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Modulation of spore adhesion of the hyperparasitic bacterium *Pasteuria penetrans* to nematode cuticle

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S.B. SHARMA AND K.G. DAVIES. 1997. Monoclonal antibodies (mAb) raised to the cuticule surface of second-stage juveniles (J2) of the nematode *Heterodera cajani* were partially characterized by immunofluorescence and Western blot analysis. Five antigens with relative molecular weights (M_r) 55, 80, 110, 180 and 210 kDa were identified with six mAb. *Pasteuria* spores, originating from the same population of *H. cajani* to which the antibodies were raised, were tested for their ability to attach to J2, which had been pretreated with each of the mAb. Monoclonal antibody HC/129 was found to reduce spore attachment by 42%, whereas HC/145 increased spore attachment by 124%. This is the first record of an antibody binding to the cuticle and increasing spore attachment, and suggests that components of the cuticle involved in inhibiting spore attachment may be masking the *Pasteuria* receptor present on the cuticle.

INTRODUCTION

The cyst nematode *Heterodera cajani* is one of the most important nematode pests of pigeonpea in the semi-arid regions of tropical India, and resource poor farmers need economical and environmentally sound management tactics (Sharma *et al.* 1992). *Pasteuria penetrans* is an obligate Gram-positive bacterium with the potential to control plant-parasitic nematodes (Stirling 1991). The majority of research with *P. penetrans* has focused on its ability to control root-knot nematodes (Stirling 1984; Oostendorp *et al.* 1991) and cyst nematodes (Nishizawa 1986; Davies *et al.* 1990). However, one of the major problems in developing this organism as a biological control agent is its host specificity, with one population of the bacterium being able to adhere to and infect one population of a nematode species but not another (Stirling 1991; Davies *et al.* 1991).

Immunological studies with polyclonal antibodies found that interspecific variation in the cuticle of root-knot nematodes was related to the ability of the bacterium to attach to the nematode (Davies and Danks 1992). Recently, a population of *Pasteuria* has been described that adheres to the cuticle of second-stage juveniles (J2) of *H. cajani*, infects developing females, and has a life cycle similar to *P. penetrans* (Sharma and Davies 1996a). A comparison between this population of

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Pasteuria isolated from *H. cajani* and another population of *Pasteuria* recovered from root-knot nematodes from the same field site showed that the host range of the *H. cajani Pasteuria* spores was much broader than those of the host range of *Pasteuria* from the root-knot nematode population (Sharma and Davies 1996b). Immunological heterogeneity in the surface of *Pasteuria* spores was related to their affinity for different populations of root-knot nematode and it was conjectured that this heterogeneity reflected heterogeneity in the nematode cuticle (Davies *et al.* 1994). This preliminary investigation describes the production of monoclonal antibodies (mAb) to the cuticle of J2 of a population of *H. cajani* and reports their effect on the attachment of spores of *Pasteuria*.

MATERIALS AND METHODS

Nematodes and bacterial endospores

Pasteuria was collected from females of *H. cajani* and designated PHc. Nematode females were recovered from soil samples collected from a pigeonpea field at the research farm of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Asia Centre, Andhra Pradesh, India (Sharma and Sharma 1989). The bacterial population was cultured for over a year on *H. cajani* growing on pigeonpea cultivar ICPL 87 in 15 cm diameter pots in a glasshouse.

Spores of *Pasteuria* were obtained by crushing infected *H. cajani* females in 0·5 ml of water in 1·5 ml Eppendorf tubes, and counted using a haemocytometer.

