CDR Project No TA-MOU-CA13-031· Studies on the Adhesion of *Pasteuria penetrans* to phytophagous Nematode Surface Coat as a Model to Provide New Strategies for Biocontrol

A Final Scientific Report

By

Prof Klara Akhmedievna Tulemisova (P I), and Dr **Irina Mamleeva** (C I), National Academic Center of Agrarian Research, 79 Abylay Khan Ave , Almaty, 480091, and The Kazakh research Institute of Fruit Growing and Viticulture, 238-a, Gagarin Ave , Almaty, 480060, **Republic of Kazakhstan** Tel +73272627891, Fax +73272623831 E-mail nacar@itte kz and **Prof Ludmila Mamonova,** The Kazakh research Institute of Fruit Growing and Viticulture, 238-a, Gagarin Ave , Almaty, 480060, **Republic of Kazakhstan**
Prof Yitzhak Spiegel (P I), Division of Nematology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50-250, **Israel** Tel +972-3-9683904, FAX +972-3-9604180, E-mail vpspigl@netvision net il and **Prof Ilan Chet** (C I), Department of Microbiology & Plant Pathology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, **Israel** Tel +972-8-9481236

This CDR project was ended, officially, in August 1996 From various reasons, an extension of the duration of the project was requested, and approved This extension had allowed us to complete some parts of this work and to strengthen the cooperation between the countries

During the project, two scientists from kazakhstan (Dr Irina Mamleeva and Mrs Dzhaklhaeva Gulnar Turarovna) had spent 6-month-training period, at Volcani Center, together with the research team working on this project Both were specially interested in immunolabelling procedures using light (fluorescent-) and electron microscope

In 1997, the team from Kazakhstan (Prof Tulemisova & Dr Mamleeva) and the Israeli P I (Y Spiegel), represented parts of their results in the IOBC meeting held in Bonn, Germany

It is aimed, at future, to continue the cooperation by submission an AID/CDR proposal which will focus on the biocontrol agent *Trichoderma*

A Attachment of *Pasteuria penetrans* spores to the surface of *Meloidogyne javanica* second-stage juveniles

Introduction

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 themeselves (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 Davies (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 due to 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 has been recently characterized by Sharon and Spiegel (unpublished results) 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, unpublished)

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 the 250-kDa polypeptide in the attachment mechanism

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 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 (1000g, 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 a flat-bottomed microtiter plates Each plate contained 30 live J2 mixed with a suspension of 10^6 spores/ml, in 50 ml PBS/2 containing Ca^{++} and Mg^{++} ions and 10 ml Coomassie Brilliant Blue (2) Each treatment was replicated in three

wells Plates were shaken horizontally at 100 RPM, room temperature, and monitored under an inverted microscope After 1 to 2 hours, when sufficient attachment had been achieved (20 to 30 spores/J2), 10 ml of 37% formaldehyde solution was added to kill and fix the nematodes The number of spores adhered 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 I Detergents J2 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 species were further incubated for 2 days, at 4 C or 25 C, and the number of adhering spores was recorded

II Carbohydrates J2 were incubated for 2 hours in Ca^{++}- and Mg^{++}- containing PBS/2, with 0 1 M of the following carbohydrates fucose, galactose, glucose, a-methyl mannoside, N-acetylgalactosamine or N-acetylglucosamine J2 were then washed with PBS/2 prior to the attachment assays

III Lectins J2 were incubated for 2 hours in PBS/2 containing Ca^{++}, Mg^{++} and Mn^{++} ions with 0 1 mg/ml Con A or WGA J2 were then washed with PBS/2 prior to the attachment assays

IV Spore treatment with M javanica *SC extract* SC 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 mg/ml leupeptin (Boehringer) and 0 1 mM N-tosyl-2-phenylalanine chloromethyl ketone (TPCK) (Sigma Chemical Co , St Louis, MO) Nematodes were then pelleted by centrifugation at 250g and the supernatant was filtered through a 0 2-mm 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 blocked for 1 hour with 1% bovine serum albumin (BSA) dissolved in PBS 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 mg/ml according to 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 injected 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 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 Non-treated nematodes or nematodes treated with one of the following reagents nonimmune serum, secondary antibody, or silver enhancement solution, served as controls Silver enhancement was not performed for transmission

electron microscopy (TEM) Non-treated nematodes or nematodes treated with either secondary antibody or nonimmune serum served as controls

