Chapter 9
Interactions Between Bacteriophage DinoHI and a Network of Integrated Elements Which Control Virulence in *Dichelobacter nodosus*, the Causative Agent of Ovine Footrot

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9.1 Introduction

Dichelobacter nodosus is an anaerobic, non-spore-forming, non-flagellated gram-negative rod (Egerton et al. 1988) with terminal knob-like swellings and polar type 4 *N*-methylphenylalanine fimbriae (Dalrymple and Mattick 1987). It has been assigned to the family *Cardiobacteriaceae* in the gamma subdivision of the *Proteobacteria* (Dewhirst et al. 1990). *D. nodosus* is the principal causative agent of footrot (Beveridge 1941), a mixed bacterial infection of the hooves of sheep, goats, deer and cattle. The genome of one strain of *D. nodosus* has been sequenced (Myers et al. 2007) and is very small (1.3 Mb) with an unusually small proportion of genes devoted to regulation. We have identified seven mobile genetic elements which integrate into the *D. nodosus* genome and have proposed that the virulence of this bacterial pathogen is modulated by these integrated genetic elements (Katz et al. 1991; Katz et al. 1994; Cheetham et al. 1995; Bloomfield et al. 1997; Whittle et al. 1999; Cheetham et al. 2008; Tanjung et al. 2009). We discuss here evidence for interactions between these mobile genetic elements and possible mechanisms for coordinate control.

9.2 Transmission of Ovine Footrot

D. nodosus is free-living and may be cultured under anaerobic conditions in the laboratory. The bacteria may persist and multiply for months or years in footrot lesions in hooves of infected animals. Although the cells die rapidly away from the host due to oxygen sensitivity, they may survive in soil or faeces or on the pasture

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for 4 to 5 days (Laing and Egerton 1978). This period of survival in the soil is crucial for the transmission of footrot, since infective material is transferred from exposed lesions on the feet of one animal to the soil or pasture, thereby contaminating the feet of previously unaffected animals. Transmission is favoured by warm, moist conditions and long pasture, which may damage the interdigital skin and facilitate penetration of the bacteria. Sheep tend to walk in single file, thus contributing to the spread of footrot (Stewart 1989). Footrot infections in sheep start in the interdigital cleft of the hoof and progressively spread down the inside wall of the claw and across the sole. This eventually results in severe underrunning and separation of the hoof from the underlying epidermis, causing severe pain and lameness which interferes with the ability of the sheep to graze, leading to loss of body condition and reduced wool production.

9.2.1 *Virulence of* D. nodosus

Different strains of *D. nodosus* cause disease of differing severity, ranging from benign to virulent. Benign footrot results in only mild lameness, which usually heals spontaneously when dry environmental conditions return. By contrast, virulent footrot spreads rapidly within a flock of sheep and results in severe lameness. Extracellular proteases produced by virulent strains are more thermostable than those produced by benign strains (Depiazzi and Rood 1984) and have greater elastolytic activity (Stewart 1979) which may aid in penetration of the hoof. In addition, virulent strains show greater twitching motility, generated by polar fimbriae (Depiazzi and Richards 1985) and twitching motility is essential for virulence (Kennan et al. 2001; Han et al. 2008).

9.3 Mobile Genetic Elements in the *D. nodosus* Genome

In an attempt to identify genes with a role in virulence, DNA sequences present in the virulent strain, A198, but absent from the benign strain, C305, were isolated (Katz et al. 1991). This work led to the identification of the *intA* (formerly *vap*) element (Katz et al. 1992; Katz et al. 1994; Cheetham et al. 1995) and the virulence-related locus, *vrl* (Haring et al. 1995; Billington et al. 1999). Further analysis of the integration site for the *intA* element in different strains led to the isolation of the *intB* (Bloomfield et al. 1997), *intC* (Whittle et al. 1999) and *intD* elements (Tanjung et al. 2009). Bacteriophage induction experiments led to the isolation of the bacteriophage DinoHI (Cheetham et al. 2008) and analysis of sequences adjacent to the integrated bacteriophage indicated the presence of another integrated genetic element, designated element X. The properties of these integrated genetic elements are discussed in detail below.

9.3.1 *The* intA *Element*

The *intA* element (Cheetham et al. 1995) is found in most virulent strains, but is also found in about 30% of benign strains. It consists of an integrase gene, *intA*, together with *toxA* and a series of *vap* (virulence-associated protein) genes, *vapA-vapH* (Fig. 9.1). Although these genes were isolated as potential virulence genes, they show no similarity to genes involved in virulence in other bacteria. Instead, *vapB-vapH* are similar to genes involved in the replication or maintenance of plasmids or bacteriophages and the *vapA*/*toxA* operon encodes a plasmid addiction system (Bloomfield et al. 1997) highly related to the *higB*/*higA* system from the killer plasmid Rts1 of *E. coli* (Tian et al. 1996).