Immunization and antibody production

Mice (BALB/c) were immunized with 5000 J2, recovered from cysts of *H. cajani*, in 500 μl of phosphate-buffered saline (PBS). After 3 weeks, 50 μl blood samples were obtained and tested for their ability to recognize cryostat sections of *H. cajani* J2 by immunofluorescence (see below). A mouse exhibiting a strong immunological response to the nematode was subsequently boosted with J2 4 d prior to fusion. All injections were administered into the peritoneum in 0·5 ml PBS. Myeloma cells (SP2/0-Ag) were grown in DMEM medium supplemented with 20% foetal bovine serum (20-DMEM), 1 mmol l^{-1} glutamine and 1% 100 × penicillin/streptomycin (all medium and reagents supplied by Gibco, Grand Island, NY, USA). SP2/0-Ag and splenocytes were fused in the presence of 30% polyethylene glycol (PEG 1500; Boehringer-Mannheim, Mannheim, Germany) by spinning (Harlow and Lane 1988), and the cells were selected in DMEM containing 1% 50 × hypoxanthine-azaserine (HAZA; Sigma, St Louis, MO, USA) in the presence of peritoneal macrophages.

ELISA, immunofluorescence screening and cloning

J2 (approximately 40 000) were crushed in 500 μl PBS, spun in a centrifuge (10 000 g, 5 min), the supernatant fluid removed, and a BioRad (BioRad Laboratories, Hemel Hempstead, UK) protein assay performed according to manufacturer's instructions. The supernatant fluid was then stored at −40 °C until required. Microtitre plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with *H. cajani* protein at 2 μg ml^{-1} and the hybridoma tissue culture supernatants tested for antibody production by ELISA according to Davies and Lander (1992). Cryostat sections of *H. cajani* J2 were prepared using an aluminium capsule containing Bright Cryo-M-Bed mounting medium (Bright Instruments Ltd, Huntingdon, UK) according to the manufacturer's instructions. After the block containing the nematodes was frozen, it was stored at −40 °C. Sections of nematodes were cut using a microtome (Bright Instruments Ltd) then placed on slides precoated with poly L-lysine (Harlow and Lane 1988). Sections were fixed in cold acetone (10 min) and allowed to air dry. Sections were stored in the dark at −40 °C. Immunofluorescence was done by washing the sections in PBS (three times), blocking in 20-DMEM for 30 min, and incubating in hybridoma tissue culture supernatant fluid for 2 h. The sections were then rewashed (three times) and incubated for 2 h in goat anti-mouse antiserum (1:50) conjugated to fluorescein isothiocyanate (FITC; Sigma) at 37 °C. After incubation the sections were again washed (three times)

and mounted in Citifluor™ (Agar Scientific, Stansted, UK). All preparations were examined using an Olympus BH-2 microscope fitted with an epifluorescence illumination with a 455 nm excitation filter and a 520 nm barrier filter. Hybridoma cell lines producing antibodies positive to the nematode were cloned by limiting dilution (Harlow and Lane 1988) and retested.

Polyacrylamide gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS–PAGE) was performed using a 12% (w/v) separating gel (pH 8·8) and 4% (w/v) stacking gel (pH 6·8). Cuticle extracts (100 μl) were prepared (Davies and Danks 1992) and diluted 1:1 (v/v) with sample buffer (50 mmol l^{-1} Tris/HCl, pH 6·8, 2% SDS w/v, 2% 2-mercaptoethanol, 10% glycerol and 0·002% bromophenol blue w/v) and heated for 2 min at 100 °C before 20 μl of the solution was loaded onto the stacking gel. Prestained SDS–PAGE molecular weight markers (number SDS-7B; Sigma) were run on each gel. After electrophoresis, the gel slabs were electroblotted onto nitrocellulose membranes (Bio-Rad) in continuous buffer (Davies and Lander 1992) using a Semiphor™ blotting system (Hoeffer Scientific Instruments, Newcastle-under-Lyme, UK). Membranes were blocked with 2% bovine serum albumin (BSA) in PBST (2% BSA w/v and 0·05% v/v Tween in PBS).
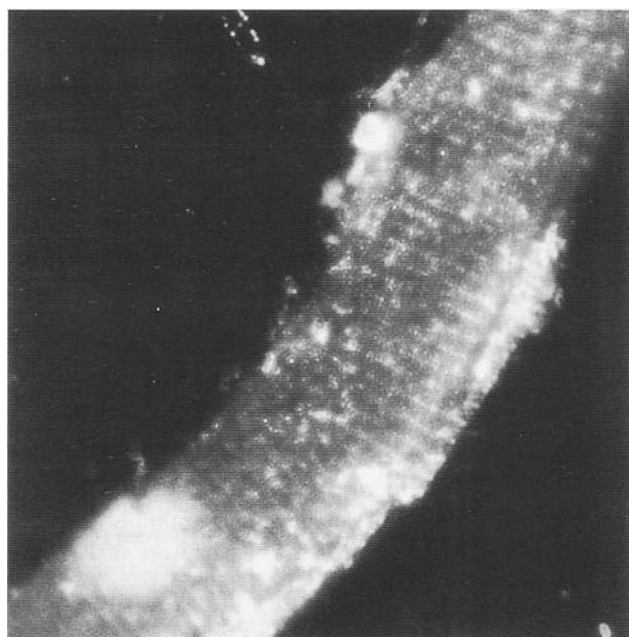
Effect of mAb on *Pasteuria* attachment

