Infectivity of Bacillus penetrans in Plant-parasitic Nematodes

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Abstract: Larvae of *Meloidogyne* spp. were readily infected with the endoparasite *Bacillus penetrans* by exposure to an aqueous suspension of spores from infected root-knot nematode females, or by passage of larvae through a shallow layer (50 cm^3) of spore-infested soil. Infection severely reduced motility of second-stage larvae through soil. *Bacillus penetrans* exhibited a distinct host specificity in that only 5 of 16 nematode species tested became infected with the population used. *Meloidogyne javanica, M. arenaria,* and *M. incognita* became more heavily infected than *M. hapla* or *Pratylenchus scribneri* under similar conditions, but all of these species were also good hosts. Ultrastructural changes in the endospore within sporangia adhering to the cuticle are similar to those reported for other germinating *Bacillus* endospores. *Key Words:* nematode pathogen, endoparasite, host specificity.

Bacillus penetrans (Thorne) Mankau, 1975 (= *Duboscqia penetrans*), an obligate parasite of some plant-parasitic nematodes, has shown potential as an effective biological control agent in previous studies (4, 5, 8, 10). The results have been sufficiently promising to justify a concerted effort to study the applied biology of this parasite. The life cycle of the organism has been described recently (6), and the pertinent taxonomic literature has been reviewed (3).

The mode of nematode infection by *B. penetrans* is unique among bacterial parasites of invertebrates, and a clear understanding of the process is essential to further investigations. Some of the initial results on infectivity and host range are reported herein.

MATERIALS AND METHODS

The population of *B. penetrans* used in these studies was found infecting greenhouse cultures of *Meloidogyne javanica* that originated from fields in the vicinity of Riverside, CA. One-week-old tomato seedlings potted in steam-sterilized soil were inoculated with infected larvae from these cultures. Infected *M. javanica* populations were maintained in the greenhouse by replanting with tomato seedlings and reinoculating with noninfected *M. javanica* larvae approximately every 6 months. Infected populations were maintained for several years, and experimentally utilized soil from them was designated as spore-infested soil (SIS). SIS was air-dried in approximately 2.5-cm layers on trays for 1 week or longer at 25-30 C and was stored for periods of several years without loss of infectivity.

Adult *M. javanica* females were dissected from the tomato roots to obtain spores of the parasite. Infected females were identified by their dull creamy white to amber color and absence of egg masses. Infection was confirmed by crushing the females in a drop of water and observing the spores with a compound microscope. About 2×10^6 spores were obtained from an average infected female.

The host range of this population of *B. penetrans* was determined by two methods. In the first method, 10 infected *M. javanica* females were dissected from root galls, placed in 0.5 ml of distilled water in a small watch glass, and crushed with the ground end of a glass rod. After cuticle pieces were removed with forceps, the suspension was diluted with 3.5 ml of distilled water and mixed. A droplet of this suspension was then examined under a microscope to confirm the presence of a large number of spores. The spore suspension was distributed into four small syracuse watch glasses; a similar set, containing only distilled water, served as a control. Forty hand-picked individuals of the test nematode were rinsed in distilled water and placed in each watch glass with 10 noninfected *M. javanica* larvae, and these served as an additional control. The watch glasses were incubated on wet filter paper within petri dishes at room temperature and examined daily for 3 consecutive days. Ten of the test nematodes were picked from each watch glass, rinsed in distilled water, and microscopically examined in a temporary water mount at 1000 X. With

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the second method, a suspension of approximately 2,000 nematodes of the species tested were poured over 50 cm^3 of infested soil, as in the standard infection technique described later, and collected after 36 h. Nematodes obtained were also examined for infection in a temporary water mount as previously described.

All nematodes used in this study were obtained from greenhouse pot cultures utilizing appropriate host plants except the following: *Tylenchulus semipenetrans* was collected from feeder roots (incubated in aerated water for 48 h) of infected citrus trees from a local orchard. *Pratylenchus vulnus* was obtained by washing and screening soil collected around old walnut trees. *Aporcelaimus* sp. was from field soil known to have high populations, but because their relative numbers were not large, only approximately 200 were tested. *Aphelenchus avenae* and *Aphelenchoides* sp. were extracted from monoxenic cultures growing on *Rhizoctonia solani* in potato-dextrose agar in petri dishes. A reliable technique for inoculation was developed in which 50 cm^3 soil were placed on Kim-Wipes® (Kimberly-Clark Corp. Neenah, WI) in a formed screen basket resting in a petri dish partially filled with water. A suspension of 500 *M. javanica* larvae was added to SIS and to steam-sterilized soil in four units each. This technique, with some adjustment of the parasite titer by dilution of SIS with noninfested soil, was adopted as a standard technique for infecting suspensions of root-knot larvae.

