Sequence of Pilin from *Bacteroides nodosus* 351 (Serogroup H) and Implications for Serogroup Classification

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The nucleotide sequence of the pilin gene from *Bacteroides nodosus* strain 351, currently classified as serogroup H, subgroup 2 (H2) has been determined. The gene encodes a single polypeptide (prepilin) of 160 amino acids and M_r 17 150. However, pilin isolated from *B. nodosus* 351 migrates as two distinct bands in sodium dodecyl sulphate-polyacrylamide gel electrophoresis, due to an internal peptide bond cleavage. Amino acid sequence studies of pilin from *B. nodosus* 351 have established that the cleavage occurs between $_{72}$ Ala and $_{73}$ Ser of the mature protein sequence. Comparisons of gene and amino acid sequences of pilin from *B. nodosus* 351 with the corresponding sequences from strains of serogroups D and H1 indicate that these sequences share a close relationship. However, the level of sequence identity between *B. nodosus* 351 pilin and pilin from strain 265 of serogroup H1 is lower than anticipated for strains within a serogroup and suggests that *B. nodosus* 265 and *B. nodosus* 351 should not be classified within the same serogroup.

INTRODUCTION

The Gram-negative rod *Bacteroides nodosus* is the principal causative agent of ovine footrot (Beveridge, 1941), a disease of considerable economic importance in the sheep grazing industry. Strains of *B. nodosus* can be classified into a number of serogroups based on pilus-mediated cellular agglutination. Several serotyping schemes exist. However, the one most frequently used in Australia (Claxton *et al.*, 1983; Claxton, 1986) classifies strains into nine serogroups designated A to I. Serogroups can be further divided, based on cross-tube agglutination studies (Claxton *et al.*, 1983), into a current total of 18 subgroups. An alternative classification system (Day *et al.*, 1986) divides *B. nodosus* isolates from British sheep into 17 serotypes, based on the incomplete removal of agglutinating activity from antisera after absorption by antigens of heterologous serotypes. Vaccination with either killed bacteria or purified pili, in a suitable adjuvant, will protect sheep against footrot when challenged with the same strain of *B. nodosus* (Egerton & Burrell, 1970; Egerton & Roberts, 1971; Stewart, 1978*a, b*; Every & Skerman, 1982; Stewart *et al.*, 1983). Since protection against infection is limited to within a serogroup, multivalent vaccines are required for the control of footrot in the field.

The polar pili of *B. nodosus* are comprised of a subunit polypeptide, pilin (M_r ~17000), the size of which is strain dependent. Amino acid or gene sequences of pilin from *B. nodosus* strains representative of serogroups A to H have been published (McKern *et al.*, 1983, 1985, 1988; Elleman & Hoyne, 1984; Elleman *et al.*, 1986*c*; Elleman & von Ahlefeldt, 1987; Dalrymple & Mattick, 1987; Finney *et al.*, 1988). On the basis of sequence similarity, *B. nodosus* pilins can be divided into two easily distinguishable sets (Finney *et al.*, 1988). The A-set includes pilins from serogroups A, B, C, E, F and G, and the D-set includes pilins from serogroups D and H. Pilins from serogroup I, based on known serological reactivities (Claxton, 1986), are most probably members of the A-set. Although pilins within a set exhibit a high degree of amino acid sequence

identity (>60%), the degree of identity between pilins from different sets (<40%) is no better than that between pilins from *B. nodosus* and those from other bacterial genera including *Neisseria*, *Moraxella* and *Pseudomonas* (Elleman, 1988). Pilins from these genera and those of *B. nodosus* are collectively known as the *N*-methylphenylalanine (mePhe) group as they all possess the unusual amino acid at the amino-terminus.

Within the H serogroup of *B. nodosus*, two subgroups have been distinguished on the basis of low level cross agglutination (Claxton *et al.*, 1983). Pilins from representatives of both subgroups show two subunits in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Anderson *et al.*, 1987). In order to define further the H serogroup and the D-set pilins, the gene sequence of pilin from *B. nodosus* 351 (Bn351) of serogroup H, subgroup 2 (H2) has been determined.

