Analysis of sequences flanking the vap regions of *Dichelobacter nodosus*: evidence for multiple integration events, a killer system, and a new genetic element

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INTRODUCTION

*Dichelobacter nodosus* (formerly *Bacteroides nodosus*), a Gram-negative anaerobic bacterium with polar type 4 fimbriae, is the principal causative agent of ovine footrot (Beveridge, 1941). Strains of *D. nodosus* are classified as virulent, intermediate or benign, according to the severity of the disease which they cause in sheep. The vap regions of the *D. nodosus* genome may have arisen by the integration of a genetic element and may have a role in virulence. The virulent *D. nodosus* strain A198 has multiple copies of the vap regions. In the present study, sequences to the left and right of vap regions 1, 2 and 3 of strain A198 were analysed by Southern blotting and DNA sequencing. The results suggest that vap regions 1 and 2 arose by independent integration events into different tRNA genes. The discovery of a second integrase gene (intB), a gene with similarity to bacteriophage repressor proteins (regA), and a gene similar to an ORF from a conjugative transposon (gepA), suggests that a second genetic element, either a bacteriophage or a conjugative transposon, is integrated next to vap region 3 in the *D. nodosus* genome. The arrangement of intB and the vap regions in three other virulent strains and one benign strain was determined using using Southern blotting and PCR. One strain, H1215, contained vapE and not vapE, and thus resembles vap region 3, suggesting that vap region 3 also may have arisen by an independent integration event. In all strains, a copy of intB was found next to the vap regions. The vap regions contain two genes, vapA and toxA, with similarity to the hig genes of the killer plasmid Rts1. Evidence is presented that vapA and toxA have a similar function in *D. nodosus*.

Keywords: footrot, virulence, horizontal gene transfer, conjugative transposon

benign strains (Rood et al., 1996), suggesting that these DNA sequences may have a role in virulence. The vap regions appear to have arisen by the integration of a genetic element into a tRNA gene in the *D. nodosus* genome (Cheetham et al., 1995). A plasmid carrying the vap genes has been isolated from one strain of *D. nodosus* (Billington et al., 1996b). The major genes identified within the vap regions are a series of virulence-associated protein (vap) genes, designated vapA, vapB, vapC, vapD and vapE, and an integrase gene, intA, which has amino acid similarity to the integrases of some bacteriophages of *Escherichia coli*, *Shigella flexneri* and *Vibrio cholerae* (Katz et al., 1992; Cheetham et al., 1995). The absence of a transformation system for *D. nodosus* precludes the direct testing of the role of the vap genes in virulence.

Genes similar to vapA, vapC and vapD have been identified in the genome of *Haemophilus influenzae*, and
The virulent strain in which it was found, and it has been proposed that the role of vapC and vapD is to co-ordinate the replication of the virulence plasmid with cell division (Pullinger & Lax, 1992). In H. influenzae, the vapC gene, which is similar to vapB from D. nodosus, is located adjacent to vapC. VapB and VapC also show amino acid similarity with putative proteins encoded by the trbH region of the E. coli F plasmid (Katz et al., 1992).

The virulent D. nodosus strain A198 contains three copies of the vap regions, designated vap regions 1, 2 and 3 (Katz et al., 1994; Fig. 1). Regions 1 and 3 are adjacent in the genome of D. nodosus, while region 2 is located elsewhere. The complete sequence of vap regions 1 and 3 has been determined (Katz et al., 1992, 1994; Cheetham et al., 1995). vap region 1 contains the intA and vapA–E genes, together with several smaller ORFs, and a gene, vapA' (formerly vapI; Cheetham et al., 1995), encoding a protein with 36.6% amino acid similarity to VapA. vap region 3 contains vapD, vapA', whose product has 57.3% amino acid similarity to VapA, and vapE', encoding a protein with 62.6% amino acid similarity to VapE. Southern blotting and partial DNA sequencing has been used to show that vap region 2 contains vapA–D (Katz et al., 1994) but the composition of the remainder of vap region 2 was unknown.

