JOURNAL OF INVERTEBRATE PATHOLOGY 66, 52–59 (1995)

A New Microsporidium, *Nosema empoascae* n. sp., from *Empoasca fabae* (Harris) (Homoptera: Auchenorrhyncha: Cicadellidae)

XINZHI NI,*[,1] ELAINE A. BACKUS,* AND JOSEPH V. MADDOX†

*Department of Entomology, University of Missouri, Columbia, Missouri 65211; and †Illinois Natural History Survey, 607 East Peabody, Champaign, Illinois 61820

Received October 12, 1993; accepted February 28, 1995

A previously undescribed microsporidium is associated with both the cytoplasm and nucleus of *Empoasca fabae* tissues. We describe this microsporidium as a new species of *Nosema*, the first to be associated with an auchenorrhynchan insect. The rod-shaped fresh spores measured 5.07 × 1.66 μm, while stained spores were 4.18 × 1.48 μm. Spores had three to six coils of isofilar polar filament at the posterior end. The microsporidium infected four different tissues: the salivary glands, the anterior midgut, the Malpighian tubules, and the reproductive systems (both ovarioles and testes). Both vegetative forms and spores of the microsporidium were observed in all four tissues. We describe morphological characters of the microsporidium by both light and electron microscope studies and propose a possible developmental cycle.

KEY WORDS: Microsporidia, *Nosema*, ultrastructure, developmental cycle, *Empoasca*.

INTRODUCTION

Insects are considered one of the most common hosts for microsporidia, but with one exception, there is no report of any microsporidium associated with the Homoptera. Fantham and Porter (1958) reported a *Toxoglugea* sp., which was later identified as *Toxoglugea fanthami* Weiser, 1961 (Weiser, 1961; Sprague, 1977b), in the bean aphid, *Aphis rumicis* L. (Homoptera: Sternorrhyncha: Aphididae) from Canada.

There is no report of any microsporidium associated with auchenorrhynchan homopterans. During an examination of our laboratory colony of the potato leafhopper, *Empoasca fabae* (Harris), we found a microsporidium associated with the salivary glands, anterior midgut, the Malpighian tubules, and ovarioles and testes. We present herein the morphological characters and developmental cycle of this microsporidium and propose the creation of a new species of *Nosema*.

[1] Current address: Department of Entomology, Montana State University, Bozeman, MT 59717.

MATERIALS AND METHODS

Insect Colony

A microsporidium-infected laboratory colony of *E. fabae* was established from field-collected insects during the summers between 1984 and 1989, primarily 1987, from alfalfa fields near Columbia, Missouri and during 1989 near Urbana-Champaign, Illinois. Leafhoppers were reared on broad bean (*Vicia faba* L.) seedlings, under fluorescent lights in growth chambers, with photoperiod 16:8 (L:D) and temperature 26°C: 20°C (L:D). This microsporidium was transmitted transovarially. Horizontal transmission (i.e., oral transmission, venereal transmission) was never observed (Ni, 1993).

Light Microscope Observations

Adult leafhoppers sampled from the laboratory colony were squashed, smeared on glass coverslips, and examined with a compound microscope for presence of spores. One hundred twenty spores were measured under the light microscope using the wet-mount preparation described by Hazard *et al.* (1981). To investigate developmental stages of the microsporidium, infected insect tissues were smeared on glass coverslips, air-dried, fixed in absolute methanol for 10 min and stained in a 10% (v/v) aqueous solution of Giemsa stain (Sigma Chemical, St. Louis, MO) for approximately 30 min at room temperature. The stained specimens on the coverslips were then washed in running tap water (pH 7) for 2 min. After air-drying, the coverslips with specimens were mounted with Permount on clean slides.

Transmission Electron Microscope (TEM) Observations

Leafhoppers were dissected and tissues were identified under a stereomicroscope in a mixture of 1% (w/w) saline (NaCl) solution and 3% (w/w) glutaraldehyde. Because the leafhopper organs were small, they were

0022-2011/95 $12.00

embedded individually in 2% (w/w) warm agar, to avoid loss or damage to the tissues during the preparation. The tissues in agar blocks were fixed overnight in 3% glutaraldehyde with 0.1 M phosphate buffer (pH 7) at 4°C. Salivary glands, alimentary canal, the Malpighian tubules, and ovarioles were prepared, since preliminary findings suggested those tissues would be infected. After glutaraldehyde fixation, tissues were rinsed repeatedly in phosphate buffer for 30 min, postfixed in 1% (w/w) OsO_4 overnight at 4°C, rinsed again in the phosphate buffer for 30 min dehydrated in a standard acetone series, and then infiltrated and embedded in Epon 812 resin. Tissues were sectioned with a LKB 7800-045 Sapphatome knife on a LKB III microtome and stained with 0.5% (w/w) uranyl acetate followed by 1% (w/w) lead citrate. Sections were examined and photographed with a JEOL JEM-100B electron microscope in the Electron Microscopy Laboratory, College of Agriculture, Food and Natural Resources (University of Missouri, Columbia, MO) and a Zeiss 9S-2 electron microscope at the Insect Pathology Laboratory, Illinois Natural History Survey (Champaign, IL).

