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Attachment of *Pasteuria penetrans* Endospores to the Surface of *Meloidogyne javanica* Second-stage Juveniles[1]

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Abstract: *Pasteuria penetrans* spore adhesion to *Meloidogyne javanica* second-stage juveniles (J2) was examined following several different pretreatments of the latter. The detergents sodium dodecyl sulfate and Triton X-100, the carbohydrates fucose and α-methyl-D-mannoside, and the lectins concanavalin A and wheat germ agglutinin reduced spore attachment. Spores exposed to *M. javanica* surface coat (SC) extract exhibited decreased adherence to the J2 surface. Second-stage juveniles that had been treated with antibodies recognizing a 250-kDa antigen of J2 SC extract had fewer spores attached to their surfaces, as compared to nontreated J2, except in the head region. This inhibition pattern was similar to that of antibody-labelling on *M. javanica* J2 as observed by electron microscopy. It is suggested that several SC components, such as carbohydrate residues, carbohydrate-recognition domains, and a 250-kDa antigen, are involved in *P. penetrans* spore attachment to the surface of *M. javanica*.

Key words: biological control, concanavalin A, glycoconjugate, *Meloidogyne javanica*, nematode, neoglycoprotein, *Pasteuria penetrans*, surface coat, wheat germ agglutinin.

Pasteuria penetrans is an obligate parasite of the root-knot nematode, *Meloidogyne* spp. Variations in bacterial spore adherence to the surface of second-stage juvenile (J2) surfaces have been attributed to differences in the surface composition of different species, races, and populations of root-knot nematodes (6) as well as to the heterogeneity of the spore surfaces themselves (8,9,12).

To improve the ability of *P. penetrans* to serve as a biocontrol agent, attempts have been made to understand the nature of its spores' adhesion to *Meloidogyne* J2. Such knowledge would further contribute to nematode surface characterization and to the development of other, novel control strategies. Davis (5) demonstrated in vitro binding of spore extract to a 190-kDa glycoprotein derived from an extract of *M. javanica* J2 cuticle. Earlier studies by Stirling et al. (19) could not confirm the involvement of a lectin-carbohydrate mechanism in spore attachment. Later, however, Bird et al. (3) reported that the lectins concanavalin A (Con A) and wheat germ agglutinin (WGA) inhibit spore attachment to *M. javanica* J2, probably because of binding of the lectins by the spores. Davies and Danks (6) also demonstrated that a carbohydrate-protein mechanism is involved in *P. penetrans* spore attachment to *M. incognita:* N-acetylglucosamine residues on the spore surface recognized carbohydrate-recognition domains (CRD) on the nematode surface. These authors also reported that polyclonal antibodies raised against cuticular extracts of *M. incognita*-recognized by 43- and 80-kDa polypeptides-inhibit spore attachment to the surface of *M. incognita* J2 (6).

The surface coat (SC) of *M. javanica* J2 recently has been characterized by Sharon and Spiegel (unpubl.). A polyclonal antibody raised against SC extract recognized a 250-kDa polypeptide on an immunoblot; visualization under light microscopy revealed binding of the antibody to the J2 surface and amphids, but not the head region (Sharon and Spiegel, unpubl.).

This report describes the effect of different factors, including detergents, carbohydrates, and lectins, on *P. penetrans* spore attachment to the surface of *M. javanica* J2, and suggests the involvement of a 250-kDa antigen in the attachment mechanism.

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Materials and Methods

Nematodes: The root-knot nematode *M. javanica* was propagated on tomato (*Lycopersicon esculentum* Mill cv. Hosen Eilon) in the greenhouse, or aseptically in petri dishes containing excised root cultures of tomato (10). Eggs were separated from egg masses with sodium hypochlorite (0.5%, 1 minute) and hatched in twice-diluted 0.1 M phosphate buffer saline, pH 7.2 (PBS-2) to obtain infective J2.

Bacteria: A local isolate of *P. penetrans* was cultured on root-knot nematode females propagated in the greenhouse. Females dissected from roots were washed thoroughly, crushed in a tube with PBS, and vortexed vigorously. The spore suspension was removed, concentrated by centrifugation (1,000*g*, 10 minutes), and sonicated for 30 seconds. The suspension was kept at −20 °C until use.

