Identification of three gene regions associated with virulence in *Dichelobacter nodosus*, the causative agent of ovine footrot

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(Received 5 March 1991; revised 23 May 1991; accepted 12 June 1991)

Dichelobacter nodosus (formerly *Bacteroides nodosus*) is a Gram-negative strict anaerobe and is the primary pathogen involved in ovine footrot. A comparative hybridization strategy was used to isolate recombinant clones which hybridized to DNA from a virulent strain of *D. nodosus* but not with a benign isolate. Three virulence-associated gene regions were identified and one of these regions was shown to be present in multiple copies in the *D. nodosus* genome. Hybridization studies on 101 clinical isolates of *D. nodosus* showed that these strains could be divided into three hybridization categories which could be correlated with the virulence of the isolates. The recombinant clones have considerable potential for the development of a gene-probe-based method for the differential diagnosis of ovine footrot.

Introduction

Virulent ovine footrot is a highly contagious disease characterized by separation of the hoof from the underlying epidermal tissues, resulting in severe lameness, loss of body condition and reduced wool production (Egerton *et al.*, 1969; Stewart *et al.*, 1984). There is a benign form of the disease which causes only mild lameness (Egerton & Parsonson, 1969). An intermediate form of ovine footrot also exists (Stewart *et al.*, 1982). In the early stages of infection the three forms of footrot are clinically indistinguishable (Stewart, 1989).

The principal causative agent of both virulent and benign footrot in sheep is the anaerobic Gram-negative bacterium *Dichelobacter nodosus* (Beveridge, 1941), which was formerly known as *Bacteroides nodosus* (La Fontaine & Rood, 1990; Dewhirst *et al.*, 1990). *D. nodosus* strains isolated from virulent footrot infections have been shown to differ from benign isolates. Virulent isolates produce relatively heat-stable proteases (Depiazzi & Rood, 1984) which have a characteristic isoenzyme pattern after electrophoresis (Every, 1982) and have elastolytic activity (Stewart, 1979). Virulent isolates of *D. nodosus* also exhibit greater twitching

motility, which is measured by determining the colony diameter (Depiazzi & Richards, 1985). Because of these differences, the proteases, which degrade a variety of substrates found in the skin and hoof of the sheep, and the fimbriae (or pili) which are responsible for twitching motility, are thought to be virulence determinants in *D. nodosus* (Depiazzi *et al.*, 1986).

The differences in protease thermostability, electrophoretic zymogram pattern, elastase activity and colony diameter form the basis of present diagnostic tests used to differentiate benign from virulent footrot infections. The tests are expensive and as they require the preliminary isolation and culture of *D. nodosus* from infectious tissue, they are both laborious and time consuming. The diagnosis is also complicated by the occurrence of strains of intermediate virulence (Stewart *et al.*, 1982). *D. nodosus* isolates can also be divided into nine different serogroups based on their fimbrial antigens (Claxton *et al.*, 1983). Virulent, intermediate and benign strains are found in each serogroup.

Rood & Yong (1989) suggested that a diagnostic test based on monoclonal antibodies or DNA probes could provide a rapid means of identifying virulent footrot infections directly from lesion material. In an effort to isolate a DNA probe, or probes, which could be used to develop a rapid method for the diagnosis of virulent ovine footrot, we have sought regions of the *D. nodosus*

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genome unique to virulent strains. We describe here the isolation from a virulent *D. nodosus* strain of three DNA fragments that are associated with virulence. We present data showing that the three regions could represent previously unidentified virulence determinants in *D. nodosus* and may be useful in the differential diagnosis of virulent, intermediate and benign footrot in sheep.

