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In Vivo and In Vitro Development of the Protist *Helicosporidium* sp.

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ABSTRACT. We describe the discovery and developmental features of a *Helicosporidium* sp. isolated from the black fly *Simulium jonesi.* Morphologically, the helicosporidia are characterized by a distinct cyst stage that encloses three ovoid cells and a single elongate filamentous cell. Bioassays have demonstrated that the cysts of this isolate infect various insect species, including the lepidopterans, *Helicoverpa zea, Galleria mellonella,* and *Manduca sexta,* and the dipterans, *Musca domestica, Aedes taeniorhynchus, Anopheles albimanus,* and *An. quadrimaculatus.* The cysts attach to the insect peritrophic matrix prior to dehiscence, which releases the filamentous cell and the three ovoid cells. The ovoid cells are short-lived in the insect gut with infection mediated by the penetration of the filamentous cell into the host. Furthermore, these filamentous cells are covered with projections that anchor them to the midgut lining. Unlike most entomopathogenic protozoa, this *Helicosporidium* sp. can be propagated in simple nutritional media under defined in vitro conditions, providing a system to conduct detailed analysis of the developmental biology of this poorly known taxon. The morphology and development of the in vitro produced cells are similar to that reported for the achorophyllic algae belonging to the genus *Prototheca.*

Key Words. Black fly, entomopathogens, Helicosporidia, *Helicosporidium,* insect pathogen, pathogenic protist, Simuliidae, *Simulium jonesi.*

HELICOSPORIDIUM parasiticum, a pathogenic protist detected initially in a ceratopogonid (Diptera), was described and named by Keilin (1921) and was subsequently placed in a separate order, Helicosporidia, within the Cnidiospora (Kudo 1966). Weiser (1970) examined both the type material and a new isolate from a hepialid larva, and proposed that this organism be transferred to the Ascomycetes. Later work by Kellen and Lindegren (1974) described the life cycle of a *Helicosporidium* sp. [isolated originally from *Carpophilus mutilatus* (Nitidulidae, Coleoptera) Kellen and Lindegren 1973] in a lepidopteran host, the navel orangeworm *Paramyelois transitella,* and agreed that this organism belonged with the primitive ascomycete group. Kellen and Lindegren (1974), conducting lab transmission studies, reported that cysts ingested by host navel orangeworm released the cyst contents into the gut lumen; both sporoplasms (ovoid cells) and filaments were found in the lumen at 3 h post-infection. Elongate cells 11.5 × 3.5 μm, believed to develop from the released sporoplasms, were detected in the hemocoel at 24 h post-challenge. These cells divided to form 4 daughter cells within a pellicle. By 72 h, spherical cells 3.5 μm in diam. were observed in the hemolymph. Between d 3–6 these cells divided and developed into additional spherical cells. After this, the daughter cells underwent sporogony, secreted a thick spore wall (or pellicle) and differentiated into the cassette of a filament and three sporoplasms. Lindegren and Hoffman (1976) proposed that the developmental stages of this organism placed it closer to the Protozoa than to the Fungi. Because of this uncertain taxonomic status, the helicosporidia have not appeared in classification systems of either the Protozoa or the Fungi and have been unclassified since 1931 (Cavalier-Smith 1998; Patterson 1999). Fukuda et al. (1976) isolated a *Helicosporidium* sp. from the mosquito *Culex territans* and determined that larvae were infected per os and that the disease may persist though adult eclosion. Additional helicosporidia have been detected in mites, cladocerans, trematodes, collembolans, and pond water samples (Avery and Undeen 1987a; Pekkarinen 1993; Purrini 1984; Sayre and Clark 1978).

Recently, a *Helicosporidium* sp. was isolated from larvae of the black fly *Simulium jonesi* Stone and Snoddy, collected in Florida. This identification was based on the presence of a cyst stage composed of three ovoid cells and an elongate filamentous cell. Preliminary assays demonstrated that this *Helicosporidium* sp. was capable of infecting *Helicoverpa zea* larvae challenged per os. The ability to produce quantities of this pathogen in a lab insect provided an opportunity to conduct a series of laboratory experiments. We present data on the discovery, host range, and in vivo development of this *Helicosporidium* sp. and provide new evidence on the invasion process. We also demonstrate that this pathogen has very simple nutritional requirements and can be cultivated in vitro on a wide range of media and maintain infectivity for insect hosts.

MATERIALS AND METHODS

Insect methods. The test insects, including various dipteran and lepidopteran hosts, were accessed from established lab colonies and were maintained according to standard protocols as previously described (Avery and Undeen 1987a, 1987b).

Field isolation. Black fly larvae were collected from Hatchet Creek, Alachua County, Florida (N 29° 43′ 50.4′′, W 82° 14′ 56.6′′) in the fall of 1998. Larvae from the sample were examined for signs of infection and for species identification. The total number of larvae and the prevalence of infection was estimated from the samples.

