CHAPTER ONE:

General introduction.

1.1) Footrot

1.1.1) Pathology

Ovine footrot is a mixed bacterial infection of the epidermal structures of the hoof. Bacteria which contribute to the disease include *Dichelobacter nodosus* (formerly *Bacteroides nodosus*, Dewhirst *et al.* 1990), *Fusobacterium necroforum*, *Actinomyces pyogenes*, *Bacillus* spp, *Clostridium perfringens*, *Staphylococcus aureus* and *Spirochaeta penortha* (Beveridge, 1941; Depiazzi and Penhale, unpublished). *D. nodosus* is the specific causative agent of ovine footrot since it is the only organism of the footrot flora which is capable of reproducing the disease when applied as a pure culture (Beveridge, 1941). Within the affected hoof, *D. nodosus* is found primarily at the advancing margin of the lesion (Egerton *et al.*, 1969). *D. nodosus* and *F. necrophorum* are the predominant organisms found in the footrot lesion with *D. nodosus* penetrating fresh tissue which is then made necrotic by *F. necrophorum* and invaded by the host of other microbes (Roberts and Egerton, 1969; Egerton *et al.*, 1969). *Clostridium* spp and *F. necrophorum* may aid the survival of *D. nodosus* in dormant lesions by repressing its growth, thereby enabling *D. nodosus* to survive within the hoof without causing disease (Depiazzi and Penhale, unpublished). *D.*

nodosus, unlike *F. necrophorum*, is not a member of the normal bacterial flora of the hoof (Stewart, 1989).

Footrot infection starts in the interdigital cleft of the hoof causing irritation and swelling of the interdigital skin (Egerton and Parsonson, 1969). The disease, depending on the severity, progresses down the inside wall of the hoof then under the hoof horn and may cause underrunning of the sole of the hoof and ultimately separation of the horn from the sole (Figure 1.1). In the most severe cases the lesions may continue up the outside wall of the hoof causing further separation of the horn from the sole (Stewart, 1989). A footrot infection is characterised by the foul smelling necrotic exudate produced from the necrotic tissue (Beveridge, 1941)

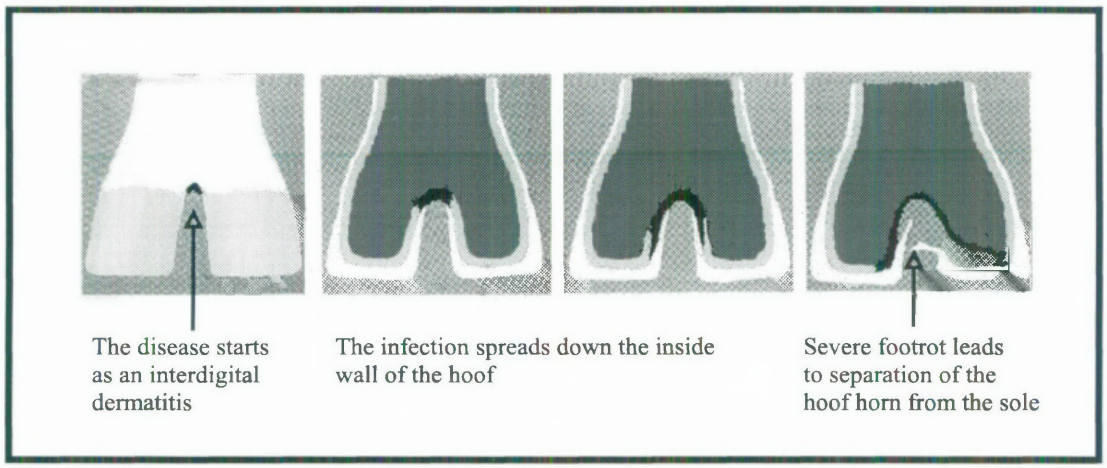

Figure 1.1: Diagrammatic representation of the ovine hoof showing the progression of footrot depending on the virulence of the *D. nodosus* strain involved in the infection (modified from Walker, 1988).

1.1.2) Clinical manifestations

D. nodosus causes a spectrum of disease in sheep (Alexander, 1962; Stewart *et al.*, 1982a, 1984, 1986), ranging from an interdigital dermatitis (benign footrot), through underrunning lesions in a couple of feet (intermediate footrot) to severe

necrotic separation of the horn of the hoof in most feet (virulent footrot). The clinical forms of the disease are dependent on the invasiveness of the *D. nodosus* strain which causes the infection. Thus, strains of the bacterium are classified as benign, intermediate or virulent.

Benign footrot is characterised by an interdigital dermatitis with, very rarely, underrunning to the edge of the sole (Figure 1.1). Many members of the flock may be infected but lameness is not usually apparent (Glynn, 1993) with each animal having only one or two feet affected (Stewart, 1989), and self-curing tends to occur as the season dries. With an intermediate footrot infection, a small proportion of the flock have severe lesions with underrunning and separation of the sole and horn of the hoof. This separation may extend to, and sometimes continue along, the outside wall of the hoof, causing lameness (Figure 1.1; Stewart, 1989). Some financial losses occur but most sheep recover as the season dries, although some may remain chronically infected unless treated.

Virulent footrot is a persistent chronic condition with severe extensive necrotic underrunning of the whole hoof (Figure 1.1), usually involving more than one foot of each animal, in a high percentage of sheep within the affected flock (Stewart, 1989). The foul-smelling necrotic exudate produced in the severe lesions allows the rapid spread of the disease. Lameness is a prominent feature of virulent footrot and significant loss of condition has been observed (Stewart *et al.*, 1982a, 1984, 1986). Spontaneous recovery does occur in a proportion of sheep but others if untreated may maintain chronic infections through to the next season and beyond (Beveridge, 1941; Stewart, 1989). Under appropriate conditions *D. nodosus* may persist for many years in clinical lesions (Beveridge, 1941).

1.1.3) Epidemiology

Footrot is an endemic disease affecting the sheep industry world wide, in temperate climate regions enjoying good rainfall (Walker, 1988; Stewart, 1989). In Australia, in New South Wales (NSW) alone there are at least 5,000 properties with footrot (Egerton and Raadsma, 1993). Figure 1.2 indicates the regions within NSW where the risk for the expression of footrot is highest and lowest (Egerton and Raadsma, 1993). The areas of NSW with the lowest risk of a footrot outbreak tend to be the drier regions while the highest risk areas enjoy the most rainfall or are extensively irrigated (Locke and Coombes, 1994). Within regions of Australia endemic to footrot, outbreaks usually occur seasonally when there are warm, moist conditions and lush or long pasture. Outbreaks may also occur in Australia in late autumn, particularly when good rains have fallen, but predominantly occur in late spring with the onset of warm, moist conditions (Stewart, 1989). In times of drought the prevalence of footrot is greatly reduced but the disease does not disappear (Egerton, 1983).

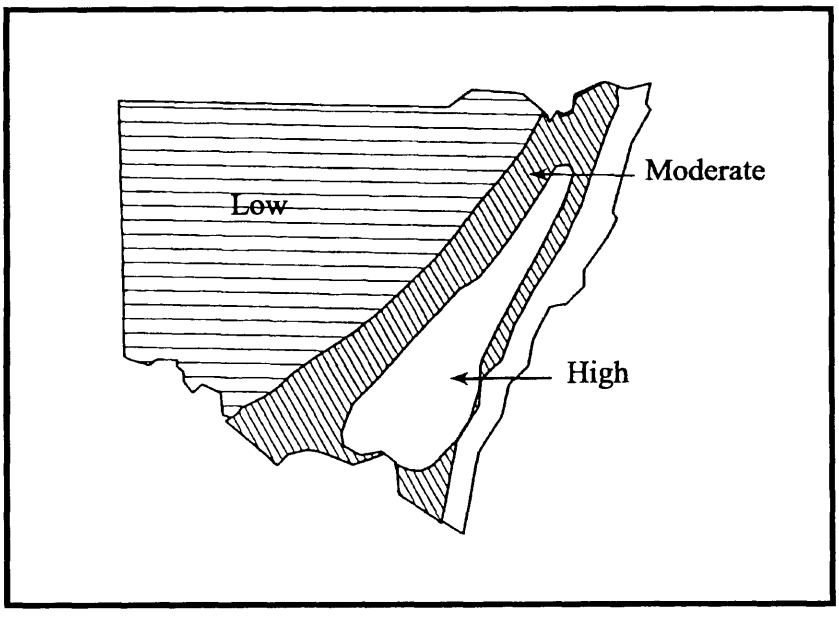

Figure 1.2: Map of NSW, Australia, showing the low, moderate and high risk regions for the expression of footrot (Egerton and Raadsma, 1993).

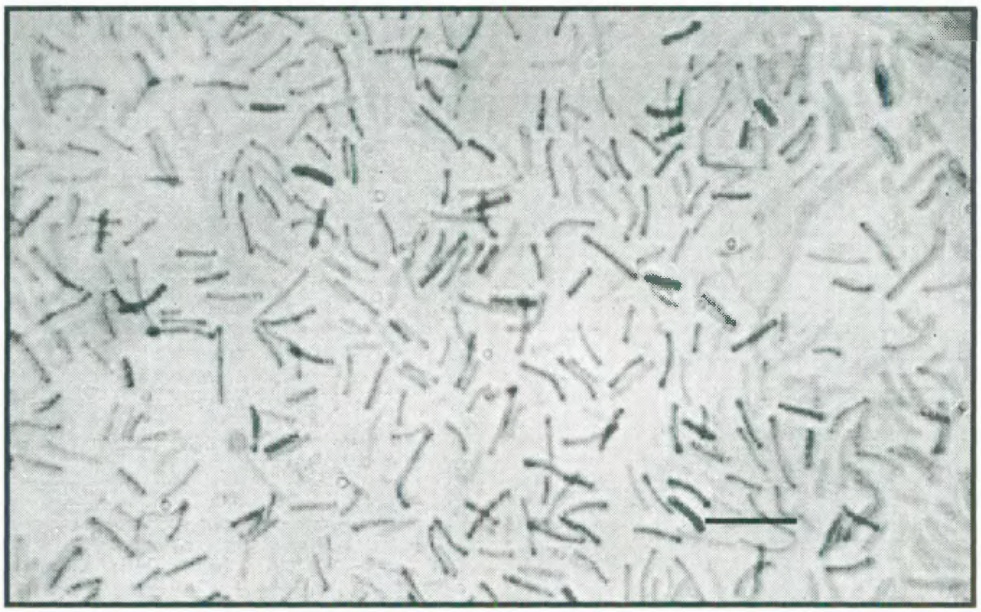

Figure 1.3: Gram stain of a pure culture of *D. nodosus* strain C305. Bar = 10μm

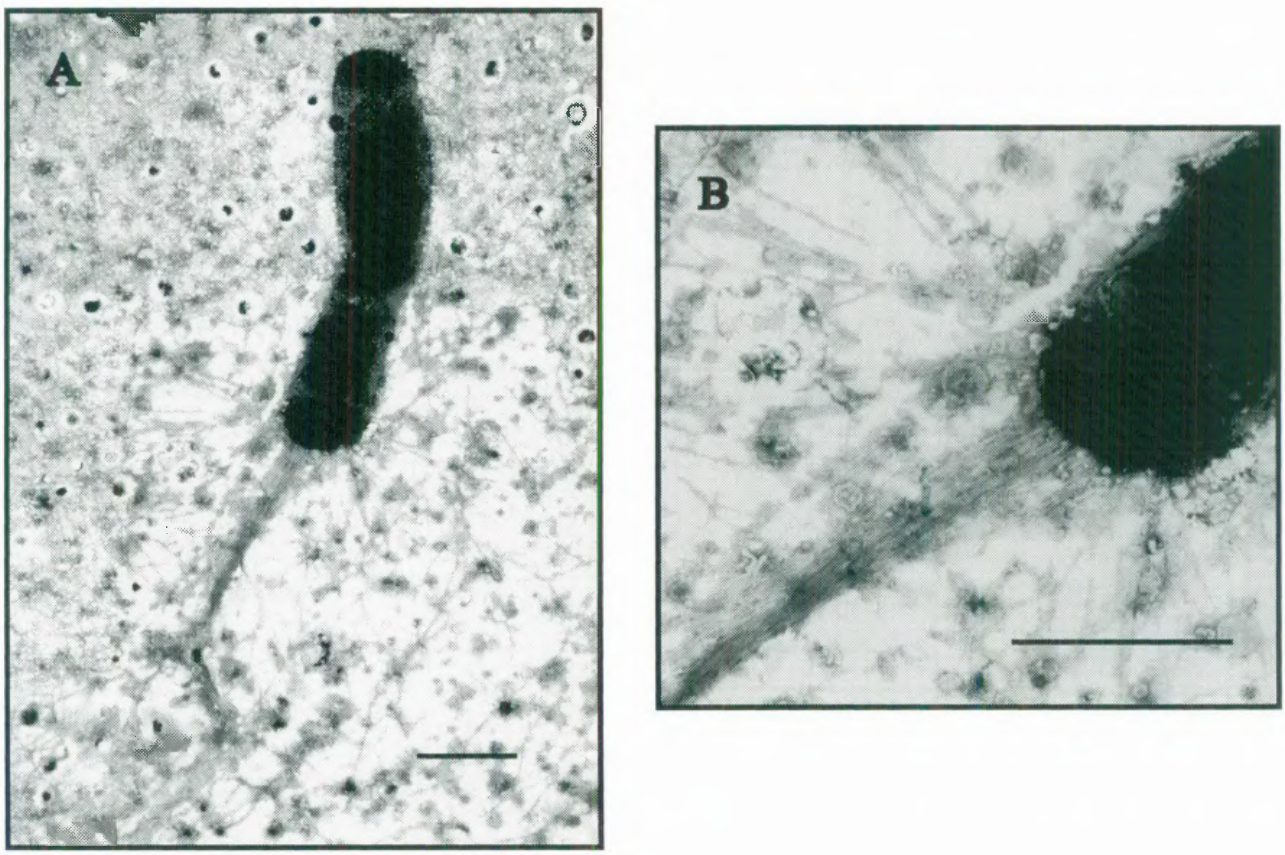

Figure 1.4: **A** and **B** - Electron micrographs of a *D. nodosus* cell, strain H1215, stained with phosphotungstic acid. Bars = 1 μm.

