JOURNAL OF INVERTEBRATE PATHOLOGY 65, 230–236 (1995)

Identification and Isolation of Dimorphic Spores from *Nosema furnacalis* (Microspora: Nosematidae)[1]

HIDETOSHI IWANO[2] AND TIMOTHY J. KURTTI

Department of Entomology, University of Minnesota, St. Paul, Minnesota 55108

Received March 23, 1994; accepted January 10, 1995

Spore morphology of *Nosema furnacalis* and infectivity for a clone of the *Helicoverpa zea* cell line were examined by both light and electron microscopy. Dimorphic spores were found in cultured cells infected with *N. furnacalis*. One type having a few coils of the polar filament (FC spore) germinated spontaneously inside or outside the cell after formation. The other type had many coils of the polar filament (MC spore). The FC spores possessed four coils of the polar filament and MC spores had nine coils on the average. This spore dimorphism was demonstrated to be a characteristic not limited to *N. bombycis*. With continued passage, the proportion of FC spores in cultured cells sharply increased. Spores isolated after passage 85 to 90 were used to infect cultured cells without prior priming at an alkaline pH. In cultures inoculated at a rate of 10 spores/cell, the prevalence of infected cells was 45% 24 hr postinoculation and 98% by 96 hr postinoculation. © 1995 Academic Press, Inc.

KEY WORDS: ***Nosema furnacalis;*** **Nosematidae; microsporidia; spore dimorphism; spore germination; *in vitro* culture.**

INTRODUCTION

Nosema furnacalis (Wen and Sun) was described as a parasite of the Asian corn borer, *Ostrinia furnacalis* (Guenee) by Wen and Sun (1988), who observed that this microsporidium infected a wide range of tissues and insects. A culture of *N. furnacalis* has been maintained for over 70 serial transfers in a cell line of a *Helicoverpa zea* (Boddie) (Kurtti *et al.*, 1994), but the way in which *N. furnacalis* is maintained within cultured cells over many transfers has not been elucidated. Spontaneous intracellular germination of spores, which developed early after inoculation of the host insect, has been observed in *Nosema algerae* Vavra and Undeen (Vavra and Undeen, 1970; Avery and Anthony, 1983) and *Nosema apis* Zander (Fries *et al.*, 1992). In addition, forms resembling sporoplasms have been observed in cultured cells (Ishihara, 1969; Kurtti *et al.*, 1983) but their origin was not determined. Iwano and Ishihara (1991a,b) recently demonstrated that *Nosema bombycis* Naegeli, the type species in the genus *Nosema*, produced two morphologically distinct spores *in vivo* and *in vitro*. One type (FC spore) had few coils of the polar filament germinated intracellularly and the discharged sporoplasm invaded neighboring cells to spread *N. bombycis* among cultured cells. The other type of spore (MC spore), common in insects with older infections, had many coils of the polar filament and thicker endospore and was refractory to spontaneous germination.

This paper describes the differentiation of two types of spores of *N. furnacalis in vitro* and demonstrates the potential of spores to disseminate the microsporidium by spontaneous intracellular and extracellular germination. Taken together, we interpret these findings to indicate that this form of spore dimorphism is a determinant of microsporidian cross-infectivity between cells and a basic feature of the genus *Nosema*.

MATERIALS AND METHODS

Propagation of N. furnacalis in H. zea

Helicoverpa zea larvae were used to produce spores for comparison with those harvested from cell cultures. Larvae were reared on a modification of the Vanderzant–Adkisson diet (Chippendale *et al.*, 1965) and infected with *Nosema furnacalis* as stated by Kurtti *et al.* (1994). Neonate larvae were placed on food surfaces contaminated with spores ($1–2 \times 10^5$/50 μl: 260 spores/mm^2) and incubated at 27°C until just before pupation. Infected larvae were homogenized and the spores purified by density gradient centrifugation using 70% Percoll as described by Kurtti *et al.* (1990).

Propagation of N. furnacalis in Vitro

Cultures of *N. furnacalis*, previously isolated by Kurtti *et al.* (1994), were propagated in a clone (G5) of

[1] This is Publication No. 21,566, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Present address: Faculty of Agriculture and Veterinary Medicine, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252, Japan.

0022-2011/95 $5.00

the *H. zea* cell line BCIRLHZAM1 (McIntosh and Ignoffo, 1983). Cultures were maintained at 31°C in L15B medium (Munderloh and Kurtti, 1989) containing 80 mM glucose and supplemented with 3-[*N*-morpholino]propane-sulfonic acid (MOPS, 25 mM, pH 7.5), 0.1% of bovine lipoprotein concentrate (ICN, Levine, CA), heat-inactivated fetal bovine serum (5%), and gentamycin (50 μg/ml). *Nosema furnacalis* was subcultured when the percentage of infected cells reached 70 to 90%. Transfers involved mixing infected cells (5×10^5) with uninfected cells (2.5×10^5) in 10 ml of fresh medium. Infected cultured cells, frozen and stored in liquid nitrogen after 2 and 14 passages of subculture, were regenerated to compare the extent of spore development with that maintained for 85 to 90 passages in continuous culture.

