

Adhesion of *Pasteuria penetrans* to the cuticle of root-knot nematodes (*Meloidogyne* spp.) inhibited by fibronectin: a study of electrostatic and hydrophobic interactions

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(Received 22 September 1995; revised 4 November 1995 and 20 December 1995; accepted 20 December 1995)

SUMMARY

Pasteuria penetrans is a bacterium with the potential to control plant-parasitic nematode populations; the mechanism of spore adhesion, however, is poorly understood. Attachment assays were performed in media supplemented with various concentrations of fibronectin and in the presence and absence of KSCN which modulates hydrophobic interactions. A reduction in the strength of the hydrophobic effect prevented spores from binding to the cuticle as did 20 µg/ml fibronectin. It was also shown directly utilizing a newly-developed technique which utilizes 3-hexadecanoyl-7-hydro-coumarin as an indicator of the fibronectin binding to the spore surface that the presence of KSCN prohibited binding. This effect was interpreted to indicate that the reduction of binding was the direct result of the influence of hydrophobic interactions between the fibronectin and the spore surface. Western blot analysis of cuticle extracts of *Meloidogyne incognita* and *Caenorhabditis elegans* revealed small amounts of fibronectin to be present. Fibronectin, or a similar receptor, present in the cuticle could be responsible for the adhesion of *P. penetrans* by hydrophobic interactions.

Key words: bacterial adhesion, fibronectin, extracellular matrix, Nematoda, *Meloidogyne*, *Pasteuria*, cuticle, biological control, electrostatic potential, hydrophobic surface.

INTRODUCTION

Plant-parasitic nematodes are important pests which are largely controlled through the application of nematicides and by growing non-host crops (Sasser & Freckman, 1987). Due to environmental concerns over nematicides, however, alternative methods are urgently being sought (Thomason, 1987). *Pasteuria penetrans*, a Gram-positive bacterium, has been identified as a potential biological control agent of root-knot nematodes (Stirling, 1991; Oostendorp, Dickson & Mitchell, 1991), the most important nematode pests. The *Pasteuria* group of bacteria are obligate parasites which produce resistant endospores. The first stage in the infection process is when 2nd-stage juveniles, after hatching from eggs, migrate through the soil and become encumbered with spores of the bacterium. The spores subsequently germinate and proliferate throughout the pseudocoelom of the developing nematode prohibiting the nematode from producing eggs (Sayre & Starr, 1988, 1989). There are two main factors prohibiting the use of *Pasteuria* as a biocontrol agent; its host specificity and the inability to mass culture the bacterium (Stirling, 1991). In standard attachment assays, one population of spores of the

bacterium will adhere to one population of nematode better than another (Stirling, 1991; Davies, 1994). Immunofluorescence and enzyme digestion studies of the cuticle surface of 2nd-stage juveniles of root-knot nematodes have shown interspecific differences in the cuticle related to the adhesion of spores of *Pasteuria* and a carbohydrate/protein interaction may be involved (Davies & Danks, 1992, 1993). More recent studies with a panel of monoclonal antibodies raised against the surface of *Pasteuria* spores have demonstrated a high degree of heterogeneity both in the surface of the spore and also the nematode cuticle (Davies, Pearson & Redden, 1994). In order to control nematode populations it is important to ensure that the nematode pest is susceptible to the bacterium being applied (Channer & Gowen, 1992) and, therefore, an understanding of the biochemical mechanism of attachment is essential to being able to select effective spore populations for biological control. The recent technical developments in nematode fermentation (Georgis & Manweiler, 1994) and nematode transformation (Grant, 1992) may lead to a potential mechanism for *in vivo* mass culture of the bacterium on an alternative host nematode; an understanding of the mechanism by which spores adhere to the nematode cuticle would also be fundamental to such an approach. Recent studies using a newly-developed fluorescence technique indicate that the adhesion of

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Pasteuria spores is probably dependent on a balance between electrostatic and non-polar interactions between the nematode cuticle and the surface of the spores; the spores of *P. penetrans* were demonstrated to possess electrostatic properties which may be modulated by the presence of different concentrations of electrolyte (Afolabi, Davies & O'Shea, 1995). It was also concluded that the spore surface is equipped with other influential non-polar properties and that both polar and non-polar properties may play some role in the adhesion mechanism with a prospective host surface.

