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Molecular Characterization of a Genomic Region Associated with Virulence in *Dichelobacter nodosus*

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The major pathogen implicated in footrot, a highly contagious disease of sheep, is the strict anaerobe *Dichelobacter nodosus* (formerly *Bacteroides nodosus*). Sequence analysis of a 2,262-bp segment of the *D. nodosus* genome which is more prevalent in virulent isolates than in other isolates showed the presence of four open reading frames which appeared to have consensus transcriptional and translational start signals. These virulence-associated genes have been designated *vapABCD*. Two of the three copies of the *vap* region in the genome of the reference strain *D. nodosus* A198 were shown to carry all of the *vap* genes, whereas one copy contained only the *vapD* gene. The VapD protein was gel purified, shown to contain the predicted amino-terminal sequence, and used to raise rabbit antibodies. Western blots (immunoblots) showed that all of the *D. nodosus* strains tested that contained the *vap* region produced the VapD protein. The VapD protein had significant amino acid sequence identity with open reading frame 5 from the cryptic plasmid of *Neisseria gonorrhoeae*, and the *vapBC* operon had sequence similarity with the *trbH* region of the *Escherichia coli* F plasmid. It is proposed that these gene regions evolved from the integration of a conjugative plasmid from another bacterial species into the *D. nodosus* chromosome.

The strict anaerobe *Dichelobacter nodosus* (formerly known as *Bacteroides nodosus* [10]) is the causative agent of footrot, a highly contagious and economically significant disease of sheep. The extent of the disease in infected sheep is dependent on climatic conditions and the virulence of the invading microorganism. As a result, isolates of *D. nodosus* often are classified as virulent, intermediate, or benign, depending on the severity of the disease they would cause under optimal climatic conditions (37). Potential virulence factors produced by *D. nodosus* isolates include polar type 4, or N-MePhe, fimbriae (11) and extracellular proteases (27), although the roles of these factors in pathogenesis have not been determined. Virulent and benign isolates have been shown to differ in the amount of elastase that they produce (36, 38) and in the thermostability (6, 9, 13) and isoenzyme profiles (12, 20) of their respective proteases. Other studies have revealed differences in their levels of twitching motility (7, 8).

Studies in this laboratory have been aimed at the development of DNA probes to be used in diagnostic tests for ovine footrot. We have constructed a gene bank from the virulent reference strain *D. nodosus* A198 and by using a comparative hybridization procedure have identified three recombinant plasmids (pJIR318, pJIR313, and pJIR314B) which hybridize primarily with nonbenign isolates of *D. nodosus* and therefore could possibly be used as diagnostic gene probes (17).

The plasmid pJIR318 hybridized with almost all of the virulent and intermediate strains of *D. nodosus* tested. The results indicated that it contained sequences which correlated with virulence and were absent from 67% of the benign isolates. Southern hybridization experiments also showed that there were three copies of the pJIR318 region in the *D. nodosus* A198 genome (17). In this paper, we report the entire nucleotide sequence of pJIR318 and the identification of several open reading frames (ORFs). One of these ORFs encoded a cytoplasmic *D. nodosus* protein which had an amino acid sequence similar to that of a protein encoded by the cryptic plasmid from *Neisseria gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains. All *Escherichia coli* strains were derivatives of DH5α (Bethesda Research Laboratories) or TG1 (Boehringer Mannheim) and were grown in 2× YT medium (26) supplemented with ampicillin (100 μg/ml). *D. nodosus* strains were from J. Egerton, University of Sydney (strains B1006 and C1008); L. Depiazzi, RVL Bunbury (strains AC419, AC424, AC554, AC6, and AC176); and W. Yong, RVL Hamilton (strains A198, 305, HA343, HA352, HA337, HA390, HA386, HA393, HA389, HA340, and HA652) and were grown in TAS broth (34) at 37°C in an atmosphere of 10% H_2 and 10% CO_2 in N_2. *N. gonorrhoeae* strains MS-11A, Gc var40, and JKD109 were from T. Meyer (25), R Demarco de Hormeache (5), and J. Davies (Monash University), respectively. Strain JKD109 was used because it did not carry the cryptic plasmid.