Spore attachment assay: The attachment assays were conducted in flat-bottomed microtiter plates. Each plate contained 30 live, 2-day-old J2 mixed with a suspension of 10^6 spores/ml, in 50 µl PBS-2 containing Ca^{++} and Mg^{++} ions and 10 µl Coomassie Brilliant Blue (2). Each treatment was replicated in three wells. Plates were shaken horizontally with an orbital shaker at 100 RPM, room temperature, and monitored under an inverted microscope. After 2 hours, when sufficient attachment had been achieved (20 to 30 spores/J2), 10 µl of 37% formaldehyde solution was added to kill and fix the nematodes. The number of spores adhering to 25 J2 surfaces was counted under a light microscope. Separate counts were performed on the whole J2 body, on the area between the tail and the median bulb, and on the anterior region.

Factors affecting spore attachment. Detergents: Second-stage juveniles were incubated for 30 minutes in PBS-2 containing 1% sodium dodecyl sulfate (SDS) at 25 °C, or 1% Triton X-100 at 0 °C, and then washed three times with PBS-2 to remove excess detergent. SDS-treated and non-treated specimens were further incubated for a recovery-period of 2 days, at 4 °C or 25 °C, and the number of adhering spores was recorded.

Carbohydrates: Second-stage juveniles were incubated for 2 hours in Ca^{++}- and Mg^{++}-containing PBS-2, with 0.1 M of the following carbohydrates: fucose, galactose, glucose, α-methyl mannoside, N-acetylgalactosamine, or N-acetylglucosamine. Second-stage juveniles were then washed with PBS-2 before conducting attachment assays.

Lectins: Second-stage juveniles were incubated for 2 hours in PBS-2 containing Ca^{++}, Mg^{++}, and Mn^{++} ions with 0.1 mg/ml Con A or WGA. Second-stage juveniles were then washed with PBS-2 before conducting attachment assays.

Spore treatment with M. javanica *SC extract:* Surface coat was extracted by gentle agitation of 1 ml *M. javanica* J2 for 1 hour at 25 °C, in a 2-ml solution of 1% SDS dissolved in PBS-2. The reaction mixture contained the following protease inhibitors: 1 mM Pefabloc (Boehringer Mannheim, Germany), 1 µg/ml leupeptin (Boehringer), and 0.1 mM N-tosyl-2-phenylalanine chloromethyl ketone (TPCK) (Sigma Chemical, St. Louis, MO). Nematodes were then pelleted by centrifugation at 250 *g* and the supernatant was filtered through a 0.2 µm cellulose-acetate membrane (Corning, Corning, NY). The detergent was finally excluded from the supernatant by overnight dialysis against distilled water at 15 °C, or by using Detergent Absorber Gel (Boehringer). Proteins were concentrated by lyophilization.

Pasteuria penetrans spores were incubated for 1 hour with 1% bovine serum albumin (BSA) dissolved in PBS, to block unspecific binding to proteins, and then washed with PBS. Half of the blocked spores were incubated at 25 °C for 1 hour with the SDS-SC extract; protein concentration was 300 µg/ml according to the method reported by Bradford (4). The other half of the spores were incubated in PBS and served as a control.

Labelling of nematodes with antibodies: Polyclonal antiserum was produced in mice in-

jected with SDS-extracted SC proteins. Antiserum from nonimmune mice was collected as well. Nematodes were first blocked for 1 hour at 25 °C with 1% BSA dissolved in PBS-2, washed with 0.1% BSA (washing solution), and then incubated for 1 hour at 25 °C with the antiserum (immune or nonimmune), diluted 1:500 in the washing solution. After washing three times with the washing solution, some of the nematodes were used for spore attachment assays and the others were further labelled to visualize antibody-binding under a light microscope. Nematodes were incubated for 1 hour in a 1:200-diluted solution of gold-conjugated secondary antibody (goat anti-mouse IgG, 5-nm particles; BioCell, Cardiff, UK), washed with the washing solution and the distilled water, and then exposed to a silver-enhancement solution (BioCell) for 5 to 15 minutes until labelling could be visualized under a light microscope. To visualize nematodes by scanning electron microscopy (SEM), J2 were labelled as described, except that the exposure to silver enhancing solution was reduced to 2 minutes. Nontreated nematodes or nematodes treated with one reagent, including nonimmune serum, secondary antibody, or silver enhancement solution, served as controls. Silver enhancement was not performed for transmission electron microscopy (TEM). Nontreated nematodes or nematodes treated with either secondary antibody or nonimmune serum served as controls.