In vivo propagation of the *Helicosporidium* sp. Cysts of the *Helicosporidium* sp., extracted from infected black flies *Simulium jonesi,* were fed per os to second and third instar corn earworm *Helicoverpa zea* larva. Eight test larvae, initially starved for 24 h prior, were each allowed to imbibe a 10 μl droplet containing 4×10^5 cysts. Treated larvae were placed in individual cups containing artificial diet and incubated at 26 °C under a 16-h light:8-h dark period. At 10–14 d post-challenge, cream-colored hemolymph was harvested from infected larvae and subjected to several cycles of low-speed centrifugation (600 *g,* 1–2 min). Cysts, located in the pellets, were suspended in 50 mM Tris-HCl buffer pH 8.0 and purified on a linear gradient of Ludox (DuPont Chemical, Boston, MA) following Undeen and Vavra (1998). The band containing the cysts was collected, diluted in 10 vols. of water, and subjected to several cycles of low-speed centrifugation to remove residual gradient material. Cysts were counted using a hemacytometer and measured with a split image micrometer. They were placed either at 4 °C or −70 °C for short and long-term storage, respectively.

Insect bioassays. The viability of the purified cysts was assessed by per os challenge of early instar *H. zea* and tobacco hornworm *Manduca sexta* larvae and by hemocoelic injection into late instar waxmoth *Galleria mellonella* larvae with 10^5 cysts/insect. Test insects were provided with their respective diet and incubated at 26 °C.

Bioassays were conducted with several species of Diptera to determine their susceptibility to per os challenge with purified

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cysts of the *Helicosporidium* sp. Mosquito assays were conducted with groups of 100 one- to two-day-old (1st instar) colony larvae exposed at 27 °C in 3.5 oz plastic cups in 100 ml of water with 2% alfalfa and potbelly pig chow mixture (2:1) for 24 h. Mosquitoes and exposure media were then transferred to enamel pans with 500 ml of water and fed according to standard protocols. Mosquitoes (*Aedes taeniorhynchus*, *Anopheles albimanus*, *An. quadrimaculatus*, and *Culex quinquefasciatus*) were exposed to the *Helicosporidium* sp. at doses ranging from 3.5×10^2 to 5.0×10^5 cysts/ml. Because *An. quadrimaculatus* was highly susceptible, a 30% serial dilution of 5.0×10^4 cysts/ml (6 doses, three replicates) was conducted to determine the 50% infective concentration (IC_{50}). Mosquitoes were examined for infection as larvae 5 to 6 d post-exposure and as adults 1 to 2 d after emergence. In addition, 100 three-day-old (3rd instar) *Musca domestica* larvae were continuously exposed to 7.8×10^8 cysts (total) mixed with their diet. Fly larvae were examined for infection 6 to 7 d post-exposure for the presence of cysts and as adults 1 to 2 d after emergence.

In vitro dehiscence of Helicosporidia cysts. The *Helicosporidium* sp. used in these experiments was harvested from infected *H. zea* larvae. Initial experiments addressed the ability of fluids extracted from the midgut lumen to stimulate release of the filamentous cell from the cyst stage. The midgut fluid used to stimulate cyst dehiscence was from dissected midguts of late instar *H. zea* larva. Midguts were homogenized gently and centrifuged at 16,000 *g* for 15 min. The supernate was than passed through an MC centrifugal filter unit (0.45 μm, Millipore Corp., Bedford, MA) and frozen at −20 °C. The influence of midgut extract on the release of the elongate cells was replicated twice and monitored over time. Approximately 10^4 cysts, rinsed in deionized H_20, were suspended in either 50 μl of water or in 25 μl of water and 25 μl of midgut fluid. Various other physical (e.g. exposure to −70 °C freeze-thaw cycle, vacuum or sonication) and chemical treatments (4 h incubation in either protease K (1 mg/ml), pH 10 buffer, pH 4.0 buffer, or in 50 mM $CaCl_2$) were assessed for the ability to dehisce cyst preparations. Microscopic examination was used to estimate cyst dehiscence in the various treatment and control preparations.

In vitro cultivation of Helicosporidia. Preliminary experiments determined if either the cyst or the dehisced cyst preparations could develop in insect cell cultures. Dehisced cyst preparations were prepared by placing an aliquot of purified cysts between sterile glass slides. The pressure exerted by the top slide induced cysts to dehisce within minutes releasing the ovoid cells and filamentous cells from the pellicle. Released cells were collected by rinsing glass surfaces with sterile PBS. Aliquots of the intact cysts and the dehisced cyst preparations were added to replicate wells containing 1 ml of insect tissue culture medium TC100 + 10% fetal calf serum (FCS) seeded with either vesicular cell lines derived from imaginal discs of *Trichoplusia ni* and *Spodoptera frugiperda* (Lynn et al. 1982) or conventional SF9 insect cells established from pupal ovarian tissue of *S. frugiperda* (Catalog No. CRL1711 ATCC, Rockville MD). Inoculated plates were incubated at 26 °C. Growth and development of *Helicosporidium* sp. in the tissue culture plates was monitored using Hoffman modulation optics.

A second series of experiments was conducted with cysts harvested after 14 d incubation in the imaginal disc cultures. Aliquots of the cysts were added to TC100 + 10% FCS media or media plus the *T. ni* vesicle cell line at an initial concentration of 8×10^4 and 1×10^4 cysts ml^{-1}. The 24-well plate was incubated at 26 °C. Wells were examined daily using Hoffman modulation optics to monitor development. At intervals, cells were resuspended and aliquots quantitated with a hemacytometer.

