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Characterization of the surface coat from infective juveniles of a root-knot nematode, *Meloidogyne incognita*

Lin, Hao-jan, Ph.D.

The University of Arizona, 1993

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CHARACTERIZATION OF THE SURFACE COAT FROM INFECTIVE JUVENILES OF A ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

by

Hao-jan Lin

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PLANT PATHOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Hao-jan Lin

entitled Characterization of The Surface Coat from Infective Juveniles of A Root-knot Nematode, *Meloidogyne incognita*

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Date 8/5/93

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Date 8/5/93

M. McClure — Date 5 Aug 93

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

M. McClure — Dissertation Director — Date 5 Aug 93

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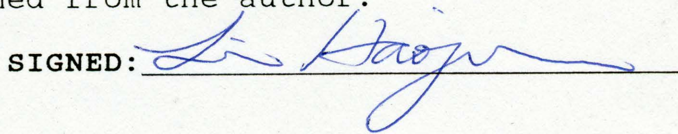
SIGNED: 

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Abstract

The nematode surface coat is defined as an extracuticular component on the outermost layer of the nematode body wall. Surface coat proteins of Meloidogyne incognita race 3 infective juveniles were characterized by electrophoresis and Western blotting of extracts from radioiodine and biotin-labeled nematodes. Extraction of labeled nematodes with cetyltrimethylammonium bromide yielded a principal protein band with a molecular weight larger than 200 KD and several faint bands of lower molecular weight ranging from 31 KD to 179 KD. Western blots of unlabeled proteins were probed with a panel of biotin-lectin conjugates, but only Concanavalin A bound to the principal band. Nematodes labeled with radioiodine and incubated in water for 20 hours released ^{125}I into the water, indicating that surface coat proteins may be loosely attached to the nematode. Antiserum to the principal protein reacted with the surface of live nematodes and with surface proteins previously separated by electrophoresis. Differential patterns of antibody labeling were obtained on Western blots of extracts from M. incognita race 1, 2, and 3, Meloidogyne hapla race 2, and Meloidogyne arenaria race B. One biological role of the surface coat may be to act as the receptor site for attachment of the nematode bacterial parasite, Pasteuria penetrans. This possibility is suggested because antiserum raised against surface coat proteins of Meloidogyne incognita race 3 reduced bacterial attachment.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are worldwide in their distribution and attack almost every type of crop, causing considerable losses of yield or affecting the quality of the product. Staple food crops in the tropics and virtually all vegetable, fruit and ornamental plants grown in the Mediterranean and subtropical countries are subject to attack by species such as *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne hapla* and *Meloidogyne arenaria*. In addition to direct damage caused by root knot nematodes, many species predispose plants to infection by fungal and bacterial pathogens (Taylor 1979 for review). The parasitic behavior of second-stage juveniles (J2) of *M. incognita* recently has been documented by the video-enhanced interference contrast light microscopy in *Arabidopsis thaliana* (Wyss 1992), the J2 invades the root tip, or the sites where lateral roots are formed in the region of elongation, close to the meristematic zone. Invasion is intracellular and then intercellular. After a J2 penetrates the root, it migrates acropetally between cortical and meristematic cells without injuring the cells. When the J2 reaches the apex of the root, it turns around and migrates to the differentiating vascular cylinder, where it becomes sedentary and begins to feed. Feeding initiates the induction of giant cells surround the J2 head region and normal plant

cell functions are altered to supply food for the nematode. Giant cells degenerate when the nematode dies. Cells surrounding the giant cells increase in size and begin to multiply at a faster rate than normal and this results in a gall or knot being formed around the nematode.

The life cycle of the southern root-knot nematode, Meloidogyne incognita, consists of an egg stage, four juvenile stages and an adult stage. The juvenile stages are separated by four molts during which the cuticular portion of the body wall is shed. The nematode's body wall is composed of the cuticle, a hypodermis or epidermis, and the longitudinally oriented somatic musculature. The cuticle is a proteinaceous, multilayered and non-cellular structure that encloses the nematode. It is synthesized by an underlying layer, the hypodermis (Jansson 1987, Wright 1987 and Zuckerman 1983). The cuticle of plant-parasitic nematode consists typically of an outer epicuticle, a cortical zone, a median zone, and a inner basal zone. The structure and thickness of the cuticle varies among nematode species and among developmental stages of the same species. In the free-living nematode, Caenorhabditis elegans, the cuticle has been shown to be composed principally of collagenous protein extensively cross-linked by disulfide bonds (Cox 1981).

An extra-cuticular component on the outer surface of the nematode, that ranges in thickness from 5 nm to 20 nm and can

be visualized only by electron microscopy (EM), is termed a surface coat (SC) or glycocalyx. In my study the term "surface coat" is used in preference to "glycocalyx", because the latter was originally used to refer to the carbohydrate moieties on the outside part of a cell membrane (Luft 1976). Foley et al (1986) showed that the nematode surface behaves differently than cell membranes. However, Bird & Zuckerman (1988) argued that the term 'glycocalyx' might be more appropriate, because the surface coat is considered to be a secretion from the nematode rather than an integral part of the cuticle.

Cetyltrimethylammonium bromide (CTAB) and other cationic detergents efficiently dissolve the surface coat (McClure & Spiegel 1991, Pritchard 1985 and 1988), but anionic ones have little effect, perhaps owing to the strong negative charge of the surface (Page 1992b). Surface coat proteins are heterogenous with regard to molecular weight (Maizels 1984). Glycosylation of the SC, which was detected by a variety of lectin conjugates has been shown in a number of nematodes (Kaplan 1992, McClure 1988, Spiegel 1982, 1988 & 1983, and Spiegel et al. 1991). The entire surface, or parts of it, were labeled with the lectin (Spiegel et al. 1991, McClure 1988). Surface carbohydrates appear to be part of glycoproteins in the animal parasitic nematodes, free-living nematodes, or plant parasitic nematodes, such as Tylenchulus

semipenetrans and Anguina species, (Bird 1988 and Spiegel 1988).

The presence of lipid in the surface of plant parasitic nematodes has been demonstrated by pretreatment of nematodes with lipase which increased the surface-labeling intensity of lectin (Spiegel 1988). An extraction with organic solvents followed by TLC analysis revealed the presence of polar, apolar and complex glycolipid on the surface of C. elegans (Blaxter 1992). Additionally, fluorescent lipid analogs were used to probe the surface of the animal parasitic nematodes, Trichinella spiralis and Toxocara canis (Kennedy 1987, Proudfoot 1990 and 1991). Lipid was detected on the nematode's surface and fluorescence was recovered after photobleaching.