Scanning and transmission electron microscopies 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 which had not been processed for electron microscopy The observations were made under a light microscope

Results

Factors affecting spore attachment I Detergents Less spores adhered to the surfaces of detergent-treated J2 than to those of non-treated 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 non-treated J2, whereas no adhesion of spores was recorded with the SDS-treated J2 kept at 4 C for 2 days (Table 1)

II Carbohydrates Less spores adhered to J2 pretreated with fucose or a-methyl mannoside, except for the head region where no inhibition was recorded (Table 2) Galactose, glucose, N-acetylgalactosamine and N-acetylglucosamine pretreatments, on the other hand, did not significantly affect the intensity of spore adhesion as compared to the non-treated J2 (Table 2)

III Lectins Pretreatment of J2 with Con A or WGA reduced spore attachment, with Con A, the reduction recorded in the head region was minor but significant (Table 3)

IV 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)

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 alae 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* J2 stripping this layer with detergents (without affecting nematode viability), or pretreatment of spores with an extract of this layer, 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 Zuckerman et al (20) A similar recovery phenomenon has been observed with human red blood cells adhering to *M javanica* J2 surfaces (Sharon and Spiegel, unpublished) Previous studies with *M incognita* have revealed that only drastic procedures reduce spore binding (13), for example, Davies and Danks (6,7) reported that *M incognita* J2 treatment with SDS for 2 hours does not result in less adherence, but in fact increases 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 a-methyl mannoside adhered less spores than non-treated 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)

The CRD on J2 surfaces were related to the C-type group, a Ca^{++}-dependent animal lectin (17,18) Binding of *P penetrans* has also 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 less spores (Table 3), suggesting the involvement of surface carbohydrate residues in the attachment process Bird et al (3) reported 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 non-treated 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, unpublished data) and can therefore not 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 which has been previously reported for germ-tube 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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Table 1 *Pasteuria penetrans* spore attachment to *Meloidogyne javanica* second-stage juveniles (J2) as affected by detergent treatments

Treatment	after incubation at 25 C‡	after incubation at 4 C‡	no incubation
	spores/J2	spores/J2	spores/J2
Control	20 8 ± 6 8 a	22 3 ± 5 2 a'	18 5 ± 5 4 a"
Triton X-100	8 4 ± 4 6 b	19 5 ± 4 8 a'	6 4 ± 5 6 b"
SDS†	5 5 ± 3 9 b	21 2 ± 5 6 a'	6 2 ± 4 7 b"

‡J2 were incubated for 48 hours

†SDS = sodium dodecyl sulfate

Data are means of 25 replicates 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 5 a	16 8 ± 8 2 a'
Methyl mannoside	4 7 ± 2 2 a	9 7 ± 5 5 b'
Fucose	6 2 ± 1 6 a	11 5 ± 4 8 b'
Glucose	6 3 ± 2 9 a	18 7 ± 9 6 a'
N-Acetylglucosamine	6 0 ± 3 3 a	15 4 ± 7 3 a'
Galactose	4 6 ± 2 0 a	15 0 ± 5 1 a'
N-Acetylgalactosamine	5 1 ± 2 1 a	15 1 ± 5 2 a'

Data are means of 25 replicates Means within a column followed by a common letter are not different, according to Tukey's HSD (P= 0 05)

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 5 a	22 4 ± 9 7 a'
WGA	5 2 ± 3 5 ab	14 2 ± 7 6 b'
Con A	3 2 ± 3 1 b	6 5 ± 4 6 c'

WGA = wheat germ agglutinin

Con A = concanavalin A

Data are means of 25 replicates 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 3 a	19 3 ± 9 9 a'
SC-treated spores	5 0 ± 2 4 a	4 5 ± 3 1 b'

Data are means of 25 replicates Means within a column followed by a common letter are not different, according to Tukey's HSD (P= 0 05)

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 4 a	14 1 ± 9 4 a'
Nonimmune serum	5 7 ± 2 3 a	10 1 ± 5 7 ab'
Antibody	5 4 ± 2 9 a	5 0 ± 4 9 c'

Data are means of 25 replicates Means within a column followed by a common letter are not different, according to Tukey's HSD (P=0 05