DNA sequencing. General molecular techniques used in cloning and in the analysis of DNA molecules were as described previously (3, 30). DNA sequencing was performed by using T7 DNA polymerase kits and double-stranded DNA templates in the dideoxy-mediated chain termination method (31) as described by the manufacturers (Pharmacia; Promega).

Southern blot analysis. Samples (1 μg) of *D. nodosus* genomic DNA prepared as previously described (1) were digested with *Hin*dIII. The DNA was fractionated by electrophoresis through a 0.8% agarose gel and transferred (35) to a nitrocellulose membrane (BA-85; Schleicher & Schuell). Southern blots were done at high stringency as before (17).

PAGE of proteins. Cell extracts were prepared from approximately 100 μg (wet weight) of pelleted *E. coli* or *D.*

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nodosus cells by boiling the cells for 5 min in the presence of 0.1 M Tris-HCl buffer, pH 6.8, containing 10% glycerol, 1.25% sodium dodecyl sulfate (SDS), and 5% β-mercaptoethanol. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (21, 32), transferred to nitrocellulose (39) and reacted in immunoblots (2) with anti-VapD antiserum diluted 1/100 and sheep anti-rabbit immunoglobulin (Silenus).

Purification of VapD and production of antiserum. A 1.5-ml overnight culture of JIR1793 [DH5α(pJIR318)] was pelleted by centrifugation (all centrifugation steps were at 12,000 × *g*) for 5 min, and the cell pellet was extracted in 6 M urea–50 mM sodium acetate (pH 6.0) for 12 h at 22°C. The extract was centrifuged for 30 min, and 1.0 ml of supernatant was precipitated with 9.0 ml of methanol, cooled to −20°C, and kept at −20°C overnight. The precipitate was collected by centrifugation, dissolved in sterile phosphate-buffered saline, and separated on a preparative 15% (wt/vol) SDS-PAGE gel. The band containing VapD was visualized with PAGE blue 83 (BDH Ltd), and VapD was electroeluted by using a Biotrap (Schleicher & Schuell) electroeluter. The eluted protein was precipitated with 9 volumes of methanol as described above. The purified VapD protein (100 μg) was used with an Applied Biosystems 470A Gas-Phase Protein Sequencer to derive the amino-terminal sequence. To prepare specific antibodies, 100 μg of purified VapD was emulsified in Freund incomplete adjuvant and injected twice, 10 weeks apart, intramuscularly into a healthy adult male rabbit. The rabbit was exsanguinated 4 weeks later.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence determined in this study is M74565.

RESULTS

DNA sequence analysis of pJIR318. The virulence-associated plasmid pJIR318 has been mapped and shown to contain a 2.3-kb insert of *D. nodosus* A198 DNA (17). The complete nucleotide sequence of pJIR318 was determined on both DNA strands and is shown in Fig. 1. Analysis of this sequence showed the presence of four short ORFs preceded by consensus ribosome-binding sites (33). These ORFs have been designated *vapA* through *vapD* (virulence-associated proteins). A fifth ORF (ORF118), 118 amino acids in length, which lacks a Shine-Dalgarno sequence is also present. Consensus promoter sites (15) are located upstream of *vapA*, *vapB*, and *vapD* but not *vapC* (Fig. 1).