Methods

Strains and media. Prototype virulent strains of each serogroup of *D. nodosus* were obtained from J. Egerton, University of Sydney, Australia (Claxton *et al.*, 1983). Other virulent, intermediate and benign isolates of *D. nodosus* were obtained from J. Egerton, or were isolated at the Regional Veterinary Laboratories in Albany, Western Australia, or Hamilton, Victoria. All *D. nodosus* isolates were cultured for 2–7 d on hoof agar (Thomas, 1958) and TAS agar or TAS broth (Skerman, 1975) at 37 °C in an atmosphere of 10% (v/v) H₂ and 10% (v/v) CO₂ in N₂. *Actinomyces pyogenes*, *Fusobacterium necrophorum*, *Peptostreptococcus* sp., *Corynebacterium* sp., *Bacillus cereus*, Beveridge organism and *Clostridium* sp., were cultured on blood agar as described by Depiazzi (1988). All *Escherichia coli* strains were derivatives of DH5 α (F ϕ 80dlacZAM15 Δ (lacZYZ-argF)U169 recA1 endA1 hsdR17 supE44 λ -thi-1 gyrA relA1; Bethesda Research Laboratories) and were cultured in 2 $\times$ YT medium (Miller, 1972) or LB (Maniatis *et al.*, 1982) supplemented with 100 μ g ampicillin ml⁻¹.

DNA isolation and general molecular techniques. DNA was prepared by the method of Anderson *et al.* (1984) from *D. nodosus* cells grown in TAS broth. Plasmid DNA was prepared from *E. coli* by the method of Birnboim & Doly (1979) or Holmes & Quigley (1981). General molecular techniques used in cloning and analysis of DNA were as described by Maniatis *et al.* (1982).

Southern and dot-blot hybridization analysis. For Southern blotting, 1–2 μ g of *D. nodosus* genomic DNA was digested with the appropriate restriction endonuclease and fractionated by electrophoresis through a 0.8% agarose gel. The DNA was transferred to a nitrocellulose membrane (BA-85, Schleicher and Schuell) by the method described by Southern (1975).

Dot-blots were prepared using *D. nodosus* DNA or from whole cells grown on TAS agar. Cells were removed from the plates by suspension in phosphate-buffered saline. For DNA dot-blots, 0.5–1 μ g *D. nodosus* DNA (in 0.25 ml H₂O) was denatured by boiling for 2 min. The DNA samples were cooled and 0.25 ml of a solution containing 1.8 M-NaCl, 0.18 M-sodium citrate and 4.4 M-formaldehyde was added. The DNA (0.1 ml) was applied to a nitrocellulose membrane with the aid of a dot-blot apparatus (Schleicher and Schuell). The membrane was rinsed in 2 $\times$ SSC (1 $\times$ SSC contains 0.15 M-NaCl and 0.015 M-sodium citrate) before baking for 4 h at 65 °C. For dot-blots using whole cells, 10⁶–10⁷ cells were applied to a nylon membrane (BioTrace RP) as described by Korolik (1990).

Southern and dot-blots were hybridized with plasmid DNA labelled with [³²P]dATP (Amersham) using a random primer labelling kit (Bresatec). Prehybridization and hybridization solutions contained 50% (v/v) formamide, 4 mM-EDTA, 32 mM-NaOH, 40 mM-NaH₂PO₄, 0.72 M-NaCl, 1% (w/v) SDS, 0.5% skim milk and 0.3 mg denatured salmon sperm DNA ml⁻¹. Hybridization was carried out at 37 °C for 16–20 h. The blots were washed twice for 30 min in 2 $\times$ SSC, 0.1% SDS and twice for 30 min in 0.1 $\times$ SSC, 0.1% SDS. All washes were carried out at 65 °C.

Cloning of *D. nodosus* DNA. Five 25 μ g samples of genomic DNA from the *D. nodosus* reference strain A198 (Dewhirst *et al.*, 1990) were

treated with various amounts (0.8–15 U) of the restriction endonuclease *Sau*3A for 1 h at 37 °C. The digests were heated to 65 °C for 15 min and samples analysed on a 0.8% agarose gel. The digested DNA was pooled and layered on a 10–40% (w/v) sucrose gradient (in 1 M-NaCl, 20 mM-Tris/HCl pH 8.0, 5 mM-EDTA). The gradient was subjected to ultracentrifugation in a Beckman SW40 Ti rotor for 24 h at 26000 r.p.m. at 20 °C. Fractions (0.5 ml) were collected and the size of the DNA fragments determined by agarose gel electrophoresis. Fractions containing DNA in the size ranges 0.25–1 kb, 1–2 kb and 2–3 kb were diluted and ethanol-precipitated (Maniatis *et al.*, 1982). The DNA was then ligated with *Bam*HI-digested, phosphatase-treated pUC18 DNA (Yanisch-Perron *et al.*, 1985).