Effect of Continuous Passage on Infectivity

Spores, recovered after 85 to 90 passages in culture, were tested for their ability to infect cells without prior activation in a solution having an alkaline pH. Infected cultures (30 ml) were harvested by centrifugation (670*g*, 10 min) and the pellet was resuspended in sterile water for storage (3 days, 4°C). Spores released from the lysed cells were purified using Percoll gradient centrifugation (Sato and Watanabe, 1980). The lysate was centrifuged (670*g*, 10 min) and the resuspended pellet (in 0.2 ml water) layered onto the surface of 100% Percoll 2.8 ml (neutralized with concentrated HCl). After ultracentrifugation (39,000*g*, 40 min) the supernatant fluid was discarded and the layer of spores on the Percoll shelf washed in sterile water. Purified spore suspensions were diluted in complete L15B medium (1.4×10^7 and 1.4×10^8 spores/ml) and 1-ml aliquots inoculated into uninfected *H. zea* cultures (1.8×10^7 cells/ml). Cultures were incubated at 31°C and sampled daily for light microscopy. Samples were centrifuged onto glass slides, fixed, and stained (see below) to determine the proportion of infected cells. Prevalence of infected cells was determined for 1000 cells in every three replicate slides using light microscopy.

Light and Electron Microscopy

Light and electron microscopy were used to compare spores developed *in vitro* with those from infected larvae of the European corn borer, *Ostrinia nubilalis*. Spores were examined in cultures after passages 2 and 14 (both 7 days after subculture), after passage 81 (4 days after subculture), and after passage 91 (5 days after subculture). Six neonate larvae of *O. nubilalis* were placed on Vanderzant–Adkisson diet coated with spores (1×10^5 spores/larva) and incubated at 29°C. Nineteen days after inoculation, infected silk glands were dissected out of last instar larvae to examine MC spores formed *in vivo* for comparison with the stages found in cultured cells.

For light microscopy, cell suspensions were centrifuged onto glass slides using a cytospin centrifuge (Shandon Southern Instruments, Sewickley, PA) and dissected silk glands were smeared on slides. Cell spreads and silk gland smears were fixed in methanol and stained using Giemsa stain (8%, pH 6.5 in Sørenson buffer).

For electron microscopy, infected culture cells and silk glands were fixed overnight with glutaraldehyde (2.5%, 0.05 M sodium cacodylate, pH 7.4, 5°C). Following a 0.05 M sodium cacodylate (pH 7.4) wash, they were postfixed with osmium tetroxide (1%, 0.1 M sodium cacodylate, 5°C) for 90 min, dehydrated through an ethanol series, and then embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut with a diamond knife mounted on an ultramicrotome (RMC, MT-7000), stained with uranyl acetate and lead mixture (Takagi *et al.*, 1990), and examined with an electron microscope (Phillips, CX12) operated at 60 kV. Longitudinal sections of spores (n = 125) were used to count the number of polar filament coils.

RESULTS

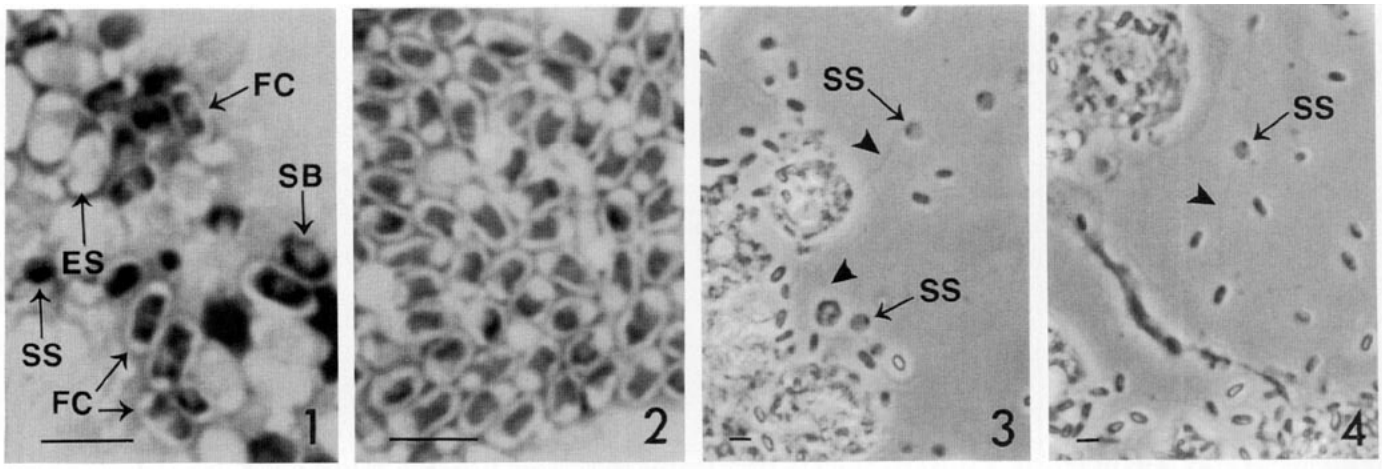
Comparison of Spores from Cultured Cells with Those from Silk Glands

