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Temporal Formation and Immunolocalization of an Endospore Surface Epitope During *Pasteuria penetrans* Sporogenesis[1]

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Abstract: The synthesis and localization of an endospore surface epitope associated with the development of *Pasteuria penetrans* was determined using a monoclonal antibody (MAb) as a probe. Nematodes, uninfected or infected with *P. penetrans*, were harvested at 12, 16, 24, and 38 days after inoculation (DAI) and then examined to determine the developmental stage of the bacterium. Vegetative growth of *P. penetrans* was observed only in infected nematodes harvested at 12 and 16 DAI, whereas cells at different stages of sporulation and mature endospores were observed at 24 and 38 DAI. ELISA and immunoblot analysis revealed that the adhesin-associated epitope was first detected at 24 DAI, and increased in the later stages of sporogenesis. These results indicate that the synthesis of adhesin-related proteins occurred at a certain developmental stage relative to the sporulation process, and was associated with endospore maturation. Immunofluorescence microscopy indicated that the distribution of the epitope is nearly uniform on the periphery of each spore, as defined by parasporal fibers. Immunocytochemistry at the ultrastructural level indicated a distribution of the epitope over the parasporal fibers. The epitope also was detected over other structures such as sporangium and exosporium during the sporogenesis process, but it was not observed over the cortex, inner-spore coat, outer-spore coat, or protoplasm. The appearance of the adhesin epitope first at stage III of sporogenesis and its presence on the parasporal fibers are consistent with an adhesin-related role in the attachment of the mature endospore to the cuticle of the nematode host.

Key words: biological control, ELISA, immunoblot, immunofluorescence, immunogold labeling, *Meloidogyne arenaria*, monoclonal antibody, nematode, *Pasteuria penetrans*, root-knot nematode, SDS-PAGE.

Pasteuria penetrans (Thorne) Sayre & Starr is a gram-positive, endospore-forming procaryotic parasite of *Meloidogyne* spp. Endospores attach to second-stage juveniles (J2) as they move through soil pore spaces. After the endospore-encumbered juvenile enters a host root and begins feeding, the endospore germinates at some point in development, presumably before the J2 molts to the third-stage juvenile (Sayre and Starr, 1985, 1988; Sayre and Wergin, 1977; Serracin et al., 1997). The infective tube is analogous to a germ tube that penetrates the nematode cuticle and hypodermal tissue, and then enters the pseudocoelom (Sayre and Starr, 1988), where unknown growth factors promote vegetative growth, differentiation, sporulation, and maturation of endospores.

There are certain attributes that make *P. penetrans* a desirable biological control agent: (i) encumbered juveniles have reduced activity and ability to infect roots (Sturhan, 1985); (ii) infected juveniles complete their life cycle, but females have low or no fecundity (Bird, 1986; Bird and Brisbane, 1988); (iii) infection of the nematode host results in propagation and amplification of endospores; and (iv) endospores, released into the soil, are resistant to desiccation, high temperature, and most nematicides, and are persistent and virulent for more than a year or longer (Dickson, 1998; Dickson et al., 1994; Dutky and Sayre, 1978; Stirling, 1985; Williams et al., 1989). This prokaryote completes its life cycle and produces virulent endospores only within the pseudocoelom of *Meloidogyne* spp., which must be reared on intact plant hosts either in pots or on excised-root systems (Verdejo and Jaffee, 1988). Attempts to culture *P. penetrans* in vitro have failed to produce virulent endospores (Bishop and Ellar, 1991; Reise et al., 1988; Williams et al., 1989). The physiological aspects of its life cycle have been studied and are reasonably well understood (Chen et al., 1997b; Freitas, 1997; Giannakou et al., 1999; Hatz and Dickson, 1992; Nakasano et al., 1993; Serracin et al., 1997; Stirling, 1981). However, biochemical events that occur during the development of *P. penetrans*, leading to the formation of virulent endospores within the pseudocoelom, are poorly understood.

The molecular basis for the recognition and attachment of endospores to their nematode hosts has been the subject of investigation in several laboratories. Lectin-carbohydrate interactions have been suggested to be involved in the attachment of *P. penetrans* to its nematode host. Previous studies have shown that wheat-germ agglutinin (WGA) inhibited the attachment of endospores (Bird et al., 1989; Charnecki, 1997; Charnecki et al., 1998; Davies and Danks, 1993). Also, proteins extracted from endospores of *P. penetrans* were recognized, not only by monoclonal antibodies (Charnecki, 1997; Charnecki et al., 1998; Davies and Redden, 1997) and polyclonal antibodies selected against whole endospores of *P. penetrans* (Charnecki et al., 1998; Chen et al., 1997a; Davies et al., 1992; Persidis et al., 1991) but also by WGA (Bird et al., 1989; Charnecki, 1997; Persidis et al., 1991). These results indicate that one or more epitopes detected by the antibodies may be glycosylated with β-1,4 linked-N-acetylglucosamine. The ability of

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antibodies and lectins to inhibit attachment of the endospores to nematode hosts has provided the basis for considering a role for glycan ligands as determinants in adhesins involved in the attachment of endospores to nematode hosts (Charnecki, 1997; Charnecki et al., 1998; Persidis et al., 1991; Preston et al., 2003).

Understanding the processes that lead to the growth, differentiation, sporulation, and maturation of *P. penetrans* within the pseudocoelom likely will provide a basis to establish the conditions required for its mass production in vitro. The objectives of this study were to (i) determine the synthesis of spore-associated proteins (adhesins) as a function of *P. penetrans* development within the pseudocoelom of the nematode host, *M. arenaria* race 1; (ii) determine the distribution of an adhesin-associated epitope on the surface of virulent endospores; and (iii) detect and localize an adhesin-associated epitope during the sporogenesis process.

Material and Methods

Nematode source: Meloidogyne arenaria (Neal) Chitwood race 1 used in this study was originally isolated from peanut (*Arachis hypogea L.*) at Green Acres Research Farm, University of Florida, Alachua County, Florida. Nematodes were reared on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) maintained in a greenhouse. Eggs of the nematodes were extracted from galled roots with NaOCl (Hussey and Barker, 1973). Second-stage juveniles were obtained by hatching the eggs in a modified Baermann funnel (Pitcher and Flegg, 1968).

