Budding: A new stage in the development of *Chytridiopsis typographi* (Zygomycetes: Microsporidia)

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**Abstract**

*Chytridiopsis typographi* Weiser, 1954, the microsporidian pathogen of the spruce bark beetle, *Ips typographus* (Coleoptera: Scolytidae), has an early developmental period with plurinucleate mother cells, each of which produces a single bud. The globular bud is connected with the mother cell by a collar and the cellular constituents are pushed to the distant end of the bud. Both the mother cell and the bud continue to develop; the bud then separates from the mother cell and grows to produce a cell of the same type. Both cells then continue sporogonial development and produce sporophorous vesicles with 16–32 spores. The process of a single mother cell producing a single bud that grows to an identical stage is new in the development of *C. typographi* and has no analogy in other Microsporidia.

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**1. Introduction**

The production of the microsporidium *Chytridiopsis typographi* for biological control of *Ips typographus* (Tonka et al., 2009) provided an opportunity to study the early phases in the development of the pathogen. Beetles infected with *C. typographi* were examined to obtain ultrastructural information in addition to details previously provided by Purrini and Weiser (1984, 1985). Specifically, we were interested in the sporogonial stage ending with two types of sporophorous vesicles (thin-walled and thick-walled) and two types of spores. Vesicles of the thin-walled type dehisce in the gut of the host and release thin-walled spores that extrude their sporoplasms under the effect of the gut juices. These spores serve primarily to spread the infection in the gut of the same host. The rest of the thin-walled spores exit the gut with the faeces and soon lose their activity in the external environment. The thick-walled vesicles are cyst-like structures with resistant walls containing 16–32 spherical thick-walled spores. These vesicles do not release spores but exit the host in the faeces of the beetle, serving to infect new hosts. In our experiments the suspension of stages used for the laboratory infection was freshly prepared from dissected infected beetles and contained both types of sporophorous vesicles. The composition of the spore suspension and immediate transfer to susceptible hosts evidently resulted in increased development of a specific type of early-stage sporogony. These stages were present to a degree exceeding their normal occurrence in field-collected infections. The cells, which are evidently products of sporoplasms from thin-walled spores, form specific mother cells that produce a single bud. The bud grows to a size and structure identical to that of the mother cell. This process is described in the present study.

**2. Materials and methods**

The microsporidian *C. typographi* is frequently recovered in collections of bark beetles, *Ips typographus* from the Šumava National Park, near to the southwest border of the Czech Republic. Beetles collected from the bark galleries of spruce trees were dissected and the midgut was excised and inspected in a drop of insect saline under a cover slip (Wegensteiner et al., 1996). For production of the pathogen, infected gut tissues were crushed in saline and used to inoculate a group of young adult *Ips typographus* reared in the laboratory. Young (brown) beetles were kept in plastic vials 8 mm in diameter, 100 mm long, with a chip of bark exposed at the end of the tube. The tubes, containing a maximum of 10 beetles per tube, were held at 25 ± 1 °C in a 16:8 light/dark regime. A drop of saline with suspended stages of *C. typographi* from crushed infected gut tissues was placed on the chip of bark fed to the beetles. New clean bark chips were provided every 2 days and dead beetles were removed. The vials were held for 50 days with periodic replacement of the bark. Mortality usually began after day 12 with maximum mortality occurring on day 25 and a second peak on day 35. Mortality ceased after day 45. Dead...
beetles were dissected in saline and positive midguts were stored in water at 4 °C until use.

For the present study, beetles were inoculated as above and active beetles were removed from the rearing tube on day 8 after inoculation. They were decapitated in a droplet of saline and the gut tissues were excised and inspected for infection. The infected tissues were transferred into 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer of pH 7.4, cut into segments, transferred into fresh glutaraldehyde solution, and held overnight at 4 °C. After several washes in cacodylate buffer, the material was post-fixed in 2% aqueous OsO₄ at 4 °C for 2 h. Fixed tissues were embedded in Polybed 812 epoxy resin, stained with uranyl acetate and lead citrate and examined and photographed using a JEOL-JEM 1010 electron microscope at 80 kV equipped with Megaview 3 CD camera.

3. Results

3.1. Pathology

Multiple thin-walled developmental stages were observed in the anterior midgut epithelial cells of the host. The stages were scattered throughout the midgut tissues from the epithelial brush border cells to the basal membrane (Figs. 1–3, bb, bm) and were not concentrated in any layer. Late sporogonial stages, sporophorous vesicles of thin-walled and thick-walled type and mature spores were rare. In Fig. 1, more than 46 early developmental stages in the development to budding mother cells (Bm) and buds (b) are present, and only two thick-walled sporophorous vesicles (SpT) are observed. All stages of the mother cell have a distinct type of cytoplasm that contrasts with the higher density midgut tissue cells of the bark beetle. The initial stages (x) are small spherical stages, 1–2 \( \mu m \) in diameter, with a single nucleus and appear in groups. These stages grow to oval mother cells, each approximately 7 \( \times \) 5 \( \mu m \), with four nuclei. The thin cytoplasm of these cells contains a system of endoplasmic reticulum and multiple vacuoles enclosed within a thin plasma membrane.

