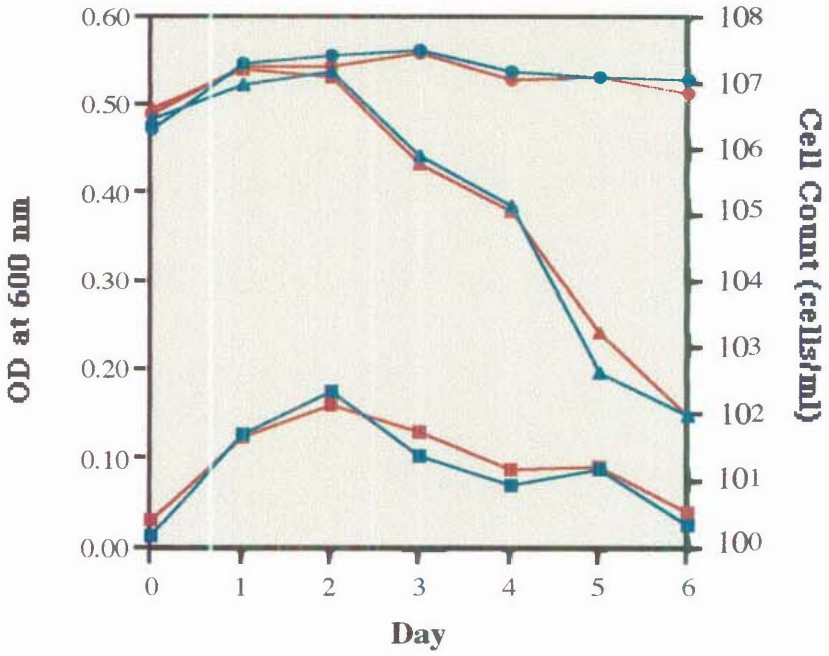
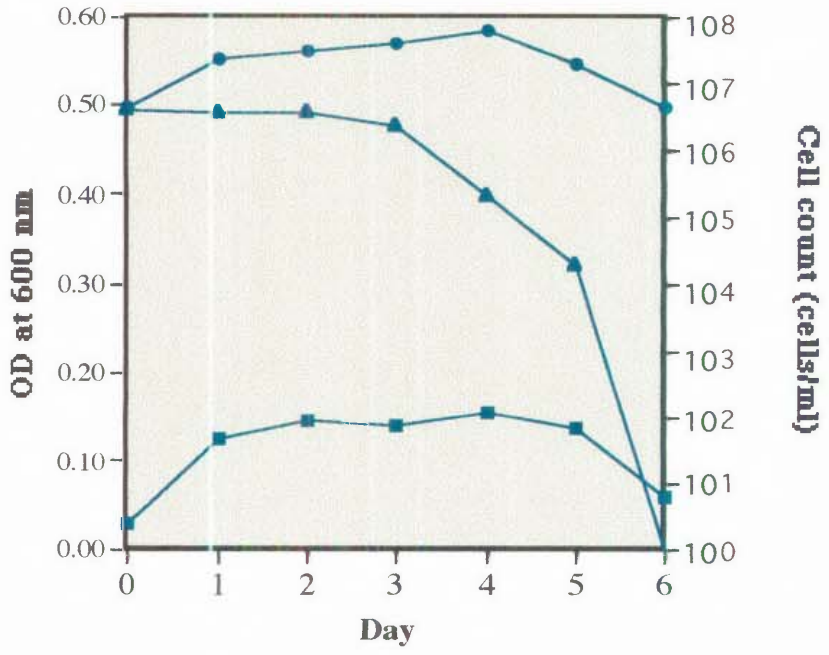
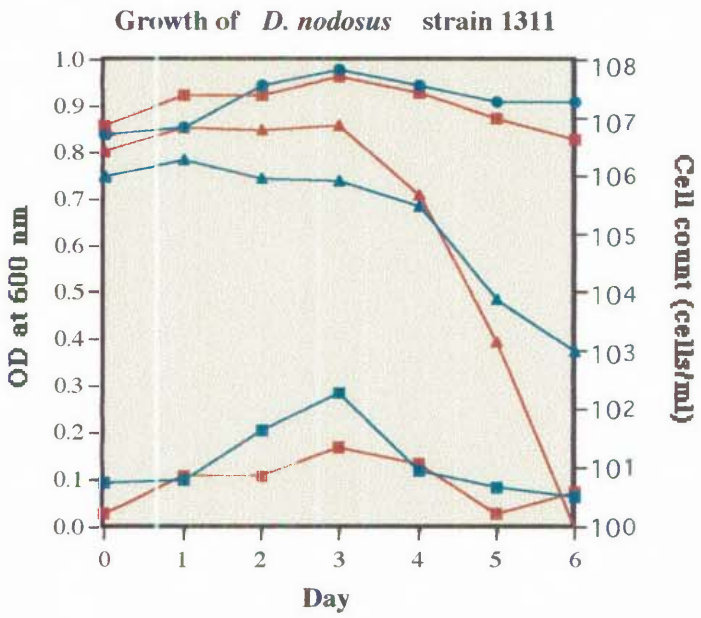
A **Growth of *D. nodosus* virulent strain A198**