METHODS

Preparation of *B. nodosus* DNA and pili. *B. nodosus* 351 (Bn351) (CSIRO Animal Health Collection), originally designated *B. nodosus* VCS1057 (Department of Veterinary Clinical Studies, University of Sydney) and classified as serogroup H, subgroup 2 (Claxton *et al.*, 1983), was grown anaerobically in modified Eugon broth at 37 °C for 48 h. DNA and pili were prepared from the bacterial culture by methods previously described (Elleman *et al.*, 1984; Stewart *et al.*, 1983). Purification of pili was monitored by SDS-PAGE (Laemmli, 1970) using 15% (w/v) acrylamide in a Bio-Rad Mini-Protean II system. In some cases 2-mercaptoethanol was omitted from the sample incubation buffer.

DNA hybridization procedures. The probe used to identify the pilin gene of Bn351 was derived from a 620 bp *Dra*I/*Bgl*I fragment containing the coding region of the pilin gene from *B. nodosus* 265 (Bn265) DNA cloned in M13mp7. Radiolabelled DNA corresponding to the Bn265 pilin gene, prepared using [α - 32 P]dATP (Elleman *et al.*, 1986c), was used both to screen *Escherichia coli* clones (Maniatis *et al.*, 1982) for the presence of the *B. nodosus* 351 pilin gene and also to identify the pilin gene in endonuclease digested DNA from Bn351 or from recombinant plasmids after electrophoretic separation of DNA fragments in submerged agarose (1%) gels and transfer to nitrocellulose membranes (Smith & Summers, 1980). Nitrocellulose membranes were prehybridized in 50% formamide, 1 $\times$ Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 5 $\times$ SSC (SSC: 150 mM-NaCl, 15 mM-sodium citrate, pH 7.0), 20 mM-potassium phosphate, pH 7.0, and 0.1% sodium dodecyl sulphate at 43 °C for 5 h and then hybridized with the radiolabelled probe under the same conditions for 16 h. Membranes were washed in 2 $\times$ SSC at room temperature for 1 h prior to autoradiography.

Cloning the pilin gene. *B. nodosus* DNA (10 μ g) was digested with *Hind*III restriction endonuclease and fractionated by electrophoresis in a submerged agarose (1%) gel. DNA fragments of 2.0 to 3.5 kbp were isolated using NA45 membrane (Schleicher & Schuell) and ligated with *Hind*III cut and dephosphorylated pBR322. This material was used to transform competent *E. coli* RRI. *E. coli* transformants harbouring the pilin gene were identified by hybridization to 32 P-labelled DNA probe. Plasmid DNA (pPAH107) was extracted from one of the positive clones and purified by CsCl/ethidium bromide density gradient centrifugation (Radloff *et al.*, 1967).

Nucleotide sequence determination. Nucleotide sequence was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977). The 3.1 kbp *Hind*III fragment and the derivative 1.2 kbp and 1.7 kbp *Hind*III-*Nhe*I fragments from pPAH107 were inserted into phage M13 DNA (Messing, 1983) for the preparation of templates. The sequence was determined on both strands and traversed the single *Nhe*I restriction site located within the pilin gene. Oligonucleotides were synthesized either manually (Elleman & Hoyne, 1984) or automatically on an Applied Biosystems 381A DNA synthesizer and were purified by high performance liquid chromatography.

Separation of polypeptide chains of pilin. To determine the cleavage point within the pilin molecule, the polypeptide sub-chains were separated on the basis of their differential solubility under acidic conditions using the following procedure. A solution of Bn351 pili (~ 1 mg ml $^{-1}$) in 0.05 M-sodium phosphate/0.15 M-NaCl, pH 7.5, was adjusted to 1% with respect to trifluoroacetic acid and kept at 4 °C overnight. The solution was centrifuged (10000 g) at 4 °C for 2 min. The pellet, after washing with 1% trifluoroacetic acid, and the supernatant were further analysed.

Analysis of peptides. Amino acid analysis was performed as previously described (McKern *et al.*, 1983). The pellet and the supernatant were digested with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) as described elsewhere (McKern *et al.*, 1988) and the digestion products were separated by reverse phase liquid chromatography using a HiPore TP-318 column. Peptide fragments, purified by this method, were initially characterized by amino acid analysis and those of interest were further characterized by gas phase sequencing (McKern *et al.*, 1988).

