

## Endospore heterogeneity in *Pasteuria penetrans* related to adhesion to plant-parasitic nematodes

K.G. Davies, M. Redden and Tracie K. Pearson

Entomology and Nematology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK

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K.G. DAVIES, M. REDDEN AND T.K. PEARSON. 1994. Hybridoma cell lines were screened by indirect immunofluorescence for the secretion of monoclonal antibodies (Mabs) to the surface of a population of endospores of the obligate nematode hyperparasite *Pasteuria penetrans*. Whereas polyclonal antibodies from test sera samples recognized 100% of the spores, five selected Mabs recognized different proportions of the spores ranging from 10 to 90% showing that the spore population was highly heterogeneous. Probing spores adhering to different nematode populations with the five Mabs showed that different subpopulations of the spores were specific to different nematode populations indicating cuticular heterogeneity among the nematode populations. Each of the five Mabs recognized a far larger proportion of the spores adhering to the nematode population on which the *Pasteuria* population was originally cultured than of those adhering to other populations.

### INTRODUCTION

The *Pasteuria* group of Gram-positive endospore-forming bacteria have potential to be developed into biological control agents of plant-parasitic nematodes (Stirling 1991). The taxonomy of the hyperparasite remains unclear but it is probably made up of a number of species and isolates which differ in their host ranges and virulence; all the economically important genera of plant-parasitic nematodes have an association with these bacteria (Sayre and Starr 1988). Three species of *Pasteuria* have been described so far: (1) *P. penetrans* parasitic on *Meloidogyne incognita* and probably other root-knot nematodes (Sayre and Starr 1985); (2) *P. thornei* on *Pratylenchus brachyurus* (Starr and Sayre 1988); and (3) *P. nishizawae* which parasitizes cyst nematodes (Sayre *et al.* 1991). A major obstacle to the commercial development of these bacteria as biocontrol agents is their limited host ranges; isolates of *P. penetrans* were found not only to adhere to a particular species of root-knot nematode but also to different populations within *M. incognita* (Stirling 1985; Davies *et al.* 1988). As nematodes often occur as mixed populations, the deployment of an isolate not compatible with the nematode populations present would undoubtedly lead to a failure to control the nematodes. It is therefore of fundamental importance to be able to assess the heterogeneity of both the nematodes and the pasteuria spores to ensure their compatibility.

Correspondence to: Dr K.G. Davies, Entomology and Nematology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, UK.

The biochemical mechanism by which spores of the bacterium bind to the nematode cuticle is poorly understood. The pretreatment of bacterial spores with a series of enzymes, lectins and sugars suggested that *N*-acetylglucosamine on the spore surface was acting as an adhesin (Davies and Danks 1993). A polyclonal antibody raised to a population of pasteuria spores showed quantitative differences in avidity between the antibody and the surface of different populations of spores and this in turn correlated to the ability of the antibody to inhibit the different populations of spores from binding to the nematode cuticle (Davies *et al.* 1992). From this study it was concluded that different distributions of epitopes were present on the surface of the spores, and that these may be adhesins which, in part, account for the differences observed in host specificity (Davies *et al.* 1992). The present study builds on this work and reports the production of monoclonal antibodies (Mabs) to the surface of spores of *Pasteuria penetrans*.

### MATERIALS AND METHODS

#### Nematodes and bacterial cultures

Single egg mass lines of *M. incognita* races 1, 2, 3 and 4, and *M. arenaria* races 1 and 2 (originating from North Carolina State University) were cultured in a glasshouse at 25°C on tomato plants, cv. Pixie, in a peat/sand (1 : 1, v/v) compost. Second-stage juveniles were hatched from egg

masses by placing them in tap water on a small tray at room temperature (Hooper 1986). *Pasteuria penetrans* (population PP1 provided by Dr S.R. Gowen, Natural Resources Institute, Chatham, UK) was cultured on *M. incognita* race 2 using the method of Stirling and Wachtel (1980) and the tomato root powder produced stored dry at 4°C. Suspensions of spores were prepared by grinding *Pasteuria* infested tomato root powder in tap water with a pestle and mortar, and the spores filtered with a 10- $\mu$ m sieve before they were counted using a haemocytometer slide. The concentrations of the suspensions were adjusted to  $10^6$  spores ml<sup>-1</sup>. Purified suspensions of spores, for immunization, were obtained by loading spores (PP1), obtained from crushed infected females of *M. incognita* race 2, on a two-step sucrose gradient (4 ml, 40% and 2 ml, 90%) spun at 25 000 *g* at 4°C for 60 min; they were washed in PBS (10 mmol l<sup>-1</sup> sodium phosphate buffer, pH 7.4, 0.9% sodium chloride). The numbers of spores in the clean suspensions were counted and the suspensions stored at -20°C.

