

CARBOHYDRATE/PROTEIN INTERACTIONS BETWEEN THE  
CUTICLE OF INFECTIVE JUVENILES OF *MELOIDOGYNE*  
*INCOGNITA* AND SPORES OF THE OBLIGATE HYPERPARASITE  
*PASTEURIA PENETRANS*

BY

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Standard assays were done to test the effects of various preincubation treatments on the attachment of spores of *Pasteuria penetrans* to the cuticles of second-stage juveniles (J2) of *Meloidogyne incognita*. Either J2s or spores were preincubated in a series of proteolytic, lipolytic and glycolytic enzymes, carbohydrates, detergents and solubilising agents and attachment assays performed. J2 and spores were also incubated in a series of fluorescent labelled lectins and examined using a fluorescence microscope. The surface of the J2 appears to contain carbohydrate recognition domains which are probably not collagen and these interact with N-acetylglucosamine moieties on the spore surface which are linked to either glycoproteins or peptidoglycans. As exposing the spores to lysozyme (muramidase) weakens attachment the spores also contain N-acetylmuramic acid.

The *Pasteuria* group of Gram-positive endospore-forming bacteria consists of at least three species, of which *P. penetrans* is an obligate parasite of root-knot nematodes such as *Meloidogyne incognita* (Sayre & Starr, 1988; Starr & Sayre, 1988); *Pasteuria* has potential as a biological control agent (Mankau, 1975; 1980; Stirling, 1984; Brown *et al.*, 1985; Oostendorp *et al.*, 1990). The first stage in the infection process commences when J2 migrating through the soil become encumbered with spores of the bacterium. Individual populations of the bacterium exhibit a restricted host range with spores from one population of *P. penetrans* having a greater affinity to the cuticle of J2 from one egg mass than from another, even where the egg masses were from the same site (Davies *et al.*, 1991). The nature of the surface of the second-stage juvenile cuticle and its interaction with spores of *P. penetrans* is of fundamental importance in understanding the host specificity of the bacterium. However, the mechanism by which spores of the bacterium adhere to nematode cuticle is still poorly understood. A number of studies have investigated the role of lectin/carbohydrate interactions between the spore and its nematode host: early studies failed to implicate such interactions (O'Brien, 1980; Stirling *et al.*, 1986). However, a more recent study (Bird *et al.*, 1989) has shown that wheat germ agglutinin (WGA) inhibited the attachment of spores of *P. penetrans* to *M. javanica* by about 70%; spores were prevented from attachment in the presence of an inhibitory

hapten sugar. Similarly, concanavalin A (Con A) inhibited the attachment of the bacterium to the nematode cuticle, but this was not reversed when the inhibitory hapten sugar was included in the attachment assay (Bird *et al.*, 1989). The electrophoretic profiles of spores visualised by WGA were very similar to those visualised with a polyclonal antibody, raised to spores of *P. penetrans*, suggesting that the proteins were highly glycosylated with N-acetylglucosamine (Persidis *et al.*, 1991). Differences in the amount and nature of the proteins on the surface of spores of different populations of *P. penetrans*, visualised by the same polyclonal antibody, may account for differences observed in host specificity (Davies *et al.*, 1992). Spores treated with periodate, which denatures glycosyl residues, also resulted in decreased attachment (Bird *et al.*, 1989). These results indicate that glycosyl residues occurring on the surface of *P. penetrans* were attaching to lectins (carbohydrate binding proteins) on the nematode surface. In a study of the binding of red blood cells to the cuticles of nematodes preincubated in a series of sugars, attachment was shown to involve carbohydrate moieties on the cells which recognised carbohydrate domains on the nematode surface (Spiegel *et al.*, 1991). The presence of carbohydrate residues has been reported on the surfaces of several free-living and plant parasitic nematodes (Zuckerman *et al.*, 1979; McClure & Zuckerman, 1982; Jansson *et al.*, 1986, Robertson *et al.*, 1989; Ibrahim, 1991; Spiegel *et al.*, 1991). Also, several fungi possess carbohydrate binding proteins on their spores or trapping devices which are thought to be involved in the adhesion of these fungi to the nematode cuticle (Nordbring-Hertz & Mattiasson, 1979; Nordbring-Hertz & Chet, 1986; Jansson & Nordbring-Hertz, 1988). Furthermore attachment of a coryneform bacterium was inhibited when dauer larvae of *Anguina agrostis*, which contain glycoproteins with N-acetylglucosamine residues were incubated in proteolytic enzymes, indicating that these glycoproteins play a crucial role in the adhesion of the bacterium (Bird & Zuckerman, 1989). Because of the subtle differences in recognition exhibited by spores of *P. penetrans* to different populations of the same species of root-knot nematode the mechanism of attachment is unlikely to be simple and may involve several different types of ligand. The present study describes the preincubation of either J2 of *M. incognita* or spores of *P. penetrans* in a number of denaturing reagents, enzymes, hapten sugars and lectins to establish some aspects of the biochemical nature of the mechanism of attachment.