The cuticle of animal-parasitic nematodes and to a lesser extent plant-parasitic nematodes has generated much interest as it is the interface between parasite and host and may be important in the elicitation of immune responses (Blaxter *et al.* 1992; Politz & Philipp, 1992). The cuticle is a component of the extracellular matrix secreted by the hypodermis and as such would be expected to contain collagens, and other large glycoproteins such as laminin, fibronectin and nidogen (Kramer, 1994). Although much research has been undertaken to investigate cuticular collagen and cuticlin much less is known about the other associated antigens (Politz & Philipp, 1992).

Fibronectin has been found to have an important role in the pathogenicity of many bacteria (Hasty *et al.* 1989; Courtney, Hasty & Ofek, 1990) by binding to microbial surface components which specifically recognize adhesive matrix molecules (Patti *et al.* 1994). It is a member of a large family of heterodimeric glycoproteins ($M_r \sim 440$ kDa) present in mammalian circulatory fluids, the extracellular matrix, basement membranes and cell surfaces of many animals, and is capable of interacting with a wide variety of other macromolecules such as actin, collagen, fibrin, heparin and gelatin (Carson, 1989). Much research has been aimed at understanding the adhesion of the medically important Gram-positive *Staphylococci* and *Streptococci* groups of bacteria in relation to fibronectin. The mechanism by which group A *Streptococci* are thought to adhere to host cells is through hydrophobic interactions with fibronectin and as little as 1 $\mu\text{g/ml}$ of fibronectin can block the attachment of *Streptococci* in attachment assays (Courtney *et al.* 1990). The present study seeks to identify the importance that electrostatic and non-polar interactions may play in adhesion, and to determine the particular role that fibronectin may play in the adhesion mechanism using fluorimetric and immunological techniques.

MATERIALS AND METHODS

Nematodes and Pasteuria penetrans endospores

Second-stage juveniles of *Meloidogyne incognita* (Race 2, originating from North Carolina State University), routinely cultured on tomato plants cv.

Pixie, in a peat/sand (1:1, v/v) compost, grown in a glasshouse at 25 °C, were hatched in tap water from egg masses, using a hatching tray, and counted in a Hawksley nematode counting slide (Hooper, 1986). *Caenorhabditis elegans* (wild type, strain N2) were kindly provided by Dr Julie Ahringer, MRC Laboratory of Molecular Biology, Cambridge, UK) were grown on agar plates with *Escherichia coli* OP50 (Brenner, 1974); live nematodes, of mixed stages, were washed off the agar plates with PBS and washed by repeated centrifugation and resuspension in PBS to remove *E. coli* and counted as above. Endospores of *Pasteuria penetrans* (population PP1 originating from Dr Simon Gowen, University of Reading, UK) were collected by homogenizing infected females of *M. incognita* (Race 2) which had been dissected from galls of tomato roots (Stirling & Wachtel, 1980). The spores were filtered through a 20 μm nylon filter, washed with 50 ml of Millipore water (Millipore Ltd, UK) and dialysed against millipore water for 2 to 3 h. The spores were counted in a haemocytometer and a stock suspension of 5×10^6 spores/ml was stored at 4 °C.