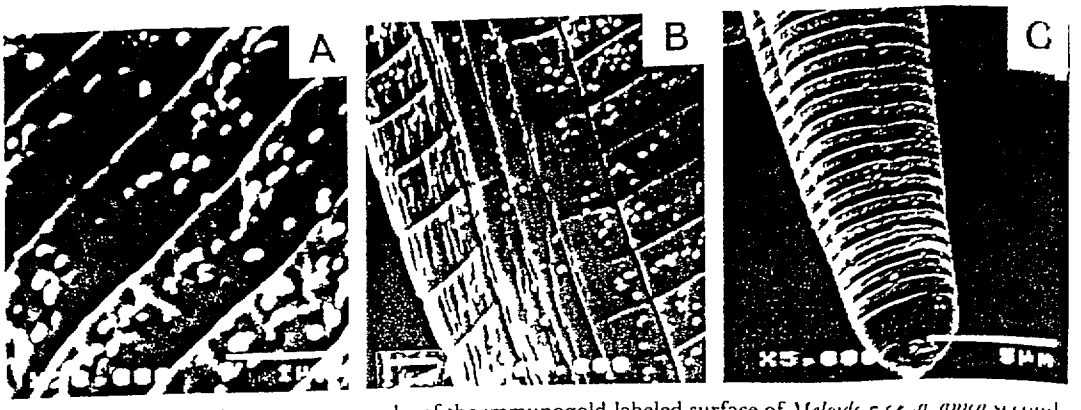

Fig 1 Scanning electron micrographs of the immunogold labeled surface of *Meloidogyne javanica* second stage juveniles labelling was enhanced by silver enhancement reaction A) Transverse anterior section B) Lateral field C) Anterior region

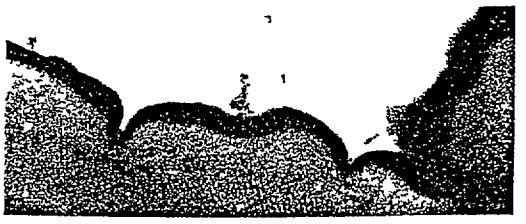

Fig 2 Transmission electron micrograph of the immunogold labelled surface of *Meloidogyne javanica* second stage juveniles cut longitudinally (×40 000)

B *Meloidogyne javanica* surface proteins Characterization and lability

Introduction

The presence and characterization of carbohydrate-binding proteins (CBPs) in animals has been investigated intensively over the last few years The 'animal lectins' are classified into several groups, one of which is Ca^{+2}-dependent (C-type) Several mannose-binding proteins belong to this group, which can also bind fucose, glucose and N-acetylglucosamine (glucNAc) (Drickamer & Taylor, 1993)

Animal lectins have scarcely been reported on nematode surfaces Human red blood cells (HRBC) have been reported to bind to the surface of several plant-parasitic nematodes, and preincubation of *Meloidogyne javanica* pre-parasitic second-stage juvenile (J2) with a series of carbohydrates inhibited this binding, which was Ca^{+2}- and Mg^{+2}-dependent (Spiegel *et al* 1995) HRBC also adhered to nylon fibers coated with SC extracted from *M javanica* J2 binding decreased when the nylon fibers were pre-incubated with fucose or mannose (Spiegel *et al* 1995) These experiments support a working hypothesis that HRBC adhesion involves surface carbohydrate residues of HRBC and CBPs on the nematode surface A direct labelling of *M javanica* J2 surfaces with neoglycoproteins further supported this hypothesis (Sharon & Spiegel, 1996)

The epicuticle of many nematodes is covered by a thin layer, characterized by a net negative charge and recognized by electron microscopy as a fuzzy coating This layer has been termed 'surface coat' (Bird & Bird, 1991, Wright, 1987) Surface coat (SC) investigations have focused mainly on animal and free-living nematodes, and the labile nature of the SC has been explored (Proudfoot *et al* 1991) In plant-parasitic species, the labile and transitory nature of the SC were demonstrated for *M incognita* using radioiodinated and biotinylated nematodes (Lin & McClure,