The multiple copies of the vap regions could have arisen by chromosomal duplications, or by multiple independent integration events. In this study, Southern blotting was used to determine the composition of vap region 2, and the DNA sequences flanking vap regions 1, 2 and 3 were analysed. In addition, the arrangements of the vap regions in four other strains of D. nodosus was determined. The results suggest that vap regions 1, 2 and 3 arose by independent integration events.

**METHODS**

**General methods.** Methods for the growth of D. nodosus, preparation of DNA, and cloning and analysis of DNA have been reported elsewhere (Katz et al., 1994).

**Dichelobacter nodosus strains.** Virulent D. nodosus strains A198, B1006, G1220 and H1215, and benign strains C305 and H1204, were provided by Dr J. I. Rood, Monash University, Clayton, Victoria, Australia.

**Southern blot hybridizations.** The methods for the preparation of probes, stringent hybridization conditions, and chemiluminescent detection have been reported elsewhere (Cheetham et al., 1995). The probes were restriction enzyme.
Polymerase chain reactions. The 25 μl amplification reactions contained 1 μmol of each oligonucleotide primer, 1.8 mM MgCl₂, 0.2 mM dNTPs, 1 x reaction buffer (supplied with the enzyme by the manufacturer, BresaTec); 10 mM Tris/HCl, pH 8.8, 400 mM (NH₄)₂SO₄, 2 mg gelatin ml⁻¹, 4.5% Triton X-100), 1.0 unit Taq DNA polymerase, and approximately 1 ng genomic DNA. The reaction mixtures were amplified for 30 cycles (each cycle consisting of 90 s at 94 °C, 60 s at 60 °C and 120–300 s at 72 °C) in a Corbett FTS-320 thermal cycler (Corbett Research). A 10 μl sample of the amplification products was analysed by agarose gel electrophoresis. Oligonucleotide primers were 20 nucleotides long. Primers A, B, D, F and G were identical to sequences starting at positions 2143, 4512, 9250 and 12535 (GenBank accession number L31763) in vap regions 1 and 3. The approximate positions of the primers are shown in Fig. 1. The primer pairs used were: A and C, A and E, B and E, D and E, D and I, F and I, and G and H.

DNA sequence analysis. DNA sequencing was done using T7 DNA polymerase kits and double-stranded DNA templates, as described by the manufacturer (BresaTec). The nucleotide sequence of both DNA strands was determined in an overlapping manner and across all internal restriction sites. GenBank searches were carried out using the FASTA-TRANS (Pearson & Lipman, 1988) program through the Australian Genome Information Service (ANGIS). In all cases, the Kolmogorov–Smirnov statistic was less than 0.1, and all amino acid similarities reported were highly significant, with probability values of < 0.005 (Gaeta, 1996). Sequences related to IntB were aligned using the CLUSTALW program (Higgins et al., 1992).

RESULTS

Comparison of sequences to the left of vap regions 1 and 2

A comparison of the restriction maps of the left-hand ends of vap regions 1 and 2 suggests that divergence between the two regions occurs to the left of the leftmost KpnI site (Fig. 1). Southern blotting using probes 1 and 2 showed that vap region 2 contains the genes intA and vapE (data not shown). However, probes 3 and 4 each detected only one band, suggesting that there is a single copy of these sequences in the genome of D. nodosus strain A198. To confirm these results, the DNA sequence of a 1·1 kb Xhol-PvuII fragment from the left-hand end of vap region 2 was determined. Comparison of the sequences (Fig. 2) shows that there is 97% sequence identity between vap regions 1 and 2 and in the first 190 nucleotides to the right of the attachment site. There is 79% conservation of the sequence of the attachment site. An 89 bp tRNA₅₅ gene was identified at the left-hand junction of vap region 2, with 83.5% identity to the ser-tRNA-V gene of E. coli (Grosjean et al., 1985). This tRNA gene is similar to the previously identified tRNA gene at the left-hand end of vap region 1 (Cheetham et al., 1995), having 81% identity over 89 bp. However, the tRNA gene from vap region 2 has the anticodon GGA instead of GCU, has a shorter extra arm, and has base substitutions in the stem of the WC loop. These results suggest that vap region 2 arose by the integration of the vap element into a tRNA gene different from the one in which vap region 1 is inserted.