A final series of assays addressed the ability of *Helicosporidium* sp. to develop in conventional lab media including Czapek Dox broth (CD), CD + 2% yeast extract (CDY), *Candida* liquid broth, Vogel-Bonner minimal broth, TC100, TC100 + 10% FCS, and Sabouraud dextrose (SD) broth. These media were tested for their ability to support the development of *Helicosporidium* sp. The cells produced in TC100 + FCS were inoculated into broth cultures (5×10^3 cells/ml^{-1}) and incubated without and with shaking (New Bruswick gyro-rotory shaker, 250 rpm) at both 25 °C and 35 °C. At intervals, aliquots were removed and cell replication assessed by hemacytometer counts.

Transmission electron microscopy. Gradient-purified cysts produced in *H. zea* were prepared for ultrastructural examination by primary fixation in 2.5% glutaraldehyde plus 1% acrolein at 60 °C for 2 h, post-fixing in 2% osmium tetroxide at RT, dehydrating in an ethanol series, and embedding in Spurr's resin. Thin sections, stained in uranyl acetate and lead citrate, were observed and photographed at 75 kV.

Scanning electron microscopy (SEM). In vitro and in vivo cell preparations purified in Ludox gradients were mounted on slides coated with poly-L-lysine and fixed in buffered 2% glutaraldehyde, rinsed three times, and fixed in 1% aqueous osmium tetroxide. Samples were dehydrated in an ethanol series and subjected to critical point drying. Samples were coated with gold and viewed with a Hitachi S-4000 FS scanning electron microscope operating at 6 kV. Digital images were processed and measured with NIH-image software.

RFLP analysis. The integrity of the in vivo- and in vitro-produced *Helicosporidium* sp. was assessed by conducting RFLP reactions on the genomic DNA. Two regions of the ITS1-5.8S-ITS2 and the 18S rDNA have been recently PCR-amplified and sequenced in our lab (Tartar, A., pers. commun.). Gradient-purified helicosporidial cells produced under both in vivo and in vitro conditions were centrifuged at 8,250 *g* for 10 min. The pellet, ~ 35- to 50-μl, was extracted according to the protocol outlined for Masterpure™ Yeast DNA extraction kit (Epicentre Technologies, Madison, WI). The final pellet, suspended in molecular biology-grade water, was frozen at −20 °C. The ITS1-5.8S-ITS2 of the helicosporidial ribosomal DNA was amplified with a mixture of *Taq* DNA polymerase (Promega, Madison, WI) and PFU polymerase (Stratagene, La Jolla, CA), using the primers TW81 and AB28 (Curran et al. 1994) producing products of approximately 950 bp. Of 844 bases sequenced, there is an *Eco*RV recognition site at 435 and a *Nde*I site at 216. The 18S region was amplified using the forward primer 18S-69F (5′-CTGCGAATGGCTCATTAAATCAGT-3′) and the reverse primer 18S-1118R (5′-GGTGGTGCCCTTCCGTCAA-3′), which resulted in a product of approximately 1100 bp. In the sequenced section (from the forward primer), there is a recognition site for *Hind*III at 413 (Tartar et al., pers. commun.).

RESULTS

Isolation and biology. A species of *Helicosporidium* was isolated from larvae of *Simulium jonesi* in September 1998. Prevalence was low with only three of 200 larvae infected. Cloudy areas in the posterior regions of the abdomen identified infected individuals. Examination of fresh *S. jonesi* tissue with phase contrast microscopy revealed discoid cysts that measured $6.5 \pm 0.2 \times 5.9 \pm 0.3$ μm. Light and electron microscope studies demonstrated that the cysts contained a core of three ovoid cells and a single filamentous cell (Fig. 1A). The filamentous cell makes 3 to 4 coils, forming a helix around the ovoid cells within the mature cysts. The outer wall or pellicle