#### Immunization and antibody production

Mice (Balb/C) were immunized with suspensions of clean spores of *P. penetrans* population PP1 ( $10^7$ ). After 3 weeks 50- $\mu$ l blood samples were obtained and tested for their ability to bind to the surface of spores of *P. penetrans* (PP1) by immunofluorescence (see below). A mouse exhibiting a strong immunological response to the bacterium was subsequently boosted with  $10^7$  spores (PP1) 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% fetal bovine serum (20-DMEM), 1 mmol l<sup>-1</sup> glutamine, 1% 100  $\times$  Pen/Strep (all medium and reagents supplied by Gibco). A fusion was done, between SP2/0-Ag and splenocytes in the presence of 30% polyethylene glycol (PEG 1500 Boehringer, Mannheim) by spinning (Harlow and Lane 1988) and the cells were selected in DMEM containing 1% 50  $\times$  HAZA (hypoxanthine-azaserine, Sigma) in the presence of peritoneal macrophages.

#### Immunofluorescence screening and cloning

Bacterial spores (15  $\mu$ l of tomato root suspensions) were allowed to adhere to multitest slides coated with poly-L-lysine (Harlow and Lane 1988). After washing in PBS (3 times) spores were blocked in 20-DMEM for 30 min and then incubated in hybridoma tissue culture supernatant for 2 h, rewashed (3 times) and then incubated for 2 h in goat anti-mouse antiserum (1 : 50) conjugated to fluorescein isothiocyanate (FITC) (Sigma) at 37°C. After incubation the spores were again washed (3 times) and mounted in Citi-

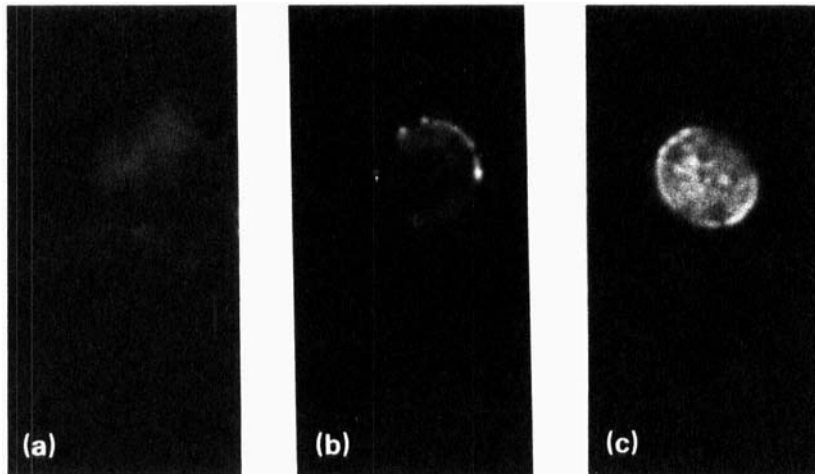
fluor™ (Agar Scientific). All preparations were examined using an Olympus BH-2 microscope fitted with epifluorescence illumination with a 455 nm excitation filter and a 520 nm barrier filter. Hybridoma cell lines producing antibodies positive to the spore surface were cloned by limiting dilution and retested.

#### Experimental

- (1) Multitest slides coated with spores of *P. penetrans* population PP1 were incubated separately with five Mabs and visualized as above. Two sets of 15 spores, taken at random, were assessed for the ability of each Mab to recognize each spore using the following scale: -, no recognition; +, weak recognition; ++, strong recognition. Control spores were incubated in 20-DMEM and anti-mouse FITC conjugate as above.
- (2) Spores of *P. penetrans* population PP1 were attached to several second-stage juveniles of root-knot nematodes (*M. incognita* races 1, 2, 3 and 4, and *M. arenaria* races 1 and 2) by centrifugation (Hewlett and Dickson 1993). Nematodes (*ca* 500) encumbered with spores were then transferred to wells in a Multiscreen™ (Millipore) filtration plate (0.45  $\mu$ m, hydrophilic) and incubated with Mabs in tissue culture supernatant. The spore encumbered nematodes were washed (3 times) by the addition of 100  $\mu$ l PBS followed by vacuum filtration and then incubated in the anti-mouse FITC conjugate as above. After incubation the spore encumbered nematodes were again washed and mounted on a slide for microscopical examination as above.

