Characterisation of potential adhesins of the bacterium *Pasteuria penetrans*, and of putative receptors on the cuticle of *Meloidogyne incognita*, a nematode host

A. PERSIDIS, J. G. LAY, T. MANOUSIS, A. H. BISHOP and D. J. ELLAR*

University of Cambridge, Department of Biochemistry, Tennis Court Rd, Cambridge CB2 1QW, UK

* Author for correspondence

Summary

Pasteuria penetrans **spores were fragmented by glass bead vortexing, producing exosporial membranes and spore fragments, which consisted of fibre bundles. Both exosporia and spore fragments are capable of host-specific attachment to the cuticle of *Meloidogyne incognita*, a root-knot nematode host. Putative *M. incognita* receptors appear to be soluble in β-mercaptoethanol (BME) but not SDS, and are also sensitive to tryptic digestion and deglycosylation by endoglycosidase F. Polyclonal antibodies against intact spores and spore fragments inhibited attachment up to 25% and Fab' fragments of anti-spore antibodies produced 100% inhibition. The antibodies, however, did not show preferential staining of particular spore structures in thin section immunolabelling studies. Exposure of *Pasteuria penetrans* spores to HCl or urea–SDS–dithiothreitol renders them incapable of attachment to their host juveniles and extensively disrupts fibres that surround the spore core. Protein extracts from spore fragments or from exosporial membranes are identical, and urea–BME extracts from either structure, but not SDS extracts, can inhibit the attachment of spores to juveniles by 60–80%. An inhibitory BME extract from spore fragments was analysed by anion-exchange chromatography and adsorption onto host cuticle followed by immunoblotting. It appeared to contain six potential spore adhesins of approximate M_r 24–29, 38–47, 59, 89, 126, and 190 ($\times 10^3$). Lectin affinity blotting with wheat germ agglutinin and concanavalin A showed that all of these proteins bear terminal *N*-acetylglucosamine residues and the 38–47 kDa band also bears terminal Glc/Man residues. A BME extract from strain Pp 1 inhibited the attachment of strain PNG to *M. incognita* and *vice versa* and the PNG strain had a greater amount of two proteins of 98 and 126 kDa than the Pp 1 extract.**

Key words: *Pasteuria penetrans*, *Meloidogyne incognita*, adhesins, receptors, blotting, spore attachment.

Introduction

The *Pasteuria* genus of Gram-positive mycelial endospore-forming bacteria consists of two species, *P. penetrans* and *P. thornei*, which are obligate parasites of root-knot nematodes such as *Meloidogyne incognita*, and root-lesion nematodes such as *Pratylenchus brachyurus*, respectively (Sayre *et al.* 1988; Starr and Sayre, 1988).

P. penetrans is a potential biocontrol agent of the economically important root-knot nematodes (Stirling, 1984; Brown *et al.* 1985). The current lack of *in vitro* culture methods (Williams *et al.* 1989), and the large number of bacterial isolates available has limited the number of systematic studies on host specificity, although it appears that individual populations have narrow host ranges (Sayre *et al.* 1988), attaching to one or two species of nematodes in high numbers or to more nematode species in lower numbers (Davies *et al.* 1988).

Nematode parasitism is initiated when *P. penetrans* endospores attach in a host- and stage-specific manner to the cuticle of nematodes (Sayre, 1980). This adhesion phase is poorly characterised at present. It is believed to be facilitated by fibres that surround the spore core (Sayre and Wergin, 1977). Although lectin-carbohydrate interactions appear to be involved in the adhesion of nematophagous fungi to their host cuticle (Jansson *et al.* 1985; Nordbring-Hertz, 1988), early studies did not demonstrate similar interactions operating between *P. penetrans* and its host cuticle (O'Brian, 1980; Stirling *et al.* 1986). In a more recent study, however, (Bird *et al.* 1989), it was shown that WGA (wheat germ agglutinin) inhibited the attachment of *P. penetrans* to *M. javanica* by about 72%, this effect being completely reversed when *N,N'*-diacetylchitobiose was added. Similarly, Con A (concanavalin A) inhibited the attachment of the bacterium to *M. hapla* by about 55%, although the inhibition in this case was not reversed when methyl-α-D-mannoside was included in the attachment assay medium (Bird *et al.* 1989). These authors also found that pretreating spores with periodate resulted in about 90% fewer spores attaching to *M. javanica*. They concluded from their data that lectin–carbohydrate interactions were involved in the adhesion of *P. penetrans* to its nematode hosts.

The literature to date on the characterisation of putative nematode cuticle receptor(s) for *P. penetrans* is poor. The nematode cuticle is a complex multilayered structure, which is replaced at each stage of the life cycle and which exibits stage-specific ultrastructural and

compositional differences (Reddigari *et al.* 1986; Robinson *et al.* 1989). BME-soluble and -insoluble fractions from cuticle of 2nd stage juveniles and also adult females of *M. incognita* were shown to consist of collagens that differed in amino acid composition between the two stages (Reddigari *et al.* 1986). These workers showed that the BME-soluble fraction from juveniles contained 12 collagen species of which 7 were glycosylated, whereas the same fraction from adult females contained 9 collagen species none of which was glycosylated.

P. penetrans adheres to the cuticle of *M. incognita* juveniles but not to adult females. A number of micro-organisms adhere in a species and stage-specific fashion to plant-parasitic nematode cuticle. Apart from *P. penetrans*, they include nematophagous fungi such as *Arthrobotrys oligospora* and *A. conoides* (Rosenzweig and Ackroyd, 1983), *Meria coniospora* (Jansson *et al.* 1985), and *Dilophospora alopecuri* (Bird and McKay, 1987), and also coryneform bacteria such as *Corynebacterium rathayi* (Bird *et al.* 1989). The only reported isolation of a molecule with potential as an adhesin for nematode cuticle is that of a 20 kDa calcium-binding lectin with an affinity for GalNAc, isolated from *A. oligospora* (reviewed by Nordbring-Hertz, 1988). Here we report on the characterisation of two polyclonal antibodies against whole *P. penetrans* spores and against the parasporal fibres, in an effort to develop reagents that inhibit attachment and selectively recognise spore structures involved in attachment, such as the fibres. We also present data on the nature of putative receptor(s) on *M. incognita* juvenile cuticle, and of potential *P. penetrans* adhesins.

Materials and methods

Nematodes

Second-stage juveniles of *M. incognita* (Race 2, Accession number 135, from North Carolina State University, Raleigh, USA), and *M. arenaria* (Race 1, Accession number 351, from North Carolina State University, Raleigh, USA), were obtained from eggs hatching in a hatching tray at room temperature in tap water (Hooper, 1986). The eggs were dissected from roots of tomato plants (*Lycopersicon esculentum*, cv. Moneymaker or Rotegnon), that were maintained in glasshouses at the Rothamsted Experimental Station, Harpenden, UK, and were provided by Dr B. R. Kerry.

