the exception of pIE1107 and pDN1, all plasmids that contain IncQ core sequences belong to the *E. coli* incompatibility group Q.

The absence of antibiotic resistance determinants in pDN1 suggests that pDN1 is a minimal broad-host-range replicon, being comprised only of genes that in RSF1010 have been shown to be necessary for the initiation of plasmid replication and mobilisation (Frey *et al*, 1992; Haring *et al*, 1985; Meyer *et al*, 1982; Meyer *et al*, 1984; Scholz *et al.*, 1984; Scholz *et al.*, 1989).

Four putative promoter sequences were identified in pDN1 (Figures 3.3 and 3.4). Although the consensus sequences of these promoter regions do differ from those identified as P1, P2, P3 and P4 (Scholz *et al.*, 1989) in RSF1010, their location is the same, suggesting that they have an equivalent function. No putative terminator sequences were identified in pDN1 using the GCG Terminator program (ANGIS). It is therefore possible that the *oriV* may act as a transcriptional terminator, due to the dyad symmetry characteristic of this region.

3.2.6 Comparison or *oriV* and *oriT* regions of pDN1, pIE1107 and RSF1010

The putative origin of replication (*oriV*) from pDN1 (position 1 to 451) was compared to the *oriV* of RSF1010 (position 2348 to 2743), to the two *oriV* regions identified in pIE1107, designated here as *oriV-1* (position 145 to 519) and *oriV-2* (position 5202 to 5657), and to the iteron region of the *oriV* from pTF-FC2. Sequence analysis indicates that although *oriV* of pDN1 is similar to the *oriV* regions of pIE1107, RSF1010 and pTF-FC2 (Figure 3.5), it is most similar to *oriV*-2 of pIE1107. pDN1 has 95.0% nucleotide identity to the *oriV*-2 of pIE1107 compared with 90.8% and 83.7% nucleotide identity to *oriV*-1 and *oriV* of plasmids pIE1107 and RSF1010 respectively. In addition, both the *oriV* of

