Extraction and Purification of *Pasteuria* spp. Endospores[1]

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Abstract: Pasteuria penetrans is an endospore-forming bacterial parasite of root-knot nematodes that has potential as a biological control agent. Biochemical investigations of *P. penetrans* are limited because of difficulty in obtaining large quantities of endospores free of plant debris and contaminating microorganisms. Our objective was to develop a technique for extraction and purification of *P. penetrans* endospores from root-knot nematodes. Tomato roots infected with *Meloidogyne arenaria* that was parasitized by *P. penetrans* were digested with cytolase. The nematode females along with plant debris were washed with a jet stream of water onto an 800-µm-pore sieve nested on a 250-µm-pore sieve. The materials retained on the 250-µm-pore sieve were centrifuged through a 20% sucrose solution. The resulting loose pellet fraction was collected on a 250-µm-pore sieve and then centrifuged through a 47% sucrose solution. Endospore-filled females were handpicked from the 47% sucrose pellicle fraction. Endospores were released by grinding the females with a glass tissue grinder. The endospores were then filtered through a nylon filter with 8-µm openings, collected by centrifugation, and subjected to buoyant density centrifugation in different media. Further purification by buoyant density centrifugation in a linear gradient of sodium diatrizoate resulted in a preparation of endospores free of debris. This additional step may be desirable for the further characterization of components unique to the endospores.

Key words: bacterium, biological control, endospore, extraction, *Meloidogyne* spp., nematode, *Pasteuria penetrans,* purification, root-knot nematode, sodium diatrizoate.

Pasteuria spp. belong to a group of endospore-forming bacteria that are obligate parasites of nematodes and cladocerans. Except for one species that parasitizes water fleas, all others have been found on nematodes. Some isolates of *Pasteuria* spp. have shown potential for biological control of several important genera of plant-parasitic nematodes (Dickson et al., 1994). The endospores of the bacteria are both the survival and infective stage, and they can exist in soil for long periods apparently without loss of viability. Once the endospores contact a host nematode, they may attach to the cuticle and penetrate the nematode. Host-parasite recognition is determined by the interaction between compounds on the surfaces of both the nematode cuticle and the endospores (Bird et al., 1989; Chen et al.,

1997; Davies and Danks, 1993; Davies et al., 1992; Persidis et al., 1991). Detailed studies in biochemistry and molecular biology of endospores may provide crucial information for development of the bacteria as biological control agents and for species delimitation. Such studies require the preparation of endospores free or nearly free from components of host nematodes, microbial contaminants, and plants. The objective of this study was to develop techniques to purify *Pasteuria* spp. endospores for definitive analysis of their macromolecular composition.

Materials and Methods

Specific gravity of endospore-filled females: Isolate P100 (Oostendorp et al., 1990) of *Pasteuria penetrans* (Thorne) Sayre & Starr was cultured on *Meloidogyne incognita* (Kofoid & White) Chitwood race 1 on tomato (*Lycopersicon esculentum* Mill cv. Rutgers) reared in the greenhouse for about 2 months. The infected roots were placed in a beaker containing a 12.5% solution of cytolase PCL 5 (Genencor International, Rochester, NY) and incubated on the laboratory bench for 1 week at ambient room temperature (23–24 °C). The enzyme-treated root materials were washed with a jet stream of water onto an 800-µm-pore sieve nested on a 250-µm-pore sieve. The females and plant debris

caught on the 250-µm-pore sieve were centrifuged at 1,500g in a 60.3% (w/v, sp. gr. 1.22 g/cm^3) sucrose solution in a 50-ml polypropylene conical centrifuge tube for 5 minutes. The loose pellet was centrifuged again in 68.5% (w/v, sp. gr. 1.25) sucrose, and the resulting supernatant and pellicle fraction (floating pad) were collected and examined for presence of females. The pellet from the 68.5% sucrose step contained no females and was discarded. The females in the supernatant and pellicle fraction from the 60.3% sucrose step were collected by filtration with a 250-µm-pore sieve and then centrifuged through a series of sucrose solutions with specific gravities of 1.11, 1.12, 1.13, 1.14, 1.15, 1.16, and 1.17 g/cm^3. At each step of centrifugation, the pellicle fraction was collected and the pellet was centrifuged in the next sucrose solution. After the final step, both pellicle and pellet were collected. Endospore-filled females, conspicuous by their opaque and white appearance when illuminated from above with a stereomicroscope, were handpicked with forceps from each fraction and counted. The experiments were performed once.