Live J2 of *H. cajani* (approximately 500) were then transferred to wells in a Multiscreen™ filtration plate (0·45 μm, hydrophilic) and incubated with 100 μl of tissue culture supernatant fluid containing a selected mAb for 2 h at 37 °C. The nematodes were then washed by the addition of 100 μl PBS followed by vacuum filtration, and the process repeated three times. The treated nematodes were suspended in 100 μl PBS and transferred to an Eppendorf tube together with 200 μl of a suspension of PHc spores (5 × 10^5 ml^{-1}). An attachment test was done using the centrifugation technique described by Hewlett and Dickson (1993). The spore encumbered nematodes were removed from the Eppendorf tubes and mounted on slides for microscopical examination (× 400); 14 nematodes were selected at random and the number of spores adhering to each J2 was recorded. The data were analysed by analysis of variance using GENSTAT (1987)

RESULTS AND DISCUSSION

The fusion resulted in the production of 182 cell lines that were able to produce mAb when tested by ELISA against homogenates of *H. cajani* J2. Further testing of these positive

cell lines showed that only 7% were positive in immuno-fluorescence assays, with 3% recognizing the cuticle or body wall, and 2% or less labelling the intestine, pharyngeal glands, amphids and stylet. Several of the mAb to the cuticle revealed the annulations (Fig. 1) and these were tested for their effect on the attachment of spores of *P. penetrans*. The effect of exposing J2 to the mAb before undertaking spore attachment tests was variable (Table 1); most of the cell lines did not have a significant effect (HC/11, HC/66, HC/146 and HC/179). However, cell line HC/129 produced a 42% reduction in attachment, while HC/145 produced an increase in attachment of 124%. Western blot analysis of these mAb (Fig. 2) showed that HC/129, which reduced attachment, recognized antigens with an M_r of 180 and 55 kDa, the same antigens as those recognized by HC/11, which had no effect on attachment. Monoclonal antibody HC/145, which increased attachment, recognized antigens of M_r 210 and 80 kDa. Monoclonal antibodies HC/146 and HC/179 recognized single antigens with an of M_r 80 and 110 kDa, respectively; pretreatment of J2 with these antibodies increased spore attachment by 17% and 28%, respectively, but these differences were not statistically significant.

The cuticle acts as the interface between the nematode and its environment and is a selectively permeable, physiologically active barrier that serves as an exoskeleton. The cuticle surface of nematodes is thought to be important in the ability of

Table 1 The mean number of spores of *Pasteuria penetrans* adhering to second-stage juveniles (J2) of *Heterodera cajani* ($n = 14$) in a standard attachment assay in which juveniles were pretreated with several monoclonal antibodies

Treatment	Number of spores per J2
Control TCS*	20·7
Control no spores	0·2
HC/11	19·4
HC/66	26·5
HC/129	12·1[a]
HC/145	46·4[b]
HC/146	24·2
HC/179	26·5