The origin of the SC is not known. Bird and Zuckerman (1988) have hypothesized that SC is deposited by secretions released by excretory/secretory pores. Moreover, the SC is capable of renewal after degradation by proteolytic enzymes (Bird 1988). However, the evidence presented for these contentions is mostly circumstantial. Surfaces of phytonematodes stained with protein-specific stain were unstained except in areas adjacent to the amphids and excretory pore (Premachandram 1988). Another hypothesis proposed by Spiegel and McClure (1991) is that the SC originates from host materials and coats the nematodes as they

emerge from the eggs. A more target-specific labeling system for the SC is required before the origin of the SC can be demonstrated conclusively.

Information regarding the biological role of the SC is scarce. Limited information is available concerning the involvement of the SC in interactions between animal parasites, free-living or phytophagous nematodes with other microorganisms or the parasite's host.

Infection structures (traps or spores) of nematophagus fungi are covered with an adhesive material that firmly attaches to the nematode surface. The attachment is mediated by a lectin (Jansson 1987,and Nordbring-Hertz 1979). In some cases, the surface carbohydrate is non-specifically located over the entire cuticle, but in other cases they are specifically located on either the head or tail, thus explaining the frequent attachment of the fungal traps to the cephalic and caudal region.

Anguina agrostis, a seed-gall nematode of annual ryegrass, is the vector for Corynebacterium rathayi (Clavibacter sp.). Livestock grazing on infected ryegrass are poisoned by corynetoxins produced by the bacteria. The bacteria adhere strongly to the surface of the nematode's cuticle, and the mechanism of adhesion may be lectin mediated (Bird 1989). McClure and Spiegel (1991) confirmed the contribution of the nematode's SC to the process of adhesion

by EM and cytochemical studies. However, juveniles that naturally resisted bacterial adhesion did not lack a SC. It was hypothesized that the SC of individuals, to which bacteria do not adhere naturally, lacks crucial components that can not be defined by conventional electron microscopy.

Pasteuria spp. are host-specific obligate parasites of a number of plant parasitic nematodes. Their development and life cycle is highly synchronized to the nematode's life cycle (Sayre 1985,1988 and Stirling 1985). Nematicidal efficacy and its capacity to survive in soil under extreme conditions make Pasteuria penetrans a promising candidate as an alternative to nematicidal chemicals (Sayre 1980 and McClure 1991). The process of Pasteuria spore attachment to the nematode cuticle is not understood. Stirling et al.(1986) proposed that a biochemical interaction involving P. penetrans spores and the nematode surface layer is responsible for binding. Stirling et al. (1986) concluded that lectins are not involved in P. penetrans spore attachment after investigations with different lectins. However, Davies and Danks (1992 and 1993) claimed that the lectin on nematode surface might interact with N-acetylglucosamine moieties on the spore surface. Unfortunately, a recent attempt using potential antigens on the nematode surface failed to prove this assumption (Davies 1992).

Another role of the SC may be its function in recognition

phenomena (Zuckerman 1984) where it could operate similar to a glycocalyx anchored in plasma membranes. McLaren (1976) hypothesized a dual role for the surface coat; one being that of a receptor-effector in the chemotactic process and the other as a monitor of the output of gland secretions. Spiegel et al (1982) showed the presence of sialic acid residues in the nematode body wall by reacting nematodes with DNP-DABH (Dinitrophenyl-L-2,4 diaminobutyric acid hydrazide) and labeled DNP with primary IgG, and secondary anti-rabbit goat fluorescein. However, a chemotactic function related to neuraminic acid and sugar residues, whether located on sensillum membranes or the surface coat, has not been established. Furthermore, Wright (1987) claimed that the epicuticle immediately underlying the SC is more likely an envelope than a plasma membrane.

The objective of my study was to characterize the SC with respect to constituent proteins, including their molecular weights, glycosylation, and possible release into the environment. In addition, the biological role of the surface coat as the receptor for P. penetrans endospores was investigated. The SC proteins from the J2 of M. incognita race 3 were characterized by electrophoresis and Western blotting with radioiodine and biotin-avidin labeling systems. ^{125}I-Bolton Hunter reagent, labeling the lysine residues, was used to iodinize protein components (Bolton and Hunter 1973).

Nonradioactive biotin-avidin labeling has been widely used in the isolation of biotin-derivatized materials and cytochemistry because of the high affinity constant ($K_D = 10^{-15}$) between avidin and biotin (Bayer 1980). Reactive biotin derivatives are available that attack lysine amino groups (N-hydroxysuccinimide-ester) and aldehydes (biotin hydrazide). Radiolabeling of animal parasitic nematodes has shown that surface molecules are released over time into culture supernatant (Maizels 1983,1984 and Philipp 1980). The release of surface coat from Meloidogyne incognita was investigated by following the radioactivity removed over different periods of times with the J2 labeled by ^{125}I-Bolton-Hunter reagent. Polyclonal antibodies were raised against the SC components to demonstrate antigenic differences of the SC extracts from Meloidogyne spp. and to show if the SC is a receptor for attachment of Pasteuria penetrans, a bacterial parasite of root-knot nematodes. My studies enhanced the understanding of the basic biology of root-knot nematodes in regards to their interaction with P penetrans. Such knowledge may facilitate the development of future biological control strategies for plant parasitic nematodes.

Materials & Methods

1. Nematode Cultures

Egg mass of Meloidogyne spp. were obtained from J. N. Sasser, Department of Plant Pathology, North Carolina State University, and propagated on eggplant, Solanum melongena cv. Black Beauty, in the greenhouse. Eggs were collected from infected plants (McClure et al. 1973) and hatched on nylon mesh (pore size 20 μm) in distilled water to obtain infective J2.

2. Bacterial Cultures

A dry soil culture of Pasteuria penetrans, strain P100, was provided by Dr. D. Dickson (University of Florida, Gainesville FL). Bacteria were increased by infecting M. incognita race 3 J2 an aqueous suspension for up to two days at 30 C in the dark. The suspension of J2 with bacterial spores was mixed into steamed sandy (50%) soil. A tomato (Lycopersicon esculentum cv. Florida Petit) seedling was then transplanted into the soil. After 4 weeks, tomato roots with galls containing infected nematodes were washed with running tap water and air dried at room temperature on paper towels. Dried galls placed in 0.9% NaCl saline buffer were cut with a scalpel to release bacterial endospores. The spore suspension was filtered through nylon mesh (20 μm pore size) and concentrated by centrifugation at 10,000xg for 5 minutes at room temperature. The reconstituted spore suspension (1 cc in

0.9% NaCl) was then layered on the top of a centrifuge tube containing 9 cc of 90% sucrose and centrifuged for 30 min at 1,000xg. The layer containing the spore suspension and some debris was pipetted into 60 ml of 0.9% NaCl and concentrated at 10,000xg for 5 minutes. Bacterial spores were kept at -20 C for long term storage or at 4 C in the dark for short term storage.

