Isolation of the gene encoding pilin of *Bacteroides nodosus* (strain 198), the causal organism of ovine footrot

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The gene for pilin, the monomeric protein subunit from which the pilus of *Bacteroides nodosus* is constructed, has been isolated. Isolation was achieved by cloning the fragmented genome of *B. nodosus* in *Escherichia coli* RR1 using the plasmid vector pBR322. Pilin-producing colonies were identified by screening with a colony immunoassay using antiserum from a sheep immunized against purified pili from *B. nodosus* strain 198, and were further characterized by immunoblot analysis. Final confirmation of the presence of the pilin gene was by nucleotide sequence data which translated to the known pilin amino acid sequence.

Pilin Bacteroides nodosus *Footrot* *Cloning*

1. INTRODUCTION

Pili are filamentous surface appendages occurring in many genera of Gram-negative bacteria. Bacterial species as diverse as *Neisseria gonorrhoeae, Pseudomonas aeruginosa, Moraxella nonliquefaciens* and *Bacteroides nodosus*, show a high degree of similarity in pilus structure [1–4]. Each pilus is composed of identical monomeric subunits of the protein pilin of M_r ~18000, the NH_2-terminal amino acid sequence of which is highly conserved between the genera. Vaccines prepared from purified pili have been shown to provide prophylaxis against infection. In the case of *N. gonorrhoeae*, human volunteers were protected against gonorrhoea from urethral challenge [5], while sheep vaccinated with pili from *B. nodosus* were protected against infection with footrot incurred from strains of the homologous serogroup [6–8].

Current commercial vaccines against footrot contain piliated bacteria from each of 8 major serogroups of *B. nodosus* [9]. This vaccine is relatively expensive due to the inclusion of such a large number of serogroups of *B. nodosus*, as well as the fastidious growth requirements, relatively sparse growth and variable pilus expression of this anaerobe when grown in bulk liquid culture. As an approach to reduce the cost of vaccination we are attempting to produce the pilin protein by recombinant DNA techniques. This paper reports the isolation, by cloning, of the gene encoding the pilin protein of *B. nodosus* strain 198.

Abbreviations: PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair

2. EXPERIMENTAL

2.1. *Preparation of DNA from* B. nodosus

B. nodosus (strain 198) was harvested from modified Eugon broth [9] after anaerobic growth for up to 2 days at 37°C. Cells from a 1-l culture were washed in 0.15 M NaCl, 0.1 M EDTA (pH 8.0), and resuspended in 4 ml of 25% sucrose in 50 mM Tris–Cl, 10 mM EDTA (pH 8.0). Lysozyme (1 ml of 10 mg/ml), EDTA (1.25 ml of 0.25 M, pH 8.0) and ribonuclease (66 μl of 10 mg/ml, heat-treated) were added with incubation of 0°C for 30 min. Proteinase K (1 mg) was

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added, and cell lysis was brought about by the rapid addition with mixing of SDS (2 ml of 2%). After 15 min at 0°C, the mixture was incubated overnight at 37°C. The lysate was extracted for 40 min at 0°C with an equal volume of neutralized phenol by rotating the emulsion at 60 rpm. To the separated aqueous phase, two vols ethanol at −20°C were gently added; DNA was removed by spooling as it precipitated at the interface. The precipitate was washed in 70% ethanol, air-dried, and dissolved in 1 ml of 10 mM Tris–Cl, 1 mM EDTA (pH 8.0). The DNA preparation was banded in CsCl/ethidium bromide by equilibrium density gradient centrifugation [10].

2.2. *Construction of a clone bank from* B. nodosus

DNA was digested for 1 h with sufficient *Sau*3AI to produce the maximum number of 10 kbp fragments as judged by electrophoresis on 1% agarose gel. To ensure a representative distribution of the genome within this size range, digestion times of 1 and 2 h were also used and the total mixture was size-fractionated on a 10–40% neutral sucrose gradient for fragment sizes of 7–15 kbp [11].