The sequences to the left of the tRNA genes in vap regions 1 and 2 are not related (Fig. 2). Part of an ORF, designated pnpA, with approximately 67% identity over 239 amino acids to the polynucleotide phosphorylase genes of E. coli (Regnier et al., 1987), Photobacterium luminescens (Clarke & Dowds, 1994) and H. influenzae (Fleischmann, 1995) was identified to the left of the tRNA gene in vap region 2. Partial sequencing of the Xhol-NruI fragment at the left of vap region 1 revealed part of an ORF which had 48.2% amino acid identity with the Mycobacterium smegmatis aspartokinase gene (data not shown; Cirilio et al., 1994).

Identification of an integrase gene to the right of vap region 3

The sequence of 4·8 kb of DNA beginning at the SacI site at the right-hand end of region 3 (Fig. 1) was determined, and five potential genes were identified. One of these, designated intB, encodes a protein with deduced amino acid similarity to several bacterial integrases. However, intB appears to be a pseudogene, as there is a stop codon at position 130. Similarity to other integrase genes continues after this stop codon, in a different reading frame. The amino acid sequence of intB, adjusted to remove this stop codon, was compared to the amino acid sequences of other integrases. The highest level of amino acid identity was to E. coli retropathage εR73 (Sun et al., 1991), with 33.8% amino acid identity over 403 amino acids. There was 32.6% amino acid identity with the integrase from bacteriophage SF6 of Shigella flexneri (Clark et al., 1991), and 31.3% amino acid identity with an integrase from Vibrio cholerae (Kovach & Peterson, 1994). A multiple alignment of the amino acid sequences of these integrases (data not shown) revealed that 15·6% of amino acid residues are identical in all four proteins, including His¹⁶⁶, Arg²⁸⁶ and Tyr²⁹⁹, which are conserved between a number of bacteriophage integrases (Argos et al., 1986). The intB gene product also shows 31·0% amino acid identity with the intA gene product from vap region 1 of D. nodosus (Cheetham et al., 1995).

The ORF designated regA adjacent to intB has amino acid similarity to bacterial DNA-binding proteins and bacteriophage repressor proteins. The level of amino acid identity was 40.5% to the pectin lyase regulator RdgA of Eruwina carotovora (Liu et al., 1995), 39.2% to the regulatory protein PrtR of Pseudomonas aeruginosa (Matsui et al., 1993), and 36.2% to the repressor (cl gene
product) of bacteriophage ψ80 of E. coli (Ogawa et al., 1988). A putative helix–turn–helix motif and a putative leucine-zipper domain were identified in the regA gene (data not shown; Brennan & Matthews, 1989).

Three further potential genes were identified in this region (Fig. 1). The gene designated gepA (genetic element protein A) encodes a protein with 33.3% amino acid identity to ORF4, an ORF downstream from the rteC gene near the origin of transfer of a conjugative transposon. Bacteroides thetaiotaomicron (Stevens et al., 1993). The gepB gene encodes a protein with 64.9% amino acid similarity over 188 amino acids to an unidentified ORF in the cob gene cluster from Pseudomonas denitrificans (Crouzet et al., 1991), while the gepC gene shows no significant similarity to any genes in the databases.

The presence of an integrase gene followed by a DNA-binding regulatory gene suggests that the sequences to the right of vap region 3 are part of a second integrated mobile genetic element, which could be an integrated bacteriophage. However, the similarity of gepA to an ORF downstream from the rteC gene of B. thetaiotaomicron and the fact that the integrases of some conjugative transposons are members of the lambda integrase family (Salyers et al., 1995), as is intB, raises the possibility that this second genetic element is a conjugative transposon.

Comparison of sequences to the right of vap regions 2 and 3

The sequence of vap region 2 from toxA to just past the attachment site (Fig. 1) has been determined previously (Katz et al., 1994). We determined the sequence from the attachment site to the next EcoRI site (Fig. 1). Comparison of this sequence with the sequence to the right of vap region 3, beginning at the attachment site, is shown in Fig. 3. The two sequences are 92.5% identical over the first 560 nucleotides, with 80.7% identity over the first 140 amino acids of the IntB proteins encoded by each region. However, similarity between the sequences ends at this point. It is of interest that the intB gene adjacent to vap region 2 is not interrupted by the stop codon found in the intB gene adjacent to vap region 3. However, there appears to be only a partial or truncated copy of the intB gene next to vap region 2. Thus, although there are two copies of this gene in D. nodosus strain A198, neither copy appears to be functional.