Purification and fragmentation of Pasteuria penetrans *spores*

The 2 strains of *P. penetrans* used were Pp 1, Australian isolate 1, originally obtained from S. R. Gowen, University of Reading, UK, and PNG, Papua New Guinea (PNG) isolate, originally obtained through J. Bridge, Commonwealth Institute of Parasitology, St Albans, UK. Both strains were provided by Dr B. R. Kerry, Rothamsted Experimental Station, Harpenden, UK, in the form of tomato plants infected with spore-laden *M. incognita* females. The roots were extensively washed in running tap water and then cut into 2 cm pieces, which were homogenised with a pestle and mortar in distilled water. The homogenate was filtered through muslin and a 20 μm nylon filter prior to centrifugation at 10 000 ***g*** at room temperature for 5 min. The pellet was resuspended in saline (0.9%, w/v, NaCl) and loaded onto a 2-step sucrose gradient (3 parts 80%, 1 part 40%), which was centrifuged at 25 000 ***g*** for 3 h at 4°C. A band of spores (Pp 1 or PNG) was obtained at the interface and these were washed several times with water. The spores were stored as a concentrated suspension in water at −20°C until used, or they were sonicated with a sonic probe (Dawe Soniprobe 7532A, Dawe Instruments Ltd., UK) according to Stirling *et al.* (1986), prior to attachment assays or fragmentation by glass bead vortexing, by a modification of the method of Hancock and Poxton (1988). Briefly, 5×10^8 spores in 1.5 ml fragmentation buffer (50 mM Tris–HCl, pH 7.0, 1 mM EDTA, 1 mM PMSF), were added to a 10 cm×1 cm Pyrex test tube together with 0.4 g of glass beads (0.10–0.11 mm, B. Braun Melsungen, FRG). The tube was vortexed for 30 min at 4°C and the supernatant was collected after the beads had been allowed to settle. The supernatant contains a mixture of spore fragments and exosporia, as judged by light and electron microscopy (see below). The mixture was resolved by pulse centrifuging the suspension for 4 s in a minifuge operating at maximum speed. The pellet contained spore fragments and the supernatant exosporia. This step was repeated 3 times.

Antibodies

Pre-immune serum was collected from 2 half-lop rabbits maintained in the Departmental animal house. They were immunised subcutaneously with a 200 μg suspension of Pp 1 spores or spore fragments in saline 1:1 with Freund's complete adjuvant, followed by 2 booster doses of 100 μg spores or spore fragments, 1:1 with Freund's incomplete adjuvant, at 10-day intervals.

Fab' production was carried out by a modification of the method of Johnstone and Thorpe (1987). Briefly, IgG was purified from immune serum by Protein A–Sepharose CL 4B affinity chromatography and digested with pepsin. This was followed by DTT (dithiothreitol) and iodoacetamide reduction to produce the Fab' fragments, which were used in attachment assays. The intact antibodies were used in thin-section immunolabelling studies as described below and also in attachment assays. Both pre-immune and immune sera were serially diluted 1:4 (v/v) within the range 1:4 to 1:1024 with PBS (50 mM sodium phosphate, pH 7.4, 0.9% (w/v) NaCl). Control assays in PBS were also carried out.

Attachment assays

These were carried out in 96-well plates (Alpha Laboratories, UK), in 200 μl of the relevant medium for each experiment, containing 5×10^6 ml spores and about 50 one-day-old juveniles. The plate was incubated in an orbital shaker at 100 revs min^{-1} and 25°C overnight. After attachment a 50 μl sample of the suspension was placed on a microscope slide and 10 μl of 5% formaldehyde in water was added to kill the nematodes. The number of spores attached to the visible top side of the nematodes was counted by phase-contrast or Nomarski optics of an Olympus BH 1 light microscope.

Electron microscopy of attached spore fragments and exosporia

An attachment assay was carried out between a mixture of Pp 1 spore fragments and exosporia and *M. incognita* or *M. arenaria* juveniles, in 50 mM Mops buffer, pH 7.0. After attachment the juveniles were washed 3 times in the previous buffer prior to being fixed in 2% glutaraldehyde, pH 7.3, post-fixed in 2% osmium tetroxide, dehydrated through an ethanol series (25% to 100%), washed in polypropylene oxide and infiltrated with Spurr's resin. Silver–gold sections were cut and collected on Formvar/carbon-coated copper grids, which were stained with aqueous uranyl acetate (20 mg ml^{-1}) followed by Reynold's lead stain. Sections were observed with an AEI 801S electron microscope operating at 60 kV.

Negative staining

A drop of a water suspension of spores was placed on Formvar/carbon-coated copper grids and allowed to air-dry. The grid was floated face down onto stain solution (0.2% uranyl acetate or 0.5% ammonium molybdate in water, centrifuged prior to use), and then washed by floating face-down onto water. An AEI 801S electron microscope operating at 60 kV was used for observation.

Thin-section immunolabelling

Unsonicated spores or a mixture of spore fragments and exosporia were fixed in 2% glutaraldehyde, pH 7.3, dehydrated through an ethanol series (25–100%), washed in polypropylene oxide and

infiltrated with Spurr's resin. Silver–gold sections were cut and collected on Formvar/carbon-coated copper grids which were incubated with anti-spore antibody, anti-spore fragment antibody or pre-immune serum at a 1:500 dilution in Tris/BSA buffer (20 mM Tris–HCl, pH 7.4, 0.5% BSA, 225 mM NaCl, 0.1% Triton X-100, 20 mM azide). The grids were washed with Tris/BSA buffer and then incubated with Protein A–gold conjugate (Janssen) at 0.4 $\mu g\,ml^{-1}$ in Tris/BSA. The grids were washed in Tris/BSA, post-fixed with 2% glutaraldehyde, washed in water and contrasted with uranyl acetate (20 $mg\,ml^{-1}$) and Reynold's lead stain prior to observation with an AEI 801S electron microscope operating at 60 kV.

Nematode cuticle extraction

M. incognita cuticle fragments were produced according to Reddigari *et al.*(1986), and were extracted as follows. (a) SDS extraction–delipidation. Cuticle fragments produced from about 1000 juveniles were boiled in 1% SDS in water for 3 min, washed 5 times in fragmentation buffer (see section on spore fragmentation), and delipidated according to Finne and Krusius (1982), with methanol:chloroform (1:2, v/v) for 2 h at room temperature. (b) SDS–BME extraction. After the SDS extraction and wash described in (a), the cuticle pieces were boiled for 2 min in 5% aqueous BME. Cuticle from (a) and (b) was washed several times in 50 mM Mops buffer, pH 7.0, prior to an attachment assay with Pp 1 spores in the previous buffer.

Enzyme digestions of cuticle

About 1000 *M. incognita* juveniles were digested with 0.1 $mg\,ml^{-1}$ trypsin (bovine, SIGMA) in 50 mM Mops buffer, pH 7.0, or with endoglycosidase F (from *Flavosporium meningosepticum*, obtained from NEN), 60 m units ml^{-1} in 20 mM glycine/NaOH buffer, pH 9.3, 1 mM EDTA. The incubations were carried out for 4 h at 37 °C after which the juveniles were washed several times in 50 mM Mops buffer, pH 7.0, prior to attachment assays with Pp 1 spores in Mops buffer. Controls included juveniles incubated in the relevant buffers alone.

Acid extraction of spores

Two Pp 1 spore samples were resuspended in 0.1 ml of 1.2 M HCl or in 0.1 ml of water. The suspensions were placed in Pasteur pipettes, which were sealed at both ends and heated at 60 °C for 24 h. At the end of the incubation the spores were washed 3 times in 50 mM Mops buffer, pH 7.0, prior to negative staining and attachment assays with *M. incognita* juveniles.

Extraction of spores and spore fragments

The following extraction media were used: (a) 6 M urea, 1% SDS, 50 mM DTT, pH 8.0, unbuffered; (b) 1% SDS (w/v) in water; (c) 10% BME (v/v) in water; (d) 9 M urea in 10 mM Tris–HCl buffer, pH 8.8; (e) 9 M urea in 10 mM Tris–HCl buffer, pH 8.8, 10% BME (v/v), (f) 6 M urea, 5% BME (v/v), in 0.1 M sodium acetate buffer, pH 5.0. A sample of intact Pp 1 spores was extracted with (a) or with water by boiling for 10 min and was washed with water prior to negative staining. Spore fragments or exosporia produced from 5×10^8 Pp 1 or PNG spores were extracted by boiling in 0.2 ml of media (b)–(f). The extracts were acetone-precipitated by adding 4 volumes of ice-cold acetone and incubating at −80 °C for 20 min. The suspensions were centrifuged in a minifuge operating at maximum speed for 5 min and the pellets were air-dried prior to being suspended in SDS–PAGE sample buffer (50 mM Tris–HCl, pH 7.5, 1% SDS (w/v), 25 mM DTT, 1 mM EDTA, 10% glycerol (v/v) and 0.0025% bromophenol blue (w/v) for electrophoresis and immunoblotting. Alternatively, extracts were dialysed against 2 l of 50 mM sodium phosphate buffer, pH 7.4 (dialysis buffer), for 4 h and against a further 2 l of the same buffer overnight at 4 °C prior to attachment assays. The numbers of spore fragments and exosporia were equalised prior to extraction. Appropriate control extractions including extraction media boiled in the absence of spores and dialysed were carried out.