#### RESULTS

One-hundred and fifty hybridoma cell lines were produced from the fusion, of which five cell lines were positive to the surface of the spores when screened by immunofluorescence. The polyclonal test bleed recognized 100% of the spores and this contrasted with the Mabs which showed differential recognition of the spores within the population of PP1 (Fig. 1 and Table 1). Mabs PP1/12 and PP1/134 recognized over 90% of the spores examined but the majority only at a low level. This contrasts with PP1/84 which recognized less than 10% of the spores. The other two Mabs, PP1/53 and PP1/117, recognized 84% and 70% of the spores respectively. These results indicate that the surfaces of the spores are heterogeneous in their reaction to the different Mabs. Probing the spores adhering to the different populations of nematodes showed that different subpopulations of the spores were adhering (Table 2); PP1/12 recognized 45% of the spores adhering to *M. incognita* race 2 but did not recognize any of the other populations of spores adhering to any of the other nematodes; PP1/53 and



**Fig. 1** Indirect immunofluorescence of spores recognized by monoclonal antibody PP1/12 showing (a) no recognition, (b) weak recognition and (c) strong recognition

PP1/134 recognized between 5 and 50% of the spores on all races of *M. incognita* but none adhering to either race of *M. arenaria*; PP1/84 recognized over 90% of the spores on *M. arenaria* but none of the spores adhering to the *M. incognita* populations with the exception of *M. incognita* race 2, the population upon which the original pasteuria population PP1 had been cultured; PP1/117 was the only antibody which recognized spores adhering to all the *M. incognita* populations and cross reacted with 15% of the spores on *M. arenaria* race 2.

## DISCUSSION

Host specificity in populations of *Pasteuria penetrans* is well documented (Stirling 1985; Davies *et al.* 1988) and the deployment of *P. penetrans* for nematode control may produce a selection pressure in favour of nematode sub-

populations which are resistant to *P. penetrans* (Channer and Gowen 1992). If *Pasteuria* is to be used successfully in the control of nematodes it is therefore important to know the variation present both in the nematode and the hyperparasite. Polyclonal antibodies have shown that the surfaces of different spore populations of *Pasteuria* (Davies *et al.* 1992) and cuticles of root-knot nematodes (Davies and Danks 1992) exhibit both qualitative and quantitative differences and it was hypothesized that these differences may account for differences in host range. However, the use of cross-reactive polyclonal antibodies, and the difficulty in obtaining single spore isolates, due to the obligate nature of the bacterium, made it impossible to assess, in detail, the heterogeneity of the populations of spores. The work presented here shows that, even within what was regarded as a relatively homogeneous spore population, there is a high degree of variation and that this variation is also exhibited

Antibody	No recognition	Weak recognition	Strong recognition
PP1/12	0	30	0
PP1/53	5	18	7
PP1/84	27	1	2
PP1/117	9	21	0
PP1/134	2	28	0
PP1/PC	0	0	30

**Table 1** Indirect immunofluorescence of 30 spores of *Pasteuria penetrans* population PP1 with five monoclonal antibodies (PP1/12, PP1/53, PP1/84, PP1/117 and PP1/134) and a polyclonal control (PP1/PC) assessed as: no recognition, weak recognition, strong recognition

Nematode	PP1/12	PP1/53	PP1/84	PP1/117	PP1/134
<i>M. incognita</i> race 1	0	3	0	7	1
race 2	9	10	10	6	10
race 3	0	10	0	5	1
race 4	0	5	0	10	3
<i>M. arenaria</i> race 1	0	0	18	0	0
race 2	0	0	19	3	0