Tissue Specificity

One hundred 2-week-old adults were dissected in 1% (w/w) saline (NaCl) solution under a stereomicroscope. All leafhopper organs were identified according to the description of internal morphology by Berlin and Hibbs (1963), Helms (1968a,b), and Ammar (1985). While whole testis tissues were only prepared and examined in wet-mounted slides for fresh tissues under a compound light microscope, the four other types of most heavily infected tissue were prepared individually for transmission electron microscopy. Semithin sections of these four types of insect tissues were stained with toluidine blue and were examined and photographed under a compound light microscope.

RESULTS

Light Microscope Observations

Uninucleate and diplokaryotic meronts (Figs. 1A, 1B, and 1C) were present. Diplokaryotic meronts were more numerous than uninucleate meronts. Multiplication was by binary division of diplokaryotic cells (Figs. 1B and 1C). Binucleate sporonts (Fig. 1D) were typically smaller than meronts. Sporonts divided to form two sporoblasts (Fig. 1E) and immature spores (Fig. 1F).

The spores were rod-shaped. Fresh spore size averaged 5.30 ± 0.06 × 1.27 μm (n = 120; range: 4.44–6.35 in length and 1.27 uniform in width) under the compound microscope and averaged 5.07 ± 0.07 × 1.66 ± 0.02 μm (n = 30; range: 4.56–5.70 and 1.43–1.71) from micrographs, while, from microphotographs, the Giemsa-stained spores (Fig. 1G) averaged 4.18 ± 0.06 × 1.48 ± 0.02 μm (n = 30; range: 3.84–4.80 and 1.44–1.92). Spores and meronts of the microsporidium were frequently found in host cell nuclei in Giemsa-stained insect tissue smears (Fig. 1H)

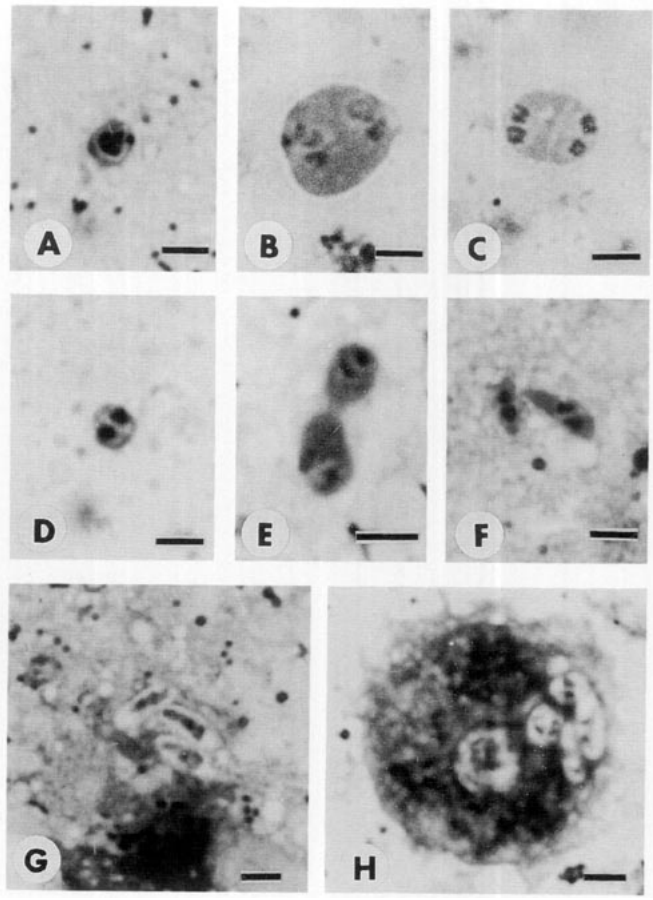

FIG. 1. Light micrographs of life stages (bars, 2.5 μm). (A) Uninucleate meront. (B) Merogonic plasmodium with two diplokarya. (C) Dividing merogonic plasmodium, giving rise to two diplokaryotic merozoites. (D) A single binucleate sporont. (E) Dividing sporont to form binucleate sporoblasts. (F) Two associated immature binucleate spores. (G) Giemsa-stained spores. (H) Spores and meronts of the microsporidium associated with a host cell nucleus.