Fluorometric studies of the spore surface using HEXCO

Characterization of the electrostatic nature of the endospore surface using 3-hexadecanoyl-7-hydrocoumarin (HEXCO) has been described in detail (Afolabi *et al.* 1995) and, using this technique, it is possible to study the effect of fibronectin on the hydrophobic properties of the surface of the spore by exposure to thiocyanate, which is an inhibitor of hydrophobic interactions (Hatefi & Hanstein, 1969). A fluorescence quenching curve for HEXCO bound to endospores was made by titration with potassium thiocyanate (KSCN). Stock solutions of 100 mM KCl and 2 M KSCN in 5 mM HEPES at pH 7.05 were prepared and 10^6 spores were incubated with 25 μM HEXCO in 100 mM KCl for 1 h at 37 °C. The spores labelled with HEXCO were eluted using a PD-10 column to remove unbound HEXCO. Different aliquots of 2 M KSCN were added to obtain different concentrations ranging from 0 to 100 mM KSCN and changes in fluorescence intensity were observed with an excitation wavelength of 381 nm and an emission wavelength of 438 nm. A second titration was performed against different concentrations of purified human fibronectin (kindly provided by Dr Ian Critchley of Smith Kline & Beecham, Brockham Park, Betchworth, Surrey, UK) in the presence and absence of thiocyanate; stock solutions were 1 mg/ml fibronectin and 80 mM KSCN in 1 mM HEPES at pH 7.05. *Pasteuria* spores (5×10^5) were incubated with 25 μM HEXCO in 1 mM HEPES and 80 mM KSCN respectively for 1 h at 37 °C. The spores were eluted to remove unbound

HEXCO, as described above, and different concentrations of fibronectin, ranging from 0 to 26 $\mu\text{g/ml}$, were titrated against HEXCO-labelled spores in 1 mM HEPES and 80 mM KSCN. Fluorescence intensity changes were measured as before.

Endospore adhesion assays

Stock solutions of 20 $\mu\text{g/ml}$ fibronectin 5 mM and 100 mM KCl and 100 mM KSCN were made up in 5 mM HEPES at pH 7.05. Endospores (10^6) were concentrated by centrifugation (1000 g for 5 min at room temperature) in 4 siliconized Eppendorf tubes and treated by resuspension in 200 μl of 20 $\mu\text{g/ml}$ fibronectin, 5 mM and 100 mM KCl and 100 mM KSCN respectively. Each of the treatments was serially diluted to give a range of spore concentrations from 10^6 to 10^3 spores/ml which were incubated overnight at 4 °C. Freshly hatched 2nd-stage juveniles (1000/ml) were concentrated by centrifugation and resuspended in each of the treatments as described above and incubated for 1 h at room temperature. Following incubation, 100 μl of the treated nematode suspensions were added to each of the respective spore treatments and an attachment test performed by centrifuging the treated 2nd-stage juveniles together with the treated *Pasteuria* spores in siliconized Eppendorf tubes for 2 min (Hewlett & Dickson, 1993). The spores adhering to the nematodes were counted on 20 individual 2nd-stage juveniles using a high powered microscope ($\times 400$).

Immunization, monoclonal antibody production and screening

Two Balb/C mice were immunized with 4000 freshly hatched 2nd-stage juveniles (J2) of *M. incognita* (Race 2). Four weeks later a second immunization of 4000 J2 was administered. After a further 3 weeks blood samples were obtained and tested for immunological response by ELISA using microtitre plates (Nunc, Maxisorb) coated with soluble protein (2 $\mu\text{g/ml}$) extracted from 2nd-stage juveniles (Davies & Lander, 1992). A mouse exhibiting a strong immunological response was subsequently boosted with 2000 J2 4 days prior to fusion. All injections were administered into the peritoneum in 0.5 ml of PBS. Myeloma cells (SP2/0-Ag) were grown in DMEM medium supplemented with 20% foetal bovine serum (20-DMEM), 1 mM glutamine, 1% $100\times$ Pen/Strep (all medium and reagents supplied by Gibco). A fusion was made, between SP2/0-Ag and splenocytes in the presence of 30% polyethylene glycol (PEG 1500 Boehringer, Mannheim) by spinning (Harlow & Lane, 1988) and the cells were selected in DMEM containing 1% $50\times$ HAZA

(hypoxanthine-azaserine, Sigma) in the presence of peritoneal macrophages. Hybridoma cell lines were screened by ELISA using microtitre plates (Nunc, Maxisorb) coated with 2 $\mu\text{g/ml}$ fibronectin (Davies & Lander, 1992). Hybridoma cell lines producing antibodies were cloned by limiting dilution and retested.

