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Aspects on the attachment of *Pasteuria penetrans* on root-knot nematodes

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Summary. Variability in spore attachment of the bacterial parasite *Pasteuria penetrans* to second stage juveniles of *Meloidogyne javanica* and *M. incognita* populations originating from different locations were recorded. Culture of an isolate of *Pasteuria penetrans* on a *Meloidogyne* population did not reveal a high compatibility with the nematode population in subsequent attachment tests. However, culture of *Pasteuria* on a mixed *Meloidogyne javanica / M. incognita* population resulted in high attachment ability on both species. Serological studies revealed differences between sub-populations of *Pasteuria* that were created by passaging the same *Pasteuria* isolate with different *Meloidogyne* species.

Key words: *Meloidogyne javanica*, *M. incognita*, monoclonal antibodies, biological control, spore adhesion.

The first stage of development of *Pasteuria penetrans* is its attachment to the cuticle of second stage juveniles of root-knot nematodes. Attachment does not necessarily result in subsequent germination of the spores and development of the vegetative and reproductive phases within the nematode body. Also, a high attachment level decreases the ability of encumbered juveniles to find and invade the root system (Adiko & Gowen, 1999; Davies *et al.*, 1988; Stirling, 1984). *Pasteuria penetrans* has been shown to differ in its attachment level to different species of nematodes (Oostendorp *et al.*, 1990; Subbotin *et al.*, 1994; Vouyoucalou & Gowen, 1995) and reproduction of the nematode on different hosts can also affect spore attachment (Tzortzakakis *et al.*, 1995). Stirling (1985) in agreement with results reported by Spaull (1984) suggested that *P. penetrans* exhibits intrageneric specificity because the spores of the population he used did not adhere readily to all *Meloidogyne* populations used in his study. Even if the same species of nematode is used, the attachment level of a single isolate of *Meloidogyne* spp. is not uniform (Stirling, 1985). The difficulty in predicting the encumbering behaviour of the spores results from a lack of detailed knowledge at the molecular level for the spores and nematode surfaces. Nematode parasites have unusual physiochemical properties (Kennedy *et al.*, 1987) and this might be a reason why it is difficult to understand the fundamental procedure of spore attachment on the surface coat of nematodes.

Davies *et al.*, (1992), working with polyclonal antibodies, reported that surfaces of different spore populations of *P. penetrans* exhibit quantitative and qualitative differences. Subsequent studies with monoclonal antibodies revealed heterogeneity of the spores according to their reaction to five Mabs (Davies *et al.*, 1994). However, the procedure of attachment appears complicated as physical and chemical procedures are involved that might be affected by environmental conditions. Davies *et al.* (1996) concluded that extracellular matrix components of the nematode cuticle such as fibronectin could be responsible for the adhesion of *P. penetrans* spores by hydrophobic interactions. These hydrophobic forces might have been removed by the presence of potassium thiocyanate (KSCN) which greatly reduced the attachment ability of spores to nematode cuticle (Davies *et al.*, 1996). The spore surface has been found to have a net negative charge depending on the pH, salt concentration, and valency of the cation present in the electrolyte medium. Thus, it may be concluded that electrostatic interactions may be important in the binding of spores to nematode cuticles (Afolabi

Table 1. Origins of *Pasteuria penetrans* populations used in the immunofluorescence test.

Isolate	Originally isolated from	Passaged on	Designated as population
*Pp*E	*M. incognita / javanica*	*M. javanica*	A
*Pp*3	*M. javanica*	*M. javanica*	B
*Pp*3	*M. javanica*	*M. incognita*	C
*Pp*E	*M. incognita / javanica*	*M. incognita*	D

et al., 1995).

The experiments reported here investigated if the original nematode host in which *P. penetrans* spores were produced influences the rate of attachment of these spores in subsequent studies with the original and other nematode hosts. Attachment of spores of four different populations of *P. penetrans* on three isolates of *M. javanica* and two of *M. incognita* was also investigated. There are contradictory results in the bibliography for the influence of the species in which a *Pasteuria* population was originally isolated, when this population is encumbering another isolate of the same species. Consequently serological studies were conducted to determine any differences between different isolates of *P. penetrans* spores.