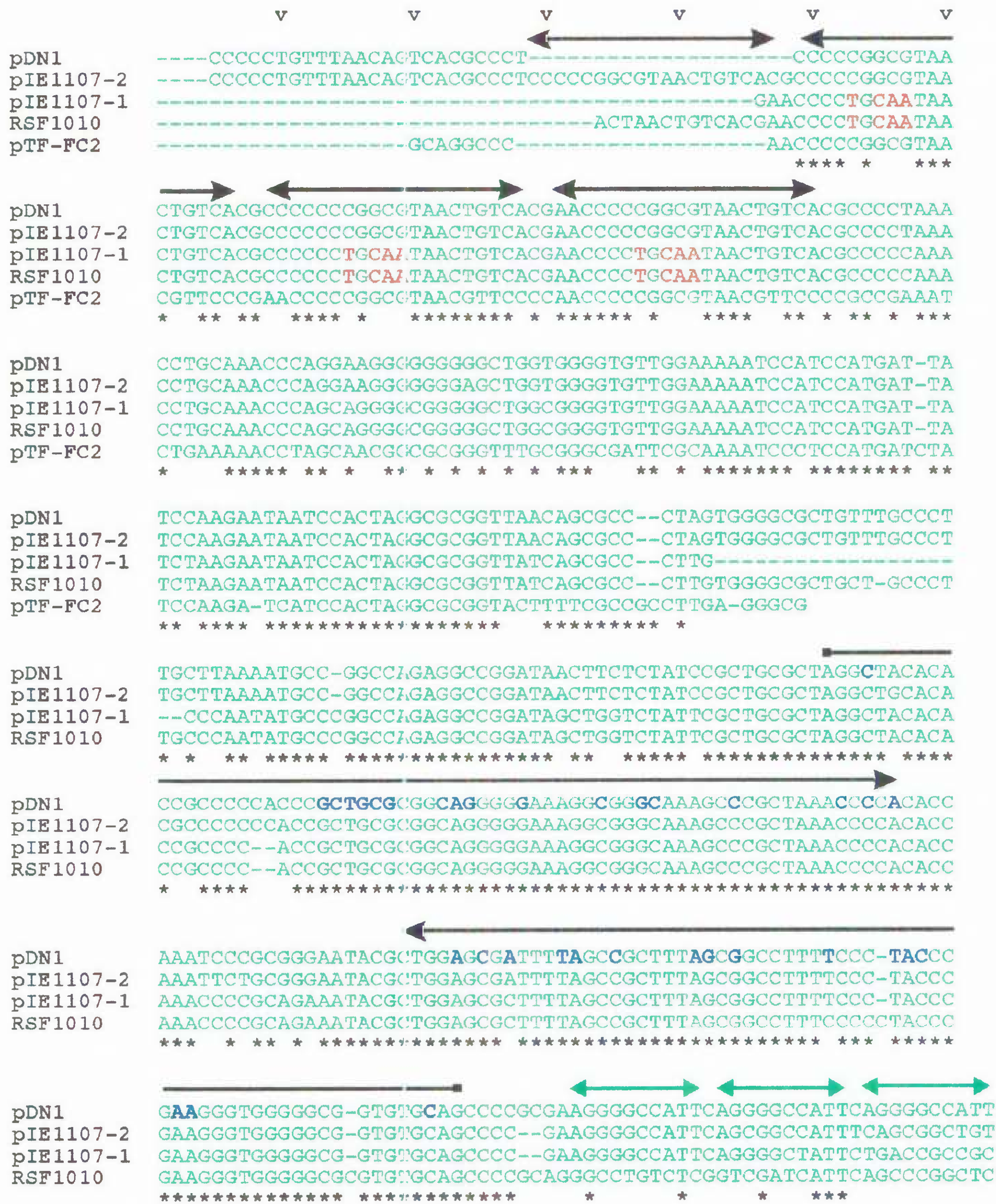

Figure 3.5: A ClustalW alignment of the *oriV* regions from *D. nodosus* plasmid pDN1 and related plasmids. Sequences are from the *oriV* regions of plasmid pIE1107 (Tietze, 1998) (*oriV-2* and *oriV-1*), RSF1010 (Scholz *et al.*, 1989) and from pTF-FC2 (Dorrington & Rawlings, 1990). The nucleotides which are identical in all four sequences are indicated (*). Repeats are identified as follows: four 20 bp direct repeats (iterons, black double-headed arrows) and three 10 bp direct repeats (green double-headed arrows); 63 bp (arrow right) and 60 bp (arrow left) inverted repeats each containing a plasmid-specific single-stranded DNA replication initiation (*ssi*) signal (Sakai & Komano, 1996). 10 bp intervals are shown (v) above the aligned sequences. Nucleotide differences within the inverted repeats are shown in blue.

pDN1 and *oriV*-2 are located immediately after the stop codon of *repC*, and are not separated by antibiotic resistance genes, unlike *oriV*-1 of pIE1107 and the RSF1010 *oriV* (Figure 3.4).

The *oriT* region of RSF1010 (position 3083 to 3170) was localised to an 88 bp segment (Derbyshire *et al.*, 1987; Nordheim, Hashimoto-Gotoh & Timmis, 1980) and has 76% identity to position 804 to 891 of pDN1. The corresponding region of pIE1107 (position 893 to 980) has 100% identity to the *oriT* region of pDN1.

3.2.7 Spontaneous loss of pDN1 from *D. nodosus* strain 1311

After routine laboratory growth and a subsequent genomic DNA preparation from *D. nodosus* strain 1311, plasmid DNA was no longer visible, suggesting that the native plasmid pDN1 had been lost spontaneously from *D. nodosus* virulent strain 1311, generating a daughter strain designated as 1311A. Southern blot analysis, using pDN1 as a probe, confirmed that pDN1 had been lost from strain 1311 to give a daughter strain, designated 1311A (Figure 3.6, panel I).

To eliminate the possibility that strain 1311 had been inadvertently mixed with another strain of *D. nodosus* available in our laboratory at the time of plasmid loss, Southern blot analysis of strains 1311 and 1311A was performed and subsequently compared to that previously determined for strains B1006, G1220, H1204 and H1215 (Bloomfield *et al.*, 1997). Genomic DNA from both strain 1311 and 1311A was probed with DNA fragments corresponding to *vap* genes *A-D*, *vapE*, *vapE'* and *intB* (Appendix 4). In both strain 1311 and 1311A the *vapE* probe hybridised to an 8.9 kb *Eco*RI fragment and a 4.7 kb *Hin*dIII fragment; *vapA-D* hybridised to 3.2 kb and 1.95 kb *Eco*RI/*Hin*dIII fragments corresponding to *vapsA-D*; neither strain 1311 or 1311A hybridised to *vapE'*.