TEM Observations

Meronts were observed within the host cell nuclei (Fig. 2A) and cytoplasm (Figs. 2B and 2C), but nuclear infections were comparatively rare. No dividing meront was observed in host cell nuclei. The plasmalemma of the meronts was surrounded by extracellular vesicles (microvillosities of Vávra, 1976). There were fewer extracellular vesicles surrounding meronts in nuclei (Fig. 2A) than in cytoplasm (Fig. 2C). Karyokinesis of meronts in the host cell cytoplasm was indicated by the presence of two centriolar plaques (Fig. 2B; arrows). Two daughter cells of meronts after cytokinesis were also surrounded by scattered extracellular vesicles (Fig. 2C). Three diplokaryotic meronts

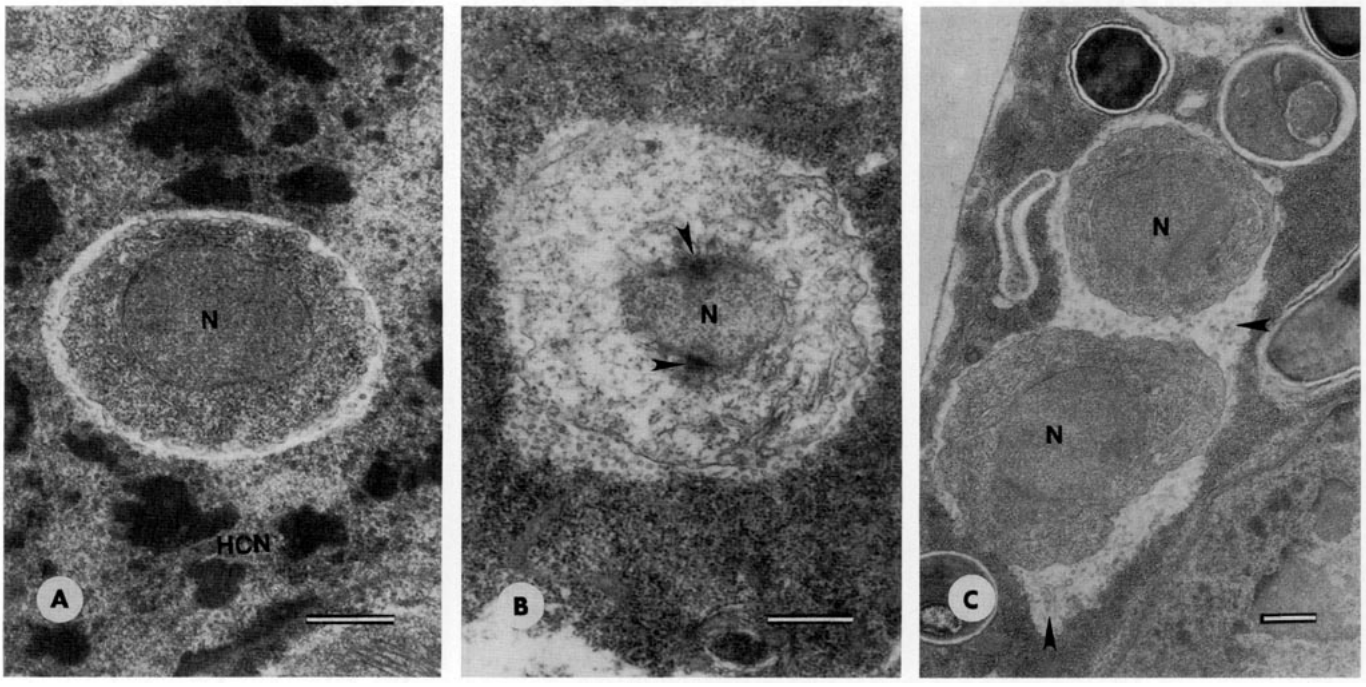

FIG. 2. Merogonic stages of the microsporidium (bars, 0.5 μm). (A) Meront with single nucleus (N) visible within a host cell nucleus (HCN). (B) Meront with a single nucleus visibly undergoing mitosis indicated by two centriolar plaques (arrows). (C) Two meronts surrounded by extracellular vesicles (arrows).

were surrounded by the extracellular vesicles (see arrow) in Fig. 3A. A thickening plasmalemma indicated the transition to sporogony (Fig. 3B; arrows). Sporonts further developed into sporoblasts, indicated by polar filament morphogenesis (Fig. 3C; arrows).

Sections of microsporidian spores are shown in Fig. 4. Spores were binucleate (Fig. 4B). The polar filament was isofilar with three to six coils (Fig. 4A). Other characters of spore ultrastructure are shown in Figs. 5A and 5B. These included endospore (0.25 μm) and exospore (0.01 μm), the anchoring disc of the polar filament, and two regions of polaroplast, the lamellar and vesicular regions, the manubroid part of the polar filament, and endoplasmic reticulum. Coils of the polar filament had three visible layers, and a membranous polar body was present at the posterior end of the spores (Fig. 5B). Fresh spores without any appendage were shown in the two light micrographs: Fig. 5C with phase contrast and Fig. 5D with Nomarski differential interference contrast.

