# Chapter 1. Introduction

## 1.1. Ovine footrot

Ovine footrot is an infectious disease, which affects the skin connecting the claws of small ruminants. The disease is economically important in sheep-rearing countries with mild climates. The economic loss is due to the cost of the treatment and production loss resulting from reduced body weight and reduction of wool production. Ovine footrot is a mixed bacterial infection in which *Dichelobacter nodosus*, formerly known as *Bacteroides nodosus,* is the essential transmitting agent (Beveridge, 1941; Egerton *et al.*, 1969; 1983; Dewhirst *et al.*, 1990; La Fontaine and Rood, 1990; Jimenez *et al.*, 2003).

Footrot occurs in all major sheep-producing countries. It was introduced to the Southern hemisphere with animals imported from Europe in the 19$^{th}$ and early 20$^{th}$ century (Stewart, 1989). In Australia and New Zealand, footrot is a major disease of the Merino breed, but also occurs in different breeds of British sheep, e.g. Romney and Cheviot; sheep export from Australia and New Zealand have been associated with outbreaks of footrot in Bhutan, Malaysia and Nepal (Egerton, 1983; Kelly, 1990; Ghimire *et al.*, 1998; Zakaria *et al.*, 1998). In Australia, in NSW alone the cost of the disease due to loss of production, treatment, control, prevention and eradication was about 43 million AUD annually with 6000 flocks infected in the 1980s (Egerton and Raadsma, 1990). However, due to an intensive management program, in 2005, only 128 flocks were infected, though the number of flocks has also been reduced by 50% (Seaman, 2006).

*D. nodosus* is a slow growing and fastidious anaerobe in culture, making its isolation and detection from footrot samples difficult. The extensive diversity of the bacterium makes typing even more complicated (Zhou *et al.*, 2001). In sheep, three different clinical forms of the disease have been described: virulent, intermediate and benign. Virulent footrot (VFR) is economically one of the most important infections of sheep. Because of its impact on production and welfare, it is often the target of expensive control and/or eradication programs, either on a property or on a regional basis (Egerton, 1989; Abbott and Egerton, 2003a). Intermediate footrot is a mild footrot observed in between virulent and benign, however, NSW footrot policy considers intermediate as virulent footrot (Seaman, 2003). Benign footrot causes little or no production loss and does not warrant intervention. There is therefore a need to identify accurately the form of footrot present in a flock (Ghimire *et al.*, 2002), however, there is a spectrum of disease, from benign to virulent. A contagious ovine digital dermatitis (CODD) was recently reported and found to be different from footrot, it does not respond to footrot treatments and the role of *D. nodosus* in the clinical process is not known (Moore *et al.*, 2005).

*D. nodosus* naturally infects cattle, goats, deer and sheep. Isolates from cattle cause only benign disease in sheep; however there are no reports on the severity of disease caused by cattle strains in goats (Beveridge, 1967). *D. nodosus* isolates from goats display a range of virulence, similar to that of sheep isolates. It has been shown that strains from sheep and goats can naturally transmit between these two species. This has not only substantiated the long suspected role of goats as a reservoir of VFR for sheep, but has emphasised the necessity of tests or criteria for differential diagnosis of the forms of footrot in goats (Ghimire *et al.*, 2002).

## 1.2. Pathology

Footrot lesions of sheep are due to a combined invasion by *D. nodosus* together with a gastrointestinal inhabitant *Fusobacterium necrophorum* (Roberts and Egerton, 1969; Egerton, 1983). However, *D. nodosus* is the only organism capable of reproducing the disease when applied as pure culture and lesions of footrot do not develop in its absence (Beveridge, 1941; Stewart, 1989).

## 1.3. Clinical signs

Lameness is the sign that usually indicates the presence of footrot in the flock. The proportion of affected animals exhibiting lameness and the degree of lameness are determined by the severity of the outbreak. Even in severe outbreaks, some affected animals may not be lame. Characteristically, sheep with severe footrot are recumbent for much of the time as feeding is restricted, body weight reduces and wool quality is adversely affected. Affected rams do not mate readily and ewes in poor body condition have lower lambing percentages (Egerton, 1989).

The bacterial invasion results in a recurrent degeneration of the stratum granulosum of the epidermis associated with degeneration of the epidermis and vacuole formation. Leucocytes invade the affected areas of the epidermis. The role of the different bacteria in the pathogenesis of footrot has yet to be elucidated, but the elimination of *D. nodosus* from lesions either by specific immunization or antibiotic therapy will result in a cure of most affected animals (Egerton, 1989).

Footrot lesions vary from mild inflammation of the interdigital space to extensive separation of the soft horn of the heel and sole. Clinical examination of sheep with fully developed footrot caused by virulent strains can be readily differentiated from those infected with benign strains. Thus, in individual flocks it is possible to recognise clinically different manifestations of footrot.

### 1.3.1. Virulent footrot (VFR)

Virulent footrot is an outbreak which leads to a high prevalence of infection. Among affected animals, a high proportion has severe lesions, which separate the sole from the wall and toe of the hoof (Fig. 1.1) Some animals are unaffected and a small proportion will have lesions only of the interdigital space (Egerton and Parsonson, 1969).

### 1.3.2. Benign footrot (BFR)

Benign footrot is an outbreak in which, even when the prevalence is high, the proportion of animals with severe, i.e. under-running, infections is very low. Of the affected sheep, 1 – 2 percent will have separation of the horn, but most will have infections confined to the interdigital space (Egerton and Parsonson, 1969).

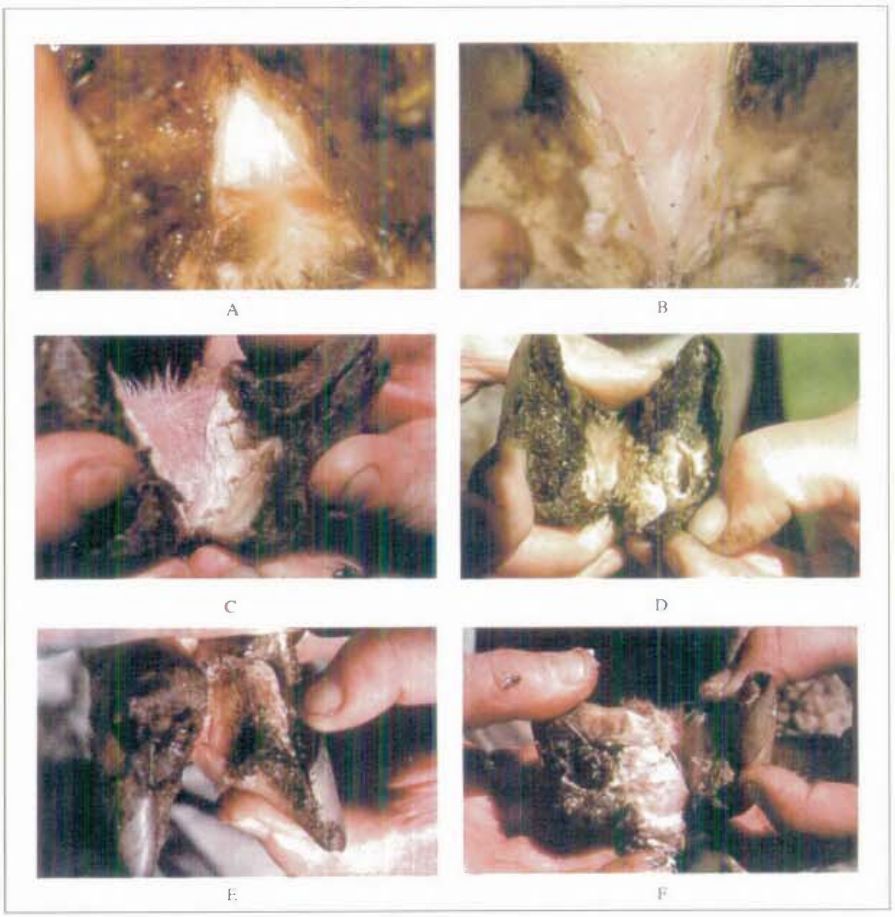



**Fig. 1.1:** Appearance of the hoof at different stages of footrot infection. (A, B) Benign footrot: (A) Intergital dermatitis of posterior interdigital skin and skin-horn junction. (B) More pronounced dermatitis. (C to F) Virulent footrot: (C) Severe dermatitis of interdigital skin and axial walls of the hoof horn. (D) Separation of the soft horn of the hoof. (E) Underrunning of the hard horn of the hoof. (F) Advanced virulent footrot with underlying tissue beneath the separated horn (Egerton, 1989).

