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Spore Dimorphism and Some Other Biological Features of a *Nosema* sp. Isolated from the Lawn Grass Cutworm, *Spodoptera depravata* Butler[1]

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A microsporidian isolated from the lawn grass cutworm, *Spodoptera depravata* Butler had two nuclei in diplokaryotic arrangement throughout its development. A sporont gave rise to two spores, and there was no sporophorous vesicle. This microsporidium produced two types of spores, those with 2 to 3 coils and those with 11 to 12 coils of the polar tube during development in *Antheraea eucalypti* cells. Larvae of the silkworm, *Bombyx mori*, were not infected by the microsporidium when the spores were administered perorally. Spores of the predominant type, with 11 to 12 polar tube coils, were longer and narrower than those of the corresponding spores of *Nosema bombycis*. Furthermore they did not react with latex particles, which had been sensitized with a monoclonal antibody against spores of *N. bombycis*, another isolate of *Nosema* (M11) or an isolate of *Vairimorpha* (M12). The new isolate is distinct from *N. bombycis* and is the second species to be isolated from *S. depravata*.

Key words: Microsporidia, *Nosema* sp. Sd-NU-IW8701, *Nosema bombycis*, spore dimorphism, cell culture

INTRODUCTION

IWANO and ISHIHARA (1991 a) have reported that two types of spores are produced in cultured cells by *Nosema bombycis* and a *Nosema* sp. isolated from the lawn grass cutworm, *Spodoptera depravata* BUTLER. The latter was later identified as a strain of *N. bombycis* (IWANO and ISHIHARA, 1991 c). One type of spore is characterized by 3 to 5 coils of the polar tube, thin endospore (35 nm), early development (36 h postinoculation), and the spontaneous discharge of a short polar tube. The other type is characterized by 10 to 12 coils of the polar tube, thick endospore (91 nm), and late development (54 h postinoculation). The second type predominates in the later stages of cell culture and is similar to the purified spores harvested from diseased insects (IWANO and ISHIHARA, 1989, 1991 a). The two types of spores will be referred to in this paper as those with few coils (FC) and those with many coils (MC). It is presumed that FC, which eject their polar tubes into neighboring cells by spontaneous germination

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inside the host cell, are important in cell-to-cell transmission, while MC, which persist longer in the ecosystem, are important in insect-to-insect transmission (IWANO and ISHIHARA, 1991 c). In this paper we report on spore dimorphism in cultured cells, of another *Nosema* sp. from *S. depravata*.

MATERIALS AND METHODS

Microsporidia. A microsporidium (*Nosema* sp. Sd-NU-IW8701. This isolate will be referred to NSD in this paper.) was isolated from a moth of the lawn grass cutworm *S. depravata* that had been captured by a light trap in August, 1987 at Sayama city, Saitama. Larvae of *S. depravata* reared on young shoots of Italian rye grass were perorally infected with the microsporidium and the resulting spores in infected individuals were purified by repeated centrifugation ($1,500 \times g$, 5 min) followed by Percoll density gradient centrifugation ($73,000 \times g$ for 30 min). They were stored at 5°C until use. Two isolates of *N. bombycis* that had been maintained in silkworms, *Bombyx mori*, were used to compare the spore size. One isolate, Bm-NU, was obtained from the National Sericultural Experiment Station, Suginami, Tokyo more than 30 years previously. The other isolate, *Nosema* sp. Sd-NU-IW-8401 was isolated in 1984 from *S. depravata* (IWANO and ISHIHARA, 1991 b). This isolate will be referred to Sd-200 in this paper.

Cell line. A cell line from *Antheraea eucalypti* was cultured at 27°C in GRACE's medium supplemented with 5% fetal bovine serum (Gibco), 5% heat inactivated *B. mori* hemolymph, 200 units/ml penicillin and 200 μg/ml streptomycin. To infect the cultures, 0.2 ml of a suspension containing 2.0×10^9 spores was combined with an equal volume of 0.1 M KOH and, after 40 min incubation at 27°C was added to 12 ml of *A. eucalypti* cell suspension. Aliquots (0.8 ml) of the mixture were placed in 60 ml plastic flasks (Corning) and after 1 h incubation, the supernatant was replaced with 2 ml of fresh medium.