Arrangement of the vap regions in four other strains of D. nodosus

To further investigate the evolution of the vap regions, the arrangements of the vap genes in four strains of D. nodosus, from serogroups B (virulent strain B1006), G (virulent strain G1220) and H (benign strain H1204 and virulent strain H1215) were compared with the arrangement of the vap genes in virulent strain A198 (serogroup A). These experiments were also designed to investigate the composition of the vap region of strain H1204, which is one of the 30% of benign strains which have been shown previously to contain at least part of the vap region. Preliminary Southern blots (Katz et al., 1991) had suggested that the vap regions in these strains were different from those in strain A198. Maps of the vap regions in these strains were constructed by analyzing the results of Southern blot experiments, using probes (Fig. 1) which would detect intA, intB, vapA−E, vapA′, vapA′′ and vapE′, and PCR experiments, using
The vap regions of Dichelobacter nodosus

primers complementary to several regions of the genome of D. nodosus strain A198 (Fig. 1).

The results from the Southern blot experiments are summarized in Fig. 4. All strains contained at least one copy of vapa, vapB, vapC and vapD, which were located adjacent to each other. However, not all strains contained a copy of vapE. The virulent strain H1215 lacks vapE, but contains a copy of vapE (Fig. 5), located in the same position as vapE in all of the other strains tested. This is similar to the arrangement in vap region 3 in strain A198, except that vap region 3 does not contain vapA-C or toxA. Further similarity between vap region 3 of strain A198 and the vap region of strain H1215 was shown by hybridization experiments with probe 14 (Fig. 1). This probe detects a sequence which is absent from vap region 1, located between vapD and vapC in vap region 2, and located to the left of vapD in vap region 3. In strain H1215, this sequence is also located to the left of vapD. The similarity between the vap region of strain H1215 and vap region 3 of strain A198 suggests that vap region 3 of strain A198 could have arisen by integration of a separate vap element, containing vapE, followed by the loss of vapA-C, rather than by partial duplication of vap region 1 and subsequent divergence of the vapE gene.

The absence of vapE from strain H1215 suggests either

Fig. 3. Alignment of the sequence from the right-hand end of vap region 2, beginning at the attachment site (reg.2, top sequence) with the sequence from the right-hand end of vap region 3, beginning at the attachment site (reg.3, bottom sequence). The amino acid sequences of the intB genes are shown above (reg.2) or below (reg.3) the DNA sequences. The stop codon at position 586 which interrupts the intB coding region is shown in bold and underlined. The first 214 nucleotides of the sequence from the right-hand end of region 2 have been reported previously (Katz et al., 1994).
that VapE is not essential for virulence, or that VapE' can perform the same function. Strain H1215 has a single copy of all of the genes in the vap region, so multiple copies are not required for virulence, and does not contain vapA', suggesting that this is not required for virulence. By contrast, the benign strain H1204 has one copy of all of the genes identified in the vap regions, so the lack of virulence in this strain must be attributable to factors outside the vap region, unless one or more of the vap gene products is non-functional in this strain.

The two copies of intB which have been identified in strain A198, together with one copy of intB in strains B1006, G1220, H1204 and H1215, are located adjacent to the vap regions (Fig. 4). Strains H1204 and H1215 contain an additional copy of intB, which does not appear to be associated with the vap regions.

PCR experiments were used to confirm the maps produced from the Southern blot results. The results of these experiments are summarized in Table 1. No product was obtained using DNA from strain C305 as a template, as this strain does not contain most of the sequences in the vap regions. Primers D and E, which flank the vapD gene, gave a product of approximately 300 bp in all strains. We have previously shown that the three copies of the vapD coding region in strain A198 are identical (Katz et al., 1992, 1994; Cheetham et al., 1995).