Polyacrylamide gel electrophoresis and immunoblotting of cuticle extracts

Freshly hatched 2nd-stage juveniles (40000) of *M. incognita* or mixed stages of *C. elegans* grown on OP50 were washed, as previously described, and were pelleted by centrifugation. Protein was extracted by incubating nematodes at 100 °C for 2 min in 500 μl of extraction buffer (0.125 M Tris-HCl, pH 6.8, containing 1% SDS and 5% 2-mercaptoethanol (2-ME) and 10% glycerol (Hemmer *et al.* 1991)); the nematodes were removed by centrifugation (1000 g for 2 min) and the supernatant fraction was stored at -45 °C. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) was performed using a 10% (w/v) separating gel (pH 8.8) and 4% (w/v) stacking gel (pH 6.8). Cuticle extracts (100 μl) were diluted 1:1 (v/v) with sample buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2% (v/v) 2-ME, 10% (v/v) glycerol and 0.002% (w/v) bromophenol blue) and heated for 2 min at 100 °C before 20 μl of the solution was loaded onto the stacking gel. Pre-stained SDS-PAGE molecular weight markers (Sigma; Cat No SDS-7B) were run on each gel. After electrophoresis, the gel slabs were either fixed (40% methanol/10% acetic acid) and silver stained (Bio-Rad Silver Stain) or electroblotted onto nitrocellulose membranes (Bio-Rad) in continuous buffer (Davies & Lander, 1992) using a Semi-phor[®] blotting system (Hoeffer Scientific Instruments). Membranes were blocked with 2% BSA in PBST (2% (w/v) bovine serum albumen and 0.05% (v/v) Tween in PBS).

Following transfer and blocking, the blots were probed with either a rabbit anti-human fibronectin polyclonal antibody (Sigma; Cat No F-3648) or one of two tissue culture supernatants that were positive to human fibronectin, washed ($\times 2$), and then incubated with either goat anti-rabbit (Sigma; Cat No A-8025) or rabbit anti-mouse (Sigma; Cat No A-2418) IgG whole molecule alkaline phosphatase conjugate (1:500 in 2% BSA in PBST for 2 h) as appropriate. After a further 2 washes, the membranes were incubated in alkaline phosphate substrate (0.03% (w/v) nitroblue tetrazolium, 0.02% (w/v) 5-bromo-4-chloro-3-indolyl phosphate, in Tris-HCl, pH 9.5, substrate buffer); the reaction was terminated with several washes in distilled water.

RESULTS

The effects of the nature and concentration of various electrolytes on the adhesion of P. penetrans to 2nd-stage juveniles (J2)

The effect of electrolyte concentration on the ability of spores to adhere to J2s is shown in Fig. 1. There is a clear difference between the adherence profiles determined in 5 mM and 100 mM KCl suggesting that electrostatic repulsion is much larger at lower salt concentrations and dramatically reduces the attachment of the spores to the J2s. At the higher salt concentration (100 mM KCl), with the electrostatic repulsion much reduced, the attractive forces are clearly dominating. From this experiment, together with earlier studies (Afolabi *et al.* 1995), it would seem that 2 opposing interactions are involved in the attachment mechanism of *P. penetrans* to the nematode surface. The first consists of repulsive electrostatic interactions, which are influenced by the ambient electrolyte concentration, and the second are attractive forces, which presumably have their origin in the hydrophobic effect.