Light and electron microscopy. To observe infected cells, one culture flask was examined every 6 h postinoculation. For light microscopy, smears were prepared and stained with Giemsa solution after fixation with absolute methanol. One hundred infected cells were used to determine the abundance of developmental stages of the microsporidium in cultured cells.

For electron microscopy, cells were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate (pH 7.5) at 5°C for 90 min, postfixed in 1% osmium tetroxide in 0.05 M sodium cacodylate (pH 7.5) at 4°C for 50 min. Samples were subsequently dehydrated in a graded series of ethanol and finally were embedded in Epok 812. Ultrathin sections were stained with uranium acetate and lead citrate mixture (TAKAGI et al., 1990) and observed with JEM 1200EX electron microscope. A drop of spore suspension (10^7/ml) was mounted on a slide precoated with 0.1% protamine sulfate in order to prevent Brownian motion of spores (MARSOT and COUILLARD, 1973). Fifty spores were measured in size with an image splitting microscope (Vickers-Union Kogaku).

Immunological test. A commercial kit (Yakult Co., Tokyo) of latex particles for diagnosis of microsporidia in the silkworm, *B. mori* was used. The kit consisted of three kinds of latex beads suspension. One kind of beads was coated with monoclonal antibody (Mab) against spores of *N. bombycis*, another was coated with Mab against spores of an isolate of *Nosema* (M11) and the third was coated with Mab against spores

of an isolate of Vairimorpha (M12). According to the report by MIKE et al. (1988) who developed the kit, all microsporidia were obtained from the National Institute of Sericultural and Entomological Science (previously the National Sericultural Experiment Station). The *N. bombycis* used to sensitize the beads is tentatively referred to *N. bombycis* (Bm-NIS-YK). When three or more particles were adsorbed to each spore, the reaction was considered to be positive. Test spores were considered to be immunologically identical to the reference spores, when 80% or more spores in three fields examined had adsorbed latex particles.

Infection of silkworms. Twenty larvae of 2nd instar *B. mori* were fed with a slice of diet (Nihon Nosan-Kogyo, Yokohama) painted with 4.8×10^6 spores of *Nosema* sp. (NSD). When the insects had consumed the diet, they were raised with fresh diet. Five larvae were dissected at the early fifth instar, to check for the presence of spores in mid-guts, Malpighian tubles, fat bodies, trachea and silkglands. The remainder were not used in present experiment.

RESULTS

Spore morphology

Fresh spores of *Nosema* sp. (NSD) from *S. depravata* were ovocylindrical (Fig. 1) and measured $4.04\pm0.19\times2.14\pm0.09$ μm ($n=50$) with a length/width ratio of 1.89.