PCR assays using primer G, which anneals just before vapG', and primer H, which is complementary to a
sequence within \textit{vapF}, gave bands of 450 bp in strains A198 and H1215, and 780 bp in strains B1006 and G1220. This confirms the similarity between \textit{vap} region 3 and the \textit{vap} region of strain H1215, and shows that there is variation in the region around \textit{vapG'} and \textit{vapF} in some strains.

Assays using primers D and I confirmed the presence of a copy of \textit{vapD} near \textit{intB} in strains B1006 and G1220, and not in the two H strains. The product obtained using primers F and I and DNA from strains B1006 and G1220 was 1-8 kb, slightly larger than that obtained using the two H strains, 1-5 kb. This suggests that there has been an insertion or deletion of about 300 nucleotides between \textit{vapA'} and \textit{intB} in these strains. The use of primers B and E led to a much shorter product in strains B1006 and G1220 than in the other strains. This suggests that \textit{vapH} is located on the left side of \textit{vapE} (or \textit{vapE'}) in strains A198, H1204 and H1215, and on the right side of \textit{vapE} in strains B1006 and G1220. Alternatively, there may be copies of \textit{vapH} and \textit{vapG} on both sides of \textit{vapE} in these two strains. The longer products obtained from these strains using the primers A and E is consistent with this second explanation. The 4.0 kb product in strain H1215 is consistent with the location of \textit{vapE'} in this position in this strain. Finally, primers A and C gave a product of the same size in all strains. In summary, the PCR experiments have confirmed the maps derived from the Southern blotting data, provided further evidence that \textit{vap} region 3 of strain A198 and the \textit{vap} region of strain H1215 are related, and shown that there is diversity between the strains in the areas around \textit{vapH}, \textit{vapG}, \textit{vapG'} and \textit{vapF}. Small deletions, insertions, or duplications appear to have occurred in these areas.

### Table 1. Summary of the results of PCR experiments

DNA from the strains indicated was used in PCR assays, as described in Methods. The sizes of the bands, determined by agarose gel electrophoresis, are shown in kb. A dash indicates that no product was obtained.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>A198</th>
<th>B1006</th>
<th>C305</th>
<th>G1220</th>
<th>H1204</th>
<th>H1215</th>
</tr>
</thead>
<tbody>
<tr>
<td>D and E</td>
<td>0:30</td>
<td>0:30</td>
<td>–</td>
<td>0:30</td>
<td>0:30</td>
<td>0:30</td>
</tr>
<tr>
<td>G and H</td>
<td>0:45</td>
<td>0:78</td>
<td>–</td>
<td>0:78</td>
<td>–</td>
<td>0:45</td>
</tr>
<tr>
<td>D and I</td>
<td>1:0</td>
<td>1:0</td>
<td>–</td>
<td>1:0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F and I</td>
<td>–</td>
<td>1:8</td>
<td>–</td>
<td>1:8</td>
<td>1:5</td>
<td>1:5</td>
</tr>
<tr>
<td>A and E</td>
<td>3:0</td>
<td>3:6</td>
<td>–</td>
<td>3:6</td>
<td>3:0</td>
<td>4:0</td>
</tr>
<tr>
<td>A and C</td>
<td>0:60</td>
<td>0:60</td>
<td>–</td>
<td>0:60</td>
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<td>0:60</td>
</tr>
</tbody>
</table>

A 1-86 kb XhoI fragment from \textit{vap} regions 1 and 3, containing \textit{toxA'}, \textit{vapA'} and part of the \textit{toxA} gene, was inserted into the \textit{SalI} site of the plasmid pUC18 (plasmid pBC105; Fig. 6) and used to transform \textit{E. coli} to ampicillin resistance. DNA was prepared from transformants, digested with \textit{SphI}, diluted, religated, and used to transform \textit{E. coli}. This deletion removes part of the \textit{vapA} gene (Fig. 6). The transformation frequency using the religated plasmid was much lower than expected, and, even though the plasmid DNA appeared to have been cut to completion before ligation, the few transformants obtained, with one exception, contained the complete 1-86 kb XhoI fragment. DNA from one

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### A killer system in \textit{D. nodosus}?