#### MATERIALS AND METHODS

##### *Nematodes*

*Meloidogyne incognita* race 2 (obtained from North Carolina State University) was cultured on tomato cv. Pixie plants, in peat/sand (1:1) compost in the glasshouse at 25°C. J2 were hatched from egg masses by placing them on a small tray in tap water at room temperature (Hooper, 1986).

*Pasteuria* spores

Spores of *P. penetrans* population PP1 (provided by Dr S.R. Gowen, University of Reading) were concentrated by homogenising infected females, that had been cultured using the method of Stirling & Wachtel (1980), in a 1 ml iniform homogeniser (Jencons). Spores were counted using a haemocytometer and suspensions of  $2 \times 10^6$  spores ml<sup>-1</sup> water produced and stored at 4°C.

*Enzyme, sugar solutions, denaturing reagents and lectins*

The enzymes (Table I; Sigma) and reagents were made up in either 50 mM Tris-HCl, pH 7.4 or 50 mM sodium acetate, pH 5.8 depending on whether the pH of their optimum activity was basic or acidic. A series of stock sugar solutions, 0.4 M, (D-glucose, D-galactose, D-mannose, L-fucose, D-xylose, L-arabinose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetylneuraminic acid, N-acetyl muraminic acid) were prepared in 50 mM Tris-HCl, pH 7.4. A 10mM periodic acid solution was prepared in the sodium acetate buffer. Cuticle denaturing reagents, 1% sodium dodecylsulphate (SDS) and 5% β-mercaptoethanol (BME) were prepared in 10mM sodium phosphate buffer (PBS) pH 7.4, containing 0.9% NaCl. Delipidation was carried out using a chloroform:methanol:water mixture (8:4:3 v/v)(Finne & Krusius, 1982). Fluorescein-labelled lectins (Vector Laboratories) were reconstituted at 1 mg ml<sup>-1</sup> in PBS containing 15 mM NaCl, 10 mM CaCl<sub>2</sub>, and 10 mM MgCl<sub>2</sub>.

*Preincubation of nematodes and attachment assay.*

Prior to the commencement of any incubation Eppendorf tubes (1.5 ml) were blocked overnight at 37°C with 1% (w/v) solution of bovine serum albumin

TABLE I

*Enzymes and their unit concentrations in which infective second-stage juveniles of Meloidogyne incognita and spores of P. penetrans were incubated*

Enzymes	Units per ml	Enzymes	Units per ml
<b>Glycolytic</b>			
α Glucosidase type VI	70.0	β Glucosidase type VII	4.8
β Galactosidase grade VI	450.0	α Mannosidase	25.0
β N-Acetylglucosaminidase	35.0	Hyaluronidase type I-S	290.0
Neuraminidase type V	1.1	Mutanolysin	3200.0
Muramidase (egg white)	62500.0	Chitinase	0.5
<b>Proteolytic</b>			
Pepsin A	3300.0	Proteinase K	14.0
Trypsin type II	1540.0	Collagenase 1A	380.0
α chymotrypsin type II	39.0		
<b>Lipolytic</b>			
Lipase type XI	405.0		