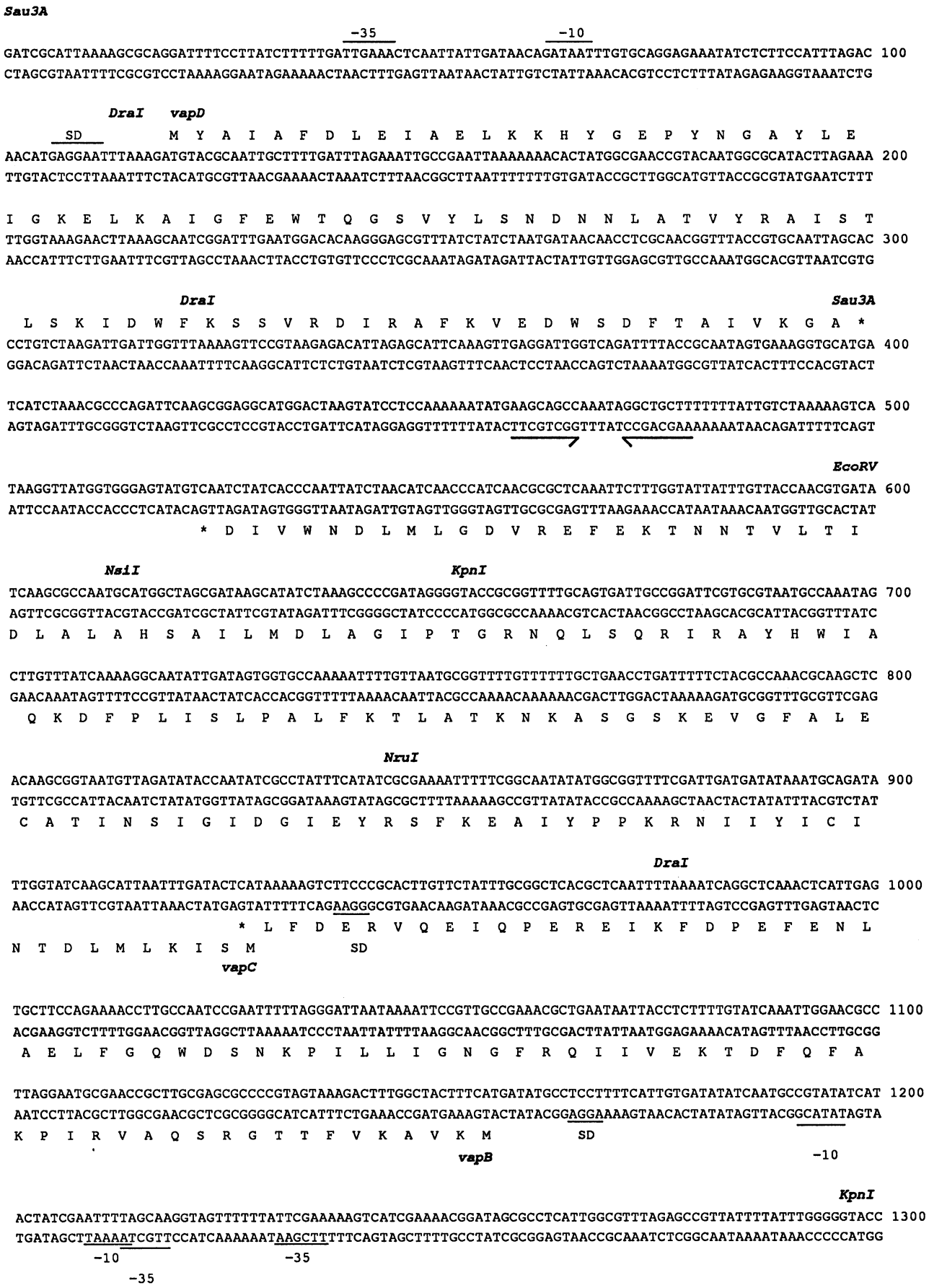
Southern hybridization analysis of *vap* region. There are three copies of the *vap* region in *D. nodosus* A198, represented by the 6.2-, 4.6-, and 3.5-kb *Hin*dIII fragments which hybridize with pJIR318 (17). To see whether these copies each contained the entire *vap* region, Southern blots of genomic DNA from the virulent strain *D. nodosus* A198 and the benign strain *D. nodosus* 305 were hybridized with probes specific to each *vap* gene. Blots were prepared by using *D. nodosus* A198 DNA digested with *Hin*dIII, which does not cut within the pJIR318 insert. Multiple hybridizing bands were present in each A198 blot, whereas no hybridization was observed in the strain 305 lanes. The results showed that although the 6.2- and 4.6-kb *Hin*dIII fragments hybridized with each of the *vapA*-, *vapB*-, *vapC*-, and *vapD*-specific gene probes, the 3.5-kb fragment hybridized only with the *vapD*-specific gene probe (data not shown). This fragment therefore does not contain a copy of the *vapABC* genes.

