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The site-specific integration of genetic elements may modulate thermostable protease production, a virulence factor in *Dichelobacter nodosus*, the causative agent of ovine footrot

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The Gram-negative anaerobe *Dichelobacter nodosus* is the causative agent of footrot in sheep. The authors have previously characterized two genetic elements, the *intA* (*vap*) and *intB* elements, which integrate into the genome of *D. nodosus*. In the virulent strain A198 there are two copies of the *intA* element. One copy is integrated into the 3′ end of the *tRNA-ser*$_{GCU}$ gene, close to the aspartokinase (*askA*) gene, and the second copy is integrated into the 3′ end of the *tRNA-ser*$_{GGA}$ gene, next to the polynucleotide phosphorylase (*pnpA*) gene. In this study, a new genetic element was identified in the benign strain C305, the *intC* element, integrated into the 3′ end of the *tRNA-ser*$_{GCU}$ gene, next to *askA*. The *intC* element was found in most *D. nodosus* strains, both benign and virulent, which were examined, and was integrated into *tRNA-ser*$_{GCU}$ in most strains. Between the *askA* and *tRNA-ser*$_{GCU}$ genes, a gene (designated *glpA*), was identified whose predicted protein product has very high amino acid identity with RsmA from the plant pathogen *Erwinia carotovora*. RsmA acts as a global repressor of pathogenicity in *E. carotovora*, by repressing the production of extracellular enzymes. In virulent strains of *D. nodosus* the *intA* element was found to be integrated next to *pnpA*, and either the *intA* or *intC* element was integrated next to *glpA*. By contrast, all but one of the benign strains had *intB* at one or both of these two positions, and the one exception had neither *intA, intB* nor *intC* at one position. The loss of the *intC* element from the virulent strain 1311 resulted in loss of thermostable protease activity, a virulence factor in *D. nodosus*. A model for virulence is proposed whereby integration of the *intA* and *intC* genetic elements modulates virulence by altering the expression of *glpA, pnpA, tRNA-ser*$_{GCU}$ and *tRNA-ser*$_{GGA}$.

Keywords: footrot, virulence, pathogenicity, *vap*, pathogenicity island

INTRODUCTION

The Gram-negative anaerobic bacterium *Dichelobacter nodosus* is the principal causative agent of ovine footrot (Beveridge, 1941). *D. nodosus* strains are classified as virulent, intermediate or benign depending on the severity of the disease which they cause in sheep. The *vap* regions of the *D. nodosus* genome arose by the integration of a genetic element, the *intA* (*vap*) element, into a *tRNA* gene. This element is found in almost all virulent strains, but is absent from the majority of benign strains (Katz *et al.*, 1991; Rood *et al.*, 1996), suggesting that the *vap* sequences may have a role in virulence.

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Abbreviation: HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone.

The GenBank accession number for the *intC* element in strain C305 beginning at the left-most *Eco*RI site (Fig. 1) is Y15939.

Multiple copies of the *intA* element, termed *vap* regions 1, 2 and 3, are present in the virulent *D. nodosus* strain A198 (Katz *et al*., 1994). Regions 1 and 3 form part of a contiguous 11·9 kb virulence-associated region (Fig. 1; Cheetham *et al*., 1995). Region 1 is integrated into a *tRNA-ser*$_{GCU}$ gene close to the aspartokinase (*askA*) gene, while region 2 is integrated into a different *tRNA-ser* gene, *tRNA-ser*$_{GGA}$, next to the polynucleotide phosporylase (*pnpA*) gene (Bloomfield *et al*., 1997).

The genes which have been identified in the *vap* regions of the virulent *D. nodosus* strain A198 are designated *intA*, *vap* (virulence-associated protein) *A–H*, and *toxA* (Fig. 1, Katz *et al*., 1992; Cheetham *et al*., 1995; Bloomfield *et al*., 1997). The *intA* gene product is an integrase belonging to the lambda family of site-specific recombinases (Cheetham *et al*., 1995). VapA and ToxA show amino acid similarity to the HigA and HigB proteins from the killer plasmid Rts1 (Tian *et al*., 1996), which encode a toxin and antidote molecule, respectively, and act to prevent loss of the plasmid Rts1 in *Escherichia coli*. VapA and ToxA may have a similar function in *D. nodosus* (Bloomfield *et al*., 1997). The *vapB* and *vapC* genes are similar to *vagC* and *vagD* from the virulence plasmid of *Salmonella dublin*. The products of these genes are believed to coordinate replication of the virulence plasmid with cell division (Pullinger & Lax, 1992). VapD and VapE have amino acid similarity to the products of plasmid-borne genes of unknown function from a variety of bacterial species (Katz *et al*., 1992; Cheetham *et al*., 1995). The remaining genes, *vapF*, *vapG* and *vapH*, have no similarity to previously identified genes.

A native plasmid which contains *vap* region 1 and an insertion sequence (IS*1253*) has been isolated from strain AC3577 of *D. nodosus*, and may be the progenitor of the chromosomal *vap* sequences found in other strains (Billington *et al*., 1996). IS*1253* is not found in the *vap* regions of strain A198, but there is a copy near the outer-membrane protein (*omp*) gene cluster, which is responsible for production of the major outer-membrane protein of *D. nodosus* (Moses *et al*., 1995).

A second genetic element, the *intB* element, which may be part of a prophage or a conjugative transposon, is integrated next to *vap* region 3 in strain A198 (Bloomfield *et al*., 1997). The end of this element contains an integrase gene, *intB*, a regulatory gene, *regA* (Fig. 1), and three genes of unknown function, *gepA*, *gepB* and *gepC*.

In this paper, we describe a new genetic element, the *intC* element, in *D. nodosus*. In strain C305, the *intC* element is integrated into *tRNA-ser*$_{GCU}$, next to *askA*. We show that loss of this element from one virulent strain resulted in a concomitant loss of thermostable protease activity, a virulence factor in *D. nodosus* (Depiazzi *et al*., 1990). Mechanisms by which the loss of the *intC* element might result in the loss of thermostable protease activity are discussed.