FPLC of spore extract

Pp 1 spore fragments from 5×10^8 spores were extracted with 10% BME (v/v) in water as above, and the extract was dialysed as above against 20 mM Tris–HCl buffer, pH 9.0. The extract was diluted to 0.5 ml with the Tris buffer and loaded onto a Pharmacia MonoQ HR 5/5 column, pre-washed and equilibrated with the above Tris buffer. The column was eluted with a linear gradient of NaCl from 0.0 M to 1.0 M in the previous Tris buffer, at a flow rate of 1 $ml\,min^{-1}$ at room temperature; 39×1 ml fractions were collected, and absorbance at 280 nm was monitored; 1 ml fractions were pooled in pairs and the 2 ml pooled fractions were processed using Centricon-10 (Amicon) centrifuge tubes according to the manufacturer's instructions, to exchange the alkaline Tris buffer for 50 mM sodium phosphate buffer, pH 7.4, and to concentrate the fractions to 0.4 ml. Half of the resulting sample was acetone precipitated prior to immunoblotting and the other half was used in attachment assays with an appropriate buffer control.

Adsorption of spore extract onto cuticle

Pp 1 spore fragments from 5×10^8 spores were extracted with 10% aqueous BME and the extract (200 μl) was dialysed against dialysis buffer as previously described. A sample of that extract was removed (sample 1). The remainder of the extract was incubated with approximately 500 000 *M. incognita* juveniles, pre-washed in dialysis buffer, for 2 h at room temperature in a 1.5 ml Eppendorf tube shaking at 100 revs min^{-1} on an IKA-VIBRAX-VCR shaker. After incubation, the nematodes were pelleted by centrifugation and the supernatant was removed (sample 2). The juveniles were washed 4 times in dialysis buffer and then boiled for 10 min in SDS–PAGE gel sample buffer. The extracted juveniles were pelleted and the supernatant removed (sample 3). A control supernatant (sample 4) was produced from juveniles not exposed to spore extract. Samples 1–4 were acetone precipitated and boiled in SDS–PAGE gel sample buffer and all 4 samples were analysed by immunoblotting.

SDS–PAGE

This was carried out according to Laemmli (1970), using a 5% stacking gel and 10 or 13% separating gels. Gels were silver stained according to Wray *et al.* (1981) and identical gels were electroblotted as described below.

Immunoblotting

Proteins separated by SDS–PAGE were transferred to nitrocellulose filters (Schleicher and Schuell, FRG) by electrophoresis according to Towbin *et al.* (1979), using a Bio-Rad 'Trans-Blot' apparatus. The filters were blocked by incubation with 3% BSA (w/v) in TBS buffer (10 mM Tris–HCl, pH 7.4, 0.9% NaCl, w/v), for 20 min on a rocking platform at room temperature. 100 μl of neat antibody against Pp 1 spores or spore fragments was added and the filters were incubated as before for a further hour. They were washed 3 times in TBS for 5 min per wash and then incubated in TBS with 3% BSA (w/v) containing 0.1% (v/v) of goat anti-rabbit IgG, peroxidase conjugated (SIGMA), for 30 min as before. The filters were washed as before and developed by incubation in 0.05% (w/v) 4-chloro-1-napthol (SIGMA) in 20% methanol in TBS, with 0.02% hydrogen peroxide. The reaction was terminated with several rinses in distilled water.

Lectin affinity blotting

This was carried out by a modification of the method of Johnstone and Thorpe (1987). Filters obtained as above were treated as for immunoblotting, except that, instead of antibodies, peroxidase conjugates of WGA and Con A (SIGMA) were used. The lectins were dissolved at a 1 $mg\,ml^{-1}$ concentration in TBS buffer, and 100 μl of this stock was used in 50 ml of TBS, 3% BSA (w/v). Incubation was for 30 min at room temperature on a rocking platform. The filters were washed and developed as in the previous section.

Results

Fragmentation of P. penetrans *spores*

Intact Pp 1 spores vortexed with glass beads for 30 min

were effectively broken into fragments. Membranes were also released, which were readily separated from the fragments by pulse centrifuging the suspension. Two to three repetitions of this centrifugation produced preparations of spore fragments and membranes that appear to be essentially pure, as judged by light microscopy (Fig. 1). The micrographs show intact spores with refractile central cores (Fig. 1A), fragments that are dark, lacking the central core and are often horse-shoe shaped (Fig. 1B), and finally membranes that are transluscent and of uniform size and shape (Fig. 1C). Below we show that extracts from the two preparations also differ quantitatively in their ability to inhibit attachment of spores to juveniles.

We believe that the membranes obtained are exosporia and not sporangia, for the following reasons. The membranes were obtained from spores that had been sonicated with a sonic probe prior to fragmentation by glass bead vortexing. Sonication has been shown to remove the sporangial membrane from *P. penetrans* spores (Stirling *et al.* 1986), leaving behind the exosporium surrounding the spore core with the fibres, and it was this sonicated material that was subsequently fragmented. When observed by light microscopy the membranes appear to be of uniform size and shape (Fig. 1C). The sporangia of *P. penetrans* vary, however, in size and shape depending on the extent of maturation of the endospores. Fig. 2A shows an electron micrograph of a sample from the glass bead fragmentation mixture obtained after 15 min of vortexing, when fragmentation is not yet complete. No sporangia are seen around the spores and an exosporium is seen peeling off from a spore (arrow).

A mixture of Pp 1 fragments and exosporia was incubated with juveniles of *M. incognita*, the latter were washed and then observed by thin-section electron microscopy. Fig. 2B shows that both exosporia and fragments are capable of attachment. Fragments appear to consist of fibre bundles on their own or attached to pieces of core cortex but devoid of spore cytoplasm. The same mixture of Pp 1 fragments and exosporia will not attach to *M. arenaria* juveniles (data not shown), which agrees with the findings of Davies *et al.* (1988), that Pp 1 shows a preference for *M. incognita* over *M. arenaria*.

Antibodies against P. penetrans

Intact sonicated Pp 1 spores and also Pp 1 fragments were used to raise two polyclonal rabbit anti-sera, which were used in thin-section immunolabelling studies of Pp 1 spores. Fig. 3 shows the results obtained with anti-fragment antibody. No single spore structure appears to be preferentially labelled. This pattern was identical when antibody against intact spores was also used (data not shown). Pre-immune sera produced the same background labelling as that of the Protein A–gold control in Fig. 3 (data not shown).

In attachment assays with *M. incognita* juveniles both antibodies inhibited the attachment of Pp 1 spores up to a maximum of 20–25 %, as shown in Table 1. The antibodies also produced spore agglutination. Fab′ fragments of the antibody against intact Pp 1 spores did not agglutinate Pp 1 spores (data not shown) and inhibited their attachment to *M. incognita* juveniles by 100.3 % (S.D. 7.9), relative to a PBS control assay.