Amino acid sequence analysis. The amino acid sequences of the putative VapA (11.3 kDa), VapB (8.9 kDa), VapC (15.2 kDa), and VapD (10.6 kDa) proteins and ORF118 were analyzed for membrane-spanning domains by using the ALOM program (18). The results indicated that only the VapC protein had the potential to be an integral membrane protein. The amino acid sequences also were compared with the protein sequences in various data banks. Homology was detected between VapD and the sequence, found in the Protein Data Base of Japan, of ORF5 from the *N. gonorrhoeae* cryptic plasmid (19). The sizes of the *D. nodosus* and *N. gonorrhoeae* ORFs are similar (93 and 92 amino acids, respectively), and 46% of the amino acids are identical (Fig. 2A). No significant amino acid sequence similarity with the other Vap proteins was found.

However, when the nucleic acid sequence of pJIR318 was compared with the DNA sequences in GenBank by using the FASTA search program (28), it was discovered that the *vapB-vapC* region of pJIR318 showed 53% identity in 706 bp of overlap with the *traD-traI* intergenic region of the *E. coli* F plasmid. This region of the F plasmid contains an ORF (*trbH*) which could encode a 26-kDa protein (4, 16). No ORF similar to *trbH* is present in pJIR318. Examination of the *trbH* region shows that two ORFs with homology to *vapB* and *vapC* are encoded by the DNA strand complementary to *trbH* (Fig. 2B). The two F-plasmid ORFs, which we have designated TRAORF1 and TRAORF2, are both preceded by Shine-Dalgarno sequences, and an RNA polymerase-binding site has been identified upstream of TRAORF1 (16, 24). Just as the initiation codon of *vapC* overlaps the stop codon of *vapB*, the initiation codon of TRAORF2 overlaps the stop codon of TRAORF1.

Production of the VapD protein in *E. coli*. To determine whether any of the *D. nodosus vap* genes are expressed in *E. coli*, cell extracts prepared from JIR1793 [DH5α(pJIR318)] were fractionated on polyacrylamide gels. An abundant, low-molecular-weight protein was visible in extracts from cells harboring pJIR318 (Fig. 3). No protein of a similar size was visible in extracts from cells carrying the vector pUC18. The low-molecular-weight protein was tentatively designated the *vapD* gene product, as it was expressed in *E. coli* cells carrying pJIR330, a deletion derivative of pJIR318 which contained only *vapD* (Fig. 3). The apparent size of the putative *vapD* gene product (approximately 6 kDa) was much lower than the molecular weight predicted from the deduced VapD amino acid sequence (10.6 kDa). To verify that the 6-kDa protein was the *vapD* gene product, the protein was gel purified and the N-terminal sequence was determined. The protein sequence that was generated, Met-Tyr-Ala-Ile, correlated with the putative start codon and N terminus of VapD that was deduced from the DNA sequence.