## 1.4. Characteristics of growth

*D. nodosus* is a slow growing anaerobe. It is an obligatory parasite, for which the only known habitat is the hooves of ruminants affected with footrot. It survives with a minimal carbohydrate requirement and largely on the keratins of the hoof, and it does not persist in the environment for more than a week (Skerman, 1975; Egerton, 1989).

## 1.5. Epidemiology and transmission

Outbreaks of footrot are determined by a number of host, environmental and microbial factors. Exposure to environmental conditions that devitalise the interdigital skin is necessary for the initiation of footrot. When the hoof is damaged it is susceptible to invasion by *F. necrophorum*. Damage resulting from the invasion allows the establishment of *D. nodosus* if it is present and the environment is favourable. Transmission of footrot requires adequate moisture and a mean ambient temperature above $10^0$C (Graham and Egerton, 1968). The severity of footrot depends partly on virulence factors in the infecting strains of *D. nodosus*. Multi-strain infections may occur (Egerton, 1989). The incubation period of footrot is 10 – 14 days under the favourable conditions of high humidity and mild temperatures. Significant damage to the hoof may occur within 14 days. Thereafter, again depending on the host, the environment and the infecting bacteria, cases may persist, regress or heal completely. There is evidence that some animals that apparently recover from footrot, either naturally or after treatment are carriers of *D. nodosus*. These carriers may relapse and re-infect unaffected members of the flock when environmental conditions are suitable.

## 1.6. Virulence determinants of *D. nodosus*

### 1.6.1. Fimbriae

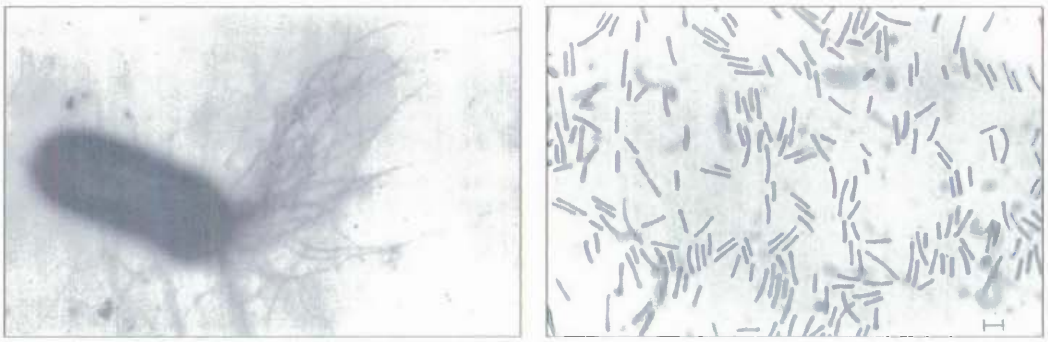
*D. nodosus* cells show twitching motility, which is a form of locomotion which helps bacteria to move independently in a random direction. Twitching assists *D. nodosus* in penetration of the hoof, and virulent strains generally have higher twitching motility than benign strains (Depiazzi and Richards, 1985; Elleman, 1988; Depiazzi *et al.*, 1990; Billington *et al.*, 1996).

Twitching motility is caused by the polar type-IV fimbriae (Fig. 1.2), which are assembled from a protein subunit called pilin. This subunit is exported from the cell and polymerised to form the fimbrial strand by a complex multi-component pathway. The fimbrial strand is 6 nm in diameter and can reach up to 25 μm in length. This plays a major role in both pathogenicity and the immunological response of the host (Anderson *et al.*, 1986; Johnston *et al.*, 1995; Doughty *et al.*, 2000).

Several genes involved in fimbrial biogenesis in *D. nodosus* have been cloned and sequenced and found to be homologues of genes in other bacteria that produce type IV fimbriae (Hobbs *et al.*, 1991; Johnston *et al.*, 1995; Johnston *et al.*, 1998). These genes are not localised to one area of the *D. nodosus* chromosome and, therefore, do not appear to be located in pathogenicity islands (La Fontaine and Rood, 1997). The sequence of the whole genome of the virulent strain VCS1703A shows 21 putative genes required for fimbrial biogenesis, together with 10 fimbrial regulatory genes (Myers *et al.*, 2007). Insertional inactivation of *fimA*, which encodes a fimbrial subunit, resulted in a reduction of virulence, showing that fimbriae are essential virulence determinants (Kennan *et al.*, 2001).

Beveridge (1941) observed antigenic variation between the *D. nodosus* strains. These variations are due to the fimbriae and there are 10 serogroups (Claxton *et al.*, 1983; Stewart, 1989). Serogroups can be further subdivided into serotypes. Antigenic diversity in the fimbriae is due to sequence variation in the fimbrial gene (*fimA*). A PCR based test to identify serogroups was developed by Zhou and Hickford (2000). Variation in the carboxy-terminal region of *fimA* has been used to develop a PCR-based test for rapid identification of *D. nodosus* serotypes (Dhungyel *et al.*, 2002).

Cagatay and Hickford (2005) studied the fimbrial proteins isolated from *D. nodosus* strains and identified the process of post translational glycosylation of the amino acid from in and out of the variable region of FimA. The type-IV fimbriae of *Neisseria gonorrhoea* and *N. meningitidis* (Power and Jennings, 2003) and *Pseudomonas aeruginosa* 1244 (Comer *et al.*, 2002; Smedley *et al.*, 2005) have also been found to be glycosylated and fimbrial proteins were found to be glycosylated only on the surface of meningococci (Virji *et al.*, 1993). Several reports suggest that fimbrial glycosylation may have direct functional consequences in pathogenesis (Virji, 1997; Upreti *et al.*, 2003).



(a) (b)

**Fig. 1.2:** (a) Electron micrograph of *D. nodosus* showing the polar location of the pili (fimbriae) [McKern *et al.*, 1989]. (b) *D. nodosus* cells stained by Gram straining. Note the predominance of slightly curved rods with swollen ends. Scale bar equals 5 μm [Skerman, 1989].

### 1.6.2. Extracellular proteases

*D. nodosus* secretes proteases which act to break down tissues within the hoof (Stewart, 1979; Kortt *et al.*, 1982; Green 1985; Depiazzi *et al.*, 1990). Protease characteristics have been correlated with the virulence of *D. nodosus* for a long time (Depiazzi and Richards, 1979; Kortt *et al.*, 1982). The proteases from virulent strains are much more stable than proteases from benign strains when heated at 60°C for 10 minutes. In addition, different

banding patterns have been observed when comparing virulent and benign strain extracellular proteases on polyacrylamide gels (Kortt *et al.*, 1983).

