Journal of Nematology 38(1):165–167. 2006.

A Method for Isolation of *Pasteuria penetrans* Endospores for Bioassay and Genomic Studies

Jenora T. Waterman, David McK. Bird, Charles H. Opperman

Abstract: A rapid method for collection of *Pasteuria penetrans* endospores was developed. Roots containing *P. penetrans*-infected root-knot nematode females were softened by pectinase digestion, mechanically processed, and filtered to collect large numbers of viable endospores. This method obviates laborious handpicking of *Pasteuria*-infected females and yields endospores competent to attach to and infect nematodes. Endospores are suitable for morphology studies and DNA preparations.

Key words: endospores, method, *Pasteuria penetrans*.

Pasteuria penetrans is a gram positive hyperparasite of root-knot nematodes (RKN: *Meloidogyne* spp.) and other nematodes. This bacterium is a member of the *Bacillus-Clostridium* clade, and it is most closely related to *Bacillus halodurans* and *B. subtilis* (Charles et al., 2005). In nature, endospores of *P. penetrans* attach to the cuticle of second-stage juveniles (J2) migrating through soil. *Pasteuria penetrans* endospores germinate when the nematode establishes a feeding site in the host vasculature (Sayre, 1993). A germ tube is extended into the pseudocoelom of the nematode, which gives rise to microcolonies, thalli, and, eventually, endospores. Proliferation of the endoparasite within the pseudocoelomic cavity of the nematode causes degeneration of the reproductive tissues, greatly reducing fecundity. Endospores are released into the surrounding soil upon decay of the female cadavers and root tissue. Due to its ability to control nematode populations, *P. penetrans* has potential as a biocontrol agent for RKN (Chen and Dickson, 1998). Although the use of *P. penetrans* to control RKN is promising, its fastidious nature has inhibited mass production of endospores.

Current methods for collecting endospores of *P. penetrans* require handpicking infected females (Oostendorp et al., 1990; Chen et al., 2000). Here we present a rapid method for collecting endospores that eliminates the need for handpicking females. Our method includes mechanical processing of digested root material and filtration to collect quantities of viable *P. penetrans* endospores orders of magnitude higher than previously reported methods.

Materials and Methods

Pasteuria penetrans (Gainesville, Florida, Isolate; Pasteuria Bioscience, Alachua, FL) endospores were attached to *Meloidogyne arenaria* Race 1 J2 using a modified method of Hewlett and Dickson (1993). Briefly, endospores of *P. penetrans* were attached to 10,000 J2 at a ratio of 100 endospores/J2 by centrifugation at 3,220*g* for 5 min at room temperature in 10 ml distilled water in a 50-ml tube. Average attachment, average number of endospores attached to nematodes, and percentage attachment were determined. Ten thousand J2, with 97% ± 6% (mean ± 1 standard deviation for three replicates) having endospores attached, were used to inoculate each 4-wk-old tomato plant (*Lycopersicon esculentum* 'Rutgers large red') maintained at approximately 30°C in a greenhouse. After about 60 d, roots were harvested from the plant, rinsed with room temperature water, and soaked for 4 hr at room temperature or overnight at 4°C in a 1:5 dilution of 100X Crystalzyme (Valley Research, South Bend, IN) pectinase solution.

Softened galls (with excess root material removed) were placed in a mortar and gently smashed with a pestle to crush galls and, consequently, endospore-filled females. The smashed root material was transferred to a glass jar filled with 50 ml distilled water and then shaken for 2 min. Endospores were collected by pouring the resultant slurry over a series of stacked sieves: a 250-µm-pore sieve, a 75-µm-pore sieve, and a 25-µm-pore sieve on the bottom. The material passing through the 25-µm-pore sieve was recovered and transferred to a glass jar, and the shaking step was repeated once with an additional 50 ml distilled water. The slurry was again filtered through the series of stacked sieves and the eluate filtered through a 5-µm polycarbonate filter (GE Osmonics, Minnetonka, MN) under vacuum; the filtrate was recovered and stored at 4°C. This sample was visually inspected for the presence of endospores using a light microscope at ×400. The endospore concentration was determined using a Brightline hemacytometer (Hausser Scientific, Horsham, PA). Differential interference images were collected using a Leica DM1RB microscope with a x100 oil immersion objective (1.3 NA).