After an outbreak of footrot a proportion of apparently-healed sheep may harbour virulent strains of *D. nodosus* in the hoof but show no obvious signs of the disease (Thomas, 1962; Egerton and Raadsma, 1993). Infection in a small focus under healthy horn is difficult to identify and poses an important source of reinfection (Stewart, 1989). This relapse of inapparent cases into clinical disease may occur up to two years after supposedly-successful treatment and healing (Egerton and Raadsma, 1993). Benign strains of *D. nodosus* were also found to be present in a significant proportion of sheep which had apparently healed after a footrot outbreak. The interdigital area was identified as another site where these bacteria could survive from year to year (Glynn, 1993). Although *D. nodosus* is not a part of the normal healthy hoof flora, the bacterium is totally host-dependent for multiplication and long-term survival (Stewart, 1989).

1.1.4) Transmission

Transmission requires warm, moist conditions, some form of damage to the interdigital skin of the hoof (usually water-softened skin) and a source of infection (Stewart, 1989). The disease is transmitted through a flock by the necrotic exudate from exposed lesions being transferred onto pasture or mud and thereby contaminating the feet of other sheep (Beveridge, 1941). The infectivity, on muddy or wet pasture, of the exudate from affected sheep was found to last no more than 7 days (Beveridge, 1941; Laing and Egerton, 1978). Any factors which increase the number of sheep in a given area, such as high stocking rates and limited access to creek flats or waterways, increase the likelihood of transmission. Sheep in feedlots or housed indoors under wet, crowded conditions are also at high risk (Stewart, 1989) and

transmission has been observed between flocks in damp sheep yards (Whittington, 1995). The transmission of *D. nodosus* by mechanical means, i.e. by kangaroos, rabbits, birds, blowflies, water runoff, tractor tires or farmers' boots, is unlikely. However, mechanical transmission was suspected to have occurred in one study (Stewart *et al.*, 1984) but this was not conclusively proven. Hoof paring implements pose the highest risk of accidental transfer of the bacteria between sheep (Whittington, 1995).

Environmental factors play a large role in the progression of the disease (Stewart *et al.*, 1984). Depending on the season, the transmission period, in Australia, for *D. nodosus* can be as short as 3 to 4 weeks but is usually 6 to 12 weeks (Egerton *et al.*, 1972, 1983). Under ideal conditions (wet and warm weather) transmission may continue for at least 16 weeks (O'Meara *et al.*, 1993). Transmission ceases when pastures dry (Stewart, 1989). The type of pasture is also important due to the effects of trauma on the interdigital skin (Beveridge, 1941; Woolaston, 1993) since normal, healthy, dry skin is not susceptible to infection. Long seed-bearing grass may cause more physical damage to water-softened interdigital skin than waterlogged short grass, thus increasing the animals' vulnerability to *D. nodosus* (Woolaston, 1993).

1.1.5) Host susceptibility

Some breeds of sheep are naturally more resistant to footrot (Beveridge, 1941; Emery *et al.*, 1984). Merinos, the major breed of sheep in Australia, tend to have more severe infections compared to their crosses with British breeds (Egerton *et al.*, 1972). The lesions in the feet of British breeds tend to be confined to the interdigital cleft and are generally of short duration as compared to Merinos, where a much larger number of feet develop severe underrunning of the sole of the hoof (Emery *et al.*,

1984). The integrity of the interdigital skin appears to be the major reason for the resistance of some breeds of sheep to footrot. When the interdigital skin was scarified and challenged with lesion material, previously-resistant sheep developed similar necrotic lesions to the susceptible breeds (Stewart, 1989). Under natural conditions the moisture-softened interdigital skin of the hoof could easily become more susceptible to *D. nodosus* infection after damage by stones, sticks, thistles, stubble (Stewart, 1989) or long seed-bearing grasses (Woolaston, 1993).

The age and sex of the sheep is also known to affect the susceptibility to infection and the severity of infection (Egerton and Raadsma, 1993). Lesions were found to increase in severity with an increase in age of the sheep (Woolaston, 1993). Footrot infections are also substantially worse in ram lambs when compared to ewe lambs (Skerman, 1986). Individual sheep within the same flock can have varying degrees of the disease ranging from chronic footrot, through to transient infections and a minority with no infection at all (Egerton *et al.*, 1983). A strain of sheep have been selectively bred for this innate resistance to footrot (Skerman and Moorhouse, 1987). A breeding program over 15 years has produced sheep which tend to have less severe infections and lesions which heal more rapidly than lesions in similar breeds. This method of artificially infecting sheep with footrot and selectively breeding from more resistant individuals has been proposed as having a role in long term strategies for the control of footrot (Skerman and Moorhouse, 1987). However, the feasibility of breeding for resistance to footrot has to be carefully balanced with the breeding for other desired traits, such as wool quality.

The severity of ovine footrot is thus the net result of three factors: virulence of the strain of *D. nodosus*, environmental conditions and innate resistance of the host (Emery, 1988; Egerton and Raadsma, 1993).

1.1.6) Footrot in ungulates other than sheep.

Cases of footrot have been identified in cattle (Thomas, 1962; Egerton and Parsonson, 1966a), goats and deer although the disease is less severe than the ovine equivalent (Egerton, 1989). Within the ungulates, other than sheep, the lesions tend to be confined to the interdigital skin with underrunning and separation of the hoof horn from the sole of the foot occurring less frequently (Egerton, 1989). Footrot is clinically and economically important in the cattle and goat industries due to lameness, production losses and treatment costs. Footrot lesions may also predispose the feet of cattle to more damaging, deeper infections (Egerton, 1989).

Benign strains of *D. nodosus*, capable of causing footrot in sheep, have been found in bovine interdigital lesions indicating that cattle may act as carriers of ovine footrot (Richards *et al.*, 1980). A strain of *D. nodosus*, which is virulent in sheep, has also been isolated from a bovine hoof (Egerton and Parsonson, 1966a) thus indicating that cattle may act as a source for a virulent footrot outbreak in sheep. This inter-species transmission has important implications for the control and eradication of virulent ovine footrot (Richards *et al.*, 1980). The treatment and prevention costs of footrot, therefore, have to be extended to all ungulates on an infected property thus greatly increasing the economical cost of the disease. Goats also constitute a reservoir for all variants of *D. nodosus* (Egerton and Raadsma, 1993) with transmission from goats believed to have been responsible for footrot outbreaks in the New England area of New South Wales, Australia, during 1984 (Scott-Orr, 1986). Therefore, wild or feral ungulates may act as a constant source of reinfection making the eradication of virulent footrot a virtual impossibility.

1.1.7) Economic importance

Infection with virulent footrot leads to significant body-weight loss, resulting from loss of appetite, with the amount of weight lost related to the severity of the lesions (Stewart *et al.*, 1984; Marshall *et al.*, 1991a). Over a 2 year footrot infection trial, an overall 12-15% loss of sheep body weight, a 5% decrease in wool fiber length and a 7% loss in fleece weight in infected animals was observed (Walker, 1988). Marshall *et al.* (1991a) obtained similar results. The infected feet of sheep were also attractive to sheep blowfly (*Lucilia cuprina*). When the sheep was at rest the fly-blown hoof was in contact with the fleece promoting an area of body strike (Marshall *et al.*, 1991a) thus causing further loss in the animals' condition. Also observed was a decrease in mean wool fiber diameter. Since fineness is a desired quality of wool, it has been suggested that the total value of the wool may be enhanced to more than compensate for the decrease in quantity (Marshall *et al.*, 1991a). However, this was not found to be the case in trials with intermediate and benign footrot which have also been found to cause wool production losses in infected sheep. The value of the fleece from infected sheep was 7% less per head than the value of the wool from the uninfected group (Glynn, 1993).

For the producer, there are many additional costs of footrot, resulting from death of animals (in the most severe untreated cases), decrease in quantity, quality and value of the wool produced, treatment and control of the disease (including equipment, drugs, vaccines, and chemicals), treatment of flystrike, labour and time spent on these extensive treatments (Marshall *et al.*, 1991a) and the reduced value of all sheep on an affected property (Egerton and Raadsma, 1993) plus possible quarantine of the property. More indirect losses due to footrot include disruption of normal farm

operations, a need for surveillance of sheep, lost market opportunities, animal welfare considerations and the social stigma associated with having sheep with footrot (Stewart, 1989).

1.1.8) Treatment and prevention

1.1.8.1) Antibiotics

Treatment of sheep with a single antibiotic injection significantly reduced the number of footrot-infected feet and sheep. A penicillin/streptomycin antibiotic combination was found to help cure 80% of experimental cases of footrot (Egerton and Parsonson, 1966b). Furthermore, a lincomycin/spectinomycin combination has a 92.5% cure rate in clinical trials (Venning *et al.*, 1990). Erythromycin has since been found to be equally effective and a cheaper treatment for virulent footrot than the aforementioned combinations of antibiotics (Webb Ware *et al.*, 1994). Antibiotic treatment can be costly when a large number of sheep need to be treated, therefore the use of antibiotics is only justifiable in valuable animals, such as rams and stud sheep (Stewart, 1989). Widespread use of antibiotics also promotes the development of antibiotic-resistant bacteria with potential for transfer of resistance between organisms thereby presenting a health risk to both sheep and humans.

1.1.8.2) Footbathing

Formalin has been used as an antiseptic footbath for many years and has been reasonably effective and is relatively inexpensive (Stewart, 1989). The toxicity of formalin is a major drawback to this treatment. Prolonged exposure is harmful to the sheep and the farmer (Stewart, 1989), and safe disposal of the formalin solution is an important environmental concern. The labour-intensive process of foot paring, the cutting away of necrotic hoof material to expose all infected areas, is required with

formalin treatment as formalin does not penetrate hoof keratin and hence only acts as a surface disinfectant (Malecki and McCausland, 1982).

Footbathing in zinc sulphate was found to give a marked improvement in recovery from footrot lesions (Demertzis *et al.*, 1978). The addition of the surfactant sodium lauryl sulphate (SLS) was found to greatly increase the penetration of zinc into ovine hoof horn (Malecki and McCausland, 1982). Zinc sulphate is also more environmentally-friendly than formalin, is comparable in price, has a longer shelf life and is safer for the sheep and the farmer (Stewart, 1989). Zinc sulphate/SLS foot baths prior to exposure to *D. nodosus* can also offer protection from footrot infections for 2 - 3 weeks (Marshall *et al.*, 1991b) but continued prophylactic use is required to protect sheep for the entire transmission period and the cost may be difficult to justify.

A cure rate of 84% in footrot-affected sheep treated by footbathing in zinc sulphate/SLS has been observed (Malecki and Coffey, 1987). Foot paring prior to bathing feet was not found to improve the cure rate compared to bathing only (Malecki and Coffey, 1987; Casey and Martin, 1988). While zinc sulphate foot baths reduce the number, duration and severity of footrot lesions it does not penetrate interdigital tissues sufficiently to eliminate *D. nodosus* (Glynn, 1993) which may therefore survive to the next favourable season.

1.1.8.3) Vaccination

In Australia a vaccine consisting of whole *D. nodosus* cells derived from isolates representing all serogroups (see section 1.2.2 below) has been commercially available (O'Meara *et al.*, 1993). However, the complex antigenic mixtures required in a vaccine against all serogroups of *D. nodosus* results in a poor response from the immune system of the sheep, due to antigenic competition, and therefore can only offer short-term protection (O'Meara *et al.*, 1993). Protection from infection is

unlikely to last 12 weeks and under the correct environmental conditions for transmission of footrot may not last 8 week post vaccination (O'Meara *et al.*, 1993).