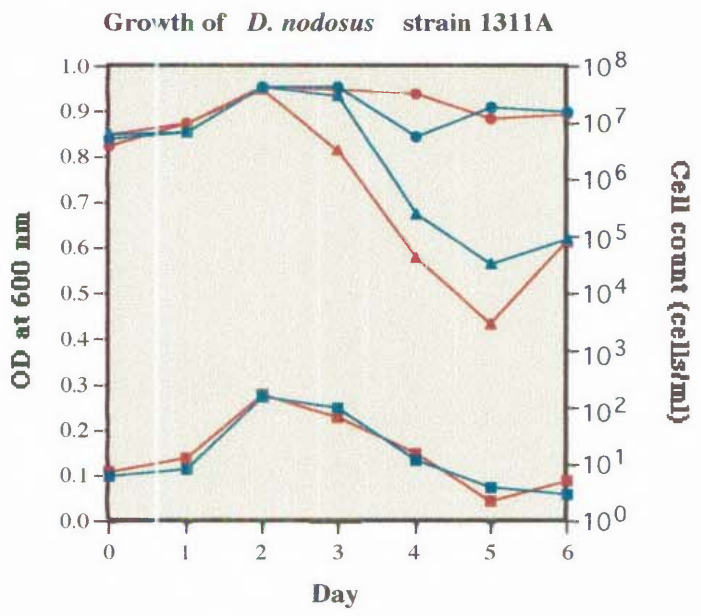
B Growth of *D. nodosus* benign strain C305



C


Growth of *D. nodosus* strain 1311
OD at 600 nm
Cell count (cells/ml)
Day
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
108
107
106
105
104
103
102
101
100
0
1
2
3
4
5
6

D


Growth of *D. nodosus* strain 1311A
OD at 600 nm
Cell count (cells/ml)
Day
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
1
2
3
4
5
6

3.2.11 Transformation experiments with *D. nodosus*

Electroporation was chosen as the means by which to attempt to transform *D. nodosus* because, at least in *E. coli*, electroshock-induced transformation is the most efficient method available (Chassy, Mercenier & Flickinger, 1988; Hanahan & Bloom, 1996). When chemically-induced competent cells are used for transformation, approximately 10% of the cells are competent for DNA uptake, whereas for electroporation-competent cells, 100% of cells are thought to be competent (Hanahan & Bloom, 1996). In either case, only about 10% of competent cells actually yield transformants (Hanahan & Bloom, 1996). Furthermore, electrotransformation has been demonstrated to be useful in the transformation of a wide range of bacterial cells previously thought to be untransformable (Chassy *et al.*, 1988; Shigekawa & Dower, 1988). The electroporation process involves the discharge of a voltage potential across a cell suspension, which results in the transient depolarisation of the cell membranes, inducing the formation of pores which allow the influx and efflux of macromolecules from the cells (Calvin & Hanawalt, 1988; Chassy *et al.*, 1988; Hanahan & Bloom, 1996).