3. Solubilization of Surface Coat Protein

About 1 cc. of M incognita, Race 3 J2 were collected as described above and were washed by pelleting in distilled water several times at 1,000xg for 3 minutes. Four cc of 0.25% cetylmethylammonium bromide (CTAB) containing proteinase inhibitors (5 μg/ml aprotinin, 2 mg/ml iodoacetamide, 0.2 mg/ml phenylmethylsulfonyl fluoride and 50 μg/ml soybean trypsin inhibitor) was added to the J2 pellet (Pritchard 1985 and 1988) and the suspension was incubated up to 4 hrs at 37 C with agitation for 10 seconds every 30 minutes. After concentration by centrifugation, the supernatant was filtered through a 0.22 μm Millipore (Millex-GV) membrane and concentrated with a 10,000 NMWL Immersible-/CX Ultrafilter (Millipore Corp.). The concentrated extract was then diluted with 3 volumes of 2% SDS in 0.5 M Tris-HCL, pH 6.8, and concentrated to approximately 200 μl. The crude extract was stored at -70 C.

4. Live Nematode Surface-labeling

A. Biotinylation

Freshly hatched J2 were washed several times in distilled water and incubated with 0.3 mg/ml biotin-N-hydroxy-succinimide-ester (NHS-biotin, Calbiochem-Novabiochem Corp.) for 1 h at room temperature. The excess biotin was removed by centrifuging and resuspending the biotinylated nematodes 4 times in distilled water (Keith 1990).

B. Radio-iodination

Freshly hatched J2 were labeled using ^{125}I-Bolton Hunter reagent according to the instructions given by the supplier (E.I. Du Pont de NEMOURS & CO. INC). The reaction was carried out at pH 8.0 in 0.01 M phosphate buffered saline (PBS) with 250 μCi of ^{125}I for 1 hour at room temperature. Uncombined iodine at the end of incubation period was removed by reaction with excess lysine and the nematodes were washed repeatedly with PBS (Robinson 1989).

C. Lectin-FITC

M. incognita, Race 1 were centrifuged at 1,000xg in 1.5 ml microcentrifuge tubes. Supernatant was aspirated and the pellet of nematodes, containing 1,000-2,000 J2, was resuspended in 0.2 ml of Ulex europaeus agglutinin-I (UEA), provided by E-Y Laboratories as a conjugate of fluorescein isothiocyanate (FITC). Lectin-FITC conjugate was diluted to a concentration of 0.1 mg/ml with 0.01 M PBS, pH 7.2. After incubation for 90 minutes, nematodes were washed three times

with PBS. The lectin-treated nematodes were mounted under coverslips on glass microscope slides and examined by incident fluorescence microscopy with an excitation wavelength of 450-490 nm and barrier filter of 520 nm (McClure 1988).

D. Anti-rabbit FITC:

Juveniles, washed as described above, were incubated in rabbit serum (immune or pre-immune) for 2 hours. Treated nematodes were then washed three times with PBS and incubated in goat anti-rabbit antiserum-FITC (Sigma) for one hour. After incubation, the treated nematodes were washed to remove unbound antibody conjugates and examined by fluorescence microscopy.

5. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed using either 4-20% (w/v) gradient precast gels (Jule Inc.), or 6% or 12% non-gradient gels (pH 8.8) prepared on a Hoefer SE 250 gel casting form, according to Laemmli (1970). A stacking gel (4% acrylamide, pH 6.8) was pipetted on top of the resolving gel prior to use. For each 8 μl sample of extract per track, 2 μl glycerol and 1 μl of 0.1% tracking dye (bromophenol blue) was added and the mixture was boiled for 90 seconds to denature the protein. Gradient gels, 0.75 mm thick, were run at a constant 10 mA for 2 hours while 6% gels, 1.0 mm thick, were run overnight at 3 mA. Coomassie Blue prestained standard protein markers (14.4 Kd to

97.4 Kd; BIO-RAD) were run on each gel as a reference. Following electrophoresis, the gel slab was silver stained (Morrissey 1981) or used unstained for Western blotting. For silver staining, the gel was fixed in 50% methanol for 3 minutes followed by 30 minutes in 10μg/ml dithiothreitol. The gel was then incubated with 0.1´ (w/v) silver nitrate for 30 minutes and placed in the developer (50 μl of formaldehyde in 100 ml of 3% sodium carbonate) until the desired level of staining was attained. Staining was stopped by adding 5 ml of 45% (w/v) citric acid.

6. Immuno-Blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electrophoretic transfer (Towbin 1979), on a BIO-RAD 'Trans-Blot' apparatus operated at 0.8 mA/cm^2 for 8 hours. The transfer buffer contained 50 mM Tris, 380 mM glycine, 0.1% wt/vol SDS, and 20% methanol. The membrane was then blocked in Blotto/Tween (5% nonfat dry milk (w/v) and 0.2% Tween 20) in 0.01 M pH 7.2 PBS with agitation on an orbital shaker for two hours at 23 C. The blot was washed with PBS once for 5 minutes and incubated in polyclonal anti-SC antiserum (1:100), containing 0.2% Tween 20, for at least two hours at 23 C. Blotted proteins labeled with the anti-SC antibodies were detected by incubation in alkaline phosphatase secondary anti-rabbit antiserum (1.1 μg/ml, Sigma) in Tris-buffered Saline (TBS)(25 mM Tris-HCl, 0.8% NaCl,

0.002% KCl, pH 8.0) for 1 hour at 23 C. The blot was washed 3 times in TBS between each incubation and then developed in bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) until the desired intensity of color was attained. The NBT substrate solution was prepared from stock solutions. NBT stock was prepared by dissolving one NBT tablet (10 mg substrate content, Sigma) in 1 ml of deionized water. BCIP stock was prepared by dissolving one BCIP tablet (25 mg substrate content, Sigma) in 0.5 ml 100% dimethyl formamide. Alkaline phosphatase buffer contained 100 mM NaCl, 5 mM $MgCl_2$, and 0.1 M Tris-HCl at pH 9.5. Substrate solution was prepared by adding 66 μl NBT stock to 10 ml alkaline phosphatase buffer, mixing well, then adding 33 μl of BCIP stock. It was used within one hour. Development was stopped by rinsing with PBS containing 20 mM EDTA.