Pasteuria penetrans source: Pasteuria penetrans strain P-20 (Oostendorp et al., 1990) used in this study was originally collected from females of *M. arenaria* race 1 parasitizing peanut in Levy County, Florida, and reared on *M. arenaria* race 1 growing on tomato in a greenhouse. One to three-day-old J2, with endospores attached to their cuticles, were obtained by incubating them with a suspension containing 1×10^5 endospores/ml overnight with constant aeration at room temperature. Endospores were exposed to a mild sonification (Sonic water bath, Fisher Scientific, Suwanee, GA) for 5 minutes before attachment. Twenty spore-encumbered J2 were chosen randomly from a glass-slide mount, and the number of endospores attached per J2 was estimated with an inverted compound microscope at ×400. The percentage of J2 with endospores attached was 100%, with an average of 7 ± 3 endospores per J2. Tomato plants (45-day-old seedlings) growing in a 15-cm-diam. clay pots were inoculated with endospore-attached J2 (3,000 J2/plant) and maintained in a greenhouse. Three days later, the plants were again inoculated as before. At 45 to 60 days after inoculation (DAI), the root systems were harvested, washed with tap water, and weighed. Roots were cut into pieces 2 to 5 cm long and subjected to digestion in a 1-liter Erlenmeyer flask containing Rapidase Pomaliq 2F at 1:5 (g/v) (Gist Brocades Pomaliq product number 7003-A/DSM, Food Specialities, Menominee, WI) previously optimized with a buffer system (Charnecki, 1997), and agitated on a shaker at 120 oscillations per minute for approximately 24 hours at room temperature. Softened roots were placed in a 600-µm-pore sieve nested in a 150-µm-pore sieve (Hussey, 1971). Females were obtained by centrifugation through sucrose gradients (Chen et al., 2000). The supernatant containing the females was collected in a beaker, and the females were examined for *P. penetrans* infection with an inverted microscope at ×100. Endospores were collected by grinding the females with a sterile pestle, and the suspension filtered through a nylon filter either with 21-µm or 18-µm openings (Spectra/Mesh). The concentration of endospores was determined by counting three 10-µl aliquots using a hemocytometer at a magnification of ×450.

Experimental design: Two sets of *M. arenaria* J2—one exposed to *P. penetrans* endospores and the other unexposed—were compared with respect to development. These were arranged randomly, with four replications per treatment per each designated ''window of *P. penetrans* development" (harvest time: 12, 16, 24, and 38 DAI). The windows of development were based on those reported by Hatz and Dickson (1992) and Serracin et al. (1997). 'Rutgers' tomato seedlings were inoculated with 3,500 J2/plant (≤2 days old) with and without endospores attached. Plants were maintained in a growth chamber at 25 °C to allow the nematodes to enter roots. After 48 hours, the plants were removed from pots and the roots washed thoroughly with tap water to remove any J2 that had not penetrated. The seedlings were replanted in clay pots (15-cm-diam.) and placed in a growth chamber at 35 °C. Plants were harvested at 12, 16, 24, and 38 DAI. The root systems harvested from plants were washed in tap water, dried with a paper towel, weighed, cut into pieces 2 to 5 cm long, and incubated in an aqueous solution of commercial Rapidase Pomaliq 2F (Charnecki, 1997). Nematodes and softened roots were collected and washed as before. Twenty nematodes were hand-picked from each root system. To determine the percentage of nematodes infected by *P. penetrans*, and the stage of development of the bacterium, the nematodes were crushed individually in a 2.5-µl drop of 5% lactophenol and 1% methyl blue (w/v) (Sigma, St. Louis, MO) (Serracin et al., 1997) under a cover glass on a glass slide and examined with an inverted microscope at ×400 magnification. The remaining uninfected and infected nematodes from each harvest time were hand-picked, washed with deionized water at 6,000g three times for 6 minutes each, and stored in 1.5-ml siliconized microtubes containing 10 µl PBS (10 mM phosphate buffer, 0.15 M sodium phosphate, pH 7.2) at −20 °C.

Extraction and determination of proteins: Noninfected and *P. penetrans*-infected nematodes harvested at each

interval after inoculation, and mature endospores (2×10^6 spores/10 µl PBS, pH 7.2) used as a control, were obtained as described previously. Nematodes in 10 µl PBS (pH 7.2) were disrupted with a pestle, and then 30 µl of the extraction solution containing ×1.33 UDC (8 M urea, 0.04 M dithiothreitol, 6.65 mM CHES buffer, pH 10) was added to each microfuge tube containing the samples. Microfuge tubes were placed into a water bath for 2 hours at 37 °C and treated with 20 seconds of sonication (Brankson Cleaning Equipment, Shelton, CN) every 15 minutes. Extracts were centrifuged at 10,000g for 5 minutes at room temperature, and aliquots of the supernatant were collected and stored at –20 °C prior to ELISA and SDS-PAGE analyses. Protein estimation was performed by a micro-protein assay, based on the Bradford's method (Bradford, 1976), according to the manufacturer's instructions (BioRad, Hercules, CA). Standard curves were generated using bovine serum albumin, and colorimetric measurement was performed at 595 nm (Hewlett Packard 8451A Diode Array spectrophotometer, Palo Alto, CA).

Monoclonal antibody: The anti-P-20 IgM monoclonal antibody (IgM MAb) used in this study was raised in mice against whole endospores of *P. penetrans* P-20 strain and selected for its ability to bind to the surface of endospores (Preston and Rice, unpubl.). This monoclonal antibody recognized an epitope shared on several polypeptides resolved by SDS-PAGE following buffered 6 M urea extraction of endospores from different biotypes of *P. penetrans* as well as other *Pasteuria spp.* (Brito et al., 1998, 2000; Charnecki, 1997; Charnecki et al., 1998; Preston et al., 2003) but did not react with polypeptides extracted from endospores of other soilborne species (Schmidt et al., 2002). The ability of the antibody to block attachment of *P. penetrans* (P-20 strain) to the cuticle of *M. arenaria* race 1 supports the conclusion that it recognizes a key determinant on one or more adhesins involved in host recognition and attachment (Charnecki, 1997; Charnecki et al., 1998). The hybridoma generating this antibody has been deposited with the American Type Culture Collection as HL 1325 (2A4-1D10). The IgM was purified from mouse ascites by gel filtration from a Sephacryl S-300 column (100 × 2.5 cm) equilibrated and eluted with 0.02 M sodium phosphate buffer, pH 7.2; 0.5 M NaCl.

Epitope quantification by ELISA: Proteins (100 ng/well) extracted from *P. penetrans*-infected nematodes (either 13 infected nematodes harvested at 12 and 16 DAI or 5 infected nematodes harvested at 24 and 38 DAI) at each harvest interval, or from P-20 strain endospores alone as a positive control (2×10^6 endospores/µl), were applied to appropriate wells of a multi-well plate with 100 µl/well of coating buffer (15.0 mM Na_2CO_3, 33.4 mM $NaHCO_3$, and 0.2% NaN_3) and incubated overnight at 4 °C. After washing the wells four times with PBST (10 mM sodium phosphate buffer, pH 7.6, 154 mM NaCl, 0.2% Tween 20), the primary antibody, anti-P-20 IgM MAb diluted to 1:100,000 in PBST, was added to the appropriate wells (100 µl/well) and incubated for 1.5 hours at room temperature. Wells were washed with PBST, and the secondary antibody, an anti-mouse IgM-alkaline phosphatase conjugated (Sigma) diluted at 1:4000 in PBST, was added to all wells, incubated for another 1.5 hours at room temperature, and the wells were washed with PBST as before. Alkaline phosphatase substrate, 0.1% *p*-nitrophenyl phosphate (w/v) (Sigma) in alkaline phosphatase substrate buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, 0.5 mM $MgCl_2$), was added to all wells, and color development was measured with an automated microplate reader at 405 nm (BioRad model 2550, Hercules, CA).