3.2.11.1 Parameters varied in *D. nodosus* transformation experiments

Electrocompetent *D. nodosus* cells were produced using a protocol modified from that described for production of electrocompetent *E. coli* cells (Ausubel *et al.*, 1995) (Section 2.13.5). Standard conditions for electroporation of *D. nodosus* competent cells are described elsewhere (Section 2.13.6). No transformants were obtained with pDN2 using the standard method ☹, although *D. nodosus* cells were viable since on plates on which no antibiotics had been incorporated into the medium >1000 *D. nodosus* colonies were visible.

Since the mechanisms involved in bacterial transformation are not well understood, development of a transformation system is dependent on a large number of empirical

factors (Chassy *et al.*, 1988; Mercenier & Chassy, 1988). Consequently, in transformation experiments with *D. nodosus* numerous parameters were altered in an effort to achieve the transformation of *D. nodosus*, and these have been summarised in Table 3.3.

Table 3.3: Summary of parameters in *D. nodosus* electrotransformation experiments

Before Pulse	Strains of *D. nodosus* *	A198, C305, 1311, 1311A
	Molecules electroporated*	pDN2 (amp^R), pDN3 (tet^R), pDN4 (amp^R/kan^R)
	Wash Solutions	deionised water
	Cell growth medium	eugonbroth
	Growth phase*	late log or stationary phase
	Pre-pulse incubation*	fresh cells or cells frozen in 10% glycerol at -70°C
Pulse	Electroporation temperature	4°C
	Electroporation media*	10% glycerol or 1mM HEPES/10% glycerol
	Volume of cells*	40 µl or 100 µl
	DNA concentration*	0.5 µg or 5 µg
	DNA resuspension buffer	TE buffer
After Pulse	Recovery medium	eugonbroth
	Recovery period*	4 hours or 16 hours
	Selection method*	ampicillin or tetracycline or kanamycin
Instrument	BioRad *E. coli* Pulser™	
	Cuvette gap (cm)	0.1 cm
	Voltages (kV)*	1.2 kV to 2.5 kV, in 0.1 kV increments
	Field Strengths(kV/cm^{-1})*	12 kV/cm^{-1} to 25 kV/cm^{-1}, in 1 kV/cm^{-1} increments
	Capacitor	25 µF
	Resistor	200 Ω
	Time constant	4.5 to 5.0 msec

(*) indicates those parameters in which varied conditions were utilised in an effort to achieve electroporation of *D. nodosus* cells. All variables that were used are indicated in the third column.

(i) DNA concentration and quality

The frequency of transformation in *E. coli* is highly dependent on DNA concentration, increasing linearly from 5 ng/µl to 5 µg/µl (Shigekawa & Dower, 1988). At the same time however, with increasing DNA concentrations the concentration of contaminants associated with any DNA preparation (including phenol, EDTA, SDS) also increases, and may have a deleterious effect on cells. Thus in *D. nodosus* transformation

experiments, either 0.5 µg or 5 µg of plasmid DNA was added to the electroporation medium and plasmid DNA was prepared by both the method of Birnboim & Doly (Birnboim & Doly, 1979) and Holmes & Quigley (Holmes, Nuttall & Dyall-Smith, 1991) for transformation experiments.

The best choice of DNA for transformation is a native plasmid isolated from an isogenic strain of the same bacterium (Chassy *et al.*, 1988; Mercenier & Chassy, 1988). pDN1 is a native *D. nodosus* plasmid, though it was necessary to modify pDN1 for use in transformation experiments. Three different plasmids constructed from the native plasmid pDN1, were used in transformation experiments, including pDN2 (amp^R), pDN3 (tet^R) and pDN4 (amp^R/kan^R) (Section 3.2.8.3). One of the four strains used in these transformation experiments, strain 1311A, was also close to isogenic with the natural host strain of the native plasmid, strain 1311 (Section 3.2.7). Although the size of the DNA molecules may also influence electrotransformation efficiency, the sizes of these pDN1 derivatives is well within these limits, since plasmids from less than 3 kb up to 44 kb have been inserted at high efficiencies *via* electroporation (Shigekawa & Dower, 1988).