The recombinant clones were screened using a modified version of the colony hybridization procedure of Gergen *et al.* (1979). Bacterial colonies containing plasmid DNA were replicated in triplicate to LB agar containing 100 μ g ampicillin ml⁻¹. The clones were preserved on one of the plates. After 5 h at 37 °C, Whatman 541 filters were layered on two of the plates. The filters were removed after a further 3 h incubation at 37 °C and placed, colony side down, on LB agar containing 250 μ g chloramphenicol ml⁻¹. After incubation for 16–20 h the filters were removed, treated in 0.5 M-NaOH for 7 min, washed twice for 5 min with 1 M-Tris/HCl pH 7.2 and once with 0.5 M-Tris/HCl pH 7.2, 1.5 M-NaCl for 5 min. The filters were rinsed several times with 95% (v/v) ethanol and dried at 65 °C. Duplicate filters were hybridized separately with ³²P-labelled genomic DNA from the benign *D. nodosus* strain 305 and the virulent strain A198 in a hybridization solution containing 0.5 M-NaCl, 0.1 M-sodium phosphate buffer pH 7, 6 mM-EDTA, 0.1% (w/v) SDS and 1 mg denatured salmon sperm DNA ml⁻¹, using 3 ml hybridization solution per filter. The *D. nodosus* genomic DNA preparations were labelled using a random primer labelling kit (Bresatec). The filters were washed under stringent conditions as described above for Southern and dot-blots. The filters were dried and exposed to X-ray film at –70 °C for 3–5 d.

Results

Cloning and identification of virulence-associated fragments of *D. nodosus* DNA

To identify virulence-specific fragments of the *D. nodosus* genome we constructed several sets of *E. coli* recombinant clones which contained DNA from the virulent strain A198. These clones were screened to detect recombinant plasmids which hybridized with labelled genomic DNA from A198 but did not hybridize with labelled DNA from the benign *D. nodosus* strain, 305. Clones containing virulence-specific sequences would be expected to hybridize only with A198 DNA whereas almost all of the other recombinants should hybridize with both A198 and 305 DNA.

It was anticipated that the differences between virulent and benign isolates of *D. nodosus* might be small. Therefore, the clones containing the smallest inserts (0.25–1 kb *Sau*3A fragments) were chosen initially for testing. Duplicate sets of colonies were transferred to filters and hybridized separately with ³²P-labelled total A198 DNA and ³²P-labelled 305 DNA. However, the majority of the clones failed to hybridize strongly with labelled A198 DNA; no difference was seen between

Fig. 1. DNA dot-blots of virulent and benign *D. nodosus* isolates. Isolates from each serogroup were probed with (a) plasmids obtained in screening *E. coli* clones containing DNA from the virulent *D. nodosus* strain A198, and (b) two subclones of pJIR314. DNA was prepared from prototype virulent (V) *D. nodosus* strains (Claxton *et al.*, 1983) of serogroup A (A198), B (1006), C (1008), D (1172), E (1137), F (1017), G (1220), H (1215) and I (1623) and from benign (B) isolates of serogroup A (AC176), B (AC6), C (305), D (AC390), E (1689), F (1045), G (1029), H (1204) and I (1040). Samples of the 18 *D. nodosus* DNA preparations were applied to nitrocellulose membranes and probed with ^{32} P-labelled plasmid DNA.

clones containing small inserts and controls containing only pUC18. Only one clone which hybridized with A198 DNA more strongly than 305 DNA was identified. In an effort to increase the sensitivity of the colony hybridizations a variety of hybridization and washing conditions and membranes were tested. None of the procedures

allowed the reliable detection of clones with inserts of *D. nodosus* DNA of less than 1 kb.