Virulent isolates of *D. nodosus* produce several extracellular serine protease isoenzymes, which are encoded by the *aprV2*, *aprV5* and *bprV* genes (Lilley *et al.*, 1992; Riffkin *et al.*, 1993; 1995). The major thermostable protease is encoded by *aprV2*, while *aprV5* encodes the major thermolabile protease (Kennan, R. M, and Rood, J. I., personal communication). Closely related protease genes, *aprB2*, *aprB5* and *bprB*, are found in benign isolates (Riffkin *et al.*, 1993; Lilley *et al.*, 1995; Riffkin *et al.*, 1995).

Biochemical studies of virulent strains have identified four extracellular acidic serine protease isoenzymes (V1, V2, V3 and V5) and a basic protease, BprV. Benign stains secrete five acidic serine proteases (B1 to B5) and a basic protease (Kortt *et al.*, 1994). Since the number of isoenzymes is greater than the number of genes, post-translational modification of the proteases is likely. The proteases secreted by virulent strains have greater activity against elastin than proteases secreted by benign strains. The difference in biochemical properties of the proteases forms the basis of several diagnostic tests (Depiazzi *et al.*, 1991; Palmer., 1993) as discussed in section 1.8.4.

## 1.7. Disease treatment and control

The objective of footrot treatment and control is to eradicate the infection and disease from the flock and to maintain and protect the sheep from new infections. This approach provides the best outcome for the welfare and productivity of the animal because it prevents the painful and debilitating underrunning footrot lesion. Affected and unaffected animals must be separated, and unaffected animals should be kept in an environment

known to be free of *D. nodosus*. Affected animals should be culled, or treated. To confirm their freedom from footrot, the unaffected animals should be re-examined twice at intervals of 3 to 4 weeks (Egerton, 1989).

### 1.7.1. Foot-bathing

In the early stages of infection, the disease is amenable to control by antibacterial solutions applied in a footbath. If it remains uncontrolled, the infection extends by under-running the horn of the hoof. At this stage, foot paring is necessary if topical antibacterial treatment is going to be effective. Sheep may be walked through a formalin solution, but repeated walking of sheep through footbaths (6 m long and 10 cm deep) which contain 15 – 18% zinc sulphate solution, and then allowing sheep to dry on concrete or metal grating is adequate (Jelinek *et al.*, 2001). The other widely used treatment is Radicate®, a copper salt solution used similarly to zinc sulphate (Seaman, 2003). Sheep foot paring can become damaging, as it can lead to development of chronic lameness and stress, so it should be used sparingly. The penknives used for pairing should be cleaned and disinfected after every use. However, after paring good cure rates can be achieved by walking sheep through footbaths (Abbort and Egerton, 2003a; 2003b).

### 1.7.2. Antibiotic treatment

Topical antibiotic treatment using an oxytetracycline spray is effective for early footrot. Treated animals should be kept in a dry place for a minimum of 30 minutes, as returning animals to wet pasture after treatment inhibits diffusion of antibiotics to the affected tissues (Egerton, 1989). Animals treated with topical antibiotics do not require foot bathing. The topical administration of a solution of lincomycin and spectinomycin either

via a hand-held garden sprayer or footbath is reported to produce excellent results (Davies *et al.*, 1999). For topical treatment, all the overlying necrotic tissue of the hoof must be removed either with secateurs or a sharp knife. This procedure is slow and laborious and it is also painful.

Parenteral antibiotic treatment can be more helpful than foot paring and topical treatment during severe footrot. Antibiotic treatments such as oxytetracycline, tilmicosin and a combination of penicillin and streptomycin have been used successfully (Watson, 1999). These antibiotics are long acting. Animals which do not respond to the antibiotics should be identified and culled immediately (Hosie, 2004).

Both topical and parenteral treatment when properly administered will cure 90 – 95 percent of cases. Treated animals should be examined after 3 – 4 weeks to identify animals that have failed to respond and these should be culled (Egerton, 1989).

### 1.7.3. Vaccination

Vaccines against *D. nodosus* fimbrial antigens have been produced, but the large number of serogroups makes vaccine preparation difficult. Egerton and Merritt, (1970) found that *D. nodosus* is highly susceptible to bactericidal antibody in-vitro. The efficacy of footrot vaccines containing whole *D. nodosus* cells was tested in infected and uninfected sheep, by injecting *D. nodosus* intramuscularly (Egerton and Roberts, 1971). Resistance to footrot was observed in the vaccinated sheep. Three commercial vaccines were patented by CSIRO (Commonwealth Scientific and Industrial Research Organisation), Australia in 1971 and 1972 and used for the control of ovine footrot. These contained cells with

mineral oil adjuvant. However, these were prepared from a single serotype (monovalent) (Egerton and Burrell, 1970; Egerton and Roberts, 1971).

Subsequent field trials of these vaccines conducted in 1975 were unsuccessful (Keogh, 1976). This could be due the presence of multiple serogroups present in a single flock or in a single sheep (Claxton, 1981). This identification led to the development of bivalent and multivalent vaccines (Claxton *et al.*, 1983; Stewart *et al.*, 1991). A multivalent, piliated, alum-oil adjuvanted *D. nodosus* vaccine was also produced containing nine serogroups A – I (Hunt *et al.*, 1994; Raadsma *et al.*, 1994), but the duration of immunity for the multivalent vaccine was relatively short, about 12 weeks and booster doses were required. Monovalent vaccines produced longer lasting immunity (Thorley and Egerton, 1981; Stewart *et al.*, 1986; Hunt *et al.*, 1995).

As the monovalent vaccines were found to be more potent, inducing persistently higher antibody titres and were both curative and protective, a serogroup specific vaccination scheme was developed. The first priority is to identify the serogroups(s) present in the affected population, which can be done by PCR using primers from *fimA* (Dhungyel *et al.*, 2002). It is also essential to monitor the specific vaccination program with regular checks on footrot status and it is important to identify the serogroups from new isolates in cases of non-response to the vaccine (Dhungyel *et al.*, 2002). Specific vaccination has been successful in eradicating virulent footrot from mixed flocks of sheep and goats in Nepal and from sheep in Bhutan (Egerton *et al.*, 2002; Gurung *et al.*, 2006). The results of trials conducted in Australia were found to be promising (Dhungyel *et al.*, 2006).

In the UK farmers are found to be more concerned when the disease is at its peak, and use parenteral and topical antibiotics and vaccines (Wassink *et al.*, 2003; 2005; Green *et al.*, 2007). Footrot elimination would only be feasible with the help of government supporting the treatment and control measures taken by the farmers (Walker, 1997). Progress could be achieved by small workshops to increase the awareness of the farmers about footrot disease control (Thompson *et al.*, 1999).

Thus, the impact of footrot on a flock can be reduced to an acceptable level by regular foot-bathing with or without vaccination. If control is the goal, paring should be limited to those sheep in which the flock control system has failed. Diagnosis of virulent footrot is essential because the expense of eradication of benign footrot in commercial flocks is not warranted. When eradication is the goal, its achievement depends upon complete removal of *D. nodosus* from the flock. This can be done most effectively by disposal of the whole flock, resting pasture and barns for 7 days, and then replacing with sheep known to be free of virulent footrot, as *D. nodosus* does not persist in the environment for more than 7 – 10 days (Egerton, 1989). Vaccinating a flock of sheep with serogroup-specific vaccines could eradicate that particular serogroup from the flock, but eradication could be expensive when multiple virulent serogroups are found in the same flock.