RESULTS

Identification and isolation of the pilin gene

Bn351 DNA was digested with the restriction endonucleases *Hind*III, *Eco*RI, *Sph*I, *Pst*I or *Bam*HI and fragments were electrophoretically separated in a submerged agarose (1%) gel. After transfer of the enzymic digests to nitrocellulose and hybridization with radiolabelled DNA corresponding to the pilin gene of Bn265, autoradiography of the nitrocellulose membrane revealed single positive bands of 3.1, 13, 11, 13 and 16 kbp for the respective digests. Fragments of 2.0 to 3.5 kbp from a *Hind*III digest of Bn351 DNA were subsequently ligated to pBR322 and the mixture used to transform *E. coli* RR1. An *E. coli* transformant containing a 3.1 kbp insert in pBR322 was detected using a radiolabelled pilin gene probe.

On completion of DNA sequence studies, radiolabelled DNA probes corresponding to the identified coding sequence of pilin from Bn351 were prepared. Hybridization studies using these probes on Bn351 DNA digested with *Hind*III, *Pst*I, *Eco*RI, and *Bam*HI and fractionated by agarose gel electrophoresis identified only a single band in each digest, thus indicating the presence of a single copy of the pilin gene within the genome of Bn351. This finding is consistent with the identification of a single-copy pilin gene in other *B. nodosus* strains (Elleman *et al.*, 1986a, b; Finney *et al.*, 1988).

Nucleotide sequence of pilin gene

The nucleotide sequence derived from Bn351 (Fig. 1) contains an open reading frame encoding a single chain polypeptide of 160 amino acids and M_r 17150 from the most likely initiation codon of the pilin gene, based on the rules of Stormo *et al.* (1982). A putative promoter has been proposed for the pilin gene of Bn198 (Johnson *et al.*, 1986) based on sequence similarity with promoters from genes which require the *ntxA* gene product for transcription (Ausubel, 1984). A sequence ₂₈₈TTGGCATC. . . ACGCA, almost identical to the putative promoter of Bn198 pilin, is present in the pilin gene of Bn351. A potential transcription termination signal for RNA polymerase in the form of a region of hyphenated dyad symmetry and multiple thymidine residues (Platt, 1986) is present downstream of the coding region (Fig. 1). The potential stem and loop of the terminator is identical for Bn351, Bn340 and Bn265 and possesses a free energy ($\Delta G_{25^\circ\text{C}}$) of -64 kJ mol^{-1} . The nucleotide sequence of the pilin gene shares many common features with the pilin genes of other *B. nodosus* strains and bears greatest overall similarity to Bn340 of serogroup D and Bn265 of serogroup H1. Comparison of nucleotide sequence of the pilin gene from Bn351 with the pilin genes of Bn340 and Bn265 shows 77% and 84% sequence identity respectively within the coding regions. Comparison of sequence from Bn351 with comparable sequences from Bn340 and Bn265 shows that upstream of the pilin coding region Bn351 exhibits 99% and 96% sequence identity respectively while downstream the levels of identity are 99% and 86% respectively (Fig. 2).

Codon usage within the pilin gene of Bn351 corresponds closely with that observed for pilin genes in other strains of *B. nodosus* (Elleman & Hoyne, 1984; Elleman *et al.*, 1986b; Finney *et al.*, 1988). There is a higher occurrence of T relative to C as the third base in the quartet codons and a higher occurrence of A relative to G in the third base position of both duet and quartet codons.

Predicted amino acid sequence of pilin

Comparisons of the predicted amino acid sequence of pilin (Fig. 3) from Bn351 with pilin sequences from Bn340 and Bn265 show that they are identical for the first 57 residues and relatively well conserved up to residue 77. The remainder of the sequences have regions of high variability interspersed with relatively small regions which are identical in all three sequences. The predicted amino acid sequence of pilin from Bn351, in common with all *B. nodosus* prepilins, includes an amino terminal leader sequence of seven amino acid residues (Met. Lys. Ser. Leu. Gln. Lys. Gly) which is absent from the mature polypeptide. The predicted mature pilins from strains Bn351 and Bn265 from the H serogroup have a greater degree of sequence identity (80%) than either of them has with the mature pilin of Bn340 of the D serogroup (69% and 68% respectively).