Tissue Specificity

Microsporidian spores were present in low numbers throughout the hemocoel, but higher concentrations of spores occurred in four tissues: salivary glands, alimentary canal, the Malpighian tubules, and gonadal tissue of both female and male. Both vegetative forms and spores of the microsporidium were found primarily in cytoplasm of all four tissues. Extracellular vesicles were associated with vegetative forms of the microsporidium. In addition, both ovarioles and testes contained spores. The spores were often found in clusters in the germarium area of leafhopper ovarioles.

DISCUSSION

Identification

This is the first report of a microsporidium associated with the suborder Auchenorrhyncha of Homoptera, and the microsporidium from the potato leafhopper is different from *T. fanthami* from the bean aphid (Weiser, 1961). The spores of *T. fanthami* form within an interfacial envelope and are horseshoe-shaped (Sprague, 1977b).

Both light and electron microscopic studies of the microsporidium from the potato leafhopper revealed characteristics that are typical for the genus *Nosema* (Sprague, 1978; Larsson, 1988); most developmental stages are binucleate, and sporogony is apansporoblastic and disporoblastic. Vegetative reproduction appeared to be by binary fission, as evidenced by both light and transmission electron microscopy. The spores were rod-shaped, binucleate, with three to six coils of isofilar polar filament and polaroplast laminate—bipartite. We assign this microsporidium to the genus *Nosema*. Even though we realize that the genus *Nosema* probably contains many heterogeneous species, we believe that at this time the establishment of a new genus for this new species is not justified.