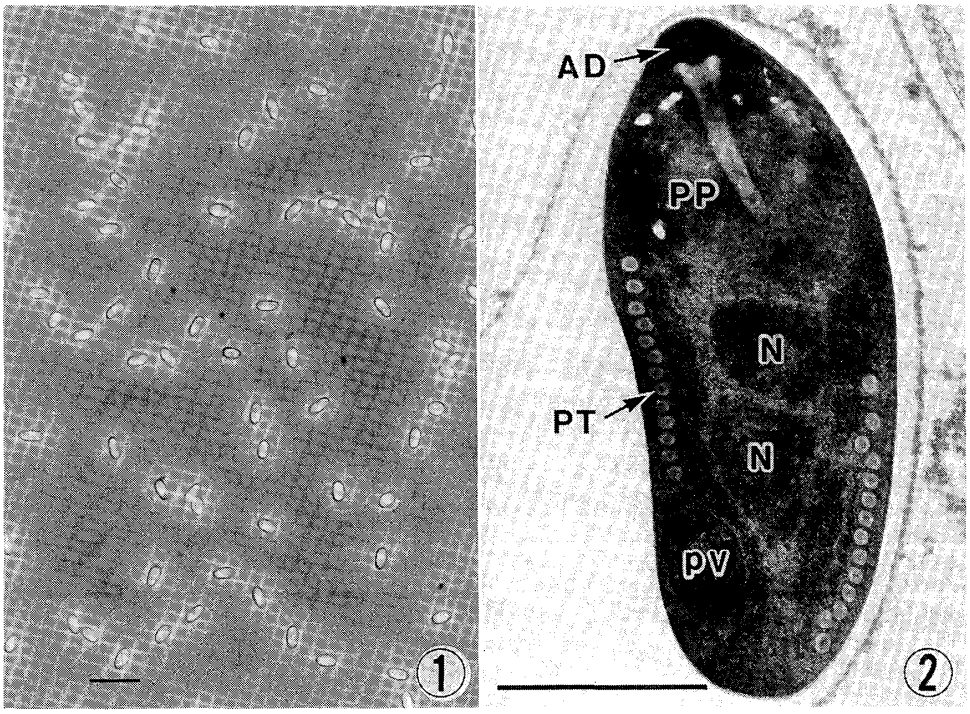

Fig. 1. Fresh spores isolated from the lawn grass cutworm, *Spodoptera depravata*. Bar= 10 μm.

Figs. 1, 2, 4–10. Stages of *Nosema* sp. (NSD) in cells of *Antheraea eucalypti*.

Fig. 2. Fine structure of a spore from *S. depravata* showing 11–12 sections of coils of the polar tube. AD: anchoring disc, N: nucleus, PP: polaroplast, PT: polar tube, PV: posterior vacuole. Bar=1 μm.

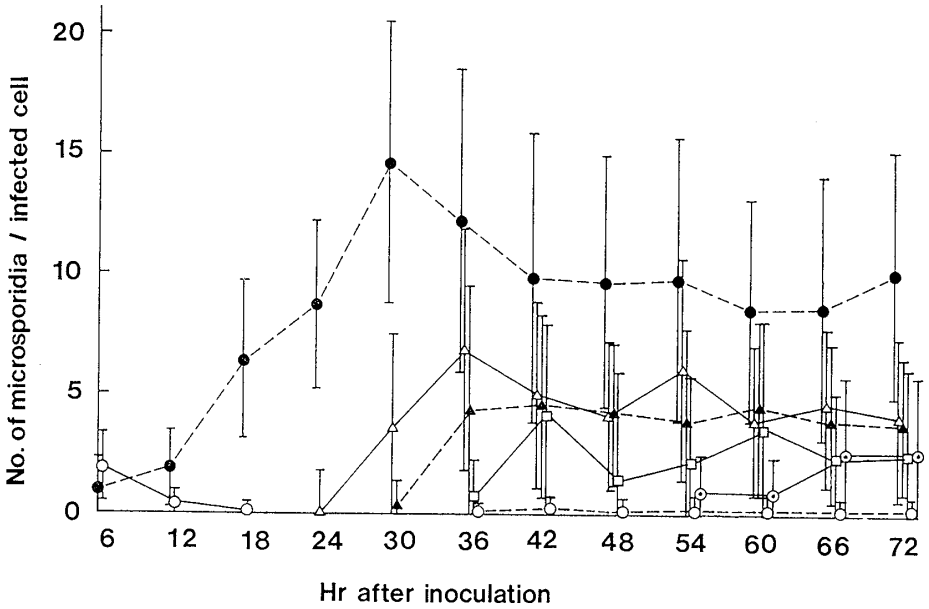

Fig. 3. Abundance of various stages during the course of development of *Nosema* sp. (NSD) in *A. eucalypti* cell. ○ : sporoplasms, ● : meronts, △ : sporonts, ▲ : sporoblasts, □ : FC, ◉ : MC. Vertical lines indicate standard deviations (s.d.).

Those of *N. bombycis* were $3.62\pm0.11\times2.28\pm0.10$ μm ($n=50$) for Bm-NU isolate and $3.60\pm0.19\times2.21\pm0.11$ μm ($n=50$) for Sd-200 isolate, respectively. Electron microscopy of the new isolate revealed an anchoring disc, two nuclei in diplokaryotic arrangement in the central region, 13 to 14 coils of the polar tube, and a posterior vacuole (Fig. 2).

Immunological test

Spores of *Nosema* sp. (NSD) did not react with any of the three kinds of latex particles which had been sensitized respectively with monoclonal antibodies against the Bm-NIS-YK isolate of *N. bombycis*, the M11 isolate of *Nosema* or the M12 isolate of *Vairimorpha*. However, spores of the Bm-NU isolate of *N. bombycis* specifically bound to the latex beads which had been sensitized with the monoclonal antibody for the Bm-NIS-YK isolate of *N. bombycis*, but not with antibody for M11 or M12.