1996), and with *Anguina agrostis* where surface proteins were replaced after treatments with proteolytic enzymes (Bird & Zuckerman, 1989) In animal parasites the SC is believed to have an important role in adaptation and survival in paratenic hosts because recognition of exposed surface molecules by the host immune system can contribute to development of an effective immune response (Kennedy, 1991) In plant-parasitic nematodes, studies of the interactions with their parasites (nematophagous fungi or *Pasteuria* spp , for example) or other microorganisms (e g *Clavibacter*) demonstrated the biological role of the nematode SC in those interactions (Bird, Bonig & Bacic, 1989, Davies & Danks, 1993, Spiegel & McClure, 1995) Recently it has been shown that the SC is released during the infection process, the SC was detected in infected host tissues with antibodies raised to *M incognita* SC (Gravato Nobre et al 1995, McClure & Gravato Nobre, personal communication)

In this paper we describe I Characterizations of CBPs and other surface proteins of a plant-parasitic species, the root-knot nematode *M javanica*, and II The dynamic properties attributed to the plant-parasitic nematode SC

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 Eggs were separated from egg masses with sodium hypochlorite (0 5%, 1 min) and hatched in two-fold-diluted phosphate buffered 0 1 M saline, pH 7 2 (PBS/2) to obtain infective J2 that had no direct contact with host tissues Unless otherwise specified, 2- to 3-day-old J2 were used

SC proteins extraction

Proteins were extracted with sodium dodecyl sulfate (SDS) according to a method described previously (See 'first annual report')

Gel electrophoresis and western blots

Analytical - SDS polyacrylamide gel electrophoresis (SDS-PAGE) through 4-15% gradient gels was performed in a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA), using 1 5-mm-thick vertical slab gels essentially as described by Laemmli (1970) Pre-stained molecular-weight standards (Sigma) were run on gels to destine for electroblotting

For western blots, proteins were transferred onto nitrocellulose sheets using a Gelman Sciences BioTrance midi-apparatus (Ann Arbor, MI, USA) General protein-staining on blots was performed with Protogold (BioCell, Cardiff, UK) according to the manufacturers' instructions

Labelling of glycoproteins on blots

Labelling of glycoproteins was performed using gold-conjugated lectins Blots were blocked overnight at 4^0C with PBS containing 1% Bovine serum albumin (BSA) and 0 05% Tween-20 (blocking solution) and then washed three times with PBS containing 0 1% BSA and 0 05% Tween-20 (washing solution) Gold-conjugated Con A and WGA lectins (E Y Laboratories, San Mateo, CA, 1 /ml) dissolved in the washing solution containing 1 mM Ca^{+2}, Mg^{+2} and Mn^{+2} ions, were incubated for 1 hr at RT with the blots Binding specificity was verified by pre-incubating the lectins for 30 min with 0 1 M of the appropriate hapten -methyl mannoside for Con A, and glucNAc or chitobiose for WGA Blots were washed with the washing solution

and then distilled water and labelling was visualized with a silver-enhancing kit for blots (BioCell)

Labelling of CBPs on blots

CBPs were labelled on western blots, following the aforementioned blocking and washing steps Gold-conjugated neoglycoproteins (E Y Laboratories), fucosylated-, mannosylated- and glucosylated-BSA, were diluted to a concentration of 1 /ml in washing solution containing Ca^{+2} ions and incubated with the blots for 1 hr at 37°C BSA-gold was used as a negative control Specificity was verified by 1 Pre-incubating the blots under the same conditions, with the following competitive haptens (0 1 M) fucose, -methyl mannoside, glucose and N-acetylgalactosamine (galNAc) or galactose 2 Pre-incubating the neoglycoproteins with the appropriate lectins (20 /ml) *Ulex europaeus* agglutinin (UEA-I) with fucosylated-BSA, and Con A with mannosylated or glucosylated-BSA

Labelling SC proteins with biotin localization on J2 and on western blots

Meloidogyne javanica J2 were incubated for 15 min with each one of the following biotin reagents 1 sulfo N-hydroxysuccinimide (NHS)- biotin (Pierce), 2 -aminocaproic acid NHS-biotin (Sigma) Stock solutions dissolved in 0 05 M carbonate buffer (pH 9 2) were diluted in PBS/2 to a working concentration of 500 /ml Each of these biotin treatments was followed by washing the nematodes three times with PBS/2 containing 0 1% BSA

The biotin label was localized by incubating nematodes for 2 hr with gold-conjugated goat anti-biotin (1-nm particles, BioCell) diluted (1/100) in the washing solution Non-labelled J2 which were incubated with the antibodies served as controls Nematodes were then prepared for

light microscopy, using the silver enhancement method, as described in the '**third annual report**'