Nature of the cuticle receptor(s)

M. incognita juvenile cuticle was extracted with SDS followed by delipidation or by SDS followed by BME, prior to attachment assays with Pp 1 spores. Fig. 4 shows that

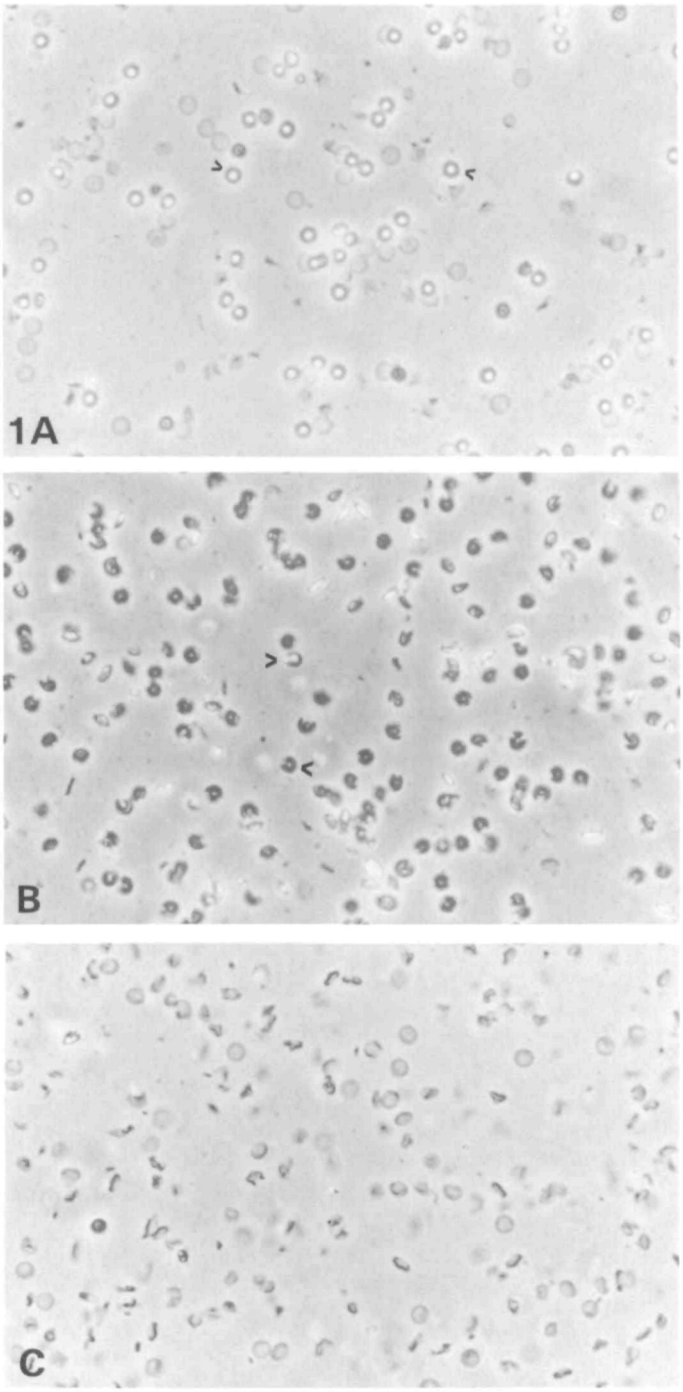

Fig. 1. Fragmentation of Pp 1 spores after glass bead vortexing. Phase-contrast light micrographs; ×500. (A) Mixture of intact spores, spore fragments and membranes after 15 min of vortexing. Arrows show intact spores with refractile central cores. (B) Purified spore fragments after 30 min of vortexing. Arrows show dark horse-shoe-shaped fragments lacking the refractile central core. (C) Purified membranes, which are transluscent and uniform in size and shape when viewed from above or below.

juvenile cuticle does not bear a spore load after SDS–BME extraction but does so after SDS treatment and delipidation. It appears, therefore, that BME-soluble components of the cuticle act as receptor(s) for *P. penetrans* spores.

Digestion of intact juveniles with trypsin or with

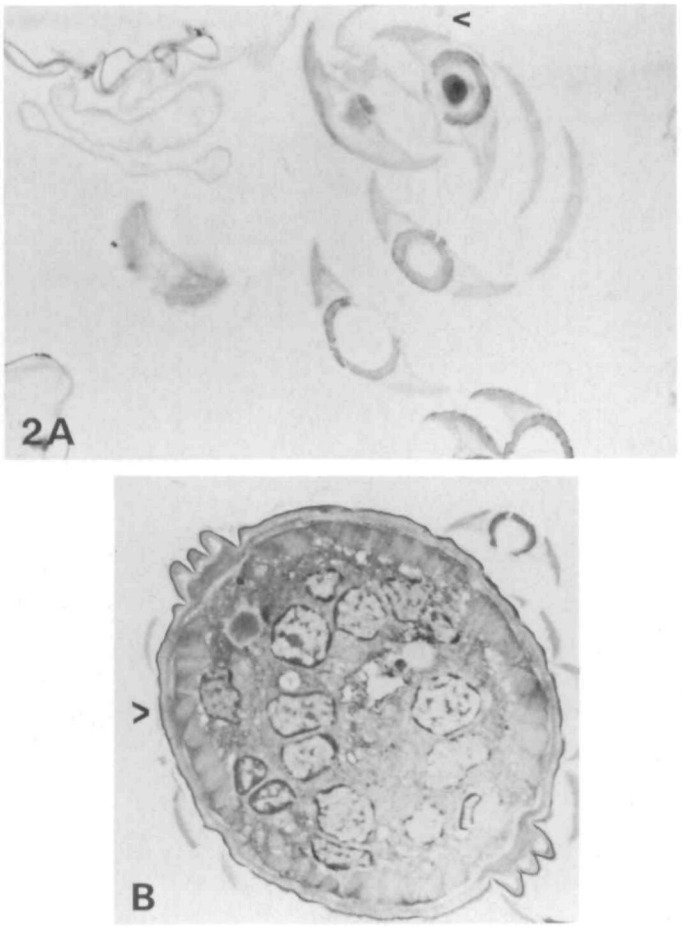

Fig. 2. Electron micrographs of spore fragments and exosporia; ×4400. (A) Thin-section micrograph of Pp 1 spore fragments and membranes after 15 min of vortexing with glass beads. The arrow shows an exosporium peeling off a spore. (B) Thin-section micrograph of Pp 1 spore fragments and exosporia attaching to an *M. incognita* juvenile. The nematodes were washed prior to observation. The arrow shows a segment of exosporium attached to cuticle and extending from a spore fragment.

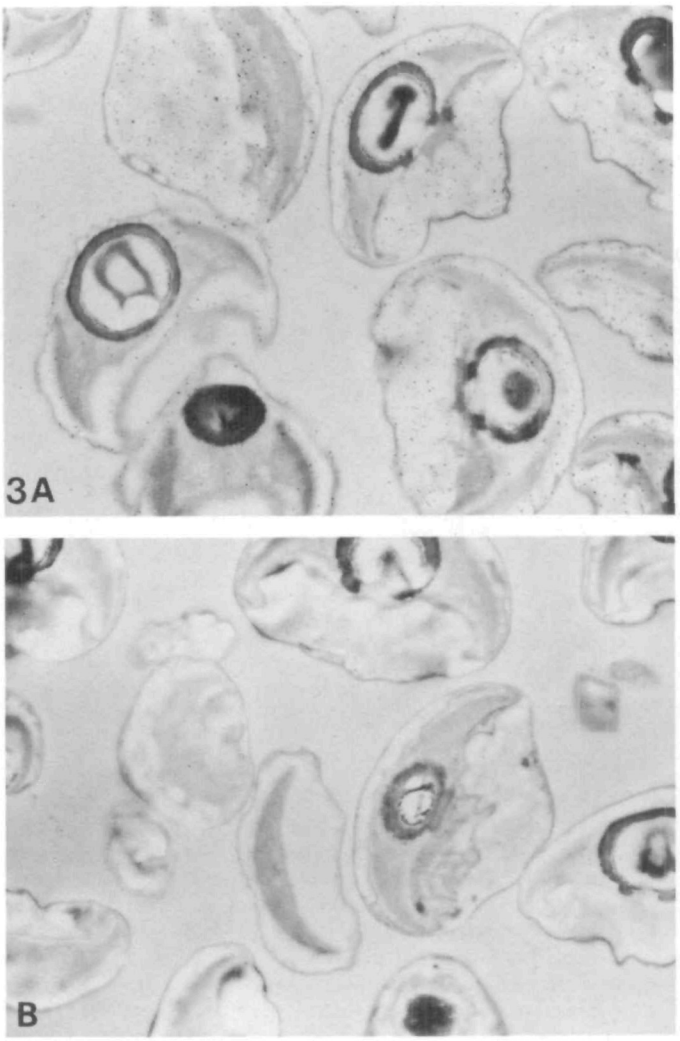

Fig. 3. Thin-section immunolabelling of Pp 1 spores with anti-Pp 1 fragment antibody; ×11 600. (A) Antibody labelling; (B) control Protein A–gold conjugate labelling.