The effects of parasite infection on root-knot larvae was demonstrated by the following experiment. Three sets of soils (A, B, & C, in which A & B consisted of SIS containing root-knot nematodes and C consisted of citrus grove soil that was known to contain numerous citrus nematodes but not the bacterial parasite) with four replications were prepared. Nematodes were initially extracted from the soils and the soils were air-dried. The soils were moistened prior to the addition of 500 root-knot nematode larvae, air-dried again, and rehydrated again prior to the addition of 500 citrus nematode larvae. In a final treatment, SIS (B) was steam-sterilized, whereas the other soils were air-dried as before, rehydrated, and 800 *M. javanica* larvae added. In each treatment, nematodes recovered 24 h after being set upon the screens in petri dishes were examined (Table 1).

Ultrathin sections of infected larvae were prepared by fixing specimens in a mixture of formol-calcium fixative (1) and 20% DMSO (dimethyl sulfoxide) in distilled water (11). The two components were mixed in a 5:1 ratio (v/v) of fixative to DMSO immediately prior to use. Specimens were fixed 1 h at room temperature, rinsed three times in tap water, punctured, and post-fixed 2.5-3.0 h in phosphate buffered OsO_4. They were then rinsed 3 times in tap water and embedded in 2% agar made in 0.9% saline (13). The material was dehydrated through an acetone series and embedded in a mixture of 9 parts Maraset resin 655 and 1 part Cardolite resin NC513 with 1.0 ml of MM dimethylbenzylamine/50 ml resin. Sections were cut with glass knives on a Sorvall MT-2 ultramicrotome and stained 20 min in a saturated ethanol solution of uranyl acetate

TABLE 1. Recovery of *Meloidogyne javanica* larvae from *Bacillus penetrans*-infested and control soil.

Larvae recovery per soil	Soil treatment					
	None (Initial extraction)		A, B, C (Air-dried)[y]		A, C (Air-dried)[z] B (Steam-sterilized)	
	Healthy	Infested	Healthy	Infested	Healthy	Infested
Spore-infested soil (A)	0	48[x]	2	42	21	232
Spore-infested soil (B)	5[x]	50[x]	1	65	777	0
Citrus soil control (C)	2,079[w]	0	475	0	470	0

[w]Citrus nematode larvae.
[x]Root-knot larvae originally in soil.
[y]Five-hundred *M. javanica* added/15-cm pot.
[z]Eight-hundred *M. javanica* added/15-cm pot.

and then 5 min with lead acetate (9). The sections were examined and photographed on a Phillips-300 transmission electron microscope operated at 80 Kv.

RESULTS

Of the 500 *M. javanica* added to replicate SIS and control units, an average of 8 healthy and 61 infected larvae were recovered from units with SIS and an average of 430 larvae were recovered from the sterile soil after 48 h. The total number of larvae recovered from the SIS was only 16% of the number recovered from the controls and 88% of these were infected with the parasite.

In a test on infectivity of the parasite on root-knot nematode larvae in three soils, most of the larvae recovered from soils A & B were infected after 24 h (Table 1). An average of 2,079 citrus nematode larvae were recovered from soil C, but none were infected with the parasite. After being air-dried for 48 h, the screen baskets containing soils A, B, and C were returned to clean petri dishes partially filled with water. A suspension of 500 healthy *M. javanica* larvae was added to each soil replicate. Extractions examined after 24 h showed only about a 10% recovery of the larvae added to SIS soils A and B as compared to about 95% recovery of those added to soil C. Over 95% of larvae recovered from A and B were infected. To determine whether the impaired motility or recovery of larvae in soils A & B may have been caused by some factor other than parasite infection, such as physical properties of soils A & B, all soil replicates were again air-dried for 48 h and 500 citrus nematode larvae were added. The latter are similar in size and motility to *M. javanica* but are not hosts of the parasite. After 24 h extraction, an average of almost 95% of the citrus nematode larvae were recovered from all soils. Of the 800 *M. javanica* larvae added to all soil replicates in the final treatment, about 97% of those added to the steamed SIS (B) were recovered, but only 30% of those added to the air-dried SIS (A) were recovered and over 95% of the latter were infected with the parasite. None of the larvae from steamed soil B were infected (Table 1). Recovery of larvae from the citrus grove soil (C) was about 59% of those added.