In the prototype virulent strain A198 there are three copies, or partial copies, of the *intA* element, designated *vap* regions 1, 2 and 3 (Fig. 9.1). *Vap* region 1 is integrated into *tRNA-ser*$_{GCU}$ immediately downstream from *csrA* (formerly *glpA*, (Whittle et al. 1999) with the integrase gene, *intA1* (Myers et al. 2007) located about 200 nucleotides downstream from the end of *tRNA-ser*. *Vap* region 2 is integrated into a different tRNA-ser gene, *tRNA-ser*$_{GGA}$, immediately downstream from *pnpA*. *Vap* region 3 is located adjacent to *vap* region 1. A 19-nt sequence from the ends of both the tRNA-ser genes is duplicated at the ends of *vap* regions 2 and 3 and forms the attachment (*att*) site for integration. In most strains studied, the *intA* element, if present, is found integrated at one or both of these sites. However, in strain AC3577, the *intA* element is maintained stably as a plasmid (Billington et al. 1996).

9.3.2 *The* intB *Element*

The *intB* element (Bloomfield et al. 1997) is found in all strains tested, both benign and virulent. It is located immediately after *vap* region 3 in strain A198 and contains an integrase gene, *intB*, followed by *regA*, which has high similarity to the repressor proteins from several bacteriophages. This is followed by three genes of unknown function, *gepA*, *gepB* and *gepC* and then by genes encoding a potential sulphate transporter. The limits of the *intB* element have not been determined as the attachment sites have not been identified. Furthermore, Southern blot analysis showed that *gepA* was absent and *regA* was not directly adjacent to *gepB* in many strains. It is likely that the *intB* element at the end of *vap* region 3 in strain A198 has been disrupted. In this strain, but not some others, *intB* is a pseudogene.

A partial copy of *intB* is found at the end of *vap* region 2 in several strains. In some strains which lack the *intA* element, *intB* is located immediately downstream from either *tRNA-ser*$_{GCU}$ or *tRNA-ser*$_{GGA}$. Thus, the *intA* and *intB* elements have a common integration site and tandem integrations are possible.

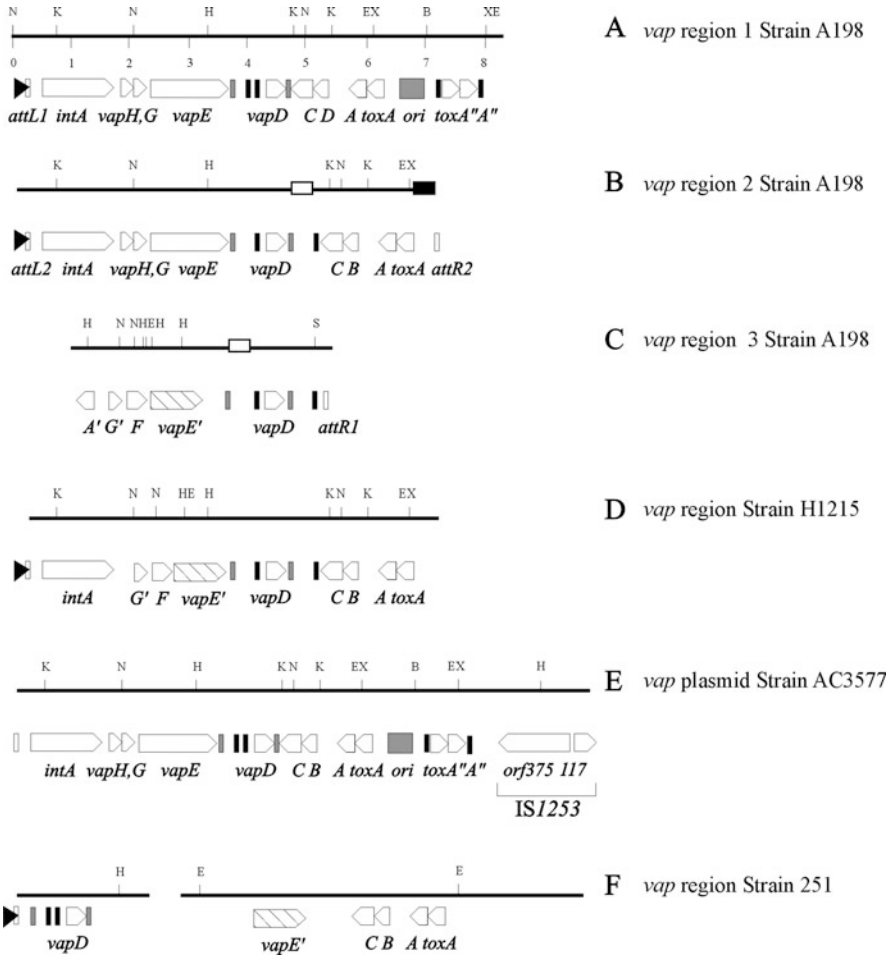


Fig. 9.1 Different forms of the *intA* element. The numbers show the distance in kb from the leftmost *Nru*I site in *vap* region 1, strain A198. The restriction sites shown are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nru*I (N), *Sac*I (S) and *Xho*I (X). Not all restriction sites are shown for strain 251. The major potential genes are shown by *open arrows*. The 19 bp *att* sites are indicated by small *open boxes*, 103 bp repeats or partial copies are indicated by small *shaded boxes*, 102 bp repeats or partial copies are indicated by small *solid boxes* and the putative origin of replication is indicated by a large *shaded box*. The tRNA-ser genes are indicated by *solid triangles*. DNA sequences found in *vap* region 2 but not in *vap* region 1 are indicated on the *scale line* for *vap* region 2. The complete DNA sequences of *vap* regions 1 and 3 have been determined. The maps of the remaining strains are based on partial DNA sequencing, Southern blotting and PCR experiments. The maps A–E have been published previously (Cheetham et al. 1999)