(ii) Electroporation medium

The medium in which the cells and DNA are suspended during electroporation is also an important factor in the success of electrotransformation (Mercenier & Chassy, 1988). A medium of low ionic strength is required to obtain reasonably long pulses and to limit the current at the high voltages during electrotransformation. Media buffered with 1-10 mM HEPES, pH7 (Dower, Miller & Ragsdale, 1988) or phosphate (Chassy & Flickinger, 1987; Fiedler & Wirth, 1988; Powell *et al.*, 1988), has been used with as much success as unbuffered media (Calvin & Hanawalt, 1988). In addition osmotic agents including sucrose (Fiedler & Wirth, 1988), raffinose, and glycerol (a cryoprotectant) have been added to electroporation media however, the importance of these osmotic agents has not yet been established (Shigekawa & Dower, 1988). In transformation experiments with *D. nodosus* two different electroporation media were used including 10% glycerol and H_2O

or 1 mM HEPES/10% glycerol. In future transformation experiments, a wider range of electroporation mediums could and should be utilised.

(iii) Field Strength

The optimal voltage for electropermeabilisation is unique to each bacterium (Chassy *et al.*, 1988). In general the percentage of the population that survives decreases as voltage increases, however the extent in the loss of viability varies so widely that there is no rule for determining how much cell death is optimal (Chassy *et al.*, 1988). For *E. coli* the optimal electrotransformation range is from 12.5 to 16.7 kV/cm (Hanahan & Bloom, 1996). Consequently in experiments with *D. nodosus* cells a wide range of voltages (1.2 to 2.5 kV in 0.1 kV increments) were used in an effort to achieve electrotransformation. All experiments were carried out using in 0.1 cm cuvettes, and thus field strengths ranged from 12 kV/cm^{-1} to 25 kV/cm^{-1} in 1 kV/cm^{-1} increments. The average time constant was 4.5-5.0 msec.

(iv) Growth phase

Electrocompetence is also related to growth phase. For example it has been established that for *E. coli* (Shigekawa & Dower, 1988), *Campylobacter jejuni* (Miller, Dower & Tompkins, 1988) and *Streptococcus lactis* (Powell *et al.*, 1988) mid-log phase cells are most efficiently electrotransformed, whilst stationary phase cells are not. In contrast, other bacteria such as *Lactococcus lactis* (McIntyre & Harlander, 1989; McIntyre & Harlander, 1989) are most efficiently transformed in stationary phase. Consequently in *D. nodosus* transformation experiments both log phase and stationary phase cells were utilised in transformation.

(iv) Strain of *D. nodosus*

Optimal conditions for electroporation are known to vary between strains of a given species (Mercenier & Chassy, 1983). In order to this address this, four different strains of *D. nodosus* were utilised in these experiments including strains A198, C305, 1311, 1311A.

In spite of the wide range of conditions which were tried, transformation was not successful. Future experiments should include a wider range of *D. nodosus* strains since this may increase the chance of obtaining transformants. Also, since *D. nodosus* grows so poorly in broth medium, the possibility that cells harvested directly from a plate culture of *D. nodosus* might be more suitable for electrotransformation should also be investigated.

3.3 Discussion

3.3.1 pDN1, a broad host range mobilisable plasmid

D. nodosus plasmid pDN1 is a 5.1 kb replicon of moderate copy number, and is very closely related to plasmids belonging, or related to, the *E. coli* incompatibility group Q. At both the nucleotide and amino acid levels pDN1 is most closely related to pIE1107 (Tietze, 1998) and then to IncQ plasmid RSF1010 (Scholz *et al.*, 1989) which is similar or identical to plasmids R1162 (Guerry *et al.*, 1974) and R300B (Barth & Grinter, 1974).