It is possible to identify the putative role that such hydrophobic interactions may play by performing similar experiments to that in Fig. 1 but comparing the binding profile of the spores to J2s in the absence and presence of KSCN. This reagent, although water-soluble, possesses a very high entropy of hydration (Hatefi & Hanstein, 1969) and, therefore, will interfere strongly with hydrophobic interactions in aqueous solution. Thus, binding experiments performed with this electrolyte may be used to dissect the role that hydrophobic interactions may play in an adhesion assay. In our experience, about 100 mM KSCN is necessary to produce a clear effect. This means, however, that the effective electrolyte concentration is the same as the higher concentration shown in Fig. 1, and in order to make an appropriate comparison, the binding profile in the presence of KSCN should be compared to that performed in 100 mM KCl. This data is shown in Fig. 2 and illustrates that at the same electrolyte concentration the binding of the *P. penetrans* spores to the J2s is much reduced in the presence of 100 mM KSCN. Thus it would appear that hydrophobic interactions must be involved and actively promote attachment.

The effects of the nature and concentration of various electrolytes on the binding of fibronectin to spores of P. penetrans

We have suggested that fibronectin may play a role in the attachment mechanism of the spores of *P. penetrans* to the nematode surface. It is possible to test this hypothesis using a newly-developed fluorescence technique outlined by Wall, Ayoub & O'Shea (1995) as directed towards *P. penetrans* by Afolabi *et al.* (1995). Essentially, this involves the

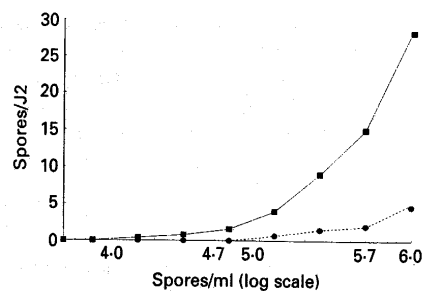


Fig. 1. Spore attachment assay in which 2nd-stage juveniles (J2) were exposed to different concentrations of spores in 5 mM (●) and 100 mM (■) KCl (standard error less than ± 2 spores).

labelling of a biological surface with a fluorescent probe which is responsive to the electrostatic field which exists on the surfaces of virtually all biological particles. We have already reported that one such probe, known as HEXCO, is highly suitable for use with bacterial spores (Afolabi *et al.* 1995) and Fig. 3 shows the results of such a study. For these experiments, spores are labelled with indicator-amounts of HEXCO and then challenged with increasing amounts of a macromolecule suspected to be involved in the adhesion. In Fig. 3, fibronectin was titrated into the spore suspension and the resulting fluorescence changes plotted as the ordinate; the results are indicative of the binding of fibronectin at the concentrations indicated. Figure 3 also illustrates the effects of KSCN on the ability of the spores to bind fibronectin; there is a clear reduction in the amount bound in the presence of KSCN compared to the amount bound in 100 mM KCl. It is known that, at the concentrations we have employed, 100 mM KSCN does not result in the denaturation of fibronectin (Hatefi & Hanstein, 1995). This implies, therefore, that hydrophobic interactions appear to be involved in the binding of the fibronectin molecule to the spore surface.

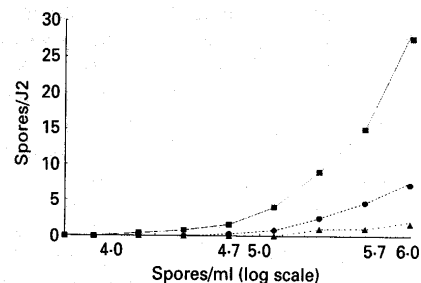


Fig. 2. Spore attachment assay in which 2nd-stage juveniles (J2) were exposed to different concentrations of spores in 100 mM KCl (■), 100 mM KSCN (●) and 20 µg/ml fibronectin (▲) (standard error less than ± 2 spores).

