Ultrastructure, Morphology, and Sporogenesis of *Pasteuria penetrans*

Z. X. Chen, D. W. Dickson, L. G. Freitas, and J. F. Preston

First and second authors: Entomology and Nematology Department, University of Florida, Gainesville 32611-0620; third author: Plant Pathology Department, University of Florida, Gainesville 32611-0680; fourth author: Microbiology and Cell Science Department, University of Florida, Gainesville 32611-0700.

Accepted for publication 22 November 1996.

ABSTRACT

Chen, Z. X., Dickson, D. W., Freitas, L. G., and Preston, J. F. 1997. Ultrastructure, morphology, and sporogenesis of *Pasteuria penetrans*. *Phytopathology* 87:273-283.

Pasteuria penetrans is a bacterial parasite of root-knot nematodes that shows great potential as a biocontrol agent. Scanning and transmission electron microscopy were used to study the ultrastructure, morphology, and sporogenesis of four isolates of *P. penetrans*. The effects of different *Meloidogyne* spp. and tobacco cultivars on sporangium size and morphology of endospores attached to the cuticle of second-stage juveniles (J2) of root-knot nematodes also were investigated. The *P. penetrans* isolates and their origins were P-20 from *M. arenaria* race 1 in Levy County, FL;

P-100 from *Meloidogyne* sp. in Pasco County, FL; B-4 from *Pratylenchus scribneri* in Seminole County, FL; and P-120 from *Meloidogyne* spp. in Alachua County, FL. Sporangia of the four isolates were identical morphologically and similar in their dimensions, ranging from 2.39 to 3.42 μm in diameter and from 1.38 to 2.38 μm in height. Different *Meloidogyne* spp. and tobacco cultivars had no effect on sporangium size. Endospores attached to J2 were visualized in three forms: endospores retaining the sporangium wall, endospores covered with a thin exosporium, and endospores without covering. Sporogenesis of *P. penetrans* was similar to that of other gram-positive bacteria and generally matched the seven-stage scheme reported for *Bacillus thuringiensis*.

Pasteuria penetrans is a widespread mycelial and endospore-forming bacterium that has shown great potential as a biocontrol agent of plant-pathogenic nematodes (4,6,9,34). *P. penetrans* has been reported on 205 nematode species belonging to 96 genera from 51 countries on 5 continents and various islands in the Atlantic, Pacific, and Indian oceans (27,36). *P. penetrans* isolates have a rather limited host range and exhibit considerable morphological diversity, particularly in the size and shape of sporangia and endospores (26,27).

Four species of *Pasteuria* have been described. *P. ramosa*, which parasitizes water fleas of the genus *Daphnia* (25), is the type species of the genus. The other three species of *Pasteuria* are parasites of plant-parasitic nematodes: *P. penetrans* on *Meloidogyne* spp. (26), *P. thornei* on *Pratylenchus* spp. (33), and *P. nishizawae* on cyst nematodes of the genera *Heterodera* and *Globodera* (31). Two other proposed new species of *Pasteuria* have been isolated from *H. goettingiana* in Münster, Germany (37) and *Belonolaimus longicaudatus* in Florida (13). Criteria for the differentiation of these species include size, shape, and structure of endospores and sporangia, developmental morphology of the bacterium, and host specificity (13,29,31,33,37).

Individual isolates of *P. penetrans* exhibit narrow host ranges (22,29,35), but the reasons for the host specificity of *P. penetrans* have not been elucidated fully (22,35). Several isolates of *P. penetrans* from Florida have varying host specificity (22) and currently are being evaluated as biocontrol agents of root-knot nematodes. The objective of this experiment was to determine whether the ultrastructure of endospores and sporangia or sporogenesis among four Florida isolates of *P. penetrans* are different.

MATERIALS AND METHODS

***P. penetrans* isolates.** The designations and origins of the *P. penetrans* isolates were P-20 from *M. arenaria* (Neal) Chitwood

race 1, Levy County, FL; P-100 from *Meloidogyne* sp., Pasco County, FL; P-120 from *Meloidogyne* spp., Alachua County, FL; and B-4 from *Pratylenchus scribneri* Steiner, Seminole County, FL (22). Isolate P-20 was propagated on *M. arenaria* race 1, and P-100 and B-4 were propagated on *M. incognita* (Kofoed & White) Chitwood race 1 growing on tomato (*Lycopersicon esculentum* Mill. 'Rutgers') in a greenhouse. Endospores of each *P. penetrans* isolate were attached separately to newly hatched second-stage juveniles (J2) of root-knot nematodes by a centrifugation technique (14). J2 with approximately five endospores attached were inoculated on potted tomato (45 to 60 days old) at a rate of 3,000 J2 per pot. Tomato plants were grown in 15-cm-diameter pots and were provided with fertilizer and water as needed. Sporangium-filled females were harvested 55 to 60 days after inoculation. For P-120, sporangium-filled root-knot nematode females were collected from galled tobacco (*Nicotiana tabacum* L.) roots growing in a naturally infested field at the Green Acres Agronomy Farm, University of Florida, Gainesville.