Collection of females for extraction of endospores: Air-dried roots of 21 tomato plants (2 months old) infected with *M. arenaria* (Neal) Chitwood race 1 females that were parasitized by *P. penetrans* isolate P20 (Oostendorp et al., 1990) from a greenhouse culture were treated with 12.5% cytolase, and the digested roots were passed through sieves as described above. The materials retained on the 250-µm-pore sieve were divided into two equal samples, each having a moist weight of 156 g. Two methods were used to extract females filled with endospores from these two samples. In method 1, the females along with plant debris from one sample were placed in phosphate-buffered saline (PBS, i.e. 10 mM sodium phosphate buffer, pH 7.6, 0.9% sodium chloride). The endospore-filled females were handpicked with forceps and placed in PBS. Care was taken to avoid plant tissue. In method 2, the second 156-g sample was distributed into 50-ml polypropylene conical centrifuge tubes, each with about 10 g of

material suspended in a 20% (w/v) sucrose solution (sp. gr. 1.073) to give a final volume of 50 ml, and centrifuged for 5 minutes at 1,500g. The loose pellet (pellet fraction) was separated from the pellicle fraction by a clear supernatant. The pellicle fraction was collected on a 250-µm-pore sieve and suspended in 50 ml of 10% sucrose (sp. gr. 1.036) and centrifuged as above. The resulting pellicle and pellet fractions from the 10% sucrose step were collected by filtration with a 250-µm-pore sieve, and diluted with PBS. The females were handpicked and counted. The loose pellet fraction derived from the initial 20% sucrose step was collected by filtration with a 250-µm-pore sieve, suspended in 47% (w/v) sucrose (sp. gr. 1.17), and centrifuged as above. The pellicle fraction was collected by filtration, and the females were handpicked and counted. The loose pellet fraction was collected by filtration, suspended in 61% (w/v) sucrose (sp. gr. 1.22), and centrifuged as above. After this step, all of the remaining females were in the pellicle fraction. This pellicle fraction was collected by filtration, and the females were handpicked and counted.

The females collected by method 1, or collected from each fraction of method 2, were suspended in PBS and ground with a glass tissue grinder to release the endospores. The number of endospores in each uniform suspension was determined in a hemacytometer at ×400. The experiments were performed once.

Buoyant density centrifugation of endospores: Endospores that were extracted from handpicked females collected by either method 1 or 2 were combined and passed through a nylon filter with 8-µm openings (Nytex, Tetko, Elmsford, NY) to remove debris. The endospores were then collected in a pellet by centrifugation for 5 minutes in a microfuge at 12,000g. Endospores were further purified in one of the following media: (i) percoll—8.1 ml of percoll (Sigma, St. Louis, MO), 0.9 ml of PBS, and 0.5 ml of the filtered endospore suspension in PBS were mixed in a 10-ml polycarbonate (Oak Ridge type) centrifuge tube; (ii) sucrose solution—a linear gradient was prepared from 0

to 85% (w/v) sucrose (sp. gr. 1.31) in a 10-ml centrifuge tube; an endospore suspension in 0.5 ml of 85% sucrose was loaded with a syringe into the tube bottom; (iii) sodium diatrizoate—a linear gradient was prepared from 0 to 0.8 M sodium diatrizoate (renografin), sp. gr. 1.30 (Winthrop Laboratories, New York, NY; Sigma) in a 10-ml centrifuge tube; an endospore suspension in 0.5 ml of 0.8 M sodium diatrizoate solution was loaded into the bottom of the gradient with a syringe via a 20-gauge spinal needle, and care was taken to avoid disturbance of the performed gradient (Preston et al., 1972).