(BSA) in PBS. About 100 J2 were concentrated at the bottom of such tubes by centrifugation (1000 *g* for 3 min) and supernatant removed. The nematodes were resuspended in 200  $\mu$ l of the respective treatment solution and incubated for 3 h at 37°C (unless otherwise stated) after which the nematodes were concentrated by centrifugation and the supernatant removed. The nematodes were washed three times in PBST (0.05% *v/v* Tween in PBS); untreated (control) nematodes were incubated in buffer. Attachment tests were conducted by resuspending the nematodes in 100  $\mu$ l buffer (PBS containing 10 mM MgCl<sub>2</sub> and 10 mM CaCl<sub>2</sub>, unless otherwise stated) and transferring them to a well in a flat bottomed microtitre plate (Dynatech). All nematodes were heat killed by placing the microtitre plate in a water bath at 100°C for 2 min after which aliquots of 50  $\mu$ l of PP1 spores were added to each well and the microtitre plate shaken for 3 h at room temperature. Spore attachment was assessed by examining J2 using a microscope (X400; Olympus, BH-2) and counting the spores adhering to the cuticle of 10 individual juveniles.

#### *Preincubation of spores and attachment assay*

Spores of *P. penetrans* population PP1 from a stock suspension of 10<sup>7</sup> spores ml<sup>-1</sup> were concentrated by centrifugation (1000*g* for 10 min at room temperature) in a 10 ml centrifuge tube and the supernatant removed. The spores were resuspended in 200  $\mu$ l of each of the treatment solutions for 3 h at 37°C. They were further diluted in 10 ml PBST and concentrated by centrifugation (1000*g* for 10 min) and the supernatants removed. The spores were again resuspended in a small volume of buffer (as above) and the density of spores measured in a haemocytometer; their concentration was adjusted to 2 × 10<sup>6</sup> spores ml<sup>-1</sup> in buffer. About 100 J2 suspended in 100  $\mu$ l of attachment buffer were then placed in wells of a microtitre plate and heat killed (as above); 50  $\mu$ l of the treated spores were then added to the wells and an attachment assay performed as described above.

#### *Fluorescence Microscopy*

J2 and spores of *P. penetrans* were concentrated using the respective methods described above and incubated for 2 h in one of the fluorescent labelled lectins (Table V) or a PBS buffer control. Following incubation the second-stage juveniles and spores were washed in PBS, as before, and mounted on a glass slide in a polyvinyl, alcohol-glycerol mounting medium containing p-phenylenediamine (Johnson & Nogueira-Araujo, 1981). 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.

## RESULTS

The preincubation of J2 in several denaturing agents affected attachment of *P. penetrans* spores (Table II). Incubation of J2 in 1% SDS, 5% BME and delipidiser for 2 h at room temperature all significantly ( $P < 0.05$ ) increased attachment. However, increasing the temperature and the time of incubation led to a significant ( $P < 0.05$ ) reduction on the attachment of the spores to the cuticle. These decreases in attachment were similar (between 65 and 71%) for J2 incubated for 10 min at 100°C; PBS alone was as effective as 1% SDS, 5% BME or delipidiser (Table II).

The incorporation of 10 mM CaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub> into the buffer significantly ( $P < 0.01$ ) increased the number of spores which attached to nematode cuticle (Table III). Preincubation of J2 in different glycolytic enzymes resulted in fewer *P. penetrans* spores adhering to the cuticle than those incubated in buffer solution. However, periodic acid had no effect on attachment (Table III; Fig. 1). Similar treatments with pepsin, proteinase K, chymotrypsin and lipase cause a significant ( $P < 0.01$ ) reduction in the attachment of spores of between 31 to 81% (Table III, Fig. 2). Pretreatment of juveniles with N-acetyl-glucosamine, fucose and xylose led to fewer spores ( $P < 0.01$ ) attaching to their cuticles (Table IV, Fig. 3).