endoglycosidase F resulted in 37.2% (S.D. 22.5) and 63.5% (S.D. 25.9) attachment, respectively, in assays with Pp 1 spores. A number of exoglycosidases and proteases were tried but only the above two had any effect.

Electron microscopy of spores

Fibrils present on various Gram-negative bacteria and shown to mediate attachment to host surfaces can be solubilised with acid or denaturing and reducing agents (Handley *et al.* 1984; Hanson and Brinton, 1988). These methods were applied to *P. penetrans* spores to investigate a possible role for the spore fibres in adhesion to nematode cuticle. Fibre solubilisation was monitored by negative staining of the extracted spores.

Fig. 5 shows the results obtained. After exposure to HCl the spores lose their central core and fragment and fibres are stained positively. Extraction with a urea–SDS–DTT mixture, however, renders the fibres clearly visible by negative staining and this treatment is seen to produce extensive dissociation of the fibres. In an attachment assay with *M. incognita* juveniles HCl-treated spores gave 1.4% (S.D. 1.1) attachment and urea–SDS–DTT-extracted spores gave 5.9% (S.D. 2.0), relative to control untreated spores, suggesting that these treatments resulted in extensive disruption and/or solubilisation of spore adhesin(s).

Production and comparison of spore extracts

Fig. 6A shows the immunoblots obtained when Pp 1 exosporia and fibre bundles were extracted with SDS or with a urea–BME mixture. The two structures gave the same immunoblot profiles, and extraction of both with urea–BME produces two bands of M_r 89 and 126 ($\times 10^3$) not seen in the SDS extract.

The SDS and urea–BME extracts from Pp 1 spore fragments and exosporia were dialysed prior to an attachment assay with Pp 1 spores and *M. incognita* juveniles. Controls included dialysed SDS, urea–BME and buffer alone. All three of the controls gave similar levels of attachment (data not shown), indicating that the dialysis did not leave any SDS or urea–BME behind that might interfere with the attachment assay. Table 2A shows that SDS did not produce an inhibitory extract from either exosporia or from fragments, whereas urea–BME produced an extract inhibiting attachment by about 80%

Table 1. *Inhibition of attachment of Pp 1 spores to* M. incognita *juveniles by the Pp 1 anti-spore and anti-fragment antibodies*

A. *Anti-spore antibody*

Dilution	% Attachment (S.D.) Pre-immune serum	Immune serum	% Inhibition
1:4	8.7 (4.3)	10.5 (9.0)	–
1:16	37.8 (8.6)	19.2 (10.7)	18.6
1:64	52.4 (9.0)	55.4 (14.6)	–
1:256	65.2 (10.6)	67.6 (12.3)	–
1:1024	93.4 (13.7)	99.6 (17.1)	–

B. *Anti-fragment antibody*

Dilution	% Attachment (S.D.) Pre-immune serum	Immune serum	% Inhibition
1:4	9.9 (4.0)	14.3 (12.1)	–
1:16	41.0 (21.4)	23.5 (11.6)	17.5
1:64	53.3 (16.2)	29.2 (11.3)	24.1
1:256	81.5 (18.6)	69.8 (10.1)	11.7
1:1024	101.6 (12.8)	106.9 (18.8)	–

The data are presented as % of control attachment assays in PBS. The % inhibition of each dilution is calculated as follows. Inhibition (%)= 100−% attachment. The % inhibition of the pre-immune serum is then subtracted from that of the immune serum to give the latter's actual % inhibition, which thus is corrected for the effects of the pre-immune serum. Standard deviations (S.D.) are shown in parenthesis.

from fragments and about 57% from exosporia. The same number of spore fragments and exosporia were used to produce the extracts; this further suggested that the two preparations are pure.

Pp 1 spore fragments were extracted with urea or BME, or with a mixture of the two, the extracts were dialysed and then used in an attachment assay with Pp 1 spores and *M. incognita* juveniles. The results are shown in Table 2B. BME alone seems to produce the most potent extract and consequently this extract was chosen for further study.

Partial characterisation of inhibitory spore extract

An attempt was made to characterise the inhibitory BME extract by anion-exchange chromatography. As Fig. 6B shows, four small peaks were obtained, eluting at 0.12, 0.27, 0.50 and 0.80 M NaCl. Fractions were pooled in pairs and this resulted in the four peaks being obtained as individual fractions. Pooled fractions 14–15, corresponding to the 2nd peak of the chromatogram, which was eluted with 0.27 M NaCl, resulted in 52.0% (S.D. 6.4) attachment of Pp 1 spores to *M. incognita* juveniles, relative to a buffer control, whereas no other pooled fraction was found to be inhibitory. Fig. 6C shows the immunoblot profile of the fractions, where lane 5 corresponds to inhibitory fraction 14–15. Lane 5 shows a band of M_r 45×10^3 and a smeared band of M_r range 24–29 ($\times10^3$) found in the inhibitory fraction, but not visible in the other lanes, containing the non-inhibitory fractions.

To obtain more direct evidence on the nature of likely adhesins, inhibitory BME extract was dialysed and then exposed to a large number of *M. incognita* juveniles. The juveniles were pelleted and the supernatant was collected. The juveniles were then washed and extracted with SDS–PAGE solubilisation buffer. This extract should contain juvenile proteins and also potential spore adhesins that were adsorbed onto the cuticle. Immunoblot analysis was then carried out on this adsorbed extract, on neat spore extract prior to adsorption and also on the supernatant after adsorption. The latter should be depleted in spore components interacting with the juveniles. Fig. 6D shows the immunoblot profile from this exper-

Table 2. *Effect of dialysed Pp 1 extracts on the attachment of Pp 1 spores to* M. incognita *juveniles*

A. *Extracts from spore fragments and exosporia*

	% Attachment (S.D.) SDS	Urea–BME
Fragments	100.9 (10.3)	21.2 (9.9)
Exosporia	101.8 (17.0)	43.2 (12.4)

B. *Extracts from spore fragments*

	% Attachment (S.D.) Urea	Urea–BME	BME
Fragments	64.2 (15.7)	46.1 (5.3)	18.5 (12.4)

(A) 1% SDS (w/v) in water and 6 M urea (w/v), 5% BME (v/v), in 0.1 M sodium acetate buffer, pH 5.0. The data are presented as % of control extractions of Pp 1 fragments in water or in sodium acetate buffer. (B) 9 M urea in 10 mM Tris–HCl buffer, pH 8.8, the previous urea solution also containing 10% BME (v/v), 10% BME (v/v) in water. The data are expressed as % of control extractions in Tris–HCl buffer or in water. Standard deviations (S.D.) are in parenthesis.