## 1.8. Diagnosis and testing

### 1.8.1. Foot scoring

Footscoring assesses the severity of damage to the hoof (Fig. 1.1) where 1 is an inflammation between the claws and 5 is severe underrunning of the hoof (Whittington and Nicholls, 1995). Scores of 1 and 2 may indicate benign footrot or could be due to an

initial stage of VFR. Early in an outbreak of VFR, most cases have lesions confined to the interdigital skin. Re-examination a few weeks later will help to confirm or modify the first diagnosis (Egerton, 1989) as the footrot lesions may advance to score 4 and 5 under favourable conditions, if it is a virulent footrot infection. Generally, in virulent footrot, both the claws are affected and more than one foot (Egerton and Roberts 1971; Seaman and Evers, 2006). This scoring system was modified to include scores of 3a, 3b, 3c, 4a and 4b by Stewart *et al.* (1982; 1983; 1984; 1985) and several other researchers, depending on the degree of severity of underrunning. In NSW, virulent footrot is usually diagnosed if >=10% of the sheep have severe lesions (scores 4 or 5) or a significant proportion of the flock show underrunning. However, favourable environmental conditions can result in high scores for benign footrot, and unfavourable conditions can result in lower scores for virulent footrot. For these reasons, laboratory tests are used to back up the diagnosis.

### 1.8.2. Clinical pathology

Confirmation of footrot in individual cases can be achieved by microscopy of Gram stained smears of necrotic material thinly spread on a slide. *D. nodosus* has a characteristic morphology, a large rod usually with terminal swellings (Fig. 1.2). The presence of *D. nodosus* does not necessarily indicate the occurrence of VFR. If VFR cannot be inferred by clinical examination alone, *in vitro* tests of *D. nodosus* may assist diagnosis (Egerton and Parsonson, 1966).

### 1.8.3. PCR methods to detect ribosomal RNA

PCR tests were developed to detect *D. nodosus* 16S rRNA genes (La Fontaine *et al.*, 1993). These were successful and were used to detect the presence of *D. nodosus* directly from lesion material from an infected sheep. This test allows the rapid identification of *D. nodosus*, however, there were several PCR inhibitory organic matters present in the samples when collected directly from lesions. In the UK, Moore *et al.* (2005) modified and standardised the 16S rRNA based PCR detection of *D. nodosus* isolates, by adding BSA and thus increasing the efficiency of PCR and overcame the inhibitory organic matter present in the lesions. However, identification of 16S rRNA does not help in differentiating between VFR and BFR. Laboratory tests used to distinguish VFR from BFR are mostly based on different properties of the proteases.

### 1.8.4. Gelatin gel tests (Protease thermostability assay)

The thermostability of extracellular proteases is greater in virulent strains than in benign strains (Depiazzi and Richards, 1979; Kortt *et al.*, 1982; 1983) Protease thermostability analysis using hide powder azure as a substrate was developed by Rinderknechet *et al.* (1969) and modified by Depiazzi and Richards (1979). Its been found that the $Ca^{2+}$-dependent protease stability assay is very reliable in differentiating between benign and virulent *D. nodosus* strains (Depiazzi *et al.*, 1990).

Palmer (1993) introduced a modified diagnostic test called the gelatin gel test, which is used to measure the thermostability of extracellular proteases. The gelatin gel test is a rapid qualitative test which gives a distinct differentiation between gel stable and unstable strains (Links and Morris, 1996). The gelatin gel test is widely used in footrot diagnosis

in Australia, especially in NSW and WA. The main drawback is that it is expensive and requires the preliminary isolation and culture of *D. nodosus* from infected tissue (Depiazzi *et al.*, 1991).

### 1.8.5. Elastase test

In 1979, Stewart identified differences in elastase production between benign and virulent strains. This was observed 7 days after culturing *D. nodosus* strains, by the digestion of elastin particles in TAS plates (Section 2.14). The virulent strains were elastase positive, showing clearing of elastin, while benign strains were not. Elastase tests were assessed by Liu and Yong, (1993) and found to be very useful to differentiate virulent, intermediate and benign *D. nodosus* strains. The gelatin gel assays and elastase assays show some correlation, as the elastase positive strains have increased protease production and increased thermostability (Liu and Yong, 1993). However, the elastase test is time consuming and results can vary in fresh and stored and passaged cells (Stewart, 1979; Depiazzi *et al.*, 1991). The elastase test is used occasionally in conjunction with the gelatin gel test in NSW.

### 1.8.6. Zymograms

The zymogram test examines the electrophoretic mobility of extracellular proteases of *D. nodosus* (Kortt *et al.*, 1983; Depiazzi *et al.*, 1991; Liu and Yong, 1993). Zymogram patterns for virulent and benign strains are different. Zymogram profiling is routinely used in Western Australia.

### 1.8.7. Gene probing

Katz *et al.* (1991; 1992) identified DNA sequences present in the virulent strain A198 but absent from the benign strain C305. Similarly, Liu and Yong identified sequences found primarily in benign, rather than virulent strains (Liu and Yong, 1993). Comparative analysis of the use of gene probes compared with other diagnostic methods (Rood *et al.*, 1996) showed that the gene probes were quite reliable. However, they have not been applied in diagnostic laboratories, presumably because they are not superior to the gelatin gel test.

### 1.8.8. Conclusions on diagnosis

The gelatin gel test is the most widely used laboratory diagnostic test for footrot in Australia. Zymogram and elastase tests are also used. However these tests are expensive and time consuming. In addition, there are false positives in the gelatin gel test, i.e. strains which secrete thermostable proteases, but do not cause virulent footrot (Cheetham *et al.*, 2006). Further analysis of genes involved in virulence may lead to improved diagnostic tests. This will be discussed in section 1.9.1.

## 1.9. Genomic islands in *D. nodosus*

### 1.9.1. The *intA* element

Katz *et al.* (1991; 1992) isolated DNA fragments from the prototype virulent strain A198 which are absent in the benign strain C305. One of these fragments was subsequently shown to be part of an integrated genetic element, the *intA* element (Cheetham *et al.*, 1995), which consists of an integrase gene, *intA*, followed by a series of *vap* (virulence-

associated protein) genes, *toxA* and an origin of replication. Integrase is an enzyme that facilitates prophage integration into or excision from a bacterial chromosome. The *vap* genes have similarity to genes involved in plasmid maintenance or replication. ToxA and VapA have amino acid similarity to HigA and HigB from the killer plasmid RtsI (Tian *et al.*, 1996a; 1996b), and are believed to comprise a toxin and antidote molecule, respectively, which act to prevent loss of the *intA* element (Bloomfield *et al.*, 1997).

The *intA* element is believed to be derived from the integration of a plasmid or bacteriophage into the *D. nodosus* chromosome. Multiple copies are present in some strains, and two integration sites have been identified: a *tRNA*-ser$_{GCU}$ gene close to the asparokinase (*askA*) gene and *tRNA*-ser$_{GGA}$ next to the polynucleotide phosphorylase (*pnpA*) gene (Bloomfield *et al.*, 1997). In strain A198, a copy of the *intA* element is integrated at both of these positions. In addition, a partial copy of the *intA* element is found immediately to the right of the *intA* element integrated into a *tRNA*-ser$_{GCU}$. These three copies have been designated *vap* regions 1, 2 and 3.

The *intA* element is present in 98% of virulent strains and present only in 28% of the benign strains (Billington *et al.*, 1999). Recently, Cheetham *et al.* (2006) investigated strains of *D. nodosus* which are classified as benign by field clinical diagnosis, but are stable in the gelatin gel assay. These strains, designated gel stable, field benign were found to lack *intA*. In contrast, gel stable, field virulent strains contained *intA*. PCR to detect *intA* is now in use to assist in footrot diagnosis in NSW.