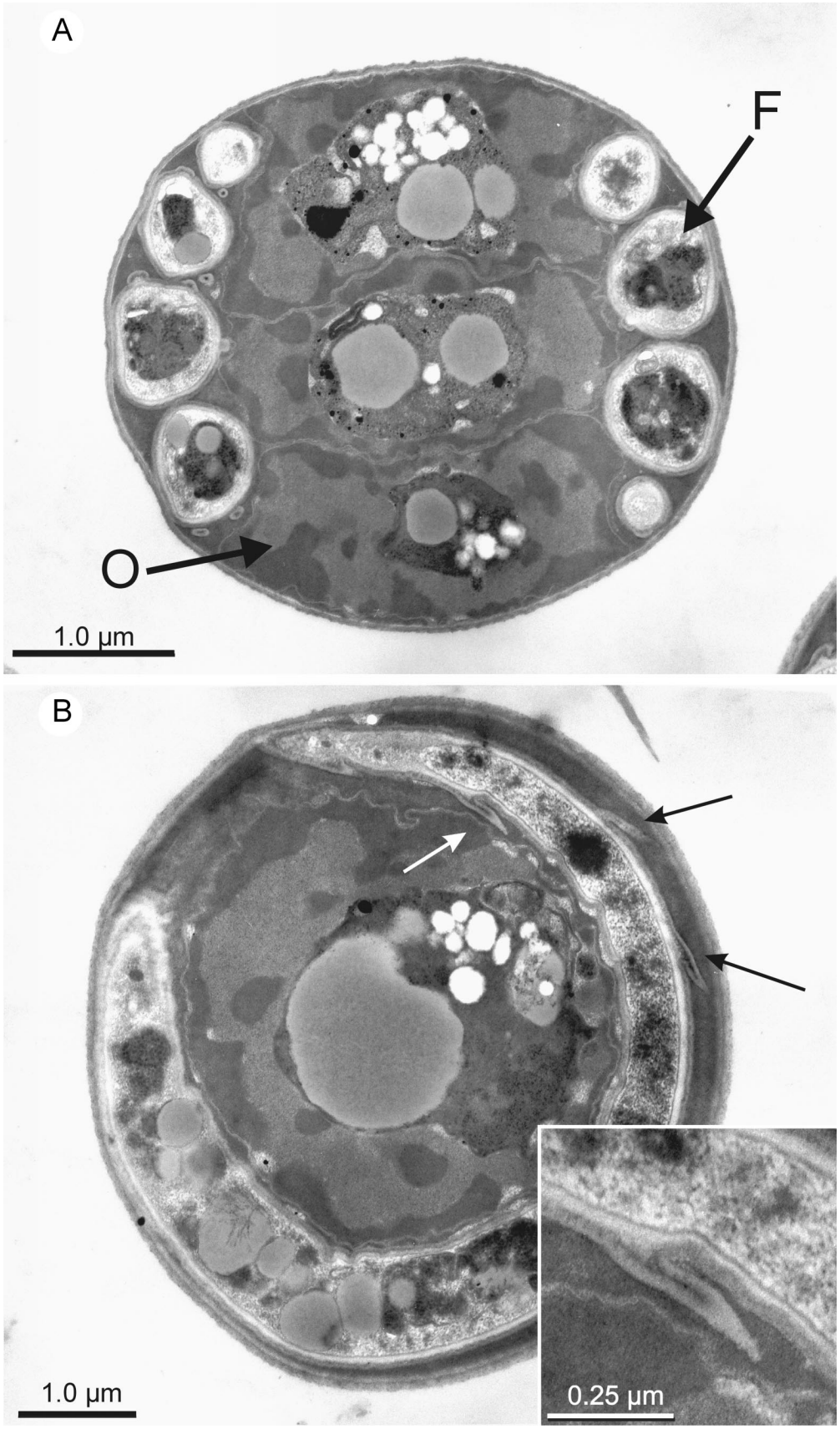

Fig. **1**. Transmission electron micrographs of the cysts of *Helicosporidium* sp. from the black fly *Simulium jonesi*. **(A)** Mature cyst composed of three central ovoid cells (O) and the peripherally located filamentous cell (F) contained within a multi-layered cyst wall. **(B)** Sagittal section of the filamentous cell within the cyst demonstrating several of the projections (arrows) on the cell wall. **Inset.** High magnification showing that the projections are modifications of the outer two layers of the cell wall.