**Table 2** Indirect immunofluorescence of 20 spores of *Pasteuria penetrans* population PP1 baited by six root-knot nematode populations (*Meloidogyne incognita* races 1, 2, 3 and 4, and *M. arenaria* races 1 and 2), probed with five monoclonal antibodies (PP1/12, PP1/53, PP1/84, PP1/117 and PP1/134), numbers given are those assessed as positive

by the different nematode hosts. It is interesting to note that the Mabs recognize a far larger proportion of the spores adhering to the nematode host on which the PPI populations of bacteria were originally cultured (*M. incognita* race 2); culturing the bacterium on a particular nematode host will therefore reduce the variability present within the original *Pasteuria* population. It is possible that the restricted host range of populations of the bacterium, reported in the literature, are to some degree an artefact of the method by which the bacterium has been cultured. The extent to which the variation reported here is due to phase variation is difficult to judge without the production of single spore isolates.

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#### REFERENCES

- Channer, A.G. De R. and Gowen, S.R. (1992) Selection for increased host resistance and increased pathogen specificity in the *Meloidogyne-Pasteuria penetrans* interaction. *Fundamental and Applied Nematology* 15, 331-339.
- Davies, K.G. and Danks, C. (1992) Interspecific differences in the nematode surface coat between *Meloidogyne incognita* and *M. arenaria* related to the adhesion of the bacterium *Pasteuria penetrans*. *Parasitology* 105, 475-480.
- Davies, K.G. and Danks, C. (1993) Carbohydrate/protein interactions between the cuticle of infective juveniles of *Meloidogyne incognita* and spores of the obligate hyperparasite *Pasteuria penetrans*. *Nematologica* 39, 53-64.
- Davies, K.G., Kerry, B.R. and Flynn, C.A. (1988) Observations on the pathogenicity of *Pasteuria penetrans*, a parasite of root-knot nematodes. *Annals of Applied Biology* 112, 491-501.
- Davies, K.G., Robinson, M.P. and Laird, V. (1992) Proteins involved in the attachment of a hyperparasite, *Pasteuria penetrans*, to its plant-parasitic nematode host, *Meloidogyne incognita*. *Journal of Invertebrate Pathology* 59, 18-23.
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 726. Cold Spring Harbour.
- Hooper, D.J. (1986) Extraction of nematodes from plant material. In *Laboratory Methods of Work with Plant and Soil Nematodes* ed. Southey, J.F. p. 51-58. Ministry of Agriculture Fisheries and Food. London: HMSO.
- Hewlett, T.E. and Dickson, D.W. (1993) A centrifugation method for attaching endospores of *Pasteuria* spp. to nematodes. *Supplement to Journal of Nematology* 25, 785-788.
- Sayre, R.M. and Starr, M.P. (1985) *Pasteuria penetrans* (ex Thorne, 1940) nom. rev., comb. n., sp. n., a mycelial endospore forming bacterium parasitic in plant-parasitic nematodes. *Proceedings of the Helminthological Society of Washington* 52, 149-165.
- Sayre, R.M. and Starr, M.P. (1988) Bacterial diseases and antagonists of nematodes. In *Diseases of Nematodes*, Vol. 1. ed. Poinar G.O. and Jansson, H.-B. pp. 69-101. Boca Raton, FL: CRC Press.
- Sayre, R.M., Wergin, W.P., Schmidt, J.M. and Starr, M.P. (1991) *Pasteuria nishizawae* sp. nov., a mycelial endospore-forming bacterium parasitic on cyst nematodes *Heterodera* and *Globodera*. *Annales Institut Pasteur/Microbiologie* 142, 551-564.
- Starr, M.P. and Sayre, R.M. (1988) *Pasteuria thornei* sp. nov. and *Pasteuria penetrans sensu stricto* emend., Mycelial and endospore forming bacteria parasitic respectively, on plant-parasitic nematodes of the genera *Pratylenchus* and *Meloidogyne*. *Annales Institut Pasteur/Microbiologie* 139, 11-31.
- Stirling, G.R. (1985) Host specificity of *Pasteuria penetrans* within the genus *Meloidogyne*. *Nematologica* 31, 203-209.
- Stirling, G.R. (1991) *Biological Control of Plant-Parasitic Nematodes: Progress, Problems and Prospects*. Wallingford: CABI.
- Stirling, G.R. and Wachtel, M.F. (1980) Mass production of *Bacillus penetrans* for the biological control of root-knot nematodes. *Nematologica* 26, 308-312.