Fimbriae have been identified as the primary protective antigens in *D. nodosus* vaccines (Stewart *et al.*, 1982b, Every and Skerman, 1983a). Recombinant fimbrial vaccines have been shown to be as protective and therapeutic as whole cell or purified fimbrial vaccines although a fimbrial vaccine against all *D. nodosus* serogroups still results in only short-term protection (Egerton *et al.*, 1987; Stewart and Elleman, 1987). The main advantage of the whole cell vaccine is that it provides partial protection against serotypes not contained in the vaccine, most probably due to shared surface antigens (Morck *et al.*, 1994), whereas the fimbrial-based vaccines do not confer protection across serogroups (Stewart and Elleman, 1987).

Vaccines are better used as a preventative treatment, since protection rates are higher than cure rates (Glenn *et al.*, 1985). Used in conjunction with other treatments, vaccination may reduce the number of cases of footrot to a level where eradication is possible (Stewart, 1989). Currently footbathing in zinc sulphate/SLS combined with vaccination is the most widely used method of treating footrot (Stewart, 1989).

1.1.8.4) Control and Eradication

Control of the spread of footrot from one flock to another may be facilitated by the use of an ELISA (enzyme-linked immunosorbent assay) test which detects sheep antibodies to *D. nodosus*. This assay may indicate whether the animals of a flock have been exposed to *D. nodosus* and are therefore potential carriers (Whittington *et al.*, 1990; Whittington and Marshall, 1990). Evidence of a footrot vaccine having been used is also detected by an ELISA, indicating prior flock exposure to *D. nodosus*. Since treated sheep are not necessarily free of *D. nodosus* the possible spread of the disease may be curtailed by containment and careful observation of the

suspicious animals. Development of more specific ELISA tests may enable them to be more widely used for the diagnosis of virulent ovine footrot (Whittington and Egerton, 1994).

Severe disease reduces wool quality and production (Marshall *et al.*, 1991b) therefore control and eradication is directed at the most virulent strains of *D. nodosus* (Stewart, 1989). It has been suggested that low-intermediate strains be classified as benign and high-intermediate strains as virulent for ease of control, treatment and eradication (Allworth, 1988). In New South Wales the footrot strategic plan requires quarantining of properties diagnosed as having virulent footrot and compulsory eradication is required by culling or treatment of affected sheep. In the high rainfall areas of southern NSW and Victoria, eradication programs have been run over a number of years with mixed success (Egerton and Raadsma, 1993). Over a four year period a survey of 105 properties in NSW running eradication programs found that only 35% of these flocks completely eradicated *D. nodosus*, 44% of flocks had residual *D. nodosus* infections, which were mostly benign, but some intermediate strains may have been present, and 20% of properties failed to eradicate virulent footrot (Egerton and Raadsma, 1993). By contrast South Australia and Western Australia, which have drier climates, have used statewide eradication programs to reduce the prevalence of virulent footrot to relatively low levels (Stewart, 1989). The probability of eradicating virulent footrot in endemic regions, excluding whole flock disposal, has been estimated to be only 50% (Allworth, 1988).

1.2) *Dichelobacter nodosus*

1.2.1) Bacteriology

D. nodosus is a Gram negative, rod shaped, obligate anaerobe with knob-like ends (Beveridge, 1941). Figure 1.3 shows a Gram stain of a pure culture of *D. nodosus* strain C305. The bacterium is relatively small, 1 - 1.7 x 3 - 6 μm (Skerman, 1989), and is a fastidious, slow-growing organism. *D. nodosus* produces extracellular proteases (Skerman, 1977) which are associated with the penetration of the hoof tissue (Depiazzi *et al.*, 1991). These proteases also have some elastolytic activity (Stewart, 1979) and proteases from virulent and benign strains differ in their properties (Kortt *et al.*, 1983; Kortt *et al.*, 1994).

D. nodosus has type 4 polar fimbriae, or pili, (Every, 1979) similar to other pathogenic bacteria such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Moraxella bovis*, *Moraxella nonliquefaciens* and *Vibrio cholerae* (Ottow, 1975; Dalrymple and Mattick, 1987; Elleman, 1988; Tonjum *et al.*, 1991). Fimbriae from this class have an unusual amino acid, N-methylphenylalanine, as the first residue of the mature protein and are therefore often referred to as MePhe type fimbriae or pili (Marrs *et al.*, 1985). *D. nodosus* fimbriae are filamentous projections concentrated at one pole of the bacterium and are up to 5 μm long and 4.5 - 6 ηm wide (Stewart, 1973). Figure 1.4 shows electron micrographs of a *D. nodosus* cell from strain H1215 negatively stained with phosphotungstic acid (PTA). The mass of fimbriae are visible at one pole. Type 4 fimbriae are involved in movement by 'twitching' (MacRae *et al.*, 1977) with a retraction of the fimbriae into the cell

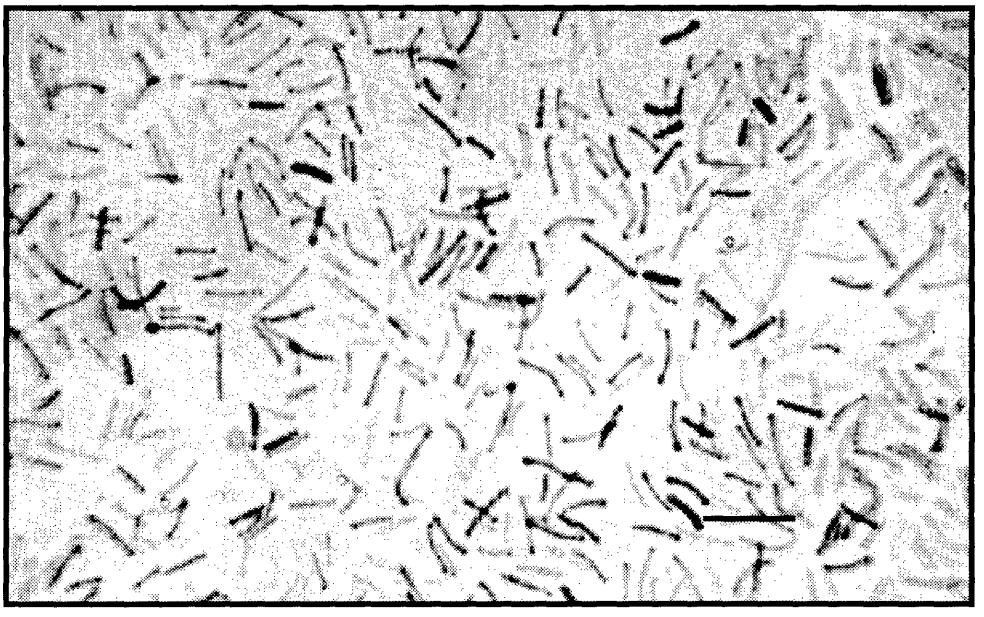

Figure 1.3: Gram stain of a pure culture of *D. nodosus* strain C305. Bar = 10μm

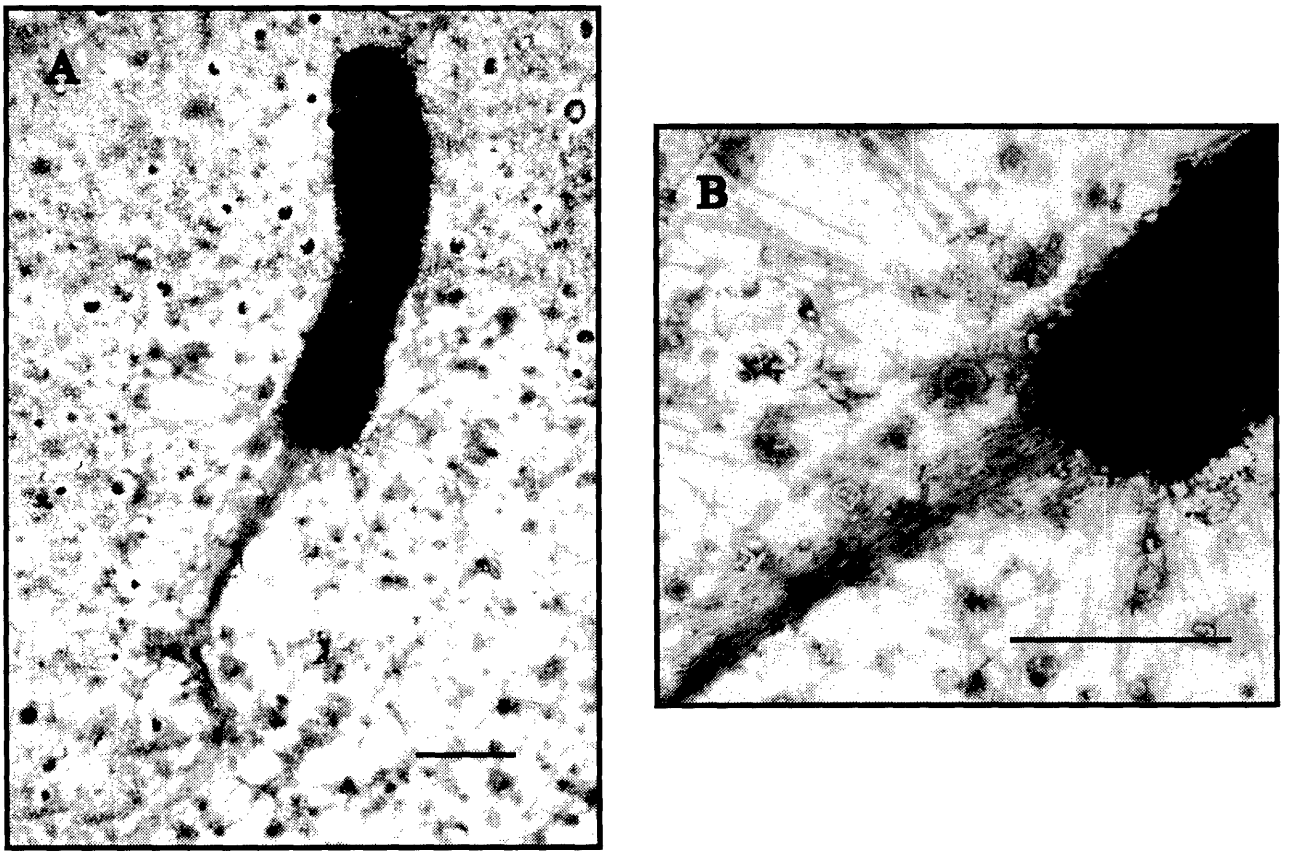

Figure 1.4: **A** and **B** - Electron micrographs of a *D. nodosus* cell, strain H1215, stained with phosphotungstic acid. Bars = 1 μm.

believed to form the basis of the twitching motility (Bradley, 1980). This twitching motility aids the spread of the bacteria through the epidermal matrix of the hoof (Walker *et al.*, 1973).

1.2.2) Identification and classification

Preliminary identification is by direct Gram stain of lesion material and location of the characteristic slightly-curved Gram-negative rod (1 to 1.7 x 3 to 6 μm) with polar swellings, which may be more deeply stained (Figure 1.3; Skerman, 1989). Colony morphology is the other main distinguishing feature of *D. nodosus*. The colonies vary depending on the media but primarily consist of a central zone with a prominent smooth dome, a narrow smooth mid-zone and a peripheral finely granular zone (Skerman, 1989). A series of standard microbiological tests can be carried out to positively confirm that the organism is *D. nodosus* (Skerman *et al.*, 1989). Recently a PCR test based on 16s rRNA has been developed for the rapid identification of *D. nodosus* from a lesion (La Fontaine *et al.*, 1993).

When *D. nodosus* was originally identified it was given the name *Fusiformis nodosus* (Beveridge, 1941). The bacterium was subsequently classified into the *Bacteroidaceae* family (Holdeman, 1984) and given the genus name *Bacteroides*. More recently, *Bacteroides nodosus* was reclassified, based on 16s rRNA sequence comparisons, into the family *Cardiobacteriaceae* and the genus *Dichelobacter* (Dewhirst *et al.*, 1990).

D. nodosus isolates are classified into 9 serogroups, A to I, based on the fimbrial antigens (Claxton, 1981; Claxton *et al.*, 1983; Claxton, 1986). Each serogroup is further divided into serotypes, to give a total of 18 serotypes, with serogroup B the most complex containing 4 serotypes (Claxton *et al.*, 1983; Stewart *et al.*, 1991). Due

to the complexity of serogroup B this group is often divided into two groups, B1 and B2 effectively classifying *D. nodosus* into 10 serogroups (Raadsma *et al.*, 1994). Classification systems vary worldwide but this is mainly due to serotypes being classified as serogroups (Stewart *et al.*, 1991; O'Meara *et al.*, 1993). Significant proportions of outbreaks of ovine footrot involve mixed serogroups with up to 6 serogroups in a flock and as many as 4 serogroups in a single animal (Claxton *et al.*, 1983). The fimbrial antigens are not virulence-associated so each serogroup contains benign, intermediate and virulent strains of *D. nodosus*. It is not uncommon for strains of *D. nodosus* with varying degrees of virulence to be associated with a single outbreak of footrot (Claxton *et al.*, 1983).