DNA fragments (15 μg/ml) were ligated to *Bam*HI-cleaved, dephosphorylated pBR322 (5 μg/ml) and the mixture was used to transform *E. coli* strain RR1 [12]. Recombinant clones sensitive to tetracycline were grown to saturation in the presence of 50 μg/ml ampicillin in individual wells of microtitre plates in a medium which permitted free storage of cells without further additions [13]. These cultures were stored frozen at −70°C and could be thawed and refrozen several times. Approx. 1500 colonies were screened by the immunoassay.

2.3. *Screening of recombinant clones*

Colonies grown overnight on YT agar plates [14] containing 50 μg/ml ampicillin were transferred by 'lifting' to nitrocellulose filters. Following cell lysis [15] colonies were screened for the presence of pilin by sheep IgG1 anti-pili (strain 198) antiserum, followed by rabbit IgG anti-sheep IgG antiserum (Nordic, Tilburg, The Netherlands) and finally 10 μCi ^{125}I-labelled protein A from *S. aureus* (Amersham). Positive clones from this assay were further analysed by immunoblotting [16]. These clones together with a pili standard from *B. nodosus* strain 198 were subjected to SDS–PAGE in 12.5% acrylamide under reducing conditions using the discontinuous buffer system in [17]. Proteins were electrophoretically transferred to nitrocellulose and the membrane probed with the antisera described above.

2.4. *Preparation of plasmids from* E. coli

Plasmids were prepared from clone bank cultures grown to saturation overnight in YT media containing 50 μg/ml ampicillin. Harvested cells were resuspended in 25% sucrose (3% of the growth volume), and lysozyme–EDTA spheroplasts were prepared [18]. Cell lysis was accomplished by an equal volume of 0.1% Triton X-100. Plasmid was isolated from the cleared lysate either by CsCl/ethidium bromide equilibrium density gradient centrifugation [11], or by phenol extraction followed by 3 ethanol precipitations [19] – the second precipitation in the presence of ammonium acetate [20] to improve restriction of the DNA.

2.5. *Subcloning and nucleic acid sequence determination*

Restriction endonuclease digests of plasmids were fractionated by electrophoresis in submerged, horizontal agarose gels (1% agarose in 0.04 M Tris–acetate buffer (pH 8.3) containing 20 mM sodium acetate, 1.0 mM EDTA and 0.5 μg/ml ethidium bromide). Fragment sizes were determined relative to a *Hin*dIII/*Eco*RI digest of phage λ.

DNA was isolated from the gel by electrophoresis onto DEAE membrane slotted in front of the band [21]. Fragments were ligated into pBR322 and used to transform *E. coli* strain RR1. Certain fragments containing sufficient pBR322 vector for replication and antibiotic selection were self-ligated. For sequence studies fragments were ligated into M13mp8 [22] to prepare single-stranded template for the dideoxy chain-termination method of sequence determination [23].

2.6. *Fractionation of cellular components of* E. coli

The cellular location of pilin expressed in *E. coli* was determined by separating the cellular com-

ponents into periplasmic, cytoplasmic and membrane fractions [24]. Lysozyme–EDTA spheroplasts were prepared and lysed by osmotic shock. The membrane pellet was further fractionated by equilibrium density gradient centrifugation in 30–50% sucrose. Separated cellular components were assayed for pilin by immunoblotting.

3. RESULTS

DNA prepared from *B. nodosus* strain 198 showed only a single band of linear chromosomal DNA in CsCl/ethidium bromide gradients. Screening of 1500 colonies from the clone bank prepared from this DNA yielded 12 positive clones as judged by the colony immunoassay. Eight produced a protein product which had an electrophoretic mobility corresponding to pilin on immunoblotting, while the other 4 produced proteins of a lower M_r. The recombinant plasmids from the probable pilin-producing colonies were analysed by restriction endonuclease digestion (*Hin*dIII, *Eco*RI, *Bam*HI and *Pst*I in various combinations) and were found to produce band patterns having several bands in common. Analysis of the restriction maps deduced from these digestions (fig.1) indicated a common region of 6.5 kbp in the inserts. This region was present in both orientations relative to pBR322 vector.