9.3.3 The intC *Element*

The *intC* element (Whittle et al. 1999) is present in most strains, both benign and virulent. It has been fully characterised from the benign strain C305 and contains an

integrase gene, *intC*, whose predicted product has 55% amino acid similarity with *intA*, followed by two genes of unknown function, *orf242* and *orf171* and then genes highly related to *vapGH* from the *intA* element (Fig. 9.2). These are followed by an insertion sequence, IS*1253*, which is also found on the *vap* plasmid (Billington et al. 1996). However, as with the *intB* element, Southern blot analysis of the *intC* element from different strains suggests that the *intC* element in strain C305 has been disrupted and may have lost genes. A longer form of the *intC* element which does not contain the insertion sequence is present in some strains. The integration site for the *intC* element is *tRNA-ser*$_{GCU}$. No strains with the *intC* element integrated into *tRNA-ser*$_{GGA}$ have been identified.

9.3.4 *The* intD *Element*

The *intD* element (Tanjung et al. 2009) is found in only about 20% of strains, almost all of which are benign. It is 32 kb in size and contains an integrase gene, *intD*, genes related to *vapGH* of the *intA* element, a gene related to *orf242* and *orf171*of the *intC* element, together with genes encoding a putative type IV secretion system and a mobilisation region (Fig. 9.2). Thus, it has the features of an integrated conjugative plasmid. In all strains examined so far, the *intD* element is integrated into *tRNA-ser*$_{GGA}$.

The integrase genes *intA*, *intB*, *intC* and *intD* encode integrases with about 50% amino acid identity. These integrases are generally more highly related to each other than to other integrases in the databases, but have diverged a long time ago.

9.3.5 *The Virulence-Related Locus,* vrl

The *vrl* (Haring et al. 1995; Billington et al. 1999) is a 27-kb region which has many features of an integrated genetic element, but lacks an integrase gene. It is found in most virulent strains and is absent from most benign strains. As with the *intA*, *intB*,

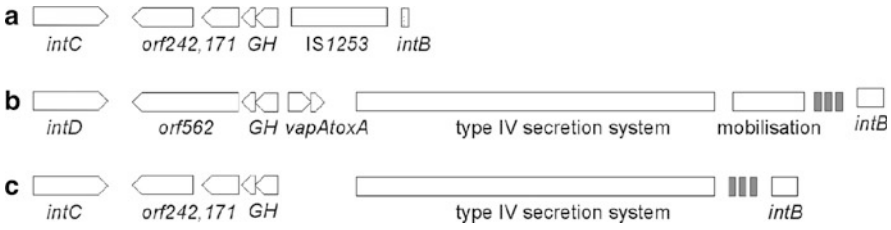


Fig. 9.2 The *intC* and *intD* elements of *D. nodosus*. (**a**) The *intC* element from strain C305. (**b**) The *intD* element from strain 819. (**c**) The proposed hybrid *intC*/*intD* element. The diagram is not drawn to scale. *Grey boxes* represent 102 bp repeated sequences. *GH* refers to the *vapG* and *vapH* genes

intC and *intD* elements, no genes with similarities to known bacterial virulence determinants were identified. The integration site for the *vrl* is *ssrA*, which encodes a potential regulatory 10Sa RNA molecule.

9.3.6 The Bacteriophage DinoHI

To investigate whether any of the integrated genetic elements were prophages, *D. nodosus* strains were treated with agents known to induce prophage excision. These experiments did not induce any of the known integrated genetic elements, but resulted in the isolation of a novel bacteriophage, DinoHI (Cheetham et al. 2008) from *D. nodosus* strain H1215. The DinoHI genome is 40 kb in size and contains an integrase gene, *intP*, which has approximately 32% amino acid identity with the integrases from the *intA*, *intB*, *intC* and *intD* elements. Although it is found predominantly in virulent strains, no genes with similarity to genes associated with virulence in bacteria were identified. The integration site for DinoHI is distinct from the integration sites for the *vrl* and the *intA* elements.

9.3.7 Element X

Analysis of sequences adjacent to DinoHI in strain H1215 indicates the presence of another integrated element, which has been designated element X (Cheetham et al. 2008). A 4 kb segment of DNA from one end of this element has been sequenced and shown to encode a type I restriction-modification system.