MATERIALS AND METHODS

The attachment rate of *P. penetrans* spores as affected by the nematode host in which they were produced. One isolate of *M. javanica* from Malawi and one of *M. incognita* (race 4 from Kenya) were used in this study. The extracting trays containing eggs dissolved on NaOCl were kept in the incubator at 28 °C for 8 days. Juveniles collected during the first two days were discarded and each day thereafter the appropriate number of juveniles was collected and the remainder discarded. The extracting trays were refilled with fresh water. These two nematode populations were used for attachment studies with two original populations of *Pasteuria*, *Pp*E originally isolated from a mixture of *M. javanica* and *M. incognita* in Ecuador and *Pp*3 originally isolated from *M. javanica* in Malawi, and two sub-populations derived from each original population. One ml of tap water containing 200 juveniles was pipetted into a 9 cm Petri dish. The volume in the Petri dish was made up to 10 ml by adding 9 ml of tap water. Then 1 ml of *Pasteuria* suspension containing 30000 spores was added. Each combination of nematode isolate-spore population was replicated 6 times. Petri dishes with nematodes and spore suspension were maintained at 24 °C. The number of spores per juvenile was estimated by counting the attached spores on 30 juveniles from each replicate after 24 hours.

The attachment rate of four different isolates of *P. penetrans* to J2s of *M. javanica* and *M. incognita*. In this experiment 4 different isolates of *P. penetrans* were used to encumber five different populations of root-knot nematodes, three of which were *M. javanica* and two were *M. incognita*. The *Pasteuria* isolates were obtained from both species in different locations. The experimental procedure was identical to that reported previously.

Immunofluorescence with polyclonal antibody and monoclonal antibodies (Mabs). Four batches of infected females (10-16 females each) in 2 ml of distilled water were kept in the freezer for 2 months. These females were homogenized and the resulting suspensions were placed in conical tubes. Volumes of each suspension (1, 0.3, 0.2 and 0.2 ml from the suspensions labeled A, B, C and D, Table 1) were removed and placed into plastic Eppendorf capsules. They were centrifuged at high speed for 5 mins and the supernatant was poured away leaving 5 µl of the suspension containing the spores. Fifty ml of sample buffer were added to each capsule and shaken to disperse the spores. They were boiled for 3 mins and then stored in a fridge. Ten µl of the stock suspensions were diluted with distilled water to make four new spore suspensions with a concentration of 300 spores/µl. Ten µl of these suspensions were allowed to adhere to multitest slides coated with poly-1-lysine at 37 °C overnight. Then the slides were washed in PBS (x3) and carefully dried. They were incubated in hybridoma tissue culture supernatant (10 µl in each spot of the multitest slide) for 2 h. Also, a positive (polyclonal antibody) and a negative (20 D) control were included for comparison. After another 3 washes in PBS they were incubated with anti-rabbit IgG conjugated to FITC (the positive controls) or with anti-mouse IgG conjugated to FITC (the samples and the negative controls). After incubation (2 h) they were washed (x3) in PBS and mounted in Citifluor (Agar Scientific). The

Table 2. Attachment of different isolates of *Pasteuria penetrans* to juveniles of *Meloidogyne javanica* and *M. incognita*.