The VapD protein produced in *E. coli* could have been smaller than predicted because of posttranslational cleavage of the carboxy terminal end. To determine whether the C terminus of the *vapD* gene product produced in *E. coli* was intact, plasmid pJIR515 was constructed. This plasmid was a derivative of pJIR318 in which the 3′ end of *vapD* had been deleted. The *vapD* gene product encoded by this plasmid should have been 4 amino acids smaller because of the loss of 18 amino acids from the C-terminal end of *vapD* and the addition of 14 amino acids due to continuation of the ORF into vector sequences. If posttranslational cleavage occurred, then the product of the deleted gene would be expected to be identical in size to the product of the intact gene. The results showed that the VapD product encoded by pJIR515 was of a higher apparent molecular weight than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DraI vapD
SD
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DraI
Sau3A
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NsiI
KpnI
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DraI
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N T D L M L K I S M
SD
vapC
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SD
vapB
-10
KpnI
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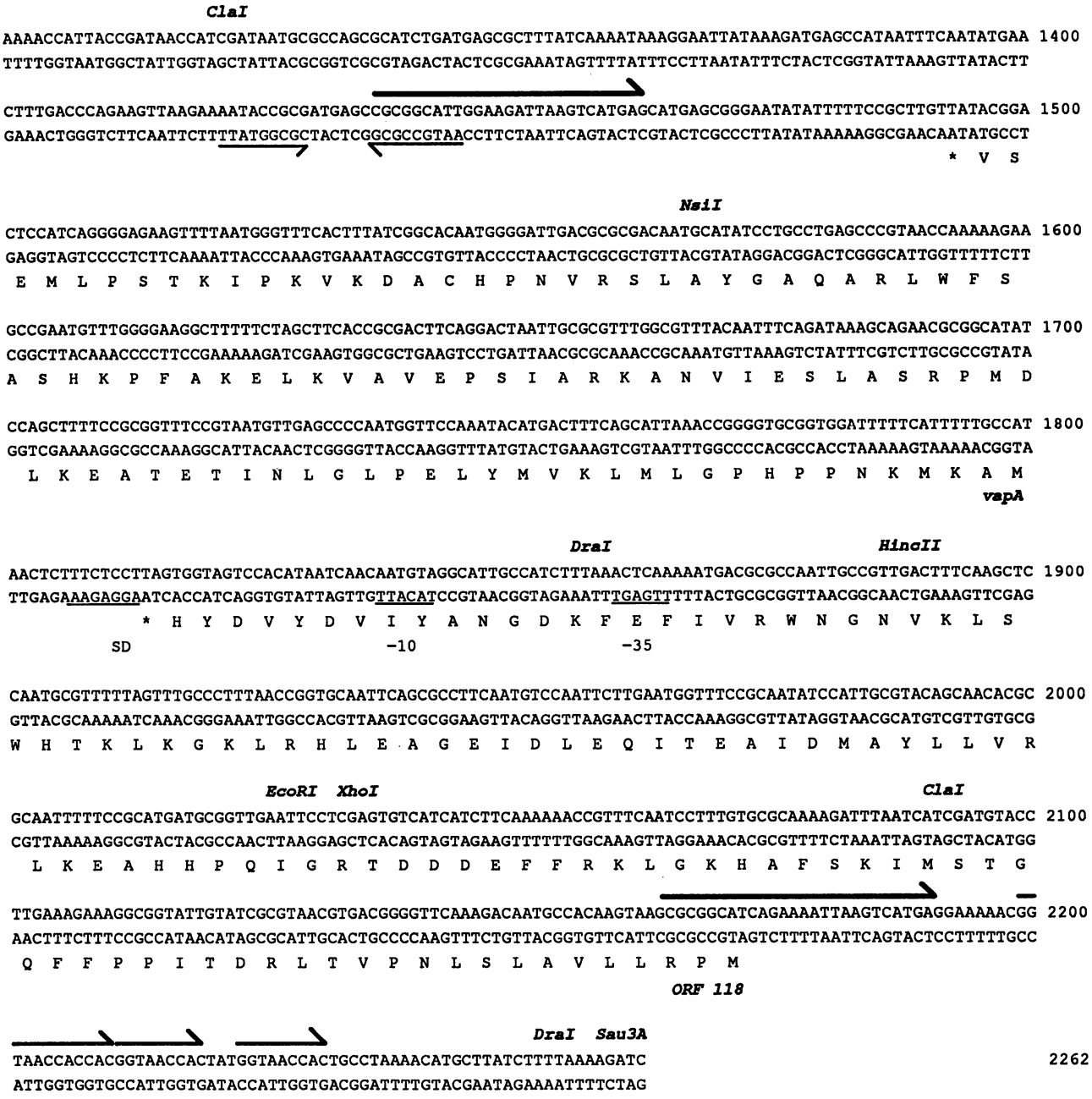

FIG. 1. Sequence analysis of the 2,262-bp *D. nodosus* DNA insert of pJIR318. Both the nucleotide sequence and the deduced amino acid sequences of ORFs from both strands are shown. Stop codons are indicated by asterisks. Potential ribosome-binding sites (33) are indicated by SD (Shine-Dalgarno), and possible −10 and −35 promoter sequences (15) are underlined. Inverted repeats which could function as transcriptional terminators are indicated with arrows below the nucleotide sequence. The inverted repeat at position 468 could act as a bidirectional terminator. Several direct repeats of unknown significance are indicated by arrows above the nucleotide sequence.

pJIR318-encoded VapD (Fig. 3). We therefore conclude that the aberrant mobility of VapD was due to the amino acid sequence of the VapD protein, in particular, sequences at the C-terminal end of the protein, and that removal of these amino acids can lead to an increase in the apparent molecular weight of the gene product.