1.2.3) Pathogenesis

Bacterial pathogenesis relies on several traits including motility, adherence, entry into host and establishment of disease (Finlay and Falkow, 1989), each of these functions requiring a number of characteristics. The two main properties of *D. nodosus* known to be responsible for increased virulence in high intermediate and virulent strains are increased motility and characteristic extracellular proteases.

Greater surface translocation, from enhanced twitching motility, is believed to be associated with virulent strains of *D. nodosus* (Depiazzi and Richards, 1985). The degree of fimbriation is not a virulence characteristic *per se* as some benign strains are well fimbriated and some virulent strains have poor fimbriation (Depiazzi and Richards, 1985; Stewart *et al.*, 1985, 1986). However, the twitching motility of the fimbriae is greater in more virulent strains (Depiazzi *et al.*, 1991). This increased twitching motility is not due to any variation in the fimbrial subunit-encoding genes

(Billington and Rood, 1991) and may therefore be due to genes of the protein export system identified in relation to twitching motility (Whitchurch *et al.*, 1990).

Type 4 fimbriae are quite common among Gram-negative bacteria and have been putatively identified in some Gram-positive species (Dalrymple and Mattick, 1987). The ability of bacteria to colonize eukaryotic cells appears to be greatly enhanced by fimbriae and twitching motility (Dalrymple and Mattick,1987). The fimbriae of *D. nodosus* do not appear to have an adhesive function (Stewart, 1975), unlike type 4 fimbriae from other bacteria (Ottow, 1975). An extra layer of the *D. nodosus* cell wall has been proposed to mediate adherence to the host surfaces (Every and Skerman, 1983b).

There are differences in extracellular proteases secreted by virulent and benign strains of *D. nodosus* (Depiazzi and Richards, 1979; Kortt *et al.*, 1983; Kortt *et al.*, 1994). Virulent strains of *D. nodosus* produce proteases which are more thermostable (Depiazzi and Rood, 1984). The thermostable proteases produced by virulent *D. nodosus* strains are associated with the greater separation of the horn of the hoof from the sole in virulent footrot (Depiazzi *et al.*, 1991). Proteases of more virulent strains also have higher elastolytic activity (Stewart, 1979) which is believed to aid in the penetration of the hoof tissues.

Footrot is a complex disease involving the interaction of one or several strains of *D. nodosus* with varying degrees of virulence, the environmental conditions, host susceptibility and the interaction with other opportunistic bacteria. The economic importance of the disease is manifest in the prevention and treatment costs, the loss of wool production, animal condition and value. The accurate diagnosis of virulence of the primary *D. nodosus* strain involved in the infection is crucial to the farm economy and the control and treatment of the disease.

1.2.4) Diagnosis of virulence

1.2.4.1) Clinical diagnosis

Clinical diagnosis of footrot gives an indication of the virulence of the causative organism involved in an epidemic. Footrot virulence, and hence virulence of the *D. nodosus* strain, are graded on the severity of the lesions (extent of underrunning), the number of feet affected in the individual animal and the number of animals affected in the flock (Whittington and Nicholls, 1995). Each clinical form of the disease starts as an interdigital dermatitis which makes it difficult to distinguish between benign and virulent forms of footrot in the early stages of infection (Egerton and Parsonson, 1969). Severe infections take two to three weeks to develop once *D. nodosus* has gained access to the epidermal structures of the hoof (Walker, 1988). The full expression of virulence is also dependent on environmental factors such as topography, moisture, temperature and the type of pasture (Stewart, 1989). Therefore under unfavourable conditions for expression of footrot a virulent strain may appear as a low intermediate or benign clinical infection (Egerton and Raadsma, 1993) thus surviving untreated through to the next more favourable season. The clinical diagnosis is still highly subjective even though scoring systems for the severity of footrot lesions are available (Liu *et al.*, 1994) and the virulence of the infecting organism is only determined after the disease is established.

1.2.4.2) Laboratory diagnosis

The primary test used to distinguish between benign, intermediate and virulent strains of *D. nodosus* involves analysis of the extracellular proteases. Colony morphology, however, can give an indication of the virulence of the *D. nodosus* isolate as virulent strains are more motile and thus have larger colonies (Depiazzi and

Richards, 1985; Depiazzi *et al.*, 1990). In estimating surface translocation Depiazzi *et al.* (1990) found that colony diameter was more accurate than quantifying the degree of cellular twitching.

A proteinase degradation test has been developed to distinguish between benign and virulent isolates of *D. nodosus* based on extracellular proteases (Depiazzi and Richards, 1979). The proteases of virulent strains of *D. nodosus* are more thermostable than proteases of benign strains. Thus, protease activity is observed after exposure to high temperatures. Results are obtained within 14 days of sampling (Kortt *et al.*, 1982; Depiazzi and Rood, 1984; Green, 1985; Depiazzi *et al.*, 1990). An elastase test, involving the clearing of elastin in the culture medium over several weeks, yields a result in 20 to 28 days from the time of sampling (Stewart, 1979). The early appearance of elastase activity is associated with the more virulent strains of *D. nodosus* while benign strains have very little, or late, activity or none at all. While, the elastase test is time-consuming, there is a good correlation with the clinical manifestations of the *D. nodosus* strain (Liu and Yong, 1993).

Iso-enzyme patterns or electrophoretic zymograms of extracellular proteases are characteristic of virulent and benign *D. nodosus* isolates. Results are obtained in around 14 days from sampling (Every, 1982; Kortt *et al.*, 1983), but this method is unable to differentiate intermediate from virulent strains (Gordon *et al.*, 1985). The benign pattern indicates the strain lacks virulence but a virulent pattern does not guarantee virulence (Kortt *et al.*, 1983). Considered together the results of colony typing, protease thermostability, elastase activity and zymogram patterns provide a broad discrimination among virulent, intermediate and benign strains (Stewart *et al.*, 1986). Virulent strains of *D. nodosus* also appear to have some agarolytic activity, with pitting observed when grown on plate media (Skerman, 1975; Stewart *et al.*,

1986). This phenomenon of agarolytic activity has also been observed in a number of other type 4 fimbriated bacteria (Froholm and Bovre, 1972; Henrichsen *et al.*, 1972; Dalrymple and Mattick, 1987).

Laboratory experiments to test the virulence of *D. nodosus* isolates can often take several weeks due to lengthy culture periods and the abundance of contaminating organisms in lesion material (Pitman *et al.*, 1994). Recently, a gelatin gel test has been developed, which measures protease thermostability and total activity (Palmer, 1993) and correlates well with the elastase test (Stewart, 1979), but is much quicker than the original thermostability test (Depiazzi and Richards, 1979; Liu and Yong, 1993). Pitman *et al.* (1994) have now modified the media and technical procedures to reduce the time of laboratory diagnosis to 8 days. However, the laboratory analysis of virulence is still time consuming and somewhat subjective (Rood *et al.*, 1996). The tests of elastase activity, the gelatin gel test and electrophoretic zymogram may give varying results or may be interpreted differently in different laboratories. Differences in results due to different brands and/or batches of elastin are also known to occur (Liu *et al.*, 1994). Different veterinary laboratories also vary in the type and number of tests conducted and there has long been a need for a more rapid, reliable test to determine the virulence of *D. nodosus* isolates (Rood and Yong, 1989).

1.3) Regions of the *D. nodosus* genome associated with virulence

1.3.1) Isolation of virulence-associated *D. nodosus* DNA

To identify virulence-associated DNA segments from the genome of *D. nodosus* a library, in the plasmid pUC18, of genomic DNA from the virulent *D. nodosus* strain A198 was screened with labelled genomic DNA from the virulent strain A198 and the benign strain C305. Katz *et al.* (1991) used this differential hybridisation method to isolate three gene regions which were present in the virulent strain and were absent from the benign strain. The clones containing these gene regions were designated pJIR313, pJIR314B and pJIR318. Hybridisation analysis of these clones to genomic DNA from 101 veterinary isolates of *D. nodosus* revealed that these sequences are present in almost all virulent strains (pJIR313 in 94% and pJIR314B, pJIR318 in 100%) and absent from most benign strains (pJIR313 and pJIR314B sequences were absent from 94% and pJIR318 sequence was absent from 67%). This situation of gene regions associated with virulence, i.e. present in virulent strains and absent from most benign strains, is often found in pathogenic bacteria (Finlay and Falkow, 1989). An interesting feature of these genomic *D. nodosus* sequences is they do not correspond to the genes for any previously identified virulence determinants (Katz *et al.*, 1991) such as the fimbriae (or pili), outer membrane proteins (*omp*) or proteases (Emery, 1988). Katz *et al.* (1991) proposed a method of screening *D. nodosus* isolates with a combination of the three plasmid clones which could be used to rapidly determine the virulence of the isolate.

Restriction endonuclease profiles of genomic DNA have been investigated as a means of determining the virulence of *D. nodosus* isolates (McGillivery *et al.*, 1989). Certain bands in the restriction enzyme profile appeared to be associated with severe clinical disease but the main use proposed for this technique has been identifying individual strains of the bacteria and thus tracing the source of the outbreak (McGillivery *et al.*, 1989). This method, as with the standard laboratory analysis, requires the isolation and growth of pure *D. nodosus* cultures, a long and time consuming process. Isolation of virulence-associated and benign-associated DNA sequences has been achieved (Liu and Yong, 1993; Liu, 1994) by a similar method as that used by Katz *et al.* (1991). However, each of the virulence-associated clones are from the same regions as those originally identified by Katz *et al.* (1991; Rood *et al.*, 1996). A polymerase chain reaction (PCR)-based assay using the newly isolated probes was introduced (Liu and Webber 1995) but there was a large overlap in the PCR products from virulent and benign strains (Liu *et al.*, 1995). It was concluded that the PCR was specific for only two of the many virulence factors of *D. nodosus* and further characterisation of virulence in this bacterium is required (Liu *et al.*, 1995).

1.3.2) Comparison of gene probes with conventional virulence diagnosis

A comparison of the diagnostic use of the gene probes isolated by Katz *et al.* (1991) and more conventional diagnosis was recently conducted (Rood *et al.*, 1996). The results from the use of the probes compared favourably to results from the primary and most widely used methods for diagnosis and identification of virulent strains of *D. nodosus*. The DNA probes were found to yield the most accurate separation of benign, intermediate and virulent strains. Compared to the clinical

designation of some strains and the elastase test the probe test was 88% accurate for the identification of virulent isolates. There was 94% successful correlation with the gelatin-gel (protease thermostability) test and 98% accuracy when compared to the electrophoretic zymogram assay. A new test for virulence, based on monoclonal antibodies to virulence-associated proteases, also correlated well with the probe tests with 95% of virulent strains identified by both tests (Rood *et al.*, 1996). The conversion of the probe-based test to a PCR test was also proposed with the aim of increasing the ease and speed of identification of the strain. Rood *et al.* (1996) concluded that a PCR test based on the virulence-associated regions (Katz *et al.*, 1991) could provide a rapid and reliable diagnostic test for the differential diagnosis of clinical isolates of *D. nodosus*. Results could be obtained within 24 to 48 hours after sampling rather than 3 to 4 weeks, as with current tests, although other diagnostic tests should be carried out to confirm and check the results of the PCR test.

1.3.3) Characterisation of the *vrl* region

Further analysis of the virulence-associated clones has revealed that pJIR313 and pJIR314B are part of a large virulence-related locus (*vrl*) 27 kb in length (Rood *et al*, 1994, Haring *et al.*, 1995). The *vrl* region has an unknown function but analysis of the limits of the virulence-associated sequences revealed a bacteriophage-like attachment site (*att*) and a gene for 10Sa RNA, suggesting that this region arose by the integration of a genetic element into the genome. The attachment site in a phage genome, *attP*, consists of a relatively large region of DNA with a core sequence of approximately 20 b.p. while an identical 20 b.p. forms the site of integration into the bacterial genome, *attB* (Campbell, 1980, 1981). The crossover at these attachment sites leaves an *attL* and *attR* at the left and right-hand ends, respectively, of the

integrated prophage. Analysis of the left-hand end of the *vrl* identified a 10Sa RNA gene, into which bacteriophages are known to insert (Kirby *et al.*, 1994), and two putative bacteriophage attachment sites, *attL* and *attL2* (Haring *et al.*, 1995), identified by their similar structure to the bacteriophage P4 *att* site. Comparison of the left-hand end of the *vrl* and sequences from the benign strain C305 showed the sequence similarity stopped just downstream of the *attL* site. The *attL2* site is located within the *vrl*, 300 b.p. downstream of *attL*, and has an unknown function. The *vrl* has been proposed to have arisen by the integration of a phage, plasmid or transposon (Haring *et al.*, 1995) but no *attR* site was identified at the right-hand virulent/benign junction. The absence of sequences hybridising to pJIR313 in some virulent strains of *D. nodosus* is possibly due to deletion of part of the *vrl* region (Rood *et al.*, 1996) in those strains.