Spores per juvenile	Encumbered juveniles											
	Meloidogyne javanica (Malawi)						*Meloidogyne incognita* (Race 4)					
	P1	P2	P3	P4	P5	P6	P1	P2	P3	P4	P5	P6
0	10	29	15	30	82	39	2	3	1	70	126	4
1-5	83	84	60	119	81	104	43	51	15	106	54	98
6-12	75	56	70	26	13	32	75	91	64	4	0	64
13-20	12	11	26	5	4	5	48	34	69	0	0	14
>20	0	0	9	0	0	0	12	1	31	0	0	0
χ^2 test	***			***			***			***		

*** P<0.001

P1: *P. penetrans* (*Pp*E originally produced on *M. incognita/javanica*);
P2: *P. penetrans* (*Pp*E passaged on *M. javanica* from Malawi);
P3: *P. penetrans* (*Pp*E passaged on *M. incognita* Race 4);
P4: *P. penetrans* (*Pp*3 originally produced on *M. javanica*);
P5: *P. penetrans* (*Pp*3 passaged on *M. javanica* from Malawi);
P6: *P. penetrans* (*Pp*3 passaged on *M. incognita* Race 4).

Table 3. Attachment of different isolates of *Pasteuria penetrans* to juveniles of different populations of *Meloidogyne javanica* (from Malawi, Kenya and Ecuador) and different races of *M. incognita* (races 3 and 4 from Kenya).

Spores per J2	Encumbered juveniles																			
	*Pp*1					*Pp*2					*Pp*3					*Pp*E				
	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
0	57	145	146	93	146	42	135	127	64	132	2	46	114	38	15	7	12	140	111	0
1-5	54	5	4	57	4	51	15	23	85	18	50	104	36	94	119	44	80	10	26	19
6-12	32	0	0	0	0	54	0	0	1	0	75	0	0	16	14	56	48	0	3	79
13-20	7	0	0	0	0	0	0	0	0	0	19	0	0	2	2	30	10	0	3	47
>20	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	13	0	0	7	5
χ^2 test	***					***					***					***				

*** P<0.001

M1: *M. javanica* (Malawi);
M2: *M. javanica* (Kenya);
M3: *M. javanica* (Ecuador);
M4: *M. incognita* (Race 3 from Kenya);
M5: *M. incognita* (Race 4 from Kenya).

*Pp*1: originally produced on *M. javanica*;
*Pp*2: originally produced on *M. incognita* but was passaged once on *M. javanica*;
*Pp*3: originally produced on *M. javanica*;
*Pp*E: originally produced on *M. incognita/javanica*.

slides were examined using a Zeiss microscope fitted with epifluorescence illumination. Fifty spores per sample were assessed for the ability for each of the five Mabs to recognize each spore using the following scale: -, no recognition; +, weak recognition; ++, good recognition; +++, strong recognition.

RESULTS

The attachment rate of *P. penetrans* spores as affected by the nematode host in which they were produced. There was strong evidence from the χ^2 test of significance for differences between the original populations (*Pp*E and *Pp3*) and the 'sub-populations' for each attachment study using *M. javanica* (Malawi) and *M. incognita* (race 4 from Kenya) (Table 2). Best attachment was obtained by P3 encumbering *M. incognita* (race 4) and poorest by P5 encumbering the same nematodes.

The attachment rate of four different isolates of *P. penetrans* to J2s of *M. javanica* and *M. incognita*. An χ^2 analysis of significance for each population of *Pasteuria* revealed that differences in

levels of attachment between the different isolates were highly significant ($P<0.001$) (Table 3). The highest attachment rate occurred with *P. penetrans* (Ecuador) encumbering *M. incognita* (race 4), and the lowest by *P. penetrans* (isolate No1) encumbering *M. javanica* (Kenya, M2), *M. javanica* (Ecuador, M3) and *M. incognita* (race 4, M5).

Immunofluorescence with polyclonal antibody and monoclonal antibodies (Mabs). When a non-specific polyclonal antibody was used, the majority of spores of each sub-population were recognized (Tables 3-6). Conversely monoclonal antibodies did not recognize spores of any of the sub-populations. Unfortunately, no monoclonal antibodies raised to the *Pasteuria* isolates used in the attachment studies were available that could clearly show differences between the *Pasteuria* sub-populations.