BN340	GCACCGTAAAAAGCCGCGGATTTCCGTTTGCTCTTCTGCTAACGCTGCTAATAATAAGGCGCGATGCGACATTGAT	
BN351	TTGCGCGTCGCCAAACAATCCGCGCACGGTAAAAAGCCGCGATTTCCGTTTGCTCTTCTGCTAACGCTGCTAATAATAAGGCGCGATGCGACATTGAT	100
BN265	TTGCGCGTCGCCAAACAATCCGCGCACGGTAAAAAGCCGCGGATTTCCGTTTGCTCTTCCGCTAACGCTGCTAATAATAAGGCGCGATGCGACATTGAT	
BN340	TTATCGCGCATATCGTTATTTGCGCGAAAGCGCTGAGACGGGCGCATGTGCCATATATT CATCATTATTCCTTTTTGTGAAACGGGCGTTATTG	
BN351	TTATCGCGCATATCGTTATTTGCGCGAAAGCGCTGAGACGGGCGCATGTGCCATATATT CATCATTATTCCTTTTTGTGAAACGGGCGTTATTG	197
BN265	TTATCGCGCATATCGTTATTTGCGCGAAAGCGCTGAGACGGGCGCATGTGCCATATATTGTCATCATCATTTCCTTTTATAAAACCAACGTTATTG	
BN340	TGCCGAAAAATAGTAGAAAAATGCAAAAAAGCGCGTGTGCCAAAAATAATTTTTAAATTATGTTTTAAATATAAAAAATATGTTGGCATTGA	
BN351	TGCCGAAAAATAGTAGAAAAATGCAAAAAAGCGCGTGTGCCAAAAATAATTTTTAAATTATGTTTTAAATATAAAAAATATGTTGGCATTGA	297
BN265	TGCCGAAAAATGGGAGAAAAATGCAAAAAAGCGCGTGTGCCAAAAATAATTTTTAACTTATTGTTTTAAATATAAAAAATATGTTGGCATTGA	
BN340	TGACGCATAATGAAAGCGTCAGGCAACTGACTCTAAACAAGATGATTTAAATGTTACATTCTTAATAGGAGAATATGATGAAAGTTTACAAAAAG	
BN351	TGACGCATAATGAAAGCGTCAGGCAACTGACTCTAAACAAGATGATTTAAATGTTACATTCTTAATAGGAGAATATGATGAAAGTTTACAAAAAG	397
BN265	TGACGCATAATGAAAGCGTCAGGCAACTGACTCTAAACAAGATGATTTAAATGTTACATTCTTAATAGGAGAATATGATGAAAGTTTACAAAAAG	
BN340	GTTTACCTTAATCGAAGTCATGATTGTAGTTGCAATTATCGGTATCTTAGCTGCAATCGCTATTCACAATACCAAACTACATCGCTCGTTCACAAGT	
BN351	GTTTACCTTAATCGAAGTCATGATTGTAGTTGCAATTATCGGTATCTTAGCTGCAATCGCTATTCACAATACCAAACTACATCGCTCGTTCACAAGT	497
BN265	GTTTACCTTAATCGAAGTCATGATTGTAGTTGCAATTATCGGTATCTTAGCTGCAATCGCTATTCACAATACCAAACTACATCGCTCGTTCACAAGT	
BN340	TAGCCGCGTTATGTCAGAACTGGACAAATGCGCACCGCCATCGAAACTTGCCTTTTGGATGGTAAAGAAGCCGATAAATGCTTCATTGTTGGACTGGT	