Although somewhat unusual, the occurrence of uni-

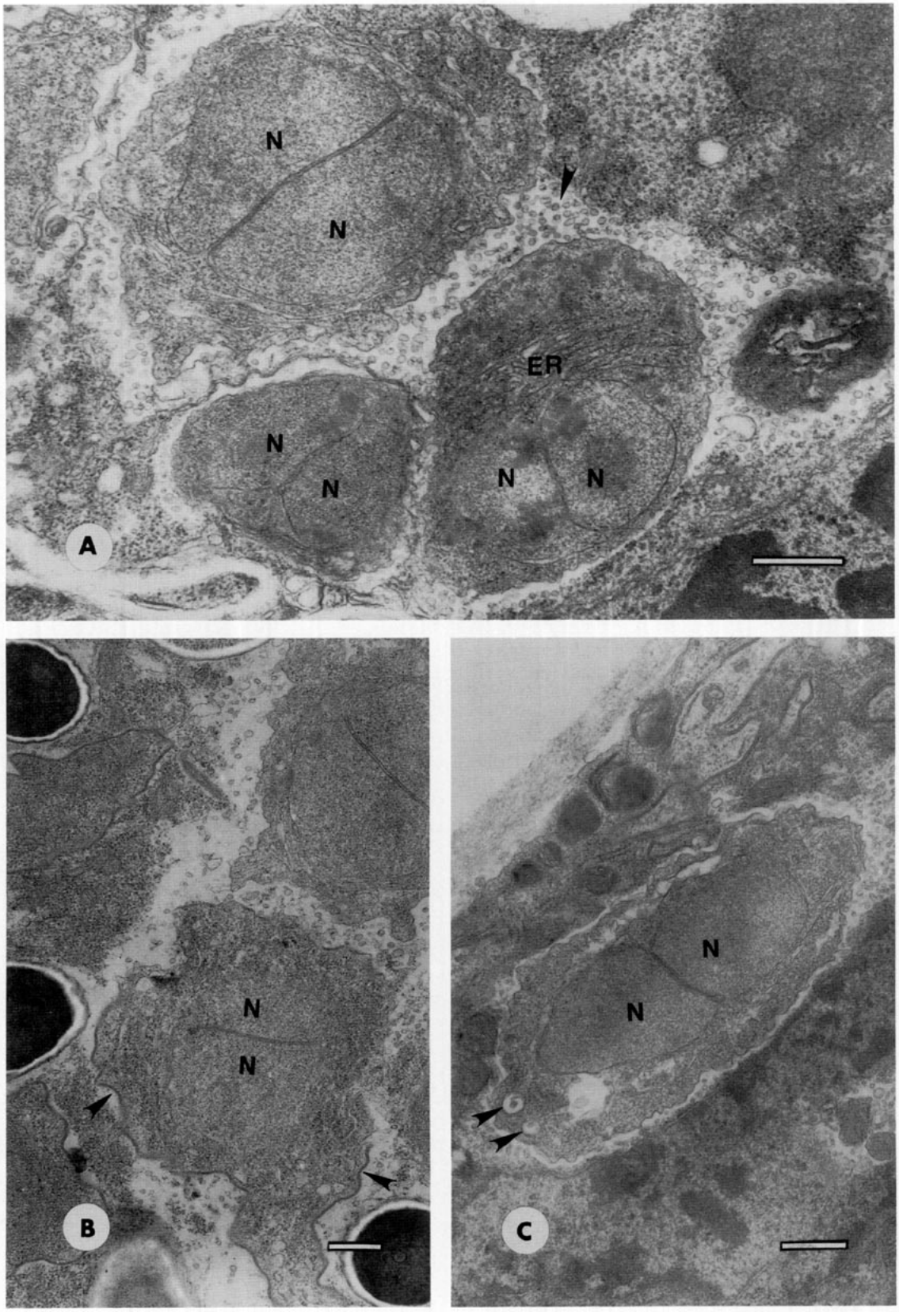

FIG. 3. Electron micrographs of further developmental stages of the microsporidium (bars, 0.5 μm). (A) Three binucleate meronts surrounded by extracellular vesicles (arrow). One of them has loosely arranged endoplasmic reticulum (ER) in cytoplasm. (B) Binucleate sporont with thickening plasmalemma (arrows). (C) Binucleate sporoblast with thickening plasmalemma and two coils of polar filament (arrows).

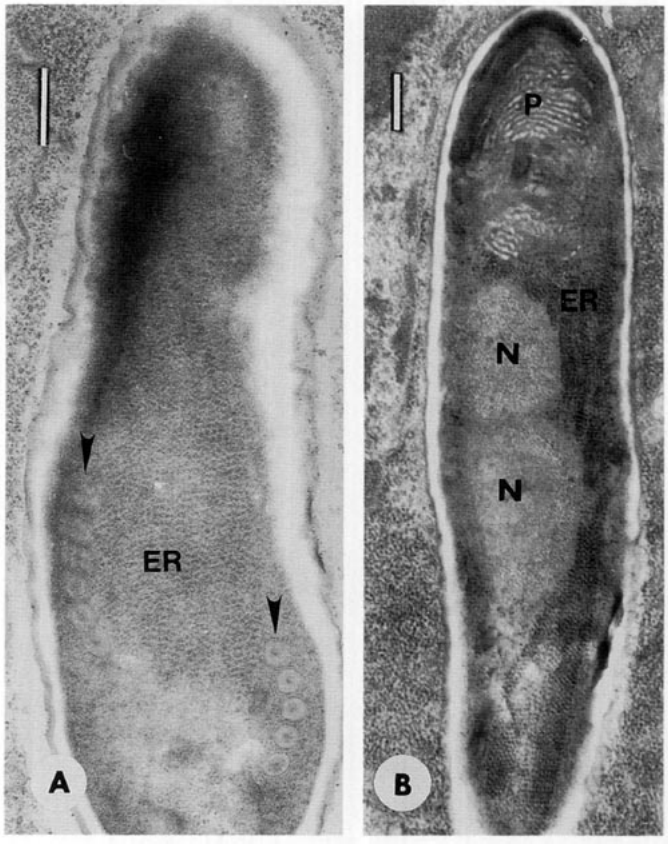

FIG. 4. Electron micrographs of nuclear configuration and polar filament of the microsporidian spores (bars, 0.25 μm). (A) Spore with five and six visible coils of polar filament in isofilar arrangement (arrows) and endoplasmic reticulum (ER). (B) Spore showing polaroplast (P), two nuclei (N), and endoplasmic reticulum.

nucleate meronts has been reported in previously described *Nosema* species, e.g., *Nosema algerae* Vávra and Undeen (Canning and Hulls, 1970; Brooks, 1988), *Nosema apicalis* Maddox and Webb (Maddox and Webb, 1983), *Nosema montanae* Wang, Streett, and Henry (Wang *et al.*, 1991), and *Nosema bombycis* Naegeli (Sprague *et al.*, 1992). In addition, variation of the number of observed coils of polar filament could be caused by three possible sources: the type of sectioned spores, the angle of section through the spores, and the maturity of the spores. Because we didn't observe any evidence for the existence of two types of spores in either light or electron microscope studies, we believe the variation of the coils of polar filament resulted from the angle of sectioning or spore maturity.

We assume that the microsporidium in the potato leafhopper is specific to Homoptera and probably to the potato leafhopper, *E. fabae*. This microsporidium is unlikely to infect hosts in other insect orders and is easily distinguished from the only other microsporidium (*T. fanthami*) described from a homopteran host. The relative rarity of microsporidia associated with homopteran insects may be related to the specific piercing–sucking feeding behavior. On crop plants, piercing–sucking insects may be less likely to ingest microsporidian spores than chewing insects (Ni, 1993).