All gradients loaded with endospores were centrifuged at 25,000*g* for 30 minutes at 20 °C in a Beckman JS 13 swinging bucket rotor. After centrifugation, the banding patterns were recorded and the contents of discernible bands removed with a syringe by means of a 20-gauge needle. The endospores in each collected fraction were diluted ca. 3× with deionized water and recovered as a pellet following brief (about 5 minutes) centrifugation in a microfuge at 12,000*g*. The endospores then were rinsed three times through deionized water with centrifugation in the microfuge, counted with a hemacytometer at ×400, and stored in a refrigerator (4 °C). The purity of the endospores was examined with microscopy at ×150 to ×600, and by analysis of individual polypeptides resolved with SDS-PAGE. *Pasteuria penetrans* endospores (3×10^5) were placed in 30 µl of sample buffer (50 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS] w/v, 10% glycerol, 0.0025% bromophenol blue w/v, and 2% β-mercapto-
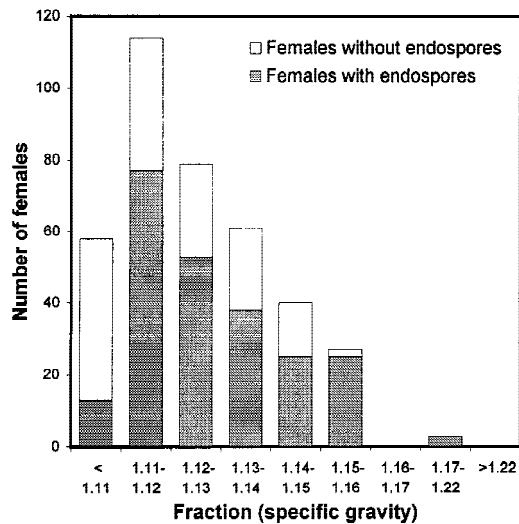

FIG. 1.   Distribution of specific gravity of *Meloidogyne incognita* race 1 females filled with endospores of *Pasteuria penetrans* isolate P100 and females without endospores.

ethanol w/v) and boiled for 5 minutes at 100 °C (Davies et al., 1992). Each sample was loaded onto a lane of 12% acrylamide and 0.32% bisacrylamide running gel. For the uninfected nematode sample, five mature females of *M. arenaria* race 1 were suspended in 30 µl of sample buffer, boiled, and loaded onto a single lane. A 7-µl sample of protein molecular mass markers of 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa (BioRad Laboratories, Hercules, CA) was run in a separate lane. Electrophoresis was carried out at constant current (25 ma) until the dye marker moved to the bottom. After electrophoresis, gels were electro-blotted to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA) for protein

Table 1.   Yields of *Meloidogyne arenaria* females and *Pasteuria penetrans* endospores following centrifugation through sucrose solutions.

Fraction	Number of females[a]	Number of endospores (millions)[a]	Endospores per female
Pellicle, 20% sucrose			
Pellicle, 10% sucrose	2,285	4.7	2,100
Pellet, 10% sucrose	1,204	10.7	8,900
Pellet, 20% sucrose			
Pellicle, 47% sucrose	2,547	40.8	16,000
Pellet, 47% sucrose	225	4.2	18,900

[a] Numbers are from 156 g of enzyme-digested moist root materials.

$$Y = 0.0000093x - 0.0128$$
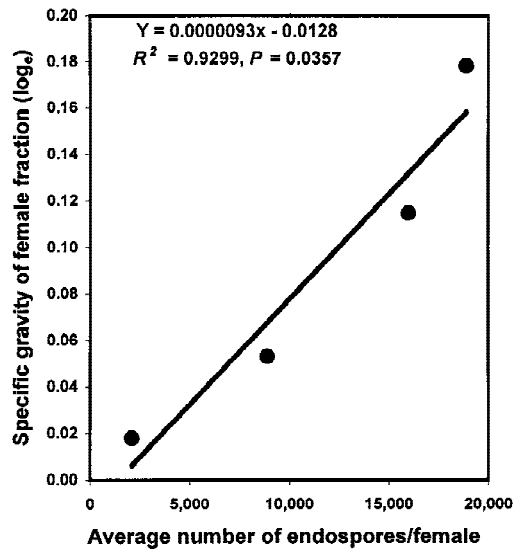$$R^2 = 0.9299, P = 0.0357$$

FIG. 2. Relationship between specific gravity of *Meloidogyne incognita* females and number of *Pasteuria penetrans* endospores per female.

visualization by immuno-detection with IgY antibodies directed against *P. penetrans* endospores (Chen et al., 1997) and alkaline phosphatase-conjugated secondary anti-IgY antibodies (Sigma).