7. Biotin-avidin Blotting:

Biotinylated juveniles were extracted with CTAB and the extract was electrophoretically separated and blotted as described above. The blot was blocked by incubation with 3% albumin, bovine fraction V (BSA) in TBS buffer (10 mM Tris-HCL, pH 7.4, 0.9% NaCl, w/v) for 2 hours at room temperature. Biotin-labeled proteins were detected by incubating the blot in 1.25 μg/ml (1:2000 of stock) alkaline phosphatase avidin (Calbiochem Inc.) for 1 hour at room temperature, followed by three washes with TBS. The blot then was developed in

NBT/BCIP substrate solution for 5 minutes.

8. Lectin-affinity Blotting:

Glycosylated SC proteins were detected by a modification of the method reported by Kaplan and Gottwald (1992). Western blots of extracts were blocked with BSA as described above for biotin-avidin blotting. Blots were then incubated with agitation for 1 hour at 23 C in biotinylated lectin (E-Y Laboratories, Inc. P.O. Box 1787 San Mateo, CA 94401): Concanavalin A (1.0 μg/ml _Canavalia_ _ensiformis_ agglutinin), GS-1 (3 μg/ml _Griffonia_ _simplicifolia_ agglutinin), SBA (2 μg/ml _Glycine_ _max_ agglutinin), WGA (2 μg/ml _Triticum_ _vulgaris_ agglutinin), LPA (10 μg/ml _Limulus_ _polyphemus_ agglutinin), BPA (2 μg/ml _Bauhinia_ _purpurea_ agglutinin), DBA (4 μg/ml _Dolichos_ _biflorus_ agglutinin), UEA (2 μg/ml _Ulex_ _europaeus_ agglutinin), PNA (0.4 μg/ml _Arachis_ _hypogaea_ agglutinin) in HEPES (N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)) buffered saline (10 mM HEPES, 150 nM NaCl, 0.1 mM $CaCl_2.2H_2O$, 0.1 mM $MgCl_2.6H_2O$, 0.01 mM $MnCl_2.4H_2O$, pH 7.5). Biotinylated lectins that bound to the blotted proteins were visualized by incubation in 1.25 μg/ml alkaline phosphatase avidin in TBS, followed by NBT/BCIP development.

9. Purification of Surface Coat Proteins:

Proteins extracted with CTAB were separated by SDS-PAGE on 6% (w/v) acrylamide slab gel. Proteins from a strip (one lane) of the gel were visualized by silver staining and the

strip was aligned with the remainder of unstained gel. Regions of the unstained gel were then excised and proteins were eluted by a electro-eluter (BIO-RAD Model 422) with constant current at 10 mA/Tube for 8 hours. After elution, the elution buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS) was removed by dialysis against PBS. The resultant partially purified SC proteins were stored at -70 C for 2-3 weeks.

10. Immunization:

Pre-immune sera were collected from rat, goat, mouse and rabbits. Rabbits maintained in the University of Arizona Animal Care facility were immunized subcutaneously (st) and intramuscularly (im) with a 2-3 μg suspension of SC proteins in saline mixed 1:1 with RIBI Adjuvant system (RIBI Immunochem Research Inc.), followed by three injections (st and im) with 0.5 μg proteins in PBS 1:1 mixed with RIBI adjuvant at 3-4 week intervals. Antibody was purified by ammonium sulfate precipitation and by Protein A-DEAE Affi-Gel Blue gel chromatography for the IgG fraction, according to the protocal provided by BIO-RAD.

11. Release of ^{125}I Surface-labeled Molecules:

Approximately 0.2 cc. of ^{125}I labeled juveniles were washed on a 4 μm-pore-size filter using distilled water to remove unbound label. Eluate was assayed for radioactivity by passing it through a 0.22 μm Ultrafree filter (Millipore) in a centrifuge at 2,000xg for one minute and counting gamma

emission from 1.0 ml in a scintillation vial containing 20 cc of water miscible scintillation cocktail (ICN Biomedicals, EcoLite). Washing continued until the counts stabilized at approximately 21000 cpm. After washing, the volume of the solution containing the labeled nematodes was brought up to 2 ml by adding distilled water and the suspension was incubated in a 15 ml glass tube with slow agitation at 23 C. Aliquots were collected at 0 hr, 1 hr, 2 hr, 4 hr, 8 hr, 16 hr, 20 hr intervals. At each interval, the nematodes were pelleted and 1 ml of supernatant was passed through a 0.22 μm filter by centrifugation at 1,000xg. The filtrate was placed in a scintillation vial containing 20 ml of scintillation cocktail and assayed for gamma emission. Distilled water (1.0 ml) was added to the glass tube containing the labeled nematodes, in order to maintain a constant volume, and the nematodes resuspended by agitation.

12. Inhibition Assay of Bacterial Attachment:

Nematodes ($4\text{-}5 \times 10^3$ J2) or P. penetrans spores (5×10^5) were washed 3 times in PBS in microcentrifuge tubes treated with 0.1% nonfat dry milk (to prevent bacterial adhesion to the tube wall) and preincubated in undiluted pre-immune and immune sera for 2 hours at 23 C. After incubation, nematodes or bacteria were washed 3 times with PBS and un-treated nematodes ($4\text{-}5 \times 10^3$ of J2) or bacteria (5×10^5) were added to the tubes of their respective counterpart. The final volume

was adjusted to 0.5 ml. The nematode and bacteria mixtures were then centrifuged for 5 minutes at 10,000xg and resuspended by using a Vortex mixer. A 10 μl sample of suspension was placed on a microscope slide and the number of spores attached to the visible sides of the nematodes was counted under a compound microscope. This experiment was conducted twice with two replications in each experiment. Results were analyzed by factorial analysis of variance (ANOVA) and followed by Duncan's multiple-range test ($P=0.05$).

Results

Identification of Surface-associated Proteins.