Comparisons of sporangium size. Scanning electron microscopy (SEM) has been used routinely to determine the size of *P. penetrans* sporangia (20,26,33). A modified procedure was used to prepare the samples for SEM (11). Tomato or tobacco roots (~20 g fresh weight) containing sporangium-filled females were incubated overnight in 100 ml of a 12.5% cytolase PCL5 solution (Genencor International, Rochester, NY) to loosen root tissue and free root-knot nematode females. After incubation, the material was decanted onto a 600- μm -pore sieve nested in a 75- μm -pore sieve and subjected to a high-pressure water spray to dislodge the females (15). Sporangium-filled females were hand-picked with forceps with the aid of a stereomicroscope at $\times 20$. They were rinsed three times in deionized water. Individual females were ruptured with fine forceps and smeared on a 1 $\times$ 0.5-cm Whatman No. 1 filter paper (Whatman International, Springfield Mill, England). Filter paper containing sporangia was immediately transferred to 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) and maintained overnight at 4°C. The fixed samples were rinsed in chilled cacodylate buffer three times for 20 min each and postfixed with 2% osmium tetroxide in buffer for 2 h at room temperature. Samples

Corresponding author: Z. X. Chen; E-mail address: zchen@gnv.ifas.ufl.edu

Publication no. P-1997-0121-06R

© 1997 The American Phytopathological Society

were rinsed three times for 10 min each with buffer to remove the excess osmium tetroxide. Samples were dehydrated through 25, 50, 75, 95, and 100% ethanol series, 1 h each. Exposure to 100% ethanol was repeated three times. After dehydration, the samples were immersed for 5 min in hexamethyldisilane (Alltech Associates, Deerfield, IL), air-dried overnight in a fume hood, mounted on aluminum stubs, coated with gold, and examined with a Hitachi S-570 (Hitachi Instruments, Danbury, CT) scanning electron microscope operating at 15 kV. The height and diameter of the

cup-shaped sporangium were determined from SEM photomicrographs taken using professional Polaroid 10 × 12.5-cm instant film no. 55 (Polaroid Corp., Cambridge, MA) or with 35-mm negative film.

Effects of nematode host and resistant crops on size of sporangia. *P. penetrans*-infected females of *M. incognita* and *M. javanica* (Treub) Chitwood were collected from a previously identified root-knot nematode-suppressive site at the Green Acres Agronomy Farm (5). The site was known to be infested with *M.*

Fig. 1. Morphology of *Pasteruria penetrans*. Scale bars = 1 μm. **A**, Mature sporangia of *P. penetrans*: dorsal view (dv) of a smooth and round sporangium. Ventral view of a wrinkled sporangium with a "connection ring" (arrowheads). **B**, Lateral view of two connected sporangia. **C**, Early sporogenous stage. **D**, Lateral view of a sporangium connected with a degenerated sporangium or stem cell (arrowhead).

incognita and *M. javanica*. Two *M. incognita* females and one *M. javanica* female filled with *P. penetrans* were collected from *M. incognita*-resistant tobacco cv. K326, and two *M. javanica* females and one *M. incognita* female filled with *P. penetrans* were collected from *M. incognita*-susceptible tobacco cv. Hicks. The samples were processed for SEM as described above. After each female was ruptured and smeared on filter paper, the cuticle of the female was processed for species identification based on perineal pattern (17).

Attachment of endospores to J2 of *M. arenaria*. Endospores of P-20 were attached to J2 of *M. arenaria* race 1 1 day after eclosion, using a centrifugation technique (14). About 50 J2 with endospores attached were picked immediately with a bristle and rinsed in three changes of deionized water. The protocol previously mentioned for SEM was used to process the samples. Morphology of endospores attached to J2 was examined with a Hitachi S-570 scanning electron microscope operating at 15 kV.

Morphological comparisons of four isolates of *P. penetrans*. Transmission electron microscopy (TEM) was used to examine the sporangium morphology of P-20, P-100, P-120, and B-4. Females containing *P. penetrans* were fixed by a microwave fixation method (19) in Karnovsky's fixative (18) in 0.1 M cacodylate buffer (pH 7.2) at 50°C. After fixation, the material was left at room temperature for 1 h. Nematodes were ruptured with a surgical knife to facilitate the penetration of solutes and infiltration of the plastic. The ruptured nematodes were embedded in a drop of 1% agarose at 45°C. The nematodes were transferred to a second fixative (40) containing 2.5% glutaraldehyde and 5% dimethyl sulfoxide in 0.1 M cacodylate buffer and kept overnight at 4°C (40).