SDS-PAGE analysis: Proteins (600 ng of total noninfected or infected nematode protein) in an appropriate volume of PBS (pH 7.2) were combined with an equal volume of sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS w/v, 10% glycerol, 0.05% bromophenol blue w/v, 2% β-mercaptoethanol), boiled for 5 minutes at 100 °C, and then centrifuged for 5 minutes at 10,000g. Endospore protein extracted from P-20 strain (2×10^6 endospores/µl) was used as a control. Twenty microliters of the supernatants were transferred into appropriate wells of a polyacrylamide gel [4% stacking gel (pH 6.8), 12% separating gel (pH 8.8)] with Tris-glycine buffer (Laemmli, 1970). A prestained molecular weight marker (SeeBlue Prestained Standards, Novel Experimental Technology, San Diego, CA) was loaded onto the same gel. Electrophoresis was carried out at 100 V for 10 minutes and then set at 200 V until the dye marker moved to the bottom of the gel. Gels were electroblotted onto nitrocellulose membranes in blotting buffer (192 mM glycine, 25 mM Tris, 20% methanol) using a Mini Transfer-blot Cell (BioRad, Hercules, CA) at a constant voltage (50 V) for 2 hours. Proteins either were stained with AuroDye according to the manufacturer's instructions (Amersham, Piscataway, NJ) or probed with anti-P-20 IgM MAb.

Immunoblotting: Following electroblotting, the nitrocellulose membranes were blocked with 0.5% nonfat dry milk (w/v) in PBST overnight at 4 °C. Polypeptides containing the epitope recognized by anti-P-20 MAb were detected as follows: membranes were incubated with anti-P-20 IgM MAb diluted at 1:2,000 in PBST (7.2) for 1.5 hours at room temperature on a shaker, washed three times for 5 minutes each with PBST, incubated with goat anti-mouse IgM MAb conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in PBST (pH 7.2) as secondary antibody for 1.5 hours at room temperature on a rotatory shaker, washed three times with PBST as before, incubated with substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) three times, 5 minutes each; and incubated with alkaline phosphatase substrate (0.1 mg/ml nitrotetrazolium blue, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl

phosphate) (Promega, Madison, WI) in substrate buffer on a shaker at room temperature until color development. The blots were washed with deionized water and dried at room temperature.

Immunofluorescence microscopy: Immunofluorescence staining was performed as described by Pogliano et al. (1995) with modifications. Fresh endospores were washed and purified as before, and then filtered through a woven polyester filter with 18-µm openings. Twenty microliters of the endospore suspension (2×10^6 endospore/µl) were transferred to a 1.5-ml siliconized microtube and fixed in 230 µl of the primary fixative containing 2.7% formaldehyde and 0.008% glutaraldehyde in PBS (10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl) for 35 minutes on ice. Endospores were suspended in 250 µl PBS (pH 7.4) and then centrifuged at 6,000g three times for 6 minutes each. After resuspending the washed endospores in 150 µl PBS, 10 µl of the suspension was transferred into each of three wells of a microscope slide that had been previously treated with 0.1% poly-L-lysine (Sigma). Each slide was incubated for 30 seconds at room temperature, and then the suspension was aspirated from the wells with a sterile-transfer pipet (Fisher Scientific). After air-drying at room temperature for 30 minutes, the endospores were incubated in 10 µl with PBST-BSA (2% BSA [w/v] and 0.05% Tween 20 [v/v]) in 10 mM PBS (pH 7.4) in a multiwell slide for 15 minutes at room temperature to block nonspecific antibody-binding sites. Primary antibody, anti-P-20 IgM MAb diluted to 1:1,000 in PBST-BSA, was added to the wells and incubated overnight at 4 °C. Wells containing the endospores were washed in PBST (pH 7.4) five times for 5 minutes each, and incubated for 2 hours in the dark at room temperature with µ-chain specific, anti-mouse IgM conjugated with fluorescein isothiocyanate (FITC, Sigma, 1:1000 diluted in PBST-BSA). Anti-P-20 IgM MAb was substituted with non-immune ascites fluid at 1:1,000 dilution as a negative control. After washing the wells with PBS (pH 7.4) 10 times for 5 minutes each, the slides were mounted in Slow Fade in a PBS-glycerol solution (Molecular Probes, Eugene, OR). Preparations were examined with differential-interference contrast and fluorescence microscopy with an excitation filter at 495 nm.

Tissue preparation for sectioning: Uninfected and *P. penetrans*-infected *M. arenaria* race 1 harvested at 20 DAI were obtained as described previously. The procedure used to carry out this study was a modification of previous work (Aldrich et al., 1995; Chen et al., 1997b; Zeikus and Aldrich, 1975). Fresh nematodes were ruptured with a surgical knife (Fisher Scientific No. 15) into 40 µl of fixative (1% glutaraldehyde, 4% formaldehyde, 5% dimethyl sulfoxide in 0.2 M sodium cacodylate buffer) (pH 7.2) to facilitate the penetration of reagents, and then embedded in 2.5% ultra-low gelling temperature agarose (Sigma) and congealed in a refrigerator (4 °C). The gel was sliced into square blocks containing individual nematodes and transferred into 12 × 75-mm culture tubes (Fisher Scientific) containing 1.5 ml of the previously mentioned fixative and incubated overnight at 4 °C. Agar blocks containing nematodes were washed four times with cold 0.1 M cacodylate buffer (pH 7.2) on ice for 30 minutes each and dehydrated in a cold ethanol series containing the following percentages: 12, 25, 38, 50, 65 for 20 minutes each, and then 75% overnight at 4 °C. This was followed by sequential incubations in 85% and 95% ethanol, and two incubations in 100% ethanol, for 20 minutes each. The specimens were embedded in LR White Resin (London Resin White, Electron Microscopy Science, Fort Washington, PA) with a graduated infiltration series of 25% and 50% resin in 100% ethanol for 3 and 6 hours, respectively, and 75%, 100%, and 100% resin, overnight each time, transferred to gelatin capsule and polymerized at 50 °C for 4 days. Sections were cut from the resin block with a diamond knife on an LKB 8800 Ultratome III microtome (Sweden) and collected on Formvar-coated nickel grids (100 mesh).