Developmental Cycle of This Microsporidium

Based on all the findings of both light and electron microscope observations, a possible developmental cycle of this microsporidium is proposed. The merogonic phase includes uninucleate and binucleate cells. Multiplication is by nuclear binary fission and cytoplasmic division. Each sporont transforms into two sporoblasts, and eventually two spores. Although uninucleate meronts were observed, their place in the developmental cycle is uncertain until further developmental studies are conducted.

Tissue Specificity

Although the extracellular vesicles surrounding the vegetative forms might suggest a host–parasite interaction, neither pathognomonic symptoms of the infection nor melanization were exhibited by the host.

Most other microsporidia cause more detrimental effects on host tissue. *N. bombycis* in *Bombyx mori* L. is a classic example of microsporidiosis showing hypertrophism. Likewise, *Ovavesicula popilliae* Andreadis and Hanula causes hypertrophied cells and melanization in both adults and larvae of the Japanese bettle, *Popillia japonica* Newman. It also causes an inflammatory response in larvae. Infected Malpighian tubules in Japanese beetle larvae are hypertrophied and white in color, while the uninfected tubules are normal and transparent (Hanula and Andreadis, 1990). There are many additional examples of microsporidia that produce pathological effects in their hosts.

In the potato leafhopper, we found extracellular vesicles surrounding the meronts and sporonts. To a lesser extent, these vesicles surrounded the sporoblasts and spores. The low density of the vesicles near sporoblasts and spores could be the residue from previous meronts and sporonts. These extracellular vesicles appeared to be secreted by the microsporidium. Extracellular vesicles can substantially increase the surface area of the meront membrane with their vesicular or tubular projections into the host cells (Vávra, 1976).

Because the tissues used for this study were from transovarially infected insects, the sequence of tissue infection cannot be completely confirmed. However, because transovarial transmission does occur (Ni, 1993), a histological time-course study of the infected insects from embryo differentiation to its adulthood would be necessary to determine which tissue, and even which portion, is infected first.

Systematics

Type host. Nymphs and adults of *E. fabae* (Harris) (Homoptera: Auchenorrhyncha: Cicadellidae).