*TCS, tissue culture supernatant fluid containing 20% bovine foetal calf serum.
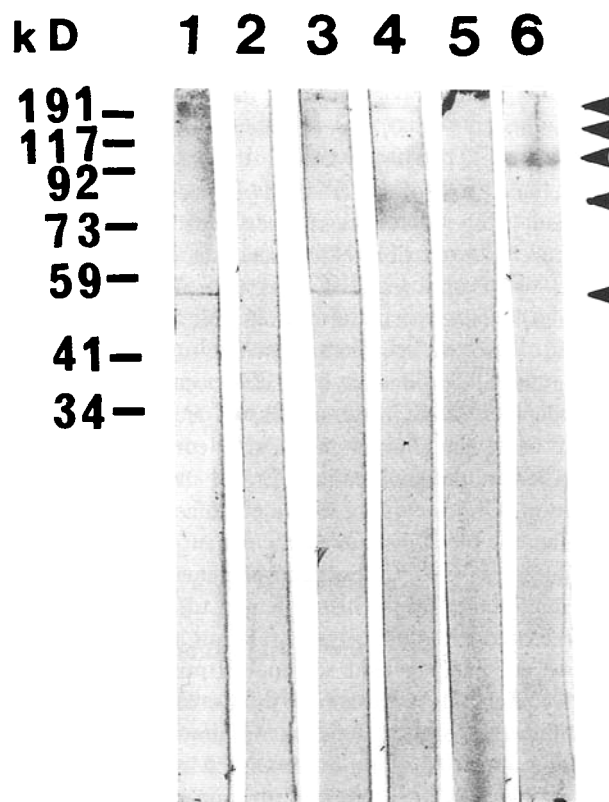ANOVA $P < 0·001$; SED $= -8·4$; [a]significant reduction; [b]significant increase.

Fig. 1 Indirect immunofluorescence of *Heterodera cajani* cuticle using monoclonal antibody HC/145, revealing cuticular annulations; preincubation of the nematode with this antibody led to an increase in attachment of spores of *Pasteuria* following subsequent exposure to spores

Fig. 2 Western blot analysis of cuticle extract from *Heterodera cajani* probed with monoclonal antibodies HC/11, HC/66, HC/129, HC/145, HC/146 and HC/179 in lanes 1–6, respectively; arrows indicate position of bands

these parasites to infect animals (Despommier *et al.* 1990; Vassilatis *et al.* 1992) and plants (Spiegel and McClure 1995; Gravato Nobre *et al.* 1995) and in hyperparasitic infection by fungi (Tunlid *et al.* 1992) and bacteria (Davies and Danks 1992). Earlier work with polyclonal antibodies to the surface of root-knot nematodes showed that the antibodies reduced the ability of spores to bind to the nematode, suggesting that they blocked the cuticle receptor (Davies and Danks 1992; Davies 1994). The mAb HC/129, recognizing a single epitope, resulted in a reduction of spores, attaching to the nematode cuticle in a similar way to that reported with polyclonal antibodies capable of recognizing multiple epitopes (Davies and Danks 1992). The observation that HC/11, which recognized the same antigens as HC/129, did not reduce attachment suggests either that HC/11 does not recognize the same epitope as HC/129 and recognizes a different epitope on the same antigen, or that the concentration of the antibody in the tissue culture supernatant fluid was insufficient to reduce spore attachment. The latter is probably the most likely because there was a tendency for HC/11 to reduce spore attachment.