METHODS

General methods. Methods for the growth of *D. nodosus*, preparation of genomic DNA, cloning and analysis of DNA, Southern blotting, DNA sequencing and DNA sequence analysis have been reported elsewhere (Katz *et al*., 1994; Bloomfield *et al*., 1997).

***Dichelobacter nodosus* strains.** Strains A198, AC390, B1006, C305, D1172, G1220, H1204, H1215, 1469, 1493 and 3138 were obtained from Professor J. I. Rood, Monash University, Clayton, Victoria 3168, Australia, and strains 819, 1169, 1311 and 2483 from Dr J. Searson, NSW Agriculture, Private Mail Bag, Yanco, NSW 2703, Australia.

Preparation and screening of a library of genomic DNA from *D. nodosus* strain C305. Genomic DNA from *D. nodosus* strain C305 was partially digested with the restriction endonuclease *Sau*3AI and fragments were ligated into the *Xho*I half-site arms of bacteriophage lambdaGEM-12 (Promega), packaged according to the directions of the manufacturer (Promega), and used to infect the lambda-sensitive *E. coli* strain LE392 (Sambrook *et al*., 1989). The library was screened by plaque-lift hybridization, on nitrocellulose membranes, using digoxigen-labelled probes and a chemiluminescent detection kit (Boehringer Mannheim), according to the manufacturer's instructions. DNA was prepared from the lambda clones by the method of Ausubel *et al*. (1989).

Oligonucleotide primers. Primer sequences were: primer A (*askA*) TAGAAGCCACGCATGTTACG; primer B (*intC*) AGCGGTTGTTCCCACATAAA; and primer C (*intB*) ATAACGCTGGCTTTAGGTGA. PCR conditions have been described previously (Bloomfield *et al*., 1997).

Assay for protease thermostability. The supernatant from liquid cultures of *D. nodosus* was assayed for proteolytic activity by measuring the release of a soluble blue dye from hide powder azure (Green, 1985). Thermostability was determined in triplicate by measuring the activity before and after heating the supernatant at 60 °C for 10 min.

RESULTS AND DISCUSSION

Isolation from strain C305 of sequences flanking *vap* regions 1 and 3 in strain A198

The benign strain C305 does not contain the genes *vapA–D* or *toxA*, suggesting that the *intA* element is not present in this strain. However, a *Hin*dIII–*Nru*I fragment located close to the left-hand end of *vap* region 3 (Fig. 1) in strain A198 has been shown previously to hybridize to genomic DNA from strain C305 (Cheetham *et al*., 1995), and repeated sequences found immediately to the left of *vapD* in strain A198 are also present in the benign strain C305 (Katz *et al*., 1994).

To investigate the hypothesis that the *vap* regions of strain A198 resulted from integrations of the *intA* element into the *tRNA-ser*$_{GCU}$ gene located between the *askA* and *intB* genes (Fig. 1), we prepared a library of genomic DNA from strain C305, and screened it with probes 1, 3 and 4A (Fig. 1). Probe 1 contained part of the *askA* gene, flanking the left-hand end of *vap* region 1 in virulent strain A198; probe 4A contained part of the *intB* gene flanking the right-hand end of *vap* region 3 in strain A198; and probe 3 contained the *Hin*dIII–*Nru*I fragment from the left-hand end of *vap* region 3 in strain