Host Range Study: Spores of the parasite readily became attached to second-stage larvae of *M. javanica;* within 24 h, 90% of the larvae were infected and most of them had more than 20 spores (sporangia) attached to their cuticles (Table 2). The larvae adhered to each other in small clusters which were visible even without a microscope. Close examination of the cluster indicated that the nematodes stuck together at a point where a spore was attached to the cuticle of one. Healthy or noninfected larvae remained free of these clusters. In small clusters the aggregation was often disrupted by the movements of the nematodes. Large numbers of infected *M. javanica* larvae often formed relatively large clusters from which they could not escape. At the end of the 72-h period, almost all of the larvae were in clusters and heavily infected. Similar behavior was noted with *M. arenaria* and *M. incognita. Meloidogyne hapla* larvae were not as heavily infected as *M. javanica,* but formed some clusters. Larvae and adults of *Pratylenchus scribneri*

TABLE 2. Relative susceptibility of host nematodes to *Bacillus penetrans.*

Species of nematode	Percent infected nematodes[a]			
	Time exposed to spore suspension (h)			Spore-infested soil
	24	48	72	
Meloidogyne javanica	90 M	100 H	100 H	78
M. incognita	88 M	100 H	100 H	79
M. arenaria	63 L	80 M	100 H	69
M. hapla	60 L	80 L	90 H	49
Pratylenchus scribneri	43 L	78 L	80 L	49

[a]Infection level (spores attached per nematode): L = light (1-10), M = medium (10-30), H = heavy (more than 30).

were readily infected but did not form the characteristic clusters observed with root-knot nematodes, and some larvae were noninfected and active at the end of the 72-h period.

Results obtained from the soil test confirmed the observations made with spore suspensions in water. Approximately 70% of the root-knot larvae recovered were lightly infected (Table 2), and a few were very heavily infected. Of the five species of nematodes recorded as hosts, *M. hapla* and *P. scribneri* showed slightly lower rates of infection than the others. *Pratylenchus brachyurus* was not infected. Two *P. vulnus* larvae were infected with a single spore, but this seemed insufficient to consider it a potential host. Even much longer periods of exposure (up to 7 days) did not increase the infection, and it was not certain that these individual spores were able to penetrate *P. vulnus* and reproduce. One specimen of *Tylenchulus semipenetrans* was also observed to have a single affixed spore, but even after populations of *T. semipenetrans* were maintained in infested soil for over 6 months, no further infection could be found. Thus, this species cannot be considered a host. None of the following species tested showed any infection in either larvae or adults: *Aphelenchoides* sp., *Aphelenchus avenae, Aporcelaimus* sp., *Ditylenchus dipsaci, Heterodera schachtii, Trichodorus christiei, Tylenchorhynchus claytoni,* and *Xiphinema index.*

Ultrastructure: Observation of the fine structure provided limited information on the general features of penetration and infection. The cup or bowl-shaped spore has almost always been observed with its concave surface affixed to the nematode cuticle. There is no ultrastructural evidence of an adhesive substance in these preparations. There is an indication of a mechanical suction-cup-like relationship of the sporangium to the cuticle (Fig. 1).