BN351	TAGCCGCGTTATGTCAGAACTGGACAAATGCGCACCGCCATCGAAACTTGCCTTTTGGATGGTAAAGAAGCCGATGAGTGCTTTATTGGCTGGACCAAG	597
BN265	TAGCCGCGTTATGTCAGAACTGGACAAATGCGCACCGCCATCGAAACTTGCCTTTTGGATGGTAAAGAAGGAAAGATGCTTCATTGTTGGACCAAG	
BN340	AGTAATTTGTTGGAGCGTGAATTTACTGCTGGTACC GAATCTACAGCAGCAGCAACAGGTCAACAGGAATCACAATTAAGTACCGGTTGCAG	
BN351	AGTAATTTGTTGGAGCTAGCGGTAGTCCAGTAGCTCTAATGACTCAACAGCTGACCACCTGGTCAAGGTGGTTAGTTCATCGACTACAAGCTAGAAG	697
BN265	AGTAATTTATAGCTGCAGCTGGTGGTGAACCTA CTAACAACGCAACAGCTGCAGATCCTGGTCAAGGAGGTTGAATATTACCTACGCACCTGAAT	
BN340	CCGACGATGAGGGAATATTGTTGCAACATTTGCTCGAAATGCTGCAGCGGCTATTAAACCTCAACATTGACATGGTCACGTAGCAAGAAGGTACTTG	
BN351	CTGATGCGACAAATGCTATTACTGCCACATTTGGTCAAAATGCTGCTGCTACACTTCACGGTAAAGCATTAAATGGACACGCGATCCGAAAGCTACTTG	797
BN265	CCACTGCTGAAAAATAGATTGAAGCTACATTTGGTCAGAAATGCTGCCGCTACACTTCATGGTAAAAAATTAACATGGACACGCGAGCCGAAAGCTACTTG	
BN340	GACATGTGCAACAACTGTTGAAGCCAAATTCACCAACTGGTTGTAAAGATGGT AAATAGTTTTCTCTCGCAAAACAATCAAGGTGTACTTC	
BN351	GTCTTGCTCAACAGAGCTTGAAGTCAAAATTCAGACCACTGGCTGTAAAGATGATCTG AAATAGTTTTCTCTCGCAAAACAATCAAGGTGTACTTC	896
BN265	GTCTTGCTCAACAGAGCTTGAAGTCAAAATTCAGACCACTGGCTGTAAAGATGATCTG AAATAGTTTTCTCTCGCAAGGCACTCAAGGTGTACTTC	
BN340	GGTACACCTTTTTTATAATTGTAGCATTTTACCATGTTAAAAAATATTGATTTTTCCGCTTTTCAATTAAAGTGATTGCCATCATTGCGATGAGCCTC	
BN351	GGTACACCTTTTTTATAATTGTAGCATTTTACCATGTTAAAAAATATTGATTTTTCCGCTTTTCAATTAAAGTGATTGCCATCATTGCGATGAGCCTC	996
BN265	GGTACACCTTTTTTATAAT GTTTTATCATGTCAAGAAATATCGATTTTTCCGCTTTTCAATTAAAGTGATTGCCATCATTGCGATGAGCTC	
BN340	GATCATTCCGCTTTGCTTTTCTTCTGATGGTAGCTGGCGC	
BN351	GATCATTCCGCTTTGCTTTTCTTCTGATGGTAGCTGGCGC	1038
BN265	GATCATTTTGCTTTACTTTTCTTCTGATGGTAGCTGGCGC	