Scanning and transmission electron microscopy: For SEM, labelled J2 were fixed for 2 hours in a solution of 2.5% (v/v) glutaraldehyde dissolved in 0.05 M cacodylate buffer, pH 7.5. Nematodes were then washed in the cacodylate buffer and serially dehydrated in ethanol (10% increments). Critical-point-drying and gold-coating were performed, and nematodes were observed in a Jeol GSM-T330A microscope.

For TEM, labelled and fixed J2 were post-fixed for 3 hours at 25 °C with 1.0% (v/v) osmium tetroxide in 0.05 M cacodylate buffer and rinsed three times with PBS. Washed specimens were transferred to 0.8% agarose blocks and serially dehydrated in ethanol (10% increments). Uranyl acetate (2% w/v for 1 hour) was incorporated at the 70% ethanol dehydration stage. Specimens were embedded in Epon by conventional procedures, and sections approximately 600 nm thick were cut, mounted on nickel grids, examined in a Jeol JEM 100CX microscope, and photographed.

The effect of ethanol dehydration on antibody labelling was examined by exposing ethanol-treated nematodes to silver-enhancing solution and comparing these to antibody-labelled nematodes that had not been processed for electron microscopy. The observations were made under a light microscope.

Results

Factors affecting spore attachment. Detergents: Fewer spores adhered to the surfaces of detergent-treated J2 than to those of nontreated J2 (Table 1). When SDS-treated J2 were kept at 25 °C for 2 days, the intensity of spore adhesion was the same as that recorded for the nontreated J2, whereas no adhesion of spores was recorded with the SDS-treated J2 kept at 4 °C for 2 days (Table 1).

Carbohydrates: Fewer spores adhered to J2 pretreated with fucose or α-methyl

Table 1. *Pasteuria penetrans* spore attachment to *Meloidogyne javanica* second-stage juveniles (J2) as affected by detergent treatments.

Treatment	Spores/J2 no recovery	Spore/J2 after a recovery at 25° C[a]	Spores/J2 after recovery at 4° C[a]
Control	20.8 ± 6.8a	22.3 ± 5.2a	18.5 ± 5.4a
SDS[b]	5.5 ± 3.9b	21.2 ± 5.6a	6.2 ± 4.7b
Triton X-100	8.4 ± 4.6b	19.5 ± 4.8a	6.4 ± 5.6b

[a]SDS-treated and nontreated J2 were incubated for a recovery period of 2 days.
[b]SDS = sodium dodecyl sulfate.
Data are means of three replicates. In each replicate, 25 specimens were counted. Means within a column followed by a common letter are not different, according to Tukey's HSD ($P = 0.05$).

TABLE 2. *Pasteuria penetrans* spore attachment to *Meloidogyne javanica* second-stage juveniles (J2) as affected by carbohydrates.

Treatment	No. of spores/ J2 head	No. of spores/ J2 body
Control	5.8 ± 2.5a	16.8 ± 8.2a
N-Acetylgalactosamine	5.1 ± 2.1a	15.1 ± 5.2a
N-Acetylglucosamine	6.0 ± 3.3a	15.4 ± 7.3a
Fucose	6.2 ± 1.6a	11.5 ± 4.8b
Galactose	4.6 ± 2.0a	15.0 ± 5.1a
Glucose	6.3 ± 2.9a	18.7 ± 9.6a
α-Methyl mannoside	4.7 ± 2.2a	9.7 ± 5.5b

Data are means of three replicates. In each replicate, 25 specimens were counted. Means within a column followed by a common letter are not different, according to Tukey's HSD ($P = 0.05$).

mannoside, except for the head region where no inhibition was recorded (Table 2). N-acetylgalactosamine, N-acetylglucosamine, galactose, and glucose pretreatments, on the other hand, did not significantly affect the intensity of spore adhesion as compared to the nontreated J2 (Table 2).

Lectins: Pretreatment of J2 with Con A or WGA reduced spore attachment on the body but not on the anterior region; with Con A, the reduction recorded in the head region was minor but significant (Table 3).

SC extract and antibodies: Pretreating spores with SDS-SC extract or pretreating J2 with antibody against the 250-kDa polypeptide caused a reduction in spore attachment to J2 surfaces; the attachment profiles followed much the same pattern as that observed with the lectins (Tables 4 and 5).