RESULTS AND DISCUSSION

The distribution of endospore-filled females and females without endospores from digested plant root debris by centrifugation in sucrose solutions with a series of densities is illustrated in Figure 1. About 61% of females were filled with *P. penetrans* endospores. The specific gravity of most endospore-filled females was between 1.11 and 1.17. Sixty-eight percent of all endospores obtained were recovered in the pellicle fraction from females that were first collected as a pellet from 20% sucrose, and then subjected to centrifugation in 47% sucrose (Table 1). The centrifugation procedure selecting the 10% sucrose pellet and the 47% sucrose pellicle fractions resulted in an accumulation of 85% of total endospores, collected from mostly endospore-filled females. The specific gravity of females was positively correlated with the average number of endospores per female, which indicates that the specific gravity of females with endospores was greater than that of females without endospores and that the specific gravity of females increased as the number of endospores per female increased (Fig. 2). Consequently, the concentration used to extract the endospore-filled females may be increased to 47% (w/v) of sucrose (sp. gr. 1.17), compared to the density of 35% (w/v) sucrose solution (sp. gr. 1.128) commonly used to extract *Meloidogyne* females (Hussey, 1971; Pableo, 1981). Our results agree with a previous study that reported a higher sucrose concentration (i.e. greater density) was needed to extract *Pratylenchus scribneri* Steiner infected with *Pasteuria* spp. than is commonly used to extract nematodes without *Pasteuria* spp. (Oostendorp et al., 1991).

The total number of endospores ex-

Table 2. Yields and relative purity of endospores of *Pasteuria penetrans* that were collected from buoyant density centrifugation in three media–sodium diatrizoate, percoll, and sucrose.[a]

Medium	Band	Percent distance of band from top	Number of endospores recovered (millions)	Percentage of endospores collected	Quality of endospores[b]
Sodium diatrizoate	1	70	2.34	11.6	Small amount of debris
	2	80	3.32	16.6	Clean, limited debris or bacterial cells
Percoll	1	21	5.40	27.0	Debris present
	2	29	0.34	1.7	Debris present
	3	33–37	0.92	4.6	Small amount of debris
Sucrose	1	88	6.80	34.0	No separation

[a] Endospore-filled females were handpicked and crushed in deionized water; endospores then were passed through a nylon filter with 8-μm openings. The filtered endospores were purified by centrifugation in density gradients of three media. Each sample contained 20×10^6 endospores.

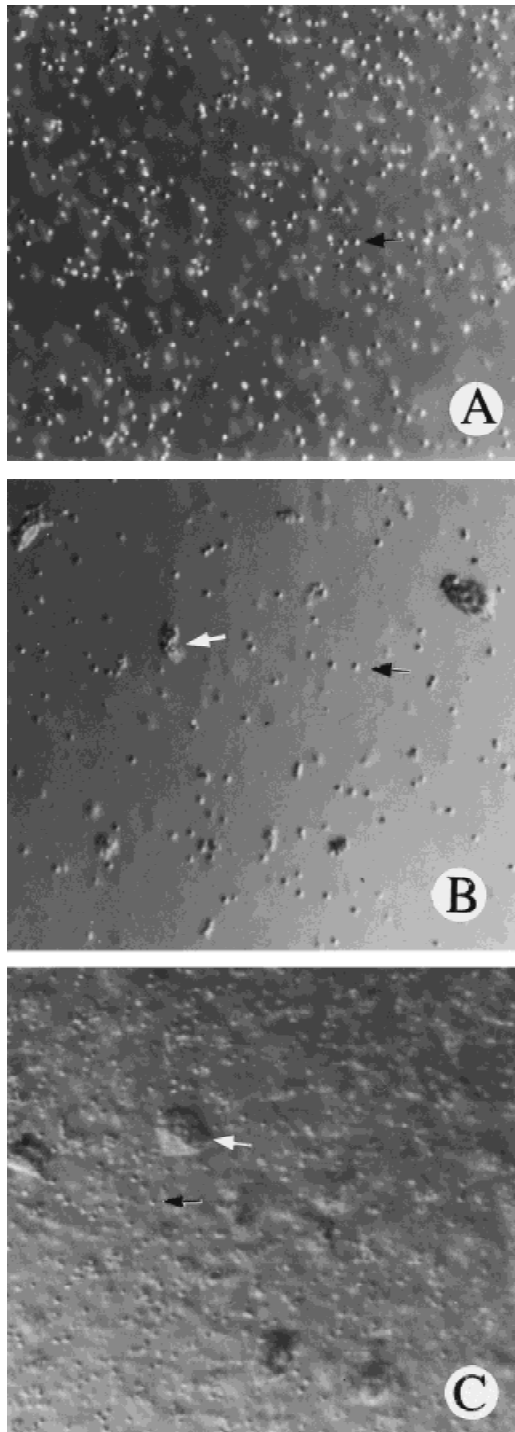[b] Quality of the preparations was estimated with a light microscope at ×150 to ×600.