9.4 A Model for the Control of Virulence by Integrated Genetic Elements

The distribution of integrated genetic elements in *D. nodosus* strains is not random. In particular, the *intA* element and the *vrl* are preferentially associated with virulent strains. However, neither of these genetic elements encodes proteins with a direct role in bacterial virulence. Analysis of the integration sites for these elements has revealed three genes with a potential role as global regulators of virulence, and we have proposed that the integrated genetic elements modulate the expression of these putative global virulence regulators (Whittle et al. 1999).

9.4.1 CsrA

The *tRNA-ser*$_{GCU}$ gene is immediately downstream from *csrA* in the *D. nodosus* genome. CsrA and the closely related protein RsmA are virulence repressors in

Salmonella enterica (Lawhon et al. 2003), *Helicobacter pylori* (Barnard et al. 2004), *Legionella pneumophila* (Molofsky and Swanson 2003) and *Erwinia carotovora* (Mukherjee et al. 1996). Virulent strains of *D. nodosus* have either *intA* or *intC* integrated next to *csrA*. Furthermore, the loss of the *intC* element, which was integrated next to *csrA* in *D. nodosus* strain 1311, resulted in a strain, 1311A, with the *intB* element next to *csrA*, which had lost protease thermostability, a virulence characteristic.

We used reverse-transcriptase PCR to show that *csrA* transcripts extend into the adjacent integrated element (Fig. 9.3). RNA was prepared from four *D. nodosus* strains which have *intA*, *intB* or *intC* next to *csrA*. A forward primer (primer 1) from within the *csrA* coding region was used in conjunction with reverse primers located within *csrA* (primer 2), within the adjacent tRNA gene (primer 3), and at three locations within the *intA* element (primers 4–6). Primer 4 is located in a sequence which is conserved between the *intA* and *intC*, but not the *intB*, elements. These experiments showed that *csrA* is expressed in all strains, and that the transcripts

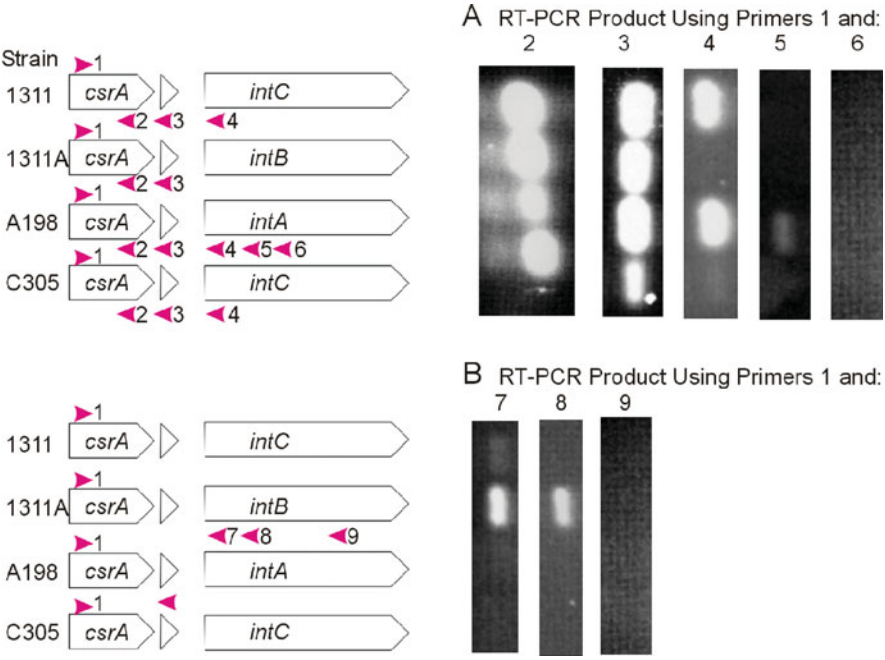


Fig. 9.3 Transcription of *csrA* extends into the adjacent integrated element. Reverse transcriptase PCR using forward primer 1 with reverse primers 2, 3, 4, 5, 6, 7, 8 or 9 was carried out on RNA prepared from *D. nodosus* strains 1311, 1311A, A198 and C305, which have *intC*, *intB*, *intA* or *intC*, respectively, next to *csrA*. The positions to which the primers bind are shown as numbered *arrowheads*. Primer 4 is complementary to a region of identical sequence in the *intA* and *intC* elements. The tRNA gene is shown as a *triangle*. Sections of agarose gels showing products of different sizes for the different primer combinations are shown in panels A and B. No products were detected when reverse transcriptase was omitted from the reaction

include the tRNA gene and extend into the *intA* and *intC* elements (Fig. 9.3a). Using a different reverse primer complementary to a sequence in the *intB*, but not *intA* or *intC* elements, we showed that transcripts also extend into the *intB* element (Fig. 9.3b). Thus, the 3′ ends of *csrA* transcripts are different in strains with different integrated elements at this locus. This may alter transcript stability or the capacity of the mRNA to be translated, thereby altering the expression of this putative virulence regulator.