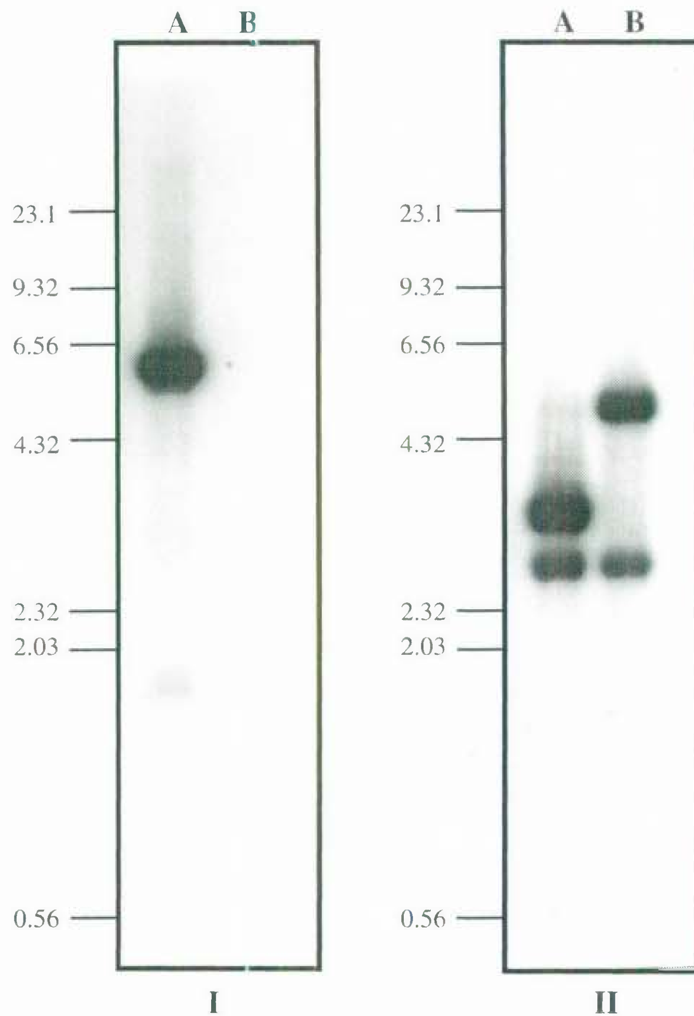

Figure 3.6: Southern blot analysis of *D. nodosus* strains 1311 and 1311A.
Genomic DNA from strains 1311 (lanes A) and 1311A (lanes B) were hybridised with probes specific for pDN1 (panel I) or *intB* (panel II) after digestion with *Eco*RI. Lambda *Hin*dIII standard fragment sizes are indicated on the left of each panel.

Unexpectedly, the pattern of hybridisation to the *intB* probe in strain 1311 was observed to be different to that observed for strain 1311A (Figure 3.6). In both strains there are two copies of *intB*. The *intB* probe hybridises to a 2.2 kb *Eco*RI fragment and a 6.7 kb *Hin*dIII fragment in both strains, whilst the second copy of *intB* hybridises to a 2.5 kb *Eco*RI and a 10.2 kb *Hin*dIII fragment in strain 1311, and a 3.3 kb *Eco*RI and 3.6 kb *Hin*dIII fragment in strain 1311A. These results suggested that the *intB* gene region had undergone rearrangement in *D. nodosus* strain 1311.

Like strains 1311 and 1311A, B1006, G1220, H1204 and H1215 all contain a single copy of the *vap* region integrated downstream of *pnpA* (Figure 1.10). However, analyses indicated that each of these strains is distinguishable from 1311 and 1311A. In contrast to 1311 and 1311A, *D. nodosus* strain B1006 has only one copy of *intB* on an 8.7 kb *Hin*dIII and 4.0 kb *Eco*RI fragments respectively. Similarly, strain G1220 contains a single copy of *intB* on a 4.0 kb *Eco*RI fragment and an 7.9 kb *Hin*dIII fragment. Strains H1204 and H1215 contain three copies of *intB*, on different sized restriction fragments (Appendix 5), and therefore these strains are not the same as strains 1311 and 1311A, which have two copies of *intB*. In addition, unlike *D. nodosus* strains 1311A and 1311, strain H1215 does not contain a copy of *vapE* and instead contains a copy of *vapE'* in the same position that *vapE* is normally found (Figure 1.9).

Results from Southern blot analyses are consistent with the hypothesis that strain 1311A is derived from *D. nodosus* strain 1311, and this is supported further by many subsequent experiments presented elsewhere in this thesis. It has been postulated previously (Bloomfield *et al.*, 1997) that *intB* may be part of a conjugative transposon (Salyers *et al.*, 1995) and this may explain the rearrangement observed in *D. nodosus* strain 1311A. The possibility that the loss of native plasmid pDN1 was associated with the rearrangement involving *intB* element in strain 1311A has been explored further (Section 7.3.4), as has the nature of the rearrangement (Section 7.2.2).

3.2.8 Modification of pDN1

In order to be useful for transformation experiments, pDN1 was modified to produce derivatives carrying appropriate antibiotic resistance markers and a multiple cloning site. In pDN1-related plasmids pIE1107 and RSF1010 the antibiotic resistance markers are located between *repC* and the *oriV*. Consequently, the introduction of an antibiotic resistance marker between *repC* and *oriV* in pDN1 was predicted to be least

likely to disrupt the expression and thus the functions of plasmid-encoded genes. Prior to the introduction of antibiotic resistance determinants to pDN1, antibiotic sensitivity assays of *D. nodosus* were carried out (Section 3.2.8.1) and expression of the proposed antibiotic resistance genes under anaerobic conditions was confirmed (Section 3.2.8.2).

3.2.8.1 Antibiotic sensitivity of *D. nodosus*

Eugonbroth cultures of *D. nodosus* strains A198, C305, 1311 and 1311A were grown for 24 and 48 hours respectively, samples were subsequently plated on appropriate media containing the antibiotic to be tested, and on a negative control plate which contained no antibiotic. Plate cultures were allowed to grow for three days under anaerobic conditions prior to assessment of growth.

In these assays the concentrations of antibiotics were as follows: kanamycin at 0.1, 0.5, 1.0, 3.0, 4.0, 5.0, 8.0, 10.0, 20.0 µg/ml; ampicillin at 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 10.0, 50.0 and 100.0 µg/ml; and tetracycline at 0.1, 0.2, 0.5, 1.0, 5.0, 10.0 µg/ml. From these assays, the highest concentration of antibiotic at which the four strains of *D. nodosus* assayed still grew were kanamycin at 3.0 µg/ml, ampicillin at 0.1 µg/ml, and tetracycline at 0.2 µg/ml.

The lowest concentration of antibiotic tested at which *D. nodosus* strains did not grow after 3 days was later used for selection of *D. nodosus* transformants, when plated on Blood-Eugonagar medium. These concentrations were determined to be 4.0 µg/ml kanamycin, 0.2 µg/ml ampicillin, and 0.5 µg/ml tetracycline.