Preincubation of spores of *P. penetrans* with  $\beta$ -galactosidase, hyaluronidase and mutanolysin all led to small increases in the numbers adhering, however, only mutanolysin caused a significant increase from the control (Table III). Fewer (<50%) spores preincubated in muramidase, chitinase and periodic acid (Table III; Fig. 1) attached to nematode cuticle than untreated spores. Preincubation of the spores in chymotrypsin significantly increased attachment (43%, Fig. 2), whereas it was significantly reduced by pepsin and lipase (Table III, Fig. 2).

TABLE II

*Mean attachment of spores of P. penetrans to the cuticle of second-stage juveniles (J2) of M. incognita following the preincubation of J2 in denaturing reagents (1% SDS: 5% BME: delipidiser) and a buffer (PBS) for 3 sets of conditions (room temperature: 2 min at 100°C: 10 min at 100°C)*

Reagent	120 min RT	2 min 100°C	10 min 100°C
1% SDS	17.0	4.8	6.8
5% BME	28.0	6.1	NA
Delipid	29.0	NA	5.7
Buffer	—	5.6	6.3
Buffer (RT)	12.4	4.6	19.6
ANOVA <i>P</i>	< 0.05	NS	< 0.05
SED	2.6	1.9	3.3

NA, not available: RT, room temperature

TABLE III

Mean attachment of spores of *P. penetrans* to second-stage juveniles (J2) of *M. incognita* following firstly the preincubation of J2s and secondly the preincubation of spores in a number of glycolytic, proteolytic and lipolytic enzymes and periodic acid

Enzymes	preincubation		Enzymes	preincubation	
	J2's	Spores		J2's	Spores
<b>Glycolytic</b>					
$\alpha$ glucosidase	7.4	16.1	$\beta$ glucosidase	13.5	19.3
$\beta$ galactosidase	15.5	22.8	$\alpha$ mannosidase	12.7	NA
N-AC gl aminidase	8.9	NA	hyaluronidase	7.9	26.1
neuraminidase	14.7	NA	mutanolysin	12.0	27.4
muramidase	9.2	9.1	chitinase	8.4	6.6
periodic acid	22.3	2.7			
<b>Proteolytic</b>					
pepsin A	7.9	10.0	proteinase K	11.0	12.5
trypsin	21.2	16.3	chymotrypsin	14.2	27.3
collagenase	20.5	14.1			
<b>Lipolytic</b>					
Lipase	3.9	10.2			
Buffer (+ salts)	20.7	19.1			
Buffer (- salts)	14.0	NA			
ANOVA <i>P</i>	0.01	0.01	NA, not available		
SED	2.10	3.90			

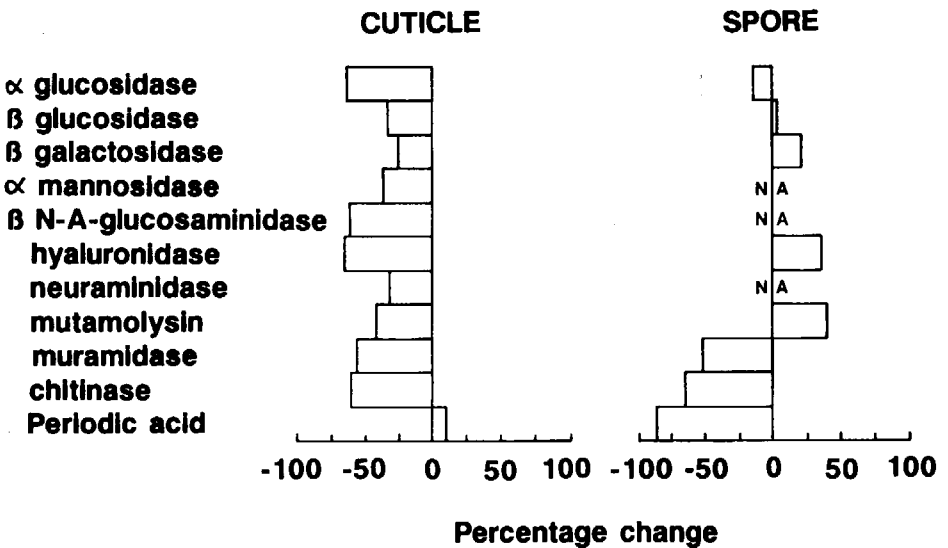


Fig. 1. The percentage change in attachment from the buffer control after preincubating second-stage juveniles of *M. incognita* and spores of *P. penetrans* in a number of glycolytic enzymes and periodic acid.