Sequence analysis of pDN1 has revealed that >90% of the pDN1 genome consists of cis-acting elements (*oriV*, *oriT*) and genes that, for IncQ plasmid RSF1010, have been shown to be indispensable for plasmid replication (*repA*, *repB*, *repC*) (Scherzinger *et al.*, 1984; Scholz *et al.*, 1984; Scholz *et al.*, 1989) and mobilisation (*mobA*, *mobB*, *mobC*) (Brasch & Meyer, 1986; Derbyshire *et al.*, 1987; Haring & Scherzinger, 1989; Meyer *et al.*, 1982; Nordheim *et al.*, 1980; Scholz *et al.*, 1989).

The high degree of sequence similarity between the pDN1 proteins and the well-characterised replication proteins of IncQ and IncQ- related plasmids, which typically are small in size, have moderate copy number and an extremely broad-host-range, suggests that pDN1 may also be a broad host range plasmid. This hypothesis was supported by the observation that antibiotic resistant derivatives of pDN1 are able to replicate stably in

E. coli strain DH5-α. Furthermore the ability of pDN1 derivatives to replicate in *E. coli* meant that it was not necessary to develop a *D. nodosus*/*E. coli* shuttle vector to transfer molecules between *E. coli* and *D. nodosus*.

The observation that pDN1 derivatives do not belong to incompatibility group Q but still have a very broad host range may be of use to other researchers; RSF1010 derivatives are often utilised as protein expression vectors due to their moderate-copy numbers and hence for some experiments it may be useful to cotransform various hosts with both an IncQ plasmid and a pDN1 derivatives to study the effects or interactions of two different proteins at once or in complementation studies. Furthermore, the features that distinguish the *oriV* of pDN1 and *oriV-2* of pIE1107 from the IncQ origin of RSF1010 may be important for the replication of pDN1 in *D. nodosus*, and hence may explain the lack of success (Billington *et al.*, 1996) in *D. nodosus* transformation experiments in which RSF1010 derived vectors were utilised.

3.3.2 How was pDN1 acquired by *D. nodosus* strain 1311?

The fact that plasmids related to pDN1 are typically not self-transmissible poses the question as to how pDN1 was transferred to *D. nodosus* strain 1311. The presence of pDN1 in strain 1311 strongly suggests that a conjugative element is or was present in the genome of strain 1311, since although plasmids can be transferred by generalised transduction the frequency of generalised transduction is generally very low, being in the order of 10^{-5} to 10^{-7} per active phage particle (Novick, Edelman & Lofdahl, 1986). It is also possible that pDN1 was transferred to strain 1311 without the co-transfer of the conjugative element.

During routine laboratory growth, pDN1 was spontaneously lost from *D. nodosus* host strain 1311, and this loss was associated with the rearrangement of *intB* element

sequences. It has been postulated previously that the *intB* gene may comprise part of an integrated genetic element, and that this element may be a conjugative transposon (Bloomfield *et al.*, 1997). Conjugative transposons (Salyers & Shoemaker, 1996), have been shown to mobilise non-conjugative plasmids that, like IncQ plasmids (Willetts & Crowther, 1981), are normally mobilised by IncP or IncIα conjugative plasmids (Shoemaker *et al.*, 1986).

The observation that pDN1 was lost and *intB* sequences were rearranged at apparently, though not necessarily, the same time in strain 1311 to generate strain 1311A, suggests that the loss of pDN1 and the rearrangement of the *intB* element sequences may be related.

3.3.3 Transformation experiments with *D. nodosus*

It is doubtful that there are any bacteria that exist that are truly untransformable (Mercenier & Chassy, 1988), including *D. nodosus*. Furthermore, in the absence of a single transformant, it is very difficult to determine precisely which parameters to alter in order to achieve transformation. It is difficult to tell whether the failure to generate *D. nodosus* transformants despite adjusting numerous parameters is due to a failure of the DNA to enter the *D. nodosus* cell, or due to the failure of transformed DNA to become established in the host cell due to restriction barriers, or due to a failure to express the antibiotic resistance genes.