9.4.2 PnpA

The second integration site for the *intA* element is *tRNA-ser*$_{GGA}$, which is located immediately downstream from *pnpA*. The *pnpA* product, polynucleotide phosphorylase, is a global regulator of virulence in *S. enterica* (Clements et al. 2002). Almost all virulent strains of *D. nodosus*, which have been tested, have *intA* at this integration site. We have shown that partial knockouts of polynucleotide phosphorylase in benign strains of *D. nodosus* result in increased twitching motility, which is a characteristic of virulent strains (Palanisamy et al., 2010). This supports the hypothesis that PnpA acts as a virulence repressor in *D. nodosus*.

9.4.3 10Sa RNA

The *vrl* is integrated into *ssrA* (Haring et al. 1995) which encodes 10Sa RNA, a tmRNA molecule that is involved in the removal of ribosomes stalled on mRNA molecules and the subsequent proteolytic degradation of the resultant peptides (Withey and Friedman 2002). In *S. enterica*, mutations in *ssrA* reduce the expression of genes from the virulence plasmid (Julio et al. 2000).

9.4.4 Model for Virulence

Comparison of the arrangement of integrated genetic elements has shown that, in general, virulent strains have *intA* or *intC* next to *csrA*, *intA* next to *pnpA* and the *vrl* next to *ssrA* (Fig. 9.4). Benign strains may have other integrated genetic elements next to *csrA* or *pnpA* or may lack the *vrl*. We have proposed that these genetic elements modulate the expression of *csrA*, *pnpA* and *ssrA*, which encode virulence regulators. The regulation of virulence by integrated genetic elements in *D. nodosus* may compensate for the dearth of regulatory genes in the very small genome (Myers et al. 2007).

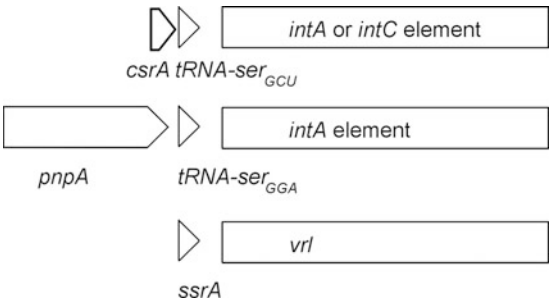


Fig. 9.4 Model for virulence of *D. nodosus*. Virulent strains have either the *intA* or *intC* element integrated into *tRNA-ser*$_{GCU}$, the *intA* element integrated into *tRNA-ser*$_{GGA}$ and the *vrl* integrated into *ssrA*. Benign strains differ in at least one integration site

9.5 Different Forms of the *intA* Element

There is considerable variation in the *intA* element, both within and between strains (Fig. 9.1). In strain A198, v*ap* region 1 contains *intA*, a complete suite of *vap* genes (*vapA-H*), *toxA*, and a second copy of the plasmid addiction system, *vapA″/toxA″*. *Vap* region 2 contains the same genes with the exception of *vapA″/toxA″*, but has a small insertion between *vapD* and *vapC* and a short additional sequence after *toxA*. *Vap* region 3 lacks *toxA* and *vapA-C* and has *vapE′* in the place of *vapE*. VapE′ has 67% amino acid identity with VapE. The plasmid form of the *intA* element found in strain AC3577 is identical to *vap* region 1, with the addition of the insertion sequence IS*1253*. In some strains, such as strain H1215, a complete set of *vap* genes is present, but *vapE′* is found instead of *vapE*. Other strains, such as strain 251, have been identified which lack *intA* and have *vapD* immediately downstream from *intA* (Tanjung, unpublished).

Alternative forms of the *intA* element may have arisen by insertions or deletions after the primary integration event. However, analysis of sequences from the *intA* element shows that these events are not simple. For example, strain 251 lacks *intA* and has *vapD* immediately downstream from the integration site. This arrangement could be generated by integration of the *intA* element, followed by the deletion of sequences between the integration site and *vapD*. However, DNA sequencing of the region between the integration site and *vapD* showed that it contains an additional sequence which is found after *toxA* in *vap* region 2. Strain 251 contains *vapE′*, which would normally be located between *vapD* and *intA*, and also contains *vapA*, *vapB* and *vapC*. The exact arrangement of these genes is unknown as Southern blot analysis does not link *vapD* with any of the other *vap* genes in this strain (Fig. 9.1). It is possible that *vapA-C* and *vapE′* are integrated at a different site.

We have identified two families of repeated sequences within *vap* regions 1 and 3 (Cheetham et al. 1995) and have proposed that these sequences may play a role in DNA rearrangements within the *vap* regions. Comparison of DNA sequences between *vap* regions 1, 2 and 3 of strain A198 and the *vap* plasmid of strain AC3577 has shown that these repeats (or partial copies of the repeats) are often located at boundaries where the sequences diverge (Cheetham and Katz 1995; Billington et al. 1996).

9.6 Interactions Between the Integrated Genetic Elements

While the *intA* element is usually integrated into the genome, it may replicate as a plasmid (Billington et al. 1996). The *intD* element has the features of a conjugative plasmid, and excision of the *intD* element has been seen in one strain (Tanjung et al. 2009). Loss of the *intC* element has also been observed (Whittle et al. 1999), so excision of this integrated element is possible. The bacteriophage DinoHI can be stably maintained in the integrated form, but may be induced to replicate by ultraviolet light (Cheetham et al. 2008). The *intB* element and the *vrl* have some bacteriophage-like features but have only been found in the integrated state. Features of these integrated elements which suggest that their replication may be co-ordinately controlled are discussed below.