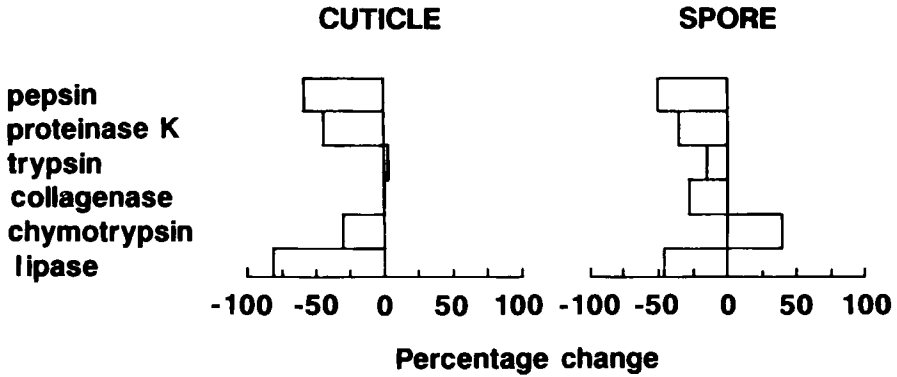


Fig. 2. The percentage change in attachment from the buffer control after preincubation second-stage juveniles of *M. incognita* and spores of *P. penetrans* in a number of proteolytic and lipolytic enzymes.

TABLE IV

Mean attachment of spores of *P. penetrans* to second-stage juveniles of *M. incognita* preincubated in a number of solutions of mono- and di-saccharides in 50 mM Tris buffer pH 7.2

sugar	attachment	sugar	attachment
L-arabinose	21.0	D-galactose	19.9
D-glucose	17.3	D-mannose	18.2
L-fucose	9.8	D-xylose	5.7
N-Ac-glucosamine	7.9	N-Ac-galactosamine	16.3
N-Ac-neuraminic acid	18.4	buffer control	20.6

ANOVA  $P < 0.001$ ; SED 1.6

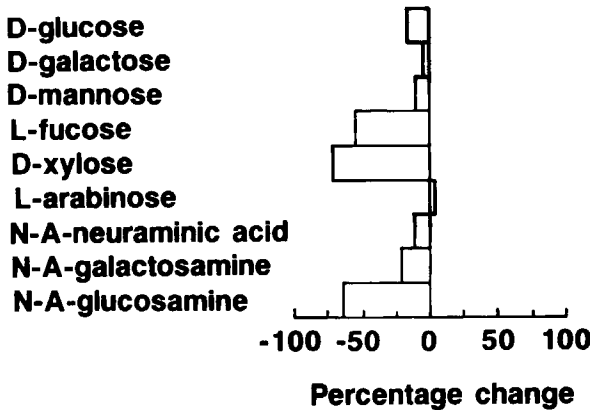


Fig. 3. The percentage change in attachment of spores of *P. penetrans*, from the buffer control, after preincubating second-stage juveniles of *M. incognita* in a number of carbohydrates.