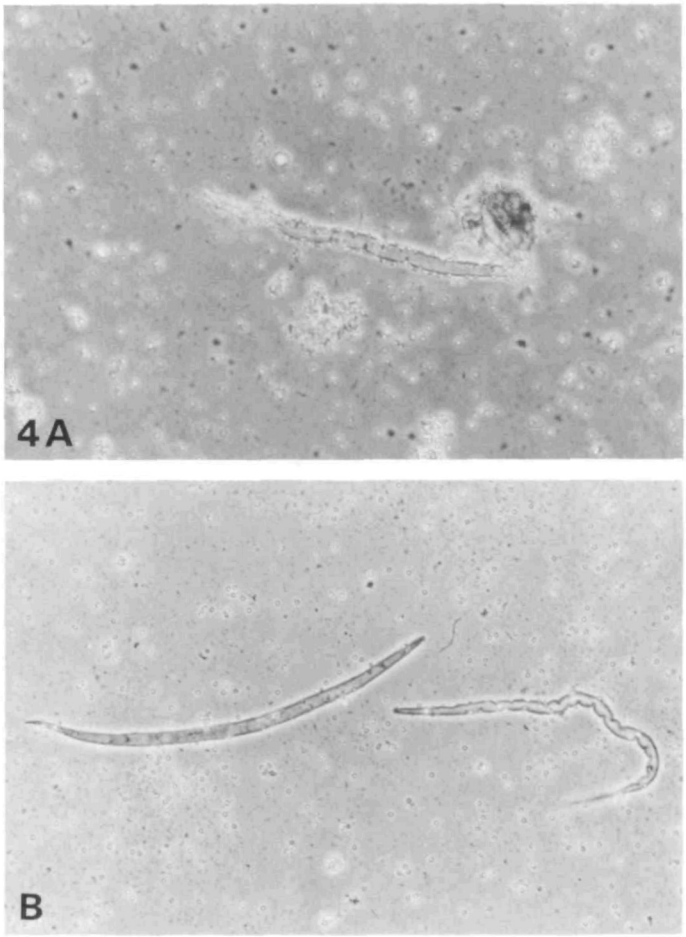

Fig. 4. Attachment of Pp 1 spores to extracted *M. incognita* juvenile cuticle. Phase-contrast light micrographs; ×120. (A) SDS-extracted and delipidated cuticle bearing attached spores; (B) SDS–BME-extracted cuticle devoid of a spore load.

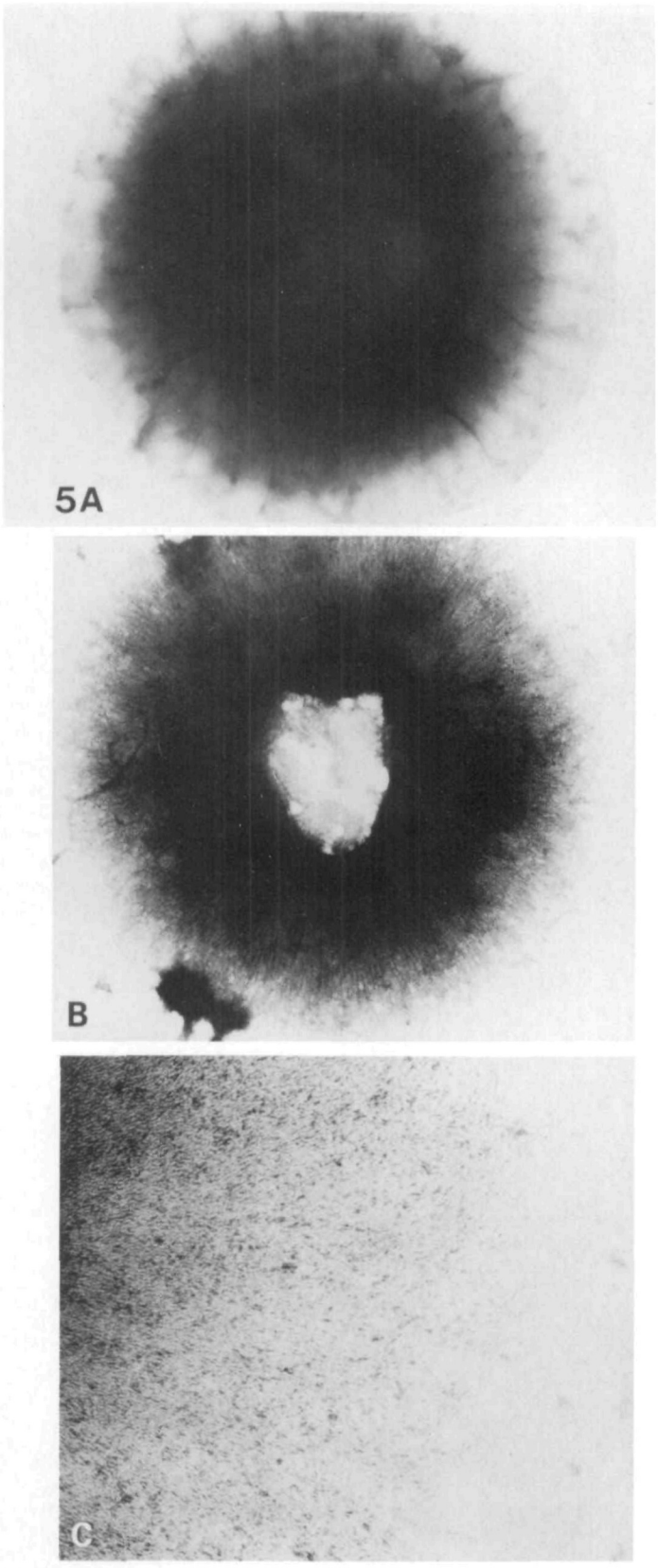

Fig. 5. Electron micrographs of Pp 1 spores, negatively stained. (A) Control spore, (×21 120) showing the exosporial membrane having trapped stain and making resolution of the undelying fibres difficult; (B) HCl-treated spore (×10 500) positively stained, showing a spore without exosporial membrane and core; (C) SDS–DTT–urea-treated spore fibres, negatively stained (×54 000).

iment, which includes a control of juveniles not exposed to spore extract.

Lane 3 shows that the cuticle-adsorbed spore extract contains three bands of approximate M_r 38, 59 and 190 $\times 10^3$ not seen in the supernatant after adsorption (lane 2), and also a band of about 28×10^3, which is of higher intensity than that in lane 2. All these bands are seen in the extract prior to exposure to juveniles (lane 1), and no bands are seen in the juvenile extract (lane 4), demonstrating the specificity of the staining reaction.

Comparison of Pp 1 and PNG spore extracts

The Pp 1 and PNG strains differ in virulence towards the same *Meloidogyne* host species, with PNG attaching to the same host in greater numbers than Pp 1 (Davies *et al.* 1988). A comparison was made between BME extracts from Pp 1 and PNG spore fragments using attachment assays and immunoblot analysis.

In an attachment assay with *M. incognita* juveniles, BME extract from Pp 1 spores resulted in 14.8% (S.D. 7.2) attachment of PNG spores relative to a control assay of PNG spores in buffer, and BME extract from PNG spores resulted in 42.7% (S.D. 6.7) attachment of Pp 1 spores, relative to a control assay of Pp 1 spores in buffer. Extract from either strain thus inhibits the attachment of the other strain to the same host nematode, suggesting a common adhesion mechanism mediated by related components.