Giemsa stain preparation. Spores produced after 81 transfers with HZAM1 cultured cells were variform (ellipsoidal or elliptic ovoidal). Two large nuclei occupied the inside of spores and were distinguished from the cytoplasm in Giemsa-stained preparations. The nuclei and cytoplasm of sporoblasts could not be discriminated. Empty spores and sporoplasms were seen together on the same slide preparation (Fig. 1). Spores recovered from silk glands of *O. nubilalis* infected with *N. furnacalis* were ovoidal to ellipsoidal. The spore shape and size were more uniform than those of cultured cells (Fig. 2). Empty spores and sporoplasms were rarely seen in smears of heavily infected silk glands of last instar larvae.

Transmission electron microscopy. Spores obtained from cell cultures intra- or extracellularly generally possessed polar filaments having three or four coils and had a depression at the posterior end (Figs. 5 and 6). This depression was presumed to be the collapsed posterior vacuole. Spores isolated from silk glands showed nine sections of the polar filament coils and no depression at the posterior end (Fig. 7). Both spore types were observed within a single infected cultured cell after 14 passages (Fig. 8).

Intra- and Extracellular Spore Germination

We observed with phase contrast microscopy the extrusion of polar filaments from a cultured cell with



FIGS. 1–4. Light micrographs of *Nosema furnacalis* as seen in Giemsa-stained preparations (1–2) and with phase-contrast optics (3–4). Bar = 5 μm. FIG. 1. *Helicoverpa zea*-cultured cell with sporoblast (SB), few-coil (FC) type spore, empty spore (ES), and sporoplasm (SS). Passage 81 (4 days after subculture). FIG. 2. Silk gland of infected *O. nubilalis* larva filled with numerous many-coil (MC) type spores. *In vivo* 19 days after inoculation. FIG. 3. Extrusion of polar filaments (arrowheads) with sporoplasms (SS) at their tips out of an infected cultured cell. Passage 91 (5 days after subculture). FIG. 4. Extracellular germination of few-coil type spore outside of cultured cell. Arrowhead, polar filament; SS, sporoplasm. Passage 91 (5 days after subculture).

sporoplasms at their tips (Fig. 3) and also sporoplasms discharged from floating spores in the culture medium without apparent stimulation (Fig. 4). Electron micrographs also confirmed the extrusion of the polar filament from spores within host cells (Figs. 9–11) and also extracellular spores liberated from cultured cell into the culture medium (Fig. 12). We recognized a sporoplasm just after its inoculation into a cultured cell after 81 transfers (Fig. 13).

Number of Coils of Polar Filament of Spores from Cultured Cells and Infected Silk Glands

The proportion of each spore type depended on the host cell and number of passages in culture (Fig. 14). The distribution pattern of polar filament coils of spores after passage 2 after inoculation exhibited two peaks, one having five and the other nine coils. The proportion of few-coil type spores (FC spore) was 50% while that of many-coil type spores (MC spore) was also 50%.

After passage 14, the number of FC spores increased considerably, while the number of MC spores decreased abruptly. The distribution pattern of coils showed a large peak at four coils and a small peak at nine coils. Spores examined after passage 81 showed only one peak at four coils. Thus, with subculture repeated, the FC spores became predominant. In contrast, most spores obtained from infected silk glands had nine sections of polar filament coil, and spores having six or less polar filament coils were not found.