To obtain a stronger hybridization signal, clones containing larger inserts of A198 DNA were screened. An improved signal-to-noise ratio was obtained and an additional 11 clones, which showed stronger hybridization with virulent DNA compared with benign DNA, were recovered by screening clones with inserts in the 1–2 kb and 2–3 kb size range.

Plasmid DNA was prepared from each of the 12 clones. The plasmids were initially tested as probes in Southern blot and DNA dot-blot hybridization experiments which used DNA prepared from a small number of virulent and benign *D. nodosus* strains. Fig. 1(a) shows DNA dot-blot analysis of virulent and benign strains of all serogroups using representative clones as probes. Based on these results, the plasmids could be divided into four groups: (1) plasmids which hybridized with all of the isolates tested (pJIR320, pJIR321 and pJIR322), (2) plasmids which hybridized with some but not all of the strains, with no apparent correlation between the pattern of hybridization and virulence or serogroup (pJIR311, pJIR312, pJIR315, pJIR316, pJIR317 and pJIR319), (3) one plasmid which hybridized more strongly with virulent strains than benign strains (pJIR314), and (4) two plasmids which hybridized with DNA from all the virulent strains tested but none or only a few of the benign strains (pJIR318 and pJIR313).

One plasmid, pJIR321, which hybridized with DNA from both virulent and benign strains, hybridized with different restriction fragments in benign and virulent isolates when used as a probe in Southern blot experiments (data not shown). This plasmid has not yet been subjected to further analysis. However, we have analysed the plasmids showing a virulence-associated pattern of hybridization (pJIR313, pJIR314 and pJIR318).

Restriction analysis of clones

Restriction maps of pJIR313, pJIR314 and pJIR318 were prepared (Fig. 2). No similarities or overlaps in restriction maps of the three clones were noted. The restriction maps were also compared to the maps of the *D. nodosus* fimbrial subunit gene region. (Anderson *et al.*, 1984; Elleman & Hoyne, 1984) and a protease gene isolated from strain A198 (Vaughan *et al.*, 1990). No similarities in the restriction maps were observed.

Using the information obtained from restriction analysis of pJIR314, two subclones of the plasmid (pJIR314A and pJIR314B) were constructed in an effort to increase the specificity of hybridization (Fig. 2). The subclones were tested by dot-blot analysis of DNA from virulent and benign strains (Fig. 1b). pJIR314B yielded a

Fig. 2. Restriction maps of the *D. nodosus* DNA inserts of the virulence-associated recombinant plasmids (pJIR318, pJIR313 and pJIR314). The *D. nodosus* fragments are inserted in the *Bam*HI site of pUC18. A *Pst*I deletion derivative of pJIR314 (pJIR314A) and a *Pst*I subclone of pJIR314 (pJIR314B) are shown. The size of each plasmid is indicated below the plasmid name. The *Eco*RI site in the multiple cloning site of pUC18 is to the left of each insert.

more virulent-specific hybridization profile, since only very faint hybridization with benign strains was observed. Therefore, all subsequent studies on this gene region were carried out on pJIR314B.

Screening of *D. nodosus* isolates

The potential of the three recombinant plasmids (pJIR313, pJIR314B and pJIR318) for use in the development of a rapid DNA probe test for the differential diagnosis of ovine footrot was tested. The plasmids were used as probes in dot-blot hybridization experiments to screen 101 virulent, intermediate and benign isolates of *D. nodosus* from Victoria, New South Wales and Western Australia. The virulence classification of each strain was determined in the laboratory of origin and was based on clinical assessment, laboratory tests, or a combination of both. Twenty-five strains were tested in dot-blot using whole bacterial cells, or purified DNA, but no differences in hybridization between the two methods were detected. The remaining strains were tested by whole-cell dot-blot. The results indicated that sequences homologous to pJIR318 were present in virtually all (97%) of the virulent and intermediate isolates of *D. nodosus* (Table 1). In contrast, 67% of the

Table 1. Hybridization analysis of *D. nodosus* isolates using virulence-associated gene probes

Virulence of isolates	No. of isolates tested	No. of isolates hybridizing to probes		
		pJIR318	pJIR313	pJIR314B
Virulent	29	29 (100%)	27 (94%)	29 (100%)
Intermediate	36	34 (94%)	13 (36%)	13 (36%)
Benign	36	12 (33%)	2 (6%)	2 (6%)

benign isolates showed no detectable homology with pJIR318 under the stringent hybridization conditions used. However, 33% of the benign strains tested did hybridize with pJIR318. The hybridization of pJIR318 with a subset of benign strains was confirmed by Southern blot hybridization; in four of the eight benign strains analysed, pJIR318 hybridized with restriction fragments which differed in size from those of the prototype virulent strains.