Biotin-labelled proteins were extracted by boiling the biotinylated and non-biotinylated nematodes in SDS-PAGE loading buffer for 15 min (Maizels *et al* 1991), followed by vigorous centrifugation to pellet the nematodes and collection of the supernatant

Biotin-labelled proteins were monitored on western blots by the following methods Blots were first blocked at 4^0C, overnight, with 5% skim milk in PBS containing 0 05% Tween 20, and washed (3 x 10 min) with PBS containing 0 1% skim milk and 0 05% Tween 20 Blots were then incubated either with 1 /ml streptavidin horseradish peroxidase (HRP) (Pierce) in the washing solution for 1 hr, or for 2 hr with anti-biotin HRP (Sigma) diluted 1 1000 in the washing solution With both methods, incubation was followed by washing twice with the washing solution and finally with only PBS Labelling was visualized with 4-chloro-1-naphthol for the streptavidin-HRP A more sensitive ECL kit (Amersham Life Science, Buckinghamshire, UK) was used as a substrate for the anti-biotin HRP, to visualize the label on x-ray film

SC lability **a** *Dynamics of HRBC* adherence to nematodes' SC

Preparation of HRBC and assay performance were according to Spiegel *et al* (1995) To evaluate the effect of J2 age on HRBC adherence, J2 were collected daily for 6 days, and each harvest was kept separately at 25^0C in PBS/2 The HRBC-binding assay was performed with each harvest on day 6, J2 used in these assays were pre-incubated for 1 hr in PBS/2 containing Ca^{+2} ions

b *Sc recovery after detergent stripping*

To test the recovery phenomenon, SDS-treated and non-treated species were further incubated for 2 days, at 4^0C or at 25^0C, and the number of adhering HRBC was recorded In addition, nematodes kept at 4^0C for 2 days were then transferred to 25^0C for another 2 days and the number of HRBC adhered to them was also monitored

To demonstrate SC-protein recovery on gels, SDS-extracted J2 were washed thoroughly and divided into two groups for a 2-day incubation at 4°C or 25°C, and a further extraction with SDS The extracts were then loaded onto SDS-PAGE gels

Results

Separation of SC extracts by SDS-PAGE

Typical SDS-PAGE pattern of the SC proteins extracted with SDS is presented in Fig 1, lane 1 The extraction yielded bands at 6, 9, 14, 22, 26, 31, 46, 49, 58, 66, 80, 205 and 250 kDa

Labelling of glycoproteins on blots

On western blots, Con A (data not shown) and WGA (Fig 2) labelled the 6-, 14-, 31-, 58- and 66-kDa bands, WGA labelled also the 22- and 26-kDa bands (Fig 2) Pre-treatment of the WGA blot with glucNAc resulted with almost complete inhibition of the labelling (Fig 2), while with Con A, labelling was only partially inhibited by -methyl mannoside (data not shown)

Labelling of CBPs on blots

The labelling pattern of the SDS-extracted SC on western blots was the same after exposure to all neoglycoproteins tested Labelling was observed at 14, 22, 26, 58 and 66 kDa (e g fucosylated-BSA in Fig 3) Preincubation with the appropriate carbohydrates did not inhibit or

reduce neoglycoprotein binding to the membrane, however, compatible lectins pre-incubated with the neoglycoproteins did reduce the
labelling intensity of the 14-, 22-, and 26-kDa bands with fucosylated- (Fig 3), mannosylated- and glucosylated-BSA When Con A was used for inhibition, a new 31-kDa band appeared, probably resulting from binding of Con A to the SC protein on the blot (see Fig 2)

Biotin labelling

The transverse annulations over the surface of second-stage juveniles, were labelled when the nematodes were exposed to the biotin reagents (e g NHS-biotin in Fig 4) When streptavidin-HRP was used to trace the biotin-labelled proteins on a western blot, the main labelling was observed at 130 kDa in both biotinylated and non-biotinylated nematodes Using anti-biotin-HRP instead of streptavidin-HRP and visualizing with ECL reagents on x-ray film revealed specific labelling at 46 and 49 kDa only with the -aminocaproic acid NHS-biotin (Fig 5)