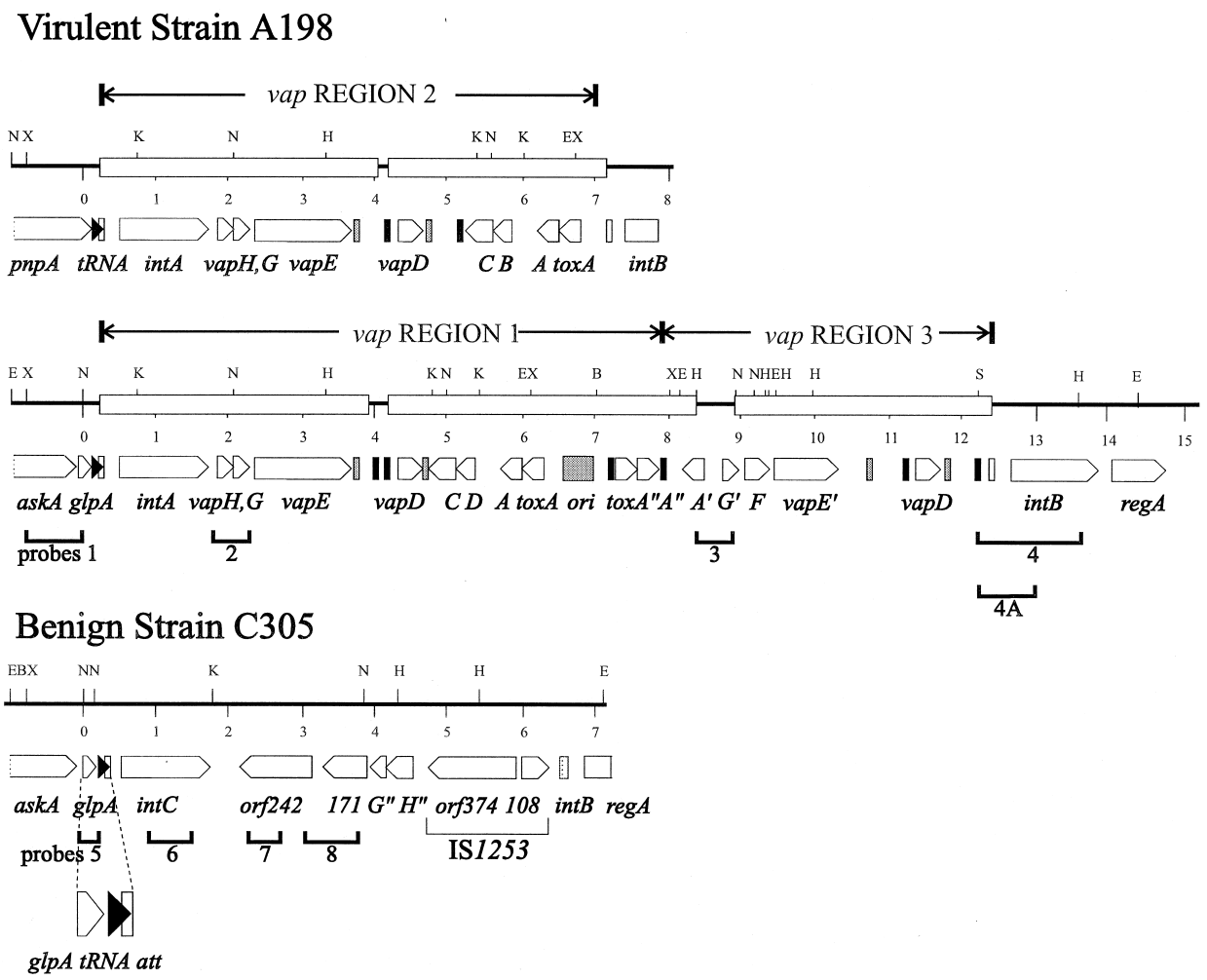


Fig. 1. *vap* regions 1, 2 and 3 of *D. nodosus* strain A198 compared with the *intC* element in *D. nodosus* strain C305. The numbers show the distance in kb from the left-most *Nru*I site in strains A198 or C305. Restriction sites shown are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nru*I (N), *Sac*I (S) and *Xho*I (X). The major potential genes are indicated by open arrows. The *tRNA-ser* genes are shown by black triangles. Repeated sequences (Cheetham *et al.*, 1995) are indicated as follows: 19 bp *att* sites (small open boxes), 103 bp repeats, or partial copies thereof (small shaded boxes), 102 bp repeats, or partial copies (small black boxes) and the putative origin of replication (large shaded box). The large open boxes show regions which are not found in strain C305.

A198. A single lambda clone to which probes 1 and 3 hybridized strongly, and probe 4A hybridized weakly, was isolated (Fig. 1). We predicted that the *askA* and *intB* genes would be close together in this clone. However, they were found to be separated by a 6·6 kb DNA segment. The DNA sequence of an 8035 bp region from this lambda clone was determined, and several ORFs were identified (Fig. 1).

A new genetic element is integrated into *tRNA-ser* in strain C305

At the left-hand end of the lambda clone from strain C305 (Fig. 1), we identified a partial ORF, designated *askA*, the DNA sequence of which is almost identical to that of the partially characterized ORF located upstream of the *tRNA-ser*$_{GCU}$ gene at the left-hand end of *vap* region 1 in *D. nodosus* strain A198 (Bloomfield *et al.*, 1997). Downstream from the *askA* gene, we identified a 92 bp *tRNA-ser* gene which had 97·8% nucleotide identity to *tRNA-ser*$_{GCU}$ from the left-hand end of *vap* region 1 of *D. nodosus* strain A198 (Cheetham *et al.*, 1995).

The predicted protein product from an ORF, designated *intC*, located 200 bp downstream from *tRNA-ser*$_{GCU}$, was found to have 54·2% amino acid identity with IntA from *vap* region 1 of *D. nodosus* strain A198 (Fig. 2a). The conserved residues included Arg^{251}, His^{336}, Arg^{339} and Tyr^{374}, which are conserved in integrases from this family (Argos *et al.*, 1986; Abremski & Hoess, 1992). The highest amino acid identity to other integrases from the databases was 40·4% with the integrase from retronphage ΦR73 (Sun *et al.*, 1991).

Fig. 2. Comparison of the amino acid sequences of (a) IntA with IntC, and (b) *Er. carotovora* RsmA with *D. nodosus* GlpA. The amino acid residues which are identical (*), amino acids with conservative substitutions (.) and amino acids conserved among many bacteriophage integrases (↓) are marked.

Two novel ORFs, designated *orf242* and *orf171*, were located downstream from *intC* in strain C305 (Fig. 1). These two ORFs had no similarity to known genes from the *vap* regions of *D. nodosus*, nor to any sequences in the databases. These were followed by two small ORFs, designated *vapG″* and *vapH″*, which showed high similarity to *vapG* and *vapH* from *vap* region 1 of strain A198. The deduced amino acid sequence of *vapG″* had 55·2% identity with VapG and VapH″ had 56·2% amino acid identity with VapH.

IS*1253* is next to *vapH″*

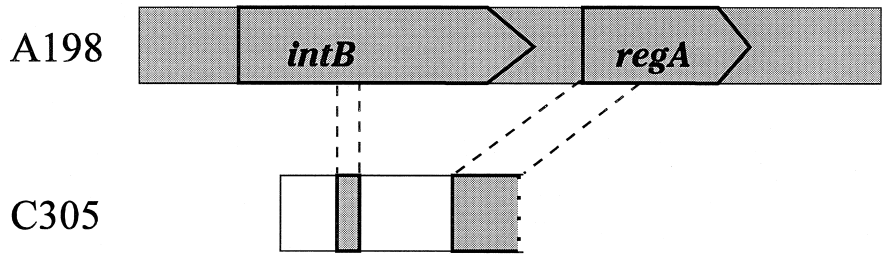
The insertion sequence IS*1253* from *D. nodosus* strain AC3577 is a 1689 bp element consisting of two ORFs, *orf375* and *orf117*, with similarity to transposase genes from a number of unusual IS elements (Billington *et al.*, 1996). In strain C305, we found a copy of this element next to *vapH″* (Fig. 1). The IS*1253* found in strain C305 had DNA identity of 98·3% over 1649 bp with the IS*1253* from strain AC3577, and consisted of two similar ORFs, *orf374* and *orf108*. The copy of IS*1253* in strain C305 appeared to be non-functional, as the coding region from *orf374* had been disrupted by two frame-shift mutations, one of which was the deletion of a GTAA repeat. A related insertion sequence is found adjacent to virulence-associated genes in several pathogenic bacteria, including *Salmonella typhimurium* (Gulig *et al.*, 1992), *Helicobacter pylori* (Censini *et al.*, 1996), *Vibrio cholerae* (Bik *et al.*, 1996), *Clostridium perfringens* (Katayama *et al.*, 1995) and *Yersinia pestis* (Hu *et al.*, 1998).