Microsporidian development

Following the inoculation of *A. eucalypti* cells, the abundance of various stages of *Nosema* sp. (NSD) in infected cells was determined by the observation of Giemsa stained smear slides (Figs. 3, 4). Sporoplasms were present in the cytoplasm 6 h postinoculation (p.i.) (Fig. 4 a). They disappeared between 24 h and 30 h p.i., but reappeared 36 h p.i. Meronts (Fig. 4 b, c) were not numerous until 12 h p.i., but began to increase from 18 h p.i. Infected cells contained 10–15 meronts from 30 h p.i. Only binary fission was observed at merogony. A few sporonts occurred 24 h p.i. and increased in number from 30 to 36 h p.i., giving rise to sporoblasts by binary fission (Fig. 4 d, e). Electron microscopy revealed that sporoblast, which already contained a few coils of

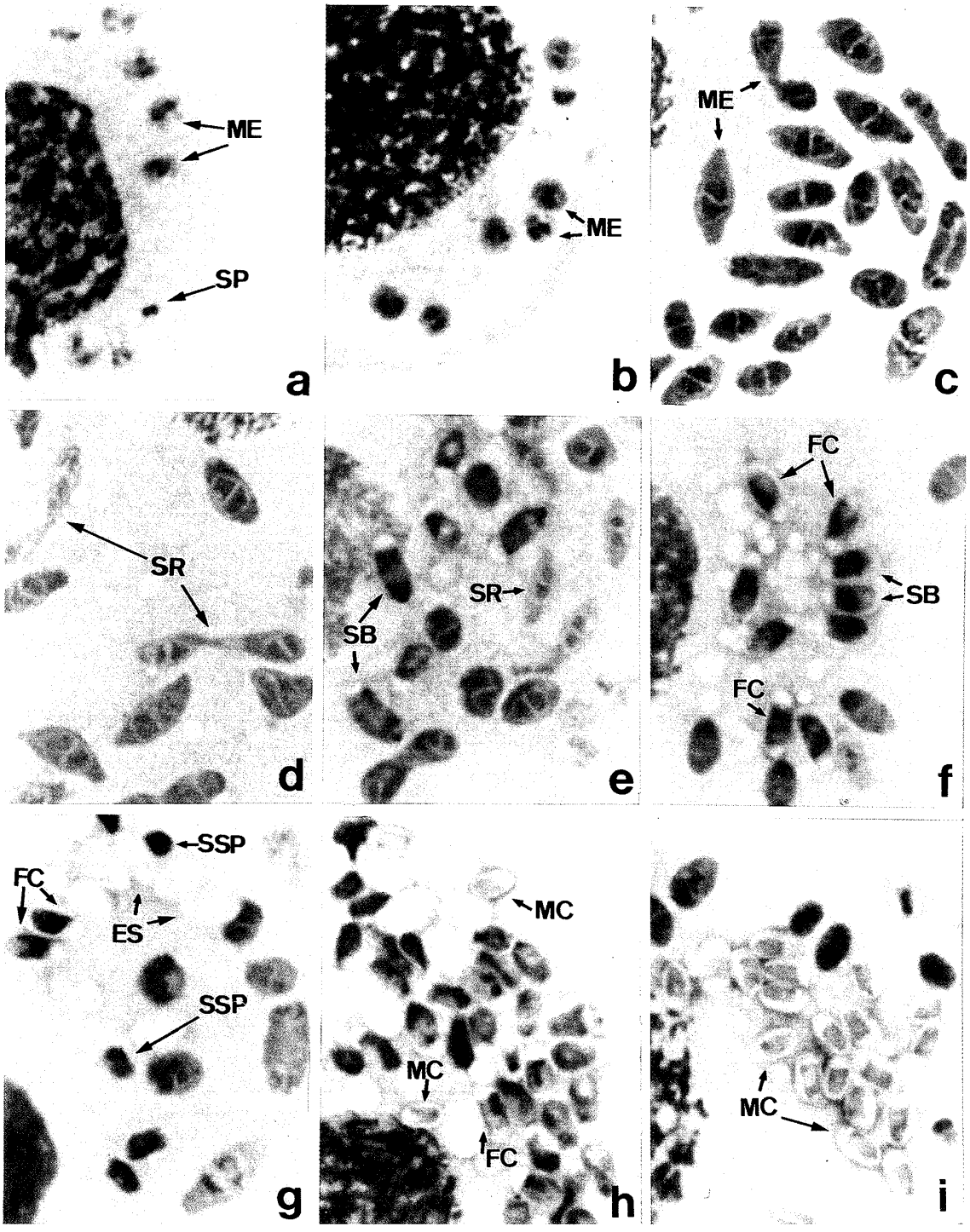

Fig. 4. Giemsa stained preparation. a: 6 h postinfection (p.i.), b: 12 h p.i., c: 24 h p.i., d: 30 h p.i., e and f: 36 h p.i., g: 42 h p.i., h: 54 h p.i., i: 96 h p.i. ES: empty spore, FC: spores with a few coils, MC: spores with many coils, ME: meronts, SB: sporoblasts, SP: sporoplasms, SR: sporonts, SSP: secondary sporoplasms. Bar=5 μm.

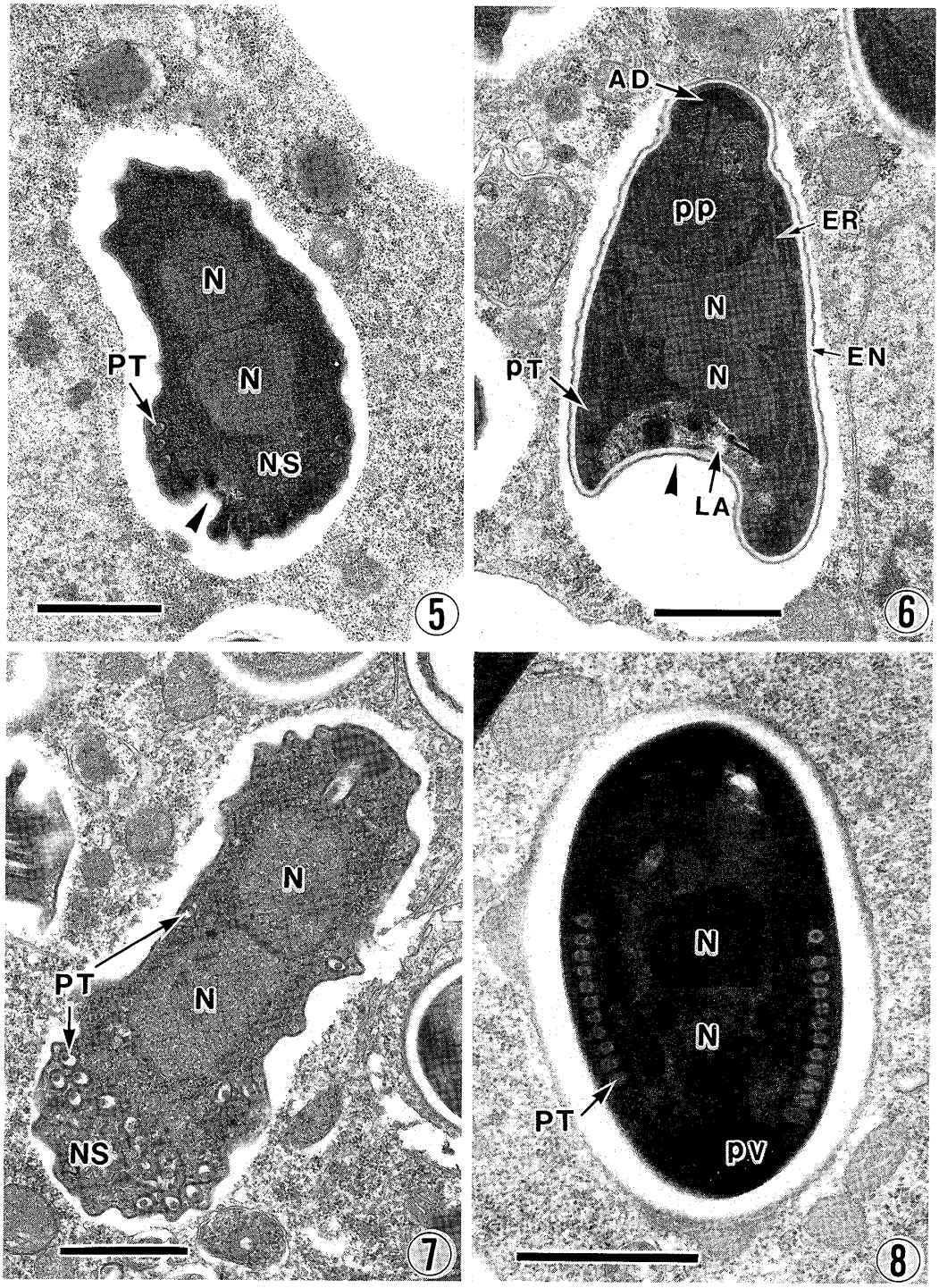

Fig. 5. Early occurring sporoblast destined to give rise to FC spore 30 h p.i. Sporophorous vesicle membrane absent. N: nucleus, NS: network structure (primitive Golgi apparatus), PT: polar tube. Bar=1 μm.