VapA has been found recently to have 22% amino acid identity with the HigA protein from the killer plasmid Rts1 (Billington et al., 1996a; Tian et al., 1996). We had previously identified an ORF upstream of \textit{vapA}, designated ORF118 (Katz et al., 1992), which lacks a Shine–Dalgarno sequence (Shine & Dalgarno, 1974). However, there is a start codon 26 codons downstream from the original proposed start codon, and this second start codon is preceded by a weak Shine–Dalgarno sequence. The predicted protein product using this new start codon is 92 amino acids long, has 45·6% amino acid identity with the 92 amino acid HigB protein from the plasmid Rts1, and has now been designated ToxA. The arrangement of the \textit{toxA} and \textit{vapA} genes in \textit{D. nodosus} strain A198 is the same as the arrangement of \textit{higB} and \textit{higA} on the plasmid Rts1. VapA', which has 36·6% amino acid identity with VapA, has 22·1% amino acid identity with HigA. \textit{vapA'} is preceded by a gene, designated \textit{toxA'}, whose predicted protein product has 31·5% amino acid identity with ToxA, and 29·3% amino acid identity with HigB. The \textit{toxA'} gene was not identified previously (Cheetham et al., 1995), as it begins with the start codon GTG. The remaining copy of \textit{vapA} found in strain A198, \textit{vapA'}, does not appear to be preceded by a copy of the \textit{toxA} gene.
transformant colony contained two plasmids, the original plasmid containing both the \textit{vapA}\textsuperscript{a} and \textit{toxA}\textsuperscript{a} genes, and the deleted plasmid containing only the \textit{toxA}\textsuperscript{a} gene. DNA from the deleted plasmid was purified from a band excised after agarose gel electrophoresis, and sequenced, confirming that this plasmid contained the \textit{toxA}\textsuperscript{a} gene alone. No transformants were obtained when DNA from the deleted plasmid was used to transform \textit{E. coli}. Dilution streaking was used to obtain single colonies from the original transformant, and all such colonies obtained contained a mixture of two plasmids. These results show that it is not possible to obtain viable transformants when \textit{toxA}\textsuperscript{a} is expressed in cells in the absence of \textit{vapA}\textsuperscript{a}, and are consistent with the hypothesis that \textit{toxA} encodes a protein which is toxic to \textit{E. coli}, and \textit{vapA} encodes an antidote protein. Northern blot analysis (data not shown) has demonstrated that \textit{toxA} is expressed in \textit{D. nodosus} strain A198.

\section*{DISCUSSION}

Similarity between the DNA sequences of the left-hand ends of \textit{vap} regions 1 and 2 begins within tRNA genes, and the sequences are almost identical after the attachment site. Since the two tRNA genes are different, this suggests that \textit{vap} regions 1 and 2 arose by independent integrations of the \textit{vap} element into the two tRNA genes. \textit{vap} region 3 could have arisen by partial duplication of \textit{vap} region 1. However, the discovery of a strain of \textit{D. nodosus}, H1215, which, like \textit{vap} region 3, has the gene \textit{vapE}\textsuperscript{a} and lacks \textit{vapE}, suggests that \textit{vap} region 3 may also have arisen by an independent integration event.

We have identified a new genetic element integrated into the \textit{D. nodosus} chromosome at the right-hand end of \textit{vap} region 3. The arrangement of an attachment site followed by an integrase gene and then a gene encoding a regulatory protein is characteristic of some lambdoid bacteriophages, such as \textit{\lambda} and P2 (Calendar \textit{et al.}, 1981), suggesting that this genetic element is an integrated bacteriophage. However, the \textit{gepA} gene which follows \textit{regA} is similar in predicted amino acid sequence to an ORF from a conjugative transposon from \textit{B. thetaiotaomicron} (Stevens \textit{et al.}, 1993). Some conjugative transposons have integrases which are members of the lambda integrase family (Salyers \textit{et al.}, 1995), as is \textit{intB}. Thus, the possibility exists that the genetic element which contains \textit{intB} is a conjugative transposon.

