Purification of Pili from *Bacteroides nodosus* and an Examination of their Chemical, Physical and Serological Properties

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Pili from *Bacteroides nodosus* were purified to greater than 99% homogeneity by precipitation at pH 4.0 and in MgCl_2 followed by chromatography on BioGel A150. The pili were composed entirely of one type of polypeptide subunit, pilin. No carbohydrates, nucleic acid, lipid, lipopolysaccharide or phosphate could be detected in purified pili preparations. The molecular weight of pilin from *B. nodosus* strains 91B and 198 was 18 400 and from strain 80 was 19 300. The isoelectric points of pili from *B. nodosus* strains 91B and 80 were both 4.5. The buoyant densities of pili from strains 91B, 80 and 198 were 1.287, 1.284 and 1.286 g ml^{-1} , respectively. The three strains of *B. nodosus* did not cross-react in K-agglutination tests and produced pili which did not cross-react in immunodiffusion tests. Antiserum to highly purified pili caused a characteristic K-type agglutination reaction. It was concluded that pili are the K-agglutinin.

INTRODUCTION

Large numbers of pili have been observed on the surface of *Bacteroides nodosus* on primary isolation from sheep affected with foot rot (Short *et al.*, 1976; Cooper, 1977). In serial subcultures of these isolates, however, bacteria bearing only low numbers of pili may appear and it has been shown that these organisms, when compared with highly piliated *B. nodosus*, form colonies of different morphology (Stewart, 1975*a, b*; Short *et al.*, 1976; Thorley, 1976), have low virulence for sheep and do not protect inoculated animals from foot rot infection (Stewart, 1975*a, b*).

Stewart (1973) and Walker *et al.* (1973) reported that sera from sheep inoculated with partially purified preparations of *B. nodosus* pili gave the typical K-type agglutination with whole bacteria as described by Egerton (1973); moreover, such sheep were resistant to experimental foot rot challenge (Stewart, 1975*a, b*, 1978*a*). Using immuno-electron microscopy, Walker *et al.* (1973) demonstrated that K-type agglutination may be caused by antibodies binding pili together and suggested that in vaccinated animals, anti-pili antibodies penetrating to the site of the hoof lesion might immobilize *B. nodosus* organisms and so prevent spread of the epidermal infection.

For further investigations of the relationships between pili, the K-agglutinin and the antigens associated with immunity, preparations free from any other cellular components were desirable. This paper describes the purification of *B. nodosus* pili to greater than 99% homogeneity and reports their chemical, physical and serological properties.

METHODS

Bacterial strains and growth conditions. The *Bacteroides nodosus* strains studied included two New Zealand isolates (strains 91B and 80) and one from Australia (strain 198). The organisms were grown anaerobically in TAS liquid medium as described by Skerman (1975). The number of subcultures was kept to a minimum to preclude selection of variant organisms which produce low yields of pili (Stewart, 1973; Short *et al.*, 1976).

Electron microscopy. Bacteria or isolated pili were suspended in drops of saline (0.15 M-NaCl) on Parafilm (American Can Company, Dixie/Marathon, Greenwich, U.S.A.) upon which collodion-coated grids were floated for 1 min; the grids were then transferred to drops of 0.5% (w/v) uranyl acetate in water at pH 4.5 for 4 min. Grids dried with blotting paper were examined in a Philips EM 201C transmission electron microscope and isolated pili were graded quantitatively as follows: (+), pili just detectable on the grid; +, low numbers of widely separated pili; 2+, many pili, only just separated; 3+, light mat of pili; 4+, dense mat of pili, obscuring almost all the grid.

Isolation and partial purification of pili. Bacteria from cultures in the late-exponential phase of growth were harvested in a continuous flow centrifuge. More pili were free in the growth medium than were attached to bacteria.

To remove attached pili, bacteria in the sediment were resuspended in ice-cold 0.01 M-phosphate buffer, pH 7.2, containing 0.147 M-NaCl (phosphate-buffered saline) and homogenized at full speed in a Sorval Omnimixer for 30 s while in an ice bath. Homogenization was repeated four times with cooling intervals between. The depiliated organisms and debris were removed by three centrifugations at 12000 *g* for 10 min.

Unattached pili were removed from the culture supernatant by overnight precipitation at pH 4.0 followed by continuous flow centrifugation at 12000 *g*.