Electrophoretic profiles of proteins from J2 labeled with either Biotin or ^{125}I showed different patterns of labeling (Fig 1 and Fig 2), but both labeled the same principal protein. The principal protein (SC_1) had a molecular weight greater than 200 KD based on comparison of its electrophoretic mobility with that of known proteins . Other, much fainter bands, ranged in molecular weight from 31.0 KD to 179 KD. The profiles of proteins labeled with biotin-avidin were not altered by the addition of a reducing agent, 2-mercaptoethanol. The SC extract from non-biotinylated J2 does not react with avidin conjugates. Bands of SC proteins were cut from electrophoretic gels and electro-eluted to obtain partially purified proteins that were used to raised polyclonal antibodies in rabbits. The resultant antibodies bound to the J2 cuticular surface and amphidial exudates.

Detection of Carbohydrate in Surface Coat Proteins.

To determine if CTAB extractable proteins were glycosylated, CTAB extracts were blotted and probed by 9 different lectin-biotins (Table 1). Only Concanavalin A (Con A) bound on SC_1 protein and two smaller size bands (Fig 3). The latter two may not be surface-associated because no corresponding band was observed in blots of biotin-labeled SC proteins (Fig 2).

Release of Surface Components

Release of ^{125}I-labeled surface components from the nematodes was demonstrated by incubating labeled J2 in water and assaying the incubation supernatant over a period of 20 hours. Radioactivity of the supernatant increased fourfold in the first hour and then decreased to the initial background level after 20 hours incubation (Table 2). The same trend were obtained in a second trial with a different experimental design in which ^{125}I-labeled J2 were incubated in separated tubes.

Specificity of Polyclonal Anti-Surface Coat Proteins Serum

Intra and interspecific cross-reactivity of polyclonal anti-SC antibodies was determined on Western blots of CTAB extracts from J2 of Meloidogyne spp. The purified IgG fraction of the antiserum reacted not only with SC proteins from Meloidogyne incognita race 3 but also with SC proteins from M. incognita races 1 and 2, Meloidogyne hapla race 2, and Meloidogyne arenaria race B (Fig 4). A common band occurred among all Meloidogyne species, but because it was not labeled with either biotin or ^{125}I, it was not considered to have originated from the surface of the J2.

Inhibition of Pasteuria penetrans Spore Attachment to M. incognita Race 3 by Anti-Surface Coat Antibodies.

The role of the SC was investigated to identify the SC as the attachment site for P. penetrans. Live J2's were treated

with polyclonal antibodies raised against SC_1 protein to see if they could block the attachment of P. penetrans endospores to the nematode. Immune serum significantly reduced the number of bacterial spores attached to M. incognita J2 when the nematodes were treated with immunized serum (Table 3). However, pre-immune serum also reduced spore attachment compared to controls. Nematodes treated with pre-immune serum obtained from goat,rat and rabbits also bound to the nematodes. Anti-SC serum bound to the bacteria (Fig 5), but the pre-immune serum did not. Treatment of bacterial spores with anti-SC serum significantly reduced the attachment of spores to the nematode, but the treatment with pre-immune serum did not (Table 3).

Discussion

Two techniques were used to verify that proteins extracted from J2 of M. incognita with CTAB originated from the surface of the nematode. Both ^{125}I and biotin labeled the same principal band, SC_1, on Western blots and antibodies against SC_1 bound to the surface of J2. Intra and interspecific reactivity of anti-SC antiserum raised from SC_1 protein of M. incognita race 3 suggested that the SC_1 protein band contained the antigenic sites which were recognized specifically by the polyclonal antibodies.

It has been reported that the biotin-avidin system may not be suitable for identifying SC proteins of all nematodes. An endogenous avidin-binding protein has been identified in C. elegans that reduces the surface specificity of biotin label (Blaxter, personal communication). Because CTAB extracts of non-biotinylated M. incognita J2 did not react with avidin, it was assumed that endogenous avidin-binding substances do not occur on the surface of this nematode.

Surface proteins of many nematodes, both parasitic and free-living, have been examined by extrinsic radioiodination and they typically represent a restricted set of molecules (Page 1992b, Maizels 1988 and Philipp 1984). In Toxocara canis, a nematode parasite of dogs, one surface glycoprotein (TES-120) is associated with the surface coat and is absent from other layers of the cuticle, while a distinct antigen

labeled the cuticle matrix as shown by immuno-electron microscopy (Page 1992b). Although I found no additional components labeled with ^{125}I in CTAB extracts, the existence of exposed proteins, either in the cuticle or the SC which contain amino acid groups that do not react with ^{125}I-Bolton Hunter reagents, can not be conclusively excluded.

When Western blots of SC proteins were probed with lectins, only SC_1 (that also labeled with ^{125}I and biotin) was labeled (Fig 3). And because the SC described for other nematodes contains carbohydrate, it is very likely that SC_1 was derived from the surface coat of M. incognita J2. The possibility that other SC bands containing sugar moieties which may bind to lectins other than those tested was not addressed. Specific binding of Con A to SC_1 demonstrated that mannose- or glucose-containing residues are present in SC_1 (Table 1). This was consistent with observations of other investigators who have shown that Con A specifically binds to the surface of live nematodes and can be displaced by treatment with competitive haptens (McClure 1988 and Spiegel & McClure 1991). Surface coat proteins have also been found on animal parasitic nematodes where, because of their lability, they are thought to play an active role in the evasion of host defence mechanisms (Vetter 1978, Grove 1987, Philipp 1980, Hammerberg 1984, Carlow 1987, Politz 1992, Ibrahim 1989, Apfel 1990, Edwards 1990 and Page 1992a). In my

investigation, ^{125}I labeled SC proteins also sloughed from the nematode's surface when viable infective J2 were incubated in water (Table 2). The release of ^{125}I at the beginning of the time-course experiment may have resulted from a rapid initial turn-over rate that soon depletes releasible SC proteins (Table 2). An important finding regarding the SC of animal parasitic nematodes is that the process of shedding is an active one, inhibitable by metabolic arrest with azide or incubation at 4 C (Smith 1981 and Edwards 1990). This indicates that there may be a specific biochemical or enzymatic pathway that induces shedding. A similar process in plant parasitic nematodes would enable them to adapt to soil biological environments as well as to hosts with resistance mechanisms that depend on interactions with the nematode's surface.

Antibodies to nematode antigens have been employed for differentiation of nematodes species (Dropkin 1988), but a reliable serological system for distinguishing populations within species has not been advanced. While the SC immunogen obtained from J2 of *M. incognita* apparently induced a more specific response in my experiment, species and subspecific diagnosis was not an objective and, therefore, not pursued further. However, based on my results, it may be possible to develop a serological system of diagnosis by utilizing more specific antibodies raised against surface components.