Clone 7A1 (fig.1) was selected for further study because of a favourable distribution of restriction sites relative to the pBR322 vector. Plasmid isolated from clone 7A1 was cleaved by *Hin*dIII or *Eco*RI digestion. The largest fragment of either digest still contained the origin of replication and β-lactamase gene of pBR322. Recircularisation of these fragments produced a plasmid which still transformed *E. coli* to pilin production. The plasmid derived from clone 7A1 by *Eco*RI digestion contained only a 2.9 kbp insert in pBR322 (see clone 7A1E of fig.1). Further subcloning of this region by isolation of the 1.8 kbp *Pst*I/*Eco*RI fragment and its insertion into pBR322 in place of the smaller *Pst*I/*Eco*RI fragment of this vector produced a plasmid which failed to transform *E. coli* to pilin production (see clone 7A1E/P of fig.1). This same 1.8 kbp fragment was 'force cloned' into M13mp8 for sequence determination.

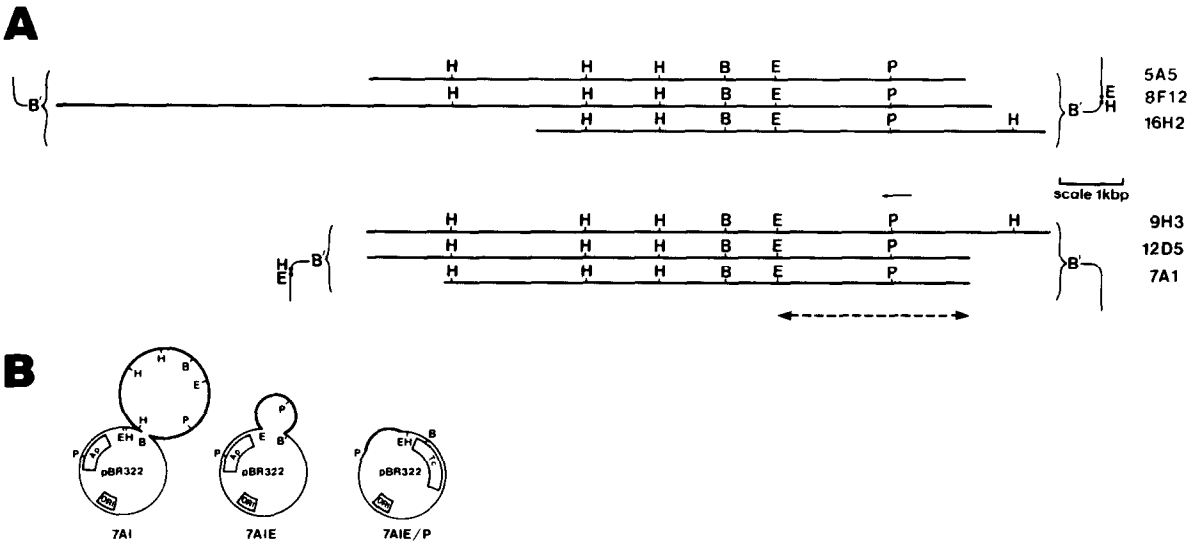

Fig.1. (A) Restriction endonuclease cleavage maps of plasmids prepared from 6 pilin-producing clones of *E. coli* (7A1, 12D5, 9H3, 16H2, 8F12, 5A5). The fragmented genome of *B. nodosus* was cloned into the *Bam*HI site (B') of pBR322 vector. The 'arms' of the pBR322 vector are shown outside the brackets. The restriction sites marked are *Eco*RI(E), *Bam*HI(B), *Hin*dIII(H) and *Pst*I(P). The insert region (2.9 kbp) of the plasmid from a pilin-producing subclone derived from clone 7A1 is also shown (◄---►). The small arrow indicates both the length of the coding sequence for mature pilin protein and the direction of transcription. (B) Plasmid from clone 7A1 together with plasmids from subclones 7A1E and 7A1E/P. Insert regions are drawn to half-scale relative to pBR322 vector.