Preliminary sequence analysis of the *vrl* has identified some open reading frames (ORFs) with similarity to genes encoded by bacteriophages and plasmids (Billington *et al.*, 1996b). The deduced amino acid sequences from the *vrl* ORFs have similarity to either the DEAH family of helicase-related proteins, DNA methylases or proteins involved in resistance to bacteriophage infection. No *vrl* ORFs have been identified that can be directly related to virulence (Billington *et al.*, 1996b).

1.3.4) Analysis of the *vap* regions

The third virulence-associated clone, pJIR318, isolated by Katz *et al.* (1991) is unrelated to the *vrl* region and has been termed the *vap* (virulence associated proteins) region(s) (Katz *et al.*, 1992). It is quite common for virulent strains of *D. nodosus* to contain multiple copies, or partial copies, of the sequences found in the pJIR318 clone. The virulent *D. nodosus* strain A198 contains two complete copies and one

partial copy of the pJIR318 sequences, termed *vap* regions 1, 2 and 3 (Katz *et al.*, 1994). Within the genome of strain A198 *vap* regions 1 and 3 have been located adjacent to one another (Katz *et al.*, 1994) and are part of one large 12 kb *vap* region (Figure 1.5; Cheetham *et al.*, 1995). *Vap* region 2 is not close to *vap* region 1/3 and is located at least 150 kb further along the *D. nodosus* chromosome (Figure 1.5; Katz *et al.*, 1994). The extensive characterisation of these *vap* regions is the primary concern of this laboratory and the results prior to and in conjunction with the work for this thesis are summarized in the following section (section 1.4).

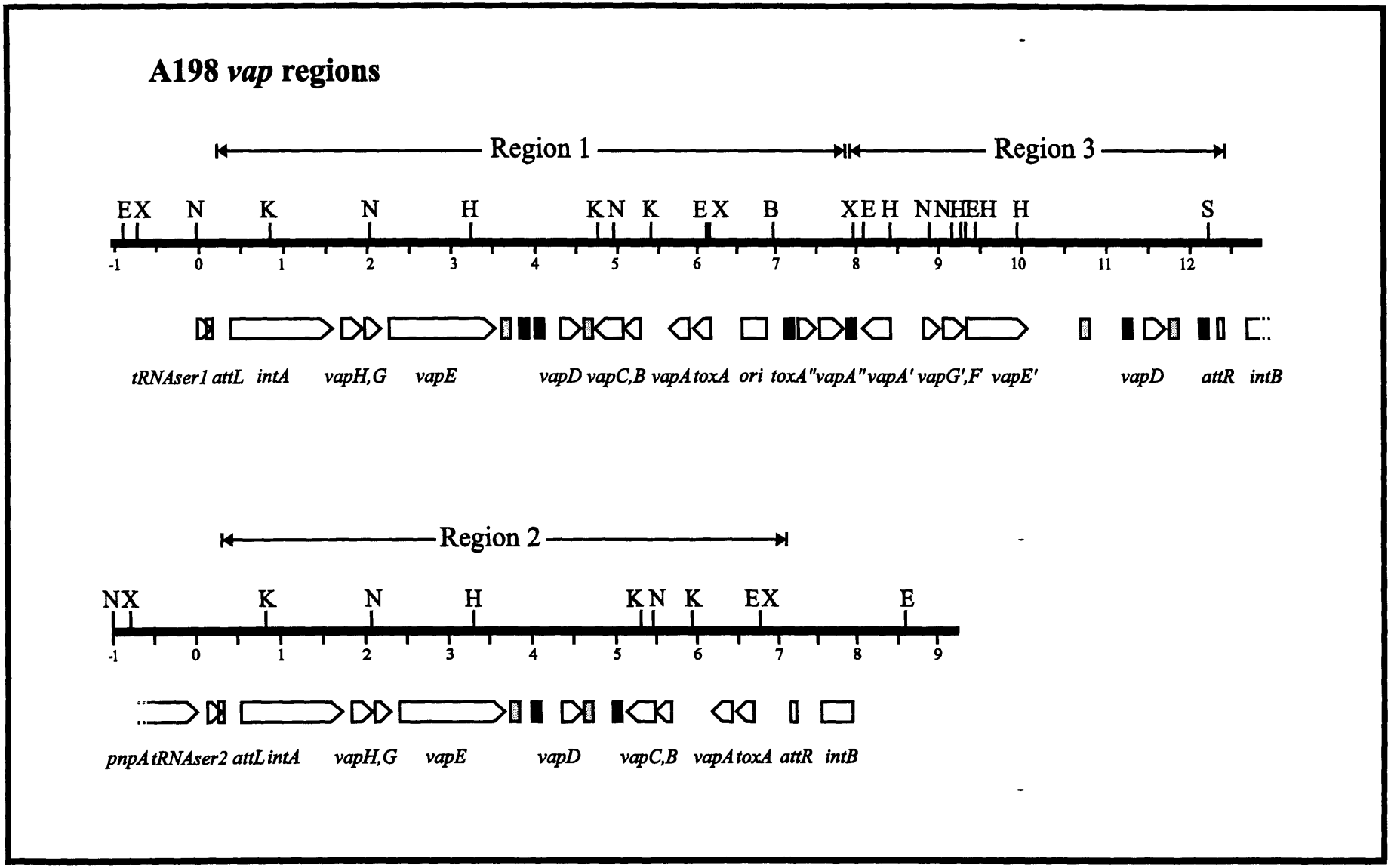

Figure 1.5: Restriction map of *vap* regions 1, 2 and 3 from *D. nodosus* strain A198. The size of the region is indicated on the map (in kb) and the restriction sites indicated are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nru*I (N), *Sac*I (S), and *Xho*I (X). The location, size and orientation of the genes are shown by open arrows. The *att* sites are represented by small open boxes, the 102 b.p. repeats are indicated by small dark shaded boxes and the 103 b.p. repeats are indicated by light shaded boxes.

1.4) Further characterisation of the *vap* regions.

The *vap* regions of the virulent *D. nodosus* strain A198 are shown in Figure 1.5. A number of the potential genes associated with the *vap* regions have been named due to their similarity to genes of known function. These include a tRNA gene, *tRNA-ser1*, integrase gene, *intA*, and toxin genes, *toxA*. The remaining potential genes have been designated *vapA-vapH*.

1.4.1) Evidence of an extra-chromosomal origin of the *vap* regions.

1.4.1.1) Integrase gene.

A gene designated *intA*, at the left-hand end of *vap* region 1 (Figure 1.5) was found to have high sequence identity to the integrase genes of the P4-like bacteriophages (Cheetham *et al.*, 1995). The similarities included 41.5% amino acid identity with the integrase of retronphage ϕR73 (Sun *et al.*, 1991), 35.4% identity with P4 integrase (Pierson and Kahn, 1987) and 35.2% amino acid identity with the integrase of bacteriophage Sf6 from *Shigella flexneri* (Clark *et al.*, 1991). This class of P4-like integrase proteins has been classified into a family of site specific recombinases including the integrases of λ, ϕ80, P22, P2, 186 and P1 (Argos *et al.*, 1986). Similarity among this family of recombination genes, from the lambdoid phages, is primarily in the C-terminal domain indicating that the *int* gene proteins are distantly related while still maintaining similar functions. This type of recombinase catalyses the joining of DNA from two replicons at specific recognition sequences or attachment sites, *att*. Divergence is also manifest in the orientation of the integrase

gene in each bacteriophage but all are located adjacent to the *attP* site, *att* site located on the phage (Leong *et al.*, 1986).

P4-like bacteriophages are small satellite phages dependent on a helper phage for head, tail and lysis genes (Six, 1975). P4 DNA replication and lysogenization, however, are independent of the helper (Lindqvist and Six, 1971; Six and Klug, 1973; Calendar *et al.*, 1981). The P4 phage is able to use any P2-like phages as a helper, such as 186, 299, PK, P3 and the ϕ-D series (Bertani and Bertani, 1971). Many other phages serologically related to P2 have been isolated suggesting they are quite numerous and widespread (Bertani and Six, 1988). Thus P4, and possibly a P4-like phage, have a wide host range including *E. coli*, *Serratia* spp, *Rhizobium* spp, *Klebsiella* spp, *Shigella dysenteriae* and *Pseudomonas putida* (Lagos and Goldstein, 1984; Bertani and Six, 1988).

Plasmids carrying this type of integrase gene are also known. The conjugative plasmid pSAM2 of *Streptomyces ambofaciens* has an *attP int* and *xis* region similar to λ, ϕ80 and P22 and is able to exist as a plasmid or integrate into the host genome (Boccard *et al.*, 1989). The pSAM2 *int* gene has C-terminal similarities to integrase proteins from bacteriophages P2, 186, P22, P1, λ, ϕ80 and P4 and N-terminal similarities with the integrases of P2, 186, P22 and P1 phages. The conjugative plasmids pSE211 and pSE101 of *Saccharopolyspora erythraea* also carry similar integrase genes (Brown *et al.*, 1990, 1994) and are able to integrate into the host genome.

Conjugative transposons from *Bacteroides* spp are also known to contain this type of site-specific recombinase (Salyers *et al.*, 1995). These transposons are an unusual group of mobile genetic elements which are found integrated into the

bacterial genome and are able to excise, circularize and transfer themselves to other bacteria (Salyers *et al.*, 1995). This class of conjugative transposons are virtually always associated with the transfer of tetracycline resistance (Tc^r) genes and hence have been called Tc^r elements (Shoemaker *et al.*, 1989). Conjugative transposons have many varied properties. They are transposon-like in that they excise from and insert into DNA, and plasmid-like in that they have a covalently closed circular transfer intermediate and are transferred by conjugation but the circular intermediate has not been observed to undergo replication (Salyers *et al.*, 1995). Conjugative transposons are also bacteriophage-like in that the excision and integration resembles that of temperate bacteriophages, due to the integrase.

This type of integrase gene is also encoded by another class of genetic element known as integrons (Stokes and Hall, 1989). Integrons consist of a 5' conserved region, approximately 1.36 kb long, a 3' conserved region, usually longer than 2 kb, and a central variable region (Stokes and Hall, 1989). The integrase gene is located in the 5' conserved region and is responsible for the insertion, deletion and rearrangement of the variable region, or gene cassette (Hall *et al.*, 1991; Collis and Hall, 1992). The variable region of the integron contains one or more genes, with the same orientation, which are most commonly antibiotic resistance genes. The antibiotic resistance genes, in both plasmids and transposons, of many Gram-negative bacteria are associated with integrons (Stokes and Hall, 1989).

Although members of this family of site-specific integrase genes are found on a variety of genetic elements, the integrases highly related to *intA* all belong to bacteriophages. The IntA protein is most similar to the integrases from bacteriophages ϕR73, P4 and Sf6.

1.4.1.2) Identification of a tRNA gene upstream of *vap* region 1.

A series of subclones were constructed from the λ clones of *D. nodosus* genomic DNA from strain A198 (Figure 1.5; Cheetham *et al.*, 1995) and were used as probes in hybridisation experiments with genomic DNA from the virulent strain A198 and the benign *D. nodosus* strain C305. Clones which hybridised to genomic DNA from A198 but not to genomic DNA from C305 were considered part of the virulence-associated, *vap*, region. In this way, the virulent/benign junctions of *vap* regions 1 and 3 were localized in the genomic DNA (Cheetham *et al.*, 1995). Examination of the DNA sequence at the left-hand end of *vap* region 1 revealed the presence of a 93 b.p. gene with 80.6% nucleotide identity to the serine tRNA genes, *serV* and *ser-tRNA-3*, of *E. coli* (Ish-Horowicz and Clark, 1973; Yamada and Ishikuri, 1973). Hence, the gene was named *D. nodosus tRNA-ser1*(Figure 1.5).

Some members of the integrase family, such as P4 and ϕR73, are known to integrate into the ends of tRNA genes such that the *int* gene is located adjacent to the 3' end of the tRNA, as with the *vap* regions (Figure 1.5). The P4 *attB* (*att* site located on the bacterial chromosome) sequence is the 3' end of a leucine tRNA gene, *leu-X*. A P4 cryptic prophage appears to be integrated at the 3' end of *leu-X* gene in *E. coli* strain K12 (Pierson and Kahn, 1987). Bacteriophage ϕR73 is known to use the selenocystyl tRNA gene in *E. coli* as an integration site (Inouye *et al.*, 1991; Sun *et al.*, 1991). This putative *vap* genetic element therefore appears to have integrated into the *D. nodosus* genome via the action of the site-specific recombinase, *intA*, using the 3' end of a serine tRNA gene as an *attB* site (Cheetham *et al.*, 1995).