The *intB* and *regA* genes are disrupted in strain C305

The *intB* and *regA* genes from strain A198 are located immediately to the right of *vap* region 3, and form part of the *intB* genetic element, separate from the *intA* element (Bloomfield *et al.*, 1997). In strain C305, an internal fragment of the *intB* coding region was located immediately following IS*1253* (Fig. 3a) – a 100 bp DNA segment with 98% identity to nucleotides 410–510 of the 1203 bp coding region of the *intB* gene next to region 3 of strain A198 (Fig. 3b). At this position in the *intB* gene seven nucleotides from the 19 bp attachment (*att*) site found at the ends of the *vap* regions were absolutely conserved (Fig. 3b). Thus, this deletion may have been catalysed by *intC*.

Following the remnant of *intB* in strain C305, there was a 344 bp segment which had no similarity to other known sequences from *D. nodosus*, or to other sequences from the databases. After this, the DNA sequence from strain C305 was almost identical to the DNA sequence from the beginning of the *regA* gene of strain A198 (Fig. 3c). However, the promoter, Shine–Dalgarno sequence, and start codon of the *regA* gene were absent from strain C305. The next 322 bp of the sequence were almost identical, except that there was a 1 bp insertion in the sequence from strain C305, resulting

(a)



(b)

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          v         v         v         v         v         v         v         v         v
GGGCGAAAGTCGCTTACATCTCCACCTACTTCCTGCTTTTCATGCGCGCCCTATTGAGTCCATCACTGCTGGAGAACTCGTCGCCCATCT  C305 7329
     :         :  :     :: : :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ATTTAACGTCAATCTGAAGAATTACATTCTTCCTGCTTTTCATGCGCGCCCTATTGAGTCCATCACTGCTGGAGAACTCGTCGCCCATCT  A198  859
 F  N  V  N  L  K  N  Y  I  L  P  A  F  H  A  R  P  I  E  S  I  T  A  G  E  L  V  A  H  L