DISCUSSION

Although *P. penetrans* originating from Ecuador (P1 isolated from *M. javanica/incognita*) did not have the best attachment rate it did reveal a very good attachment compatibility with both *Meloidogyne* species tested. In contrast *P. penetrans* (P4 isolate) isolated from a *M. javanica* population had a lower level of attachment with *M. javanica* (Malawi), and an even lower level with *M. incognita* (race 4). This result is in agreement with that from the previous experiment and confirms that *P. penetrans* isolated from a mixture of species is more likely to be compatible with a wide range of species or isolates of the species. Sub-population P2, that was obtained from passage of P1 on *M. javanica* did not have improved attachment ability when tested with *M. javanica* (Malawi), and similarly when it was tested with *M. incognita*. Conversely, sub-population P3 (passaged on *M. incognita*) had improved attachment when tested with both species, as compared to the original population. *Pp*3 (isolated from *M. javanica*) had better attachment with *M. javanica* than with *M. incognita*. Sub-population P5 (passaged once more on *M. javanica*) had a slightly lower attachment with the same species, whereas sub-population P6 (passaged on *M. incognita*) had approximately the same attachment rate. Sub-population P5 had a lower level of attachment when tested with *M. incognita*, whereas sub-population P6 (passaged on *M. incognita*) had a much higher attachment level with this nematode.

It can be concluded that when the two *Pasteuria* populations were passaged on *M. incognita* (race 4) the subsequent sub-populations had an increased attachment ability. This increase was higher when the sub-populations were tested against *M. incognita* juveniles.

It was reported that continuous culture of a *Pasteuria* population on the same isolate of root-knot nematode can create a *Pasteuria* sub-population with a higher compatibility with the homologous isolate of nematodes (Davies *et al.*, 1994). The data obtained here do not support this hypothesis as *Pp*3 cultured on *M. javanica* (Malawi) did not have an improved attachment level with its homologous isolate of nematodes.

The data presented in Table 3 do not clarify the influence of the *Pasteuria* population and nematode isolates in terms of spore attachment. *P. penetrans* from Ecuador (originally isolated from a mixture of *M. incognita/javanica*) attached best to *M. incognita* (race 4, M5). Conversely, the same population of *Pasteuria* had very low attachment on *M. javanica* that originated from Ecuador. Apart from its poor attachment to this nematode this *Pasteuria* isolate showed the highest spectrum of attachment compared to the other three isolates. The *Pp*1 isolate had a low attachment rate with all the nematode populations tested, except with *M. javanica* originating from Malawi (M1).

The *Pp*2 isolate that was originally isolated from *M. incognita,* but was passaged once on *M. javanica* (thus a selection could be obtained in favour of the last nematode species), had the best attachment, among the five nematode isolates, with *M. javanica* originating from Malawi.

P. penetrans (isolate No3) was originally isolated from *M. javanica* and maintained in the greenhouse on the same species (*M. javanica* from Malawi). This nematode was readily encumbered with *Pp*3 whereas slightly lower attachment levels were obtained on *M. incognita* (races 3 and 4). However, although this *Pasteuria* isolate was isolated and maintained on *M. javanica*, it showed a very low attachment rate with *M. javanica* originating from Kenya and Ecuador. Channer and Gowen (1992) reported that when an isolate of *P. penetrans* was cultured on a mixed population of root-knot nematodes the subsequent spore attachment was improved on all the nematode populations tested. This is confirmed here as *Pp*E was isolated from a mixture of both nematode species and gave a good compatibility with two *M. javanica* and two *M. incognita* isolates, better than any other of the *Pasteuria* populations.

The polyclonal antibody raised to *P. penetrans* (*Pp*1) (Persidis *et al.*, 1991) recognized almost all the spores of the populations tested. In contrast,

Table 4. Indirect immunofluorescence of 50 spores of *Pasteuria penetrans* population *Pp*A with five monoclonal antibodies (*Pp*1/12, *Pp*1/53, *Pp*1/84, *Pp*1/117 and *Pp*1/134), a polyclonal control (+ control) and a negative control (- control).