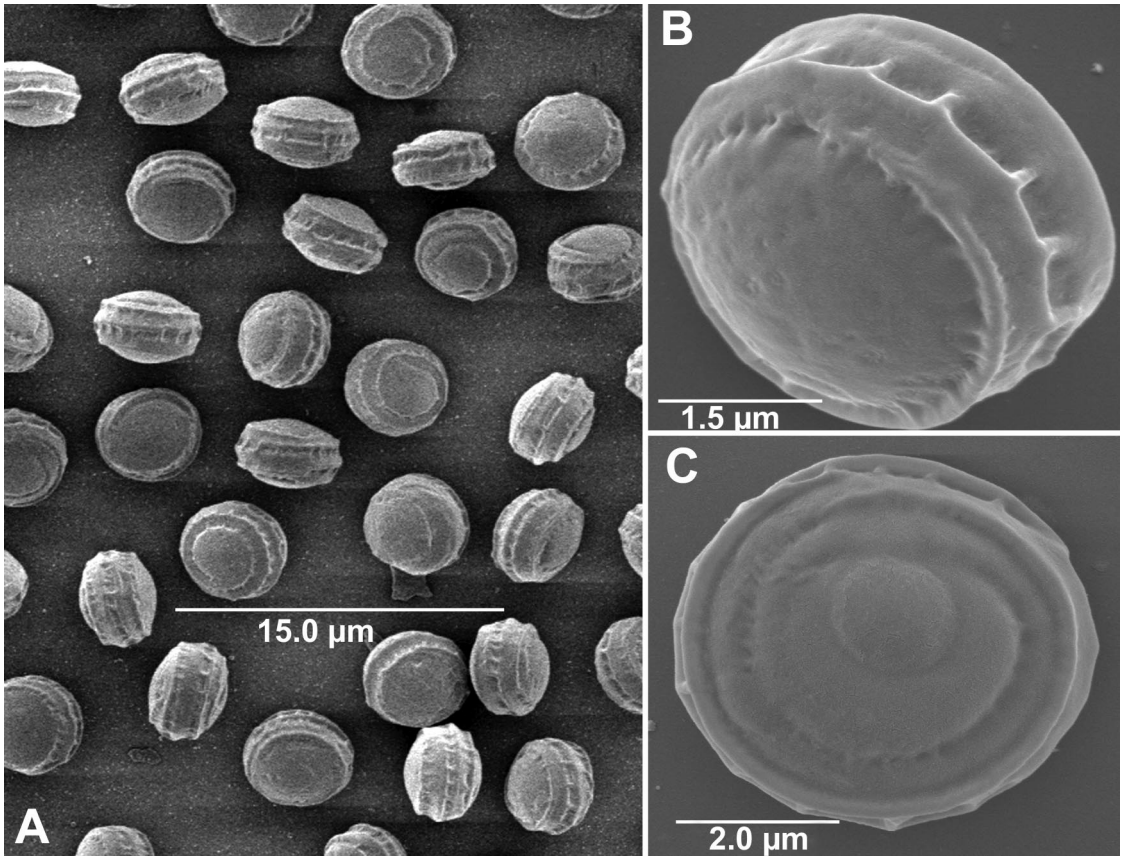

Fig. **2**. SEM of mature cysts of *Helicosporidium* sp. produced in *Helicoverpa zea* larvae. **(A)** Preparation of cysts purified by Ludox gradient centrifugation. **(B)** Narrow surface of a cyst depicting the coiled filament cell underlying the pellicle. **(C)** The broad surface of a cyst.

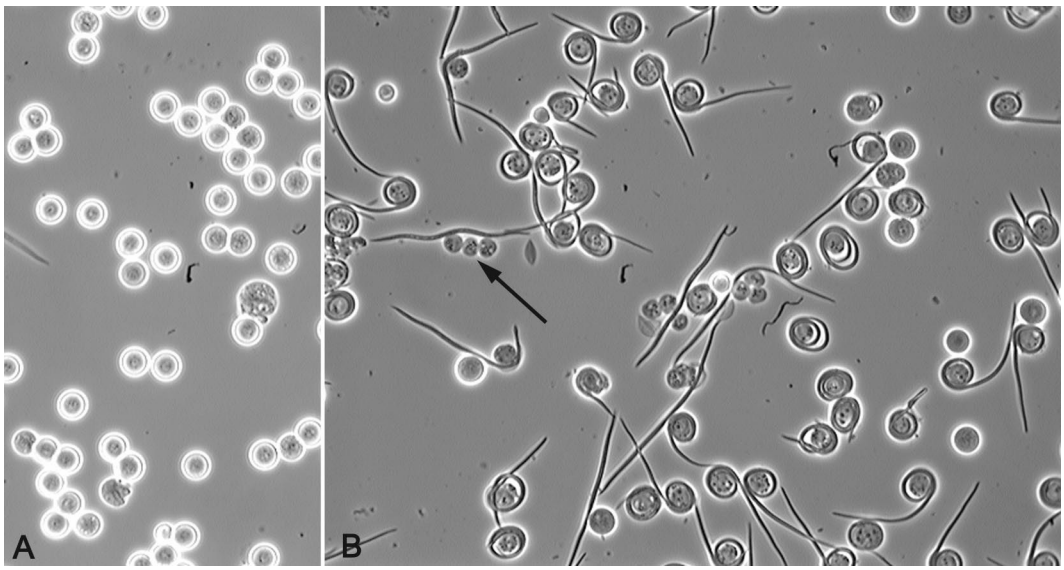

Fig. **3**. Light micrographs of purified cysts of *Helicosporidium* sp. produced in *Helicoverpa zea* larvae. **(A)** Mature discoid cysts. **(B)** Preparation where cysts have opened or dehisced releasing the filamentous cell-ovoid cell complex from the pellicle. The pressure applied to the coverslip results in a fracturing of the pellicle, a swelling of the central ovoid cells, and an uncoiling of the filamentous cells. In several cases, the three ovoid cells and the filamentous cells can be observed in proximity with the pellicle (arrow).