GCAAAACATTCCCTATGCTTACACGGCAGCCTATACGTTTCAATCTCCAATAAATGTGTACGATACACAAACAAAACTCGCTGTCTGTGT  C305 7419
::::: :::::::::::::::::: :::::::::::::  :  :  :    : ::          :: :    : : :
GCAAAGCATTCCCTATGCTTACACAGCAGCCTATACGTTGGACAATATCAAGCGCATCTATCGCCACGCGGTTAATATGCAATTGTTGGC  A198  949
 Q  S  I  P  Y  A  Y  T  A  A  Y  T  L  D  N  I  K  R  I  Y  R  H  A  V  N  M  Q  L  L  A
```

(c)

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          v         v         v         v         v         v         v         v         v
CGTATCAAAATCTGGCGTGTTAAAAGCTGAAAGAGAATTTGGTATCGATATGGGCTACATCATCGACGGCGTACAAAGACGCACAAATAA  C305 7699
  ::      : :     :: :::     :  :  ::   :    :   ::        :    ::  : :  :  ::
AATAAATGTTTTTACGTCGTAAAATCAATTATTATCATGATTTGCGTTTTACAACTTTCAGTGTTATTTTTTAACGAAAGTTAATAAATT  A198 1783

AAATCTAAAGTCAGTCTAGCTAGAAACGTAAAAATATTGCGCGAATTGAATAA CTGTCACAAGACCAACTCGCTGAAAAAATTGGTAAA  C305 7789
     : :: : :::::::::::: :::::::::::::::::::::::::::::: :::::::::::::::::::::::::::::::::::
GGTGTTGAAATGAGTCTAGCTAGCAACGTAAAAATATTGCGCGAATTGAATAATCTGTCACAAGACCAACTCGCTGAAAAAATTGGTAAA  A198 1873
         M  S  L  A  S  N  V  K  I  L  R  E  L  N  N  L  S  Q  D  Q  L  A  E  K  I  G  K

TCACAAGCGGCGATTCAAAAAATTGAAGCGGGTTTGACGCTGCGACCGCGTTTTTTGCAAGATTTGGCTAACGCGTTAGGCGTTTCAAGT  C305 7879
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TCACAAGCGGCGATTCAAAAAATTGAAGCGGGTTTGACGCTGCGACCGCGTTTTTTGCAAGATTTGGCTAACGCGTTAGGCGTTTCAAGT  A198 1963
S  Q  A  A  I  Q  K  I  E  A  G  L  T  L  R  P  R  F  L  Q  D  L  A  N  A  L  G  V  S  S 60

ATTGATTTAGAATATAAAGATTTCGAAAAAGAATTGAAGAAACAAGCTATTGAAAGCGATATCGGCACAATGGGCAAATTCCGGCTTTGG  C305 7969
:::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ATTGATTTGGAATATAAAGATTTCGAAAAAGAATTGAAGAAACAAGCTATTGAAAGCGATATCGGCACAATGGGCAAATTCCGGCTTTGG  A198 2053
I  D  L  E  Y  K  D  F  E  K  E  L  K  K  Q  A  I  E  S  D  I  G  T  M  G  K  F  R  L  W

AGCAGTAACGACCCGTTGCCTGAAGACGAATATGCTTATTTGCCGTTTTTCAAAGATGTTGAATTC                        C305 8035
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AGCAGTAACGACCCGTTGCCTGAAGACGAATATGCTTATTTGCCGTTTTTCAAAGATGTTGAATTCCAAGGCGGAACAGGTTGCTGCGAA  A198 2143
S  S  N  D  P  L  P  E  D  E  Y  A  Y  L  P  F  F  K  D  V  E  F  Q  G  G  T  G  C  C  E
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Fig. 3. Comparison of the DNA sequences of strain C305 and strain A198 containing the *intB* gene and the *regA* gene. (a) Diagrammatic representation of the *intB* and *regA* genes from strains A198 and C305. (b) Comparison of sequences containing the *intB* gene. (c) Comparison of sequences containing the *regA* gene. Nucleotides which are identical in both sequences are joined by colons. Every tenth nucleotide is indicated by v. The seven nucleotides underlined in (b) are identical to seven nucleotides from the *att* site.

in a frameshift mutation. Hence, the *regA* gene, like the *intB* gene, did not appear to be intact in strain C305.

Detection of the *intC* element in other strains of *D. nodosus*

To determine whether the genes *intC*, *orf242*, *orf171*, *vapG″* and *vapH″* were part of an integrated genetic element, 15 strains of *D. nodosus* were analysed in Southern blot experiments using probes 6, 7, 8 and 2 (Fig. 1), which detect *intC*, *orf242*, *orf171* and *vapG″H″*, respectively. The strains included five virulent strains, one intermediate strain, seven benign strains lacking the *intA* element, and two benign strains which contain the *intA* element. The results (data not shown) showed that, in ten of these strains, these five genes were

Table 1. Genetic elements integrated next to *askA* and *pnpA* in 16 strains of *D. nodosus*

Strain	Virulence classification	Located next to *askA*	Located next to *pnpA*
A198	virulent	*intA*	*intA*
B1006	virulent	*intC*	*intA*
D1172	virulent	*intC*	intA
G1220	virulent	*intC*	*intA*
1311	virulent	*intC*	*intA*
H1215	virulent	*intB*	*intA*/unknown*
AC390	benign	*intB*	*intA*
C305	benign	*intC*	*intB*
H1204	benign	*intB*	*intA*
819	benign	*intC*	*intB*
1169	benign	*intB*	*intB*
1311A	benign	*intB*	*intA*
1469	benign	*intC*	*intB*
1493	benign	*intC*	*intB*
2483	benign	*intC*	*intB*
3138	benign	*intC*	not *intA*, *intB*, *intC*

* Two copies of *pnpA* in this strain.

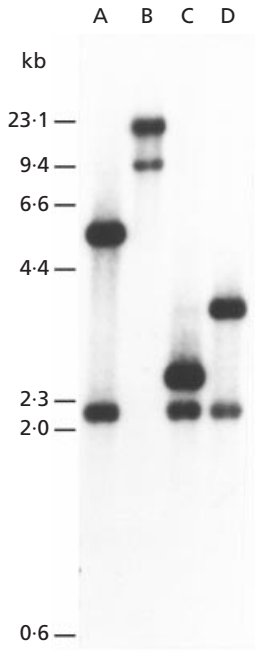

Fig. 4. Southern blot analysis of the *intB* genes of four strains of *D. nodosus*. Genomic DNA from strains A198 (lane A), C305 (B), 1311 (C) and 1311A (D) was digested with *Eco*RI and hybridized with a probe specific for *intB* (probe 4, Fig. 1).

present and found on the same-sized restriction fragment. The virulent strain A198 and the benign strain AC390 contained none of these genes. Since these genes included an integrase gene, were all located together in most strains, and were all absent from both strains A198 and AC390, these data suggest that they are part of an integrated genetic element, which we have designated the *intC* element. The virulent strain H1215 contained two copies of *intC* and one copy of *orf242*, but lacked *orf171*, *vapG″* and *vapH″*, while benign strains H1204 and 1169 contained only *intC*, suggesting that integration of this new genetic element may have been followed by deletion of some of the genes.

Site of integration of the *intC* element

The *intC* element in strain C305 was integrated into the *tRNA-ser*$_{GCU}$ gene downstream from *askA* (Fig. 1). In the virulent strain A198, *vap* region 1 is integrated into this *tRNA-ser* gene, while *vap* region 2 is integrated into *tRNA-ser*$_{GGA}$, downstream from the *pnpA* gene (Bloomfield *et al*., 1997). Probes specific for *askA* and *pnpA* were used in Southern blot experiments to determine the site of integration of the *intC* element in 13 strains of *D. nodosus* known to contain *intC* (Table 1: all strains except A198, AC390 and 1311A). In 10 of these strains, the *intC* element was located next to the *askA* gene. None of the strains tested had the *intC* element located next to *pnpA*. In strains H1215, H1204 and 1169, the *intC* element was not located next to *askA*, and its site of integration is unknown. These three strains did not have all of the genes so far recognized as being part of the *intC* element, perhaps suggesting that the *intC* element was unstable in the alternative position.

Loss of the *intC* element from virulent *D. nodosus* strain 1311