Immunogold labeling: Nickel grids with sections of noninfected and *P. penetrans*-infected nematodes, and with endospore-attached juveniles, were floated, section-side down, on 20-µl drops of 1% nonfat dry milk in PBS (pH 7.2) on a piece of Parafilm (American National Can, Menasha, WI) for 15 minutes at room temperature to block nonspecific antibody-binding sites (modified from Aldrich et al., 1992, 1995; Dykstra, 1993). Grids were transferred to 20-µl drops of primary antibody, anti-P-20 IgM MAb at 1:10,000 dilution in PBS (pH 7.2), and incubated overnight in a closed petri dish inside a moist chamber at 4 °C. Control grids were incubated on non-immune ascites fluid at 1:10,000 dilution instead of anti-P-20 IgM MAb. Grids were washed two times for 10 minutes each on 20-µl drops of high salt-Tween buffer, pH 7.2 (0.1% Tween 20 in 0.03 M Tris-HCl, pH 7.2, 0.5 M NaCl), and then two times for 10 minutes each on PBS (pH 7.2). Sections were incubated with the secondary antibody, goat anti-mouse IgM conjugated to 12-nm colloidal gold, µ-chain specific (Jackson Immuno Research, West Grove, PA), diluted 1:30 in PBS (pH 7.2), at room temperature for 1 hour. After washing as before in high salt-Tween buffer and PBS, the grids were floated on Trumps fixative (pH 7.2) (McDowell and Trump, 1976) for 10 minutes at room temperature to stabilize the antigen-antibody complex, and then washed with deionized water. Sections were stained with 0.5% aqueous uranyl acetate for 7 minutes, and Reynold's lead citrate solution (Reynolds, 1963) for 2.5 minutes, and observed on a Zeiss EM-10 transmission electron microscope at 80 kV.

Results

Microscopic examination: Vegetative growth stage of *P. penetrans* was observed only in nematodes harvested at

12 and 16 DAI (Table 1). At 24 DAI, mixed developmental stages of thalli showed advanced differentiation, including quintets, quartets, triplets, and doublets; sporulation, oval-shaped immature sporangium; and mature endospores with visible exosporium. At 38 days only various phases of sporulation and mature endospores were present in the pseudocoelom of *M. arenaria* race 1.

Epitope quantification by ELISA: The anti-P-20 IgM MAb did not recognize proteins extracted from infected nematodes harvested at 12 and 16 DAI (Fig. 1A). However, the monoclonal antibody detected proteins extracted from infected nematodes harvested at 24 and 38 DAI (Fig. 1A). Total protein content per infected nematode was determined to be 0.45 µg at 12, 0.47 µg at 16, 1.17 µg at 24, and 2.05 µg at 38 DAI (Fig. 1B). The total protein per infected nematode increased with developmental time (Fig. 1B) and was correlated with the increase in the signal detected by the anti-P-20 IgM MAb (Fig. 1A). At 24 and 38 DAI, the ELISA-based absorbance at 405 nm after 45 minutes was 1.50 and 3.20 per infected nematode, respectively, which was proportional to the amount of adhesin-associated epitope detected as *P. penetrans* reached its endospore maturation stage (Fig. 1A).

SDS-PAGE analysis and immunoblotting: Analysis of individual proteins extracted from noninfected and *P. penetrans*-infected nematodes at each window of development showed some differences in the protein profiles related to the infection of the nematode by the bacterium (Figs. 2A,B; 3A,B). The immunoblot showed that anti-P-20 IgM MAb did not recognize any protein extracted from uninfected nematodes harvested at 12, 16, 24, and 38 DAI (Fig. 2B, lanes 2, 3, 4, 5), nor were proteins extracted from infected nematodes harvested at 12 and 16 DAI detected in the immunoblot (Fig. 3B, lanes 2,3). However, the immunoblot revealed that the monoclonal antibody reacted with protein extracts of infected nematodes harvested at 24 and 38 DAI (Fig. 3B, lanes 4, 5) and with endospore proteins of the P-20 strain used as the control (Figs. 2B; 3B, lanes 6). Some proteins bearing the epitope at 38 DAI were not detected at 24 DAI; others detected at 24 DAI increased in intensity by 38 DAI, by which time the pattern was very similar to that observed for isolated endospores.

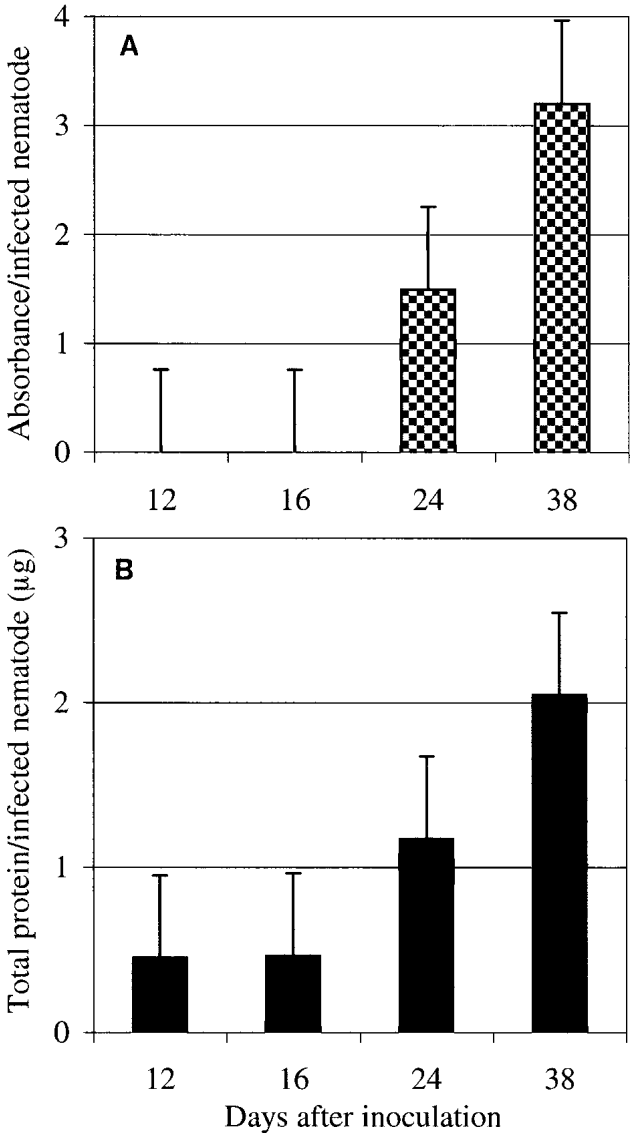

FIG. 1. Adhesin-associated epitope and total nematode protein per infected nematode as a function of the development of *Pasteuria penetrans*. A) Levels of adhesin-associated epitope determined by ELISA using anti-P-20 IgM MAb. Nematode infected total protein (100 ng/well) was applied in 100 µl/well. Values represent alkaline phosphatase substrate absorbance at 405 nm. B) Total nematode protein of infected nematodes. Lines above the bars indicate SE of the mean for six replicates per treatment.