3.2.8.2 Expression of antibiotic resistance determinants under anaerobic conditions

Prior to the introduction of antibiotic resistance genes into pDN1, *E. coli* strain DH5-α was transformed with plasmids carrying tetracycline (pBR322), ampicillin (pUC18), and kanamycin (pKT240) resistance genes, and grown on the appropriate

selective medium at 37°C under anaerobic conditions to ensure that these antibiotic resistance markers were expressed under anaerobic conditions. Control plates containing each antibiotic and plasmid-free DH5-α cells were also included in this experiment. Growth was not observed on any of the control plates, whilst *E. coli* cells harbouring the antibiotic resistance markers grew, indicating that each of the aforementioned resistance markers are expressed under the anaerobic conditions in which *D. nodosus* would be grown.

3.2.8.3 Development of pDN1 derivatives suitable for transformation of *D. nodosus*

Due to the lack of suitable restriction enzyme sites in pDN1, PCR experiments were necessary to introduce restriction enzyme sites that would allow the directional cloning of antibiotic resistance markers into pDN1 DNA. Six oligonucleotide pairs were designed for this purpose (Figure 3.7).

Oligonucleotide primers 1 and 2 were used to amplify a 3565 bp fragment (fragment 1) from pDN1 which contained a *Nsi*I site at one end and a *Cla*I site at the other end. Oligonucleotides 3 and 4 allowed the amplification of a 1490 bp fragment (fragment 2) from pDN1 which contains a *Cla*I site at one end and a multiple cloning site at the other end. The most distal site of the multiple cloning site was a *Bam*HI target site. The third oligonucleotide pair, primers 5 and 6, allowed the amplification of a 1085 bp fragment (fragment 3) spanning the ampicillin resistance gene from pUC18 (Norrander *et al.*, 1983; GenBank Accession L08752). The end of primer 5 contained a *Bam*HI site, whilst the end of primer 6 contained a *Nsi*I site. Fragment 1 was digested with *Cla*I/*Nsi*I, fragment 2 with *Cla*I/*Bam*HI and fragment 3 with *Bam*HI/*Nsi*I, after which the three restriction fragments were ligated to generate pDN2, an ampicillin-resistant derivative of pDN1 (Figure 3.8).

1 TTGATGCATCCCCCTGTTTAACAGTCACG
NsiI

2 CCAGAATATCGATAGGCTTA
ClaI

3 TAAGCCTATCGATATTCTGG
ClaI

SphI *HindIII* *StuI*
4 TCAGGATCCGCATGCGAGCTCAAGCTTCTAGAGGCCTTCAGCCTGCCGCCTTGGGCC
BamHI *SacI* *XbaI*

5 CGTGGATCCAGACGAAAGGGCCTCGTGAT
BamHI

6 CGCATGCATTATGAGTAAACTTGGTCTGA
NsiI

Figure 3.7: Oligonucleotides 1 to 6, used to construct pDN2, a derivative of pDN1 that carries an ampicillin resistance marker and a multiple cloning site. The following features of these oligonucleotides are indicated: sequences complementary to sequences from pDN1 (blue, primers 1-4) or pUC18 (blue, primers 5 and 6); to ensure sequences were able to be cut efficiently when at the end of a sequence three nucleotide sequences were added to the 5'-ends of oligonucleotides (green); restriction enzyme sites that were added to allow directional cloning of DNA sequences (black). The precise position of these oligonucleotides is described in Appendix 1, Table A1.2.

In addition, fragment 1 and fragment 2 were ligated and cloned into pUC18 to produce the plasmid pGW154(1). pGW154(1) was digested with *Nsi*I/*Bam*HI and a 5138 bp fragment ligated to a gel purified 1618 bp *Ssp*I/*Ava*I end-filled fragment from pBR322 (position 4169-1425) (Sutcliffe, 1978; GenBank Accession J01749) containing the tetracycline resistance determinant and promoter region, to generate the tetracycline resistant derivative of pDN1, designated pDN3 (Figure 3.9).

To generate the kanamycin and ampicillin resistant derivative of pDN1, pKT240 (Bagdasarian *et al.*, 1983) was digested with *Hpa*I/*Bst*EII, a 5300 bp fragment containing both a kanamycin and ampicillin resistance gene was purified, end-filled, and ligated to the 5138 bp insert from pGW154(1). This plasmid carrying both kanamycin and ampicillin resistance markers was called pDN4 (Figure 3.10).