BME extract from the two strains was dialysed as before and acetone precipitated prior to SDS–PAGE and electroblotting. The filters were visualised with anti-Pp 1 fragment antibody, followed by secondary antibody that was peroxidase-conjugated, and also with peroxidase-conjugated WGA and Con A, to identify similarities and differences in the glycosylation patterns of proteins from the two strains. Both these lectins have been shown to inhibit the attachment of *P. penetrans* spores to *Meloidogyne* hosts and also to bind to *P. penetrans* spores (Bird *et al.* 1989). Fig. 7 shows the results obtained: A is the immunoblot profile of the two strains, showing two bands of about 98 and 126 kDa (arrows) in the PNG lane (lane 2) that are present in greater amounts than in the Pp 1 lane (lane 1). Apart from this quantitative difference the profiles of the two strains are essentially identical; B shows a filter identical to that in A but visualised with WGA; and C shows another identical filter visualised with Con A. WGA produced a profile essentially identical to that of the antibody whereas Con A reacted weakly with a band of about 47 kDa present in both the other profiles.

Discussion

In this study we have attempted to characterise antibodies against *P. penetrans*, to define the nature of the cuticle receptor(s) recognised by the spores, and also of the spore adhesins mediating attachment. Antibodies were raised against both intact and fragmented *P. penetrans* spores, the fragments consisting essentially of fibre bundles occasionally attached to pieces of core cortex and devoid of spore cytoplasm. These fragments were obtained by vortexing the spores with glass beads, and the particular details of this procedure are crucial if fragments and also

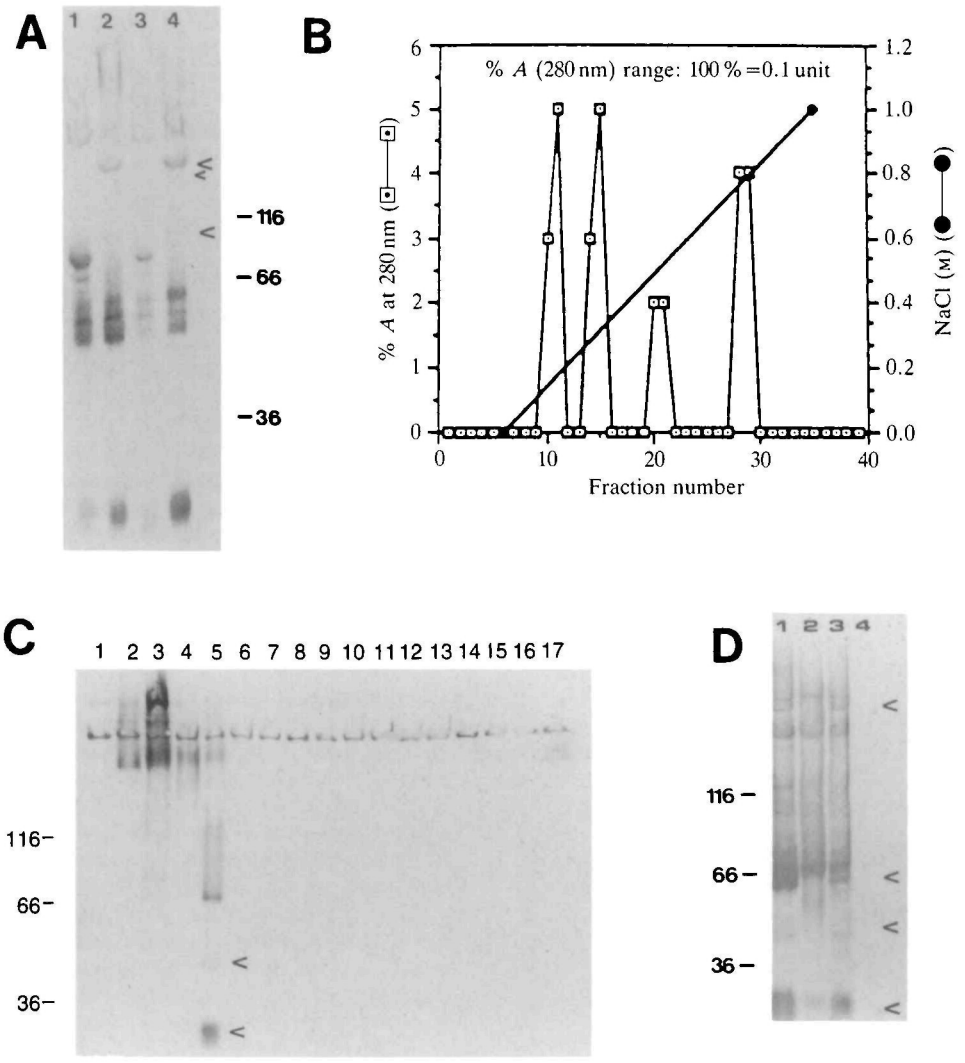

Fig. 6. Characterisation of Pp 1 spore extracts by immunoblotting and FPLC. (A) Immunoblot profile of Pp 1 fragment and also exosporium extracts after 13% SDS–PAGE, visualised with anti-Pp 1 fragment antibody. Lane 1, exosporium extract, 1% SDS; lane 2, exosporium extract, 6 M urea and 5% BME in 0.1 M sodium acetate buffer, pH 5.0; lane 3, fragment extract, 1% SDS; lane 4, fragment extract, 6 M urea, 5% BME, in 0.1 M sodium acetate buffer. Molecular weight standards ($\times 10^{-3}$) are shown on the right. The arrows show 2 bands of approximate M_r 89 and 126 ($\times 10^3$) seen in lanes 2 and 4 only. A band of approximate M_r 66×10^3 is seen in lanes 1 and 3 only as well. However, only extracts shown in lanes 2 and 4 inhibited attachment. (B) Anion-exchange chromatography of Pp 1 BME extract on a MonoQ HR 5/5 column. The column was washed and equilibrated with 20 mM Tris–HCl buffer, pH 9.0, and was eluted with a linear NaCl gradient at a 1 ml min^{-1} flow rate. Fractions 6–39 inclusive were pooled in pairs, resulting in 17×2 ml fractions, which were processed through Centricon concentrators to effect buffer exchange and concentration. (C) Immunoblot profile of the 16 fractions obtained from anion-exchange chromatography, run on a 10% SDS–PAGE gel and visualised with anti-Pp 1 fragment antibody. Lane 1, fraction 6–7 from the chromatogram; lane 2, fraction 8–9; lane 3, fraction 10–11 etc. The arrows show 2 bands of approximate M_r 24–29 and 45 ($\times 10^{-3}$) in lane 5, corresponding to fractions 14–15, which are absent from the other lanes. Only fraction 14–15 in lane 5 inhibited attachment. Molecular weight standards ($\times 10^{-3}$) are shown on the left. (D) Immunoblot profile of Pp 1 extract adsorbed onto *M. incognita* juveniles. 10% SDS–PAGE, visualised with anti-Pp 1 fragment antibody. Lane 1, spore fragment extract (sample 1 in Materials and methods); lane 2, extract after adsorption (sample 2); lane 3, cuticle-bound extract (sample 3); lane 4, control cuticle extract (sample 4). Molecular weight standards ($\times 10^{-3}$) are shown on the left. The arrows show bands of approximate M_r 28, 38, 59, and 190 ($\times 10^3$) seen in the cuticle-bound extract but not in the supernatant after adsorption.

intact exosporia are to be obtained. Williams *et al.* (1989), reported subjecting *P. penetrans* spores to sonication with glass beads and also passage through a French press, prior to catalase activity assays, but they did not describe what morphological effects, if any, these treatments had on the spores. The purity of the fragment and also exosporial preparations is shown by microscopic observation and also by biochemical assay. Our light micrographs (Fig. 1) show the fragments as dark, often horse-shoe-shaped with a defined central region where the spore core used to be, whereas the exosporial membranes are transluscent and of uniform size and shape. In addition, extracts from the same number of fragments and exosporia differ by about 27% in their ability to inhibit attachment of spores to juveniles.