The results with HC/129 contrast sharply with HC/145, which more than doubled the number of spores attaching to J2 pretreated with the antibody. This observation could be due either to the antibody forming a bridge between the spore and the nematode cuticle or to the antibody masking a part of the nematode cuticle that prohibits spores from binding. The former explanation would suggest that a part of the antibody molecule, for example the constant region of the heavy chain, contains a spore-binding site that is involved in attachment; however, this is unlikely as HC/129 would contain a similar constant region but it does not lead to an increase in spore attachment. Biochemical investigations have shown nematodes (Himmelhoch *et al.* 1977) and the surface of the bacterial spore of *Pasteuria* (Afolabi *et al.* 1995) to be negatively charged and that a balance between electrostatic and hydrophobic interactions is important in determining whether or not spores attach to the nematode cuticle (Afolabi *et al.* 1995; Davies *et al.* 1996). Therefore the results suggest that HC/145 is possibly interfering with the balance of these binding forces by reducing negative charges and/or hydrophilic configurations on the nematode cuticle. Western blot analysis of HC/146 showed that it also recognized a weak band with a M_r of 80 kDa, which it shared with HC/145; thus both these antibodies are to the same antigen but they may not recognize exactly the same epitope, so may affect spore attachment with different efficiencies.

The surface of spores of *Pasteuria* is covered in fine fibres/adhesins that are involved in the attachment process (Persidis *et al.* 1991), and the highly conserved extracellular matrix protein, fibronectin, has recently been shown to bind to spores through hydrophobic interactions (Davies *et al.* 1996). Monoclonal antibodies to the cuticle of root-knot nematodes

revealed antigenic similarities between fibronectin and nematode cuticle extracts (Davies *et al.* 1996); however, if such a ubiquitous and conserved molecule as fibronectin is responsible for attachment, the biochemical nature of host specificity, which is always seen to a greater or lesser extent in *Pasteuria* spore attachment studies, remains unclear. Therefore, the mechanism is either based on high levels of variation in the cutile receptor itself, or there are molecules capable of masking the receptor indirectly. An example of the latter mechanism has been shown in *Caenorhabditis elegans*, where *N*-acetylglucosamine sugar moieties have been unmasked in mutagenisis experiments (Politz *et al.* 1990). The results presented here suggest that the components of the cuticle that are involved in prohibiting spores from binding may be masking the cuticle receptor in a similar manner.

ACKNOWLEDGEMENTS

REFERENCES

Afolabi, P., Davies, K.G. and O'Shea, P. (1995) The electrostatic nature of the spore surface of *Pasteuria penetrans*, the bacterial parasite of root-knot nematodes. *Journal of Applied Bacteriology* **79**, 244–249.

Davies, K.G. (1994) *In vitro* recognition of a 190 kD putative attachment receptor from the cuticle of *Meloidogyne javanica* by *Pasteuria penetrans* spore extract. *Biocontrol Science and Biotechnology* **4**, 367–374.

Davies, K.G. and Danks, C. (1992) Interspecific differences in the nematode surface coat between *Meloidogyne incognita* and *Meloidogyne arenaria* related to the adhesion of *Pasteuria penetrans*. *Parasitology* **105**, 475–480.

Davies, K.G. and Lander, E.B. (1992) Immunological differentiation of root-knot nematodes (*Meloidogyne* spp.) using monoclonal and polyclonal antibodies. *Nematologica* **38**, 353–366.

Davies, K.G., Afolabi, P. and O'Shea, P.S. (1996) Adhesion of *Pasteuria penetrans* to the cuticle of root-knot nematodes (*Meloidogyne* spp.) inhibited by fibronectin: a study of electrostatic and hydrophobic interactions. *Parasitology* **112**, 553–559.

Davies, K.G., De Leij, F.A.A.M. and Kerry, B.R. (1991) Microbial agents for the biological control of plant-parasitic nematodes in tropical agriculture. *Tropical Pest Management* **37**, 303–320.

Davies, K.G., Flynn, C.A., Laird, V. and Kerry, B.R. (1990) The life-cycle, population dynamics and host specificity of a parasite of *Heterodera avenae*, similar to *Pasteuria penetrans*. *Revue de Nématologie* **13**, 303–309.

Davies, K.G., Redden, M. and Pearson, T.K. (1994) Endospore heterogeneity in *Pasteuria penetrans* related to attachment to plant-parasitic nematodes. *Letters in Applied Microbiology* **19**, 370–373.