TABLE 3. *Pasteuria penetrans* spore attachment to *Meloidogyne javanica* second-stage juveniles (J2) as affected by lectins.

Treatment	No. of spores/ J2 head	No. of spores/ J2 body
Control	7.6 ± 4.5a	22.4 ± 9.7a
Con A	3.2 ± 3.1b	6.5 ± 4.6c
WGA	5.2 ± 3.5 ab	14.2 ± 7.6b

Con A = concanavalin A.
WGA = wheat germ agglutinin.
Data are means of three replicates. In each replicate, 25 specimens were counted. Means within a column followed by a common letter are not different, according to Tukey's HSD ($P = 0.05$).

TABLE 4. *Pasteuria penetrans* spore attachment to *Meloidogyne javanica* second-stage juveniles (J2) as affected by surface coat (SC) extract.

Treatment	No. of spores/ J2 head	No. of spores/ J2 body
Control	6.0 ± 2.3a	19.3 ± 9.9a
SC-treated spores	5.0 ± 2.4a	4.5 ± 3.1b

Data are means of three replicates. In each replicate, 25 specimens were counted. Means within a column followed by a common letter are not different, according to Tukey's HSD ($P = 0.05$).

Scanning and transmission electron microscopies: By SEM, the 250-kDa antigen was detected on the transverse annulations (Fig. 1A) and lateral alae (Fig. 1B), i.e., over most of J2 surface except for the head region, where labelling was barely recorded (Fig. 1C).

The labelling pattern recorded by TEM was similar to that observed by SEM; labelling was not uniform over the entire surface, but restricted to the center of the lateral field and to the transverse annulations (Fig. 2). The preparative ethanol-dehydration step for TEM did not affect labelling, as confirmed by light microscopy.

DISCUSSION

Several mechanisms are probably involved in the *P. penetrans* adhesion process (1). For instance, studies conducted recently on physiochemical aspects suggest the involvement of electrostatic and hydrophobic interactions in spore binding (1).

In this work we demonstrate the involvement of the SC layer in *P. penetrans* spore attachment to the surface of *M. javanica*

TABLE 5. *Pasteuria penetrans* spore attachment to *Meloidogyne javanica* second-stage juveniles (J2) as affected by antibody raised against a 250 kDa-antigen.

Treatment	No. of spores/ J2 head	No. of spores/ J2 body
Control	6.3 ± 3.4a	14.1 ± 9.4a
Nonimmune serum	5.7 ± 2.3a	10.1 ± 5.7ab
Antibody	5.4 ± 2.9a	5.0 ± 4.9c

Data are means of three replicates. In each replicate, 25 specimens were counted. Means within a column followed by a common letter are not different, according to Tukey's HSD ($P = 0.05$).

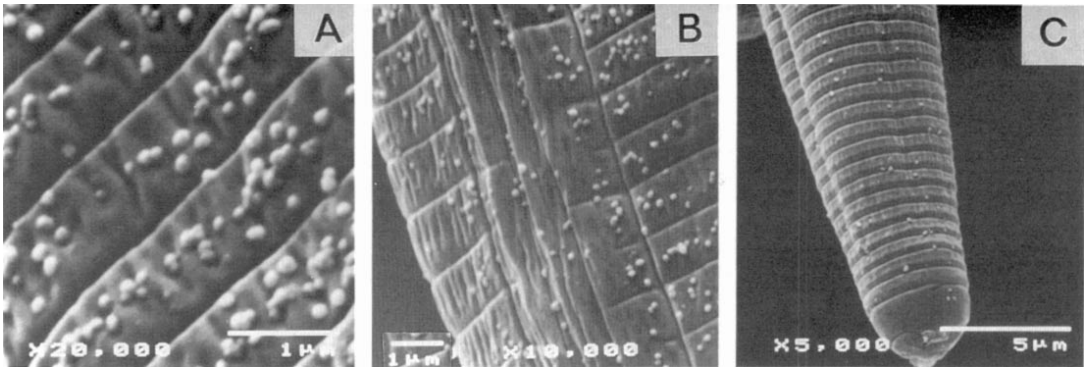

FIG. 1. Scanning electron micrographs of the immunogold-labeled surface of *Meloidogyne javanica* second-stage juveniles; labelling was enhanced by silver enhancement reaction. A) Transverse annulation. B) Lateral field. C) Anterior region.