The inhibition of attachment obtained with the antibodies and in particular with the monovalent Fab' fragments suggest that the antibodies interact with components on the spore surface, mediating adhesion to host cuticle. However, thin-section immunolabelling of

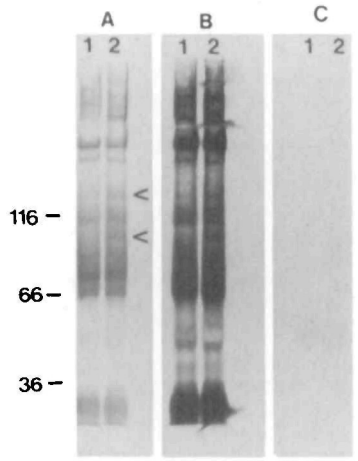

Fig. 7. Profile of Pp 1 and PNG fragment extracts after 10% SDS–PAGE, visualised in A with anti-Pp 1 fragment antibody; in B with WGA and in C with Con A. Lane 1, Pp 1 extract; lane 2, PNG extract. The arrows in A show 2 bands of approximate M_r 98 and 126×10^3, which appear in greater amounts in the PNG lane. Molecular weight standards ($\times10^{-3}$) are given on the left.

spores using these antibodies did not preferentially label any particular spore structure, and in fact it appears to have cross-reacted with the fibres and the exosporia. This suggests that the binding of the antibodies may be non-specific and/or that immunogenic epitopes present on the fibre bundles used for immunisation are abundant throughout the spore, apart from the core. Non-specificity is unlikely because the inhibition of attachment observed has been corrected for the contribution of pre-immune sera, and pre-immune sera do not show any staining after thin-section immunolabelling (data not shown).

Although the fibres surrounding the spore core are thought to facilitate the anchoring of the spore to the host cuticle (Sayre and Wergin, 1977), apart from morphological observations based on electron microscopy, no firm correlation has been made between the presence of the fibres and the ability of the spores to attach. We attempted to address this question by disrupting the fibres chemically, observing the result by electron microscopy and assaying the treated spores for their ability to attach. Spores treated with HCl, a method that removes fibres from bacterial surfaces (Handley *et al.* 1984), show extensive fibre disruption and are essentially unable to attach. Although acid extraction is a very harsh treatment, our observations do correlate the presence of intact fibres with the ability of the spores to attach. This was further substantiated when spores were treated with a urea–SDS–DTT mixture, such as the one used to solubilise type 1 fimbriae from *Escherichia coli* (Hanson and Brinton, 1988). The resulting spores again showed extensive fibre disruption coupled with a loss of attachment ability.

Since fibres thus appear to be necessary for attachment, spore fragments consisting of fibre bundles were extracted with SDS or urea–BME. The dialysed SDS extract was incapable of inhibiting attachment but the urea–BME extract and the BME extract inhibited attachment by about 80%. Similar inhibitory extracts were produced from exosporial membranes, although these extracts were not as potent as those from spore fragments, reflecting perhaps the higher concentration of adhesins in fibre bundles than in exosporial membranes. Immunoblot analysis of extracts from the two structures showed them to be identical, again reflecting the cross-reactivity observed by thin-section immunolabelling.

The spore extracts produced were analysed by immunoblotting, anion exchange chromatography and parallel attachment assays, in an attempt to identify potential adhesins. Extraction with urea–BME produced a fraction that could inhibit attachment, and one that contained two bands of M_r 89 and 126 ($\times10^3$) not present in an SDS extract that did not inhibit attachment. The two extracts were otherwise identical in immunoblot profiles. Anion-exchange chromatography produced five fractions only one of which was inhibitory and which contains bands of M_r 24–29 and 45 ($\times10^3$) not present in the non-inhibitory fractions. We propose these four species as potential adhesins because they are present in inhibitory fractions but are absent fron non-inhibitory fractions. When inhibitory extract is adsorbed onto host cuticle and the supernatant is compared with what is adsorbed onto the cuticle after washing, the supernatant is seen to be depleted in four bands that appear in the cuticle extract, of M_r 28, 38, 59 and 190 ($\times10^3$). These M_r values are approximate and electroblotting followed by lectin overlay suggests that all the components seen in the immunoblot profiles are glycosylated and contain terminal GlcNAc residues, and a species of M_r 47×10^3 also contains terminal Glc/Man residues. This glycosylation could account for the smeared appearance of some of the bands and renders estimation of M_r approximate. On the basis of this our results suggest the following species as potential adhesins: 28–29, 38–47, 59, 89, 126, and 190 kDa.

It is not unusual for a fimbriated bacterium to possess a number of adhesins. *E. coli* produces at least six types of fimbriae, all with different adhesin specificities, enabling the bacterium to attach to a number of different hosts with different receptors (Mirelman, 1986). At present we do not have data on possible proteolysis/aggregation relating the five species we propose to be *P. penetrans* potential adhesins. A quantitative difference in two species of approximate M_r 98 and 126×10^3 was observed between PNG and Pp 1 but whether or not this difference accounts for the difference in virulence between the two strains (Davies *et al.* 1988) remains to be seen.

Bird *et al.* (1989) showed that WGA and, to a lesser extent, Con A could inhibit the attachment of *P. penetrans* spores to *Meloidogyne* hosts, and that both these lectins bound onto the surface of the spores. Here we show that WGA recognises all our proposed adhesins and that Con A recognises the one of approximate M_r 47×10^3, providing an explanation for the inhibition that Bird *et al.* observed.

The observation that WGA and Con A inhibit attachment and also recognise electroblotted proteins suggests that the latter are glycosylated. Prokaryotic glycoproteins are poorly characterised, with the exception of the cell surface glycoprotein of halobacteria and also flagellins (reviewed by Lechner and Wieland, 1989), and here we provide evidence for yet another group of bacterial glycoproteins, namely the *P. penetrans* adhesin(s).

The immuno- and lectin-affinity blotting data reported here indicate that the adhesin(s) from the two strains studied are closely related. There may still be totally unrelated epitopes between Pp 1 and PNG not recognised by either antibody or lectin, but elucidation of this will have to await *in vitro* culturing and cloning studies.

We attempted to define the nature of the cuticle receptor(s) for *P. penetrans* by a combination of differential

solubilisation and enzymic digestion studies. The spores attach to cuticle components that are BME-soluble and also sensitive to tryptic digestion and deglycosylation by endoglycosidase F. BME-soluble components from *Meloidogyne* cuticle have been shown to be collagens and in the case of juveniles 7 out of the 12 collagens obtained are glycosylated, whereas none of the 9 adult female collagens are (Reddigari *et al.* 1986). These workers also showed that the BME-soluble juvenile collagens were extracted from the outermost cortical layer of the cuticle, which is also the site of *P. penetrans* attachment. Since *P. penetrans* will not attach to adult females we propose, on the basis of our observations and those of Reddigari *et al.*, that the receptor(s) for *P. penetrans* might be one or more species of stage-specific BME-soluble cortical collagens, although we do not directly show this. The partial decrease in attachment obtained after juveniles had been surface-deglycosylated with endoglycosidase F, which cleaves mannose and complex type oligosaccharides from Asn-linked glycan chains of glycoproteins (Tarentino *et al.* 1985), and the decrease obtained after tryptic digestion suggest that glycoprotein(s) with Asn-linked glycan moieties might be involved in the recognition. Partial effects after enzyme digestions, like the ones we observe, are not uncommon and could be accounted for in terms of steric hindrance (Jansson *et al.* 1984). The nature of the cuticle receptor(s) as we have defined it awaits confirmation by the actual purification of these components from cuticle.

In summary, our data suggest that BME-soluble components from spores appear to be involved in attachment (urea/BME-solubilised spores have disintegrated fibres and do not attach, and BME extracts from fibre bundles inhibit attachment), and these components are glycosylated and recognised by WGA and Con A. The juvenile cuticle components involved in attachment are also BME-soluble, suggesting that they might be collagens, and are sensitive to trypsin and endoglycosidase F.

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