Western immunoblots of VapD in *D. nodosus* and *N. gonorrhoeae*. The SDS-PAGE-purified, urea-extracted VapD protein was used to raise specific rabbit antiserum. The resultant antiserum reacted with a 6-kDa polypeptide in Western immunoblots of the virulent strain *D. nodosus* A198 but not the benign strain *D. nodosus* 305 (Fig. 4). To extend these studies, cell extracts of 16 additional *D. nodosus* isolates, which were classified as virulent, intermediate, or benign, were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-VapD antiserum in Western immunoblots. The results showed that the VapD protein was produced by all of the *D. nodosus* isolates which hybridized

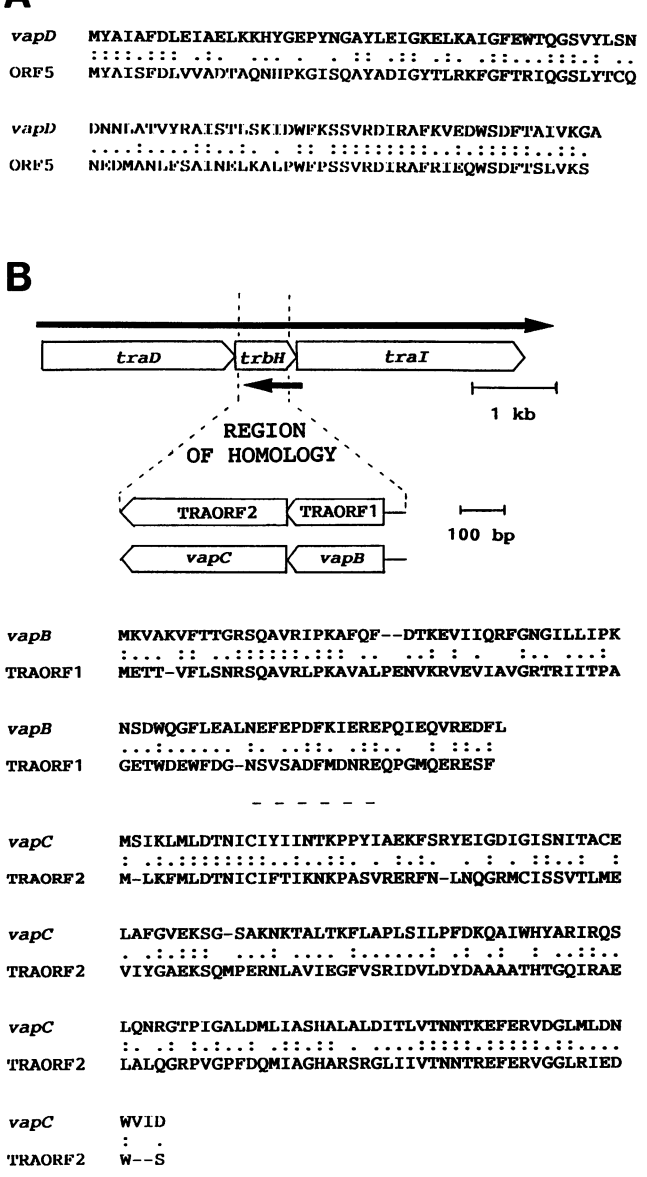

FIG. 2. Comparative amino acid sequence analysis of *vap* gene products. (A) Comparison of the *D. nodosus vapD* gene with ORF5 (19) from the cryptic *N. gonorrhoeae* plasmid. The amino acid sequences of the two ORFs were aligned by using the FASTP program (23). Identical amino acids are marked with colons, and functionally similar amino acids are marked with periods. (B) Region of the *E. coli* F plasmid that had sequence similarity with the *vapB-vapC* region of pJIR318. A comparison of two small ORFs on the complementary strand of *trbH* with *vapB* and *vapC* is shown below a map of the *E. coli traD-traI* region (16). The comparison was carried out by using the CLUSTAL program for sequence alignment (14).

with the pJIR318 probe. Isolates which did not hybridize with pJIR318 (strains AC554, AC6, and AC176) did not produce detectable levels of VapD. The virulent isolates (strains A198, B1006, and C1008) and one intermediate isolate (strain HA343) appeared to produce more VapD than the other strains (Fig. 4).