The nine *D. nodosus* serogroups did not appear to be equally represented within the group of benign isolates

Table 2. Results of dot-blot hybridization of benign *D. nodosus* isolates with pJIR318

Serogroup	No. of isolates tested	No. of hybridizing isolates
A	6	0
B	4	1
C	2	0
D	2	2
E	4	2
F	1	1
G	7	0
H	8	6
I	1	0
Unknown	1	0
Total	36	12

containing sequences homologous to pJIR318 (Table 2). For many serogroups only one or two benign isolates have been tested, so a larger sampling would be required before definite conclusions could be drawn. However, reasonable numbers of benign isolates from three Australian states (Victoria, Western Australia and New South Wales) were available for both serogroups G and H. None of the seven benign isolates belonging to serogroup G hybridized with pJIR318 while six of the eight serogroup H isolates did so, including isolates from each state. Four of these six isolates were examined in Southern blots and shown to have the same profile as the prototype H strain (data not shown).

A different hybridization profile emerged with the pJIR313 and pJIR314B probes (Table 1). These two plasmids showed nearly identical patterns; only two strains which hybridized with pJIR314B failed to hybridize with pJIR313. In contrast to the results obtained with pJIR318, only two of the 36 benign strains tested (6%) hybridized with pJIR314B or pJIR313. Both of these benign strains belonged to serogroup H and also hybridized with pJIR318. The majority of strains classified as intermediate also did not hybridize with pJIR313 and pJIR314B. Both plasmids hybridized with all the virulent isolates tested, with the exception of two strains which did not hybridize with pJIR313. This difference was confirmed by Southern blot analysis of DNA from the two strains in question (data not shown).

The virulence classification of six isolates was verified independently in blind tests at two separate laboratories. The tests confirmed that the two benign isolates which hybridized with all three DNA probes were designated correctly as benign and that the only two intermediate isolates which did not hybridize with pJIR318 were not benign. One isolate which had been classified as benign

based on clinical assessment was shown to have the characteristics of a virulent strain in laboratory tests.

Seven bacterial isolates commonly obtained from ovine footrot specimens (Depiazzi, 1988) were tested by both DNA dot-blot and whole-cell dot-blot experiments. No hybridization to *Actinomyces pyogenes*, *Fusobacterium necrophorum*, *Peptostreptococcus* sp., *Corynebacterium* sp., *Bacillus cereus*, Beveridge organism and *Clostridium* sp., was detected with any of the three clones (data not shown).

Southern blot analysis of prototype virulent *D. nodosus* strains

Each of the three clones was used as a probe in Southern blot analysis of prototype virulent *D. nodosus* strains of each serogroup (Fig. 3). Since pJIR318 contains no *Hind*III site within the insert it would be expected to hybridize with a single *Hind*III fragment in the A198 genome. Southern blot analysis of *Hind*III-digested DNA from strain A198 and prototype virulent strains of serogroups C, F and I showed that there were three fragments, of 3.5 kb, 4.6 kb and 6.2 kb, which hybridized with pJIR318 (Fig. 3a), indicating that at least three copies of sequences homologous with pJIR318 exist in these strains. The prototype D and E strains only contained the 6.2 kb *Hind*III band, whereas the serogroup B, G and H strains had larger hybridizing *Hind*III bands. The pJIR318 DNA insert contains two *Sau*3A fragments of 1.8 kb and 0.4 kb. If the three regions were identical, only two *Sau*3A fragments, of 1.8 kb and 0.4 kb, should have been present in Southern blots of A198 DNA probed with pJIR318. An additional 3.0 kb *Sau*3A fragment was detected (Fig. 3a), indicating that the three regions are not identical. Despite this diversity of *Hind*III profiles, all nine of the prototype strains had the 0.4 kb *Sau*3A fragment, and either both, or only one, of the 3.0 kb and 1.8 kb *Sau*3A fragments. No other *Sau*3A fragment which hybridized with the pJIR318 probe was detected (Fig. 3a).