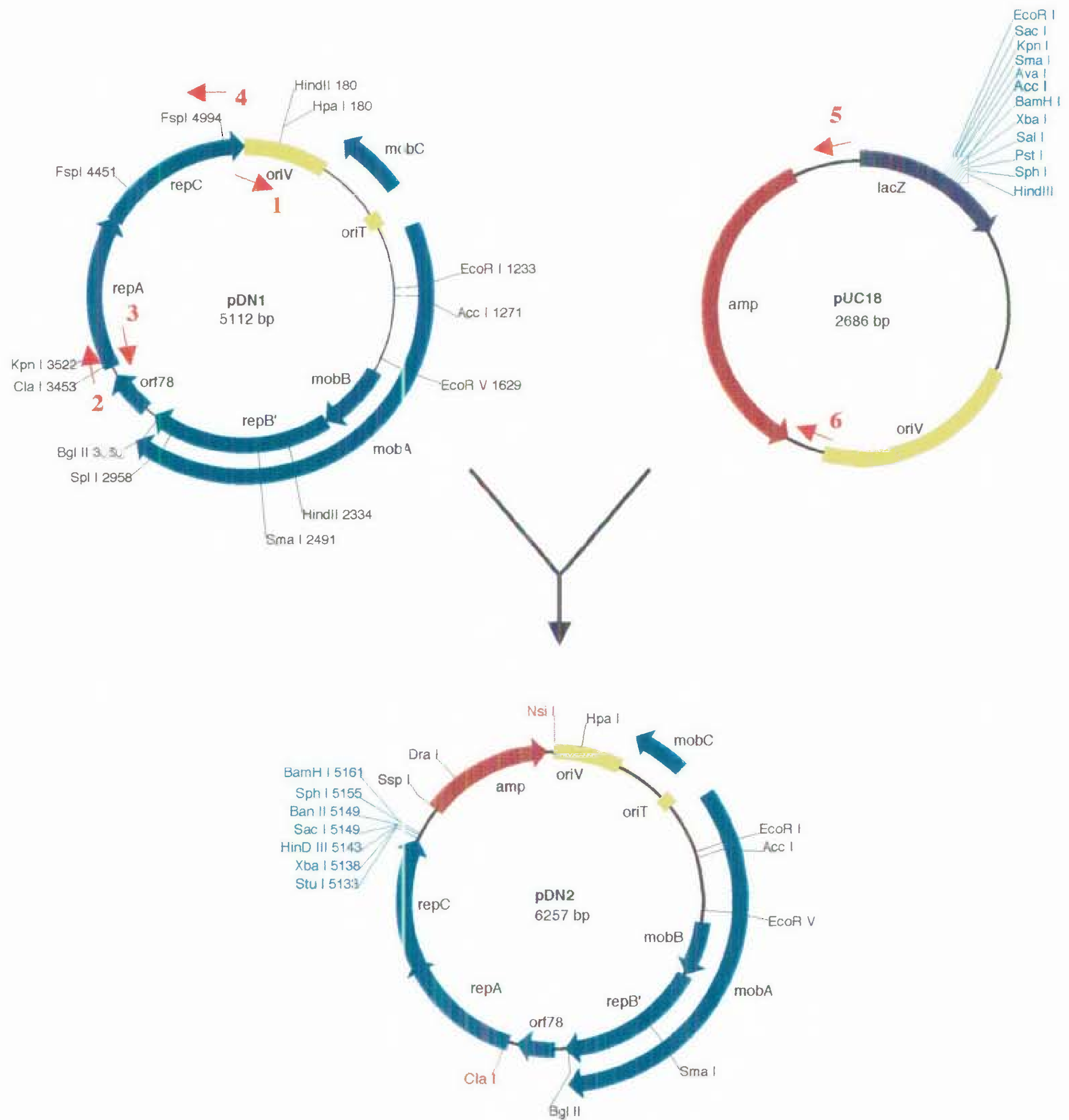

Figure 3.8: Construction of pDN2, an ampicillin resistant derivative of pDN1. The sequences complementary to oligonucleotides 1-6 and their orientation on pDN1 and pUC18 respectively are shown (numbered red arrows). The multiple cloning sites present in pUC18 and in pDN2 are shown in blue. It should be noted that plasmid sizes are not proportional.