Anti-SC antibody inhibiting the *P. penetrans* spore attachment to *M. incognita*

The role of the SC in the attachment of *P. penetrans* endospores to *M. incognita* was demonstrated by blocking the adhesion of the bacteria to the nematode with polyclonal anti-SC serum, suggesting that attachment sites were specifically occupied by antibodies. Nonspecific binding to *M. incognita* of pre-immune antibodies from other animals also occurred. The nature of the non-specific binding is not known. However, it has been found that human red blood cells (HRBC) can adhere to the surface of certain nematodes, including *M. incognita* and that the binding was reduced by preincubating J2 with different sugars (Spiegel et al. 1991). These experiments supported the hypothesis that HRBC adhesion involved carbohydrate moieties of HRBC and carbohydrate-recognition domain(s) (CRD) which may be part of a lectin (Drickamer 1988) on the nematode surface. Non-specific binding of pre-immune sera to *M. incognita* could involve such a mechanism. Davies & Danks (1992 and 1993) proposed that there may be sugar moieties, namely N-acetylglucosamine, on the spore of *P. penetrans* which is recognized by CRD on the nematode cuticle. The CRDs in animal lectins are characterized by a set of invariant residues which have been found in other proteins and are found in association with a range of the special effector domains, such as a glycosaminoglycan or epidermal growth

factor (EGF) (Drickamer 1988). It is possible that a homologous domain exists on the bacterial carbohydrate receptor and on the nematode surface, which can be recognized by anti-SC serum, but not by the pre-immune serum. Pre-immune serum may contain antibodies which bind to different domains (if any) other than CRD's and caused steric hindrance for bacterial attachment.

Another concern about the specificity of P. penetrans attachment to its nematode host can be addressed according to the results obtained from immunoblots of SC proteins (Fig 4). It was reported that the attachment frequency of endospores to nematode hosts varied with geographical regions (Spaull 1981 and Sayre 1985) and tended to adhere more easily to the original host from which spores were collected (Slana 1981). However, Stirling (1985) claimed that bacterial spore attachment was not related to the nematode species. Immunoblots (Fig 4) revealed that SC proteins from different Meloidogyne spp. can be recognized by antibodies raised against individual species (M. incognita race 3) including various non-target proteins that differ in molecular weight and antibody binding capacity. Because the SC is probably the attachment site of the endospores, the specificity of adhesion that occurred between P. penetrans and its root-knot nematode host may be determined by the differential efficiency and the quantity of SC proteins as bacterial endospore receptors.

Conclusions

This research has provided information on biochemical characteristics of the surface coat from Meloidogyne incognita and demonstrated its possible biological role as the receptor site for Pasteuria penetrans. I have identified a protein (SC_1) with molecular weight larger than 200 KD that may be surface-associated. SC_1 appears to be glycosylated and releasible from the nematode surface. Anti-SC serum raised from Meloidogyne incognita race 3 was intra and inter species-specific among Meloidogyne spp. Attachment of P. penetrans on the nematode surface coat was demonstrated by the reduction of bacterial attachment with anti-SC serum. These results set the stage for further research to improve the efficiency of P. penetrans as a biocontrol agent, and increase the biological and biochemical understanding of plant parasitic nematodes.

Appendix A: Tables and illustrations

Table 1. Carbohydrate specificity of lectin-biotin

Lectins	Carbohydrate specificity
Canavalia ensiformis (Con A)	Man, Glc, GlcNac
Arachis hypogaea (PNA)	Gal, GalNAc
Ulex europaeus I (UEA-I)	L-Fucose
Dolichos biflorus (DBA)	MAcDGal
Bauhinia purpurea (BPA)	Gal, GalNAc
Limulus polyphemus (LPA)	Sialic Acid
Triticum vulgaris (WGA)	(GlcNAc)2
Glycine max (SBA)	GalNAc, Gal
Griffonia simplicifolia (GS-I)	Melibiose, Gal

Mannose (Man), Glucose (Glc), N-acetyl-D-glucosamine (GlcNAc), Galactose (Gal), N-acetyl-D-galactosamine (GalNAc), Methyl-2-acetamido-2-deoxy-D-galactose (MAcDGal), N-Acetyl neuraminic acid (Sialic Acid), N,N-diacetylchitobiose ((GlcNAc)2).

Table 2. Release of ^{125}I from the surface of infective juveniles (J2) of _Meloidogyne incognita_

Incubation time (hours)	CPM
0	23854
1	77993
2	66100
4	59905
8	57045
16	46704
20	24517

CPM: Counts per minute (gamma emission) from a scintillation vial containing 20 cc of water miscible scintillation cocktail and 1.0 cc of supernatant from ^{125}I-labeled _M_. _incognita_ J2. Samples were drawn at intervals from a 2.0 cc pool of labeled nematodes and, after sampling, the original volume of the pool was restored by addition of 1.0 cc of sterile distilled water.

Table 3. Inhibition of *Pasteuria penetrans* endospore attachment to *Meloidogyne incognita* infective juveniles (J2) by anti-surface coat serum

Treatment	Number of attached spores/J2
Immune Serum/Nematode[u]	7.4±2.2a[z]
Immune Serum/Bacteria[v]	9.8±3.3a
Pre-immune Serum/Nematode[w]	8.2±2.8a
Pre-immune Serum/Bacteria[x]	15.8±3.6b
control[y]	20.8±5.5b

u: J2 pretreated with anti-SC serum and incubated with bacterial endospores.

v: Bacterial endospores pretreated with anti-SC serum and incubated with J2.

w: J2 pretreated with rabbit pre-immune serum and incubated with bacterial endospores.

x: Bacterial endospores pretreated with rabbit pre-immune serum and incubated with J2.

y: J2 incubated with bacterial endospores without treatment of serum.

z: Mean and standard deviation obtained from ten different nematode J2. Treatment were compared using an ANOVA ($F=7.53$, $P=0.025$), followed by Duncan's multiple-range test ($P=0.05$). Treatments differing significantly are indicated by different letters.

