*, 1988; Crosa *et al.*, 1994; Grindley, Humphreys & Anderson, 1973; Novick, 1987). *E. coli* strain DH5-α was transformed with pDN1 derivative pDN3 (Section 3.2.8.1), selecting for tetracycline resistance. Competent cells were prepared from pDN3 (tet^R) host cells and transformed with RSF1010 derivative, pKT240 (amp^R, kan^R) or pDN1 derivative pDN4 (amp^R, kan^R) and resistance to both ampicillin and tetracycline selected. Single colonies were inoculated into 2YT broth containing ampicillin (100 µg/ml) and tetracycline (10 µg/ml), grown to stationary phase and diluted 10^6-fold into antibiotic-free broth and grown until stationary phase when the culture was diluted and plated onto antibiotic-free agar to isolate single colonies. Colonies were selected and sequentially stabbed into 2YT agar containing ampicillin 100 µg/ml and then tetracycline 12.5 µg/ml. The percentage of these cells that were ampicillin sensitive and tetracycline resistant or conversely ampicillin resistant and tetracycline sensitive were equivalent to the number of pDN3 host colonies that had lost either pKT240 or pDN4 respectively after approximately 36 generations.

2.13.4 Plasmid Copy number

The copy number of pDN1 was determined using the agarose gel electrophoresis method (Crosa *et al.*, 1994). The negative of a polaroid photograph, of an ethidium bromide-stained gel of a genomic DNA preparation of *D. nodosus* pDN1 host strain 1311 was separated on a 0.8% agarose gel, and the negative was scanned using a LKB2222-020 Ultroscan XL Laser Densitometer (Pharmacia). The integrated values of the peaks corresponding to chromosomal and plasmid DNA respectively were used to determine copy number per chromosome equivalent, as follows:

$$\text{copy number} = \frac{\text{integrated value of plasmid peak}}{\text{integrated value of chromosome peak}} \times \frac{MW_c}{MW_p}$$

where MW_c and MW_p are the molecular weights of the chromosome and the plasmid respectively.

2.13.5 Preparation of electrocompetent *D. nodosus* cells

Electrocompetent *D. nodosus* cells were produced using a protocol modified from that described for the production of electrocompetent *E. coli* cells (Ausubel *et al.*, 1995). Four x 150 ml eugonbroths were inoculated with cells from 2 or 3 day BEA plate cultures of *D. nodosus*. The liquid *D. nodosus* cultures were grown to the appropriate growth phase; both log and stationary phase cells were utilised in transformation experiments. Thirty minutes prior to harvesting the cells, unopened anaerobic jars containing cell cultures were transferred to the cold room (4°C). In addition, centrifuge bottles (200 ml), centrifuge tubes (40 ml), deionised water/10% glycerol (or mM HEPES/10% glycerol), and pipette tips were cooled to 4°C.

Cells were harvested by centrifugation at 4 000 *g* in a JA14 rotor for 15 mins at 0°C. The culture supernatant was decanted, and all pellets resuspended in an equal volume (150 ml) of cold deionised water and centrifuged at 4 000 *g* in a JA14 rotor for 15 mins at 0°C. Note that all washing steps were carried out in the cold room at 4°C. The supernatant was decanted, bottles were placed on ice and each pellet resuspended in a half volume (75 ml) of cold sterile deionised water and centrifuged at 4 000 *g* in a JA14 rotor for 15 mins at 0°C.

The supernatant was decanted, and the pellets resuspended in 24 ml of deionised water/10% glycerol, and the cell suspension transferred to a 40 ml centrifuge tube and centrifuged at 4 000 *g* for 15 mins at 0°C. The supernatant was removed, and the pellet resuspended gently in an equal volume of ice cold sterile 10% glycerol in water. Forty µl aliquots of *D. nodosus* cell suspensions were transferred into microfuge tubes prechilled at −20°C and aliquots stored at −70°C until ready for use.

2.13.6 Standard method for electroporation of *D. nodosus* cells

Forty or 100 µl aliquots of *D. nodosus* electroporation competent cells were thawed on ice for 20 mins. 0.5 µg - 10 µg of plasmid DNA was added to microfuge tubes containing thawed cells (on ice) and mixed. The suspension then was immediately transferred to a prechilled 0.1 cm cuvette, the outside of the cuvette was dried before placing in the sample chamber and applying the pulse (initially 2.0 kV). Immediately after the pulse 1 ml of Eugonbroth medium was added to the cuvette and transferred to a sterile culture tube with a sterile pipette. Cells were then cultured under anaerobic conditions at 37°C to allow recovery (four hours or overnight) and gene expression. After the recovery period, cultures were transferred to microfuge tubes and centrifuged at 10 000 *g* for 2 mins. The supernatant was removed with a pipette and the pellet gently resuspended in 100 µl of Eugonbroth and plated onto the appropriate selective medium. Plate cultures were allowed to grow for three days prior to screening plates for transformants.