### 1.9.2. The *intB* element

The *intB* element, which may be part of a prophage or a conjugative transposon, is integrated to the right of *vapD* in *vap* region 3 of strain A198 (Bloomfield *et al.*, 1997). The *intB* gene is highly related to *intA*. The *intB* element contains a regulatory gene, *regA*, and three genes of unknown function, *gepA, gepB*, and *gepC* (Bloomfield *et al.*, 1997). A partial copy of the *intB* gene, designated $intB_N$ is found in all strains, either present next to *pnpA*, or elsewhere in the genome (Whittle, 1999). The *intB* element is present in all *D. nodosus* strains irrespective of virulence (Whittle *et al.*, 1999).

### 1.9.3. The i*ntC* element

In strain C305, the *intC* element is integrated into *tRNA*-$ser_{GCU}$, next to *askA*. The *intC* element has the *intC* integrase gene, an insertion sequence IS1253 and two novel ORFs, designated ORF242 and ORF171, which have no similarity to known genes from the *vap* regions of *D. nodosus*. These are followed by two small ORFs, *vapG''* and *vapH''*, which show high similarity (55.2%) to *vapG* and (56.2%) to *vapH* from the *intA* element of strain A198 (Whittle *et al.*, 1999). The majority of the genes from the *intA* and *intC* elements are quite unrelated; however integrases IntA and IntC have 54.2% amino acid identity. It has been reported that the loss of *intC* element from the virulent strain 1311 during laboratory passage resulted in a reduction of protease thermostability (Whittle *et al.*, 1999), suggesting a role in virulence. However, the *intC* element is found in most strains, both benign and virulent. This is discussed further in section 1.11. The *intC* element is usually integrated next to *askA*, and so far no strains have been found to have *intC* next to *pnpA*.

### 1.9.4. The *intD* element

The *intD* element was discovered in *D. nodosus* benign strain C305 downstream from *pnpA*. This element was lost during laboratory passage to form a strain with the *intB* element next to *pnpA*, as *intB* was located on the other side of the *intD* element. The *intD* element has 30 potential open reading frames, including a suite of genes which encode a type IV secretion system. It is likely that the *intD* element has a role in the transfer of the other genetic elements between *D. nodosus* strains (Tanjung, 2004). The *intD* element is present in 20% of *D. nodosus* strains, mostly benign, and is usually integrated next to *pnpA*.

### 1.9.5. The *vrl* (Virulence-related locus)

Experiments by Katz *et al.* (1991, 1992) identified DNA sequences present in the virulent strain A198 but not in the benign strain, C305. One set of these sequences was subsequently found to be part of the virulence-related locus (*vrl*), which is 27 kb in length. The *vrl* from strain A198 has been cloned and completely sequenced (Haring *et al.*, 1995; Billington *et al.*, 1999). The *vrl* was found to be associated with virulence as it is present in 87% of virulent strains and 6% of benign strains. The *vrl* locus contains 22 potential genes, all of which are located on the same strand, however none of the genes encode known virulence determinants. Comparison of the deduced amino acid sequences of the *vrl* gene products revealed several regions of similarity to plasmids or bacteriophages (Billington *et al.*, 1999). There is evidence that the *vrl* region was inserted by a site-specific recombination event typical of bacterial pathogenicity islands, as it is located downstream from *D. nodosus ssrA* (small stable RNA) gene. However, no integrase gene has been identified in the *vrl*.

### 1.9.6. Bacteriophage DinoHI

DinoH1 was the first inducible bacteriophage obtained from *D. nodosus*. It was induced from strain H1215 when it was treated with UV light (Bloomfield, 1997). DinoH1 was found to have an icosahedral head and a long non-contractile tail with a unique claw-like base plate when observed under electron microscopy (Fig. 1.3). The phage genome analysis indicated that it consists of a 43 kb linear dsDNA molecule. DinoHI does not contain genes found on the other integrated elements of *D. nodosus*, nor does it encode any putative virulence factors. However, it may have a role in the transfer of other integrated elements between *D. nodosus* strains by transduction. The DinoH1 integrase gene, *intP*, has been identified and sequenced. The role, if any, of DinoH1 in virulence of *D. nodosus* is not known. However, it is largely found in virulent strains.

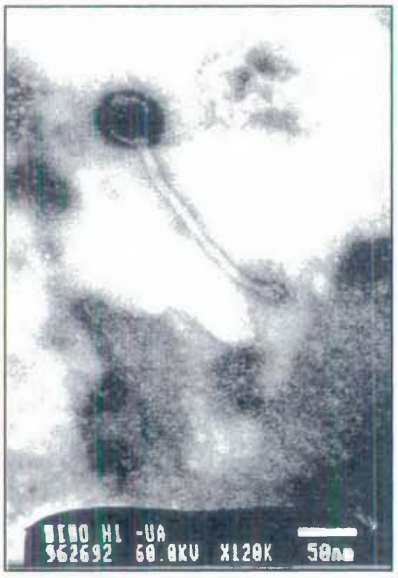



**Fig. 1.3:** Electron micrograph of DinoH1 after staining with uranyl acetate (Bloomfield, 1997).

## 1.10. Putative global regulators of virulence in *D. nodosus*

### 1.10.1. Polynucleotide Phosphorylase (PNPase)

In 1999, Whittle *et al.* discovered the *pnpA* gene in *D. nodosus* immediately upstream from *tRNA-ser*$_{GGA}$. The *pnpA* gene is present in all *D. nodosus* strains, and *tRNA-ser*$_{GGA}$ is an integration site for the *intA*, *intB* and *intD* elements.

#### 1.10.1.1. Role of PNPase

PNPase is a phosphorolytic enzyme involved in mRNA decay which is initiated by endoribonucleases. The 3' to 5' exoribonuclease activity is carried out by different ribonucleases such as PNPase, RNase II, RNase D, RNase B, RNase N, RNase T, RNase PH and RNase (Deutscher and Li, 2001). PNPase is involved in the degradation of large RNA molecules which are produced by RNase E, an endoribonuclease enzyme. In the multiprotein degradosome complex, the C-terminal RNase E interacts with PNPase to enhance degradation of mRNA and the RNase E level is maintained by autoregulation via a posttranscriptional feedback mechanism (Donavan and Kushner, 1986; Vanzo *et al.*, 1998; Ghosh and Deutscher, 1999, Diwa *et al.*, 2000). PNPase, unlike RNAase II, uses inorganic phosphate for phosphorolysis, which results in generation of nucleotide diphosphates (Deutscher and Reuven, 1991).

PNPase functions as the principal mechanism for the posttranscriptional modification of mRNAs which have been polyadenylated. During polyadenylation, poly (A) polymerase I (PAP I) adds poly (A) extensions to the 3' ends of the mRNA, tRNA as well as rRNA (Li *et al.*, 1998). This extension aid PNPase in the 3' to 5' exoribonuclease degradation. However, PNPase can also polyadenylate mRNA (Xu *et al.*, 1993; Hajnsdorf *et al.*, 1995;

Li *et al.*, 1998; Blum *et al.*, 1999; Coburn and Mackie, 1999; Goodrich and Steege, 1999; Mohanty and Kushner, 2000a; 2000b; Rott *et al.*, 2003; Sohlberg *et al.*, 2003; Khemici and Carpousis, 2004). The half life of *pnpA* transcripts depend on the amount of intracellular polyadenylated transcripts. PNPase expression itself is regulated at a posttranscriptional level by RNase III processing which affects RNase III expression level, as both PNPase and RNase III are autoregulating proteins and regulate each other's expression. The autoregulation of *pnpA* can be affected by a mutation of the RNA binding domains (Portier *et al.*, 1981; Regnier and Portier, 1986; Robert-Le Meur and Portier., 1994a; 1994b; Garcia-Mena *et al.*, 1999; Jarrige *et al.*, 2001; Jarrige *et al.*, 2002; Mohanty and Kushner, 2002).