The viability of endospores collected using the present (mortar-ground-gall) method was assessed by attachment to and infection of *M. arenaria* Race 1 J2. For infectivity assays, 10,000 J2 with endospores attached (from the present method or handpicked females disrupted in a glass homogenizer) were used to inoculate tomato plants. After approximately 60 d, 20 females

Received for publication 17 November 2005.

Center for the Biology of Nematode Parasitism, NC State University, Raleigh, NC 27695.

Supported in part by the North Carolina Agricultural Research Service and the National Science Foundation. A portion of a Ph.D. dissertation by the first author.

E-mail: warthog@ncsu.edu

This paper was edited by J. A. LaMondia.

were arbitrarily handpicked from digested root material, visually inspected for endospores, and crushed in distilled water with a glass tissue grinder to release endospores.

Endospore DNA was extracted after pelleting 5 x 10^7 endospores at 18,000*g* for 5 min. The endospores were suspended in 10 mM Tris, 1.0 mM EDTA, pH 8.0, containing 20 mg/ml lysozyme, and incubated at 37°C for 30 min. Proteinase K (20 mg/ml) and sodium dodecyl sulfate (2% w/v) were added followed by additional 30 min incubation. The pretreated endospores were transferred to a 2.0-ml impact-resistant tube containing approximately 1 g glass beads in the size range 150 to 212 µm (Sigma-Aldrich, St. Louis, MO). Phenol and chloroform were added, and the endospores were disrupted using a Savant FastPrep Bio101 bead beating instrument. The samples were extracted with four 30-sec pulses at 4.0 m/s interspersed with 1 min incubations on ice. The phases were separated by centrifugation at 18,000*g* for 10 min. The aqueous phase was further purified with subsequent phenol and chloroform extractions. Endospore DNA was precipitated by adding sodium acetate, pH 4.8, to 300 mM, 2.5 volumes 95% ethanol, and 0.014 mg/ml glycogen, followed by centrifugation at 20,000*g* for 60 min at 4°C. RNase One (Promega, Madison, WI) was used to remove contaminating RNA per manufacturer recommendations. Endospore DNA concentration was determined fluorometrically. To confirm that usable genomic DNA could be isolated from spores extracted with this method, PCR was performed on DNA extracted from endospores using *Pasteuria* 16S ribosomal DNA primers (Duan et al., 2003). Experiments were repeated twice for a total of three trials.

Results and Discussion

Davies et al. (1988) found that attachment of at least 5 endospores/J2 was necessary to ensure infection of the nematode by *P. penetrans*, but that subsequent plant-root penetration was reduced by 86% when J2 were encumbered with 15 or more endospores. In this study, we inoculated plants with 10,000 J2 with an average of six endospores attached. *Pasteuria*-infected females produce approximately 2×10^6 endospores each. Therefore, a plant infected with 10,000 *Pasteuria*-filled females contains up to 2×10^{10} endospores. Using our method, we were able to collect an average of 5.78×10^9 endospores per plant, which is approximately 30% of that theoretical maximum. This is a significant improvement over an older mortar disruption method for isolating endospores (Chen et al., 1996), which did not include enzymatic digestion or filtration to remove plant and/or nematode debris and produced 1.67×10^8 endospores/plant, or about 30-fold fewer than our method.

By eliminating the need for handpicking endospore-filled females, our method greatly reduced endospore collection time and increased the number of viable endospores that may be collected. The present method has been optimized for collecting endospores from the roots of three plants in 1 hr. However, it can easily be used to collect endospores from several more plants with a negligible increase in time.

Although noninfected and *Pasteuria*-filled *M. arenaria* females were not initially separated, passing the endospores through a 5-µm filter effectively removed most nematode and plant debris. Visual inspection of endospores collected using the present method revealed endospores with normal morphology and only a small amount of nematode and/or root debris (Fig. 1). Exosporium, spore core, and parasporal fibers are present and intact. For more sensitive assays, such as antiserum production, subsequent purification of endospores may be necessary.

Endospores collected by our method retained full biological activity and effectively attached to and infected *M. arenaria* J2. There were no differences in the average attachment or percentage attachment of endospores collected using the present method when compared to endospores from handpicked females. Average attachment was 5.6 ± 3.4 and 6.0 ± 3.1 endospores (mean ± 1 standard deviation for three replicates) per J2 for crushed galls or handpicked females, respectively. Likewise, there were no detectable differences in the infectivity of endospores collected using the present method vs. those from handpicked females. There was 95% and 97% average infectivity of J2 with endospores prepared with the present method and from 20 handpicked crushed females, respectively.