Infection of Cultured Cells with Spores Recovered from Cultures

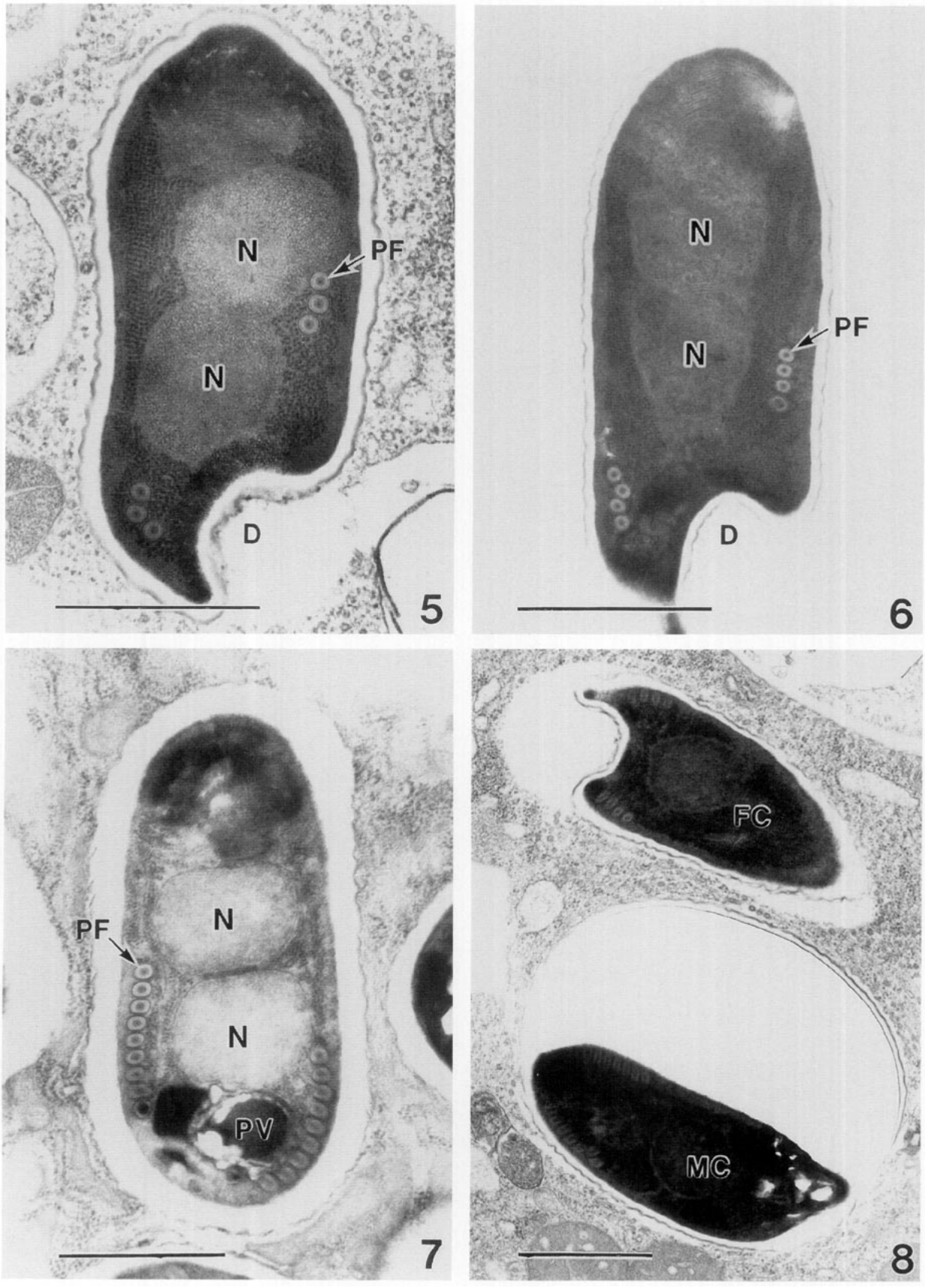
Spores were recovered after 85 to 90 subcultures from cultured cells and medium using Percoll gradient centrifugation. Microscopical observation showed that the band close to the bottom consisted of less refractile spores. Electron microscopical observations also showed that their endospore was relatively thin and spores had few coils of polar filament (not shown).

Those spores, when inoculated directly into HZAM1 cultures, successfully infected cells (Fig. 15). The prevalence of infection 24 hr postinoculation (p.i.) was high (7.9 ± 0.7%) in cultures inoculated at the rate of 1 spore/cell and reached 83.0 ± 4.2% by 72 hr p.i. Cultures inoculated with the higher dosage, 10 spores/cell, had infection prevalences of 45.0 ± 3.5 and 97.7 ± 0.2% at 24 and 72 hr p.i., respectively. With time after inoculation, the infection rates rapidly increased along equivalent proportional curves, reaching 96.4 to 98.4 ± 0.4% by 96 hr p.i. Thereafter they remained at 98%.