The pili removed directly from bacteria (bacteria-derived pili) and those derived from the culture medium (medium-derived pili) were then separately purified by the same procedure. The initial purification steps were as described by Brinton (1965): three precipitations at pH 4.0 followed by repeated precipitations with 0.1 M-MgCl₂.

Gel filtration chromatography. The pellet from the final MgCl₂ precipitation was dispersed in 5 ml 25 mM-boric acid buffer, pH 8.5, containing 2 mM-EDTA, 0.5 M-KCl, 0.5% (w/v) non-ionic detergent Lubrol WX (Sigma) and 0.02% (w/v) NaN₃, and incubated at 37 °C for 40 min. The sample was run into the top of a 2.6 × 100 cm column connected in series with a 2.6 × 40 cm column both containing BioGel A150 (Bio-Rad Laboratories). The sample was eluted downwards at 14 ml h⁻¹ with 25 mM-boric acid buffer, pH 8.5, containing 2 mM-EDTA, 0.5 M-KCl and 0.02% (w/v) NaN₃. The fractions (7 ml) collected from the column were examined for pili in the electron microscope. The ultraviolet (u.v.) absorption spectra and lipopolysaccharide (LPS) content were also determined. Fractions containing pili free of LPS were pooled and concentrated by precipitation at pH 4.0.

Isoelectric focusing. Isoelectric focusing of pili preparations was carried out in a LKB ampholine column (type 8101) at 4 °C. A sucrose gradient (0 to 50%, w/v) containing 3% (w/v) ampholine was prepared according to the manufacturer's instructions. The pili sample was dialysed against 1% (w/v) glycine in distilled water and added to the mixing tube when about one-half of the gradient had been formed. A potential difference of 400 to 600 V was applied for 48 to 72 h until the current through the column reached a constant level of about 2.5 mA and the sample bands were stationary. Fractions (2 ml) were collected, examined for pili and measurements were made of pH at 4 °C and absorbance at 280 nm.

Iso-density gradient ultracentrifugation in caesium chloride. Pili (2 to 4 mg) were dispersed in 25 mM-boric acid buffer, pH 8.5, containing 2 mM-EDTA, 0.5 M-KCl, 0.02% (w/v) NaN₃ and 0.5% (w/v) Lubrol WX. CsCl was added to give a final concentration of 26% (w/w) and the buoyant density of pili was determined by centrifugation in a 10 × 10 ml angle rotor in an MSE ultracentrifuge at 150000 *g* for 17 h at 20 °C. Fractions (0.35 ml) were collected by puncturing the bottom of the centrifuge tube and the percentage transmission was measured at 280 nm. The density was measured immediately at 20 °C by weighing every second fraction and every fraction was examined for pili by electron microscopy.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out in glass tubes as described by Shapiro *et al.* (1967) with the following modifications recommended by Wyckoff *et al.* (1977); no SDS was included in the 3% (w/v) polyacrylamide stacking gel or the 12% (w/v) separating gel, and only 0.03% (w/v) SDS was incorporated in the running buffer. Standard proteins and pili samples were dissociated with SDS and dithiothreitol and prepared for electrophoresis as described by Wyckoff *et al.* (1977). After electrophoresis, the gels were stained for protein with Coomassie brilliant blue R250 (Wyckoff *et al.*, 1977), for glycoprotein with periodic acid-Schiff's reagent (Segrest & Jackson, 1972), for phosphoprotein using methyl green (Cutting & Roth, 1973) and for lipid with oil red O (Abodeely *et al.*, 1971). Densitometric scans of Coomassie blue-stained bands in the gels were carried out on a 'Quick Scan' densitometer (Helena Laboratories, Beaumont, Tex., U.S.A.) with a white light filter. The amount of protein in each band was estimated from the peak areas of the scans by comparison with ovalbumin and bovine serum albumin standards (up to 50 mg per gel).

Molecular weights of the pilus subunits were calculated by the method of Shapiro *et al.* (1967) using the following standard proteins: lysozyme, human γ -globulin, ovalbumin (Sigma), soybean trypsin inhibitor and bovine serum albumin (Boehringer).