Most Gram-negative and many Gram-positive bacteria can best be transformed by electroporation (Chassy *et al.*, 1988; Shigekawa & Dower, 1988), however it is unknown why some strains are resistant to electrotransformation. One of the most common problems encountered is host-restriction barriers to DNA prepared from *E. coli*. In some bacteria, the DNA to be transformed is able to be passaged though a transformable intermediary

host strain in order to modify it appropriately. Alternatively, the target restriction sites can be removed from the DNA where they are known, or can be modified using the appropriate modification methylase where it has been isolated (Hanahan & Bloom, 1996; Mercenier & Chassy, 1988; Shigekawa & Dower, 1988). In many cases, such as with *D. nodosus*, these steps are not possible. The introduction of multiple cloning sites into pDN1 and of antibiotic resistance genes that also contain restriction enzyme sites, may have made the pDN1 derivatives more susceptible to degradation by *D. nodosus* restriction enzymes. Transformation experiments could be repeated by constructing a pDN1 derivative that does not contain the multiple cloning site initially, and contains antibiotic resistance markers with a minimum number of restriction sites.

Some species also secrete non-specific nucleases into the surrounding medium (Shigekawa & Dower, 1988). In an effort to minimise this problem, the DNA used for the transformation of *D. nodosus* was added immediately prior to electroporation in an effort to reduce any deleterious effects elicited by non-specific nucleases. Additional measures have been taken by others including the addition of EDTA or carrier DNA to the electroporation medium. The possibility that *D. nodosus* produces such non-specific nucleases has not yet been investigated, but perhaps in future transformation experiments these nuclease inhibitors could be employed in order to further minimise the effects of any such nuclease activity.

In addition, physical barriers including polysaccharide capsules, cell membrane composition, and cell wall thickness, density and structure may hinder the uptake of DNA (Chassy *et al.*, 1988; Shigekawa & Dower, 1988), and thus in general, Gram-negative bacteria are more easily electropermeabilised than Gram-positive bacteria (Chassy *et al.*, 1988). Hence, for Gram-positive bacteria weakening of the cell wall by the addition of L-threonine, L-lysine, cycloserine, penicillin, glycine and muramidases to growth medium, the use of lytic enzymes to remove or loosen cell wall structure, or the addition of cell wall

synthesis inhibitors are usually necessary to electropermeabilise the bacterial cells. Although such procedures are not usually required for the transformation of Gram-negative bacteria (Chassy *et al.*, 1988; Mercenier & Chassy, 1988), since no *D. nodosus* transformants were obtained it may be useful to attempt to use these cell wall-weakening agents in an attempt to achieve transformation.

Although the failure to obtain transformants may also be due to the transforming DNA molecule, it seems unlikely that pDN1 derivatives are not able to be maintained in *D. nodosus* given that pDN1 is known to replicate stably in *D. nodosus* strain 1311. It is also unlikely that plasmid functions have been disrupted by the addition of antibiotic resistance markers, since pDN1 derivatives replicate in *E. coli.*

It is possible that the promoters for the antibiotic resistance markers which are functional in *E. coli* are non-functional in *D. nodosus*. However, many promoter sequences matching the *E. coli* σ^{70} -35 and -10 consensus sequence have been identified within the *vap* regions of *D. nodosus* strain A198, and *vap* genes have been shown to be expressed at detectable levels in Northern blots (Chapter 4). This potential problem could be addressed by ligating *D. nodosus* promoter and terminator sequences to an antibiotic resistance marker, and ligating this construct to pDN1 sequences.

In addition the recovery period utilised may have been too short, and hence the antibiotic resistance genes were not expressed, or too long and so the plasmid was lost prior to selection. Two different recovery periods of four or sixteen hours were utilised in *D. nodosus* transformation experiments in order to address these possibilities.

The molecular mechanisms, genetic and biochemical, involved in the establishment of a plasmid in a host cell have not yet been determined. However, since plasmids cannot exist as naked DNA molecules, it is thought they are organised into chromatin-like structures of protein and DNA, analogous to the cellular chromosome (Hanahan & Bloom,

1996). It has been proposed that the steps involved in assembling these structures may be rate limiting or dependent upon factors that are not present continuously throughout the cell cycle, and thus only 10% of competent *E. coli* cells correctly assemble these chromatin-like structures (Hanahan & Bloom, 1996). It is possible that the analogous process in *D. nodosus* and other bacteria which are not easily transformed have a different system for assembling these chromatin structures, and perhaps this process is less efficient.