The virulent strain 1311 carries a small native plasmid, pDN1 (G. Whittle, M. E. Katz & B. F. Cheetham, unpublished), which has not been found in any other strain of *D. nodosus*. During routine laboratory growth,

pDN1 was lost spontaneously from strain 1311, generating a strain designated 1311A. Southern blot analysis (data not shown) showed that the *intC* element was not present in strain 1311A, since genomic DNA from this strain did not hybridize to DNA fragments from *intC*, *orf242* or *orf171*. *vapG″* and *vapH″* were also lost, together with one copy of IS*1253*, Thus, loss of pDN1 was accompanied by loss of the *intC* element. The concomitant loss of *intC*, *orf242*, *orf171*, *vapG″*, *vapH″* and a copy of IS*1253* provided further evidence that these genes were part of an integrated genetic element.

intB is next to *askA* in strain 1311A

If in strain 1311 the *intB* element were located next to the *intC* element, as in strain C305, then excision of the *intC* element in strain 1311 would place *intB* next to *askA*. Southern blot analysis of strains 1311 and 1311A using an *intB* probe showed that this was indeed the case (Fig. 4). Both strains contained two copies of *intB*, one of which is located in a different position in the two strains. In strain 1311, the *intB* probe hybridized to a 2·5 kb *Eco*RI fragment, while in strain 1311A a 3·3 kb *Eco*RI fragment was detected (Fig. 4, lanes C and D). In strain 1311A, the *askA* probe also hybridized to a 3·3 kb *Eco*RI fragment. These results indicated that in strain 1311, *intC* was located next to *askA* and *intB* was located at the right-hand end of the *intC* element. The loss of the *intC* element from 1311 has resulted in the relocation of the *intB* gene such that it was adjacent to *askA* in strain 1311A.

In both strains 1311 and 1311A the *intA* element was integrated into *tRNA-ser*$_{GGA}$, next to *pnpA*, and a truncated copy of *intB* was integrated into the *attR* at the right-hand end of the *intA* element. The *intB* probe hybridized to a 2·2 kb *Eco*RI fragment in both strains, indicating that the position of this copy of *intB* was unchanged by the excision of the *intC* element.

To confirm that *intC* was adjacent to *askA* in *D. nodosus* strain 1311, and that *intB* was adjacent to *askA* in strain 1311A, PCR products were amplified using primer sets specific for *askA–intC* (primers A and B), and *askA–intB* (primers A and C). As expected, a 1·1 kb *askA–intB* product was detected in strain 1311A whilst no *askA–intC* product was observed. In strain 1311, a 2·6 kb *askA–intC* product was observed, as expected.

Continuing loss of the *intC* element from strain 1311

Contrary to expectations, a 1·1 kb *askA–intB* product was also obtained in strain 1311, suggesting that *intB* was also located next to *askA* in this strain. However, when serial dilutions of 1311 template and 1311A template were used, the amount of the *askA–intB* product was much lower in 1311 than in 1311A (data not shown). This suggested that a subpopulation of cells from *D. nodosus* strain 1311 was undergoing loss of the *intC* element frequently enough to be detected. This was supported by Southern blot analysis (Fig. 4), where a faint band was detected in genomic DNA of strain 1311 by the *intB* probe at 3·3 kb. The loss of the *intC* element may be favoured under laboratory conditions.

Excision of the *intC* element does not disrupt *tRNA-ser*$_{GCU}$

The *askA-intB* PCR product from strain 1311A was subcloned, and the DNA sequence determined and aligned to the left end and right end of *vap* region 1/3 in *D. nodosus* virulent strain A198. Analysis of this junction region showed that the left-hand side of the *att* site was identical to *attL*, as would be expected if the *intC* element were excised by homologous recombination or an integrase-catalysed reaction. Analysis of the DNA sequence also showed that the *tRNA* gene was not disrupted by the loss of the *intC* element. Disruption of the *leuX tRNA* gene by the deletion of a pathogenicity island has been shown to have a major effect on virulence in uropathogenic *E. coli* (Ritter *et al*., 1995).

The *intC* element in strain C305 may be truncated

Southern blot analysis (summarized in Fig. 5) showed that the distance between *askA* and *intB* in strain 1311 was greater than 14 kb, and that all of the sequences between *tRNA-ser*$_{GCU}$ and *intB* were lost together from strain 1311 to form strain 1311A. By contrast, in strain C305, the distance between *askA* and *intB* was 6·6 kb (Fig. 1). These results suggested that in strain C305 part of the *intC* element had been deleted, perhaps due to the insertion of IS*1253*, which was located at the right-hand end of the *intC* element in strain C305.

Loss of the *intC* element from strain 1311 is associated with loss of a virulence characteristic

It has been established that the extracellular proteases of virulent strains of *D. nodosus* are more thermostable than proteases of benign strains (Depiazzi *et al*., 1990). The thermostability of the secreted proteases of virulent strain 1311 and its daughter strain 1311A was compared with that of the virulent strain A198 and the benign strain C305. The percentage loss of activity after heating ($\pm$ standard deviation) was $29{\cdot}5 \pm 5{\cdot}6$ for virulent strain A198, $74{\cdot}1 \pm 11{\cdot}9$ for benign strain C305, $20{\cdot}1 \pm 11{\cdot}4$ for strain 1311, and $84{\cdot}9 \pm 9{\cdot}0$ for strain 1311A. These results clearly showed that the proteases from strain 1311A were much less thermostable than the proteases of strain 1311, and therefore strain 1311A behaved as a benign strain in this assay. These results suggest that the loss of the *intC* element was associated with loss of protease thermostability, a trait associated with virulence in *D. nodosus*. In addition, colonies from strain 1311A were smaller and exhibited reduced exoenzyme activity on solid medium when compared to colonies from strain 1311 (data not shown). Virulent strains of *D. nodosus* have, in general, larger colonies and exhibit greater exoenzyme activity than benign strains (Depiazzi & Richards, 1985).

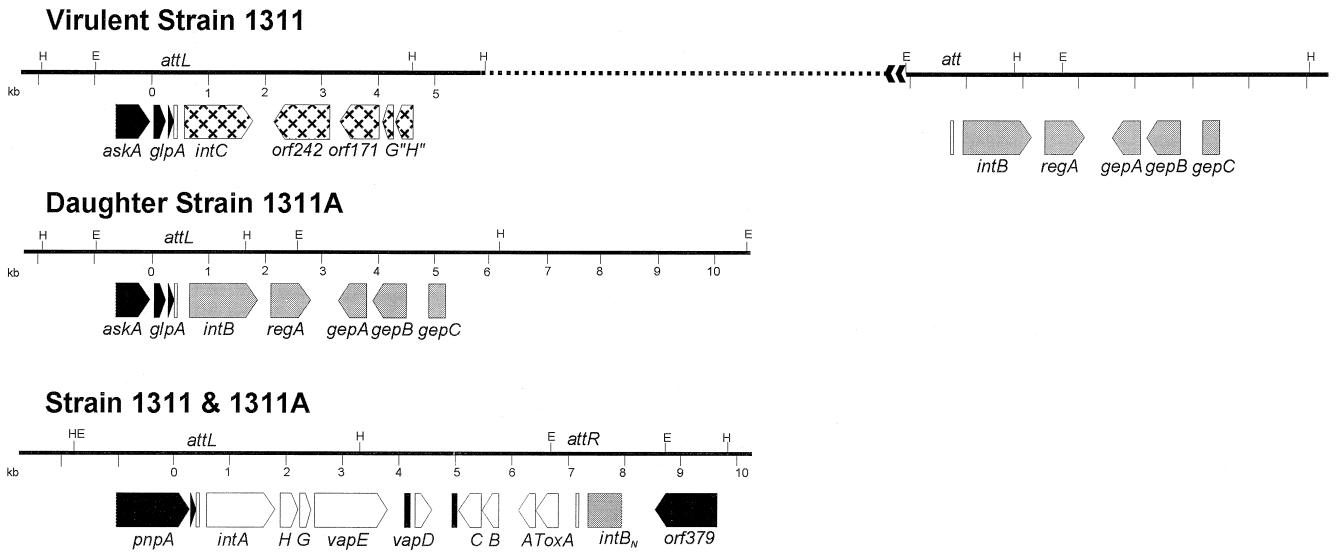