Polymerase chain reaction analysis with primers specific for *Pasteuria* 16S ribosomal DNA sequences yielded the anticipated 549-bp band. Genes isolated in this way are suitable for cloning and sequencing (Anderson et al., 1999; Atibalentja et al., 2000; Trotter and Bishop, 2003). The present method is ideal for rapidly collecting vast amounts of viable endospores suitable for attaching to and infecting nematodes, morphology studies, and DNA preparations.

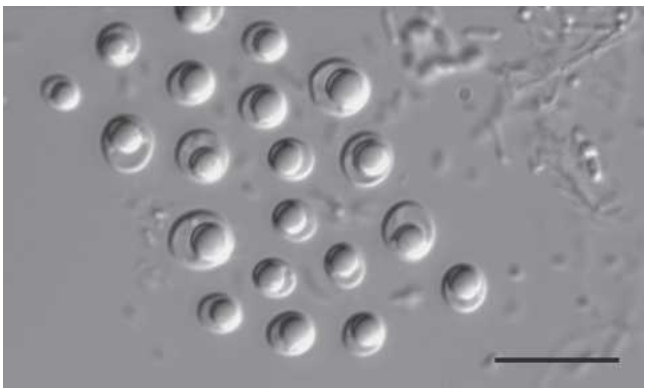

Fig. 1. Nomarski differential interference contrast microscopy of the *Pasteuria penetrans* endospore filtrate. Scale bar = 10 µm.

Literature Cited

Anderson, J. M., Preston, J. F., Dickson, D. W., Hewlett, T. E., and Maruniak, J. E. 1999. Phylogenetic analysis of *Pasteuria penetrans* by 16S rRNA gene cloning and sequencing. Journal of Nematology 31: 319–325.

Atibalentja, N., Noel, G. R., and Domier, L. L. 2000. Phylogenetic position of the North American isolate of *Pasteuria* that parasitizes the soybean cyst nematode, *Heterodera glycines*, as inferred from 16S rDNA sequence analysis. International Journal of Systematic and Evolutionary Microbiology 50:605–613.

Charles, L., Carbone, I., Davies, K. G., Bird, D., Burke, M., Kerry, B., and Opperman, C. H. 2005. Phylogenetic analysis of *Pasteuria penetrans* by use of multiple gene loci. Journal of Bacteriology 187:5700–5708.

Chen, S. Y., Charnecki, J., Preston, J. F., and Dickson, D. W. 2000. Extraction and purification of *Pasteuria* spp. endospores. Journal of Nematology 32:78–84.

Chen, Z. X., and Dickson, D. W. 1998. Review of *Pasteuria penetrans*. Biology, ecology, and biological control potential. Journal of Nematology 30:313–340.

Chen, Z. X., Dickson, D. W., and Hewlett, T. E. 1996. Quantification of endospore concentrations of *Pasteuria penetrans* in tomato root material. Journal of Nematology 28:50–55.

Davies, K. G., Kerry, B. R., and Flynn C. A. 1988. Observations on the pathogenicity of *Pasteuria penetrans*, a parasite of root-knot nematodes. Annals of Applied Biology 112:491–501.

Duan, Y. P., Castro, H. F., Hewlett, T. E., White, J. H., and Ogram, A. V. 2003. Detection and characterization of *Pasteuria* 16S rRNA gene sequences from nematodes and soils. International Journal of Systematic and Evolutionary Microbiology. 53:105–112.

Hewlett, T. E., and Dickson, D. W. 1993. A centrifugation method for attaching endospores of *Pasteuria* spp. to nematodes. Supplement to the Journal of Nematology 25:785–788.

Oostendorp, M., Dickson, D. W., and Mitchell, D. J. 1990. Host range and ecology of isolates of *Pasteuria* spp. from the southeastern United States. Journal of Nematology 22:525–521.

Sayre, R. M. 1993. *Pasteuria*, Metchnikoff, 1888. Pp. 101–111 *in* A. L. Sonenshein, J. A. Hoch, and R. Losick, eds. *Bacillus subtilis* and other gram positive bacteria: Biochemistry, physiology, and molecular genetics. Washington, DC: American Society for Microbiology.

Trotter, J. R., and Bishop A. H. 2003. Phylogenetic analysis and confirmation of the endospore-forming nature of *Pasteuria penetrans* based on the *spo0A* gene. FEMS Microbiology Letters 29:249–256.