The specimens were washed twice with cacodylate buffer for 30 min each and postfixed with 2% osmium tetroxide at 4°C overnight (11). After rinsing twice in deionized water for 20 min each, the specimens were dehydrated in an ethanol series (25, 50, 75, and 95% and three changes of 100% ethanol, 1 h each). The ethanol was replaced by 100% acetone, with two changes, 30 min each. The specimens were infiltrated through a series of Spurr's standard resin in acetone (32): 30 and 70% and two changes of 100% resin, overnight each time. After infiltration, the specimens were oriented in plastic embedding boats containing fresh 100% resin ~3 mm in depth and allowed to polymerize in a 60°C oven for 48 h.

Ultrathin sections were cut with a glass or diamond knife on a MT 6000-XL ultramicrotome (RMC, Tucson, AZ). Gray and silver sections (50 to 70 nm thick) were mounted on 100-mesh copper grids coated with Formvar film and poststained in 1% uranyl acetate for 10 min and in saturated aqueous lead citrate for 5 min (11). The specimens were examined with a JEOL 100 CX (JEOL, Tokyo) transmission electron microscope at 60 kV.

Terminology. To describe the ultrastructure of the developmental stages of *P. penetrans*, we used terms suggested by Sussman and Halvorson (38) and recently reviewed by van Itersson (39) as applied to *Bacillus* spp. The terminology is as follows: (i) the endospore is the single asexual spore that develops within a sporangium and is enclosed by an exosporium. Beginning from the inside

and working to the outside of the endospore, (ii) the plasma membrane is the innermost layer that surrounds the central protoplast and is surrounded by (iii) the cell wall, which is usually invisible in transmission electron microphotographs. (iv) The cortex appears as a wide electron-translucent zone that surrounds the cell wall and plasma membrane; the cortex is followed outside by (v) the inner coat, a much narrower multilaminar band, and (vi) the outer coat, a wide electron-dense wall. All of these structures are surrounded by (vii) the exosporium, a delicate membrane that delineates the outermost layer of a typical gram-positive bacterial spore. Within the exosporium, *Pasteuria* endospores have additional structures that include (viii) the epicortical layer, which is a discontinuous, electron-dense band around the cortex that varies in appearance from species to species and (ix) the perisporium, which largely consists of peripheral fibers, which are an essential part of the *Pasteuria* endospore and probably involved in attachment to the nematode cuticle. The central part of the entire endospore, limited by the outer coat and distinctly differentiated from the perisporium, will be referred to as the central body (37).

RESULTS

Size and shape of sporangium. Sporangia were cup-shaped and morphologically identical among isolates (Fig. 1). Dorsal sides of sporangia were smooth and round. The ventral side was wrinkled (Fig. 1A). Occasionally, two sporangia were connected. When they were separated, a trace of a "connection ring" was revealed (Fig. 1A and B). Stem cells were seldom seen in the four isolates (Fig. 1D).

The average diameter of B-4 sporangia was greater than that of other isolates ($P \leq 0.05$) (Table 1). The height of sporangia of P-120 (1.93 μm) was greater than that of P-100 (1.74 μm) ($P \leq 0.05$). The ranges of sporangium dimensions of the isolates, however, were similar, 2.58 to 3.42 μm in diameter and 1.38 to 2.38 μm in height.

Effects of nematode hosts and resistant crops on size of sporangia. Diameters and heights of sporangia differed among various

TABLE 1. Diameter and height of sporangia from four isolates of *Pasteuria penetrans*

Isolates ^x	Diameter (μm) ^y			Height (μm)		
	Average ^z	SE	Range	Average	SE	Range
P-20	3.10 b	0.04	2.58–3.34	1.81 ab	0.05	1.38–2.08
P-100	3.07 b	0.03	2.78–3.21	1.74 b	0.04	1.42–2.01
P-120	3.09 b	0.03	2.76–3.30	1.93 a	0.06	1.58–2.38
B-4	3.23 a	0.03	2.84–3.42	1.86 ab	0.04	1.58–2.13

^x P-20, P-100, P-120, and B-4 originated from *Meloidogyne arenaria* race 1 in Levy County, *Meloidogyne* sp. in Pasco County, *Meloidogyne* spp. in Alachua County, and *Pratylenchus scribneri* in Seminole County, FL, respectively.

^y Twenty sporangia each were measured from scanning electron photomicrographs at $\times 15,000$. Sporangia of each isolate were processed from a single female.

^z Means in a column followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

TABLE 2. Diameter and height of *Pasteuria penetrans* isolate P-120 sporangia grown on two tobacco cultivars and two *Meloidogyne* species

Tobacco cultivar ^x	Host nematode	Diameter (μm) ^y			Height (μm)		
		Average ^z	SE	Range	Average	SE	Range
Hicks	<i>M. incognita</i>	3.19 a	0.04	2.96–3.39	1.79 c	0.06	1.46–2.34
Hicks	<i>M. javanica</i>	3.09 a	0.03	2.75–3.30	1.93 ab	0.06	1.58–2.38
Hicks	<i>M. javanica</i>	2.90 b	0.05	2.39–3.21	1.81 bc	0.04	1.54–2.09
K326	<i>M. incognita</i>	2.97 b	0.04	2.42–3.17	1.82 bc	0.04	1.50–2.08
K326	<i>M. incognita</i>	3.09 a	0.04	2.76–3.34	1.86 bc	0.03	1.63–2.08
K326	<i>M. javanica</i>	3.14 a	0.04	2.84–3.42	2.02 a	0.03	1.76–2.17

^x Hicks is susceptible to *M. incognita* and K326 is resistant.