SC lability ***a*** *Dynamics of HRBC adherence*

HRBC did not adhere to freshly hatched 0- to 24-hr old J2 Maximum adherence of J2 kept at 25^{0}C was observed with 24- to 48-hr-old J2 and this adherence then decreased gradually (Table 1)

b *SC recovery after detergent-stripping*

Less HRBC adhered to the surfaces of detergent-treated J2 than to those of non-treated J2 (Spiegel *et al* 1995) When SDS-treated J2 were kept at 25^{0}C for 2 days, the intensity of HRBC adhesion was the same as that recorded for the non-treated J2, whereas no adhesion of HRBC

was recorded with the SDS-treated J2 which were kept at 4°C for 2 days However, when these J2 were then transferred to 25°C for 2 days, adhesion re-occurred

The electrophoretic profile of the SDS-stripped SC of nematodes kept at 25°C for 2 days was much as that of the original SDS-extraction's protein pattern, whereas the profile of the SDS-stripped SC of nematodes kept at 4°C for 2 days presented only two bands at 58 and 66 kDa (Fig 1)

Discussion

In this work we characterized surface proteins on pre-parasitic J2 of the root-knot nematode *M javanica* Politz & Philipp (1992) defined surface proteins by their accessibility to non-penetrating labelling methods and their ability to be solubilized from the cuticle by mild extraction procedures Several methods have been used to characterize and map SC proteins (e g Blaxter, 1993, Proudfoot *et al* 1991) After comparison with other detergents such as cetyltrimethylammonium bromide (CTAB), or N-octylglucoside (data not shown), we decided to choose the SDS extraction method for our analyses because it resulted in more protein fragments on an SDS-PAGE while having no effect on J2 viability

Biotin derivatives have been used to detect surface-associated proteins (Spiegel & McClure, 1995) Blaxter (1993) reported avidin-binding activity in a non-biotinylated adult *Caenorhabditis elegans* extract A similar phenomenon was observed with the biotinylated and non-biotinylated *M javanica* J2 extract using a streptavidin conjugate on western blots However, substituting an anti-biotin conjugate for the streptavidin reagent enabled the detection of specific labelling at 46 and 49 kDa These bands appeared also in the SDS SC extraction profile shown in Fig 1

Previously it has been shown that surfaces of intact J2 reacted with lectins and neoglycoproteins ('Third Annual Report') These reagents also react on western blots with the SDS-extracted SC proteins (Figs 2, 3), indicating that these labelled proteins are part of the nematode's surface complex

On the western blot, the 6-, 9-, 14-, 22-, 26-, 31-, 58- and 66-kDa bands could be related to glycoproteins because these bands reacted with lectins (Fig 2) SDS-PAGE-separated SC proteins of *M javanica* J2 reacted with both Con A and WGA, but only partial inhibition was recorded with the appropriate haptens

Evidence supporting the presence of CBPs on *M javanica*'s surface has already been presented by Spiegel *et al* (1995), using HRBC, further support was later provided where J2 were exposed to neoglycoproteins and revealed a binding pattern similar to that recorded for HRBC (**'Third Annual Report'**) In the current report, these neoglycoproteins also reacted with the SC protein extract on a western blot (Fig 3)

SC lability has been well-documented in several parasitic and free-living nematodes and it is generally accepted that one of the biological function of this feature is to avoid detection by the host's immune system (Blaxter *et al* 1992, Page *et al* 1992, Politz & Philipp, 1992)

This report explores a few aspects of SC lability in J2 of a plant-parasitic species, *M javanica* The dynamics of HRBC adherence to J2 surfaces indicates changes occurring in the SC during the juvenile life HRBC barely adhered to freshly-hatched 0- to 24-hr-old J2 (Table 1), indicating that the CBPs are probably absent at this early life stage being produced only later on Maizels *et al* (1987) reported that antibodies did not bind to freshly hatched *Toxocara canis* larvae Change in the SC during the life of *M incognita* race 3 J2 was demonstrated by Lin & McClure (1996), J2 labelled with radioiodine and biotin released labeled molecules into the

water after 20 hr incubation, indicating that the surface proteins may be loosely attached to the nematode Consequently, nematode age and SC lability should always be considered when SC-labelling or extraction are performed