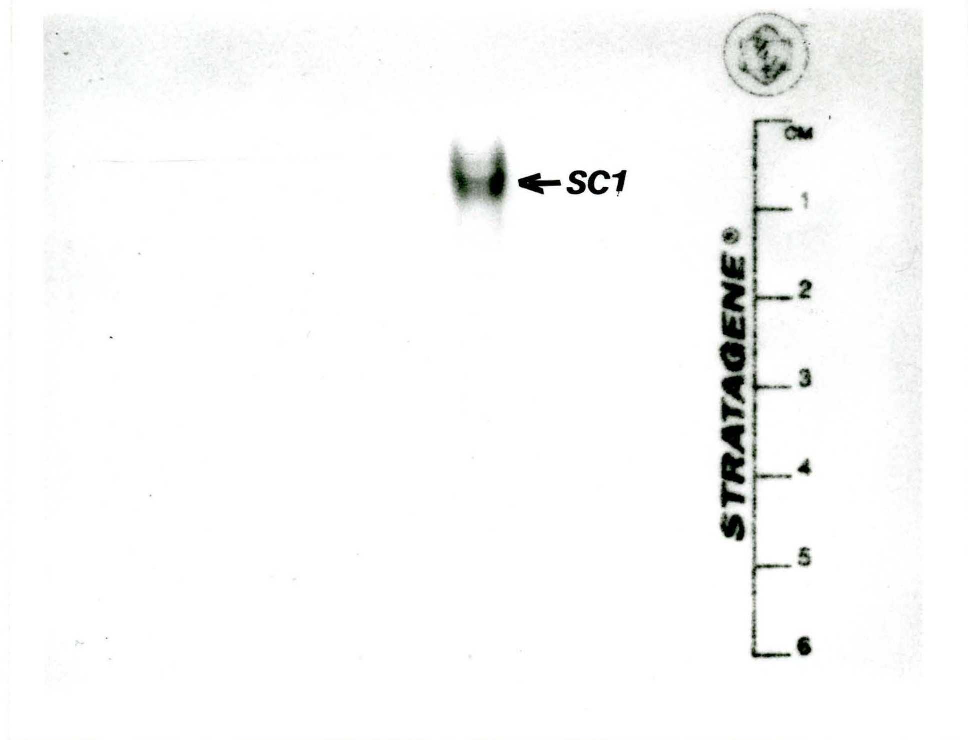

Fig 1. Western blot of CTAB extracts from <u>Meloidogyne incognita</u> race 3 infective juveniles labeled with radioiodine (^{125}I).

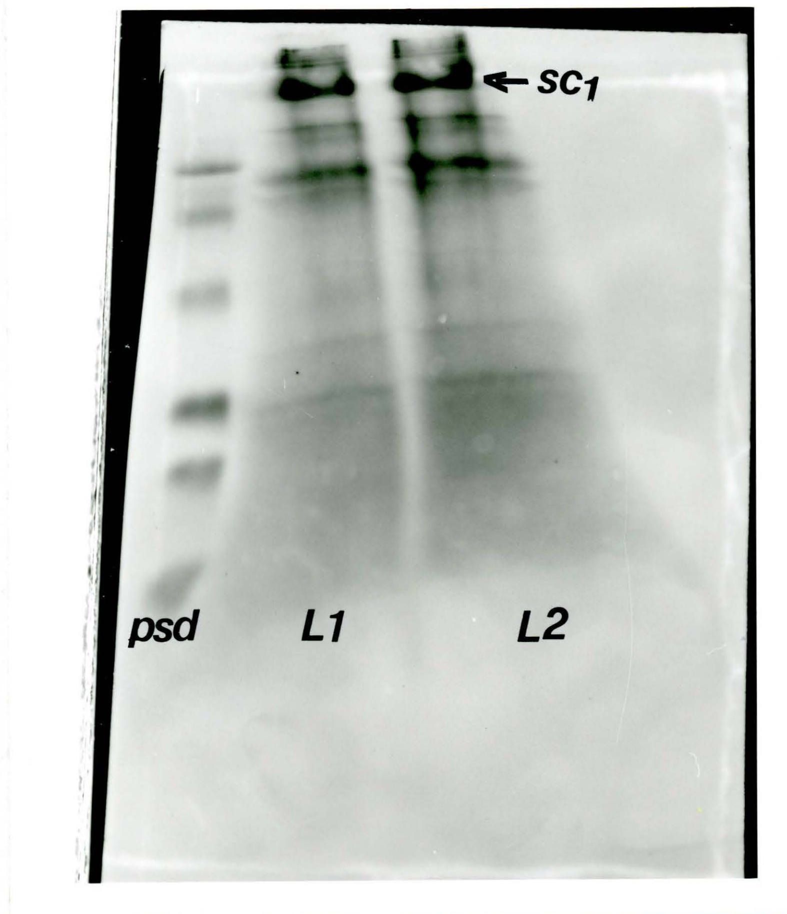

Fig 2. Western blot of CTAB extracts from Meloidogyne incognita race 3 infective juveniles (J2) labeled with biotin. Lane 1 and lane 2 were duplicate treatments. PSD was prestained standard protein markers (14.4 KD-97.4 KD).

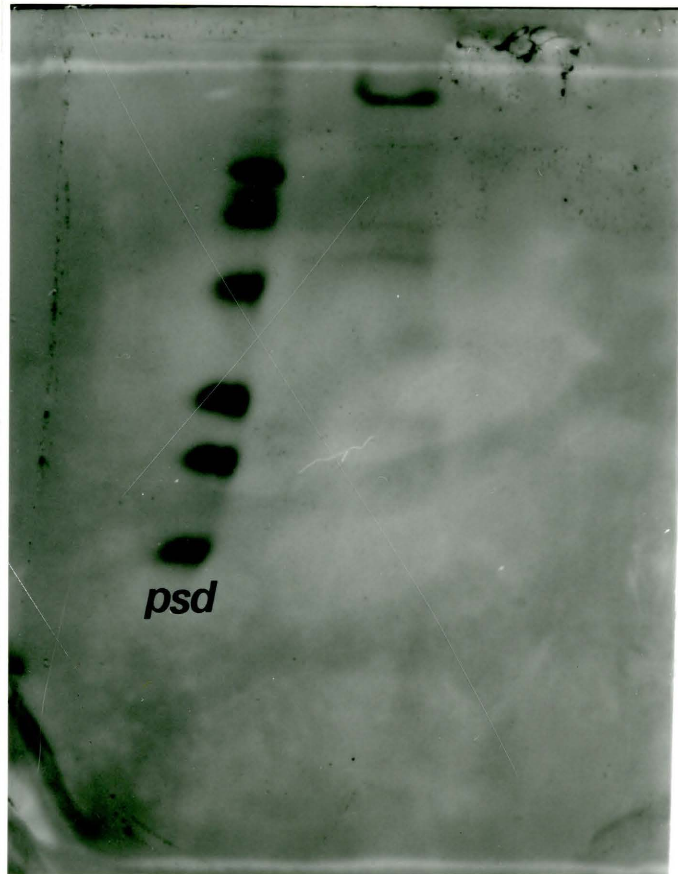

Fig 3. Western blot of CTAB extracts from Meloidogyne incognita race 3 infective juveniles (J2) labeled with Concanavalin A-biotin conjugates. PSD was prestained standard protein markers (14.4 KD-97.4 KD).