9.6.1 vapGH *and Bacteriophage Immunity*

The *vapGH* region is located immediately downstream from *intA* in *vap* regions 1 and 2 of strain A198. Related sequences are found on both the *intC* (Whittle et al. 1999) and *intD* (Tanjung et al. 2009) elements (Fig. 9.5). Sequence analysis suggests that *vapH* is not translated since there is no likely Shine-Dalgarno sequence upstream of the start codon. Blast searches revealed that *vapH* and *vapG* are related to *orf*179 of *S. flexneri*, which is in turn related to *orf199* of bacteriophage P4 (Fig. 9.6). During lysogeny in bacteriophage P4, there is constitutive transcription from the promoter P_{LE} (Fig. 9.6), which is located 36 nt downstream from the start codon for *orf199*. Within these transcripts are four

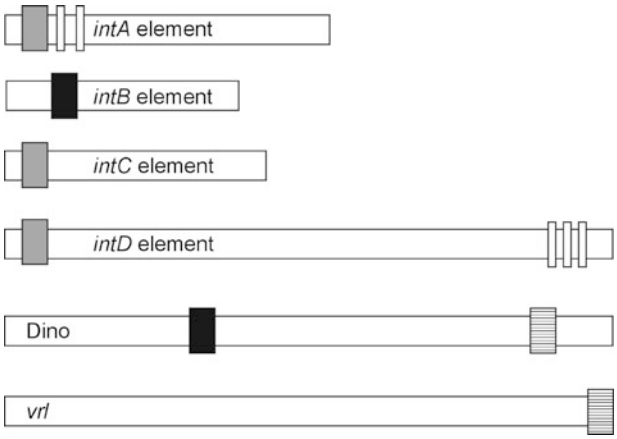
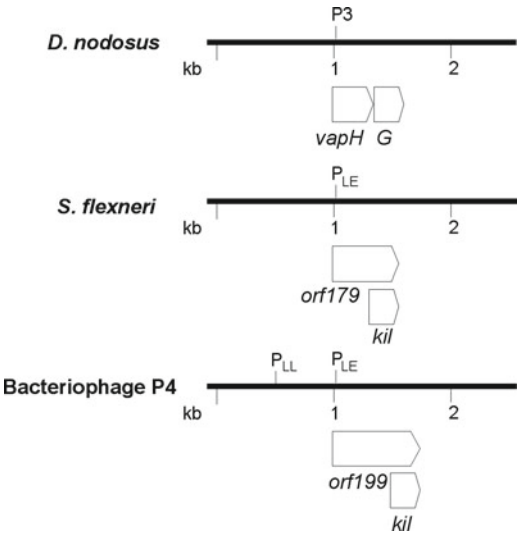


Fig. 9.5 Location of regulatory sequences shared by integrated elements of *D. nodosus*. *Grey boxes* – *vapGH*, *white boxes* – 102 bp repeats, *black boxes* – *regA*, *hatched boxes* – DinoHI packaging site. The figure is not drawn to scale

a



b

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          orf199                                        -35          P_LE          -10
P4    ATGATGATGCCCGCTCTGCAAAAACTCCCTTTTTCTGGCTTGCCTTTTTCCGGCATTTGCGGATATAGTT  50

                    orf179
Sf    -CGGTGATGAAAGGAAAGAGAAAATGTTGAATGTAGCTATTGAAAACCAGAACGGG-TGGAATTATAGTG  48

                    vapH
Dn    ATTTAAGGAGATTATTCAATAAAAATGAAGACTTATCTATTGACAAGCCAAGAGCTTTTAGCCTATAGTC  50

                                  seqA
P4    TTTCCGCTGCCGCAAAATCGGCAGCCGGGCGTGAGAACCCGAGCAATTCACAGGCGACATATGACGCGCC 100
Sf    CACCTGCACCTCATAAAACGGGTGCCGGGATTGCTACCCCGACGATGACTACTGCGCATAACCGCGCTCA  98
Dn    CACTCGCTACAGTAAGCACTGTAGTCGGGATTGGAACCCCGTTGATACGAAAGCGTGTACAGCGCTTATT 100

P4    ACGCGTCTTTTTTTGTGTCGCAATCAATGCCACAGAGCGCCAGATTATGGTGTGGTGTGCGGGGCAGCCT 150
Sf    GGCGGTTTTTTTATGCGTC------AAGCACAGCCACATTCAGATTATGGTGGGGCGTGCAGGGCAGCCG 142
Dn    AAGCCATTTTTTTATG-----CAA--AATTTGTCTATGCAAGCATACGTGGGAGAACCGAAAGGT--TCGC 141