Antiserum preparation. Anti-pili serum was prepared by inoculating adult sheep subcutaneously twice at a

4 week interval with a total of 900 µg of the most highly purified pili from strain 91B. The inoculum was prepared by emulsifying two parts of Freund's incomplete adjuvant (Arlacel/Drakeol; 1:9, v/v) with one part of pili dispersed in phosphate-buffered saline containing 0.5% (v/v) neutral formalin. Antisera against intact bacteria of strains 91B, 80 and 198 were similarly raised in sheep inoculated with preparations containing a total of 10^{10} *B. nodosus* in Freund's incomplete adjuvant. Six weeks after the second injection blood was taken from the sheep and the sera were stored at -20°C .

Bacterial agglutination. Bacterial K-agglutination tests were carried out as described by Egerton (1974).

Immunodiffusion. The double-diffusion precipitin reaction was carried out as described by Ouchterlony (1958) using 0.9% Oxoid Ionagar no. 2 in phosphate-buffered saline containing 0.04% (w/v) NaN_3 . The precipitin bands were stained with Coomassie brilliant blue R250 as described by Frost & Paranchych (1977).

Chemical assays. Protein concentrations were determined by the Lowry method with bovine serum albumin as standard. Protein concentrations and nucleic acid contamination were also estimated by the u.v. absorption method of Warburg & Christian (1941); this gave protein concentrations of pure pili preparations that were three-fifths of those obtained by the Lowry method. Carbohydrate was determined by the phenol/sulphuric acid reaction using glucose as standard (Dubois *et al.*, 1956). LPS was estimated colorimetrically by the method of Janda & Work (1971) using LPS purified from *B. nodosus* strain 80 by the method of Westphal & Jann (1965) as a standard.

RESULTS

Initial pili purification

Except where stated otherwise, the results of purification methods refer to pili from *B. nodosus* strain 91B. The pili removed from bacteria by homogenization were morphologically identical to the free pili in the growth medium; both were 5 to 6 nm in diameter and up to 15 µm long. After precipitation at pH 4.0 and precipitation with MgCl_2 five or six times, the pili preparations, whether bacteria-derived or medium-derived, were still contaminated with up to 20% nucleic acid, approximately 1% LPS and, as shown by SDS-polyacrylamide gel electrophoresis, by several proteins. In some preparations, the pili were free of nucleic acid after four or five MgCl_2 precipitations, but were still contaminated with other proteins and LPS. The reason for these differences was not determined.

These impurities could not be removed by differential centrifugation at 20000 g for 20 min, 30000 g for 1.5 h and 105000 g for 2 h. Anionic exchange chromatography on DEAE-cellulose (Whatman DE 52) and fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ or methanol also failed to remove the impurities.

Gel filtration chromatography

Chromatography on BioGel A150 (Fig. 1) separated pili from LPS, nucleic acid and, as shown by SDS-polyacrylamide gel electrophoresis, virtually all the protein contaminants. The pili in the first absorption peak eluted from the BioGel column had a u.v. absorption spectrum typical of protein with an absorption maximum at approximately 278 nm and an inflection at 285 nm. The nucleic acid was in the second peak which had an absorption maximum at 260 nm. The yield of pili at this stage was about 5 mg dry wt of protein per 10 g wet wt of bacteria.

Fractions from the first absorption peak eluted from the BioGel column were usually combined, concentrated and re-run on the same column to remove trace contaminants of LPS and nucleic acid. The final pili preparation had no detectable carbohydrates or LPS when enough pilin protein was used in the assay to detect a possible 0.1% contamination. The u.v. absorption spectrum of purified pili indicated that all nucleic acid contaminants had been removed. Finally, the pili preparation appeared to be uncontaminated with other cell components when examined by electron microscopy.

Fig. 1. Gel filtration of pili from strain 91B on BioGel A150. The pili sample applied to the column had previously been purified by acid precipitation and MgCl_2 precipitation. The fraction volumes were 7 ml. ○, Absorbance at 280 nm; □, LPS; ●, pili grade.

Fig. 2. Isoelectric focusing of pili using LKB ampholine with a pH range of 3 to 6. The pili sample applied to the column was from strain 91B purified by acid precipitation, MgCl_2 precipitation and gel filtration on BioGel A150. The fraction volumes were 2 ml. ○, Absorbance at 280 nm; □, pH at 4 °C; ●, pili grade.