#### 1.10.1.2. PNPase structural studies

PNPase is in a family of phosphate-dependent 3′ to 5′ exoribonucleases (PDX) (Mian 1997; Zuo and Deutscher, 2001). PNPase entraps the RNA substrates in its central triangular space for processing. PNPase has five conserved domains (Fig. 1.4) which consist of two core domains, which are repeats of the RNase PH domain, S1 and KH RNA binding domains and an all-α-helical domain which connects both the core domains (Symmons *et al.*, 2000).

**Fig. 1.4:** The structural arrangement of a PnpA, (repeated) RNase PH domain (green), all-α-helical domain (pink), PNPase KH domain (gray) and the S1 domain (blue).

**The all-α-helical domain**

The all-α-helical domain in PNPase is a connecter which links the two core domains and it is not highly conserved within the PNPase family (Symmons *et al.*, 2000). A mutation in the all-α-helical domain could affect RNA binding, as a potential RNA binding site is present in the central channel. However it does not affect any catalytic activity (Jarrige *et al.*, 2002).

**Core PNPase domains (RNase PH repeats)**

RNase PH (Deutscher *et al.*, 1988) is a member of a ribonuclease family, which has an overlapping function with PNPase and RNase T but not with RNase II or with RNase D (Kelly *et al.*, 1992). RNase PH is both a phosphorolytic nuclease that removes nucleotides following the CCA terminus of tRNA and a nucleotidyltransferase which adds nucleotide to the ends of RNA molecules by using nucleoside diphosphates as substrates (Jensen *et al.*, 1992; Kelly and Deutscher, 1992).

Harlow *et al.* (2004) studied RNase PH from *Bacillus subtilis* (*B. subtilis*) by protein crystallization and found that RNase PH has sequence similarity with the *Streptomyces antibioticus* PNPase second core domain which is also the RNase PH domain similar to core domain one. Though there are similarities with the RNase PH and the second core domain of PNPase, their roles are slightly different, and may be due to the presence of additional RNA binding domains (S1 and KH RNA binding domain) in PNPase (Harlow *et al.*, 2004). Jarrige *et al.* (2002) proved that the second core domain retains the catalytic activity, and a Gly(454) to Asp(454) substitution mutation from the second core domain does not affect the catalytic activity of the enzyme but affects its RNA binding, autoregulation, and degradosome stability (Regonesi *et al.*, 2004). The first core domain is not highly conserved among bacterial species compared to the second core domain.

Both core domains are involved in RNA degradation but the first core domain is not involved in polymerization (Yehudai-Resheff *et al.*, 2003).

**KH RNA binding domain**

PnpA contains two RNA binding domains: the KH domain and S1 domain. The KH domain is next to the second RNase PH core domain. The KH RNA binding protein is an evolutionarily conserved protein as it is found in bacteria, archaea and eukaryotes (Grishin, 2001). Mutation in the KH domain of the PNPase C-terminal affects the autoregulation of PNPase (Garcia-Mena *et al.*, 1999). Deletion of PNPase KH domain in *E. coli* resulted in 19-fold reduction in specific activity compared to wild type PNPase when the rate of product formation is measured by a macroarray analysis (Stickney *et al.*, 2005).

**S1 RNA binding domain**

PnpA contains the S1 RNA binding domain next to the KH domain at the rear end of the C-terminal region of PnpA. The S1 RNA binding protein, first identified in an *E. coli* ribosomal protein, is highly conserved. The S1 RNA binding domain interacts with ribosomes and mRNA with sequence specificity (Subramanian, 1983) and initiates translation (Boni *et al.*, 1991). Bycroft *et al.* (1997) determined the structure of the S1 RNA binding domain of *E. coli* PNPase using NMR. Its structure is similar to cold shock proteins and could be able to bind to various ligands by protein-RNA or protein-protein interactions. They discovered that the S1 RNA binding domain is present in one or more copies in various RNA-associated proteins (Bycroft *et al.*, 1997). In *Yersinia* PNPase, the S1 domain is important for its normal growth at lower temperatures such as $5^0$C (Rosenzweig *et al.*, 2005).

#### 1.10.1.3. Other ribonucleases which perform similar functions to PNPase

RNase II or RNase PH can also perform similar or parallel functions to PNPase. RNase II and PNPase are both ribonucleases which are equally able to degrade mRNA and in the absence of one, the other is able to take over the function (Donovan and Kushner, 1986). RNase II is also involved in 3' to 5' single stranded mRNA degradation processing and degrades an antisense RNA that regulates Tn10/IS10 transposition (Pepe *et al.*, 1994). Although RNase II is involved in the phosphorolytic degradation of mRNA, PNPase processes mRNA in a low energetically expensive fashion, as the high-energy phosphate bonds of the ribonucleotide are retained in the PNPase reaction (Deutscher, 1993b, Deutscher and Reuven, 1991). Thus, PNPase is more efficient than RNase II for mRNA degradation and is essential for low-temperature survival of *E. coli* and *B. subtilis* (Yamanaka and Inouye, 2001). As a result, PNPase is more effective than RNase II in modulating the polyadenylation of mRNAs (Mohanty and Kushner, 2000b).

RNase PH is one of the exoribonuleases, a phosphorolytic nuclease and a reversible enzyme similar to PNPase, which is essential for tRNA processing and cell viability if the cells are lacking RNase II, D, BN and T (Deutscher, 1990a, 1990b; Kelly *et al.*, 1992). Like PNPase, it is involved in the addition of residues to the 3' terminus of RNA molecules (Ost and Deutscher, 1990) and also in removal of nucleotides of tRNA (Jensen *et al.*, 1992).

### 1.10.1.4. PNPase as a global regulator of virulence

A variety of regulatory roles for PNPase have been identified. These include the regulation of bacteriophage P4 immunity in *E. coli*, competence in *B. subtilis* and adaptation to cold shock in many species (Luttinger *et al.*, 1996; Piazza *et al.*, 1996; Goverde *et al.*, 1998; Mathy *et al.*, 2001; Yamanaka and Inouye, 2001). Recently, PNPase has been shown to regulate virulence related genes in *Salmonella enterica* (Clements *et al.*, 2002).

*S. enterica* causes acute and persistent typhoid fever in humans (Hansen-Wester and Hensel, 2001; Pang *et al.*, 1995; Buchwald and Blaser, 1984; Edelman and Levine, 1986). The genetic determinants of *Salmonella* infection have been characterised. It requires SPI 1 (*Salmonella* Pathogenicity Island 1, Clements *et al.*, 2002) for its invasion and SPI 2 to grow intracellularly (Hensel *et al.*, 1998; Hansen-Wester *et al.*, 2000). Clements *et al.*, (2002) discovered the role of PNPase in *Salmonella* virulence when investigating *S. typhimurium* strain MC2, a spontaneous PNPase mutant strain. The mutation in PNPase led to a truncated PNPase protein reduced from 721 aa to 599 aa, which lost its S1 RNA binding domain and had defective 3' to 5' phosphorolytic activity. Microarray analysis of strain MC2 showed the mRNA levels of 87 ORFs were increased compared with MC1 (the wild type), and most belonged to SPI-1 and SPI-2 members. This was supported by an increase in invasion and intracellular multiplication of MC2 and also MC71 (a single point allelic mutation introduced in MC1 *pnp* gene) in MDCK cells and BALB/c mice and J774-A.1 cells. Thus PNPase was found to repress virulence by negatively controlling SPI-1 and SPI-2 expression (Clements *et al.*, 2002). In 2006, Ygberg *et al.* grew MC1 and MC71 in a medium (MM5.8) which is similar to an intravacuolar system, and carried out gene expression analysis. They found that the genes

responsible for flagella and motility were suppressed in MC1. In MC71 there was an increase in the expression of the *spv* virulence cluster present in SPI-1, whose genes are involved in the stress response and DNA repair (Ygberg *et al.*, 2006).