FIG. 3. Photomicrographs of *Pasteuria penetrans* endospores prepared by centrifugation in different media (×188). A) Sodium diatrizoate, band 2 (corresponding to the band numbers in Table 2) showing clean endospores. B) Percoll, band 3 showing endospores with a small amount of debris. C) Sucrose, mean band showing endospores with some debris; black arrows indicate separate endospores, and white arrows indicate debris.

tracted from handpicked endospore-filled females collected by centrifugation (method 2) was $60.4 \times 10^6/156$ g moist plant root material, compared to 130×10^6 when endospore-filled females were handpicked directly from the same weight of enzyme-digested root material (method 1). Thus, approximately half of the endospores may be lost during the centrifugation procedures. While handpicking is necessary for obtaining endospore-bearing females free of plant tissue, even after treatment with centrifugation protocols, this time-consuming step is shortened considerably after the endospore-bearing females have been selected by centrifugation in sucrose solutions. Passing the endospore suspension derived from infected females through a nylon filter with 8-µm openings removed most of the nematode tissues. This step also aided in the separation of the endospores from each other as they tended to clump.

Yields and general appearance of *P. penetrans* endospores obtained following buoyant density centrifugation in different media are summarized in Table 2 and Fig. 3. Among the three media used, sodium diatrizoate provided the best purification of endospores and a relatively high recovery rate. A band of clean endospores was formed at about 80% from the top in the sodium diatrizoate medium (Table 2). Microscopic examination indicated this fraction was essentially free of plant debris (Fig. 3A). Similar results were obtained with different preparations of endospores used in an immunological study (Chen et al., 1997). In the preparation of samples for the immunological study, however, a band of clean endospores in the sodium diatrizoate solution was observed anywhere between 60% to 90% of the distance from the top in this medium. However, if the endospore preparation was derived from females containing significant amounts of adherent plant tissue, or from a spore preparation that had been stored for some time before centrifugation, an endospore preparation corresponding to this band might include contaminants, including bacteria and fungi. The relatively low viscosity of sodium diatrizoate is presumably
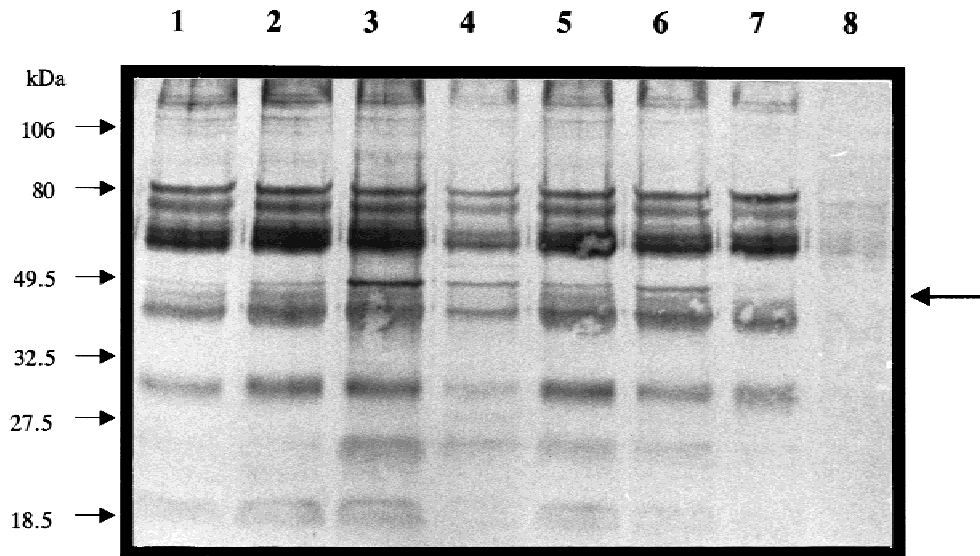
FIG. 4. PAGE gel for protein analysis of *Pasteuria penetrans* endospores prepared by centrifugation in either diatrizoate, percoll, or sucrose density media. Antigens were detected with IgY antibodies directed against whole endospores and alkaline phosphatase-conjugated anti-IgY. Lane 1: sodium diatrizoate-centrifuged endospore sample band 2 (corresponding to the band number in Table 2). Lane 2: sodium diatrizoate-centrifuged endospore sample band 1. Lane 3: percoll-centrifuged endospore sample band 1. Lane 4: percoll-centrifuged endospore sample band 3. Lane 5: sucrose-centrifuged endospore sample mean band. Lane 6: filtered endospores. Lane 7: sodium diatrizoate-purified endospores that had been used for immunological study (Chen et al., 1997). Lane 8: female nematodes not infected with *P. penetrans*. The numbers on the left side denote the positions for molecular weight markers. The arrow on the right side refers to the position for a 45-kDa protein.