Fig. 5. The *intA*, *intB* and *intC* elements of strains 1311 and 1311A. Restriction sites shown are *Eco*RI (E) and *Hin*dIII (H). The potential genes are indicated by arrows, which are cross-hatched, shaded, open or black, depending on whether they are part of the *intC*, *intB* or *intA* elements, or not part of an integrated genetic element, respectively. The *tRNA-ser* genes are indicated by black triangles, and the *att* sites at the left (*attL*) and right (*attR*) are indicated by small open rectangles. The DNA sequence of the region shown by a dotted line has not been determined.

Loss of pDN1 may occur without loss of the *intC* element

In an effort to determine whether the loss of *intC* occurred concomitantly with the loss of pDN1, the size difference between colonies of strains 1311 and 1311A was utilized to select colonies of strain 1311 that had potentially lost pDN1 and/or the *intC* element. Three small colonies were selected, and analysed. All three had lost pDN1 but still contained the *intC* element, and retained protease thermostability (data not shown). Of the 55 colonies screened, none which had lost the *intC* element were isolated. These results indicated that the loss of pDN1 may occur without loss of the *intC* element and that the loss of protease thermostability was not related to the loss of the native plasmid, pDN1, from strain 1311. However, since three colonies of reduced size were found to have lost pDN1, but not the *intC* element, the reduced size of 1311A colonies may have been due to loss of pDN1.

Sites of integration of the *intA* and *intC* elements in virulent and benign strains

There was no direct correlation between the presence of the *intC* element and virulence, since the virulent strain A198 does not contain the *intC* element, while most benign strains do contain it. Southern blot analysis of the integration sites of the *intC* and *intA* elements in 16 strains of *D. nodosus* showed that most virulent strains had the *intC* element next to *askA* and the *intA* element next to *pnpA* (Table 1). The virulent strain A198 had two copies of *intA*, one next to *askA* and one next to *pnpA*. By contrast, most benign strains had *intC* next to *askA*, but neither *intC* nor *intA* next to *pnpA*. We propose that virulent strains have the *intA* element next to *pnpA* and either the *intA* element or *intC* element next to *askA*. Strains which lack the *intA* element next to *pnpA*, or have neither the *intA* nor the *intC* element next to *askA*, would be benign if our model were correct.

Of the 16 strains studied, the only exception to the above pattern was virulent strain H1215, which has *intB* next to *askA*. Strain H1215 was atypical in several ways. Unlike all other strains studied, it contained two copies of *pnpA*, one of which had *intA* next to it. It is not known what is next to the other copy of *pnpA*. This strain also had two copies of *intC*, and their integration sites are unknown. However, it lacked *orf171, vapG″* and *vapH″*, suggesting that deletions had occurred. Strain H1215 also contains a complete integrated prophage (G. A. Bloomfield, unpublished), but the integration site is also unknown.

A putative global repressor (*glpA*) gene between *askA* and *tRNA-ser*$_{GCU}$

Between the *askA* and *tRNA-ser*$_{GCU}$ genes we identified an ORF, designated *glpA* (Fig. 1), encoding a putative product of 64 aa which had 74% aa identity over the first 50 aa with RsmA (Fig. 2b) from the plant pathogen *Erwinia carotovora* (Cui *et al.*, 1995). In *Er. carotovora*, RsmA acts as a global repressor of virulence. The *rsmA* gene product reduces the transcript level of *hslI*, a *luxI* homologue required for *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL) synthesis (Cui *et al.*, 1995), and

thereby represses extracellular enzyme production, reduces motility and colony size, and so attenuates virulence (Mukherjee *et al*., 1996). It is interesting that *D. nodosus* strain 1311A exhibits a similar phenotype to cells expressing *rsmA* in *Er. carotovora*, given the similarity between GlpA and RsmA. CsrA from *E. coli*, which is also very similar to RsmA, modulates glycogen biosynthesis by effects on mRNA stability (Liu *et al*., 1995). CsrA belongs to a family of proteins containing KH domains, which are involved in RNA binding. The *E. coli pnp* gene belongs to the same family (Liu *et al*., 1995), affects mRNA processing and decay (Alifano *et al*., 1994), and is one of the two principal enzymes involved in the degradation of bacterial mRNA to nucleotides (Li & Deutscher, 1994). Thus, in *D. nodosus*, the *intA* and the *intC* elements are integrated next to two genes whose products may be involved in gene regulation by the alteration of mRNA stability. The integration of these genetic elements may affect levels of *glpA* and *pnpA* transcripts, resulting in increased production of thermostable extracellular proteases.

Comparison of DNA sequences at the beginning of the *intA*, *intB* and *intC* elements

Analysis of the DNA sequences at the beginning of the *intA*, *intB* and *intC* elements identified several features which may be important in differential gene expression in *D. nodosus* strains containing different integrated genetic elements. These are discussed below.

Termination of transcription. The DNA sequences starting from the TGA stop codon for *glpA* for strains C305, A198 and 1311A were compared (data not shown). In all three strains, the sequence was almost identical up to the 3′ end of the *tRNA* gene (the *attL* site). Within this sequence, there was a region of dyad symmmetry (loop 1) very close to the stop codon for *glpA*, which could act as a transcriptional terminator for *glpA*. Strains C305, A198 and 1311A had the *intC*, *intA* and *intB* elements, respectively, integrated after the *attL* site. The first 42 bp of the *intA* and *intC* elements were identical, and differed substantially from the first 42 bp of the *intB* element. Within this 42 bp sequence was a second region of dyad symmetry (loop 2), which could act as an alternative transcriptional terminator for *glpA*. If this transcriptional terminator were used, the transcript would include the tRNA molecule. This dyad symmetry (loop 2) was not present in the *intB* element. Thus, termination of transcription of *glpA* may differ according to whether *intC*, *intA* or *intB* is integrated at this position.