Further Western blots were carried out with cell extracts of *N. gonorrhoeae* strains MS-11A, Gc var40, and JKD109. No reactivity in the 6- to 10-kDa region, that is, at a molecular weight consistent with the recognition of ORF5 (10.5 kDa) expressed from the gonococcal cryptic plasmid, was detected in immunoblots when anti-VapD antiserum was used (data not shown). Antiserum raised against whole piliated *N. gonorrhoeae* also failed to react with VapD expressed in *E. coli*.

DISCUSSION

Sequence analysis of pJIR318, one of three plasmids previously shown to carry gene regions associated with virulence in *D. nodosus* (17), revealed that this plasmid contains four genes, two of which, *vapA* and *vapD*, appear to be present as separate cistrons. However, the *vapC* start codon overlaps the stop codon of *vapB*. This arrangement, together with the absence of a transcription initiation site upstream of *vapC*, suggests that *vapB* and *vapC* may form an operon in which both transcription and translation are coupled. One gene, *vapD*, has been shown to be expressed in both *E. coli* and *D. nodosus*. Amino acid sequence analysis of the gel-purified VapD protein has confirmed the predicted *vapD* start codon.

In a previous study, 33% of the benign *D. nodosus* isolates that were examined hybridized with pJIR318 (17). It was not known whether these strains expressed the products of the *vap* region or were benign, because they carried homologous but nonfunctional *vap* genes. The four pJIR318-hybridizing benign isolates that have now been tested in Western immunoblots all expressed VapD. All of the hybridizing virulent and intermediate strains of *D. nodosus* that were tested also produced VapD. However, it is not possible to determine whether the VapD polypeptides produced by these isolates are functional.

The *vapD* gene product and the *vapBC* region were shown to have sequence similarity with components of the *N. gonorrhoeae* cryptic plasmid and the *E. coli* F plasmid, respectively. The significance of this sequence similarity is not clear. The function of the cryptic plasmid is unknown; however, it is present in 99% of clinical isolates of *N. gonorrhoeae* (29). The discovery of homology between ORF5 of the cryptic plasmid and a region associated with virulence in *D. nodosus* suggests that this plasmid may have a role in the virulence of *N. gonorrhoeae*. It was hoped that the VapD antiserum would provide a means by which the function of ORF5 could be investigated further. Unfortunately, anti-VapD antiserum failed to react with the ORF5 protein in *N. gonorrhoeae*, suggesting either that ORF5 was not expressed or that it did not cross-react with VapD antibodies.

The function of the *trbH* region of the *E. coli* F plasmid is also unknown. The region is unlikely to be essential for either plasmid maintenance or DNA transfer, as the *trbH* region is absent in the F-like plasmid R100 (40). The apparent conservation of the genetic organization of the *vapBC* operon and the operon encoding TRAORF1 and TRAORF2 suggests that the functional significance of the *trbH* region may reside on the strand opposite to that containing the *trbH* gene.

The presence in pJIR318 of regions homologous to two different bacterial plasmids seems to suggest that the *D. nodosus* DNA insert of pJIR318 is located on a *D. nodosus* plasmid. No plasmids have been identified in *D. nodosus*. In addition, Southern blots of *D. nodosus* DNA fractionated by pulsed-field gel electrophoresis showed that all three copies