The ability of the SC to regenerate after external removal was previously demonstrated by Bird & Zuckerman (1989) for *Anguina agrostis* These authors have shown that the SC can regain proteins lost by exposure to proteolytic enzymes when the phytonematodes are removed from these enzymes and given time to recover This renewing ability, which reflects another aspect of the SC's transitory nature, was demonstrated by us as well Detergent-treated *M javanica* J2 were capable of re-adhering HRBC after a recovery period at an optimal temperature (25^0C) Nematodes which were metabolically arrested due to incubation at low temperature (4^0C) did not re-produce all the SC components, as indicated by the absence of HRBC binding and by the electrophoretic profile of surface proteins extracted before and after incubation of J2 at different temperatures (Fig 1) This protein profile demonstrates the ability of the SC components to recover under conditions where the nematode is metabolically active SC recovery after detergent-stripping has been demonstrated with antibodies for *Nippostrongylus brasiliensis* by Preston-Meek & Pritchard (1991)

The lability of the SC is therefore an active, dynamic, metabolic event on the phytonematode surface, a property which may enable the nematode to avoid 'unnecessary' recognition events that might lead to resistance reactions by the plant

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Table 1 Binding intensity of human red blood cells (HRBC) to the outer cuticle of *Meloidogyne javanica* second-stage juveniles (J2)

Nematodes (J2) age (hrs)	Binding intensity (Number of HRBC per nematode ± s e)
0 - 24	6 3 ± 1 6 d
24 - 48	42 7 ± 5 5 a
48 - 96	37 2 ± 5 9 ab
96 - 120	28 3 ± 4 8 bc
120 - 144	19 8 ± 3 5 c
144 - 168	1 4 ± 1 4 e

Tests were run at room temperature J2 were collected daily, for 6 days, and each harvest was kept separately in phosphate buffer 0 1 M saline, pH 7 2 The HRBC binding assays for each harvest were performed on day 6 Binding was recorded 30 min after incubation with the HRBC Ten individuals were monitored per well Experiments were repeated at least three times with consistent results Means followed by the same letter are not different, according to Tukey's HSD (P = 0 05)

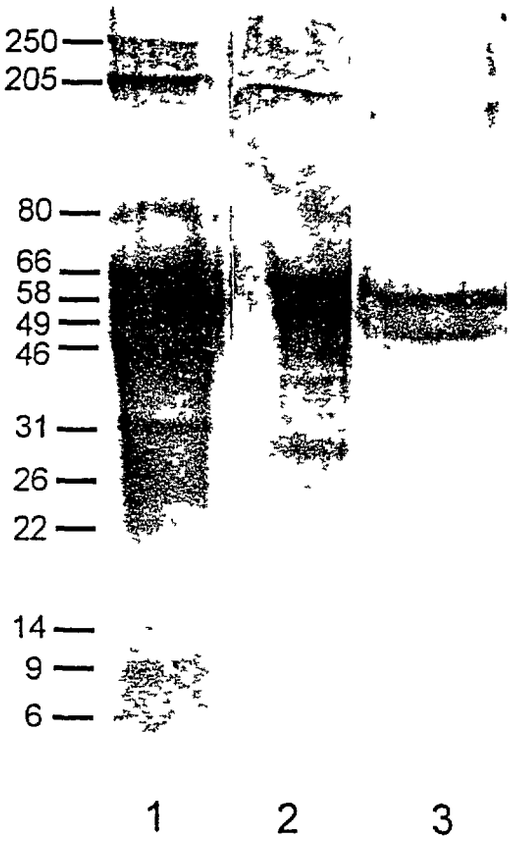

Fig 1 Protogold stained Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* 2nd stage juveniles demonstrating the temperature dependence of surface coat recovery Lane 1 SDS extraction Lane 2 further SDS extraction after 2 days at 25 °C Lane 3 further SDS extraction after 2 days at 4 °C Molecular weight markers (kDa) are indicated