*Pp*Ai	-	+	++	+++
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	46	4
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	47	3
*Pp*Aii	-	+	++	+++
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
+control	0	0	46	4
-control	50	0	0	0
*Pp*1/117	50	0	0	0
*Pp*1/134	48	2	0	0
-control	50	0	0	0
-control	48	0	2	0

Table 5. Indirect immunofluorescence of 50 spores of *Pasteuria penetrans* population *Pp*B with five monoclonal antibodies (*Pp*1/12, *Pp*1/53, *Pp*1/84, *Pp*1/117 and *Pp*1/134), a polyclonal control (+ control) and a negative control (- control).

*Pp*Bi	-	+	++	+++
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	48	2
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	49	1
*Pp*Bii	-	+	++	+++
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
+control	0	0	46	4
-control	50	0	0	0
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
-control	50	0	0	0
-control	50	0	0	0

the Mabs raised to *P. penetrans* (*Pp*1) (Davies *et al.*, 1994) did not recognize any of these populations. The population in which the Mabs were raised may be much different from those tested here. These antibodies have been sub-cultured for three years at IACR, Rothamsted. The above two reasons could explain non-recognition of the spores by the Mabs. The same weak recognition of *Pasteuria* spores by Mabs has also been reported by Espanol *et al.* (1997) following a very similar experiment to that described above.

The differences in the proteinic profiles in combination with those found using the nematode hosts suggest that each isolate of *Pasteuria* in the soil environment can be characterized as a "tank" of spores through which nematodes move. The nematodes in this spore suspension impose a selection pressure that is driven by numerous factors

Table 6. Indirect immunofluorescence of 50 spores of *Pasteuria penetrans* population *Pp*C with five monoclonal antibodies (*Pp*1/12, *Pp*1/53, *Pp*1/84, *Pp*1/117 and *Pp*1/134), a polyclonal control (+ control) and a negative control (- control).

*Pp*Ci	-	+	++	+++
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	48	2
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	49	1
*Pp*Cii	-	+	++	+++
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
+control	0	0	49	1
-control	50	0	0	0
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
-control	50	0	0	0
-control	50	0	0	0

Table 7. Indirect immunofluorescence of 50 spores of *Pasteuria penetrans* population *Pp*D with five monoclonal antibodies (*Pp*1/12, *Pp*1/53, *Pp*1/84, *Pp*1/117 and *Pp*1/134), a polyclonal control (+ control) and a negative control (- control).

*Pp*Di	-	+	++	+++
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	0	0	48	2
*Pp*1/12	50	0	0	0
*Pp*1/53	50	0	0	0
*Pp*1/84	50	0	0	0
+control	50	0	49	1
*Pp*Dii	-	+	++	+++
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
+control	0	0	48	2
-control	50	0	0	0
*Pp*1/117	50	0	0	0
*Pp*1/134	50	0	0	0
-control	50	0	0	0
-control	50	0	0	0

such as nematode species and isolate, homogeneity of the original spore population, the physical and chemical characteristics of the medium in which the encumbering procedure takes place, and the individual age of spores. Consequently, the "selected" *Pasteuria* population is likely to show a different attachment ability as compared to the parental population.

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Giannakou I. O., Gowen S. R., Davies K. G. Особенности прикрепления *Pasteuria penetrans* к галлообразующим нематодам.

Резюме. Выявлены различия в характере прикрепления спор *Pasteuria penetrans* к личинкам 2-й стадии *Meloidogyne javanica* и *M. incognita* из различных географических регионов. Культивирование изолятов *P. penetrans* на популяциях одного из видов *Meloidogyne* не выявляло повышенной прикрепляемости спор в последующих экспериментах с этим же видом нематод. Напротив, высокие показатели прикрепляемости к обоим видам мелойдогин были отмечены для изолята *Pasteuria*, культивируемого на смеси из двух видов *Meloidogyne*. Иммунологические исследования выявили различия между субпопуляциями *Pasteuria*, полученными при продолжительном культивировании на одном из видов мелойдогин.