Isoelectric focusing

Isoelectric focusing of bacteria-derived or medium-derived pili purified in a BioGel A150 column formed a single, sharply defined, opaque band in the ampholine columns at pH 4.5 in the gradient (Fig. 2). A pI of 4.5 for pili was obtained when the isoelectric focusing was carried out in gradients of pH 3 to 6, pH 4 to 6, or pH 4 to 5. If LPS was present in the sample it was not separated from the pili by isoelectric focusing. When large samples were subjected to isoelectric focusing the pili precipitated near their isoelectric point and fractionation of the column proved difficult. Pili from *B. nodosus* strain 80 also had a pI of 4.5.

Buoyant density in caesium chloride

Iso-density gradient ultracentrifugation of pili resulted in a single, sharply defined, opaque band at a density of $1.287 \pm 0.003 \text{ mg ml}^{-1}$ (mean of two experiments $\pm$ standard deviation) for pili of strain 91B (Fig. 3), a density of $1.284 \pm 0.003 \text{ g ml}^{-1}$ (four experiments) for pili of strain 80 and a density of 1.286 g ml^{-1} for pili of strain 198.

Fig. 3. Iso-density gradient ultracentrifugation of pili on CsCl. The pili sample used was from strain 91B which had previously been purified by acid precipitation, MgCl_2 precipitation and gel filtration on BioGel A150. The fraction volumes were 0.35 ml. $\circ$, Transmission at 280 nm; $\square$, density of CsCl solution; $\bullet$, pili grade.

Fig. 4. Immunodiffusion patterns of anti-pili and anti-whole bacteria sera versus various pili preparations and LPS.

(a) The centre well contained 1 μl of undiluted antiserum against pure medium-derived pili from strain 91B. Outer wells contained: (1) 30 μg of pure medium-derived pili from strain 91B; (2) crude surface antigen from 4×10^7 *B. nodosus* strain 91B prepared by homogenizing the cells for 2 min at full speed and taking the supernatant after centrifugation at 12000 g ; (3) 30 μg of pure bacteria-derived pili from strain 91B; (4) 20 μg of LPS purified from strain 91B by the method of Westphal & Jann (1965); (5) 30 μg of pili purified from strain 198; (6) 30 μg of pili purified from strain 80.

(b) The centre well contained 30 μg of pure bacteria-derived pili from strain 91B. Outer wells contained: (1) and (2) half-dilution of antiserum against whole bacteria strain 91B; (3) undiluted antiserum against whole bacteria strain 80; (4) undiluted antiserum against whole bacteria strain 198.

SDS-polyacrylamide gel electrophoresis

Only one sharply defined protein staining band per gel resulted when 10 μg of the most highly purified pili preparations were subjected to SDS-polyacrylamide gel electrophoresis. The molecular weight of this pilin band was determined as 18400 ± 300 (average of 10 gels $\pm$ standard deviation) for strain 91B, 19300 ± 400 (4 gels) for strain 80 and 18400 ± 300 (10 gels) for strain 198. Bacteria-derived and medium-derived pilin subunits were identical in molecular weight.

SDS-polyacrylamide gel electrophoresis of gels overloaded with 60 μg of purified pili from *B. nodosus* strain 91B resulted in one heavily stained band of pilin and one just detectable

Table 1. *Agglutination titres of various anti-bacterial sera and anti-pili serum with various strains of whole bacteria*

Antigen strain	Antiserum against:			
	Strain 91B	Strain 80	Strain 198	Purified pili, strain 91B
91B	10 240	< 10	20	20480
80	< 10	5120	20	10
198	40	< 10	640	20

band of protein impurity. The impurity had a molecular weight of $14\,200 \pm 200$ (average of 7 gels $\pm$ standard deviation), and densitometric protein quantification showed that it constituted less than 0.4% of the total protein. Similarly overloaded gels showed no detectable staining of the pilin band when stained for glycoprotein, lipid and phosphoprotein.

Serology

Medium-derived and bacteria-derived pili from the same strain (91B) of *B. nodosus* cross-reacted in immunodiffusion gels, but did not cross-react with pili derived from heterologous strains 80 and 198 (Fig. 4). This parallels the lack of cross-reactivity between strains 91B, 80 and 198 in agglutination reactions (Table 1). The antiserum produced against the most highly purified pili preparation of strain 91B caused the characteristically rapid, loose, floccular K-type agglutination reaction described by Egerton (1973).