There was no evidence of immune response by the host cells. The thin-walled mother cells were observed near (distance of 2–4 \( \mu m \)) the brush border of the midgut epithelial cells (Fig. 1, bb) or on the opposite side near the basal membrane of the gut (Figs. 1 and 3, bm). Thick-walled vesicles were scarce (Figs. 1 and 3, SpT), without specific orientation to the brush border and the gut lumen. Thin-walled sporonts (Fig. 2, Spt) and developing thin-walled spores were rare in this material and mature thin-walled sporophorous vesicles were not yet formed.

4. Development of bud mother cells

Vacuoles within the maturing mother cell move close to the simple plasma membrane enclosing the stage. This membrane does not thicken during maturation. Mature bud mother cells are broad oval stages 6–8 \( \times \) 5–6 \( \mu m \) in size (Figs. 1–3, Bm). They usually have four nuclei (N) grouped at one pole of the cell. The nuclei have several flat electron-dense nucleolus plaques fixed on their membrane (Fig. 4, sp) that persist during sporogonial develop-
The plaques are not paired; three to five of these structures, evident in ultrathin sections, adhere to the interior of the nuclear membrane. They are 200–400 nm in diameter, protruding up to 100 nm into the interior of the nucleus, without evidence of microtubular structures. The bud develops at one end of the oval mother cell, opposite the end at which the nuclei are grouped, which suggests that the mother cell is already polarized. The initial bud is a minute sphaerular protrusion (Fig. 1, arrow) that gradually fills with plasma and constituents (Figs. 2, 4 and 5, Bm/b), exerting pressure on the vacuolated end. The nuclei appear as membrane bound structures with electron-dense plaques (Figs. 6 and 7, N, sp). The vacuoles are concentrated around the collar (Figs. 5–7, cv). The bud is evidently extended by pressure exerted on a cap of cytoplasm at the distant end of the bud (Figs. 2, 3 and 5, Bm-b). The “propulsion system” (Figs. 6 and 7, P) seems to be composed of several vacuoles (v) closing the collar. Both the mother cell and the bud continue to differentiate independently, including maturation of the sporoblasts. The bud (b) grows to the size of the mother cell and contains several nuclei (Figs. 6 and 7, N), each with electron-dense plaques (sp). Both the mother cell and the bud form a sporont that matures with differentiation of individual sporoblasts (Figs. 6 and 8, Sb). Among the posterior vacuoles, specific spherical vacuoles (Figs. 6 and 8, po) form the posterosome with adherent coiled Golgi system membranes (G). The Golgi membranes unwind (Fig. 6, G1) and extend to the plasma membrane of the sporoblast to support differentiation of the sporoblasts. In maturing sporoblasts, the posterior vacuoles are connected with the Golgi membranes and form the posterosome system of the spores. Some metabolic material is concentrated in a “gray” vacuole (Fig. 6 g), which remains outside the sporont.

The sporogony of the mother cell and daughter cell occur at the same rate (Fig. 7, SpT, b) and the cells are in the same developmental stage when the collar closes and separation takes place. In some cases, the daughter cell is retarded in its maturation. Both cells remain in close proximity to each other. After separation of the cells (Fig. 8), sporogonial development continues and most thin-walled sporonts in Figs. 1–3 result from budding. In a specific case (Fig. 3, SpT), the budding resulted in transformation of the mother cell into a thick-walled sporophorous vesicle (SpT), whereas the bud formed a thin-walled sporont (b). The parasitophorous vacuole of the host retains the size and shape of the budding pair. The maturing thick-walled sporophorous vesicle is reduced in size and floats in the vacuole. The collar of the thin-walled bud (b) remains open and is closed off by the wall of the posterior vacuole.

5. Discussion

All microsporidia without a polaroplast and with the polar filament encased in a persistent polar sac with a honey-comb like electron-dense net replacing the polaroplast (Metschnikovellidea sensu Weiser, 1977) undergo development that is in some way different from the rest of the microsporidia. The cyst-like thick-walled sporophorous vesicles constitute part of this difference. Only a few have been investigated using electron microscopy and the authors provided no data on presporogonial development. Larsson et al. (1997) described some presporogonial stages in Intexta acarivora defined as early sporonts. He believes that sporog-
ony may be the only reproductive phase of this pathogen. Sporog- 
nial multinucleate plasmodia were the earliest stages of Buxtehu- 
deaa scanniae observed in the study of Larsson, 1980. Only 
sporogonial stages were recognized in Noelleria pulicis by Beard 
et al. (1990) and Larsson (1993) suggested that vegetative develop-
ment is absent in C. trichopterae. Our early observations (Weiser, 
1954; Purrini and Weiser, 1984, 1985) did not provide evidence 
for presporogonial development in C. typographi. Two types of 
spores were recognized in C. typographi, thick-walled spores and 
a thin-walled, less environmentally resistant type produced in 
thin-walled, temporary sporophorous vesicles that dehisce in the 
original host and are released in the gut lumen. The thin-walled 
spores spread the infection within the host by injecting sporop-
lasms into midgut epithelial cells of the original host. The thick-
walled spore type is enclosed in a persistent cyst-like thick-walled 
sporophorous vesicle, which does not release the spores in the ori-
ginal host, but exits the host in the faeces. These spores remain 
infectious for several months in storage and serve for horizontal 
transmission of the pathogen.