J2: stripping this layer with detergents (without affecting nematode viability), pretreatment of spores with an extract of this layer, or reduced spore attachment (Tables 1,4). The recovery of spore binding at an optimal temperature after 2 days indicates that the reduction in spore binding is due to surface removal and not to nonspecific blockage, as claimed by Esnard et al. (pers. comm.). A similar recovery phenomenon has been observed with human red blood cells adhering to *M. javanica* J2 surfaces (Sharon and Spiegel, unpubl.). Previous studies with *M. incognita* revealed that only drastic procedures reduced spore binding (13); for example, Davies and Danks (6,7) reported that *M. incognita* J2 treatment with SDS for 2 hours did not result in less adherence, but in fact increased it.

The involvement of CRD present on the surface of *M. incognita* in *P. penetrans* attachment has been previously suggested by Davies and Danks (7). In this report, J2 exposed to fucose or to α-methyl mannoside adhered fewer spores than nontreated J2 (Table 2). These carbohydrate moieties also reduced the adherence of human red blood cells to the J2 surface (17). The presence of fucose- and mannose-recognition domains on J2 surfaces was demonstrated using gold-labelled neoglycoproteins (16). CRDs on J2 surfaces were restricted to the area between the tail and the median bulb (16), resembling the distribution of spores following binding inhibition by carbohydrates (Table 2).

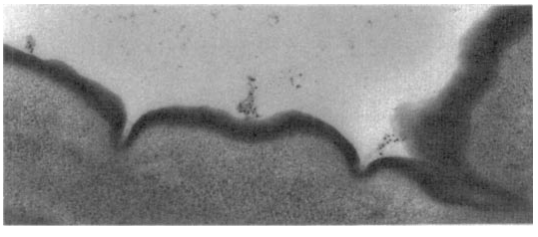

FIG. 2. Transmission electron micrograph of the immunogold-labelled surface of *Meloidogyne javanica* second-stage juveniles, cut longitudinally (×40,000).

The CRD on J2 surfaces was related to the C-type group, a Ca^{++}-dependent animal lectin (17,18). Binding of *P. penetrans* also has been reported to be enhanced by the presence of Ca^{++} ions (7), suggesting that such lectins are involved in the adhesion process. Animal lectins present in other organisms have been shown to interact with bacterial carbohydrates (11). The presence of carbohydrate residues such as N-acetylglucosamine, mannose, or glucose on the surface of *P. penetrans* was demonstrated by fluorescent lectin binding to its spores (3,7,13). Such carbohydrate residues could be candidates for binding to nematode surface CRD.

Con A and WGA lectins bind to the surface of *M. javanica* (16). In this study, J2 treated with these lectins adhered fewer spores (Table 3), suggesting the involvement of surface carbohydrate residues in the attachment process. Bird et al. (3) re-

ported inhibition of spore attachment when both J2 and spores were incubated with either Con A or WGA. However, the question of whether this inhibition is due to the lectins binding to the J2, the spores, or both was not addressed.

The 250-kDa polypeptide is one of several components in the *M. javanica* SC extract; pretreatment of J2 with an antibody recognizing this antigen reduced spore attachment as compared to nontreated J2 (Table 5), suggesting the antigen's involvement in the spore attachment process. The inhibition was recorded over the entire nematode surface except for the head region (Table 5), resembling the antigen's location on the J2 surface, as indicated by antibody labelling (Fig. 1). The binding pattern of spores pretreated with SC extract (Table 4) followed much the same pattern as that observed with antibody labelling, suggesting that the 250-kDa antigen's involvement in spore attachment is restricted to almost the entire surface, except the head region. Moreover, this antigen did not react with neoglycoproteins on a western blot (Sharon and Spiegel, unpubl. data) and therefore cannot be considered a CRD.

Electron microscopic observations of the J2 SC layer revealed the 250-kDa antigen's almost complete confinement to the center of the annulations (Fig. 2), a location that has been previously reported for germtube emergence on J2 surfaces (14,15). The similarity in these results deserves further study.

Electron microscopic observations have shown loss of the SC layer in the larvae of the animal parasite *Toxocara canis* as a result of ethanol dehydration (12). In our studies, however, labelling and(or) visualization of *M. javanica* J2 SC was not affected by the 'conventional' TEM procedure, because ethanol did not cause this layer to slough off.

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