TABLE 1. Percentage of different developmental stages of *Pasteuria penetrans* in *Meloidogyne arenaria* race 1 on tomato 'Rutgers' at 12, 16, 24, and 38 DAI at 35 °C[a].

	Days postinoculation			
Developmental stage	12	16	24	38
Vegetative growth	90	90	0	0
Differentiation	0	0	15	0
Sporulation	0	0	85	5
Mature endospores	0	0	65	95

[a]Twenty nematodes were examined at 12, 16, 24, and 38 DAI. Note that at 24 DAI more than one developmental stage was observed within the pseudocoelom of a single nematode. The developmental stages observed were: vegetative growth including mycelial colonies only within the pseudocoelom; differentiation stage, with presence of thalli differentiation, including quintets, quartets, triplets, doublets; sporulation stage, with many doublets and developing endospore with distal swollen ends connected by intercalary ends; and mature endospores, with free endospores with exosporium clearly visible.

Immunofluorescent microscopy: Just before examination with fluorescence microscope, the P-20 endospores showed a typical cup shape (Sayre, 1993) with a dark core, surrounded by the exosporium, and a translucent membrane (Sayre and Starr, 1985) (Fig. 4A). Labeling of whole endospores of *P. penetrans* isolate P-20 by anti-P-20 IgM MAb was nearly uniform (Fig. 4B), which suggests that the adhesin-associated epitope is nearly uni-

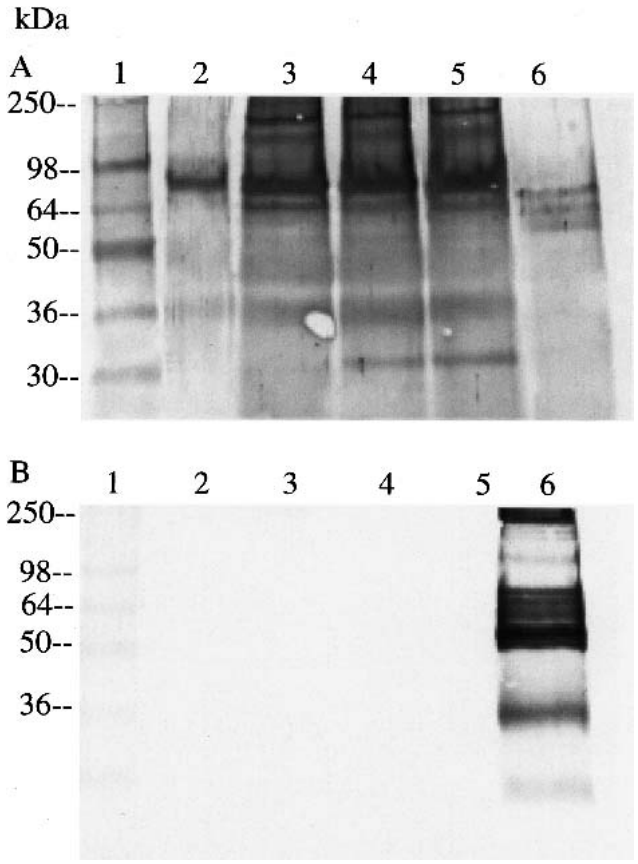

FIG. 2. Electrophoretic analysis of protein extract from noninfected *Meloidogyne arenaria* protein. Proteins were extracted at each window of development. Total proteins (600 ng) were applied per lane. A) Proteins were detected by staining with AuroDye according to manufacturer's instructions. B) Immunodetection of blotted antigens with anti P-20 IgM MAb at 1:2,000 dilution in PBST (pH 7.2). Lane 1: Molecular weight marker; lanes 2, 3, 4, and 5: Total proteins extracted from noninfected nematodes at 12, 16, 24, and 38 DAI; lane 6: Proteins extracted from *P. penetrans* P-20 endospores.

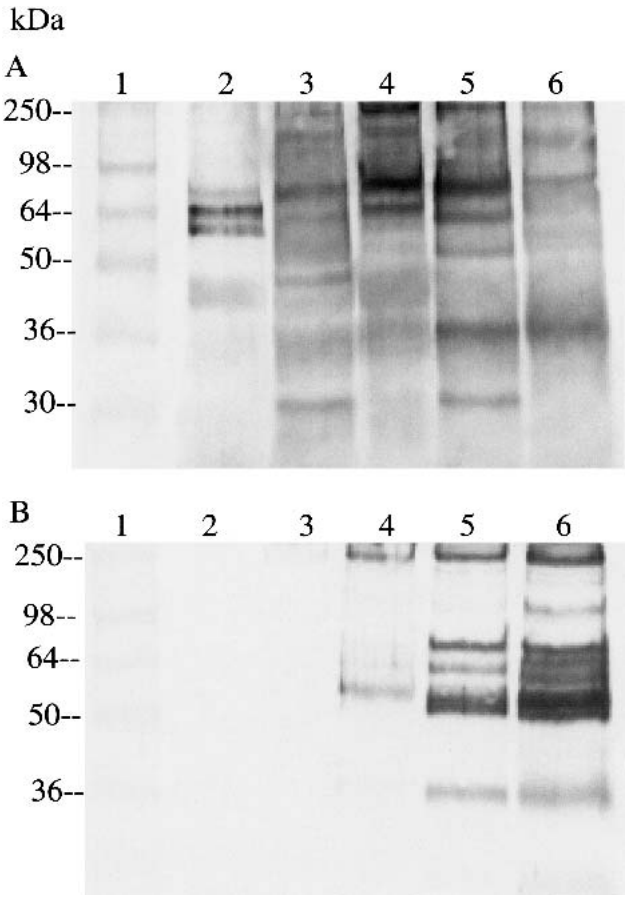

FIG. 3. Detection of *Pasteuria penetrans* adhesin-associated epitope during development within the pseudocoelom of *Meloidogyne arenaria* race 1. Nematode total proteins and endospore proteins were extracted as for Fig. 2. Protein extracts containing 600 ng were loaded into each lane. A) Detection of blotted proteins with AuroDye. B) Western blot of *P. penetrans*-infected nematodes probed with anti-P-20 IgM MAb. Lane 1: Molecular weight marker; lanes 2, 3, 4, and 5: Epitope-bearing proteins extracted from *P. penetrans*-infected nematodes at 12, 16, 24, and 38 DAI; lane 6: Proteins extracted from *P. penetrans* P-20 endospores.

formly distributed on the surface of mature endospores.