The production of two types of spores corresponds to the gen-
eral concept proposed by Iwano and Kurti, 1995, in which primary 
spores germinate to spread infection within the original host, and 
persistent spores, which remain inactive in the host until death or 
are released in the faeces for horizontal transmission. Larsson, 
1993 discussed the role of thin-walled spores in C. trichopterae 
and related species in detail without mention of any specific bud-
ding period during early-stages. In Chytridiopsis, the two types of 
spores could act as primary and persistent spores. The effect of 
internal colonization of the midgut by sporoplasms released by 
thin-walled spores is evident (in Figs. 1 and 3, x) in beetles fixed 
for TEM evaluation during the early period of infection. In our 
experiments, the immediate transfer of thin-walled spores from a 
host with an early-stage infection to a new host preserved the 
activity of the thin-walled spores and they infected the new host 
in increased numbers. This laboratory transfer procedure produced 
a different picture of the Chytridiopsis infection compared to that 
recorded for field infections. The stages distributed in the gut epi-
thelium did not cause the ulcerations observed in material col-
lected in the field (Purrini and Weiser, 1984, 1985). Ulcerations 
appear to be the final response to the production of thick-walled 
sporophorous vesicles and spores that cause lysis of host gut epi-
thelial cells for release of the stages into the gut lumen and elimi-
nation of spores in the faeces.

The budding recorded in our material has not been previously 
observed in microsporidia. In some cases, the sporogony of micro-
sporidia produce multinucleate plasmodia dividing into uninnucle-
ate sporoblasts in two different ways (Vavra and Larsson, 1999). 
One type of division corresponds to synchronous cleavage, splitting
of the plasmodium into uninucleate sporoblasts of the same maturation, which adhere in a morula- or rosette-like cluster. The process is often described as “budding”. The second type of division consists in asynchronous, consecutive maturation and separation of individual sporoblasts from a common plasmodium by “budding” or sprouting with maturation as free single spores. In all these cases, the product is different from the multinucleate plasmodium. In *C. typographi*, the budding is an unique process. It occurs at the beginning of sporogony and constitutes activity of single multinucleate cells, the mother cells, which produce one single bud protruding at the posterior end (opposite the end where nuclei are concentrated) of the broad oval cell. The whole cell is involved in the formation of the bud and produces an identical mirror-image budded cell. The pressure occurring in the initial phase of formation of the bud pushes the cytoplasm and nuclei into the growing bubble, where it is concentrated at the opposite end. The plasma membrane originating from the mother cell encloses both cells. Before the multinucleate bud separates from the mother cell, it develops to the stage the mother cell had reached at the start of budding. Prior to budding, there is no evidence of any specific nuclear division. During maturation of the bud, the mother cell continues to form sporogonial structures: the pansporoblast and sporoblast stages which mature into spores. In sections of beetles during late sporogonial development (12 or more days after inoculation), single “mother” cells are present as the initial stages in the final formation of thin-walled and thick-walled sporophorous vesicles and spores.

Examination of photographs from previous research revealed instances of mother cells containing nuclei with typical nucleolar plaques, but pairs of cells that may correspond to stages of budding are observed only exceptionally. Therefore, we suggest that the budding cycle of *Chytridiopsis* is a type of development that occurs only under specific conditions.

In ultrathin sections, the nuclei of sporonts are found to feature electron-dense plaques of nucleoli without any direct relation to spindle plaques. These structures are also observed in other Metschnikovellidea, for example, in *Noelleria pulicis* (Beard et al., 1990), *Chytridiopsis trichopterae* (designated as “nucleoli”) (Larsson, 1993), *Buxtehudea canaliculata* (Larsson, 1980) and *Intexta acarivora* (Larsson et al., 1997). In *Chytridiopsis socius*, Manier and Omnieres (1968) designated the structures as “centrosomes”. The plaques are also present in the nuclei of *Steinhau sia* (Richards and Sheffield, 1970), and appear again in *Amphiamblys capitellides* (Larsson and Koie, 2006).

It remains to be determined how to incorporate the developmental stages we describe in the life cycle of the microsporidian *C. typographi*. If we remain consistent with the general concept that there is no vegetative development in Metschnikovellidea, we must place budding in the phase of early sporogony beginning after the injection of the sporoplasm from the spore into the host gut epithelial cells as “presporogonial budding”. Its broader extension in the described case suggests an extraordinary process in transmission with viable thin-walled spores.

This stage of budding in the development of *C. typographi* is new for Microsporidia. This type of development may not occur in every infection to the extent observed and documented in the described
case, but microsporidia, and nominally Chytridiopsidae have the process of budding in their genomic memory as a possible developmental process between merogony and sporogony. The possible relationship of this type of development to the developmental processes in Zygomycetes should be investigated.

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References