Fig. 1. Comparison of the nucleotide sequence of the pilin gene and flanking regions of Bn351 (H2) with the equivalent regions of Bn265 (H1) and Bn340 (D). Sequences were aligned using the Needleman-Wunsch algorithm (Needleman & Wunsch, 1970) with a unitary matrix and a gap penalty of 2. Vertical lines indicate non-identical bases in adjacent sequences. Putative transcription initiation and termination signals are indicated by broken lines. Translation initiation and termination codons are indicated by solid lines.

Fig. 2. Strain relationships based on percentage sequence identity between Bn351, Bn340 and Bn265 in upstream (top), coding (centre) and downstream (bottom) regions of the pilin genes presented in Fig. 1 (residues 23–378, 379–858 and 859–1038 respectively). Percentage amino acid sequence identities between pilins from these strains are shown in parentheses.

Bn340	Met Lys Ser Leu Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala	1
Bn351	Met Lys Ser Leu Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala	18
Bn265	Met Lys Ser Leu Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Gly Ile Leu Ala Ala	
Bn340	Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln Met	
Bn351	Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln Met	43
Bn265	Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val Ser Arg Val Met Ser Glu Thr Gly Gln Met	
Bn340	Arg Thr Ala Ile Glu Thr Cys Val Leu Asp Gly Lys Glu Ala Asp Lys Cys Phe Ile Gly Trp Thr Gly Ser Asn	
Bn351	Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Lys Ala Asp Glu Cys Phe Ile Gly Trp Thr Lys Ser Asn	68
Bn265	Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp Gly Lys Glu Gly Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn	
Bn340	Leu Leu Asp Gly Glu Phe Thr Ala Gly Thr Glu Ser Thr Ala Ala Ala Thr Gly Gln Thr Gly Ile Thr	
Bn351	Leu Leu Gly Ala Ser Gly Ser Pro Ser Ser Ser Asn Asp Ser Thr Ala Asp His Pro Gly Gln Gly Gly Leu Val	93
Bn265	Leu Leu Ala Ala Ala Gly Gly Ser Thr Thr Asn Asn Ala Thr Ala Ala Asp Pro Gly Gln Gly Gly Leu Asn	
Bn340	Ile Lys Tyr Pro Val Ala Ala Asp Asp Glu Gly Asn Ile Val Ala Thr Phe Gly Arg Asn Ala Ala Ala Ala Ile	
Bn351	Ile Asp Tyr Lys Leu Glu Ala Asp Ala Thr Asn Ala Ile Thr Ala Thr Phe Gly Gln Asn Ala Ala Ala Thr Leu	118
Bn265	Ile Thr Tyr Ala Leu Glu Ser Thr Ala Glu Asn Lys Ile Glu Ala Thr Phe Gly Gln Asn Ala Ala Ala Thr Leu	
Bn340	Lys Pro Gln Thr Leu Thr Trp Ser Arg Ser Lys Glu Gly Thr Trp Thr Cys Ala Thr Thr Val Glu Ala Lys Phe	
Bn351	His Gly Lys Ala Leu Lys Trp Thr Arg Asp Pro Lys Ala Thr Trp Ser Cys Ser Thr Asp Val Glu Leu Lys Phe	143
Bn265	His Gly Lys Lys Leu Thr Trp Thr Arg Ser Pro Glu Ala Thr Trp Ser Cys Ser Thr Asp Val Asp Glu Lys Phe	
Bn340	Gln Pro Thr Gly Cys Lys Asp Gly Lys	
Bn351	Arg Pro Thr Gly Cys Lys Asp Asp Leu Lys	153
Bn265	Lys Pro Thr Gly Cys Lys Lys	

Fig. 3. Comparison of the predicted amino acid sequence of pilin from *B. nodosus* 351 (H2) with pilins from strains 265 (H1) and 340 (D). Sequences were aligned using the Needleman–Wunsch algorithm (Needleman & Wunsch, 1970) with a unitary matrix and a gap penalty of 2. Amino acids which differ in adjacent sequences are marked by a vertical line. Sequence numbering is relative to the first amino acid of Bn351 mature pilin. Arrowheads indicate the cleavage sites in pilins from Bn351 and Bn265.

Fig. 4. SDS-PAGE (15% acrylamide) of pilin polypeptides from *B. nodosus* strains 351 and 265, stained with Coomassie brilliant blue R250. Incubation buffer contained 2-mercaptoethanol (Laemmli, 1970) unless otherwise stated. Lanes: 1, trifluoroacetic-acid-supernatant fraction of pilin from Bn351; 2, pili from Bn351; 3, pili from Bn351, without 2-mercaptoethanol; 4, pili from Bn265; 5, M_r markers.

Separation of polypeptide chains

SDS-PAGE of strain 351 pili indicates that the pilin subunit is composed of two polypeptide chains (Fig. 4). The pilus preparation also contains contaminating membrane proteins (M_r 30000–80000) which have not been removed by repeated precipitation of pili with 0.1 M-MgCl₂. The supernatant material obtained following overnight treatment with 1% trifluoroacetic acid was found to consist almost exclusively of the slower-migrating subunit band of Bn351 pilin (Fig. 4). Further characterization of this material by amino acid analysis (peptide 1, Table 1) showed that it corresponded to the carboxy-terminal portion of the protein encoded by the DNA sequence, beginning near residue 73 of the mature pilin sequence. The occurrence of glycine, alanine and serine residues around this position (residues 71–75) precluded the precise determination of the cleavage position by amino acid analysis, since these amino acids are the most abundant 'background' residues encountered in amino acid analysis.

The predicted amino acid sequence of Bn351 pilin indicates that there are four cysteine residues in the molecule. Since the two polypeptide chains can be separated by differential solubility in 1% trifluoroacetic acid or by electrophoresis under non-reducing conditions (Fig. 4), covalent links must be absent between these polypeptide chains. The half-cystine residues of pilin from Bn265 are linked to form two intra-chain disulphide bonds (Elleman, 1988) and by analogy, the half-cystine residues of pilin from Bn351 are presumably similarly linked.