responsible for the reproducible banding patterns observed in comparison with the other media used.

Analysis of antigens following SDS-PAGE for endospores prepared in the different centrifugation media showed similar profiles with only minor differences after probing with anti-*Pasteuria* IgY. A band at 45 kDa (Fig. 4, right arrow) that was obvious in samples prepared with percoll (lanes 3 and 4), sucrose (lanes 5), and in a sample of crude endospores (lane 6) was faint (lanes 1 and 2) or very faint (lane 7) in samples prepared with sodium diatrizoate. The IgY antibody was prepared in hens using endospores that were neither centrifuged with sodium diatrizoate nor filtered with the 8-µm-opening nylon filter. The endospore preparation might have contained adhering exosporia and possibly other contaminants. This band may be derived from a contaminating organism or may represent a loosely bound component of the spore protein. In either case, the buoyant density centrifuga-

tion in the sodium diatrizoate results in the formation of endospores that are more likely free of contaminants from other organisms and are, therefore, more suitable for molecular genetic studies where contaminants could be problematic.

In summary, for the preparation of the maximum number of clean endospores, the protocol should include: (i) digestion of root tissues with cytolase; (ii) filtration through an 800-µm-pore sieve and collection of female nematodes on a 250-µm-pore sieve; (iii) differential centrifugation through 20% sucrose; (iv) centrifugation of the 20% sucrose pellet fraction through 47% sucrose to provide a pellicle fraction; and (v) collection of the 47% pellicle fractions by filtration. If 10% sucrose is used instead of 20% sucrose in the above procedure, 17% more endospores can be obtained but the female sample may contain a higher percentage of uninfected females and plant debris. Some additional purification can be achieved by handpicking at this

stage. The isolated females should be crushed with a tissue grinder in deionized water to release the endospores, which can then be filtered through a nylon filter with 8-µm openings.

To purify filtered endospores for sensitive analysis, but with significant sacrifice of yield, isopycnic centrifugation in a sodium diatrizoate gradient may be used. Sodium diatrizoate has been used successfully in the purification of mitochondria (Krawiec and Eisenstadt, 1970) and chloroplasts (Preston et al., 1972) for preparation of organelles devoid of contaminating nuclear DNA. The use of these gradients was crucial to the isolation and subsequent characterization of DNA, RNA, and proteins unique to the function of these organelles. Diatrizoate gradients also have been used for the purification of spores from *Bacillus subtilus* (Vold, 1974). In spite of some sacrifice in yield, the purification of endospores from *Pasteuria* spp. on the sodium diatrizoate gradients would be a prudent step in further studies on proteins and nucleic acids to reveal information on genes or gene products unique to these bacterial endoparasites of nematodes.

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