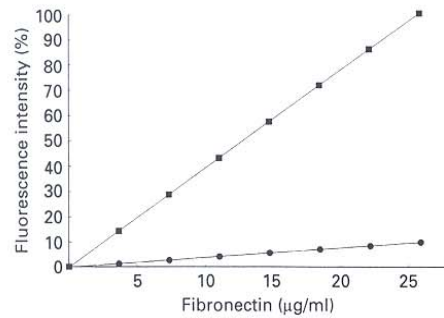


Fig. 3. Fluorescence intensity of HEXCO plotted against concentration of fibronectin in a *Pasteuria penetrans* spore suspension in the presence (●) and absence (■) of 100 mM KSCN.

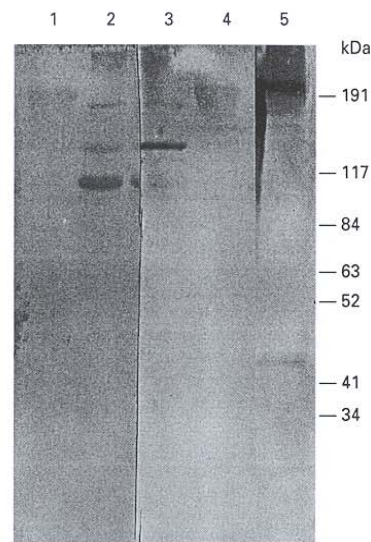


Fig. 4. Western blot analysis of human fibronectin (lanes 1 and 4), and root-knot-nematode cuticle extract (lanes 2, 3 and 5) transferred from a 10% SDS-PAGE gel probed with monoclonal antibodies 6.2 (lanes 1 and 2) and 34.2 (lanes 3 and 4) and with an anti-fibronectin polyclonal antibody (lane 5).

The effects of fibronectin on the adhesion of spores of *P. penetrans* to 2nd-stage nematode juveniles

Fibronectin is shown in Fig. 3 to bind to spores of *P. penetrans*. It was anticipated, therefore, that the presence of this molecule in the spore-nematode binding assay would affect the amount of spores binding to J2s. This was indeed found to be the case, with 20 µg/ml fibronectin causing an even greater reduction in spore binding than 100 mM KSCN (Fig. 2).

Presence of fibronectin in cuticle extracts of nematodes

Western blot analysis of nematode cuticle extract of *M. incognita* with the anti-fibronectin polyclonal

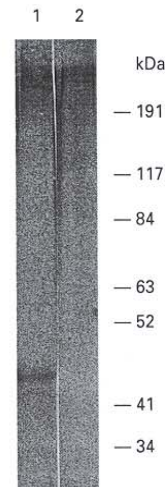


Fig. 5. Western blot analysis of root-knot nematode cuticle extract (lane 1) and *Caenorhabditis elegans* cuticle extract (lane 2) transferred from a 10% SDS-PAGE gel probed with an anti-fibronectin polyclonal antibody.

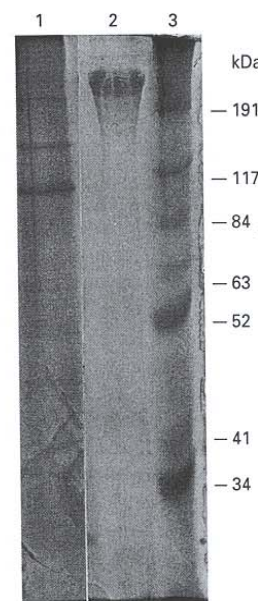


Fig. 6. SDS-PAGE mini-gel (10%) of root-knot nematode cuticle extract (lane 1) and human fibronectin (lane 2) with SDS-PAGE molecular weight markers (lane 3).