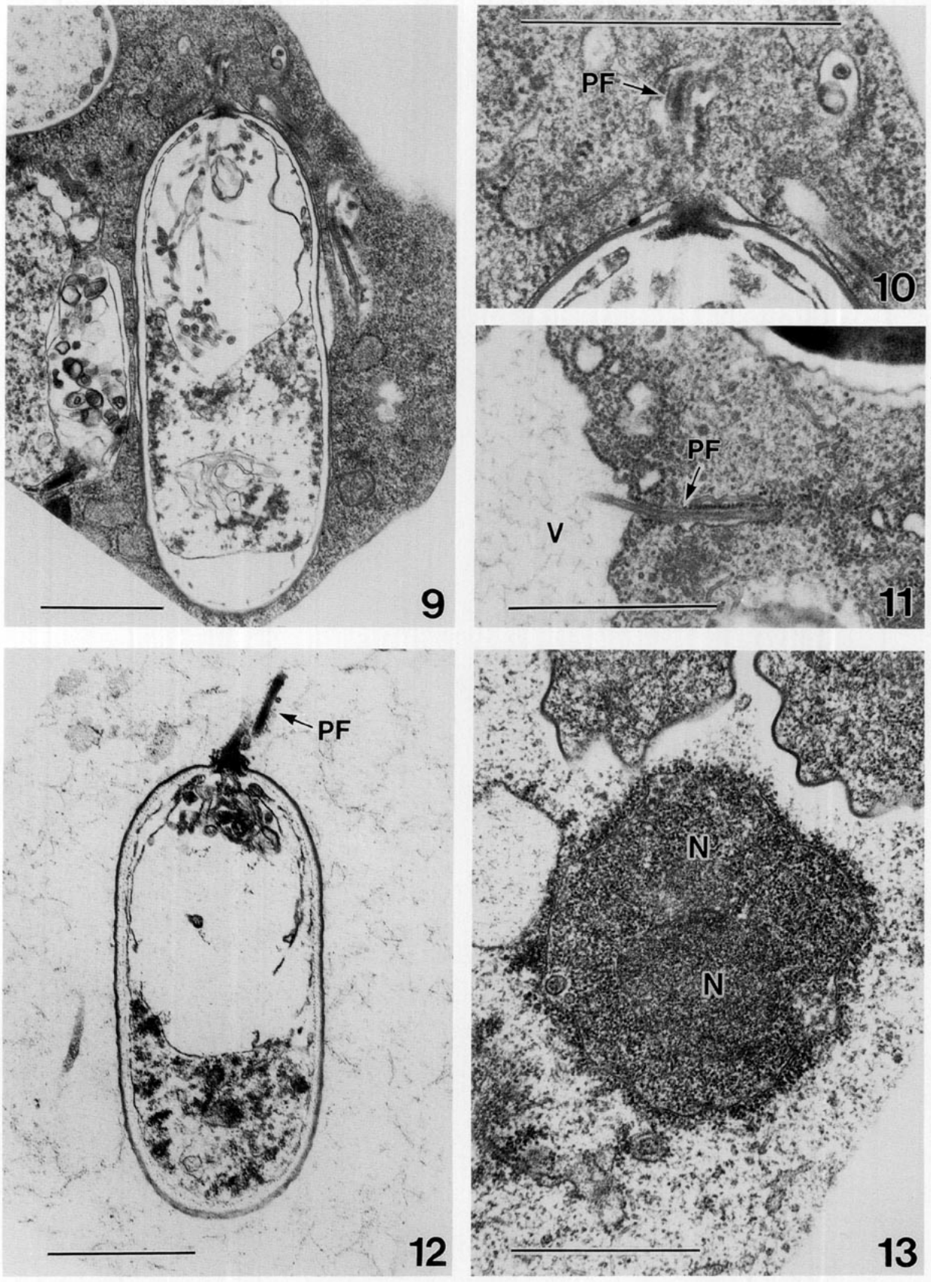
DISCUSSION

FC spores of *N. furnacalis* were isolated from HZAM1 cell cultures. MC spores were also isolated from both HZAM1 cell cultures and infected larvae. The morphological features of FC spore of *N. furnacalis* isolated from HZAM1 cell cultures closely resembled those of *N. bombycis* (Iwano and Ishihara, 1991a,b,c) or *Nosema* sp. (NSD) from *Spodoptera depravata* (Iwano *et al.*, 1994). The present results indicate that FC spores of the genus *Nosema* share common morphological characteristics, such as three to five sections of the polar filament coils, a depression at the posterior end, and relatively thin endospore, and are more variable in size. The morphological features of the MC spores confirm a previous report by Wen and Sun (1988), which does not however describe the formation of the FC spores.

FC spores of *N. bombycis* appeared at early stages of



FIGS. 5–8. Transmission electron micrographs of *Nosema furnacalis* spores. Bar = 1 μm. **FIG. 5.** Few-coil type spore in cultured cell having three coils of polar filament (PF), nuclei in diplokaryotic arrangement (N), and a depression (D) at the posterior end. Passage 81 (4 days after subculture). **FIG. 6.** Few-coil type spore outside of cultured cell having four coils of polar filament (PF), two nuclei in diplokaryotic arrangement (N), and a depression (D) at the posterior region. Passage 81 (4 days after subculture). **FIG. 7.** Many-coil type spore in a cell of infected larva showing nine coils of the polar filament (PF), posterior vacuole (PV), and diplokaryotic nuclei (N). Note that there is no depression at the posterior end. *In vivo* 19 days after inoculation. **FIG. 8.** Few-coil type spore (FC) and many-coil type spore (MC) together within a cultured cell. Passage 14 (7 days after subculture).