TABLE V

*Binding of fluorescent lectins to second-stage juveniles of M. incognita and spores of P. penetrans*

Lectin	Specificity	<i>M. incognita</i>	<i>P. penetrans</i>
Con A	D-Mann/Glc	a	-
WGA	GlcNAc	a	+
SBA	GalNAc	-	-
DBA	GalNAc	-	-/+
UEA <sub>1</sub>	L-Fuc	-/+ , a	-
PNA	D-Gal	-	-
TPA	L-Fuc/Glc	-	NA
PBS	none	-	-

No labelling = -; slight labelling = -/+; labelling = +; a = amphids; NA, not available; Con A, Concanavalin A; WGA, Wheat germ agglutinin; SBA, Soyabean agglutinin; DBA, *Dolichos biflorus* agglutinin; UEA<sub>1</sub>, *Ulex europaeus* agglutinin; PNA, Peanut agglutinin; TPA, *Tetragonolobus purpureus*; PBS, phosphate buffered saline.

Only UEA<sub>1</sub> slightly labelled the J2 cuticle when viewed with an epifluorescence microscope; WGA recognised the amphids or amphidial secretions of the nematode and also spores of *P. penetrans*. Spores of *P. penetrans* were also slightly labelled by DBA (Table V).

#### DISCUSSION

The adhesion of spores of *P. penetrans* to nematode cuticle was substantially altered by the preincubation of J2 in SDS, BME and delipidiser; this increase at room temperature was probably because the treatments removed fractions of the cuticle not involved in attachment and exposed ligands that were. Incubating the J2 at elevated temperatures was detrimental to the attachment of spores and may have caused the solubilisation and denaturation of the ligands involved.

The presence of carbohydrate residues on the surface of J2 of root-knot nematodes is well documented (McClure & Zuckerman, 1982; Davis *et al.*, 1988; Robertson *et al.*, 1989; Spiegel & McClure, 1991; Ibrahim, 1991). Because the preincubation of J2 in glycolytic enzymes had a detrimental effect on attachment of spores to the cuticle, carbohydrates on the surface of the cuticle may be involved in attachment of spores. However, this finding is inconsistent with the results obtained by the preincubation of J2 in periodic acid, where there was no difference between the treated and control nematodes. If carbohydrates on the cuticle were involved, periodic acid should have produced a reduction in attachment. The glycolytic enzymes may be binding in a nonspecific manner to the surface of the cuticle and inhibiting attachment. Several of the proteases reduced the numbers of spores on the cuticle suggesting that proteins on the surface of the cuticle are involved in attachment. Col-lagens, several of which are glycosylated, are an important component in the

structure of the J2 cuticle of *M. incognita* (Reddigari *et al.*, 1986); they have also been proposed as a receptor for *P. penetrans* (Persidis *et al.*, 1991). The inability of either collagenase or periodate to reduce attachment suggests that they are not initially involved in the attachment of spores to the cuticle, although they may be important at a later stage in the infection process. Steric hindrance by parts of the cuticle not involved in the attachment of spores may also be prohibiting access of the enzyme to the collagen. The epicuticle of some other nematodes (e.g. *Ascaris*) contains cuticlin, a non-collagenous protein matrix (Fujimoto & Kanaya, 1973), and therefore some similar non-collagenous protein may be present and involved in the attachment process.

A reduction in attachment was also caused by lipase. As periodic acid did not have an effect on attachment it is unlikely that glycolipids on the surface of the nematode cuticle are directly involved. However, the lipid domains on the surface of nematodes are highly heterogeneous and may be loosely associated with proteins (Proudfoot *et al.*, 1991). Disruption of the lipid domains by lipase could therefore indirectly interfere with the proteins involved with attachment. The lectin UEA<sub>1</sub> slightly recognised the J2 epicuticle and the others failed to bind. The inability of lectins to bind to nematode cuticle is a common phenomenon among several animal-parasitic species although they possess surface glycoproteins which are readily iodinated. The lectin may be sterically hindered from binding to the glycosyl residues by the unusual lipid composition of the epicuticle (Proudfoot *et al.*, 1991).