Immunogold labeling: The adhesin-associated epitope was not present in the ultrathin sections of vegetative cells, stage I of *P. penetrans* sporogenesis (Fig. 5A), or in stage II (Fig. 5B). A membrane (arrowhead) appears at a position one third of the way from the anterior end of the parasporium, suggesting the formation of a spore compartment distinct from the mother cell, as previously seen at this stage of sporogenesis (Chen et al., 1997b). Labeling of the adhesin-associated epitope was first observed over an ultrathin section of the stage III sporogenesis, mainly on the parasporal fibers (pf) (Fig. 6A). The antigens bearing the epitope were detected not only over the pf (Figs. 6B; 7A,B; 8A) but also over the sporangium(s) as *P. penetrans* continued to sporulate (Figs. 7A,B; 8A). The mature endospore was heavily labeled, and the epitope was localized in the sporangium(s), exosporium (ex), and pf (Fig. 8A). These results indicate that the antigens bearing the epitope, which was recognized by anti-P-20 IgM MAb, were synthesized at later stages of development associated with sporulation of *P. penetrans* within the pseudocoelom of *M. arenaria* race 1. The outer spore coat (oc), inner spore coat (ic), cortex (c), protoplasm (p), and basal ring (br) were not labeled (Fig. 8A). No labeling was observed over any structure of the mature endospore when non-immune ascites fluid was used (Fig. 8B).

DISCUSSION

Pasteuria penetrans completes its life cycle within the pseudocoelom of root-knot nematode females. Seven morphological stages of development through sporulation have been determined as I, II, III, IV, V, VI, and VII (Chen et al., 1997b). The initial step in the life cycle of *Pasteuria* is the recognition-attachment of the endospores to the cuticle of a free-living J2 root knot-nematode host. Infection of the host and germination of the endospores occur once the J2 enters the root

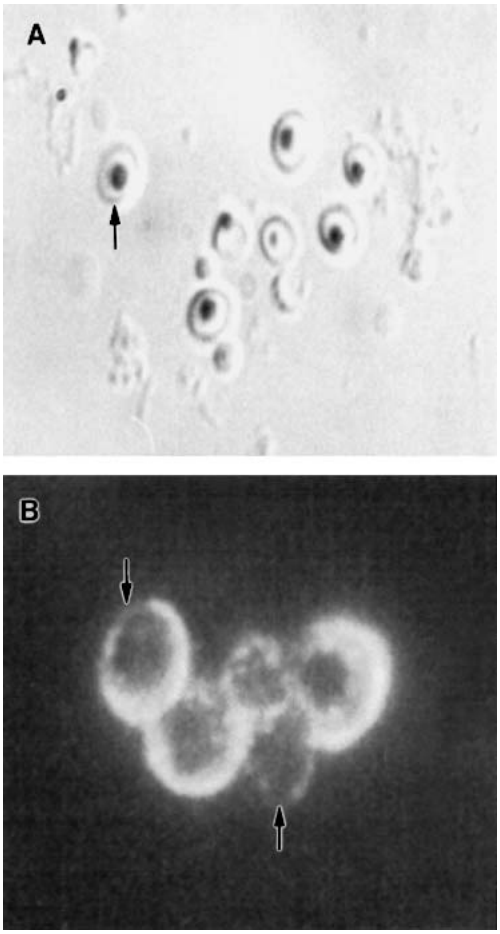

FIG. 4. Differential interference contrast (DIC) and fluorescence microscopy photomicrographs of whole endospores of *Pasteuria penetrans* P-20 strain. A) Overall shape of whole endospores using DIC. B) Labeling of an adhesin-associated epitope on the surface of whole endospores using anti-P-20 IgM MAb. Arrowheads identify regions of non-uniform labeling.

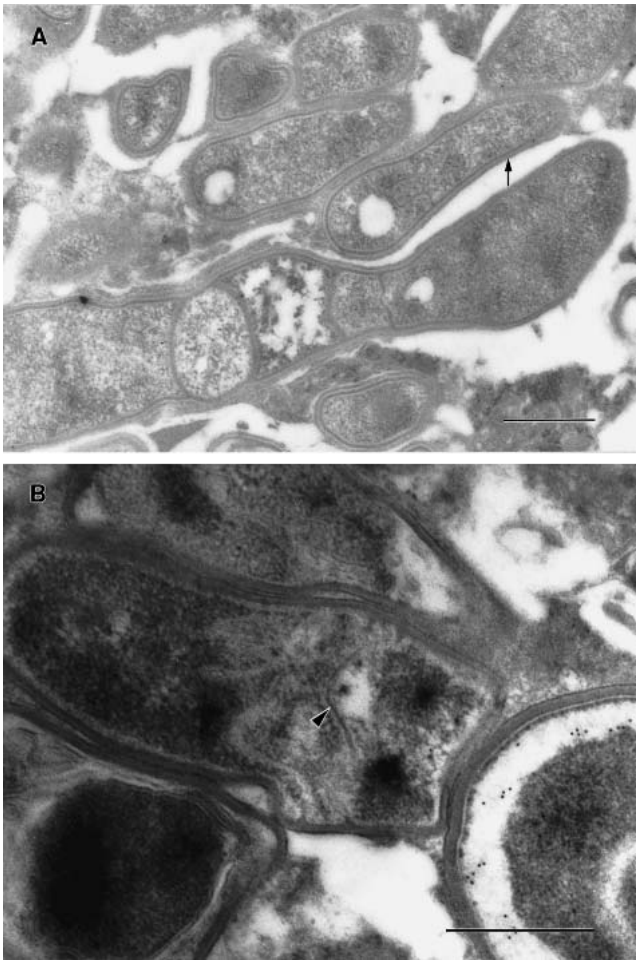

FIG. 5. Immunocytochemical localization of an adhesin-associated epitope during the development of *Pasteuria penetrans* within the pseudocoelom of *M. arenaria*. Thin sections of all stages of development of *P. penetrans* were probed with anti-P-20 IgM MAb and examined by transmission electron microscopy. Scale bars = 0.5 µm. A) Stage I of sporogenesis. A longitudinal ultrathin section of mycelial colony (arrow) of *P. penetrans* P-20 strain. No labeling is visible over the mycelium. B) Stage II sporogenesis of *P. penetrans*. Note that a membrane is forming at 1/3 distance from the anterior end (arrowhead) of the parasporium, which is characteristic of this stage.