^y Twenty sporangia each were measured from scanning electron photomicrographs at $\times 15,000$. Sporangia of each treatment were processed from a single female.

^z Means in a column followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

combinations of *Meloidogyne* species and resistant crops ($P \leq 0.05$) (Table 2). Diameters of sporangia obtained from the three *M. incognita* females, regardless of the tobacco cultivars from which they originated, averaged $3.08 \mu\text{m}$, whereas the diameter of sporangium obtained from three *M. javanica* females averaged $3.04 \mu\text{m}$. The average sporangium heights also were similar, $1.82 \mu\text{m}$ for sporangia from *M. incognita* females and $1.92 \mu\text{m}$ for sporangia from *M. javanica* females. Sporangium diameter averaged $3.06 \mu\text{m}$ for K326 and $3.07 \mu\text{m}$ for Hicks. The average sporangium heights were 1.84 and $1.90 \mu\text{m}$ for Hicks and K326, respectively. Maximum differences in sporangium diameters were less than $0.22 \mu\text{m}$ across all treatments and less than $0.23 \mu\text{m}$ for sporangium heights (Table 2). Thus, the effects of nematode hosts and tobacco cultivars on sporangium size were negligible.

Endospores attached to J2. Endospores of *P. penetrans* attached along the entire body length of J2 (Figs. 2 and 3). After attachment, the sporangial wall and exosporium of most endospores sloughed off (Figs. 2 through 4), exposing the peripheral fibers and central body. Both the endospore and the central body were round (Fig. 4D). The endospores were $2.81 \pm 0.15 \mu\text{m}$ in diameter. The central body was $1.58 \pm 0.05 \mu\text{m}$ in diameter. Peripheral fibers appeared rough when viewed with SEM (Fig. 4C).

Three distinct forms of endospores were observed attached to the cuticle of J2. In the first form, which was rare, the endospore surface was covered by the sporangium wall (Fig. 2). In the second form, the sporangium wall sloughed off, and the surface was covered by the exosporium (Fig. 4B). In the third form, both the sporangium wall and the exosporium sloughed off to reveal the intact peripheral fibers and central body (Fig. 4C and D).

Fig. 2. Endospores of *Pasteuria penetrans* attached to the anterior region of a second-stage juvenile of *Meloidogyne arenaria* race 1. One of the endospores retains the sporangium wall (arrowhead). Scale bar = $1 \mu\text{m}$.

TEM. Various developmental stages (vegetative, sporogenesis, and mature sporangia) were observed simultaneously in a single female (Fig. 5). Ultrastructure and morphology of developmental stages (mycelium and sporogenesis) of the four isolates were similar. Hyphae were septate and bounded by a $0.03\text{-}\mu\text{m}$ -thick wall consisting of an inner and outer membrane. The inner membrane constricted to form septations that delineate individual cells (Fig. 6A and B). Septations were few in this early rapid growth stage of the microcolony (Fig. 6A). The hypha terminal was dichotomously branched. In advanced developmental stages, fragmented thalli were separated from the mycelial masses, and the terminal cell was elongated (Fig. 6B). The terminal cells increased in size and became oval (Figs. 1C and 6C), measuring 1.2 to $1.7 \mu\text{m}$ long and 0.6 to $1.0 \mu\text{m}$ wide and were bounded by a $0.02\text{-}\mu\text{m}$ -thick wall. A membrane formed about $0.4 \mu\text{m}$ from the anterior end and separated the forespore from the parasporium (Fig. 6C and D). The forespore was located in the anterior end and was small compared with the parasporium. The parasporium varied in size and, in some cases, engulfed as much as two-thirds of the forespore (Fig. 6E). In these cases, the membrane was invaginated toward the cell pole, and the forespore was surrounded by an inner membrane that bounded its own protoplast and an outer membrane that bounded the mother cell's protoplast (Fig. 7A). Peripheral fibers were attached to the lower part of the forespore (Fig. 7A), and a cell wall covered the inner forespore membrane (Fig. 7B). Lamellae originated from the base and lower part of the forespore and covered the outer forespore membrane. The forespore, $1.1 \mu\text{m}$ long and $0.7 \mu\text{m}$ wide, had visible DNA and a $0.01\text{-}\mu\text{m}$ -thick boundary wall (Fig. 7C). The sporangia of this stage were egg-shaped, $2.0 \mu\text{m}$ long and $1.5 \mu\text{m}$ wide, and bounded by a $0.03\text{-}\mu\text{m}$ -thick wall.