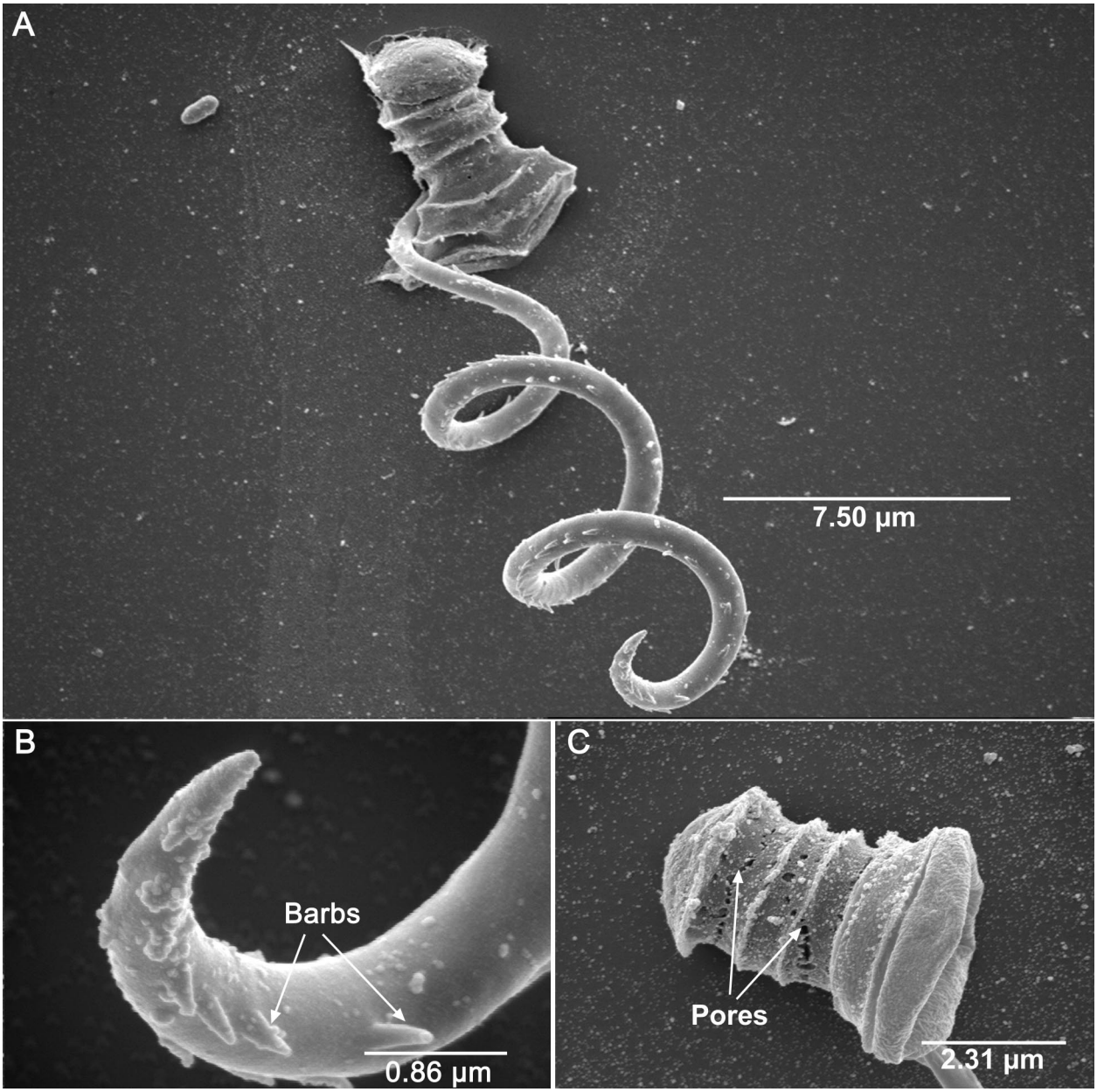

Fig. **4**. SEM of dehisced helicosporidial cyst. **(A)** The uncoiled filamentous cell extending away from the ovoid cell complex. **(B)** The surface of a filamentous cell demonstrating the orientation of the projections. **(C)** The three ovoid cells remain as an aggregate. These have expanded and no longer possess the compressed morphology observed in the cyst stage (cf. Fig 2). Distinct pores can be seen in the previously compressed region of the central ovoid cells.

is a multilaminate structure, enclosing the peripheral filamentous cell within its innermost wall layer. Within intact cysts, three centrally located ovoid cells are compressed in an accordion-like fashion. Each of these cells possesses a peripheral nucleus that encloses a cytoplasmic region that contains a variety of vacuoles and granules.

Helicosporidium sp. from *S. jonesi* produced uniform discoid cysts when grown in *H. zea* larvae (Figs. 2A, 3A). The density of these helicosporidial cysts, measuring 6.2 ± 0.3 × 5.9 ± 0.1 µm, was estimated to be ~ 1.16 g/ml from the Ludox gradient. The cysts, easily partitioned from insect hemolymph components, were virtually free of other components after being passed through the Ludox gradients. SEM revealed that the cyst possessed a ridged biscuit-shaped structure (Fig. 2A, B). On one side of the cyst the coil of the filamentous cell was discerned as a coiled ridge in the cyst wall (Fig. 2B) whereas the opposite side was rounded and possessed no coiled ridge (Fig. 2C).

When stimulated by pressure, the outer pellicle layer of the cysts (Fig. 3A) splits open or dehisces releasing the filamentous cell-ovoid cell complex from the pellicle (Fig. 3B). Release from the cyst stage produces an expanded ovoid cell aggregate and results in the uncoiling of filamentous cells (Fig. 4A, C). The filamentous cells, measuring 37 µm ± 4.3 µm in length by 0.9 ± 0.13 µm in diam., were coated with short projections (340 ± 60 nm) orientated in the same direction providing po-