Fig. 6. FC spore showing three sections of polar tube, and a thin spore wall with a depression (arrowhead) at the posterior end. AD: anchoring disc, EN: endospore, ER: endoplasmic reticulum, LA: lacuna of posterior vacuole, N: nucleus, PP: polaroplast, PT: polar tube. Bar=1 μm.

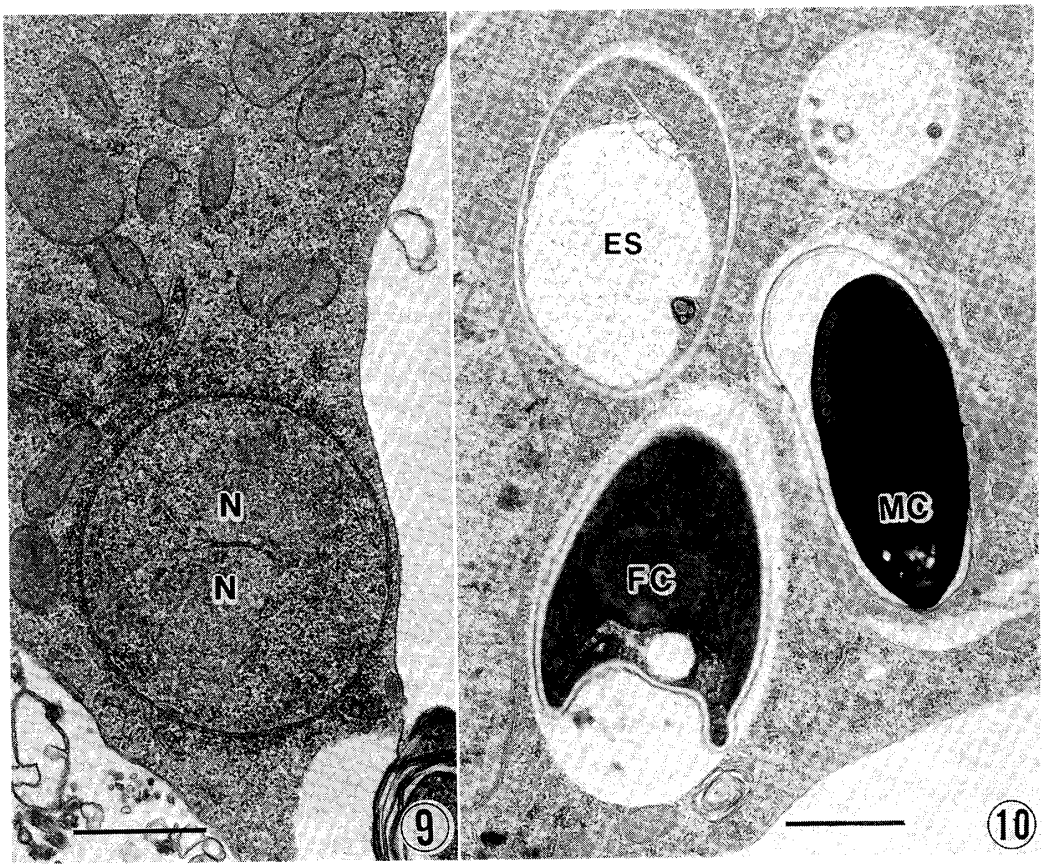

Fig. 9. Sporoplasm 48 h p.i. Note ribosomes around sporoplasm membrane and host cell mitochondria. N: nucleus. Bar=1 μm.

Fig. 10. FC, MC and empty spore lying side by side in the same cell 72 h p.i. Bar=1 μm.

the polar tube at 30 h p.i., were not bound by a sporophorous vesicle membrane. The posterior portion was depressed and the two nuclei in diplokaryotic arrangement occupied the central area. A network structure possibly a Golgi apparatus occurred between the nucleus and the posterior pole (Fig. 5).