                                  seqB                                  seqC
P4    CCGGGCTGGCCGGTATCCTGTGAAGCCGGTATTCTCACCCCCGTCTGGGCTATCGCCATCGAGCGTGAGA 200
Sf    CAAGGCTGGCCGGTTTCGGTAGTCACCGGTTGTAGCAACCCTGTGCGTCTCACCACCCATGAGATTGCTA 192
Dn    --CGTTTCTTTCGTAGCGGTAGTTCCAATCTCGCGTATGCTGTCGCCCTCTTATTTGGAACTAATGGCGG 189

                                seqC'          kil
P4    ACTCCGGCGATAGCGTATTTGCTATT-CACAGGAGGCTGCCATCATGGCTACAACCCTTACCCCCTCACA 250
Sf    CCTCTGGTGGTGAGTCATTTAAACTGACTATCGAGGCTGCCATCATGGCTACTATCCTTACCCTTTCTCA 242
                                                  vapG
Dn    CAGTCAATCCAAAATTAC----------GAAAGGAGACTGCGAATGTCTAATTCATTATCGCTGACTAT 232
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Fig. 9.6 (**a**) Similarities between the *vapGH* region of *D. nodosus*, *orf179* of *S. flexneri* and the immunity region of bacteriophage P4. The location of promoters P3, P_{LE} and P_{LL} is shown. (**b**) Alignment of DNA sequences from the immunity region from bacteriophage P4 (P4) containing *orf199* with regions containing *orf179* from *Shigella flexneri* (Sf) and *vapH* and *vapG* from

sequences designated *seqA*, *seqB*, *seqC* and *seqC′* which form secondary structures resulting in premature termination of transcription and degradation by RNases (Ghisotti et al. 1992; Deho et al. 1992; Lindqvist et al. 1993). Transcription of *orf199* can be initiated from the upstream promoter P_{LL} (Fig. 9.3), but translation of *orf199* and the in-frame gene *kil* (Fig. 9.3) is blocked by binding of the transcripts from the P_{LE} promoter to the *seqB* and *seqC* regions. This system maintains the lysogenic state and prevents superinfection since it prevents incoming P4 particles from expressing genes required for the lytic cycle and replication. In bacteriophage P4, the *kil*-encoded protein is involved in the inhibition of cell division (Ghisotti et al. 1992) and only expressed from P_{LL} during the lytic cycle.

In *D. nodosus*, there is a promoter, P3 (Fig. 9.6) located 16 nt downstream from the start codon for *vapH*. Alignment of the sequences of *vapH* with *orf179* of *S. flexneri* and *orf199* of bacteriophage P4 (Fig. 9.6b) shows that *vapH* contains sequences equivalent to *seqA*, *seqB*, *seqC* and *seqC′*, located in approximately the same positions and capable of forming secondary structures. The predicted start codon for *vapG* is in the same position as the start codons for *kil*in *S. flexneri* and bacteriophage P4. Thus, the *vapGH* region of *D. nodosus* closely resembles the *orf199* region of bacteriophage P4, and *vapH* transcripts may prevent the translation of *vapG* from integrated elements, possibly to maintain the integrated state. Although *vapG* does not share sequence homology with *kil*, it may perform a similar function. It is of interest that only one strain of *D. nodosus* has been identified in which the *intA* element is stably maintained as a plasmid and in this strain, AC3577, there is a deletion of six nucleotides together with 18 base substitution mutations within *vapH*.

The *vapGH* system is found on the *intA*, *intC* and *intD* elements. Since numerous strains contain both the *intA* and the *intC* elements, the *intA* and *intD* elements, the *intC* and *intD* elements, or multiple copies of the *intA* element, the *vapGH* system does not appear to affect superinfection.

9.6.2 *A Repressor Gene Common to Bacteriophage DinoHI and the* intB *Element*

The *intB* element contains a gene, *regA*, which is related to several bacteriophage repressor proteins, including the cI repressor from lambdoid bacteriophages (Bloomfield et al. 1997). The DinoHI genome (Cheetham et al. 2008) contains

Fig. 9.6 (continued) D. nodosus (Dn). Start codons for *orf199*, *orf179*, *vapH*, *kil* and *vapG* are *underlined* and *bold*. Sequences that can form *seqA*, *seqB*, *seqC* and *seqC′* which interact to form stem-loop structures are in *bold*. The consensus sequence for the P_{LE} promoter of bacteriophage P4 and the corresponding promoter regions from *S. flexneri* and *D. nodosus*, respectively, are *underlined*. Numbers on the *right hand side* show the nucleotide number. Accession numbers are as follows: P4 (MYP4CG), Sf (Z23101) and Dn (L31763)

a highly related gene, *regA2* (Fig. 9.5) which has 97% DNA sequence identity over 701 nt, resulting in a protein product which is identical for 231 of the 232 amino acids of the *intB* RegA. However, the start codon is absent in the DinoHI sequence. Instead, there is another potential start codon upstream which would result in an extra 17 amino acids at the N-terminus for the DinoHI RegA protein.

The likely function of RegA is to maintain the *intB* element and the DinoHI prophage in the integrated state. The high level of amino acid identity between RegA and RegA2 suggests that the two proteins may be functionally interchangeable, i.e. these repressor proteins would maintain both these genetic elements in the integrated state. Thus, excision of bacteriphage DinoHI and the *intB* element could be co-ordinately controlled.