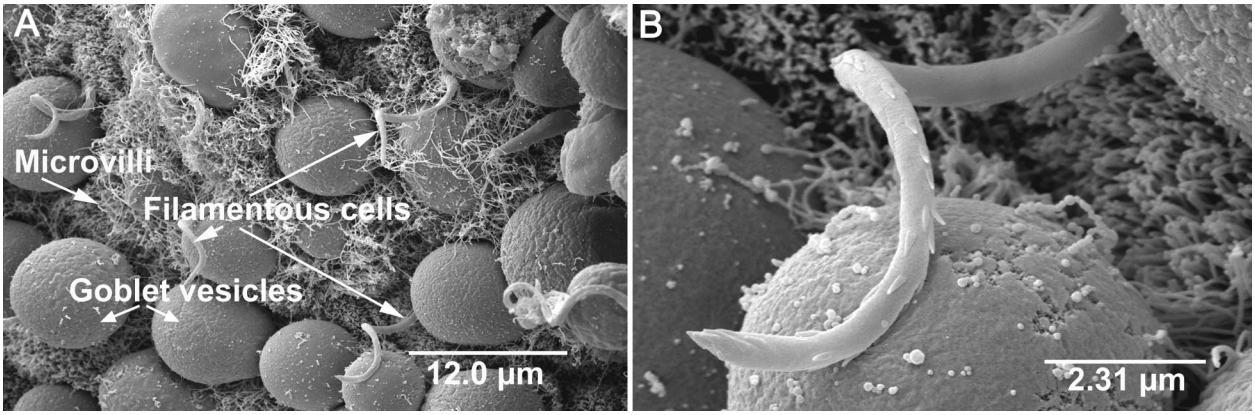

Fig. **5**. SEM of the midgut region of *Manduca sexta* at 4 h post-challenge with *Helicosporidium* sp. **(A)** Filamentous cells have penetrated the microvillar lining of these midgut cells. **(B)** A filamentous cell embedded in a midgut cell demonstrating the orientation of the surface projections.

larity to the filamentous cells (Figs. 1B, 4B). Cyst dehiscence was triggered readily by the application of gentle pressure to the coverslip covering a cyst suspension. Alternatively, the incubation of purified cysts in midgut fluid extracted from *H. zea* larvae stimulated the release of the filamentous cells from cyst suspensions. A 20 min exposure to midgut fluids resulted in more than 50% of the cysts releasing their filamentous cells. Upon activation these cysts increased in volume resulting in pellicle rupture and release of the filamentous cell. Incubated in the midgut luminal fluid the ovoid cells lysed, whereas the released filamentous cell became uncoiled and remained intact. Uncoiled filamentous cells readily clustered with other filamentous cells producing rosettes. Whether this clumping was due to a specific surface adhesion or to simply a result of entanglement of the surface barbs is unknown. The component(s) in the midgut fluid that signals dehiscence is not known; exposure of the cysts to various physical and chemical agents (see Materials and Methods) failed to stimulate the release of the filamentous cells.

In vivo host range and infectious process. Susceptibility varied among mosquito and fly species challenged with the *Helicosporidium* sp. and doses were adjusted accordingly. The dose, resulting infection rates and number of replicates for each species were: *Anopheles albimanus* (5.0×10^4, 58.3%, N eq 1), *An. quadrimaculatus* (5.0×10^4, 100%, N eq 3), *Ae. taeniorhynchus* (3.5×10^2, 14.3%, N = 1), *Cx. quinquefascaitus* (5.0×10^5, 0%, N = 2) and *M. domestica* (7.8×10^8, 80%, N = 1). The IC_{50} (95% Fiducial Limits) for *An. quadrimaculatus* was 1.0×10^4 (3.1×10^3, 3.6×10^4 cysts/ml). All susceptible species supported in vivo replication of the *Helicosporidium* sp. *Culex quinquefasciatus* larvae had a relatively high mortality level (40.0 ± 31.8%) but none of the larval or adult survivors were infected. Filamentous cells were observed in the gut lumens of *An. quadrimaculatus* and *Cx. quinquefasciatus* within 1 hr post-exposure indicating that barriers to infection in *Cx. quinquefasciatus* occurred after cysts dehisced. Examination of tissues with phase-contrast microscopy confirmed that cyst production occurred primarily in the haemocoel of *An. quadrimaculatus* larvae. Light microscopy of the *Helicosporidium* sp. in *An. quadrimaculatus* revealed that the main multiplicative phase began 3 to 4 d post-challenge and was soon followed by cyst production.

Helicosporidium sp. from *S. jonesi* is capable of infecting and replicating in a variety of insects other than Diptera. Oral