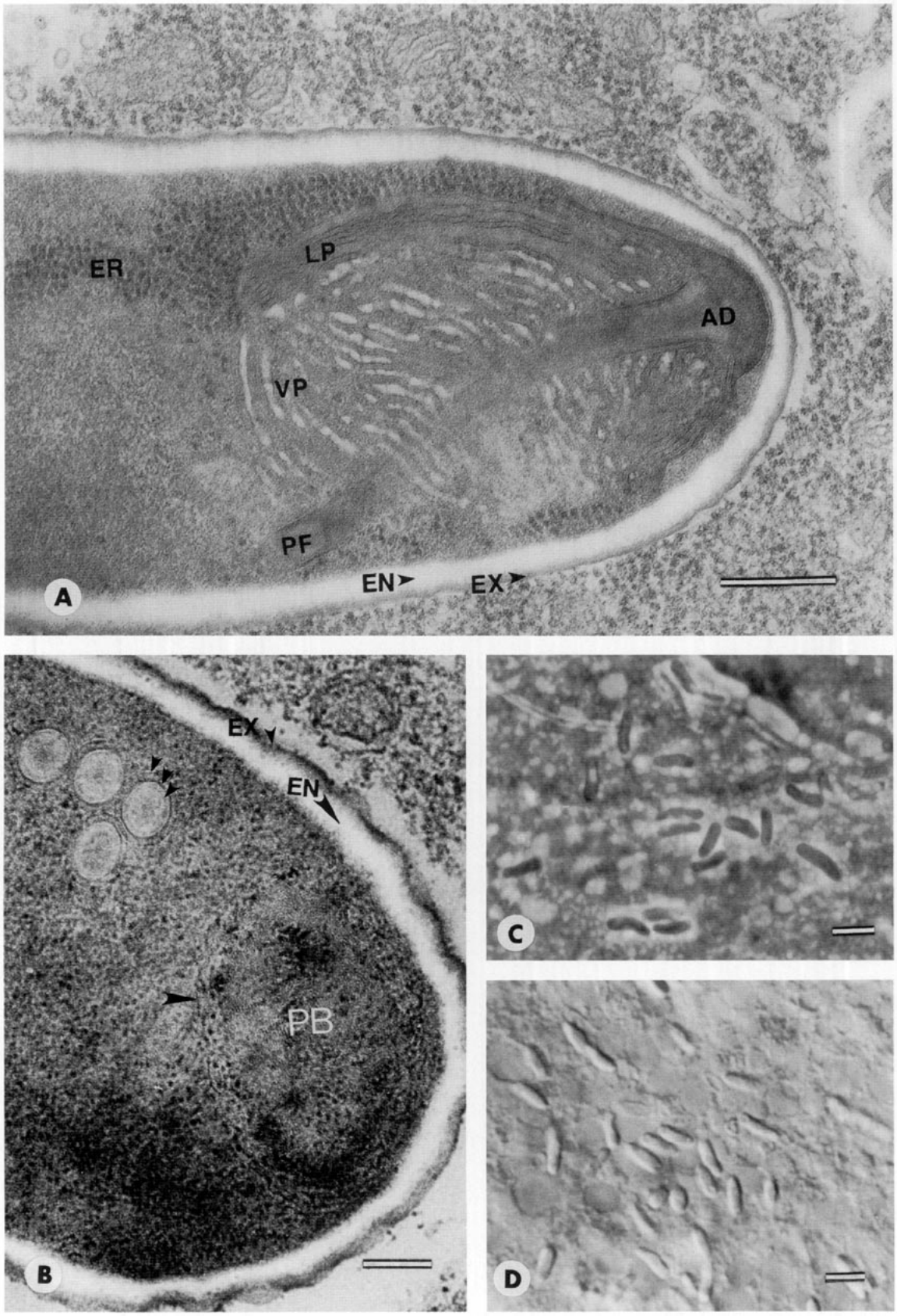

FIG. 5. Micrographs of the spore structure. (A and B) Electron micrographs showing the spore ultrastructure; (C and D) light micrographs of fresh mature spores. (A) Anterior region of a spore with endospore (EN) and exospore (EX), anchoring disc (AD), two regions (lamellar (LP) and vesicular (VP)) of polaroplast, polar filament (PF), and endoplasmic reticulum (ER) (bar, 0.25 μm). (B) Posterior region of a spore showing three layers visible of the sectioned polar filament (arrows), polar body (PB), and endospore and exospore (bar, 0.1 μm). Both light micrographs with phase contrast (C) and with Nomarski differential interference contrast (D) showing fresh spores without appendage (bars, 5 μm).

Transmission. *Per os* and venereal transmission, which could not be demonstrated, is unlikely to occur. Only transovarial transmission was confirmed (Ni, 1993).

Site of infection. Salivary glands, anterior midgut, the Malpighian tubules, ovarioles, and testes are infected with this microsporidium; however, the sequence of tissue infection could not be determined.

Interface. Meronts and sporonts of the microsporidium are surrounded by extracellular vesicles, probably of parasite origin. Extracellular vesicles also surround sporoblasts and spores but they are less abundant than for meronts.

Other parasite–host cell relations. The parasites occur in both the host cell nucleus and cytoplasm.

Haplophase. Unknown.

Meront and merogony. Meronts are either uni- or binucleate and spherical in shape. Merogony is by binary fission of diplokaryotic meronts.

Transition to sporogony. Marked by thickening of plasmalemma.

Sporont and sporogony. Sporonts are binucleate and fusiform. Sporogony ends with disporoblasts.

Spore. Rod-shaped in form. Fresh spores are $5.07 \pm 0.07 \times 1.66 \pm 0.02$ μm, while Giemsa-stained spores are $4.18 \pm 0.06 \times 1.48 \pm 0.02$ μm. Spore surface is smooth and without appendage. Spores are binucleate and have three to six coils of isofilar polar filament. The diameter of the coils of polar filament is approximately 0.10 μm with three layers visible. An anchoring disc is present. The polaroplast has two regions, an anterior lamellar region and a posterior vesicular region. A membranous polar (or posterior) body is also present.

Type locality. Champaign, Illinois, U.S.A.

Deposition of type specimens. Slides containing this microsporidium were designated as syntypes and deposited in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC, with USNMN Nos. 43222 and 43223. Paratype slides were in collection of Dr. J. V. Maddox, Illinois Natural History Survey, Champaign, Illinois.

Remarks. This microsporidium has all the characters of *Nosema*. According to its host *E. fabae*, we assign it the name *Nosema empoascae* n. sp., belonging to Subkingdom Protista, Phylum Microspora, Class Dihaplophasea, Order Dissociodihaplophasida, and Family Nosematidae (Sprague, 1977a; Levine *et al.*, 1980; Sprague *et al.*, 1992). This is the only microsporidian species reported from an auchenorrhynchan insect host—*E. fabae* (Harris).

ACKNOWLEDGMENTS

We thank Drs. Carlo M. Ignoffo, Jean R. Adams, and Arthur McIntosh for their advice on earlier work. We are greatly indebted to Drs. Jerry A. White and Merton F. Brown, and Mr. Raymond Faup, for their assistance on TEM work, and Mr. David Pinkerton for his assistance with photographic work. We also greatly appreciate three anonymous reviewers for their suggestions on the earlier draft of the manuscript. This is a contribution of the Missouri Agricultural Experiment Station, paper No. 12,023.