Conjugative transposons may have a role in the transfer of plasmids which are too small to encode the necessary transfer genes (Salyers \textit{et al.}, 1995). The \textit{vap} plasmid which has been identified in one strain of \textit{D. nodosus} (Billington \textit{et al.}, 1996b) does not appear to encode functions required for conjugation, and another non-conjugative plasmid, pEC1, of \textit{D. nodosus} has been identified recently (G. Whittle & B. F. Cheetham, unpublished). However, no conjugative plasmids have been identified so far in \textit{D. nodosus}. If the \textit{intB} element is a conjugative transposon, it may have had a role in the transfer of the \textit{vap} plasmid or pEC1 into \textit{D. nodosus}, or between \textit{D. nodosus} strains.

Six of the copies of \textit{intB} which have been identified so far in \textit{D. nodosus} strains are found in association with the \textit{vap} regions. This could be explained if the \textit{vap} plasmid and the \textit{intB} element use the same DNA sequence (attachment site) for integration. Alternatively, conjugative transposons may integrate into co-resident plasmids and mobilize them (Salyers \textit{et al.}, 1995). Thus, the \textit{intB} element and the \textit{vap} plasmid may have been transferred together. However, this hypothesis is not supported by evidence for the generation of \textit{vap} regions 1, 2 and 3 by independent integration events. The presence of a partial copy of \textit{intB} to the right of \textit{vap} region 2 of \textit{D. nodosus} strain A198 is of interest. This could have been formed by integration next to \textit{vap} region 2 of the genetic element carrying \textit{intB}, followed by loss of almost all of the sequences from this element.

The \textit{vapB} and \textit{vapC} genes of \textit{D. nodosus} are similar to the \textit{vagC} and \textit{vagD} genes from the virulence plasmid of \textit{Salmonella dublin} (Pullinger & Lax, 1992). It is of interest that the \textit{Salmonella} virulence plasmid also contains a gene required for cobalamin transport (Riouxf \textit{et al.}, 1990), as we have found similarity between the gene \textit{gepB} and a gene from the \textit{cob} gene cluster of \textit{Pseudomonas denitrificans} (Crouzet \textit{et al.}, 1991). In addition, the \textit{Salmonella} virulence plasmid contains a gene, \textit{rdS}, with similarity to the lambda family of integrases (Krause & Guiney, 1991). The \textit{vagC}, \textit{vagD}, \textit{rdS} and cobalamin transport genes are located within a 15 kb segment of the \textit{Salmonella} virulence plasmid (Dorman, 1994). The similarities between genes of this plasmid and genes from within, or near, the \textit{vap} regions of \textit{D. nodosus} suggests a possible evolutionary relationship between these genetic elements.

The \textit{vap} regions of \textit{D. nodosus} are found in 98% of virulent strains tested, but in only 30% of benign strains (Rood \textit{et al.}, 1996), and they may have a role in virulence. \textit{D. nodosus} isolates may be divided into nine serogroups, based on their fimbrial antigens, and both virulent and
benign strains are found in each serogroup (Claxton et al., 1983). In addition, most serogroups contain both strains with vap regions and strains which lack the vap sequences (Katz et al., 1991). The products of the vap genes may have a direct role in virulence, or, alternatively, the vap regions may be necessary for the maintenance or acquisition of other virulence factors. A very small number of virulent strains exist which appear to lack vap sequences, suggesting that vap sequences may be necessary only for the acquisition of other virulence factors. This proposed role for the vap sequences is supported by the observation that another genetic element associated with virulence, the vrl region, is found only in strains which contain vap sequences (Billington et al., 1996a). However, not all virulent strains contain the vrl sequences. We propose the following model for the role of the vap regions in the evolution of D. nodosus. D. nodosus strains of different serogroups may have evolved from an ancestral, benign strain. The vap sequences may have been acquired subsequently by some members of the different serogroups. This was followed by the acquisition of other virulence factors by some of the strains which had acquired the vap regions. Thus, all virulent strains would contain vap sequences, along with some benign strains, and these would be distributed throughout the serogroups. The VapA/ToxA killer system would act to maintain the vap regions in all strains. Rarely, the vap region may be lost from virulent strains.

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