antibody shows each dimer of fibronectin with M_r of ~ 220 kDa; the same antibody also recognizes a smaller antigen with M_r ~ 45 kDa (Fig. 4). Similar results were obtained with *C. elegans* cuticle extract; however, the smaller antigen had M_r ~ 58 kDa (Fig. 5). The two monoclonal antibodies each recognize human fibronectin but do not detect the presence of the nematode fibronectin (Fig. 4). A silver-stained

gel of cuticle extract does not reveal fibronectin either, suggesting it is not present in large amounts (Fig. 6). Monoclonal antibody 6.2 recognizes an antigen with $M_r \sim 117$ kDa and also recognizes to a lesser extent antigens with $M_r \sim 154$ and 190 kDa (Fig. 4). Similarly, monoclonal antibody 32.4 recognizes an antigen at $M_r \sim 154$ kDa and also recognizes to a lesser extent the antigens with $M_r \sim 117$ and 190 kDa recognized by monoclonal antibody 6.2 (Fig. 4).

DISCUSSION

The biochemical mechanism by which spores of *P. penetrans* adhere to nematode cuticle is poorly understood. Immunological studies by Persidis *et al.* (1992) and Davies (1994) have identified putative adhesins and receptors on the spore and nematode cuticle respectively. Complete inhibition of attachment by the antibodies raised against spores, however, was never obtained, suggesting that more than one mechanism or process is involved in the attachment mechanism.

More recent data suggested that electrostatic repulsive forces had to be outweighed by other attractive forces and it was suggested that hydrophobic forces could be important (Afolabi *et al.* 1995). The results reported here show that the removal of hydrophobic forces by the presence of KSCN greatly reduced the ability of spores to bind to nematode cuticle and that fibronectin, which is known to bind to *Streptococci* through hydrophobic interactions (Courtney *et al.* 1990), almost totally prohibited spore binding. This result was further strengthened by the observation that, using HEXCO as an extrinsic probe, a linear relationship was produced between fluorescence intensity and fibronectin concentration; the magnitude of which was greatly reduced in the presence of KSCN.

It was particularly interesting to find fibronectin present in cuticle extracts in Western blot analysis using an anti-human fibronectin polyclonal antibody. It was not observed on the silver-stained gel, or during immunolocalization studies, however, suggesting that it was only present at a very low concentration. There is very little information regarding fibronectin in nematodes, but recent DNA studies of twitchin, a 753 kDa polypeptide located in the muscle of *Caenorhabditis elegans*, has shown it to consist of multiple copies of fibronectin (Moreman *et al.* 1988; Benian *et al.* 1989; Benian, Hernault & Morris, 1993); the fibronectin that was observed in cuticle extracts of *M. incognita*, therefore, was probably a motif of a larger molecule present in the cuticle and not readily accessible. The two monoclonal antibodies, 6.2 and 32.4, recognize 3 antigens of ~ 117 , 154 and 190 kDa which show antigenic similarity to human fibronectin. These same antigens have been observed in purified root-

knot cuticle extracts by other workers and shown to be digested by collagenase (Reddigari *et al.* 1986). They are also recognized by Con A and possibly contain terminal mannose residues; one of these antigens with $M_r \sim 190$ kDa, together with another at $M_r \sim 45$ kDa, have also been shown to bind *Pasteuria* spore extract (Davies, 1994). Significantly, the anti-human fibronectin antibody also recognizes a antigens in the cuticle extract of *M. incognita* and *C. elegans* with $M_r \sim 45$ and 58 kDa respectively.

The analysis of the extracellular matrix of nematodes, of which the cuticle is a component, is in its infancy and the majority of studies have concentrated on cuticular collagen; many other components are yet to be identified (Kramer, 1994). The use of adhesive hyperparasites, of which *Pasteuria* is an excellent example, offers a unique approach to the study of host-parasite interactions and the molecules which may be involved. This present paper reports an investigation into the biochemical nature of spore attachment using both physicochemical and immunological techniques; the results suggest that fibronectin, or related extracellular matrix components present in the cuticle, could be responsible for the adhesion of *P. penetrans* spores by hydrophobic interactions.

IACR receives grant aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom but the work reported in this paper was supported mainly by the European Science Foundation at both the University of Essex and IACR and in part by EU Project no. T53 CT92-0098.

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