Amino acid sequence studies

A single amino acid sequence, mePhe.Thr.Leu.Ile.Glu. . . , corresponding to the amino-terminus found universally for *B. nodosus* pilins, was identified by amino-terminal sequence analysis of intact Bn351 pilus. Amino-terminal sequence analysis of the supernatant obtained following treatment of Bn351 pili with 1% trifluoroacetic acid failed to give an amino acid sequence. These data indicate that the amino terminus of the second polypeptide chain is blocked, rendering it inaccessible to amino-terminal sequencing.

Table 1. Amino acid composition of pilin peptides from strain 351 of *B. nodosus*

Peptide 1 is the carboxy-terminal polypeptide of pilin from Bn351; peptide 2 is the peptide isolated from tryptic digestion products of the amino-terminal polypeptide of pilin from Bn351; peptide 3 is the peptide isolated from the tryptic digestion products of the carboxy-terminal polypeptide of pilin from Bn351.

Amino acid	Number of residues per peptide molecule					
	Peptide 1		Peptide 2		Peptide 3	
	Found*	Predicted† (73–153)	Found*	Predicted† (67–72)	Found*	Predicted† (73–97)
Half-Cys	–	2	0	0	0	0
Asx	10.5	11	1.2	1	4.0	4
Thr	9.0‡	9	0	0	1.0‡	1
Ser	7.7‡	8	1.1‡	1	5.7‡	6
Glx	4.6	4	0	0	1.1	1
Pro	4.3	4	0	0	2.0	2
Gly	7.2	7	0.8	1	4.4	4
Ala	9.8	10	0.9	1	1.0	1
Val	1.8	2	0	0	0.8	1
Met	0.1	0	0.1	0	0	0
Ile	1.7	2	0	0	0.7	1
Leu	6.1	6	2.2	2	1.0	1
Tyr	1.0	1	0	0	1.0	1
Phe	2.0	2	0	0	0	0
Lys	6.9	7	0	0	1.1	1
His	1.9	2	0	0	1.0	1
Trp	–	2	–	0	–	0
Arg	2.0	2	0	0	0	0

–, Not quantified.

* Following hydrolysis in constant boiling HCl for 20 at 110 °C.

† Amino acid residues predicted from the pilin gene coding region.

‡ Values adjusted to compensate for losses during hydrolysis.

Following tryptic digestion of the resolubilized pellet obtained by treatment of pilin with trifluoroacetic acid, a number of peptide fragments were purified by high pressure liquid chromatography. With one exception these corresponded to peptides expected from trypsin digestion, each containing a C-terminal lysine or arginine residue. The amino acid composition of the exceptional peptide (peptide 2, Table 1) corresponded to that of the predicted sequence from residues 67–72. The absence of lysine or arginine indicated that this peptide constituted the carboxy terminus of the amino-terminal pilin subunit. The sequence of this peptide was confirmed by gas phase sequence analysis. Amino acid analysis of a tryptic peptide isolated from the supernatant fraction showed that it corresponded to residues 73–97 of the predicted mature pilin sequence (peptide 3, Table 1). Together these peptide compositions and the sequence analysis demonstrate that the cleavage point occurs between residues 72 and 73 of the mature pilin sequence, resulting in predicted polypeptides of calculated M_r values of 7200 (N-terminal polypeptide) and 9200 (C-terminal polypeptide). The discrepancy between the latter value and that observed on SDS-PAGE (Fig. 4) is similar to that previously observed with the C-terminal polypeptide of pilin from Bn265 and may reflect the unreliability of gel-derived M_r estimates for proteins of M_r less than $\sim 15\,000$ (Elleman *et al.*, 1986b).

DISCUSSION

The nucleotide sequence determined for the pilin gene of Bn351 encodes a single polypeptide of M_r 17150 which, after removal of the seven-amino-acid leader sequence and methylation of the new amino-terminal residue, would give a mature pilin of M_r 16392. However, SDS-PAGE of pili isolated from Bn351 shows two distinct bands as a result of cleavage of the pilin subunit.

The site of the peptide bond cleavage within the molecule is between $_{72}$ Ala and $_{73}$ Ser of mature pilin. This position is identical to the position of cleavage determined for pilin from Bn265, a representative of serogroup H1, in which cleavage occurs between $_{72}$ Ala and $_{73}$ Ala. The agents responsible for the breakage of the peptide bond and amino-terminal blocking have not been determined for either strain. The cleavage is probably not a result of the isolation procedure since Bn265 pili produced in *Pseudomonas aeruginosa* (Elleman & Stewart, 1988) and isolated by the same procedure as used in this study, are composed of uncleaved pilin subunits. A probable explanation of the cleavage, given the production of a number of secreted proteases by *B. nodosus* isolates (Kortt *et al.*, 1986), is an extracellular protease acting on susceptible sites within the intact pilus.