In *Yersinia spp.* the PNPase S1 domain is important for the optimal function of the type III secretion system (TTSS). A PnpA mutant lacking the S1 domain was found to be less virulent than the wild type strain (Rosenzweig *et al.*, 2005; 2007).

### *1.10.2. glpA*

In 1999, Whittle *et al.* discovered the *glpA* gene in *D. nodosus* immediately upstream from *tRNA-ser*$_{GCU}$, which is an integration site for the *intA*, *intB*, *intC*, and *intD* elements. The gene *glpA* is found in all the strains, and encodes a putative 64 aa protein that has 74% similarity to a RsmA (repressor of secondary metabolite) in *Erwinia carotovora*, a plant pathogen. GlpA and RsmA are also highly related to the carbon storage regulator (CsrA) in *E. coli* (Romeo *et al.*, 1993; Cui *et al.*, 1995).

#### 1.10.2.1. Role of CsrA

In 1993 Romeo *et al.* identified and characterized the molecular structure of the gene *csrA*, which exhibits pleiotropic effects on gene expression in *E. coli*. They found that *csrA* encodes a 61 amino-acid RNA binding protein which is involved in the regulation of glycogen biosynthesis (Romeo *et al.*, 1993). A mutation study of *csrA* suggested that *csrA* may encode a global regulatory factor (Romeo and Gong, 1993) with a role in prokaryotic messenger RNA decay (Wellington *et al.*, 1993). CsrA plays a major role in repression and activation of various genes involved in cell growth related functions such

as glycogen synthesis and catabolism, gluconeogenesis, biofilm formation, glycolysis, carbon starvation, motility flagellum synthesis and acetate metabolism (Romeo *et al.*, 1993; Sabnis *et al.*, 1995; Liu *et al.*, 1995; Yang *et al.*, 1996; Wei *et al.*, 2000; Wei *et al.*, 2001; Jackson *et al.*, 2002; Dubey *et al.*, 2003; Wang *et al.*, 2005).

CsrA regulates translation of its target genes by binding to the mRNA leaders and blocking the ribosome binding (Dubey *et al.*, 2003). The activity of *csrA* is controlled by two small RNA molecules, CsrB and CsrC. CsrB and CsrC possess similar imperfect repeat sequences (18 in CsrB, 9 in CsrC), primarily localised in the loops of predicted hairpins, which serve as CsrA binding elements. Thus, CsrB and CsrC sequester CsrA, and antagonise its activity. CsrA in turn activates CsrB and CsrC synthesis, so there is a complex autoregulatory loop (Liu and Romeo, 1997; Liu *et al.*, 1997; Romeo, 1998; Wassarman and Sortz, 2000; Gudapaty *et al.*, 2001; Pernestig *et al.*, 2001; Baker *et al.*, 2002; Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003, Dubey *et al.*, 2005).

### 1.10.2.2. Structural studies of CsrA

CsrA is a dimer with two identical subunits each composed of five strands, a small α-helix and a flexible C-terminus. An alanine-scanning mutagenesis study of *E. coli* CsrA found that the β1 and β5 sheets (Fig. 1.5) which are present parallel to each other on the opposite sides of the protein in CsrA could be involved in RNA binding and have a functional role (Gutierrez *et al.*, 2005; Rife *et al.*, 2005; Mercante *et al.*, 2006).

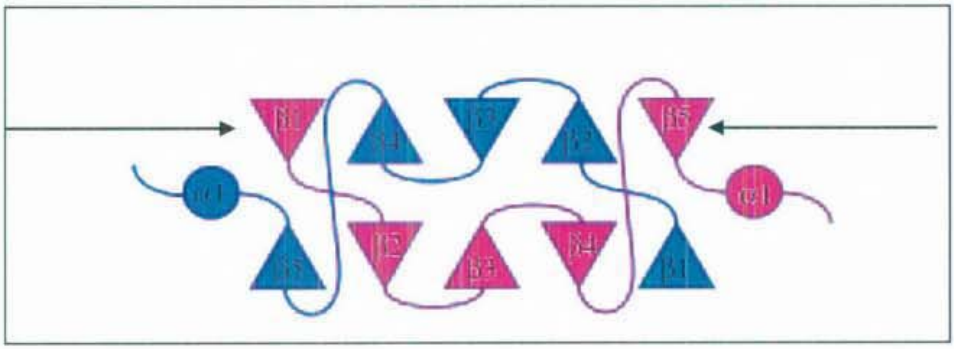


**Fig. 1.5:** CsrA topology diagram showing the connectivity between strands in the two β-sheets. Arrows show β1 (left) and β5 (right) (Gutierrez *et al.*, 2005).

### 1.10.2.3. CsrA as global regulator of virulence

In *Legionella pneumophila* loss of CsrA activates the expression of a number of virulence-associated characteristics such as cytotoxicity, motility, pigmentation, cell shape, stress resistance and evasion of phagosome-lysosome fusion. CsrA is an important factor for efficient replication in broth and macrophages. Thus, CsrA is a global repressor of virulence in *Legionella pneumophila* (Molofsky and Swanson, 2003).

In *Salmonella*, overexpression of CsrA represses virulence. However, a *csrA*⁻ mutant also showed reduced invasion activity, suggesting that CsrA is a global virulence regulator, and that the level of CsrA is critical (Altier *et al.*, 2000; Lawhon *et al.*, 2003).

### 1.10.2.4. RsmA

Mutation studies in *Erwinia carotovora* subsp. *carotovora* 71 of derepressed exoenzyme production led to the identification of a negative regulator gene, *rsmA*. RsmA, an RNA-binding protein is found to promote the decay of many mRNA species (Liu *et al.*, 1998). The Rsm system negatively regulates the production of extracellular plant cell wall degrading enzymes (PCWDEs) which are essential for pathogenicity. Overexpression of

*rsmA* suppresses motility and flagella development in *E. carotovora* subsp. *carotovora*, where the expression of *rsmA* is modulated by *rsmC*, a homologue of *csrC* (Chatterjee *et al.*, 1995; Mukherjee *et al.*, 1996; 1998; Cui *et al.*, 1999).

In *Serratia marcescens*, RsmA regulates swarming motility by regulating the *flhDC* key flagellar regulatory operon, which is involved in sensing temperature, nutrients and swarming motility. Swarming is a complex motility pattern which plays an essential role in virulence gene expression. RsmA in *Proteus mirabilis* showed high sequence similarity to CsrA in *E. coli*, and was also found to suppress swarming motility similar to *S. marcescens*. Thus, RsmA regulates gene expression by controlling mRNA stability and acts as a global regulator of gene expression and virulence in *S. marcescens* and in *P. mirabilis* (Ang *et al.*, 2001; Liaw *et al.*, 2003).