Sequence information confirmed that the coded product was pilin (fig.2), and that *Pst*I cuts within the coding sequence of the pilin gene.

Preliminary studies on the cellular location of the pilin produced by *E. coli* revealed that it is absent from the supernatant, the periplasmic space and the cytoplasm, the fractionates on sucrose density gradients with the membranous preparations.

4. DISCUSSION

The pilin gene of *B. nodosus* strain 198 has been cloned and expressed in *E. coli*. The expression of the gene when inserted in either orientation into the *Bam*HI site of pBR322 indicated that expression was from a cloned *B. nodosus* promoter. Subclones from clone 7A1, retaining the ability to express pilin, localised the gene to a 2.9 kbp fragment of DNA (fig.1). A nucleotide sequence which translated to the known pilin amino acid sequence [4] was found within this fragment, so confirming the presence of the pilin gene and pinpointing the coding region. Thus the GCA sequence of the single *Pst*I site (CTGCAG) within the plasmid insert of clone 7A1 codes for alanine residue 108 of the mature pilin protein, and the presence of coding sequence for the C-terminus of the protein within the 1.8 kbp *Pst*I/*Eco*RI fragment (fig.2) indicates that transcription must proceed downstream towards the single *Eco*RI site of the insert.

The gene for pilin production is, then, located in the *B. nodosus* genome of strain 198 between two *Hin*dIII sites 5.5 kbp apart and within the bounds of a 3.7 kbp *Hin*dIII–*Eco*RI fragment. A single *Pst*I site in the centre of this 3.7 kbp fragment is located within the protein coding sequence. Expression of pilin from the coding sequence depends on a sequence less than 1.1 kbp upstream of this *Pst*I site from a promoter recognized by the RNA polymerase of *E. coli*.

The production of pilin from *B. nodosus* by a host having less fastidious growth requirements provides encouragement for the development of a 'recombinant DNA'-produced vaccine to replace the conventional whole-cell *B. nodosus* vaccine. The latter is both expensive to manufacture, and causes vaccinal lesions at the site of inoculation through administration of a large amount of extraneous antigen in the oil adjuvant [25]. A recombinant DNA-produced vaccine could lower manufacturing costs, reduce vaccination reactions, and circumvent the problem of variable pilus expression in bulk liquid culture.

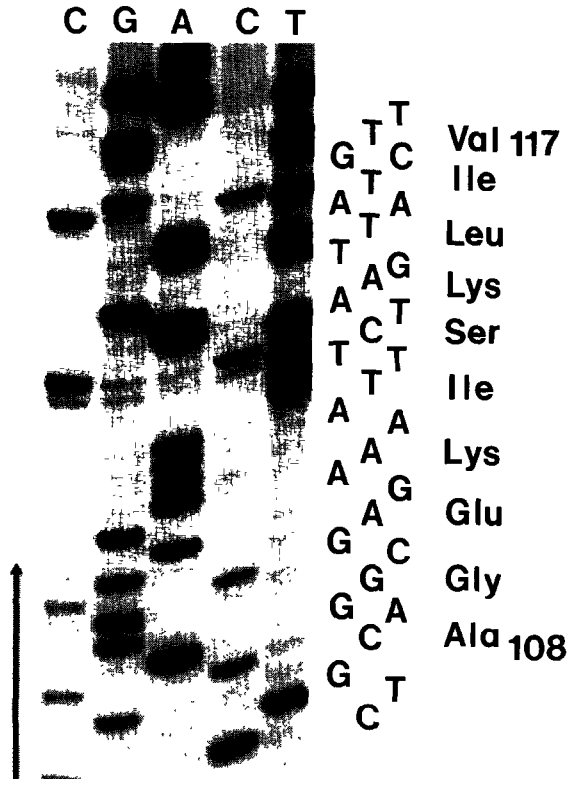

Fig.2. Autoradiograph of a portion of a sequencing gel for the 1.8 kbp *Pst*I/*Eco*RI fragment cloned into M13mp8. The sequence is read from the *Pst*I site towards the *Eco*RI site in the direction indicated by the arrow. The amino acid sequence corresponding to the nucleic acid sequence data is shown, together with the positions of these amino acid residues in the pilin sequence.