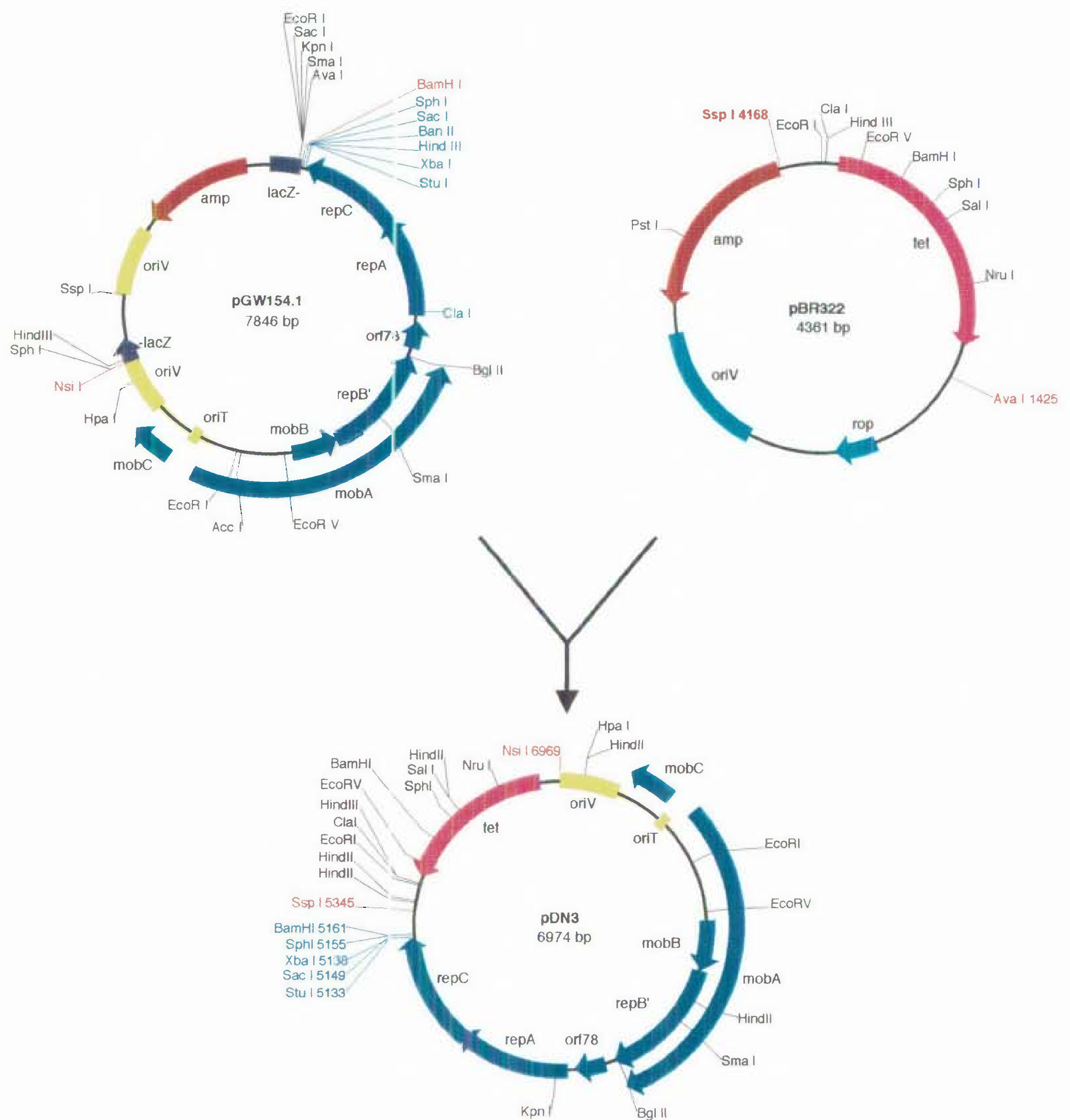

Figure 3.9: Construction of pDN3, a tetracycline resistant pDN1 derivative. The 5138 bp *Nsi*I/*Bam*HI insert from pGW154.1 was ligated to a 1618 bp *Ssp*I/*Ava*I fragment from pBR322 containing the tetracycline resistance determinant. The multiple cloning site in pDN3 is shown in blue. It should be noted that plasmid sizes are not proportional.

Figure 3.10: Construction of pDN4, kanamycin and ampicillin resistant derivative of pDN1. The 5.1 bp *Nsi*I/*Bam*HI insert from pGW154.1 was ligated to a 5.3 kb *Hpa*I/*Bst*EII fragment from pKT240 containing both kanamycin and ampicillin resistance determinants. The region of pKT240 which contains RSF1010 sequences is indicated by a narrow blue line. The multiple cloning site in pDN4 is shown in blue. It should be noted that plasmid sizes are not proportional.

These pDN1 derivatives were transformed into *E. coli* strain DH5-α, and were isolated under selective conditions. It is significant that pDN1 derivatives are able to replicate stably in *E. coli* since this means that development of a shuttle vector that can replicate in both *E. coli* and in *D. nodosus* is unnecessary, and as suggested from sequence analysis, supports the idea that the broad-host range characteristic of IncQ plasmids has been maintained in this native *D. nodosus* plasmid. Furthermore, since pDN1 derivatives can replicate in *E. coli*, the plasmid is still functional after the modifications, and this demonstrates that the selective markers and promoters are intact, since they are expressed, at least in *E. coli*.

3.2.9 pDN1 does not belong to *E. coli* incompatibility group Q

The high nucleotide similarity between *oriV* of pDN1 and *oriV-2* of pIE1107 is significant because *oriV*-2 is essential for the replication of pIE1107, and it causes strong incompatibility to derivatives carrying *oriV-2* of pIE1107 but not to RSF1010 (Tietze, 1998). *oriV*-1 (96% identity to RSF1010 *oriV*) is dispensable for the replication of pIE1107(Tietze, 1998). These observations indicate that pIE1107 belongs to a different incompatibility group than the related IncQ plasmid RSF1010, and hence suggests that pDN1 may also belong to a different incompatibility group.

Plasmid incompatibility is defined as the failure of two coresident plasmids to be stably inherited in the absence of external selection (Novick, 1987). For multiple copy plasmids, such as those belonging to the IncQ group, two different IncQ plasmids could transiently coexist in the one cell since the copy-number-control mechanism would adjust the total number of IncQ replicons, but would not distinguish between them. Eventually, after several cell divisions, and under random selection, only one of the two different IncQ plasmids would be present in any given cell. Consequently, only coresident plasmids belonging to different incompatibility groups can be maintained stably in a given host cell in the absence of selection.