Endospores were visible within the forespore. Endospores in the initial stage were round and $1.0 \mu\text{m}$ in diameter. The protoplast of the endospore, except for the light area containing DNA, was electron dense (Fig. 7D), indicating dehydration of the endospore

Fig. 3. Endospores of *Pasteuria penetrans* attached to the lateral field of a second-stage juvenile of *Meloidogyne arenaria* race 1. Scale bar = $2.5 \mu\text{m}$.

protoplast. The cortex, originating underneath the multiple lamellae, was surrounded by the endospore coat (Figs. 7D and E and 8). The exosporium was the last membrane formed to cover the endospore (Fig. 7E). When the endospore matured, a basal ring surrounded the germinal pore. The epicortical layer was located between the cortex and inner coat (Fig. 9).

In a mature endospore, the cortex and outer coat were 0.13 and 0.15 μm thick, respectively. Unlike the cortex, the outer coat tapered

toward the basal side of the endospore and was only 0.03 μm thick in the germinal pore. The germinal pore was 0.4 μm in diameter and was surrounded by a basal ring texturally similar to the outer coat (Fig. 9). The central body of the endospore was ellipsoidal, $1.4 \times 0.9 \mu\text{m}$, with the long axis lying horizontally. The epicortical layer was discontinuous and visible only on the lateral sides of the endospore. Figure 10 is a line drawing showing sporogenesis in *P. penetrans*.

Fig. 4. Endospores of *Pasteuria penetrans* attached to the cuticle of second-stage juveniles of *Meloidogyne arenaria* race 1. Scale bars = 1 μm . **A**, Sporangial wall and exosporium (arrowhead) sloughing off after attachment. **B**, Endospore with the exosporium (arrowhead). **C**, Both the sporangium wall and exosporium have sloughed off, revealing the peripheral fibers (p) and central body (c). **D**, Top view of an endospore.

DISCUSSION

Ultrastructure, morphology, and morphometrics of sporangia were similar among the Florida isolates of *P. penetrans*. Sporangia of the four isolates were 2.58 to 3.42 μm in diameter and 1.38 to 2.38 μm in height, which was smaller than those reported previously (3.0 to 4.0 μm in diameter and 2.26 to 2.6 μm in height) (33). These differences could have been caused by the different methods used to prepare samples for electron microscopy (33). Morphology of mature sporangia of our isolates was the same as those reported

for *P. penetrans* (20,26,33) but different from *P. ramosa* (25), *P. thornei* (33), *P. nishizawae* (31), and other *Pasteuria* spp. (13,37).

The difference in sporangium size of our four isolates was slight but statistically different ($P \leq 0.05$). Bird et al. (3) also found different sizes of sporangia among different isolates. We agree with their conclusion that these differences are too small to reliably differentiate various isolates.

Our isolates exhibited the same ultrastructure, morphology, and sporogenesis but differed in host specificity (22). Ciancio (7) reported that morphology and morphometrics of endospores are re-

Fig. 5. Different developmental stages of *Pasteuria penetrans* within the pseudocoel of a single root-knot nematode female. Scale bar = 1 μm .

lated to host range across nematode genera. Endospores from different nematode genera usually exhibit differences in ultrastructure, morphology, and morphometrics (7,13,20,26,31,33,37). Our isolates originated from *Meloidogyne* spp. and *Pratylenchus scribneri*, but all were reared on *Meloidogyne* spp. Stirling (35) speculated that host specificity of *P. penetrans* might be a response to nematode populations rather than to nematode species. All the isolates reported (22,35) attach to more than one *Meloidogyne* species or race. A recent study shows that *P. penetrans* can produce heterogeneous endospores (8). These heterogeneous subpopulations of endospores show specificity to different nematode populations. It also

has been reported that endospores usually attach more readily to the nematode species on which *P. penetrans* is originally obtained or cultured than to other species (8,22). Thus, the host specificity of *P. penetrans* may result from the adaptation of the bacterium to its host nematode. Such adaptations probably were not followed by changes in the ultrastructure, morphology, and morphometrics of endospores.

Two forms of endospores have been observed attached to the J2 of *Meloidogyne* spp.: endospores covered with an exosporium and bare endospores (26,28,33). The exosporium in the former, however, resembles the sporangium wall in our observation. The sporangium wall is much thicker than the exosporium. The appearance

Fig. 6. Developmental stages of *Pasteuria penetrans*. Scale bars = 1 μm. **A**, Mycelium of *P. penetrans* is dichotomously branched (arrowhead). **B**, A microcolony in stage I in the sporogenesis of *P. penetrans*. **C**, A terminal cell in stage II in the sporogenesis of *P. penetrans*. **D**, A forespore in late stage II. **E**, A forespore engulfed by the parasporium in stage III.