Spores appeared 36 h p.i. (FC in Fig. 4 f). These were identified as FC by comparison with corresponding stages of *N. bombycis* in Giemsa-stained preparations. Sporoplasms were observed together with empty spores 42 h p.i. (Fig. 4 g). Ultrathin sections of a spore 36 h p.i. showed an anchoring disc, a polaroplast and the straight part of a polar tube at the anterior pole, and two nuclei in diplokaryotic arrangement in the central region (Fig. 6). The contour of spores was generally pyriform. A coiled polar tube laid near a Golgi structure in a depression near the posterior end under the relatively thin spore wall. The number of sections of the coiled polar tube was three, hence this is the FC type of spore.

Fig. 7. Late occurring sporoblast destined to give rise to MC spore 54 h p.i. Numerous sections of polar tube (PT) around a network structure (NS). Nuclei (N) in diplokaryotic arrangement. Bar=1 μm.

Fig. 8. MC spore 54 h p.i. 11–12 sections of polar tube in a single rank beneath a thick spore wall. Nuclei in electron dense cytoplasm. Bar=1 μm.

The other type of spore (MC) which first appeared as late as 54 h p.i. These were smaller and uniform in shape and had a smooth wall without depression (Fig. 4 h). A row of 11–12 sections of the coiled polar tube was seen embedded in cytoplasm that was denser than that of FC (Fig. 8). Also MC had a thicker spore wall. Sporoblasts of MC could not be differentiated from those of FC in Giemsa-stained preparations, but only by electron microscopy. Thus the numbers of sporoblasts after 54 h p.i. are the summation of both types of sporoblast. Electron micrographs showed more sections of the developing polar tube encircling the Golgi apparatus near the posterior end than in sporoblast of FC. As with FC the sporoblasts were not bound by a sporophorous vesicle membrane (Fig. 7). From 72 h p.i., MC became more numerous than FC. Both types occurred side by side in the same cell (Fig. 10). Newly emerged sporoplasms were observed in the cytoplasm of host cells (Fig. 9). There was no parasitophorous vacuole, but the plasma membrane was encircled by host cell ribosomes and numerous mitochondria laid close by.

Infection of silkworms

Spores were not found in any of the tissues dissected out of the 5th instar *B. mori* which were administered *Nosema* sp. (NSD) spores perorally at the 2nd instar.

DISCUSSION

The sporont of the isolate NSD produced two sporoblasts which in turn, gave rise to two spores, a sporophorous vesicule membrane was absent and two nuclei in diplokaryotic arrangement were present throughout development. These features suggest that this microsporidium belongs to the genus *Nosema*. Its spores were longer and narrower than those of *N. bombycis*. Peroral administration of spores did not result in infection of larvae of the silkworm, the type host of *N. bombycis*. Moreover, the spores were not immunologically related to *N. bombycis* as shown by the latex particle adsorption tests when the latex beads were coated with a monoclonal antibody raised against *N. bombycis* spores. These results indicate that the *Nosema* sp. differs from *N. bombycis*.

The *Nosema* sp. produced two types of spores, FC and MC, in cell cultures as has been observed in the development of *N. bombycis* (IWANO and ISHIHARA, 1991 a). Thus spore dimorphism is not limited to *N. bombycis*, but is a feature shared by other *Nosema* spp. Although we did not observe the germination of the FC spores, the presence of the empty spores (Figs. 4, 10) and emerged sporoplasms (Figs. 4h, 9) suggest that spores, possibly FC, germinate spontaneously in cultured cells as do those of *N. bombycis* (IWANO and ISHIHARA, 1989, 1991 a). VAVRA and UNDEEN (1970) and AVERY and ANTHONY (1983) reported germination of *N. algerae* spores within host cells. KURTTI et al. (1983) also observed sporoplasm-like bodies of *N. disstriae* developing in cell lines. It is yet to be determined whether these *Nosema* spp. also produce two types of spore. If not, spore dimorphism may be a useful criterion for separating species of the genus *Nosema*, which SPRAGUE (1978) suspected to consist of heterogeneous groups. *Nosema scripta* (BAUER and PANKRATZ, 1993) may be an example of a monomorphic species as suggested by the authors, but further studies are needed to confirm this.

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