9.6.3 *Interactions Between the* vrl, *DinoHI and the* intA *Element*

A 1.8 kb DNA sequence from the DinoHI genome which contains the linear ends is also found at the end of the *vrl* (Cheetham et al. 2008). Thus, strains which contain both DinoHI and the *vrl* have two copies of this DNA sequence. This sequence is used for packaging of the DinoHI genome into the DinoHI bacteriophage particle. The presence of this sequence at the ends of the *vrl* suggests that the *vrl* may be transferred between *D. nodosus* strains by transduction.

The *vrl* lacks an integrase gene, but all strains which contain the *vrl* contain the *intA* element, suggesting that the *intA* integrase may be used for integration of the *vrl* (Billington et al. 1996).

9.6.4 *Mobilisation of the* intA *Element by the* intD *Mobilisation Cassette*

The *intD* element encodes a type IV secretion system with a probable role in DNA transport during conjugation (Tanjung et al. 2009). A putative origin of replication, *oriV*, and an adjacent origin of transfer, *oriT*, have also been identified. Thus, the *intD* element appears to be self-transmissible. The *intD* element contains a mobilisation region which may be involved in the transfer of smaller plasmids or other non-self-transmissible elements. Mobilisation regions typically encode proteins that function in the nicking of the *oriT* region, the subsequent piloting of the 5′ end of the nicked strand into the recipient cell and recircularisation and priming of the complementary strand for replication (Boyd et al. 1989). Four potential genes clustered at the end of the *intD* element, which contains *oriT*, share amino acid similarity with mobilisation genes. The *oriT* sequence from the *intD* element is also found on the *intA* element, next to the origin of replication (Cheetham et al. 1995). This strongly suggests that the *intA* element can be mobilised and transferred to other *D. nodosus* strains by the *intD* element.

9.6.5 *Common Repeated Sequences on the* intA *and* intD *Elements*

A 102 bp repeat is found at several locations on the *intA* element (Cheetham et al. 1995) and may have a role in rearrangements since copies or partial copies of this sequence are found at boundaries where there is sequence divergence in different forms of the *intA* element (Sect. 9.5). The *intD* element contains three copies of this 102 bp repeat, suggesting that it may be possible to exchange sequences between these two elements.

9.6.6 *Relationships Between the* intC *and* intD *Elements*

Southern blot analysis of a range of *D. nodosus* strains has shown the presence of large segments of the *intD* element in strains which lack the integrase gene, *intD* (Tanjung et al. 2009). This raises the possibility that sequences from the *intD* element may be associated with a different integrated element in other strains (Fig. 9.2). All these strains contained *intC*, and loss of the *intC* element in one of these, strain 1311, resulted in concomitant loss of the segments of the *intD* element. Thus, it seems likely that the genes from the *intD* element are found on the *intC* element in some strains. As described in Sect. 9.3.3, the *intC* element has only been fully characterised from strain C305 where it may have been disrupted by an insertion sequence. The strains which lack *intD* but contain segments of the *intD* element are those strains which appear to have a longer form of the *intC* element.

9.7 Evolutionary Significance

The analysis of *D. nodosus* strains from different sheep flocks in Australia has shown a great deal of genetic diversity. In addition, flocks of sheep or even individuals within the flock may be infected with multiple strains (Claxton et al. 1983), and it is not uncommon to isolate both benign and virulent strains from within the same flock. The persistence of both benign and virulent strains suggests that there may be evolutionary advantages under different conditions. Footrot infections are greatly influenced by environmental conditions. Under ideal conditions of moderate temperatures and high moisture levels, infections tend to be very severe and virulent strains may rapidly destroy the hoof, eventually resulting in death of the host. Under hot, dry conditions, benign footrot rapidly resolves and virulent strains may be more able to survive as isolated pockets of infection within the hoof. We have proposed that strains may switch between benign and virulent states by the excision, or acquisition and integration, of genetic elements, thereby altering the expression of three genes encoding virulence regulators (CsrA, PnpA and 10Sa RNA). This provides a mechanism for rapid and reversible adaptation of strains.

9.8 Conclusions

We have identified a series of genetic elements integrated into the *D. nodosus* genome and have proposed that these genetic elements modulate the expression of upstream genes encoding putative virulence regulators. The *intA*, *intB*, *intC* and *intD* elements integrate downstream from *csrA* while the *intA*, *intB* and *intD* elements may also integrate downstream from *pnpA*. The *vrl* integrates downstream from *ssrA*. The bacteriophage DinoHI does not appear to influence virulence directly, but may be responsible for the transfer of the *vrl* between strains. The *vapG*/*vapH* system may maintain the *intA*, *intC* and *intD* elements in the integrated state, while the putative repressor, RegA, may control both the *intB* element and DinoHI. Exchange of sequences between some of these genetic elements also appears likely. Thus, our model suggests that the integrated elements of *D. nodosus* control the expression of virulence determinants and that the elements themselves are controlled in a coordinated manner.

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