In order to determine whether pDN1 belongs to the *E. coli* IncQ group, *E. coli* DH5-α cells containing pDN3 (*tet*[R] pDN1 derivative) were transformed with an RSF1010-derived plasmid, pKT240, or pDN4 (*kan*[R] pDN1 derivative) (Section 3.2.8.3). After approximately 36 generations in the absence of antibiotic selection, 100% of pDN3/pKT240 co-transformants maintained both plasmids (Table 3.2). In contrast, pDN3/pDN4 co-transformants contained either pDN3 or pDN4, but not both plasmids, as would be expected for two plasmids belonging to the same incompatibility group (Table 3.2; Figure 3.11). Since pDN3 and IncQ derivative pKT240 are not incompatible in the same host cell, they belong to different *E. coli* incompatibility groups.

Table 3.2: Incompatibility properties of pDN1 and IncQ plasmid pKT240

Resident plasmid	Incoming plasmid	% loss of Resident plasmid	% loss of Incoming plasmid
pDN3 tet[R]	pKT240 amp[R]kan[R]	0[a]	0[a]
pDN3 tet[R]	pDN4 amp[R]kan[R]	0.7[b]	99.3[b]

Note: a total of 168[a] and 144[b] colonies were screened respectively

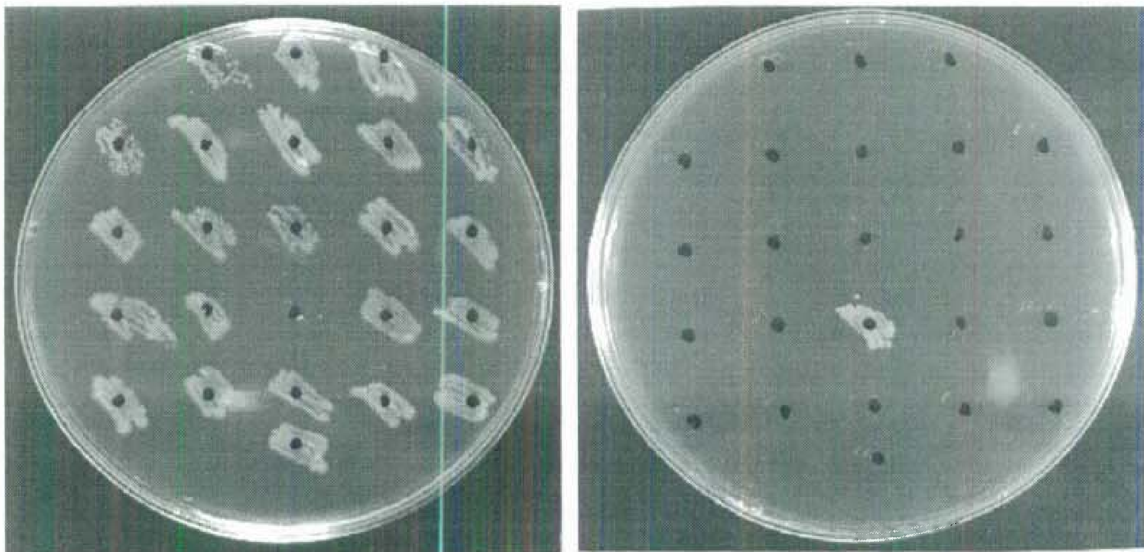

Figure 3.11: Incompatibility experiments. Single colonies of pDN1 derivatives, pDN3 (*tet*[R]) and pDN4 (*amp*[R]/*kan*[R]) cotransformants were picked and streaked on media containing tetracycline (12.5 μg/ml) **[left panel]** and media containing kanamycin (40 μg/ml) **[right panel]**. Results show that in the absence of co-selection none of the cotransformants contain both pDN3 and incoming plasmid pDN4.

Comparison of the iteron regions of pDN1 and related plasmids (Figure 3.5), indicates that the experimentally-observed difference in incompatibility, may be attributed to four base pair changes within this region that is responsible for plasmid incompatibility (Nordstrom, 1990). The *oriV* of pDN1 contains three 20 bp direct repeats with the consensus sequence 5'-CCCCCGGCGTAACTGTCACG-3', and each of these iterons is separated by two nucleotides.

The iteron region of pIE1107 (*oriV*-2) is identical to that of pDN1 except that it contains four of these direct repeats instead of three (Tietze, 1998). The iterons of RSF1010 are also similar to those in pDN1, however, the sequence 5'-CGGCG-3' within each of the direct repeats is instead 5'-TGCAA-3' in each of the three complete direct repeats that comprise the RSF1010 iterons (Figure 3.5). This indicates that these nucleotides are critical for the RepC protein to distinguish the pDN1-like iterons from the IncQ-like iterons.

It has been shown previously with R1162, that base changes within the iterons decrease the degree of incompatibility between the wild-type origin and mutated origin by altering the binding efficiency of RepC to the mutated origin (Lin & Meyer, 1984). Hence, the 4 base pair changes identified in each of the direct repeats may be responsible for the lack of incompatibility observed between IncQ plasmid pKT240 and pDN3 in this work, and between RSF1010 and pIE1107 (Tietze, 1998). The observation that the *repA*-encoded DnaA-like protein has also diverged from the RepA protein of RSF1010 (Table 3.1), suggests that the specificity of pDN1-encoded *repA* has diverged such that it specifically recognises the pDN1-iterons rather that IncQ-iterons.