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REFERENCES

[1] Hermodson, M.A., Chen, K.C.S. and Buchanan, T.M. (1978) Biochemistry 17, 442–445.

[2] Sastry, P.A., Pearlstone, J.R., Smillie, L.B. and Paranchych, W. (1983) FEBS Lett. 151, 253–256.

[3] Frøholm, L.O. and Sletten, K. (1977) FEBS Lett. 73, 29–32.

[4] McKern, N.M., O'Donnell, I.J., Inglis, A.S., Stewart, D.J. and Clark, B.L. (1983) FEBS Lett. 164, 149–153.

[5] Brinton, C.C. jr, Wood, S.W., Brown, A., Labik, A.M., Bryan, J.R., Lee, S.W., Polen, S.E., Tramont, E.C., Sandoff, J. and Zollinger, W. (1982) in: Bacterial Vaccines (Robbins, J.B., Hill, J.C. and Sandoff, J.C. eds) Seminars in Infectious Disease, vol.4, pp.140–159, Thieme-Stratton, New York.

[6] Stewart, D.J. (1978) Res. Vet. Sci. 24, 14–19.

[7] Every, D. and Skerman, T.M. (1982) N.Z. Vet. J. 30, 156–158.

[8] Stewart, D.J., Clark, B.L., Peterson, J.E., Griffiths, D.A. and Smith, E.F. (1982) Res. Vet. Sci. 32, 140–147.

[9] Claxton, P.D., Ribeiro, L.A. and Egerton, J.R. (1983) Aust. Vet. J. 60, 331–334.

[10] Radloff, R., Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci. USA 57, 1514–1521.

[11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning, pp.282–285, Cold Spring Harbour Laboratory, NY.

[12] Dagert, M. and Ehrlich, S.D. (1979) Gene 6, 23–27.

[13] Gergen, J.P., Stern, R.H. and Wensink, P.C. (1979) Nucleic Acids Res. 7, 2115–2136.

[14] Miller, J. (1972) in: Experiments in Molecular Genetics, p.431–435, Cold Spring Harbour Laboratory, NY.

[15] Kemp, D.J., Coppel, R.L., Cowman, A.F., Saint, R.B., Brown, G.V. and Anders, R.F. (1983) Proc. Natl. Acad. Sci. USA 80, 3787–3791.

[16] O'Donnell, I.J., Stewart, D.J. and Clark, B.L. (1983) Aust. J. Biol. Sci. 36, 15–20.

[17] Laemmli, U.K. (1970) Nature 227, 680–685.

[18] Kupersztoch, Y.M. and Helinski, D.R. (1973) Biochem. Biophys. Res. Commun. 54, 1451–1459.

[19] Hepburn, A.G. and Hindley, J. (1979) J. Biochem. Biophys. Methods 1, 299–308.

[20] Maxam, A.M. and Gilbert, W. (1980) in: Methods in Enzymology (Grossman, L. and Moldare, K. eds) vol.65, pp.499–560, Academic Press, New York.

[21] Elleman, T.C., Hoyne, P.A., Dyall-Smith, M.L., Holmes, I.H. and Azad, A.A. (1983) Nucleic Acids Res. 11, 4689–4701.

[22] Messing, J. (1983) Methods Enzymol. 101, 20–78.

[23] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.

[24] Osborn, M.J. and Munson, R. (1974) Methods Enzymol. 31, 642–653.

[25] Stewart, D.J., Clark, B.L., Emery, D.L., Peterson, J.E. and Fahey, K.J. (1983) Aust. Vet. J. 60, 83–84.