The reduction in attachment caused by the preincubation of J2 in L-fucose, D-xylose and N-acetyl-D-glucosamine suggests that the ligands involved in attachment can recognise these sugars, unless steric hindrance occurs by the sugars binding to neighbouring epitopes not involved in attachment. Lectin studies on the spores of *P. penetrans* show that N-acetyl-D-glucosamine, recognised by WGA, was present and that N-acetyl-D-galactosamine, recognised by DBA, was also present but to a much lesser extent. The presence of N-acetyl-D-glucosamine on the spore and its inhibition by preincubation of the nematode in its haptan sugar is consistent with the findings of other workers (Bird *et al.*, 1989). Fewer spores attached to juveniles preincubated in L-fucose and the inability to detect this sugar on spores with the lectin UEA<sub>1</sub> suggests that either L-fucose was able to sequester ligands which bind sugars on the spore or that L-fucose was present but was inaccessible to the lectin. Unfortunately, there is no commercial lectin available which recognises xylose residues; as xylose was recognised by the J2 cuticle and reduced spore attachment, nematode cuticle may be a source for such a lectin. The inability of D-mannose to inhibit attachment of spores is consistent with previous reports (Bird *et al.*, 1989). However, the binding of Con A to the spore was not observed. Such variations in the results obtained in different laboratories may reflect differences in the populations of spores under investigation. The size (Bird *et al.*, 1990) and electrophoretic banding patterns of *P. penetrans* spores (Davies *et al.*, 1992) from

different geographical locations were significantly different and may relate to the observed differences in host specificity.

Periodate pretreatment of the spores reduced the numbers that attached to nematodes and so implicated the involvement of sugars on the spores in the attachment process. The preincubation of spores in muramidase and chitinase, the substrates of which are N-acetylmuramic acid and N-acetylglucosamine respectively, also reduced attachment. These substrates are linked by  $\beta$ -1,4 glycosidic linkages and are important constituents of peptidoglycans, structures accounting for 40% or more of the cell wall of Gram-positive bacteria (Shockman & Barrett, 1983). Therefore it is likely that the ligands of the spore involved in attachment are extensions of the Gram-positive bacterial cell wall. Pepsin, proteinase K or lipase reduced spore attachment, suggesting the involvement of proteins and lipids. The proteases presumably denatured the glycoprotein and peptidoglycan components of the spore, but the reduction in attachment by lipase is more difficult to explain. Cell walls of Gram-positive bacteria do not contain glycolipid however teichoic acids can be covalently linked to membrane glycolipid (Knox & Wicken, 1973) and lipase may therefore affect spore attachment indirectly by denaturing the lipoteichoic acid.

In summary, carbohydrate recognition domains, which probably do not involve collagen, may be present on the cuticle surface and interact with N-acetylglucosamine moieties on the spore surface. These in turn are linked either to proteins or peptidoglycans which are extensions of the bacterial cell wall.

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#### ZUSAMMENFASSUNG

*Kohlehydrat/Eiweiß-Wechselwirkungen zwischen der Cuticula von Infektionsjuvenilen von Meloidogyne incognita und den Sporen des obligaten Hyperparasiten Pasteuria penetrans*

In Standardversuchen wurde der Einfluß verschiedener Vorbehandlungen auf das Anheften der Sporen von *Pasteuria penetrans* an die Cuticula von Juvenilen des zweiten Entwicklungsstadiums (J2) von *Meloidogyne incognita* geprüft. Entweder J2 oder Sporen wurden mit einer Reihe proteolytischer, lipolytischer und glykolytischer Enzyme, Kohlehydrate, Detergentien und Löslichmachern behandelt und dann Anheftungsversuchen unterzogen. J2 und Sporen wurden ferner mit einer Reihe fluoreszenzmarkierter Lektine behandelt und unter dem Fluoreszenzmikroskop untersucht. Die Oberfläche der J2 enthält anscheinend Bereiche zur Erkennung von Kohlehydraten, die wahrscheinlich kein Collagen sind. Diese Bereiche stehen in Wechselwirkung mit N-Azetylglucosamin-Einheiten auf der Sporenoberfläche, die entweder an Glykoproteine oder Peptidoglykane gebunden sind. Da die Behandlung der Sporen mit dem Lysozym Muramidase die Anheftung schwächt, enthalten die Sporen auch N-Azetylmuraminsäure.

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