FIGS. 9–13. Transmission electron micrographs showing germination of *Nosema furnacalis* spores inside cultured cell. Bar = 1 μm. **FIG. 9.** Intracellular germination of few-coil type spore in cultured cell. Passage 14 (7 days after subculture). **FIG. 10.** Higher magnification of germination of few-coil type spore from Fig. 9 showing the extruded polar filament (PF). Passage 14 (7 days after subculture). **FIG. 11.** Polar filament (PF) reaching to the vacuole (V) through host cell cytoplasm. Passage 14 (7 days after subculture). **FIG. 12.** Extracellular germination of few-coil type spore outside of cultured cell. PF, polar filament. Passage 81 (4 days after subculture). **FIG. 13.** Sporoplasm just after invasion of cultured cell. N, nucleus. Passage 81 (4 days after subculture).

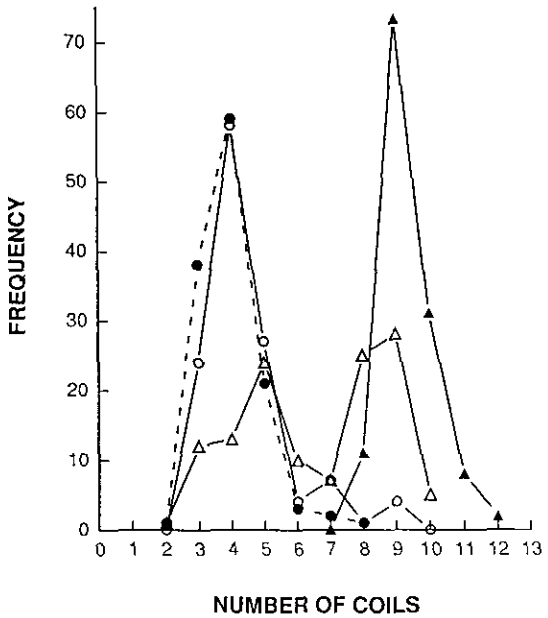


FIG. 14. Distribution pattern of *Nosema furnacalis* spores classified by number of polar filament coils, $n = 125$. (△–△) Passage 2 (7 days after subculture). (○–○) Passage 14 (7 days after subculture). (●-●) Passage 81 (4 days after subculture). (▲–▲) *In vivo* 19 days after inoculation.

infection in host insect cells (Iwano and Ishihara, 1991b) and germinated immediately after formation to discharge sporoplasms (Iwano and Ishihara, 1989, 1991b,c). It is understandable that this type of spore

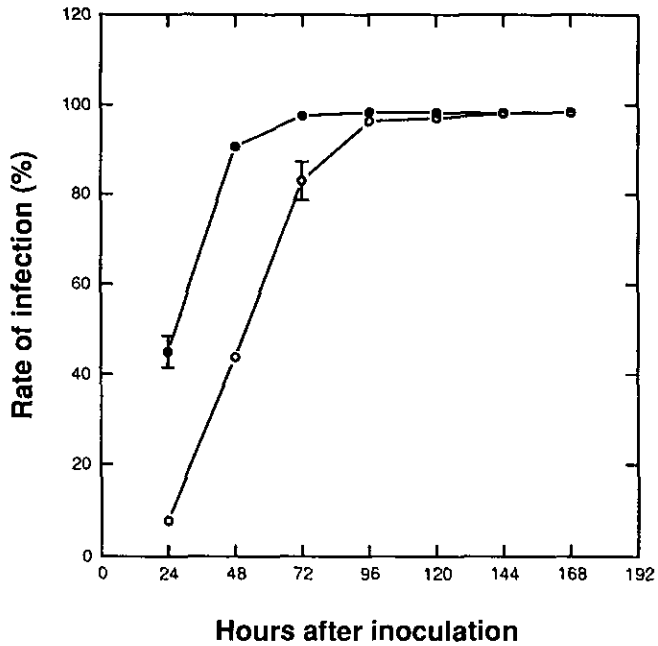


FIG. 15. Infectivity of purified and nonprimed FC spores for cultured cells. (○) 1.4×10^7 spores/ml (1 spore/cell). (●) 1.4×10^8 spores/ml (10 spores/cell). Each point is the mean ± SD of three replicate slides.

was not found in *N. furnacalis* (Wen and Sun, 1988), *N. acridophagus* (Street and Henry, 1993), and *N. scripta* (Bauer and Pankratz, 1993) since their observations were conducted on chronically infected larvae. We interpret the intracellular spore germination by spores of *N. algerae* (Avery and Anthony, 1983) to be the germination of FC spores. Odindo and Jura (1992) found that the spores of *N. marucae* discharged the sporoplasm in the fat body of legume pod borer larvae. These reports suggest that microsporidia of the genus *Nosema* generally produce a spore which plays a role in transmitting parasites from cell to cell by *in situ* germination.