1.4.1.3) tRNA genes as integration sites

The use of tRNA genes as integration sites for genetic elements enables the spread of these elements among heterogeneous bacterial populations due to the highly conserved structure of the tRNA genes (Reiter *et al.*, 1989). The multiple copies of tRNA genes within the bacterial chromosome also provide for multiple integrations (Cheetham and Katz, 1995). A number of other phages are also known to integrate into tRNA genes. These include bacteriophage SSV1 from the archaebacterial genus *Sulfolobus* which integrates into a *tRNA-arg* gene (Reiter *et al.*, 1989); HP1 phage of *Haemophilus influenzae* which integrates into a *tRNA-leu* gene (Hauser and Scocca, 1992) and P22, in *E. coli*, which uses a threonine tRNA gene (Lindsey *et al.*, 1989, 1992; Pierson and Kahn, 1987).

The conjugative plasmids pSE211 and pSE101 are known to integrate into *tRNA-phe* and *tRNA-thr* genes respectively (Brown *et al.*, 1990, 1994). The integration of these plasmids is known to involve the λ-like integrase encoded by the plasmid. Other conjugative plasmids have been found to use tRNA genes for site-specific integration. The SLP1 plasmid of *Streptomyces coelicolor* (Omer and Cohen, 1986) was found to integrate into a tyrosine tRNA gene and the *Nocardia mediterranei* plasmid pMEA100 (Madon *et al.*, 1987) integrates into a phenylalanine tRNA gene (Reiter *et al.*, 1989). This use of tRNA genes appears to be a common occurrence with examples available from eukaryotic organisms as well. The σ transposable element in *Saccharomyces cerevisiae* integrates into the 5' end of a number of yeast tRNA genes (Del Rey *et al.*, 1982). There are many other examples of genetic element integration into or near tRNA genes (Cheetham and Katz, 1995). This relatively common evolutionary mechanism appears to have led to the acquisition of the *vap* regions by *D. nodosus*.

1.4.1.4) Both *vap* and *vrl* appear to be integrated genetic elements

Another class of RNA gene known as small stable RNA, 10Sa RNA, can adopt a tRNA-like structure (Komine *et al.*, 1994), and has been reported as the site of integration of a P4-like cryptic prophage in *E. coli* (Kirby *et al.*, 1994). A 10Sa RNA gene has been identified adjacent to the virulence-associated, *vrl*, region of the *D. nodosus* genome (Haring *et al.*, 1995) and a bacteriophage-like attachment site has been identified at the left-hand junction of the *vrl* and the 10Sa RNA gene. This indicates that both of the virulence-associated genomic DNA regions of *D. nodosus* identified by Katz *et al.* (1991) have been acquired by the integration of genetic elements. No integrase gene has been identified within the *vrl*, as yet, and there does not appear to be an *attR*, right-hand attachment site, at the right-hand virulent-benign junction of the *vrl* (Haring *et al.*, 1995). Genomic rearrangements and deletions may have been responsible for the removal of the *attR* site and possibly other sections of the *vrl* integrated element (Haring *et al.*, 1995; Rood *et al.*, 1996).

1.4.1.5) *att* sites of the *vap* regions

A 19 b.p. sequence from the 3' end of the *tRNA-ser1* gene has been identified as the *att* site for integration of the *vap* region (Cheetham *et al.*, 1995). The *att* site is repeated at the right-hand end of *vap* region 3, thus defining the limits of the virulence-associated DNA. The DNA sequence between *attL* and *intA* (Figure 1.5) contains inverted repeats similar to those of P4 which are believed to be involved in integration as part of the *attP* site (Person and Kahn, 1987; Cheetham *et al.*, 1995). The arrangement of attachment sites at either end of the virulence-associated DNA is the expected end result through the action of a site-specific recombinase, *intA*, integrating a plasmid, transposon or bacteriophage into the bacterial genome.

1.4.1.6) *vap* origin of replication

A putative origin of replication, *ori* (Figure 1.5), has been identified within *vap* region 1 (Cheetham *et al.*, 1995). The *ori* consists of a series of copies of two related 21 b.p. sequences and is flanked by A + T rich DNA regions. This structure resembles the *ori* of several plasmids and bacteriophages (Marians, 1992; Nordström, 1990). For example the origin of replication of bacteriophage λ, φ80, and 82 have multiple repeats of a sequence of approximately 20 b.p. which itself contains an internal inverted repeat. The origin has a similar structure and arrangement in all three phages although the sequence of their respective repeats is unique (Furth and Wickner, 1983). Other DNA sequences, *cis*-acting elements, are often involved in the control of replication and copy number in bacterial replicons. Two potential *cis*-acting elements, a *DnaA* box and the integration host factor site, have been identified within the *vap* regions of *D. nodosus* (Cheetham *et al.*, 1995; Galli and LeBlanc, 1997). However, the significance of these sites is unknown and interactions with other replication factors has not been investigated.

1.4.1.7) Origin of the *vap* element - plasmid or bacteriophage?

The *vap* element could have arisen from a bacteriophage or a plasmid as it has features of both. At present it is not possible to distinguish between these two possibilities. The integrase is most highly related to integrases from several bacteriophages (Cheetham *et al.*, 1995) and the arrangement and expression of *vapG* and *vapH* resembles the immunity region of bacteriophage P4 (Whittle and Cheetham, unpublished). However, most of the *vap* genes show similarity to genes found on plasmids (Katz *et al.*, 1992, 1994; Cheetham *et al.*, 1995; Bloomfield *et al.*, 1997), especially *vapA*, *toxA*, *vapB* and *vapC* which are similar to genes involved with the maintenance of conjugative plasmids (section 1.4.3).

1.4.1.8) The *vap* plasmid

The recent discovery, in *D. nodosus* strain AC3577, of a circular DNA molecule consisting primarily of *vap* region 1, reveals more information about the possible evolution of these virulence-associated sequences. This molecule, designated the *vap* plasmid, pJIR896, consists of *vap* region 1, a small segment of *vap* region 3, between *vapD* and *attR* (Figure 1.5), and an insertion sequence, IS*1253*. This plasmid has been proposed as the progenitor of the chromosomal *vap* sequences (Billington *et al.*, 1996a). The entire *vap* region 1 from the *attL* site to the 102 b.p. repeat located just after *vapA"* (Figure 1.5) is contained on pJIR896. The origin of replication, *ori*, contained within *vap* region 1 and pJIR896 was found to function poorly as an origin of replication in *E. coli* (Cheetham *et al.*, 1995) but the identification of pJIR896 indicates that the *ori* must function in *D. nodosus*.

Vap region 3 from *vapA'* through to *vapD* (Figure 1.5) is absent from pJIR896 while in its place is an IS element, IS*1253* (Billington *et al.*, 1996a). IS*1253* is similar to an unusual group of insertion sequences and encodes two genes which are responsible for excision and insertion of the IS element into other DNA replicons. There are no direct or inverted repeats formed on integration and the recognition sequence for integration is unknown (Billington *et al.*, 1996a). IS*1253* is not present in the A198 *vap* regions but is located near the outer membrane protein (*omp*) gene cluster, which is responsible for the production of the major bacterial outer membrane protein (Moses *et al.*, 1995). Unlike the *vap* region, IS*1253* is not virulence-associated since it is absent from many virulent strains and present in many benign strains of *D. nodosus* (Billington *et al.*, 1996a). The pJIR896 plasmid also contains approximately 600 b.p. of *vap* region sequence from the 103 b.p. repeat just downstream of *vapD*, region 3, to the *attR* site (Figure 1.5). Thus extensive

duplications, deletions and rearrangements have occurred to separate this small section from the rest of *vap* region 1 and remove IS*1253*, assuming the plasmid is a precursor to the chromosomal sequences. Alternatively the IS*1253* element may have jumped into the *vap* region causing the deletion of most of *vap* region 3 and possibly prompting the excision and circularization of the remaining *vap* region, leading to formation of the *vap* plasmid.

1.4.2) The nature of integrated genetic elements

1.4.2.1) Is the *vap* plasmid the replicative form of a phage?

While pJIR896 appears to be maintained as a plasmid in strain AC3577, it is possible that it may be, instead, the replicative form of a defective bacteriophage (Billington *et al.*, 1996a). In a similar way, the spontaneous mutant of bacteriophage P4, *vir*1, is maintained in a stable plasmid state. This exemplifies the close evolutionary relationship between some "defective" viruses and plasmids (Goldstein *et al.*, 1982). The close relationship of *intA* to the P4 *int* also lends credence to the possibility of the *vap* element being a defective *vap* bacteriophage. P4 *vir1* can also integrate into the host genome and, through temperature induction, can move from prophage to plasmid and back again. However, the maintenance of P4 in *E. coli* as a plasmid requires an integrated copy of the P4 genome and stability of the plasmid depends on this integrated master copy (Lagos and Goldstein, 1984). *D. nodosus* strain AC3577 does not contain an integrated copy of the *vap* region.

The identification of plasmids carrying integrase genes from this recombinase family (Boccard *et al.*, 1989; Brown *et al.*, 1990, 1994) and the discovery of the *vap* plasmid (Billington *et al.*, 1996a) suggest that the *vap* regions may have been acquired by the integration of a plasmid rather than a bacteriophage. The *Streptomyces*

ambofaciens plasmid pSAM2 appears to parallel the evolution of the *vap* regions quite well with some strains of *S. ambofaciens* containing partial copies of a pSAM2-related element (Boccard *et al.*, 1989), analogous to the partial copies of the *vap* regions in different strains of *D. nodosus* (Bloomfield *et al.*, 1997). Boccard *et al.* (1989) believe pSAM2 has had a complex evolution involving large deletions, which is also similar to the *vap* regions of *D. nodosus*.

DNA rearrangements have often been caused by mobile genetic elements (Kopecko, 1980). Site-specific recombinases and IS elements have been identified as the instigators of DNA rearrangements (Sato *et al.*, 1990; Kleckner, 1981) and both are found associated with the *vap* regions (Cheetham *et al.*, 1995; Billington *et al.*, 1996a; Bloomfield *et al.*, 1997). Thus, the high fluidity of the *D. nodosus* genome at this region may be explained by the presence of the genetic elements associated with the *vap* regions.

It has been known for some time that plasmids and phages can exchange segments of DNA (Kondo and Mitsuhashi, 1964; Barksdale and Arden, 1974). A plasmid maintenance system appears to have been transferred between bacteriophage P1 and the unrelated plasmid R124 in *E. coli* (Lehnherr *et al*, 1993). Bacteriophage P4 has been known to insert into a plasmid (Bertani and Six, 1988) and a close evolutionary relationship between filamentous bacteriophages and conjugative plasmids has been proposed (Bradley, 1967) with many similarities noted. Thus, it is difficult to determine if the *vap* regions originate from a plasmid-like phage or a phage-like plasmid or some other form of genetic element.

1.4.2.2) Acquisition of the *vap* regions

If the *vap* regions are derived from a plasmid, some important questions about the origin of these sequences and their acquisition by *D. nodosus* arise. How did the

plasmid get into *D. nodosus* since the *vap* regions do not appear to encode the genes required for conjugation? One possible mode of acquisition is by a small plasmid being transferred with a conjugative plasmid or conjugative transposon (Shoemaker *et al.*, 1986; Salyers *et al.*, 1995). This hypothetical conjugative plasmid or conjugative transposon has since either integrated into the bacterial genome or been lost from *D. nodosus* leaving the *vap* regions marooned. Alternatively a bacteriophage may have transferred a plasmid to *D. nodosus* by insertion of the bacteriophage into the plasmid and then packaging of the phage/plasmid and transmission to the new host (Shimada *et al.*, 1978; Orbach and Jackson, 1982). A bacteriophage may also act as a helper phage for a possible *vap* satellite phage, similar to P4. In this case, the helper phage provides the head and tail proteins for the small satellite phage. The investigation of bacteriophages present as prophages in *D. nodosus* forms the basis of Chapter 5 of this thesis.