Only one major hybridizing *Hind*III band was seen when the blot was probed with either pJIR313 or pJIR314B. The single *Hind*III fragment which hybridized strongly with pJIR313 differed in size in the different prototype virulent strains. The serogroup A, B, C, F, G and I strains had a 2.3 kb *Hind*III fragment, the serogroup H strain had a 3.0 kb *Hind*III fragment and the serogroup D and E strains contained a fragment of approximately 12 kb (Fig. 3b). In the *Hind*III Southern blot probed with pJIR313 several faint bands are also visible. This plasmid contains a *Hind*III site very close to one end of the insert DNA, therefore one faintly hybridizing band was expected. The other bands may

represent partial *Hind*III fragments or fragments with weak homology to pJIR313. Note that the *Sau*3A hybridization profiles of all nine strains were identical (Fig. 3b).

The Southern blot probed with pJIR314B revealed a single *Hind*III fragment of about 11 kb in each prototype virulent strain, with the exception of the serogroup H strain which had a *Hind*III fragment of approximately 14 kb. The *Sau*3A profiles also were identical (Fig. 3c). The results of the Southern blot hybridizations suggest that pJIR313 and pJIR314B represent sequences found only once in the *D. nodosus* genome, although the existence of other regions with some homology to the plasmids cannot be ruled out.

In all three blots the serogroup H prototype virulent strain differed from the other prototype virulent strains in its hybridization profile. The plasmids pJIR318, pJIR313 and pJIR314B each hybridized with a unique *Hind*III fragment in the serogroup H strain. Analysis of further serogroup H isolates is required before any conclusions can be drawn from this observation.

Discussion

In this paper we have described a method for isolating genes, or parts of genes, which may play a role in bacterial virulence. The technique does not require prior knowledge of the functions of the gene products or their role as virulence determinants. Using this method we have cloned three previously unidentified regions of the *D. nodosus* genome that appear to be associated with virulence. Although sequences homologous to one of the recombinant plasmids, pJIR318, were present in 33% of the benign isolates tested, it is clear that the strains which did not hybridize with pJIR318 were not randomly distributed between the virulent, intermediate and benign strains. The plasmid pJIR318 must therefore contain a region of the *D. nodosus* genome associated with virulence. The plasmids pJIR313 and pJIR314B can also be regarded as virulence-associated since the gene regions they contained were present in virtually all of the virulent *D. nodosus* isolates and absent from virtually all of the benign isolates (Table 1).

Fig. 3. Southern blot analysis of DNA from the prototype virulent *D. nodosus* strains. DNA from the prototype virulent strains (see Fig. 1) was digested with *Hind*III or *Sau*3A, fractionated in a 1.0% agarose gel and blotted to a nitrocellulose membrane. *Hind*III-digested λ DNA was loaded on the gel as size standards. The blot was hybridized with the ^{32} P-labelled virulence-associated recombinant plasmids pJIR318 (a), pJIR313 (b) and pJIR314B (c). The same blot was used for each hybridization. Radiolabelled probe was removed from the membrane by immersion for 10 min in $0.1 \times$ SSC heated to just below boiling.

Restriction map analysis of pJIR313, pJIR314B and pJIR318 indicates that they are not part of previously cloned *D. nodosus* virulence determinants, namely the fimbrial subunit gene and a protease gene isolated from the virulent strain A198 (Elleman & Hoyne, 1984; Vaughan *et al.*, 1990). The three virulence-associated gene regions that we have identified may contain genes that are responsible for the observed differences in protease characteristics and twitching motility between virulent and benign isolates of *D. nodosus*. The gene regions may contain genes involved in fimbrial assembly, twitching motility, or protease secretion. Alternatively, the plasmids may contain part of regions whose virulence function is currently unknown. Further analysis of these regions will be required to discover the role they play in the virulence of *D. nodosus*.