REFERENCES

Ammar, E. D. 1985. Internal morphology and ultrastructure of leafhoppers and planthoppers. *In* "The Leafhoppers and Planthoppers" (L. R. Nault and J. G. Rodriguez, Eds.), pp. 127–162. Wiley, New York.

Berlin, L. C., and Hibbs, T. T. 1963. Digestive system morphology and salivary enzymes of the potato leafhopper. *Iowa Acad. Sci.* 70, 527–540.

Brooks, W. M. 1988. Entomogenous Protozoa. *In* "CRC Handbook of Natural Pesticides," Vol. 5, "Microbial Insecticides," Part A: "Entomogenous Protozoa and Fungi" (C. M. Ignoffo, Ed.), pp. 1–149. CRC Press, Boca Raton, Florida.

Canning, E. U., and Hulls, R. H. 1970. A microsporidian infection of *Anopheles gambiae* Giles, from Tazania, interpretation of its mode of transmission and notes on *Nosema* infections in mosquitoes. *J. Protozool.* 17, 531–539.

Fantham, H. B., and Porter, A. 1958. Some pathogenic bacteriform Microsporidia from Crustacea and Insecta. *Proc. Zool. Soc. London* 130, 153–168.

Hanula, J. L., and T. G. Andreadis. 1990. Comparative histopathology of infection by *Ovavesicula popilliae* (Microsporida: Pleistophoridae) in larval and adult Japanese beetles, *Popillia japonica*. *Entomophaga* 35, 247–255.

Hazard, E. I., Ellis, E. A., and Joslyn, D. J. 1981. Identification of Microsporidia. *In* "Microbial Control of Pests and Plant Diseases 1970–1980" (H. D. Burges, Ed.), pp. 163–182. Academic Press, New York.

Helms, T. J. 1968a. Postembryonic reproductive-systems development in *Empoasca fabae*. *Ann. Entomol. Soc. Am.* 61, 316–332.

Helms, T. J. 1968b. Anatomy of alimentary canal of *Empoasca fabae*. *Ann. Entomol. Soc. Am.* 61, 1604–1606.

Larsson, J. I. R. 1988. Identification of microsporidian genera (Protozoa, Microspora)—A guide with comments on the taxonomy. *Arch. Protistenkd.* 136, 1–37.

Levine, N. D., Corliss, J. O., Cox, F. E. G., Deroux, G., Grain, J., Honigberg, B. M., Leedale, G. F., Loeblich, A. R. III, Lom, J., Lynn, D., Merinfield, E. G., Page, F. C., Poljansky, G., Sprague, V., Vávra, J., and Wallace, F. G. (The committee on systematics and evolution of the society of protozoologists). 1980. A newly revised classification of the Protozoa. *J. Protozool.* 27, 37–58.

Maddox, J. V., and Webb, D. W. 1983. A new species of *Nosema* from *Hylobittacus apicalis* (Insecta: Mecoptera: Bittacidae). *J. Invertebr. Pathol.* 42, 207–220.

Ni, X. 1993. A new microsporidium associated with the potato leafhopper, *Empoasca fabae* (Harris) (Homoptera: Cicadellidae). Ph.D. dissertation, University of Missouri-Columbia.

Sprague, V. 1977a. Classification and phylogeny of the Microsporidia. *In* "Comparative Pathobiology," Vol. 2, "Systematics of the Microsporidia" (L. A. Bulla and T. C. Cheng, Eds.), pp. 1–30. Plenum, New York.

Sprague, V. 1977b. Annotated list of species. *In* "Comparative Pathobiology" Vol. 2, "Systematics of the Microsporidia" (L. A. Bulla and T. C. Cheng, Eds.), p. 161. Plenum, New York.

Sprague, V. 1978. Characterization and composition of the genus *Nosema*. *Misc. Pub. Entomol. Soc. Am.* **11**, 5–16.

Sprague, V., Becnel, J. J., and Hazard, E. I. 1992. Taxonomy of Phylum Microspora. *Crit. Rev. Microbiol.* **18**, 285–395.

Vávra, J. 1976. Structure of the microsporidia. *In* "Comparative Pathobiology," Vol. 1, "Biology of the Microsporidia" (L. A. Bulla and T. C. Cheng, Eds.), pp. 1–85. Plenum, New York.

Wang, L.-Y., Streett, D. A., and Henry, J. E. 1991. *Nosema montanae* n.sp. (Microsporida: Nosematidae), a parasite from the grasshopper *Melanoplus packardii* (Orthoptera: Acrididae). *J. Invertebr. Pathol.* 58, 211–218.

Weiser, J. 1961. Die Mikrosporidien als Parasiten der Insekten. *Monogr. Angew. Ent.* **17**, 1–149.