We have demonstrated that *N. furnacalis* also produced both FC spores and MC spores and thus showed the spore dimorphism found with *N. bombycis*. Recently, Sprague *et al.* (1992) briefly described the functions of two types of spores in *N. bombycis*. De Graaf *et al.* (1994) reported observing spore dimorphism in *Nosema apis,* but their new spores resembled incomplete sporogenesis. It is not known whether the FC and MC spores differentiate along the same or different paths of sporogenesis. More observations of sporogenesis from sporoblast to spore are needed to facilitate recognition of spore dimorphism and delineation of the FC spore.

The proportion of FC spores of *N. furnacalis* in the cultured cells increased with passaging, and after passage more FC spores were produced in cultured cells than in the host insect, where MC spores were mainly produced. Kurtti *et al.* (1994) reported spores of *N. furnacalis* from the subcultures after 77 passages infected HZAM1 cells at a higher rate than that after 4 passages. This higher infection rate is interpreted to mean that the cultures of 77 passages harbored many more FC spores than those of 4 passages. The factors leading to differences between passages or between cultured cell and host insect cell require further study.

The initial infection levels of cells by MC spore inoculation *in vitro* were low, and spread of infection was also slow among cultured cells (Kawarabarta and Ishihara, 1984; Yasunaga *et al.*, 1992), while infection rates by FC spore inoculation were much higher than MC spores (Kurtti *et al.*, 1994) and the development of sporoplasm from FC spores was much faster. Infection rates of FC spores from the culture after 85 to 90 passages reached nearly 100% at 4 days postinoculation. These facts suggest that the infectivity of FC spores for cultured cells is higher than that for MC spores.

We recovered spores from cultures in passages 85 to 90. The spores appeared to be FC spores under light and electron microscopy. Those spores, when added simply to new cultures at the rate of 10 spores per cell, infected nearly half of the cell population 24 hr p.i. However, the age of cultures used for recovering FC spores ranged over several weeks. FC spores of *N. bombycis* germinated immediately after formation; thus, FC spore of *N. furnacalis* differs somewhat from that of

N. bombycis. The exact nature of this difference remains to be determined.

The host cell type (insect and tissue of origin) and culture conditions are likely to play a role in sporogenesis. It should be pointed out that *N. furnacalis* is not known to infect *H. zea* in nature. A cell line from corn borers (*O. furnacalis* or *O. nubilalis*) was not available at the time that these studies were performed. Cell lines of *O. nubilalis* have recently been established (Kurtti, unpublished) and preliminary results indidate that there is a higher rate of *N. furnacalis* MC spore production in these lines.

ACKNOWLEDGMENTS

We thank Dr. Ulrike G. Munderloh, Department of Entomology, University of Minnesota, St. Paul, Minnesota, for laboratory assistance and valuable suggestions. We are grateful to Professor Dr. Ren Ishihara, Department of Agriculture, Nihon University, Japan, for a critical review of the manuscript. We thank Dr. Richard J. Zeyen, Mr. Gib G. Ahlstrand, and Mr. Darryl E. Krueger, Department of Plant Pathology, University of Minnesota, for use of electron microscopy facilities during these studies. We also are grateful to Ms. Maming Wang, Ms. Leanne Finholm, and Ms. Jacqueline M. Dioh, University of Minnesota, for technical assistance in the laboratory. This research was supported in part by a grant to T.J.K. from USDA under Agreement No. 90-37250-5269. This is publications number 21,566, Scientific Journal Series, Minnesota Agricultural Experiment Station. This work was also supported in part by a grant for Overseas Researchers from Nihon University and in part by the Ministry of Education, Science and Culture under a Grant-in-Aid for Scientific Research (03660057).

REFERENCES

Avery, S. W., and Anthony, D. W. 1983. Ultrastructural study of early development of *Nosema algerae* in *Anopheles albimanus*. *J. Invertebr. Pathol.* **42**, 87–95.

Bauer, L. S., and Pankratz, S. H. 1993. *Nosema scripta* N. sp. (Microsporida:Nosematidae), a microsporidian parasite of the cottonwood leaf beetle, *Chrysomela scripta* (Coleoptera:Chrysomelidae). *J. Euk. Microbiol.* **40**, 135–141.

Chippendale, G. M., Beck, S. D., and Strong, F. M. 1965. Nutrition of the cabbage looper, *Trichoplusia ni* (Hubner). I. Some requirements for larval growth and wing development. *J. Insect Physiol.* **11**, 211–223.