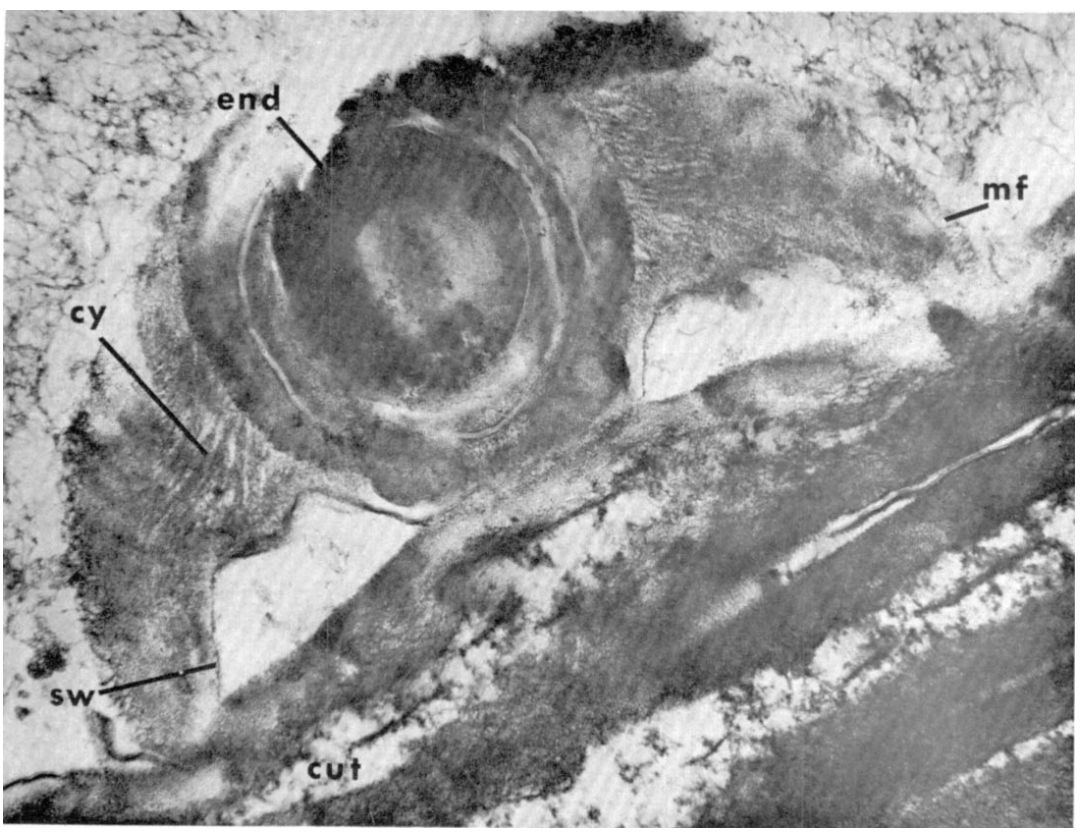

FIG. 1. Sporangium of *Bacillus penetrans* affixed to cuticle (cut) of a *Meloidogyne* second-stage larva. The raised sporangium wall (sw), fibrous sporangium cytoplasm (cy), and microfibrillar external sporangium coat (mf) are typical of this stage. The centrally-located endospore (end) has contacted the cuticle and changes can be observed in its wall structure and in the adjacent nematode cuticle.

The endospore within the sporangium becomes distinctly oriented to the nematode cuticle with some disorganization of the outer and inner spore coats, lamillar layers, and cortex adjacent to the cuticle. The nematode cuticle suggests dissolution or degradation immediately adjacent to the endospore. The sporangium cytoplasm has a fibrillar appearance, and the cytoplasm within the spore germ cell shows some distinct elements not visible in dormant spores.

DISCUSSION

Our results indicate host nematodes can be efficiently infected with *B. penetrans* by mixing nematodes with a suspension of the adhesive spores in a shallow dish of water or by allowing the nematodes to migrate through a shallow layer (ca. 15 mm) of infested soil. Infection by the parasite markedly impairs motility of root-knot larvae since only about 10% of the larvae added passed through soil infested with *B. penetrans* spores. Larvae of a non-host, *T. semipenetrans,* which passed through the same soil were almost entirely recovered, a fact indicating that infection by the parasite rather than any soil conditions caused the reduced recovery of root-knot larvae. When SIS (B) was steam-sterilized in the final treatment, almost all of the root-knot larvae added to the soil were recovered (Table 1), a result verifying the previous conclusion. The reduced recovery from the citrus soil may have been due to physical factors, such as compaction caused by repeated wetting and drying of the soil on the screen, different from A and B. We have observed that larvae with one to three spores attached to the cuticle retained normal motility, whereas the presence of more spores impaired motility through soil. The great majority of all *B. penetrans*-infected nematodes recovered from natural soil populations carried only one to two spores.

It should be possible to control experimental infection of a population of host nematodes by adjustment of the titer of spores in soil to which they are exposed. Similarly, a specific ratio of spores to numbers of root-knot larvae in an aqueous suspension should allow for controlled infection rates. Such relationships have not yet been experimentally established.

Review of infections reported in the literature and of collected infected nematode specimens indicated that a wide range of species, both plant-parasitic and free-living, are attacked by the parasite. The study of the host range of our population of *B. penetrans* among nematodes which were available to us in sufficient numbers indicated a more restricted host range than would be apparent from the literature (12). A number of the genera or species, which our parasite would not infect, have been reported as hosts; and specimens of the same or closely related nematode species occur in the senior author's collections from various areas of the world. Only 5 of 16 different species tested could be infected under the conditions used: 4 species of root-knot nematodes and 1 species of lesion nematode (Table 2). The population of the parasite studied appears to be specific to root-knot nematodes and of 3 species of *Pratylenchus* tested, only *P. brachyurus* was infected. There are undoubtedly a number of biotypes, races, or even species of this parasite. A more detailed study of the host range and host-parasite relationships of the parasite is required before the question of biotypes or species can be clarified. Our parasite population may attack other nematode species which have not yet been tested.

The penetration of *B. penetrans* through the nematode cuticle and the apparently specific relationship to the cuticle are unique among what appear to be related bacteria. The milky-disease pathogens of insects, *B. popilliae, B. lentimorbus,* and *B. larvae,* are morphologically similar but differ from *B. penetrans* in some important features (4). The former are ingested by insects and infect only certain closely related beetles of the family Scarabaeidae by penetrating the insect gut and then developing in the haemolymph. These bacteria are similar and can only be distinguished from one another by cultural and immunological methods. The ultrastructural changes which occur in *B. penetrans* during or prior to the process of infection (spore germination) are similar to those reported for *Bacillus* spores in general (2). The dissolution of an infection port in the nematode cuticle was recently verified (7).

The relatively simple infection tech-

niques we have described allow the maintenance of parasitized nematode populations and provide a basis for experimental study of the applied biology of this important nematode pathogen.

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