The fact that only two native plasmids, the *vap* plasmid (Section 1.6.3) and pDN1, have been identified in *D. nodosus* despite the large numbers of *D. nodosus* strains from which genomic DNA has been prepared (Cheetham *et al.*, 1995b; Katz *et al.*, 1992; Rood *et al.*, 1996) and the increasing number of integrated elements which are present in this organism, suggests that the establishment of a plasmid molecule may be problematic in *D. nodosus*. Perhaps, therefore, the development of a system of transformation based on integration of a transformed DNA molecule may be more successful. Such a strategy was successfully applied to the previously untransformable bacterium, *Lactobacillus acidophilus* (Mercenier & Chassy, 1988).

Numerous other strategies besides electroporation could be tried in order to develop a transformation system for *D. nodosus*, though these other methods are generally more problematic and would only be appropriate alternatives if the problem involved getting the transforming DNA into the cell rather than its establishment. Chemical methods of transformation could be used, for example a variation of the $CaCl_2$ technique has been used to transform a number of Gram-negative bacteria including: *Alcaligenes*, *Azotobacter*, *Citrobacter*, *Erwinia*, *Flavobacterium*, *Haemophilus*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia* and *Staphlococcus*. In all cases though, the transformation frequency is lower than that of *E. coli* (Mercenier & Chassy, 1988). In addition to calcium salts, strontium, barium and magnesium salts have also been utilised to induce competence (Mercenier & Chassy, 1988). In addition, polyethylene glycol (PEG) has been used to facilitate

chemically-induced competence in *E. coli* and *Saccharomyces* (Klebe *et al.*, 1983), *Rhodopseudomonas sphaeroides*, *Bacteroides fragilis*, *Bacillus brevis*, *Bacillus thurigiensis*, *Clostridium thermohydrosulfuricum*, *Streptococcus lactis* (Mercenier & Chassy, 1988).

Although natural competence has only been described in 15 diverse genera (Chassy *et al.*, 1988; Mercenier & Chassy, 1988) the possibility that *D. nodosus* is naturally competent could also be investigated. However, natural competence develops under very specific conditions that are dependent upon factors which include the pH, phase of growth, medium composition, cell density, the expression of competence factors and sequences present in the DNA molecule to be taken up (Chassy *et al.*, 1988; Mercenier & Chassy, 1988). Thus in the absence of any evidence to suggest that *D. nodosus* is naturally competent, this avenue of investigation is probably least desirable.

In the absence of a mechanism to induce transformation *via* electroporation, the possibility that pDN1 derivatives could be utilised in a conjugative transfer system for *D. nodosus* should be investigated. Initial mating experiments could be performed in *E. coli*, utilising conjugative plasmids belonging to those incompatibility groups known to be able to transfer the pDN1-related IncQ plasmids. If transfer were demonstrated in *E. coli*, one could attempt *E. coli-D. nodosus* mating experiments, using a strain of *E. coli* harbouring a conditional-lethal mutation as a donor.

In this work, the native *D. nodosus* plasmid pDN1 was isolated, sequenced and characterised. Since pDN1 is small in size, has medium copy number and is highly related to plasmids belonging or related to incompatibility group Q, pDN1 had excellent potential to be utilised and developed as a cloning vehicle to provide a suitable means by which to transform *D. nodosus*. pDN1 derivatives pDN2 (amp^R), pDN3 (tet^R) and pDN4 (amp^R/kan^R) were constructed and utilised in electrotransformation experiments in

D. nodosus, however no transformants were obtained. Further work is required in order to develop a transformation system for *D. nodosus*, since the development of a genetic system for *D. nodosus* is vital to the further analysis of the role and function of virulence-associated genes in the pathogenesis of ovine footrot.