De Graaf, D. C., Raes, H., and Jacobs, F. J. 1994. Spore dimorphism in *Nosema apis* (Microsporida, Nosematidae) developmental cycle. *J. Invertebr. Pathol.* **63**, 92–94.

Fries, I., Granados, R. R., and Morse, R. A. 1992. Intracellular germination of spores of *Nosema apis* Z. *Apidologie*. **23**, 61–70.

Ishihara, R. 1969. The life cycle of *Nosema bombycis* as revealed in tissue culture cells of *Bombyx mori*. *J. Invertebr. Pathol.* **14**, 316–320.

Iwano, H., and Ishihara, R. 1989. Intracellular germination of spores of a *Nosema* sp. immediately after their formation in cultured cell. *J. Invertebr. Pathol.* **54**, 125–127.

Iwano, H., and Ishihara, R. 1991a. Dimorphism of spores of *Nosema* spp. in cultured cell. *J. Invertebr. Pathol.* **57**, 211–219.

Iwano, H., and Ishihara, R. 1991b. Dimorphic development of *Nosema bombycis* spores in gut epithelium of larvae of the silkworm, *Bombyx mori*. *J. Seric. Sci. Jpn.* **60**, 249–256.

Iwano, H., and Ishihara, R. 1991c. Isolation of *Nosema bombycis* from moths of the lawn grass cutworm, *Spodoptera depravata* Butler. *J. Seric. Sci. Jpn.* **60**, 279–287.

Iwano, H., Shimizu, N., Kawakami, Y., and Ishihara, R. 1994. Spore dimorphism and some biological features of *Nosema* sp. isolated from the lawn grass cutworm, *Spodoptera depravata* Butler. *Jpn. J. Appl. Entomol. Zool.* **29**, 219–227.

Kawarabata, T., and Ishihara, R. 1984. Infection and development of *Nosema bombycis* (Microsporida:Protozoa) in a cell line of *Antheraea eucalypti*. *J. Invertebr. Pathol.* **44**, 52–62.

Kurtti, T. J., Munderloh, U. G., and Noda, H. 1990. *Vairimorpha necatrix:* Infectivity for and development in a lepidopteran cell line. *J. Invertebr. Pathol.* **55**, 61–68.

Kurtti, T. J., Ross, S. E., Liu, Y., and Munderloh, U. G. 1994. In vitro developmental biology and spore production in *Nosema furnacalis* (Microspora:Nosematidae). *J. Invertebr. Pathol.* **63**, 188–196.

Kurtti, T. J., Tsang, K. R., and Brooks, M. A. 1983. The spread of infection by the microsporidian, *Nosema disstriae*, in insect cell lines. *J. Protozool.* **30**, 652–657.

McIntosh, A. H., and Ignoffo, C. M. 1983. Characterization of five cell lines established from species of *Heliothis*. *J. Appl. Entomol. Zool.* **18**, 262–269.

Munderloh, U. G., and Kurtti, T. J. 1989. Formulation of medium for tick cell culture. *Exp. Appl. Acarol.* **7**, 219–229.

Odindo, M. O., and Jura, W. G. Z. O. 1992. Ultrastructure of *Nosema marucae* sp. n. (Microspora, Nosematidae), a pathogen of *Maruca testulalis* (Lepidoptera, Puralidae). *Curr. Microbiol.* **25**, 319–325.

Sato, R., and Watanabe, H. 1980. Purification of mature microsporidian spores by iso-density equilibrium centrifugation. *J. Seric. Sci. Jpn.* **49**, 512–516.

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

Street, D. A., and Henry, J. E. 1993. Ultrastructural study of *Nosema acridophagus* Henry (Microspora:Nosematidae) from a grasshopper. *Parasitol. Res.* **79**, 173–177.

Takagi, I., Yamada, K., Sato, T., Hanaichi, T., Iwamoto, T., and Jin, L. 1990. Penetration and stainability of modified Sato's staining solution. *J. Electron Microsc.* **39**, 67–68.

Vavra, J., and Undeen, A. H. 1970. *Nosema algerae* n. sp. (Cnidoptera, Microsporida) a pathogen in a laboratory colony of *Anopheles stephensi* Liston (Diptera, Cunicudae). *J. Protozool.* **17**, 240–249.

Wen, J.-Z., and Sun, C.-X. 1988. Two new species of *Nosema* (Microspora:Nosematidae). *Acta Zootaxonomica Sinica* **13**, 105–111.

Yasunaga, C., Funakoshi, M., Kawarabata, T., Aratake, Y., and Iwano, H. 1992. Isolation and characterization of *Nosema bombycis* (Microsporida:Nosematidae) from larvae of beet armyworm, *Spodoptera exigua*. *Jpn. J. Appl. Entomol. Zool.* **36**, 127–134.