RsmA is also a negative regulator of virulence in *Pseudomonas fluorescens* (Blumer *et al.*, 1999). In *P. aeruginosa*, RsmA is responsible for the pleiotropic posttranscriptional regulation of secondary metabolites by modulating the quorum sensing circuitry (Pessi *et al.*, 2001). The Rsm system also positively regulates the swarming motility, rhamnolipid synthesis and lipase production in *P. aeruginosa* PAO1 in contrast to *S. marcescens* and *P. mirabilis* where it negatively regulates swarming motility (Ang *et al.*, 2001; Liaw *et al.*, 2003; Heurlier *et al.*, 2004). Various other pathways and systems which are important for virulence, including iron acquisition, biosynthesis of the *Pseudomonas* quinolone signal (POS) and the formation of multidrug efflux pumps and motility, were altered in a transcriptome profile study between *P. aeruginosa* PAO1 and an isogenic *rsmA* mutant (Burrowes *et al.*, 2006). RsmA in *P. aeruginosa* positively regulates the virulence

associated type III secretion system during an interaction with airway epithelial cells (Mulcahy *et al.*, 2006).

The RsmA regulatory system is conserved in many enterobacterial species and it has been shown to control diverse phenotypes including motility and expression of virulence factors (Romeo *et al.*, 1993; Cui *et al.*, 1995; Mukherjee *et al.*, 1996). Overexpression of *rsmA* is generally detrimental to cell physiology and can be lethal in certain hosts (Cui *et al.*, 1999). *rsmA/csrA* is not essential in *E. coli* or *Serratia marcescens* (Romeo *et al.*, 1993; Ang *et al.*, 2001). However, a *csrA*-knockout mutation is deleterious in *Salmonella enterica* serovar Typhimurium (Altier *et al.*, 2000).

### 1.10.3. *ssrA* (small stable RNA)

In *D. nodosus*, most virulent strains contain *vrl*, which is integrated next to *ssrA*, a gene which encodes the small stable RNA, tmRNA. tmRNA has properties of both tRNA and mRNA and its structure is highly conserved in bacteria (Komine *et al.*, 1994; Hershberg *et al.*, 2003). However, there is little conservation in the size and sequence of tmRNA which varies between species (Zwieb *et al.*, 1999; Williams, 2002a, 2000b). It has similar properties to tRNA and is charged with alanine, but it does not function as a typical tRNA and does not read any codon (Karzai *et al.*, 2000). However, it plays an important role in maintaining an organised translational system, and acts on the ribosomes which are stalled during translation. tmRNA has a reading frame which is translated and adds a peptide tag to the incomplete protein in the stalled ribosome after transferring its alanyl moiety, and translation ends with the stop codon of tmRNA (Komine *et al.*, 1994; Williams *et al.*, 1999).

Bacterial tmRNA has been found to have a variety of roles, including modulation of phage growth, altering activity of DNA binding proteins in *E. coli*, and DNA replication in *Caulobacter crescentus* (Retallack *et al.*, 1994; Retallack and Friedman, 1995; Withey and Friedman, 1999; Keiler and Shapiro, 2003). In *S. enterica* serovar Typhimurium, *ssrA* plays a role in pathogenesis by inducing genes involved in infection, and is important for the integration of *Salmonella*-specific sequences (Julio *et al.*, 2000). In *Yersinia pseudotuberculosis* mutation of *ssrA* resulted in an avirulent strain, which was unable to cause mortality and survive in mice due to a reduction in the expression and secretion of virulence effector proteins at the transcriptional level (Okan *et al.*, 2006). *ssrA* is also present in the pathogenicity island of *Vibrio cholerae* and the functions are yet to be discovered (Rajanna *et al.*, 2003). Hence, *ssrA* in *D. nodosus* could be involved in altering the expression of virulence factors.

## 1.11. Model for virulence

Two putative global regulators of virulence, GlpA and PnpA, have been identified in *D. nodosus*. The *intA*, *intB*, *intC*, and *intD* elements integrate into two *tRNA*-ser genes immediately downstream from *glpA* and *pnpA*. A model for control of virulence by these integrated elements has been proposed (Whittle *et al.*, 1999) whereby integration of these elements immediately downstream from *glpA* and *pnpA* alters the 3' end of *glpA* and *pnpA* transcripts, thereby altering the activity of GlpA and PnpA. This is supported by the different arrangement of integrated genetic element arrangements next to *glpA* and *pnpA* in benign and virulent strains. In general, virulent strains have the *intA* or *intC* element next to *askA* and the *intA* element next to *pnpA* (Fig. 1.6). Strains which do not

fit this pattern are usually benign. Evidence for this model comes from the observation that virulent strain 1311, which had the *intC* element next to *glpA*, lost the *intC* element during laboratory culture. This resulted in a strain, 1311A, which had *intB* next to *glpA*, as the *intB* element was located adjacent to the *intC* element. The new strain, 1311A, had lost protease thermostability, a virulence characteristic.

Reverse transcriptase PCR studies have shown that transcripts from *glpA* and *pnpA* extend into the adjacent integrated elements (Melis, 2001; Ting, 2001). This confirms that the transcripts from *glpA* and *pnpA* are different in different strains. In addition to GlpA and PnpA, the activity of tmRNA could be modulated by the integration of the *vrl* into *ssrA*. Thus, the integration sites for five genetic elements in *D .nodosus* are located next to genes which have been identified as global regulators of virulence.

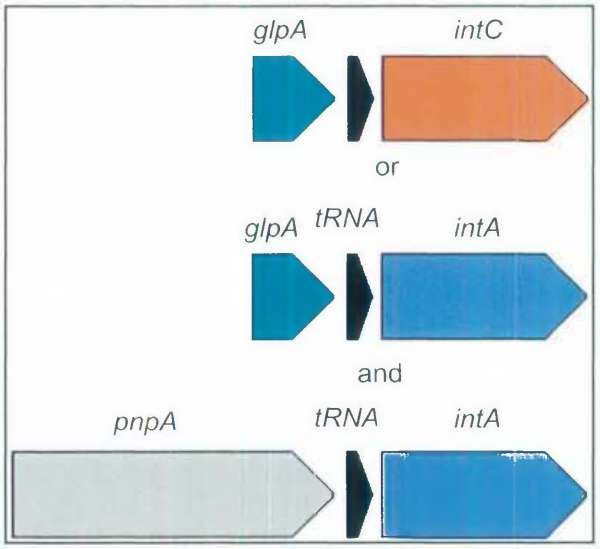



**Fig. 1.6:** Model for the arrangement of integrated elements in virulent strains of *D. nodosus* (Whittle *et. al.*, 1999). In this model, all virulent strains have either the *intA* or the *intC* element integrated next to *glpA*, and have the *intA* element next to *pnpA*.

## 1.12. Proposed Project

The aim of this project is to investigate the hypothesis that PnpA is a virulence repressor in *D. nodosus*. The work will be conducted as follows:

1. Study the role of the *pnpA* gene in both benign and virulent strains by knocking out the 3' end of this proposed virulence repressor gene. In this part of the study, a selection of benign and virulent strains will be analysed. These strains will be transformed using a construct which will truncate the C-terminus of PnpA. The parent and transformed strains will be analysed by the Southern blot technique to confirm that the truncation had been achieved.
2. The effect of the PnpA knockouts on PNPase activity as well as two virulence characteristics, protease thermostability and twitching motility, will be determined.
3. If there is a change in thermostability or twitching motility observed between the parent and *pnpA* knockout transformant strains, complementation of the *pnpA* knockout will be attempted.
4. Real-time PCR will be used to develop assays to measure mRNA levels from *pnpA* and the protease gene *aprV2*.