of the sporangium wall and exosporium is different in our scanning electron photomicrographs. The wrinkled appearance of the sporangial wall, which probably led Sayre and Starr (26,28,33) to consider it the exosporium, may result from the attachment of the endospore to the J2 cuticle. Mature endospores usually retain their sporangial wall and exosporium. When using centrifugation to attach endospores to J2 (14), we found that most of the attached endospores were without sporangial walls. Some unknown mechanisms must be involved in the rapid sloughing off of the sporangial wall and exosporium. One possible explanation is that when the endospore descends to the nematode surface, the centrifugal force ruptures the sporangial wall and allows the endospore to contact

the surface of the J2 cuticle. After this initial contact, the peripheral fibers conform with the J2 cuticle to establish a firm attachment. This process also may result in the sloughing off of the sporangial wall and exosporium because these layers are not connected to the endospore. Endospores that retain sporangial remnants probably are the intermediate or uncompleted forms of endospore attachment to J2.

Mesosomes were observed in *P. penetrans* (16,20,24–28,33), *P. ramosa* (24,25,27,28,33), and *P. nishizawae* (27,30) but not in *P. thornei* (28,33) or other *Pasteuria* spp. (13,30). We did not observe mesosomes in the four isolates studied. Mesosomes also have been observed in other bacteria (39). Based on recent evidence from

Fig. 7. Sporogenous stages of *Pasteuria penetrans*. Scale bars = 1 μ m. **A**, The forespore is surrounded by an inner membrane (i) that bounds its own protoplast and an outer membrane (o) that bounds the mother cell's protoplast. Peripheral fibers (p) have attached to the lower part of the forespore. **B**, A cell wall (arrowhead) covers the inner forespore membrane in late stage III. **C**, Lamellae (arrowhead), which give rise to the cortex, inner coat, and outer coat of the endospore, are visible in early stage IV. The nucleoid (n) contains DNA. **D**, The outer coat (arrowhead) surrounds the cortex. **E**, Exosporium (arrowhead) has formed.

freeze-fracture preparations, however, these internal membranous structures probably are fixation artifacts (10,12,21). Moreover, a recent study has proved that suboptimum fixation conditions can create the artifact (1). These conditions include any one of the following: phosphate-buffered 2.5% glutaraldehyde, 0.1% osmium tetroxide, and 0.5% glutaraldehyde plus 2.5% formaldehyde (1). Mesosomes reported in *Pasteuria* spp. always associate with material fixed with 2 or 3% glutaraldehyde in phosphate buffers (16, 20,24–28,30,33). Materials freeze-fractured, freeze-substituted, or fixed in cacodylate-buffered 2.5% glutaraldehyde lack mesosomes (1).

Transmission electron micrographs in earlier studies of *P. penetrans* only showed a few stages of sporogenesis (20,24–26,28,29, 33). Furthermore, some previous transmission electron micrographs were difficult to interpret, e.g., an opaque area in the forespore (Fig. 5 in [26] and Fig. 33.25 in [28]). Accordingly, the line drawings reported in earlier studies represented only a portion of the sporogenesis in *P. penetrans* (26). Our results provide a more complete scenario of sporogenesis than others (20,24–26,28,29,33).

The line drawings of our transmission electron micrographs reveal that sporogenesis in *P. penetrans* is similar to that of other well-studied gram-positive bacteria; here we chose *Bacillus thuringiensis* for comparison. The sporogenesis in *B. thuringiensis* has been classified into seven morphological stages (39). Stage I, defined as condensation of the replicated chromosome to form an axial filament, is not observed in *P. penetrans*. Stages II through VII are similar in *Pasteuria* and *Bacillus* and involve formation of the forespore septum (stage II); engulfment of the forespore (stage III); formation of the cortex, coat, and exosporium (stages IV through VI); and sporangium maturation (stage VII). Perisporium, termed peripheral fibers in *P. penetrans* and parasporal crystals in *B. thuringiensis*, is formed after engulfment of forespores at stage III in both organisms.

Although *Pasteuria* and *Bacillus* exhibit similar sporogenesis, they are not necessarily close relatives. Sporogenesis and ultrastructure of bacterium endospores are highly conserved (23). Sporogenesis of bacteria probably evolved only once (23). Moreover, *Pasteuria*

Fig. 8. Endospore of *Pasteuria penetrans* in the late sporogenous stage. The arrowhead indicates the position of the germinal pore of the endospore. Scale bar = 1 μ m.

Fig. 9. Mature *Pasteuria penetrans* endospores (stage VII). The epicortical layer (e) is a discontinuous membrane located inside the inner (ic) and outer coats (oc). The basal ring (br) surrounds the germinal pore. The peripheral fibers (pf) allow the endospore to attach to the nematode cuticle. Scale bars = 1 μ m.