This hypothesis is further supported by the observation that although the iteron region of pTF-FC2 is similar but divergent from both the pDN1-like iterons and the

RSF1010-like iterons, the aforementioned five nucleotide sequence within the iteron region identical to the sequence in pDN1 iterons. Despite the divergence at other places in the *oriV* region (Figure 3.5) it has been demonstrated previously that pTF-FC2 is compatible with IncQ plasmids (Dorrington & Rawlings, 1990), and it was recently demonstrated that pTF-FC2 expresses incompatibility towards pIE1107 (Tietze, 1998).

It is interesting that in pDN1, RSF1010, *oriV-1* of pIE1107 and in pTF-FC2 there are three copies of the iteron sequence, but in the *oriV-2* of pIE1107 there are 4 copies of the iteron repeat unit (Figure 3.5). The addition of the extra copies of these repeats in well characterised plasmids such as RK2 (Thomas, Stalker & Helinski, 1981), F (Tsutsui *et al.*, 1983), RK6 (Kolter, 1981) has the effect of reducing the replication frequency or copy number but does not affect plasmid stability (Nordstrom, 1990).

The *oriV* of pDN1 also contains a large inverted repeat consisting of a 67 bp (→) sequence and a 61 bp sequence (←) (Figure 3.11). The 3'-ends of these inverted repeats contain sequences of 39 bp and 35 bp respectively, that are able to assume a stem-loop structure, and correspond to the *ssi* signals A (position 377 to 411) and B (266 to 304 complement) that act as priming sites for the initiation of DNA chain elongation (Haring *et al*, 1989; Sakai & Komano, 1996) in a plasmid that is identical to RSF1010, called R1162 (Barth & Grinter, 1974; Guerry, van Embden & Falkow, 1974; Meyer, Hinds & Brasch, 1982).

In addition, pDN1 contains three 10 bp direct repeats with the consensus sequence 5'-TTCAGGGGCCA-3' that immediately follow the 3' end of the 61 bp inverted repeat. This 10 bp repeat is not present in RSF1010 and there are only two copies present in the pIE1107 origins. The significance of these 10 bp repeats is unknown.

3.2.10 Growth characteristics of *D. nodosus* strains A198, C305, 1311, and 1311A in Eugonbroth media

Four different strains (A198, C305, 1311 and 1311A) of *D. nodosus* were chosen to be utilised in transformation experiments. Strain A198 was chosen because it is a virulent strain, which contains copies of the *vap* genes, and is perhaps the best genetically characterised strain of *D. nodosus*. Transformation of strain A198 would potentially allow the selective deletion of certain virulence-associated genes, or the introduction of the putative global repressor of virulence, *glpA,* which may yield insight into virulence gene regulation in this pathogen.

Benign strain C305 contains no *vap* regions, and consequently, if it were successfully transformed one could introduce individual virulence-associated genes or regions. The introduction of virulence-associated regions would probably be more informative given the multifactorial nature of bacterial pathogenesis.

Strain 1311 is the host strain of native *D. nodosus* plasmid pDN1. Since pDN1 is known to replicate stably in this strain even in the absence of selection, the introduction of antibiotic resistant pDN1 derivatives should yield stable transformants. Transformation of pDN1 antibiotic resistant derivatives into strain 1311 would destabilise the resident pDN1 plasmid, although acquisition of an incoming plasmid is likely to occur at a reduced frequency because the incoming plasmid needs to replace the resident plasmid.

Strain 1311A was generated from strain 1311 after spontaneous loss of the native plasmid pDN1 (Section 3.2.7). Since the native plasmid replicates in parent strain 1311, pDN1 derivatives may also replicate stably in plasmid-less daughter strain 1311A. The introduction of pDN1 derivatives into strain 1311A, would not require the displacement of a resident plasmid as in strain 1311, and since strain 1311A is essentially isogenic with the strain from which the plasmid was isolated, it is likely to be an ideal recipient.

It has previously been noted that different prokaryotic cells are competent for transformation at different stages of their growth (Mercenier & Chassy, 1988; Saunders, Docherty & Humphreys, 1984). In an effort to address this, growth characteristics of the four strains of *D. nodosus* to be utilised in the transformation experiments were examined. Over a six day period the optical density at 600 nm, a cell count and cell viability were determined for *D. nodosus* strains A198, C305, 1311 and 1311A. The results of these experiments are shown (Figure 3.12).

The results of these experiments indicate that strain A198 undergoes log phase growth for the first 2 days, after which viability starts to decrease. Strain C305 has the shortest generation time, reaching the end of log phase after day 1. Strain 1311 has the slowest growth rate, ending log phase after 3 days. It is interesting that daughter strain 1311A seems to have reduced its generation time by losing pDN1, reaching the end of log phase after 2 days. After log phase, all strains show a substantial loss in viability.

Figure 3.12: (on following pages) Growth of *D. nodosus* virulent strain A198 (**A**), benign strain C305 (**B**), virulent strain 1311 (**C**) and strain 1311A (**D**) in 80 ml Eugonbroth cultures over six days. Growth measurements included: the optical density at 600 nm (squares); a cell count (circles); and a viable count (triangles). The experiment was performed twice, and the first data series (blue) and second data series (red) are distinguished in the above graph.