Cryptic prophages are very common in many bacteria. For example, prophages in *E. coli* include bacteriophage λ, which was lysogenic in the original isolates of strain K-12 (Bachmann, 1972), prophage *rac* located near the *trp* operon (Kaiser and Murray, 1979) and a P4 cryptic prophage located downstream of *leuX* gene for leucine tRNA (Pierson and Kahn, 1987). Element e14, believed to be a defective prophage, is a 14 kb region of *E. coli* strain K-12 which, when induced with UV light, is excised from the bacterial chromosome and exists in a plasmid form, lacking the genes for encapsulation and lysis (Greener and Hill, 1980). Prophages are also very common in *Bacillus* spp from soil (Reanney and Teh, 1976) and in bacteria from the rumen (Klieve *et al.*, 1989). There is evidence that *Klebsiella pneumoniae* contains a P4-like cryptic prophage (Ow and Ausubel, 1983). Phage-like particles have also been observed in *D. nodosus* preparations (Walker *et al.*, 1973; Stewart and Egerton, 1979;

Gradin *et al.*, 1991) although no phage has been isolated and characterised. A tailed phage specific for *Fusobacterium necrophorum* has been identified (Tamada *et al.*, 1985) although not well characterised. *F. necrophorum* is the bacteria which causes foot abscess in sheep and cattle. *F. necrophorum* and *D. nodosus* are also the major invaders of the epidermal tissues in ovine footrot (Egerton *et al.*, 1969).

D. nodosus during a footrot infection is in contact with a large number of other species of bacteria, and other strains of *D. nodosus* (Roberts and Egerton, 1969; Claxton *et al.*, 1983) so the scope and opportunity for lateral gene transfer is ample. Faecal contamination of the hoof also brings *D. nodosus* into contact with large numbers of bacteria from the rumen (Cheetham *et al.*, 1995), which are known to carry a multitude of bacteriophages (Klieve *et al.*, 1989). Soil bacteria harbour many different bacteriophages (Reanney and Teh, 1976) and may also come into contact with *D. nodosus*.

Transduction is possible between different species of bacteria especially among the enteric bacteria (Ackermann and DuBow, 1987). An example of this is the transfer of P1 between *E. coli* and *S. typhimurium* (Okada and Watanabe, 1968). P1 is able to adsorb to and lysogenize strains of *Shigella* spp, *Yersinia pestis*, *Y. pseudotuberculosis* (Yarmolinsky and Sternberg, 1988). In addition P1-sensitive strains of *Klebsiella* spp, *Enterbacter* spp, *Citrobacter* spp, *Erwinia* spp have been isolated (Goldberg *et al.*, 1974). P4, as previously mentioned, also has a very wide host range (Lagos and Goldstein, 1984; Bertani and Six, 1988). Transfer of plasmids between species is also very well documented (Finley and Falkow, 1989). IS elements or transposons carried by the phage or plasmid are also transferred, while conjugative transposons are able to transfer themselves between strains and species of bacteria (Kopecko, 1980). The conjugative transposon Tn916 is even able to transfer between

Gram-positive and Gram-negative bacteria (Bertram *et al.*, 1991). Thus, lateral or horizontal genetic exchange is a common feature of bacterial genome evolution (Kopecko, 1980; Campbell, 1981). Direct evidence for lateral gene transfer in *D. nodosus* and between *D. nodosus* and other species of bacteria comes from extensive analysis of the fimbrial gene subunits (Hobbs *et al.*, 1991). Mattick *et al.* (1991) have divided the fimbrial subunit genes into two distinct classes, class I and class II. Class II sequences appear to have arisen via the introduction of foreign DNA into a class I genome and there is evidence for recombinational exchange between strains of *D. nodosus* (Hobbs *et al.*, 1991).

1.4.2.3) Mobility of genes associated with virulence.

The transfer of putative virulence characteristics between bacteria has important implications for the control of disease. Both the *vap* and *vrl* regions of *D. nodosus* appear to have been acquired by the integration of genetic elements. The evolution of pathogenic traits in bacteria by the acquisition of genetic elements which encode them is quite a common theme in the study of bacterial pathogenesis. It has long been appreciated that significant numbers of genes for bacterial toxins, adhesins and other virulence traits are encoded on plasmids, bacteriophages (Elwell and Shipley, 1980) and more recently transposons (Falkow *et al.*, 1987). These elements can often be transferred between various bacterial species, increasing the pathogenicity of the recipient (Finley and Falkow, 1989). There is an ever increasing number of bacterial virulence factors found encoded on phages, plasmids and transposable elements.

Horizontal gene transfer in the evolution of pathogenic bacteria may be mediated by bacteriophage lysogenic conversion. Bacteriophages are known to be extensively involved in bacterial virulence via toxin production. Examples include diphtheria toxin expression in *Corynebacterium diphtheriae*, toxin production in entero-

pathogenic *E. coli*, toxin in *Staphylococcus aureus*, toxin of *Shigella dysenteriae*, *Clostridium botulinum* toxin and exo-toxins of *Streptococcus pyogenes* (Lubran, 1988; Dorman, 1994). A filamentous phage has been identified which encodes the cholera toxin required for pathogenesis of *Vibrio cholerae* (Waldor and Mekalanos, 1996). Shiga-like toxin of *E. coli* is usually encoded by a bacteriophage. However, in strain O111:H the toxin is located chromosomally, has been associated with IS elements, and is part of an apparently non-functional prophage (Paton *et al.*, 1993). This genomic structure resembles the *vap* or *vrl* regions of *D. nodosus*. *Mycoplasma arthritidis* is a pathogen of rats in which the MAV1 phage also presents a similar evolutionary structure to the *vap* regions of *D. nodosus*. Those bacteria infected with the lysogenic phage MAV1 are more virulent (Voelker *et al.*, 1995), as with *D. nodosus* strains carrying the *vap* and *vrl* sequences. The mechanism by which virulence is enhanced is unknown, as with the *vap* regions. MAV1 is able to integrate at multiple sites in the *M. arthritidis* genome, as is the *vap* region (regions 1 and 2, Figure 1.5). Strains without MAV1 are not completely avirulent suggesting some other necessary factors are already present in the bacterium (Voelker *et al.*, 1995) as with *D. nodosus* and the *vap* regions. The *vap* regions are present in some benign strains suggesting that other virulence factors may be absent or non-functional in these strains (Katz *et al.*, 1992).

It is quite common for plasmids to harbour genes which enhance the pathogenesis of the host organism. The toxin of *Clostridium tetani* is plasmid encoded as are some *E. coli* toxins (Lubran, 1988). A large virulence plasmid is required for some entero-invasive *E. coli* to enter mammalian cells (Falkow *et al.*, 1987). Large virulence plasmids are also necessary for full virulence of many serotypes of *Salmonella* and *Yersinia* (Woodward *et al.*, 1989). Plasmids of *Borrelia* species are also believed to

encode virulence factors (Dunn *et al.*, 1994). Virulent strains of *Haemophilus influenzae* (biogroup *aegyptius*) have a plasmid which is not found in non-virulent strains, suggesting a role in virulence (Brenner *et al.*, 1988). Only a given population of *H. aegyptius* has all the necessary virulence determinants, including the aforementioned plasmid, to cause Brazilian Purpuric Fever rather than simple conjunctivitis (Brenner *et al.*, 1988). The sudden, explosive appearance of the plague in ancient medical literature may have been due to a strain of *Yersinia* acquiring a distinctive plasmid (Falkow *et al.*, 1987).

Transposons have been identified in association with virulence genes of *E. coli*, *Salmonella* spp, *H. influenzae* and the toxin of *Vibrio cholerae* (Brynestad *et al.*, 1994). An entero-toxin of *Clostridium perfringens* has also been linked to an IS element and possible transposition (Brynestad *et al.*, 1994). Determinants of pathogenicity in uropathic *E. coli* are often found in unique segments of DNA bounded by repeated sequences (Chakraborty *et al.*, 1987) suggesting transposition. The *ail* locus of *Yersinia enterocolitica* is associated only with virulent strains (Miller *et al.*, 1989) indicating some form of transposition or transfer among strains. *N. gonorrhoea* and *P. aeruginosa* both use chromosomal recombination and transformation to increase their genetic diversity of virulence factors (Finley and Falkow, 1989) and some diversity in *Salmonella* spp is best explained by horizontal genetic transmission and chromosomal recombination (Beltran *et al.*, 1988).

This is by no means an exhaustive list of virulence factors encoded by mobile genetic elements. The examples give an indication of the broad variety of bacteria in which similar systems have been identified. The widespread occurrence of virulence factor mobility is a significant evolutionary characteristic in bacterial pathogenesis. Although in *D. nodosus* there appears to be a number of different traits required for

full virulence, at least two separate virulence-associated regions of the genome are associated with mobile elements. The continued study of the virulence characteristics and consequently the evolution of virulence in *D. nodosus* and other pathogenic bacteria is important for our overall understanding of bacterial pathogenesis and hence our ability to deal with bacterial infection and disease as new variations arise.

1.4.3) *vap* gene similarities

1.4.3.1) Lack of a transformation system

There is at present no transformation system available in *D. nodosus*. Therefore, it is not possible to test directly the role of *vap* gene products. Thus, functions have been assigned to some genes on the basis of the deduced amino acid similarities to known proteins. Attempts to develop transformation systems, based on the *vap* plasmid (Billington *et al*, 1996a) and the newly-discovered native plasmid of *D. nodosus*, pDN1 (Whittle and Cheetham, unpublished), are in progress.

1.4.3.2) *vapA* and *toxA*

There is high similarity of *toxA* and *vapA* gene products to the plasmid maintenance system of the *E. coli* killer plasmid Rts1 (Tian *et al.*, 1996; Billington *et al.*, 1996b). ToxA has 45.6% amino acid identity to HigB from Rts1 while VapA has 22% amino acid identity with HigA. The similar copies of *toxA-vapA*, *toxA"-vapA"* (Figure 1.5) with deduced amino acid identities of 31.5% and 36.6% respectively, have 29.3% amino acid identity with HigB and 22.1% amino acid identity with HigA respectively (Bloomfield *et al.*, 1997). The arrangement of *toxA-vapA* (*toxA"-vapA"*) is also the same as the arrangement of *higB-higA* on the Rts1 plasmid. These types of plasmid maintenance systems function by the production of a stable toxic molecule, HigB in this case, and an unstable antidote or inhibitor of the toxin, HigA. If during

cell division the plasmid is lost from a daughter cell the antidote is degraded more rapidly than the toxin thereby leaving the uninhibited toxin to kill the host. Only cells able to continually produce antidote, i.e. containing the plasmid and in particular the antidote gene, survive, and hence the plasmid is maintained in a bacterial population. There is some evidence that the *toxA/vapA* system is functional in *D. nodosus* (Bloomfield *et al.*, 1997). Therefore, the *toxA-vapA* locus may have played an important role in the maintenance of the *vap* regions in virulent strains of *D. nodosus* even though the region is incorporated in the bacterial genome.

The putative VapA' protein has 57.3% amino acid identity to VapA however the gene is not proceeded by a copy of the *toxA* gene (Figure 1.5; Cheetham *et al.*, 1995; Bloomfield *et al.*, 1997). This *vapA'* gene has either arisen by duplication of the *vapA* gene alone or the *toxA* gene has been deleted leaving the *vapA* antidote. This second possible situation would enable the deletion of most of the *vap* region, in a strain containing only one copy of these sequences, provided the *vapA* antidote is maintained until all the toxin proteins were degraded.

1.4.3.3) Plasmid maintenance systems.

A number of other similar but unrelated plasmid maintenance systems have been identified. The F plasmid of *E. coli* contains the *ccd* region where the *ccdB* gene product inhibits cell division thus causing cell death while the *ccdA* gene product suppress the inhibition of cell division (Hiraga *et al.*, 1986). Plasmid R1 of *E. coli* contains the *parB* locus from which the *hok* gene produces a lethal protein. The *sok* gene of this locus produces an anti-sense RNA which prevents translation of the *hok* mRNA and hence prevents the growth inhibition (Gerdes *et al.*, 1986, 1988). The *gef* and *sof* genes located on the *E. coli* chromosome are similar to the *hok/sok* system from the R1 conjugative plasmid (Poulsen *et al.*, 1991), and thus may have originated

by integration of a plasmid into the bacterial genome which may be a similar situation to the acquisition of the *vap* regions. The *E. coli* plasmid R100 contains the *pem* maintenance region where the protein produced by *pemK* blocks cell growth and is inhibited by the protein product of *pemI* (Tsuchimoto *et al.*, 1992). Bacteriophage P1 contains a system to maintain the phage in a low copy number plasmid state. The *doc* gene encodes a 'death on curing' protein and the *phd* gene product 'prevents host death' when the plasmid is present (Lehnherr *et al.*, 1993). Lehnherr *et al* (1993) found similarity to these P1 genes on the unrelated R124 plasmid of *E. coli* which suggests possible transfer among phages and plasmids. The 102 b.p. repeats, implicated in DNA mobility (Cheetham *et al.*, 1995; Bloomfield *et al.*, 1997), flanking *vapA"* and *toxA"* (Figure 1.5) may also support the mobility of maintenance genes among various replicons. The many examples of plasmid maintenance systems also present in bacterial and phage genomes parallels the evolution of the *vap* regions.