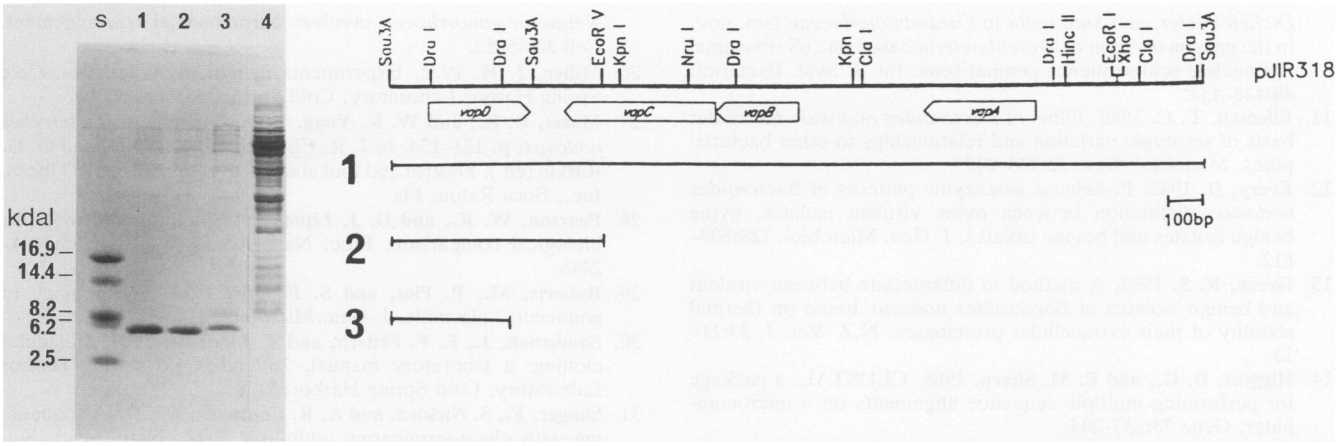

FIG. 3. Analysis of the *vapD* gene product in *E. coli*. PAGE of proteins was produced by recombinant *E. coli* cells carrying pJIR318 (lane 1), pJIR330 (lane 2), pJIR515 (lane 3), and pUC18 (lane 4). Note that 10 times as much cell extract is loaded in lane 4. Molecular weight standards are in lane S. Plasmids pJIR330 and pJIR515 are deletion derivatives of pJIR318, as shown in the accompanying diagram. Cell extracts were prepared and subjected to electrophoresis on tricine-SDS-polyacrylamide gels as described in Materials and Methods.

of the *vap* region were located on the *D. nodosus* chromosome (22). However, it is possible that this region was derived from the integration into the *D. nodosus* chromosome of a conjugative plasmid from another species. We suggest that this event was followed by gene duplications which led to the presence of three copies of the *vap* region in the *D. nodosus* chromosome. Since the Southern hybridization experiments showed that in strain A198 only two of these copies carry the *vapABC* genes, subsequent deletion events presumably have led to the evolution of the region which carries only *vapD*. Previous results showed that the number of copies of the *vap* region varies between different isolates of *D. nodosus* (17). These data suggest that the putative integration of foreign DNA into the *D. nodosus* chromosome was not a relatively recent event in evolutionary terms.

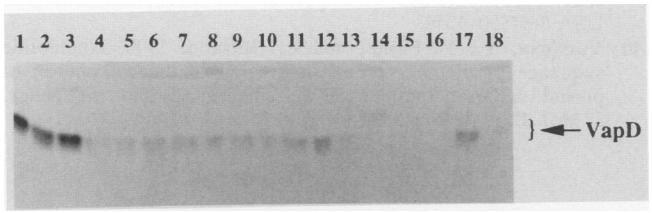

FIG. 4. Western immunoblots of *D. nodosus* cell extracts. Cells from 18 isolates of *D. nodosus* were adjusted for cell numbers, solubilized in SDS-PAGE sample buffer, and fractionated on a 15% (wt/vol) SDS-PAGE gel. The polypeptides were transferred to nitrocellulose and reacted in an immunoblot with rabbit anti-VapD antiserum. VapD reacted as a broad ca. 6-kDa band in all wells except lanes 14, 15, 16, and 18. Lanes show cell extracts from the following isolates: 1, B1006; 2, C1008; 3, HA343; 4, HA352; 5, AC419; 6, AC424; 7, HA337; 8, HA390; 9, HA386; 10, HA393; 11, HA389; 12, HA340; 13, HA652; 14, AC554; 15, AC6; 16, AC176; 17, A198; 18, 305. Strains B1006, C1008, and A198 were virulent; strains HA343, HA352, HA337, HA390, HA386, HA393, and AC554 were intermediate; and all other strains were benign. Virulence designations were made by the laboratory of origin. All of these isolates except strains AC554, AC6, AC176, and 305 hybridized with pJIR318. Strain AC554 was one of only two intermediate strains which did not hybridize to pJIR318 (17).

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