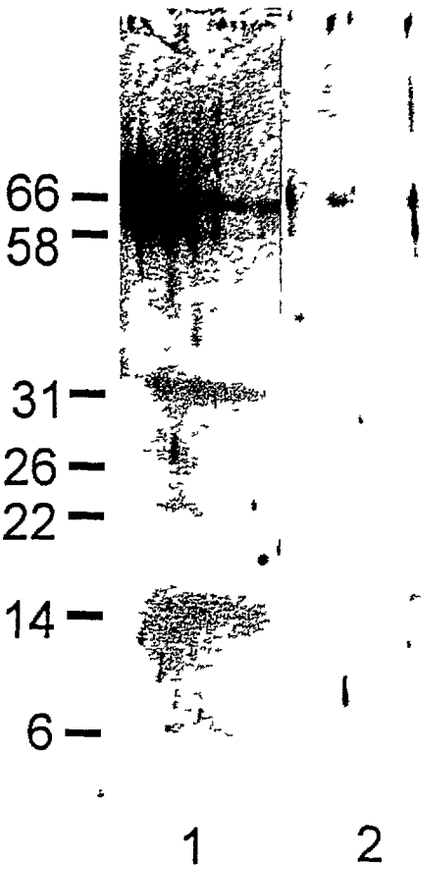

Fig 2 Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* 2nd-stage juveniles The blots were labelled with Lane 1 gold-conjugated wheat-germ agglutinin (WGA) Lane 2 pre-treatment with 0 1 M *N*-acetylglucosamine and then with gold-conjugated WGA Molecular weight markers (kDa) are indicated

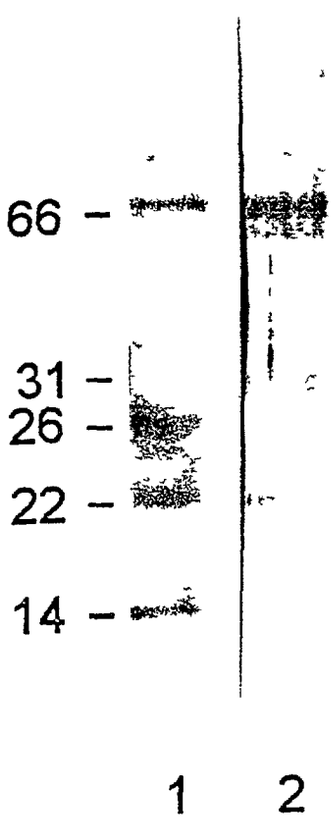

Fig 3 Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* 2nd-stage juveniles The blots were labelled with gold-conjugated neoglycoproteins Lane 1 glucosylated-bovine serum albumin Lane 2 the neoglycoprotein was pre-incubated with concanavalin A before labelling the blot Molecular weight markers (kDa) are indicated

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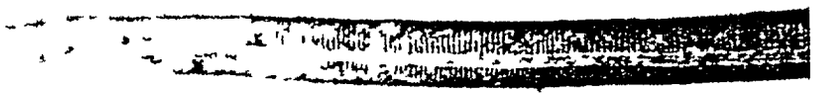

Fig 4 Anterior part of a 2nd-stage juvenile of *Meloidogyne javanica* labelled with sulfo-N hydroxysuccinimide-biotin visualized with gold-conjugated anti-biotin combined with silver enhancement

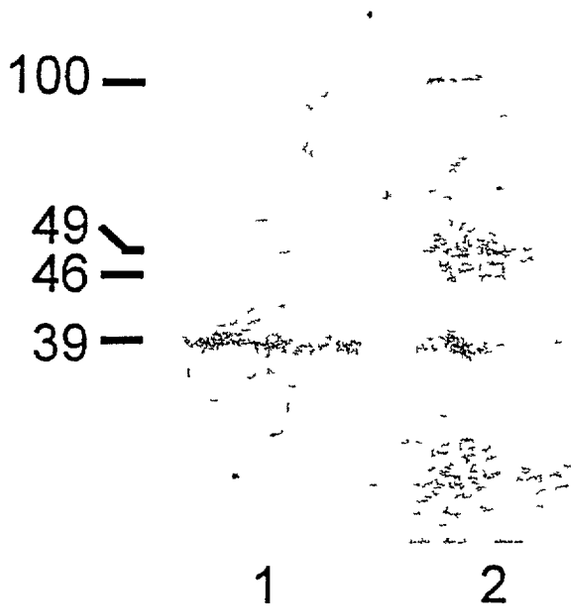

Fig 5 Western blots of biotinylated and non-biotinylated *Meloidogyne javanica* 2nd-stage juvenile extract Lane 1 biotinylation with sulfo-*N*-hydroxysuccinimide (NHS)-biotin Lane 2 biotinylation with ε aminocaproic acid NHS-biotin Molecular weight markers (kDa) are indicated

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