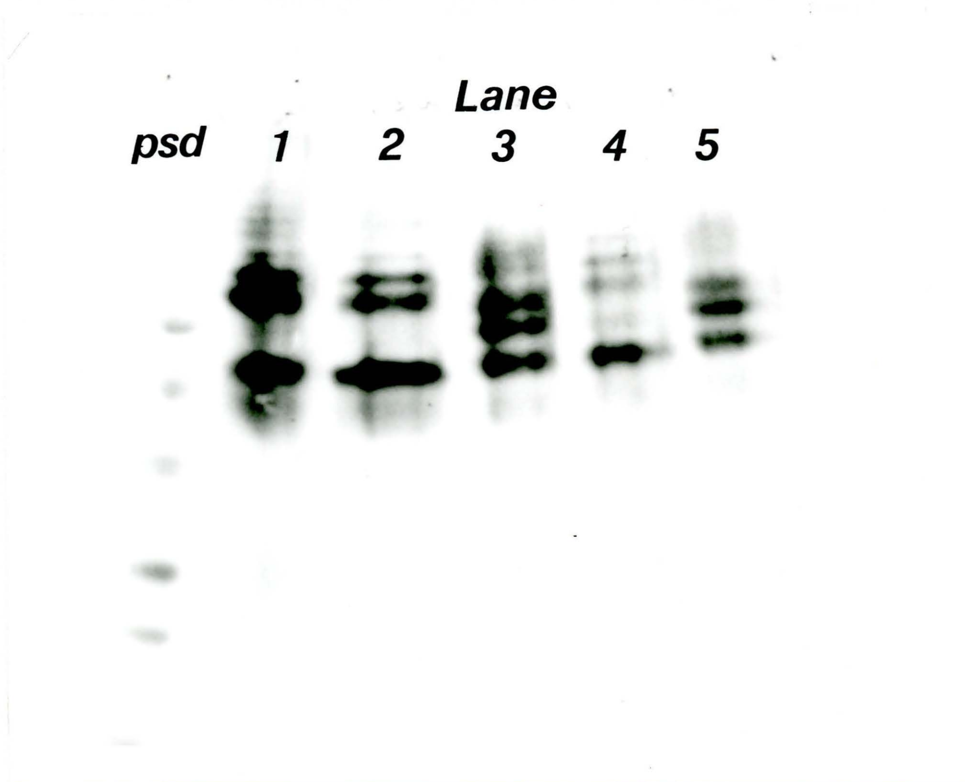

Fig 4. Western blot of CTAB extracts from unlabeled J2 of different Meloidogyne spp., probed with anti-SC antibodies against M. incognita race 3. Lane 1, extract from M. incognita race 3; lane 2, extract from M. incognita race 2; lane 3, extract from M. incognita race 1; lane 4, extract from Meloidogyne hapla race 2; lane 5, extract from Meloidogyne arenaria race B; PSD, prestained standard protein markers (14.4 KD-97.4 KD).

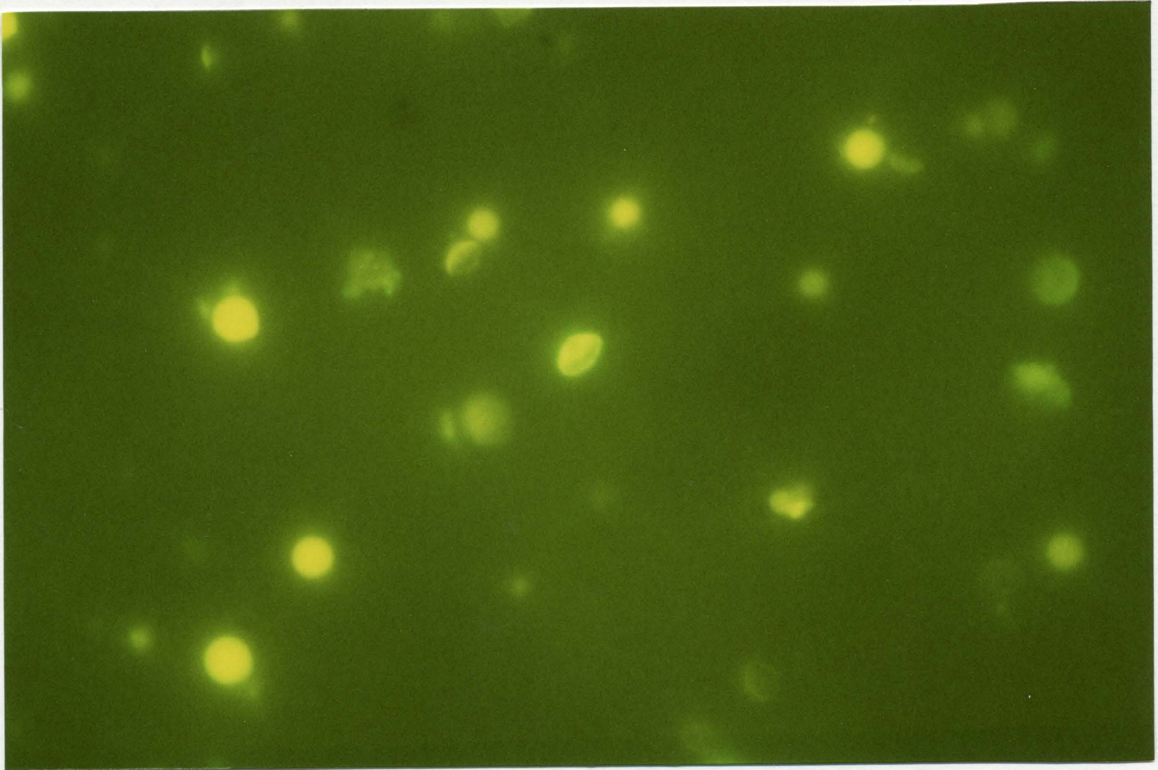

Fig 5. Photomicrograph of endospores of Pasteuria penetrans treated with antiserum to the surface coat proteins of Meloidogyne incognita race 3 and then labeled with goat anti-rabbit FITC conjugates.

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