1.4.3.4) *vapB* and *vapC*

The *vapB* and *vapC* genes (Figure 1.5) are transcribed from the same promoter, located upstream of *vapC*, and the genes overlap by 2 nucleotides (Katz *et al.*, 1992). The *vapB/C* operon has similarity to genes in the *traD-traI* intergenic region of the *E. coli* F plasmid. The two ORFs from the F plasmid were found on the complementary DNA strand to the *trbH* gene (Jalakumari and Manning, 1989; Katz *et al.*, 1992). The *trbH* region of the F plasmid has an unknown function but the genetic conservation of the *vapB/C* operon indicates the functional significance may reside on the opposite strand to the *trbH* gene (Katz *et al.*, 1992). Similarity of *vapB* and *vapC* has also been found with the *vagC* and *vagD* genes, respectively, located on the *Salmonella dublin* virulence plasmid (Pullinger and Lax, 1992). The organization of the *vagC* and *vagD* genes is almost identical to the *vapB/vapC* arrangement, as was the organization of

genes from the F plasmid *trbH* region. The function of the *vagC* and *vagD* genes of the virulence plasmid is believed to be in the coordination of plasmid replication with cell division (Pullinger and Lax, 1992).

Genes similar to *vapA*, *vapC* and *vapD*, from *D. nodosus*, have been identified in the genome of *Haemophilus influenzae* (Fleischmann *et al.*, 1995) although they are located in different parts of the *H. influenzae* chromosome and not in a '*vap* cluster' as in *D. nodosus*. *H. influenzae* also has genes similar to the *vag* genes from the *S. dublin* virulence plasmid (Fleischmann *et al.*, 1995). The *vagC* gene, which is similar to the *vapB* from *D. nodosus*, lies adjacent to the *vapC* gene in *H. influenzae* thus giving the same arrangement of *vapB/C*-like genes as in *D. nodosus*.

1.4.3.5) *vapD*, *vapE* and remaining *vap* genes

The three copies of *vapD* in *D. nodosus* strain A198 (Figure 1.5) are all identical at the nucleotide level (Katz *et al.*, 1994; Cheetham *et al.*, 1995). The *vapD* gene product has a high similarity to ORF5 of the cryptic plasmid of *Neisseria gonorrhoea* (Korch *et al.*, 1985; Katz *et al.*, 1992), the ORFA-ORFB intergenic region from pTD1 plasmid of *Treponema denticola* (MacDougall *et al.*, 1992) and an ORF from the rolling circle plasmid of *Actinobacillus actinomycetemcomitans* (Galli and LeBlanc, 1994; Cheetham and Katz, 1995). All these ORFs have unknown functions although the cryptic plasmid of *N. gonorrhoea* is found in 99% of clinical isolates (Roberts *et al.*, 1979) and may therefore have a role in virulence of *N. gonorrhoea* (Katz *et al.*,1992)

The *vapE* gene product was found to have similarity to the product of an ORF from the cryptic plasmid pMA1 of the unicellular cyanobacterium *Microcystis aeruginosa* (Juerchott and Boerner, unpublished). This ORF has an unknown function. There is also a copy of the *vapE* gene, *vapE'* (Figure 1.5; *vap* region 3),

which when translated has 62.6% amino acid identity to VapE. The remaining *vap* genes, *vapF*, *G* and *H* and the extra copy of *vapG*, *vapG'* with deduced amino acid identity of 71% to VapG (Figure 1.5; Cheetham *et al.*, 1995), have no similarity to genes in the gene banks.

1.4.4) Rearrangements within the *vap* regions

1.4.4.1) Repeats and duplications

Spread throughout the *vap* regions of *D. nodosus* strain A198 (Figure 1.5) are two different repeated sequences. The first repeat consists of a 103 b.p. sequence. There are four copies, complete or incomplete, of the 103 b.p. repeat spread throughout *vap* region 1/3 and two copies in *vap* region 2 (Figure 1.5). The second type of repeat is 102 b.p. and is present in six copies, complete or incomplete, in *vap* region 1/3 and two copies in region 2 (Figure 1.5; Cheetham *et al.*, 1995; Bloomfield *et al.*, 1997). Each copy of *vapD* is proceeded by a 102 b.p. repeat and followed by a 103 b.p. repeat. The two copies of *vapD* in region 1/3 have identical DNA sequences beginning with the 102 b.p. repeat and ending with the 103 b.p. repeat indicating that some mechanism may have duplicated the DNA between these repeats (Cheetham *et al.*, 1995). Also the sequence between the 103 b.p. and 102 b.p. repeats downstream of *vapD* in region 2 is exactly duplicated upstream, rather than downstream, of *vapD* in region 3 (Figure 1.5). This movement of segments of DNA is similar to the movement of integron cassettes although the 102 and 103 b.p. imperfect repeats do not form inverted repeats. For these DNA sequences to be identical the duplication events are either recent evolutionary duplications or there is some form of recombinational exchange maintaining multiple identical chromosomal copies of these sequences which have an unknown function. The lack of a transformation

system for *D. nodosus* makes it difficult to investigate the function of such highly conserved sequences.

The *toxA"* and *vapA"* genes are closely flanked by copies of the 102 b.p. repeat (Bloomfield *et al.*, 1997). Since this repeat appears to be involved in some form of duplication mechanism, around *vapD*, they may therefore enable the toxin-antidote system (*toxA" and vapA"*) to move throughout the *D. nodosus* genome and possibly onto any plasmids, phages or transposons that may enter *D. nodosus*. This has important implications in the evolution of these genetic elements and any future hosts which would be forced to maintain the now addictive mobile elements thereby continually expressing the newly acquired features. This scenario may explain the maintenance of the *vap* regions in *D. nodosus*.

The 102 b.p. repeat has also been found in *D. nodosus* strain C305 which does not hybridise to pJIR318, and therefore does not contain *vapA*, *B*, *C*, *D* and *toxA* (Katz *et al.*, 1994). Within the C305 genome there are three copies of the 102 b.p. repeat adjacent to sequences found at the right-hand end of the A198 *vap* region 3. It is possible that this 102 b.p. repeat originates from a benign strain and has been incorporated into the *vap* regions by extensive duplications and rearrangements (Cheetham *et al.*, 1995). There is also the possibility that C305 has previously contained the *vap* regions but has since lost all but a couple of repeats although their orientation is reversed in C305 which indicates inversions have also occurred in this region.

1.4.4.2) Divergence of genes within the *vap* regions.

The large amount of divergence between *vapA-toxA* and *vapA"-toxA"* within *vap* region 1 indicates that these sequences have been separated for a large amount of time and are not the result of recent duplications. The proteins produced by these genes

have approximately 30% identity and the DNA sequences have very little similarity (Bloomfield *et al.*, 1997). The remainder of *vap* region 1 is highly similar to *vap* region 2 in the virulent strain A198 suggesting a close evolutionary relationship between these two regions. However, *vap* region 3 appears to be very distantly related to *vap* regions 1 and 2 suggesting a long evolutionary separation. The *vap* genes of *vap* region 3, *vapA'*, *vapG'* and *vapE'*, are 57.3%, 71% and 62.6% identical at the amino acid level to their respective *vap* region 1 counterparts. The *vapF* gene is also present in *vap* region 3 and is absent from *vap* regions 1 and 2. These variations between the regions suggests that *vap* region 3 may be a separate element that has also integrated into the A198 genome at this point. The *vapD* gene, and some of its flanking sequences, appear to have arisen recently, in evolutionary terms, as the nucleotide sequence of these segments is identical between all three *vap* regions.

There is a segment of DNA within *vap* region 3, between *vapA'* and *vapG'*, including the *vapG'* gene, which is also found in benign strains. This indicates the complicated evolution of the *vap* regions in *D. nodosus* and is an area which requires extensive analysis to determine the evolutionary history of these regions. The analysis of the DNA sequences flanking *vap* regions 1 and 3, of the virulent strain A198, in the benign strain C305 form the basis of Chapter 4 of this thesis.

1.4.5) Analysis of the limits of the *vap* regions

1.4.5.1) Left-hand end of *vap* region 2

Analysis of the left-hand end of *vap* region 2 in A198 has revealed a second serine tRNA gene, *tRNA-ser2*, in *D. nodosus* (Figure 1.5; Bloomfield *et al.*, 1997). This second tRNA is 89 b.p. and has 81% nt. identity to the *D. nodosus tRNA-ser1* identified adjacent to *vap* region 1. The *tRNA-ser2* gene has 83.5% identity to *ser-*

tRNA-V of *E. coli* (Grosjean *et al.*, 1985) and is, thus, more similar to *ser-tRNA-V* than to *tRNA-ser1* from *vap* region 1 of *D. nodosus* (Bloomfield *et al.*, 1997). The *tRNA-ser2* gene has a GGA anticodon as compared to the GCU of *tRNA-ser1*. This results indicate that *vap* region 2 has arisen by the separate integration, rather than duplication, of another *vap* region genetic element into a different *tRNA-ser* gene to form multiple integrated copies of the *vap* regions (Bloomfield *et al.*, 1997).

1.4.5.2) Right-hand ends of *vap* regions 3 and 2

Adjacent and outside the right-hand end, *attR* site, of *vap* region 3 is a partial ORF, designated *intB*, with approximate equal similarity to P4-like integrase genes and *intA* from the *vap* regions. A partial copy of this gene is also present to the right of *vap* region 2 and *intB* is associated with the *vap* regions in other strains of *D. nodosus* (Bloomfield *et al.*, 1997). The presence of part of an integrase gene to the right of *vap* region 3 suggests that another genetic element may be integrated at this point. The integrase may not even be linked to the *vap* sequences and may have just integrated into the same, or similar, *att* site (Bloomfield, 1992). To determine the nature and significance of this second genetic element further studies are required. This investigation will form the basis of Chapter 3 of this thesis.

An *att* site has been identified as the limits of *vap* region 2 in strain A198 (Bloomfield *et al.*, 1997) although some *vap* sequences common to *vap* region 1 and the *vap* plasmid are absent from *vap* region 2. *Vap* region 2 is missing the iterons proposed as the origin of replication for this element (Cheetham *et al.*, 1995) as well as *vapA"* and *toxA"* (Figure 1.5). Extensive genomic rearrangements appear to have occurred in and around the right-hand end of *vap* region 2 since the *intB* gene is completely disrupted (Bloomfield *et al.*, 1997). Only the first 140 amino acids of IntB

are present at this end of *vap* region 2 whereas the P4-like integrases are usually around 400 a.a. in length (Argos *et al.*, 1986).

1.5) Aims and directions of this study

1.5.1) Investigation of the *intB* element

Identification of part of a second integrase gene outside the *vap* regions indicates another genetic element has integrated into this region. The sequence analysis of the entire *intB* gene and genes further downstream will reveal more information about this putative genetic element adjacent to the *vap* regions. The nature of this element, bacteriophage, transposon or plasmid, may give us an indication of the relationship to the *vap* regions and the type of mobile elements involved in the evolution of this remarkably fluid region of the *D. nodosus* genome. Chapter 3 of this thesis will present the results of this investigation and discuss the implications and relevance to the overall project involving the analysis of the virulence-associated protein regions.

1.5.2) Investigation of the *vap* insertion sites in a *vap*-negative benign strain of *D. nodosus*.

The second major aim of this project will be to investigate the evolution of the *vap* regions by comparison of the sequences flanking the *vap* regions in strain A198 with the same sequences from a benign strain of *D. nodosus* that does not contain the *vap* regions. A library, in bacteriophage λ, of the benign strain C305 will be constructed and screened with clones from the sequences flanking *vap* regions 1 and 3

of *D. nodosus* strain A198. Three clones will be used, these include sequences outside the left-hand end of *vap* region 1, prior to *tRNA-ser1*, the small sequence between *vap* regions 1 and 3, between *vapA'* and *vapG'* (Figure 1.5), and a clone from outside the right-hand end of region 3, *intB*. If the virulent strains of *D. nodosus* acquired the *vap* regions by a simple insertion into a more benign ancestor then the flanking sequences of the *vap* regions would be expected to be adjacent in a strain that does not contain the insertion. However, the *vap* regions appear to have had a complex evolution involving duplications, deletions and rearrangements so the organization of these flanking sequences cannot be predicted in C305. Analysis of λ clones from C305 that hybridise to the *vap* flanking sequences, from A198, will give some insight into the evolution of this region and the relationship of C305 and A198. The results of this analysis are presented in Chapter 4 with discussion of the evolution of the *vap* regions in the most widely investigated *D. nodosus* strains A198 and C305.

1.5.3) Attempts to induce bacteriophages from *D. nodosus*.

The third section of this study involves the analysis of various *D. nodosus* strains for the presence of an inducible prophage. The *vap* regions, *intB* and the *vrl* region are believed to be derived from genetic elements integrated into the *D. nodosus* genome. To investigate the possibility that one or more of these elements is an integrated bacteriophage, attempts will be made to induce bacteriophage excision from *D. nodosus*. This search for an inducible prophage is presented in Chapter 5. The resulting analysis of the first native bacteriophage identified in *D. nodosus* is discussed.