Fig. 10. Line drawing of sporogenesis in *Pasteuria penetrans* (middle row) compared to sporogenesis in *Bacillus thuringiensis* (upper row, redrawn from [39]) and previously reported sporogenesis of *P. penetrans* (lower row, redrawn from [26]).

and *Bacillus* differ in several fundamental ways. Most importantly, *Pasteuria* differs from *Bacillus* in that *Pasteuria* penetrates its host with a germ tube and forms thalli in the host body cavity. Because artificial cultivation of *Pasteuria* spp. is currently unsuccessful, the phylogenetic relationship of *Pasteuria* and *Bacillus* to other gram-positive bacteria is unknown (2).

ACKNOWLEDGMENTS

Florida Agricultural Experiment Station Journal Series R-05304. Supported in part by a grant from the Southern Region IPM: grant 93-34103-8396. A portion of a Ph.D. dissertation by Z. X. Chen. We thank H. C. Aldrich, R. M. Giblin-Davis, and G. C. Smart, Jr., for reviewing this manuscript, and H. C. Aldrich, H. Cromroy, B. Carpenter, and D. Williams for providing facilities and instructions for this study.

LITERATURE CITED

- Aldrich, H. C., Beimborn, D. B., and Schonheit, P. 1987. Creation of artifactual internal membranes during fixation of *Methanobacterium thermoautotrophicum*. Can. J. Microbiol. 33:844-849.
- Berkeley, R. C. W., and Ali, N. 1994. Classification and identification of endospore-forming bacteria. Pages 1-8 in: Fundamental and Applied Aspects of Bacterial Spores. G. W. Gould, A. D. Russell, and D. E. S. Stewart-Tull, eds. Blackwell Scientific Publishing, Oxford.
- Bird, A. F., Brisbane, P. G., McClure, S. G., and Kimber, R. W. L. 1990. Studies on the properties of the spores of some populations of *Pasteuria penetrans*. J. Invertebr. Pathol. 55:169-178.
- Brown, S. M., Kepner, J. L., and Smart, G. C., Jr. 1985. Increased crop yields following application of *Bacillus penetrans* to field plots infested with *Meloidogyne incognita*. Soil Biol. Biochem. 17:483-486.
- Chen, S., Dickson, D. W., and Whitty, E. B. 1994. Response of *Meloidogyne* spp. to *Pasteuria penetrans*, fungi, and cultural practices in tobacco. J. Nematol. 26:620-625.
- Chen, Z. X., Dickson, D. W., McSorley, R., Mitchell, D. J., and Hewlett, T. E. 1996. Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. J. Nematol. 28:159-168.
- Ciancio, A. 1995. Phenotypic adaptations in *Pasteuria* spp. nematode parasites. J. Nematol. 27:328-338.
- Davies, K. G., Redden, M., and Pearson, T. K. 1994. Endospore heterogeneity in *Pasteuria penetrans* related to adhesion to plant-parasitic nematodes. Lett. Appl. Microbiol. 19:370-373.
- Dickson, D. W., Oostendorp, M., Giblin-Davis, R. M., and Mitchell, D. J. 1994. Control of plant-parasitic nematodes by biological antagonists. Pages 575-601 in: Pest Management in the Subtropics, Biological Control—A Florida Perspective. D. Rosen, F. D. Bennett, and J. L. Capinera, eds. Intercept, Andover, England.
- Dubochet, J., McDowall, A. W., Menge, B., Schmid, E. N., and Lickfeld, K. G. 1983. Electron microscopy of frozen-hydrated bacteria. J. Bacteriol. 155:381-390.
- Dykstra, M. J. 1993. A Manual of Applied Techniques for Biological Electron Microscopy. Plenum Press, New York.
- Ebersold, H. R., Cordier, J. L., and Luthy, P. 1981. Bacterial mesosomes: Method-dependent artifacts. Arch. Microbiol. 130:19-22.
- Giblin-Davis, R. M., Williams, D., Hewlett, T. E., and Dickson, D. W. 1995. Development and host attachment studies using *Pasteuria* from *Belonolaimus longicaudatus* from Florida. (Abstr.) J. Nematol. 27:500.
- Hewlett, T. E., and Dickson, D. W. 1993. A centrifugation method for attaching endospores of *Pasteuria* spp. to nematodes. J. Nematol. 25 (Suppl.):785-788.
- Hussey, R. S. 1971. A technique for obtaining quantities of living *Meloidogyne* females. J. Nematol. 3:99-100.
- Imbriani, J. L., and Mankau, R. 1977. Ultrastructure of the nematode pathogen, *Bacillus penetrans*. J. Invertebr. Pathol. 30:337-347.
- Jepson, S. B. 1987. Identification of root-knot nematodes (*Meloidogyne* species). CAB International, Oxon, England.
- Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. (Abstr.) J. Cell Biol. 27:137A-138A.
- Login, G. R., Galli, S. J., and Dvorak, A. M. 1992. Immunocytochemical localization of histamine in secretory granules of rat peritoneal mast cells with conventional or rapid microwave fixation and an ultrastructural post-embedding immunogold technique. J. Histochem. Cytochem. 40:1247-1256.
- Mankau, R. 1975. *Bacillus penetrans* n. comb. causing a virulent disease of plant-parasitic nematodes. J. Invertebr. Pathol. 26:333-339.
- Nanninga, N. 1971. The mesosome of *Bacillus subtilis* as affected by chemical and physical fixation. J. Cell Biol. 48:219-224.
- Oostendorp, M., Dickson, D. W., and Mitchell, D. J. 1990. Host range and ecology of isolates of *Pasteuria* spp. from the southeastern USA. J. Nematol. 22:525-531.
- Priest, F. G. 1993. Systematics and ecology of *Bacillus*. Pages 3-16 in:

- Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics. A. L. Sonenshein, J. A. Hoch, and R. Losick, eds. American Society for Microbiology, Washington, DC.
24. Sayre, R. M. 1993. *Pasteuria*, Metchnikoff, 1888. Pages 101-111 in: *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics. A. L. Sonenshein, J. A. Hoch, and R. Losick, eds. American Society for Microbiology, Washington, DC.
 25. Sayre, R. M., Gherna, R. L., and Wergin, W. P. 1983. Morphological and taxonomic reevaluation of *Pasteuria ramosa* Metchnikoff 1888 and "*Bacillus penetrans*" Mankau 1975. *Int. J. Sys. Bacteriol.* 33:639-649.
 26. Sayre, R. M., and Starr, M. P. 1985. *Pasteuria penetrans* (ex Thorne, 1940) nom. rev., comb.n., sp.n., a mycelial and endospore-forming bacterium parasitic in plant-parasitic nematodes. *Proc. Helminthol. Soc. Wash.* 52:149-165.
 27. Sayre, R. M., and Starr, M. P. 1988. Bacterial diseases and antagonists of nematodes. Pages 69-101 in: *Diseases of Nematodes*. G. O. Poinar, Jr., and H.-B. Jansson, eds. CRC Press, Boca Raton, FL.
 28. Sayre, R. M., and Starr, M. P. 1989. Genus *Pasteuria* Metchnikoff, 1888. Pages 2601-2615 in: *Bergey's Manual of Systematic Bacteriology*. S. T. Williams, ed. Williams and Wilkins, Baltimore, MD.
 29. Sayre, R. M., Starr, M. P., Golden, A. M., Wergin, W. P., and Endo, B. Y. 1988. Comparison of *Pasteuria penetrans* from *Meloidogyne incognita* with a related mycelial and endospore-forming bacterial parasite from *Pratylenchus brachyurus*. *Proc. Helminthol. Soc. Wash.* 55:28-49.
 30. Sayre, R. M., Wergin, W. P., Nishizawa, T., and Starr, M. P. 1991. Light and electron microscopical study of a bacterial parasite from the cyst nematode, *Heterodera glycines*. *J. Helminthol. Soc. Wash.* 58:69-81.
 31. Sayre, R. M., Wergin, W. P., Schmidt, J. M., and Starr, M. P. 1991. *Pasteuria nishizawae* sp. nov., a mycelial and endospore-forming bacterium parasitic on cyst nematodes of the genera *Heterodera* and *Globodera*. *Res. Microbiol.* 142:551-564.
 32. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31-43.
 33. Starr, M. P., and Sayre, R. M. 1988. *Pasteuria thornei* sp. nov. and *Pasteuria penetrans* sensu stricto emend., mycelial and endospore-forming bacteria parasitic respectively, on plant-parasitic nematodes of the genera *Pratylenchus* and *Meloidogyne*. *Ann. Inst. Pasteur Microbiol.* 139:11-31.
 34. Stirling, G. R. 1984. Biological control of *Meloidogyne javanica* with *Bacillus penetrans*. *Phytopathology* 74:55-60.
 35. Stirling, G. R. 1985. Host specificity of *Pasteuria penetrans* within the genus *Meloidogyne*. *Nematologica* 31:203-209.
 36. Sturhan, D. 1988. New host and geographical records of nematode-parasitic bacteria of the *Pasteuria penetrans* group. *Nematologica* 34:350-356.
 37. Sturhan, D., Winkelheide, R., Sayre, R. M., and Wergin, W. P. 1994. Light and electron microscopical studies of the life cycle and developmental stages of a *Pasteuria* isolate parasitizing the pea cyst nematode, *Heterodera goettingiana*. *Fund. Appl. Nematol.* 17:29-42.
 38. Sussman, A. S., and Halvorson, H. O. 1966. *Spores: Their Dormancy and Germination*. Harper and Row, New York.
 39. van Iterson, W. 1984. *Inner Structures of Bacteria*. Van Nostrand Reinhold, New York.
 40. Zeikus, J. A., and Aldrich, H. C. 1975. Use of hot formaldehyde fixative in processing plant-parasitic nematodes for electron microscopy. *Stain Technol.* 50:219-225.