tissue of a plant host, and establishes a permanent feeding site (Sayre and Starr, 1985, 1988). Vegetative growth, differentiation, sporulation, and spore maturation then occur (Imbriani and Mankau, 1977; Sayre, 1993; Sayre and Wergin, 1997; Serracin et al., 1997). The mechanisms involved in the attachment have been the subject of study in several laboratories. The results of these studies have led to the establishment of a model where glycoproteins, designated as adhesins and lectins, are involved in the interaction of *P. penetrans* and the nematode host (Davies and Danks, 1993; Persidis et al., 1991). Previous studies have shown that microbial adhesins, or bacterial surface proteins, may function as virulence factors in structures such as pili, or fimbriae (Robins-Browne et al., 1994; Salyers and Whitt, 1994; Suoniemi et al., 1995) as well as afimbrial adhesins (Salyers and Whitt, 1994), and allow bacteria to attach, colonize, and invade their hosts. For instance, *Streptococcus pyogenes*, a gram-positive pathogen, has a nonfibrillar adhesin (protein F) that mediates its attachment to fibronectin, a protein found on many host cells surfaces, including the mucosa of the human throat (Salyers and Whitt, 1994). However, the mechanisms used by *Pasteuria* spp. to produce virulent endospores within the pseudocoelom of the nematode host are not well understood. Mohan et al. (2001) found that fibronectin-like proteins extracted from *M. javanica* are involved in the attachment of endospores. We determined the relative time of the synthesis of an adhesin-associated epitope during the development of *P. penetrans* within the pseudocoelom of *M. arenaria* race 1. In addition, this epitope was detected and localized during endospore development.

ELISA and immunoblot analyses revealed that only proteins extracted from *P. penetrans*-infected nematodes at 24 and 38 DAI were recognized by anti-P-20 IgM MAb and the amount of the epitope was greater at the height of sporulation (38 DAI) than at any other

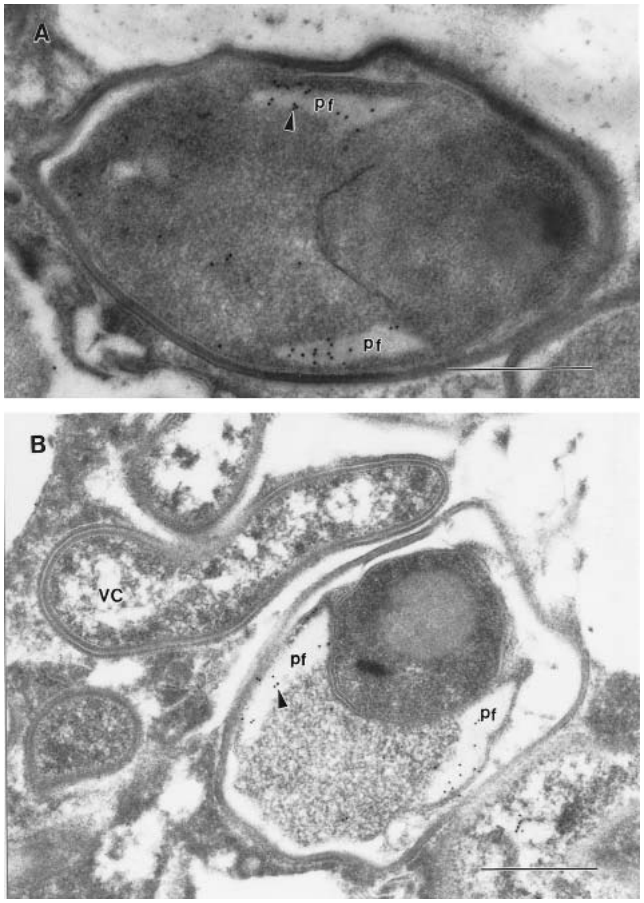

FIG. 6. Labeling of sporogenous stages of *Pasteuria penetrans*. Scale bars = 0.5 μm. A) Stage III sporogenesis showing labeling of the adhesin-associated epitope (arrowhead) mainly over the parasporal fibers (pf). B) Stage IV sporogenesis, gold particles (arrowhead) are concentrated in pf. Note that the vegetative cell (vc) was not labeled.

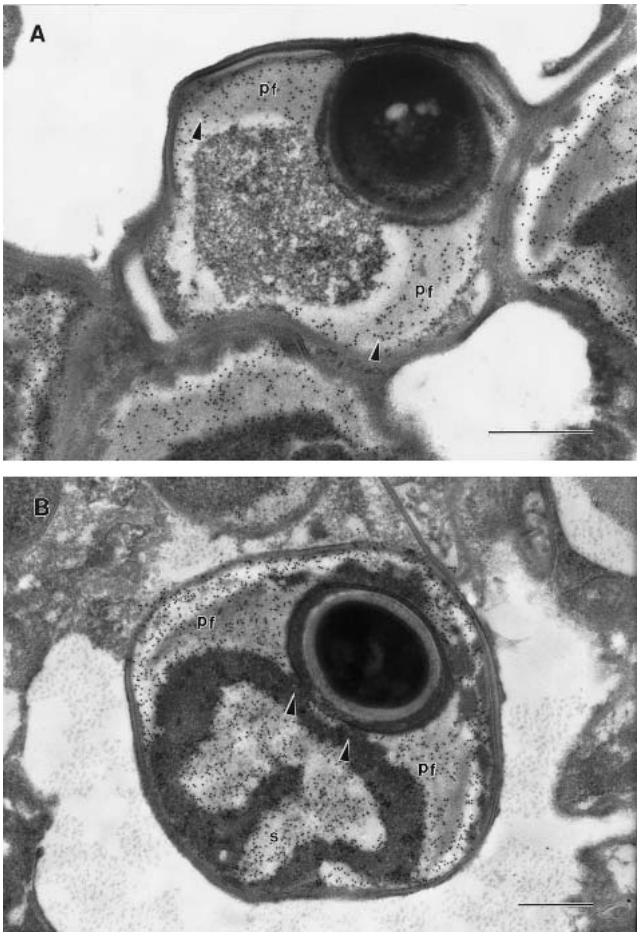

FIG. 7. Sporogenous stages of *Pasteuria penetrans*. Scale bars = 0.5 μm. A) Stage V of sporogenesis. Gold label (arrowhead) indicating antibody binding is present over the parasporal fibers (pf). B) Stage VI of sporogenesis, labeling of the adhesin-associated epitope is observed over the parasporal fibers (pf) and sporangium (s).

developmental stage (12, 16, and 24 DAI). The Western blot showed a higher degree of similarity in the protein profile of *P. penetrans*-infected nematodes at 38 DAI to the mature P-20 spore protein, used as a control, than with *P. penetrans*-infected nematodes from any other window of development. Examination of the infected nematodes harvested at 12 and 16 DAI by light microscopy revealed that only the vegetative growth stage, including clusters of mycelial colonies and thalli, were found throughout the pseudocoelom of nematodes. At 24 and 38 DAI, sporulation and maturation stages were observed within the pseudocoelom. Therefore, the synthesis of the adhesin-associated epitope occurred at a certain developmental stage relative to sporogenesis, and it was absent in vegetative growth and differentiation stages.