Virulent strains had the *intA* element integrated next to *pnpA*, while most benign strains had *intB* at this position. In the virulent strain A198, the stop codon in *pnpA* was located only one base before the *tRNA-ser*$_{GGA}$ gene, and no putative transcriptional terminators were evident before the end of the *tRNA* gene. Thus, it is likely that *pnpA* transcripts include the tRNA molecule, and would differ according to the genetic element integrated at this position. The integration of the *intA* element, but not the *intB* element, next to *pnpA* would provide a transcriptional terminator.

Antisense RNA. Within the divergent region downstream of loop 2, sequences weakly resembling the consensus sequence for the *E. coli* σ^{70} −35 and −10 promoter sequences (Hawley & McClure, 1983) were identified in the regions upstream of *intC* and *intA*, but were not present upstream of *intB*. If these promoter sequences are functional, the antisense transcript produced could potentially sequester those messages transcribed on the opposite DNA strand, and left of the *intC* element and *intA* element integration sites, including those transcripts for *glpA*, *pnpA*, *tRNA-ser*$_{GCU}$ and *tRNA-ser*$_{GGA}$ genes.

tRNA processing. Since the *intA* and *intC* elements integrate into the 3′ ends of *tRNA-ser*$_{GGA}$ and *tRNA-ser*$_{GCU}$, they could alter the expression of these genes, thereby affecting the levels of these two serine tRNAs. This could, in turn, affect the translation of genes which use the corresponding serine codons. The tRNA-ser may have a role in virulence, similar to that proposed in uropathogenic *E. coli*, where the LeuX tRNA molecule is thought to act as a global regulator of several virulence factors (Ritter *et al*., 1995).

DNA topology. It has been reported previously that the integration of genetic elements can alter gene expression by affecting DNA topology (Dorman, 1995; Ott, 1993; Zagaglia *et al*., 1991). It is therefore possible that an element of critical size, such as the *intC* or *intA* elements, must be integrated adjacent to both *glpA* and *pnpA*, in order to provide the appropriate topology for thermostable protease expression or secretion.

A model for the role of the *intA* and *intC* elements in the virulence of *D. nodosus*

Our analysis of 16 strains of *D. nodosus* has shown that the *intA*, *intB* or *intC* elements may integrate next to *glpA*, and either the *intA* or the *intB* elements may integrate next to *pnpA*. Our model proposes that virulent strains of *D. nodosus* have the *intA* element integrated next to the *pnpA* gene, and the *intC* or *intA* element integrated next to the *glpA* gene. The presence of these integrated genetic elements may alter the expression of *glpA*, *pnpA*, *tRNA-ser*$_{GCU}$, *tRNA-ser*$_{GGA}$ or *askA*, and reduce the expression of thermostable proteases through effects on mRNA stability, tRNA availability, or HSL levels.

It has been observed previously that virulent strains may have more than one copy of the *intA* element, but that strains with increased numbers of copies do not show increased virulence. This is consistent with our model, since the integration of a single copy of the *intA* element next to the *pnpA* gene and the *intC* element next to the *glpA* gene is sufficient for virulence. The presence of the *intA* element in some benign strains is also consistent with this model, since these strains would be benign if they did not have either the *intA* or the *intC* element integrated next to *glpA*, and the *intA* element integrated

next to *pnpA*. For example, the benign strain AC390 has two copies of *intA*, one of which is integrated next to *pnpA*, but does not have the *intC* element, and the second copy of the *intA* element is not integrated next to *glpA* (Table 1). Based on these results, a diagnostic test to determine the virulence of strains of *D. nodosus* could be designed, using PCR to determine which genetic elements are integrated next to *glpA* and *pnpA*.

There is one report of a small number of virulent strains which lack sequences from the *vap* region (Rood *et al.*, 1996). These strains may have a copy of the *intC* element integrated next to both *glpA* and *pnpA*. It will be of great interest to analyse such strains to determine whether genetic elements are integrated at these two locations.

Similarities between the *intA* and *intC* elements

The majority of the genes from the *intA* and *intC* elements are quite unrelated. However, the two integrases, IntA and IntC, have 54·2% amino acid identity, and *vapG* and *vapH*, from the *intA* element, and *vapG″* and *vapH″*, from the *intC* element, have approximately 55% amino acid identity, and 81% nucleotide identity. In addition, two sequences of 42 bp and 61 bp are almost totally conserved in the DNA segment between the *att* site and the beginning of *intA* and *intC*. These results suggest that there has been genetic exchange between the *intA* and *intC* elements, that the related sequences are required for similar functions and have been maintained by selection, or that the two elements have evolved from a common ancestor.

Phase variation in *D. nodosus*

In culture we have shown loss of the *intC* element from a virulent strain of *D. nodosus*, resulting in loss of a virulence factor. Loss of the *intC* element from *D. nodosus* strains in natural infections may allow switching from the virulent to the benign phenotype, and may have a selective advantage. However, this has not yet been observed *in vivo*. Loss of the *intA* element is very unlikely, due to the *vapA/toxA* maintenance system in this element (Bloomfield *et al.*, 1997). At present, we do not know whether the *intA*, *intB* or *intC* elements can be transferred between *D. nodosus* strains, although a plasmid containing the genes from *vap* region 1 has been identified in one strain of *D. nodosus* (Billington *et al.*, 1996). It is possible that strains of *D. nodosus* may be able to switch between the benign and virulent states by the loss or gain of these genetic elements. This work has significant implications for footrot eradication programmes which are currently in place in Australia.

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