Known pilin sequences from *B. nodosus* isolates clearly fall into two distinct sets, with members of a set possessing greater sequence similarity to each other than to any member of the other set. An obvious feature which distinguishes members of the two sets is the number and position of cysteine residues. The A-set pilins, comprising pilins from serogroups A, B, C, E, F, G and probably I, possess a single, centrally-located disulphide bridge, whereas D-set pilins, from serogroups D and H, have a carboxy-terminal-located disulphide bridge in addition to a more centrally located bridge. Aligned amino acid sequences of pilins from the two sets show greater than 59% sequence identity for members of the same set, but less than 39% identity for members of different sets. On the basis of sequence comparisons and the position and number of cysteine residues, pilin from strain 351 clearly is a member of the D-set.

Comparison of pilin polypeptides from the three *B. nodosus* strains 351, 340 and 265 indicates that they are closely related, with Bn351 and Bn265 sharing the closest relationship (Fig. 2). The amino-terminal regions of the three sequences are almost identical, suggesting that the important antigenic determinants are located in the carboxy-terminal regions of the molecules. The relative relationships displayed between Bn351, Bn340 and Bn265 as judged by comparison of both the amino acid sequences of pilins and the nucleotide sequences of the coding regions of the pilin genes is not reflected in comparisons of sequence flanking the coding regions. In particular, the pilins of Bn351 and Bn265 appear to be the most closely related, whereas in the regions flanking the pilin coding region, Bn351 and Bn340 exhibit the closer relationship. This disparity may indicate the occurrence of genetic rearrangements in ancestral strains, possibly through homologous recombination.

In *Neisseria gonorrhoeae*, antigenic variation of pilin has been linked to the existence of silent pilin loci with the expression of new antigenic forms facilitated by intrachromosomal recombination between these silent loci and the active expression locus (Kooimey *et al.*, 1987). However *B. nodosus*, like *P. aeruginosa* (Strom & Lory, 1986), possesses only a single detectable pilin and therefore *B. nodosus* pilin must rely on a different mechanism to generate antigenic change. Antigenic diversity among *B. nodosus* pilins could result from point mutations, insertions and deletions within the pilin gene, although, as with *P. aeruginosa* (Johnson *et al.*, 1986), the possibility of intrachromosomal recombination between regions of low sequence identity cannot be excluded.

Pilin of Bn351 (serogroup H2) has 80% sequence identity to pilin from Bn265 (serogroup H1), which is greater than the identity (69%) of Bn265 to Bn340 (serogroup D). However, the level of sequence identity between pilins from Bn265 (H1) and Bn351 (H2) is considerably lower than has been found between pilins of strains from different subgroups within a serogroup. For example, comparison of pilin sequences from Bn198 (A1) and Bn286 (A2), strains representative of the two subgroups of serogroup A, indicates 95% identity (P. A. Hoyne, unpublished data), while comparison of four sequences from strains representing the four subgroups of serogroup B reveals similarly high levels of identity between any two sequences (T. C. Elleman, unpublished data). In addition, the level of identity between the predicted amino acid sequences of pilins from Bn265 (H1) and Bn351 (H2) is lower than that found between pilins of some strains belonging to different serogroups. For example, comparison of amino acid sequences of Bn216 (serogroup E) and Bn1017 (serogroup F) indicates 81% identity. These observations suggest that Bn351 and Bn265 differ sufficiently from each other to preclude their assignment to the same serogroup. Recent serological studies support this conclusion and fail to classify Bn351 as a

member of serogroup H (S. E. J. Thorley, personal communication). Using the alternative classification system of Day *et al.* (1986), Bn265 and Bn351 are classified as different serotypes; Bn265 is classified as serotype H whereas Bn351 is classified as serotype O (S. E. J. Thorley, personal communication). Furthermore, vaccination trials precipitated by our sequence observations have shown that pili from Bn351 fail to protect sheep from footrot when challenged with Bn265 (D. J. Stewart, unpublished data). To accommodate Bn351 into the scheme of Claxton *et al.* (1983) would require extension of the system by addition of a new serogroup having Bn351 as its prototype strain.

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