The K-agglutininogen was a heat-sensitive antigen which was destroyed by heating at 100 °C for 10 min. After pure pili preparations had been heated at 100 °C for 10 min they did not react with anti-pili serum in immunodiffusion tests and contained no intact pili detectable by electron microscopy.

The appearance of a single precipitation band in the immunodiffusion tests (Fig. 4) provided further evidence of the purity of pili prepared by precipitation at pH 4.0, precipitation with MgCl_2 and chromatography twice on a BioGel A150 column.

DISCUSSION

Ottow (1975) has made provisional groups for the wide variety of non-flagellar appendages which have been recognized on many Gram-negative and a few Gram-positive bacteria. The pili of *B. nodosus* seem to fall into the category of group 4, an example of which are the pili of *Pseudomonas aeruginosa* which have the following properties: they are polarly arranged, flexible, rod-like filaments having a diameter of 6 nm and an average length of 2500 nm; they have no axial hole; they do not act as attachment organelles but do promote bacterial motion; they are composed entirely of protein with pilin subunits of molecular weight 17800, buoyant density on CsCl of 1.295 g ml^{-1} , isoelectric point of pH 3.9 and u.v. absorption maximum at 280 nm (Frost & Paranchych, 1977; Weiss, 1971). *Bacteroides nodosus* pili are very similar in that they are polarly arranged, have a diameter of 5 to 6 nm, have no axial hole, have no adhesive properties, and are composed entirely of protein with pilin subunits of molecular weight 18400, buoyant density on CsCl of 1.287 g ml^{-1} , isoelectric point of pH 4.5 and a u.v. absorption spectrum with a maximum at 278 nm and an inflection at 284 to 287 nm. The minor absorption peak at 287 nm in the u.v. absorption spectrum of pure pili type I from *E. coli* is attributed to tyrosine with the hydroxyl group either hydrogen-bonded to a carboxyl group or imbedded in a non-polar environment within the pilin molecule (Brinton, 1965).

A major difficulty in purifying pili in this study was the complete removal of LPS. The methods of sedimenting pili by acidification, MgCl_2 precipitation, $(\text{NH}_4)_2\text{SO}_4$ fractionation,

methanol precipitation and centrifugation all left a persistent LPS contamination. Similar difficulties were experienced in the removal of LPS or LPS-containing outer envelope material from pili prepared from *Neisseria gonorrhoeae* (Novotny & Turner, 1975; Robertson *et al.*, 1977) and *Moraxella nonliquefaciens* (Frøholm & Sletten, 1977). DEAE-cellulose chromatography and isoelectric focusing of *B. nodosus* pili did not remove all the LPS. The only reliable method was gel filtration on BioGel A150. It would thus seem that pili have a close association with LPS or possibly an LPS-phospholipid-protein complex similar to that excreted into the nutrient medium by *E. coli* and *Salmonella typhimurium* (Rothfield & Pearlman-Kothencz, 1969). The pili preparations used in the vaccine protection trials by Stewart (1975*a*, 1978*a*) and the K-agglutination experiments by Walker *et al.* (1973) and Stewart (1975*a*, 1978*a, b*) were only purified by repeated sedimentation by acidification, $MgCl_2$ precipitation and differential centrifugation. The preparations would probably have been contaminated with LPS and possibly nucleic acid, as indicated by the presence of carbohydrate and the u.v. absorption spectrum which was more characteristic of nucleic acid than of protein (Stewart, 1975*a*). It is noteworthy that the pili purified by Stewart (1975*a*, 1978*b*) had a polypeptide subunit molecular weight of 14500; this closely approximates the molecular weight of the trace impurity detected in the pili preparations reported in this paper.

In spite of this discrepancy, the results of Stewart (1975*a*, 1978*a, b*) and Walker *et al.* (1973), indicating an association between pili and the K-agglutinin of *B. nodosus*, have been confirmed by my results which showed that antiserum to highly purified pili gave a typical K-type agglutination with homologous *B. nodosus* strain 91B but not with strains 80 or 198. Furthermore, in the electron microscope the agglutinated bacteria appeared to be held together by antibody binding to pili. The lack of cross-reaction between different strains in the K-agglutination test corresponds to the lack of cross-precipitation in the immunodiffusion test between purified pili and heterologous antisera. Investigations of the protective immunogenic properties of highly purified pili in inoculated sheep exposed to foot rot infection will be reported elsewhere.

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