The synthesis of specific molecules at specific times during the germination, growth, and sporulation of the endospore-forming bacterium *Bacillus subtilis* has been rigorously established. For example, dipicolinic acid (pyridine-2, 6-dicarbonate) is formed during the first 5 hours of sporulation (Schlegel, 1986), whereas the small, acid-soluble spore proteins (SASPs), a group of DNA-binding proteins (at neutral to slightly alkaline pH), are synthesized after 3 to 4 hours into sporulation (Johnson and Tipper, 1981; Setlow, 1985). Both molecules are found only in endospores (Fliss et al., 1985; Schlegel, 1986). Even though some molecules are synthesized at specific stages of sporulation, it is possible that they are degraded and used to carry out a certain function at another stage. For example, during the first 5 hours of sporulation in *B. subtilis* much of the vegetative cell protein is degraded (Schlegel, 1986).

Immunofluorescence microscopy indicates that the adhesin-associated epitope is nearly uniformly distributed on the surface of virulent endospores. However, Davies and Redden (1997), working with PP1 MAb, showed that the labeling was site-specific, especially over the concave region of the endospore. The heterogeneity of the endospore surface has been observed not only between but also within populations of *P. penetrans* (Davies and Redden, 1997). Previous studies have shown that differences in the amount and nature of spore-surface proteins, as recognized by several monoclonal antibodies, may account for surface heterogene-

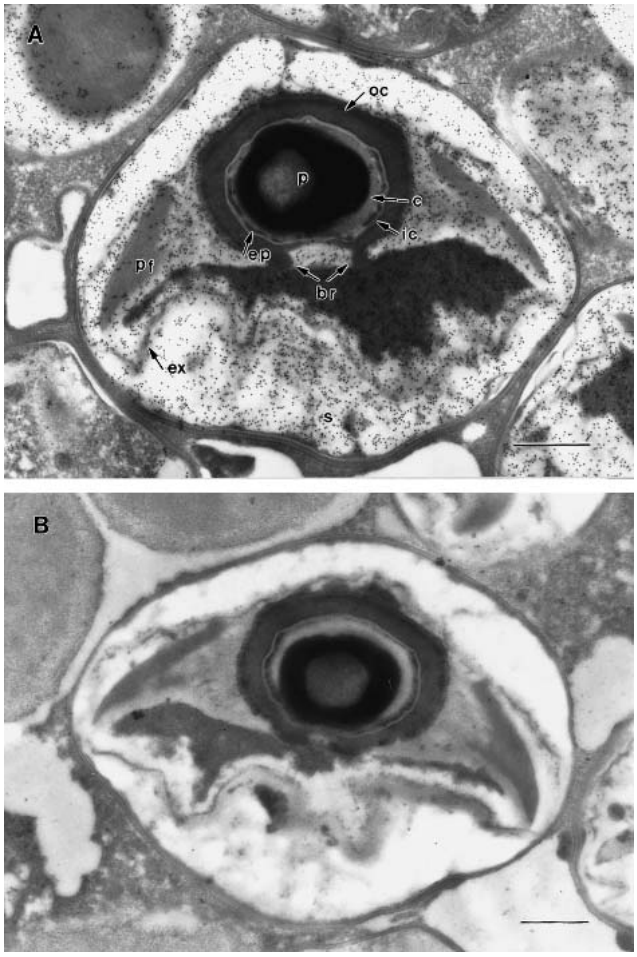

FIG. 8. Late sporogenous stage of *Pasteuria penetrans*. Scale bars = 0.5 µm. A) Stage VII of sporogenesis, a mature endospore showing the sporangium (s), exosporium (ex), and parasporal fibers (pf) heavily labeled, whereas the outer spore coat (oc), inner spore coat (ic), epicortex (ep), cortex (c), protoplasm (p), and basal ring (br) are not labeled. Note that the pf were not uniformly labeled. B) A mature endospore of the *Pasteuria penetrans*, stage VII used as control. No label is observed over the thin section of the endospore.

ity of endospores as well as host specificity (Davies et al., 1992). Davies et al. (1994), using monoclonal antibodies, showed that the surface of endospores of the PP1 strain of *P. penetrans* is highly heterogeneous. These and subsequent studies (Davies and Redden, 1997) have suggested that endospore surface properties are directly related to the virulence of *P. penetrans*.

Antigens bearing the epitope recognized by anti-P-20 IgM MAb were synthesized during sporogenesis. Labeling was first observed at stage III of sporogenesis, mainly in the pf. In contrast to stage III sporogenesis, mature endospores were heavily labeled and the adhesin-associated epitope was localized in the pf, sporangium, and exosporium.

The general pattern of the labeling of the adhesin-associated epitope over thin sections of a mature endospore was similar to that found in previous studies, where mature endospores were probed with a polyclonal antibody (Davies et al., 1992; Persidis et al., 1991). These authors concluded that the labeling showed no preference for a particular structure of the endospore and suggested that a nonspecific binding of the antibodies could have occurred. These observations may reflect a heterogeneity in the polyclonal antibody preparation and(or) selection of a single stage of development. In our study, it was shown that the adhesin-associated epitope is synthesized at a certain stage of development related to endospore formation and is localized initially in the pf early in stage III, becoming widespread throughout the sporangium and exosporium but not in the central body of stages IV, V, VI, and VII of sporogenesis. Labeling was not uniformly distributed in the pf. Also, no labeling was observed in the outer or inner spore coat, epicortex, cortex, protoplasm, or basal ring.

These observations establish a window of development in which the adhesin-associated epitope is formed and where further studies concerning the formation of this epitope should be directed. The fact that the epitope occurs in several polypeptides and is distributed over several structures of the mature endospores suggests its involvement in the recognition of the nematode host as an early event in the attachment process. It may increase the chances for a cooperative interaction between the adhesin epitope with receptors on the cuticle of the nematode host, such as carbohydrate binding proteins (Bird et al., 1989; Davies and Danks, 1993; Persidis et al., 1991) and fibronectin-like residues (Mohan et al., 2001). Cooperative processes resulting from multiple sites of recognition and interaction would be expected to drive a chance encounter toward irreversible attachment that allows the attached endospore to maintain its association with a J2 root-knot nematode as it infects a root and migrates to a feeding site. Other forces derived from hydrophobic and electrostatic interactions also may contribute to attachment (Afolabi et al., 1995; Davies et al., 1996; Esnard et al., 1997). It is also apparent that an essentially irreversible association between the *Pasteuria* endospore and its nematode host is responsible for the encumbrance that may prevent the nematode from reaching and infecting a plant host. The approach to synchronous development of *P. penetrans* in the root-knot nematodes provides a system in which to further define events, including specific gene expression, translation, and post-translational modifications, that are required for the formation of virulent endospores that can then serve as biocontrol agents for the suppression of root-knot nematode.

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