Despommier, D.D., Gold, A.M., Buck, S.W., Capo, V. and Silberstein, D. (1990) *Trichinella spiralis*: secreted antigen of infective L_1 larva localizes to the cytoplasm and nucleoplasm of infective host cells. *Experimental Parasitology* **71**, 27–38.

GENSTAT (1987) *Genstat 5 Reference Manual*. Oxford: Clarendon Press.

Gravato Nobre, M.J., von Mende, N., Dolan, L., Schmidt, K.P., Evans, K. and Mulligan, B. (1995) Immunolabelling of cell surfaces of *Arabidopsis thaliana* roots following infection by *Meloidogyne incognita* (Nematoda). *Journal of Experimental Botany* **46**, 1711–1720.

Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Spring Harbour: Cold Spring Harbour.

Hewlett, T.E. and Dickson, D.W. (1993) A centrifugation method for attaching endospores of *Pasteuria* spp. to nematodes. *Journal of Nematology* **25** (Suppl.), 785–788.

Himmelhoch, S., Kisiel, M.J. and Zuckerman, B.M. (1977) *Caenorhabditis briggsae*: electron microscope analysis of changes in negative charge density of the outer cuticle membrane. *Experimental Parasitology* **41**, 118–123.

Nishizawa, T. (1986) On a strain of *Pasteuria penetrans* parasitic on cyst nematodes. *Revue de Nematologie* **9**, 303.

Oostendorp, M., Dickson, D.W. and Mitchell, D.J. (1991) Population development of *Pasteuria penetrans* on *Meloidogyne arenaria*. *Journal of Nematology* **23**, 58–64.

Persidis, A., Lay, J.G., Manousis, T., Bishop, A.H. and Ellar, D.J. (1991) Characterization of potential adhesins of the bacterium *Pasteuria penetrans*, and of putative receptors on the cuticle of *Meloidogyne incognita*, a nematode host. *Journal of Cell Science* **100**, 613–622.

Politz, S.M., Philipp, M., Estevez, M., O'Brian, P.J. and Chin, K.J. (1990) Genes that can be mutated to unmask hidden antigenic determinants in the cuticle of the nematode *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences USA* **87**, 2901–2905.

Sharma, R. and Sharma, S.B. (1989) Sticky swarm disease of *Heterodera cajani* and *Meloidogyne javanica* caused by *Pasteuria penetrans*. *International Pigeonpea Newsletter* **10**, 26–27.

Sharma, S.B. and Davies, K.G. (1996a) Characterisation of *Pasteuria* isolated from *Heterodera cajani* using morphology, pathology and serology of endospores. *Systematic and Applied Microbiology* **19**, 106–112.

Sharma, S.B. and Davies, K.G. (1996b) A comparison of two sympatrix species of *Pasteuria* isolated from a tropical vertisol soil. *World Journal of Microbiology and Biotechnology* **12**, 361–366.

Sharma, S.B., Smith, D.H. and McDonald, D. (1992) Nematode constraints of chickpea and pigeonpea production in the semiarid tropics. *Plant Disease* **76**, 868–874.

Spiegel, Y. and McClure, M.A. (1995) The surface coat of plant-parasitic nematodes: chemical composition, origin, and biological role – a review. *Journal of Nematology* **27**, 127–134.

Stirling, G.R. (1984) Biological control of *Meloidogyne javanica* by *Bacillus penetrans*. *Phytopathology* **74**, 55–60.

Stirling, G.R. (1991) *Biological Control of Plant-Parasitic Nematodes: Progress, Problems and Prospects*. Wallingford, UK: CABI.

Tunlid, A., Jansson, H.-B. and Nordbring-Hertz, B. (1992) Fungal attachment to nematodes. *Mycological Research* **96**, 401–412.

Vassilatis, D.K., Despommier, D., Misek, D., Polvere, R., Van Gold, A.M. and der Ploeg, L.H.T. (1992) Analysis of a 43-kDa glycoprotein from the intracellular parasitic nematode *Trichinella spiralis*. *Journal of Biological Chemistry* **267**, 18459–18465.