The prototype virulent strains all appear to contain only one copy of the pJIR313 and pJIR314B gene regions. However, several of these strains have at least three copies of sequences homologous to pJIR318 (Fig. 3a). The functional significance of this finding is unclear as it appears that some prototype virulent strains may possess only a single copy of these sequences. It will not be possible to determine the degree of similarity of the regions, and whether all copies of genes present in the regions are expressed, until each region has been isolated and characterized.

In blots probed with two of the plasmids (pJIR313 and pJIR318) there was no detectable hybridization to the DNA of the majority of the benign strains. It is therefore unlikely that these plasmids contain complete virulence regions since adjacent sequences not associated with virulence would hybridize to the DNA of both virulent and benign strains. To isolate the entire virulence-associated regions pJIR313 and pJIR318 will be used to probe a bacteriophage λ library of *D. nodosus* strain A198. It is anticipated that characterization of each virulence region in its entirety will yield additional DNA probes and may enable us to determine the role of these gene regions in virulence.

It was not unexpected that some of the benign strains contained sequences homologous to pJIR313, pJIR314B and pJIR318. Although the clones may contain genes, or parts of genes, whose functions are required for virulence, small deletions or point mutations in these genes could result in a loss of virulence without a change in hybridization pattern. In addition, it is possible that the virulence classification of some of the isolates had been incorrectly assessed, despite the fact that in blind tests, in two independent laboratories, of six benign strains which had hybridized with at least one of the probes only one strain that had been misclassified was detected. Southern blot analysis of a limited number of benign strains hybridizing with pJIR318 showed that at

least some of the benign strains contained no obvious differences when compared with the prototype virulent strains. It is not known whether the prevalence of serogroup H, and possibly serogroups E, D and F, among benign isolates that hybridize with pJIR318 is of any significance.

The three virulence-associated plasmids were used to screen over 100 isolates of *D. nodosus*, a demanding task considering the slow growth rate and fastidious growth requirements of this organism. The results suggest that the clones could be used to develop a rapid diagnostic method to assess field isolates of *D. nodosus*. If we exclude pJIR313, since it failed to hybridize with two virulent strains, the isolates can be grouped by their hybridization profiles into three categories: (1) isolates that hybridize with both pJIR318 and pJIR314B (100% of the virulent isolates, 36% of the intermediate isolates, 6% of the benign isolates), (2) isolates that hybridize with pJIR318 but not with pJIR314B (58% of the intermediate isolates, 27% of the benign isolates), and (3) isolates that do not hybridize with either clone (6% of the intermediate isolates and 67% of the benign isolates). Since all of the virulent isolates tested fell into the first category, hybridization of a field isolate with both pJIR318 and pJIR314B would indicate an urgent requirement for quarantine, or other appropriate measures, until the results of other diagnostic tests were available. The vast majority of the isolates that fail to hybridize with either clone are benign. Sheep infected solely with these strains could safely be excluded from control measures applicable to virulent outbreaks. The treatment of footrot cases caused by isolates in the second category would depend on the procedures for handling cases of infection with intermediate strains.

It must also be realized that the designation of individual isolates as virulent, intermediate and benign can be somewhat variable. Different criteria often are applied in different laboratories. In addition, clinical assessments of the potential of an organism to cause a footrot lesion are influenced by the climatic conditions which apply around the time of examination and specimen collection. The virulence designations of *D. nodosus* isolates therefore must not be regarded as absolute. The three hybridization groups identified in this study may well turn out to have at least as much clinical significance as the existing classification scheme. For example, it would be of interest to determine if there is any difference in virulence between intermediate isolates in categories 1 and 2.

We thank J. Egerton for providing *D. nodosus* strains, M. Palmer, J. Vaughan and D. Stewart for the testing of strains and S. Billington for helpful discussions. We gratefully acknowledge the strong research support of the Australian Wool Corporation.

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