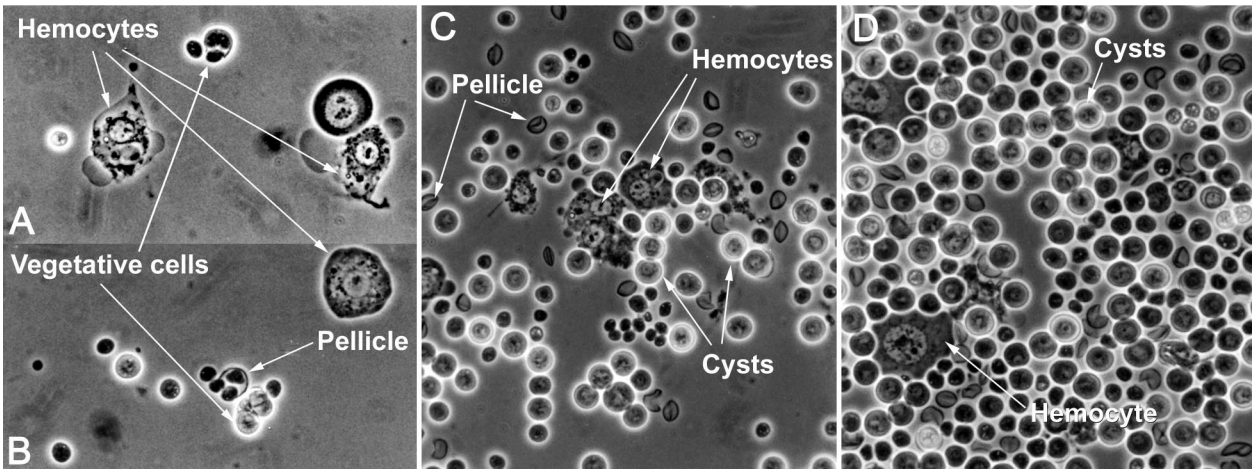

Fig. **6**. Light micrographs of hemolymph sampled from *Manduca sexta* larvae infected with *Helicosporidium* sp. **(A, B)** Two days post-infection extracellular vegetative helicosporidial cells and normal looking hemocytes. **(C)** Six days post-infection plasmodial-like hemocytes displayed cytopathic effects. **(D)** Ten days post-infection. Note the number of refractile cysts and vegetative cells as compared to the numbers of hemocytes.

challenge of *H. zea* and *M. sexta* larvae with cyst preparations was lethal to tested insects. Examination of dissected alimentary tracts revealed that ingested cysts bound initially to the peritrophic matrix in larvae of both challenged *H. zea* and *M. sexta*. Within 2 h post-ingestion, cysts dehisced releasing filamentous cells from the ovoid-cell pellicle complex. SEM of the midguts dissected from *M. sexta* larvae at 4 h post-ingestion revealed that the released filamentous cells penetrated the peritrophic matrix and attached to the midgut columnar epithelium (Fig. 5A). These filament cells penetrated the midgut with the projections oriented away from the penetration point (Fig. 5B), suggesting that these cell wall extensions may play a role in anchoring the filamentous cell to the gut epithelium. In the case of *M. sexta*, vegetative cells were observed in the hemolymph within 2 d post-ingestion (Figs. 6A, B). Vegetative cells, containing variable numbers of cells within the pellicles, were observed to be both associated with circulating hemocytes and present as freely circulating cells. By 6 d post-ingestion, infection suppressed the feeding and growth of *M. sexta* larvae. At this time, plasmodial-like hemocytes displayed marked cytopathic effects (CPE, Fig. 6C). It is unclear whether the *Helicosporidium* sp. induced a haemocyte fusion, blocked cytokinesis, or stimulated hemocyte nuclear division. Within 10–14 d, treated larvae contained massive numbers of mature cysts in the cream-colored hemolymph (Fig. 6D). At this point large numbers of cysts could be extracted easily using several cycles of centrifugation followed by high-speed centrifugation through a Ludox gradient.

The injection of purified cysts into *G. mellonella* larvae resulted in a somewhat different developmental pathway. Within minutes after injection numerous phagocytic hemocytes were observed to contain cysts. By 72 h, clusters of melanized hemocytes were attached to the basement membrane of various tissues. Dissection of these hemocytic granulomas revealed the presence of actively developing colonies of helicosporidial vegetative cells. As time progressed, *Helicosporidium* sp. continued to multiply, appearing by 10 d post-injection associated with the muscles and fat body tissues of these larvae. All larvae injected with cysts died at the larval pupal molt, whereas the control larvae pupated and molted to the adult stage.

In vitro growth and development. *Helicosporidium* sp. isolated from *S. jonesi*, in addition to infecting a range of insects, grew under in vitro conditions. Unlike the in vivo situation, inoculation of imaginal disc cell lines with purified cysts did not result in an immediate dehiscence; at 48 h post-inoculation (p.i.) only a small percentage of cysts released filamentous cells. In all tested insect cell cultures helicosporidial replication occurred in an extracellular fashion. After 10 d, wells inoculated with 5×10^3 cells and 4×10^4 cells produced 2.6×10^5 cells and 1.2×10^6 cells respectively, resulting in a 52- and 30-fold increase in cyst numbers.

Additional assays conducted with TC100 + 10% fetal calf serum (FCS) medium with and without SF-9 cells demonstrated that *Helicosporidium* sp. could develop with or without living cells (Figs. 7A, B). Daily observations of these cultures demonstrated that the helicosporidial cells did not infect the SF9 cells. By 48 h, both media stimulated vegetative growth of helicosporidial cells (Fig. 8A). Single non-motile cells, detected during the vegetative phase, possessed a textured outer surface and measured 2.6 ± 0.33 μm (N = 40) in diam. (Fig. 8C). Like the cyst stage, vegetative cells were enclosed within a pellicle (Fig. 8B), which could contain 1, 2, 4, or 8 vegetative cells. High concentrations of insect cells were observed to restrict the dispersal of progeny cells. Significantly, no lysis of SF9 cells was observed during this vegetative growth period. In most cases, the presence of helicosporidial cells and extracellular material formed a biofilm over the surface; suspended cells were not detected in these cultures. After 72 h, contiguous monolayers of helicosporidial cells were observed in wells lacking the SF9 cells. Gradient centrifugation of vegetative growing cultures (Fig. 9A) produced three distinct bands; the top band contained empty pellicles (Fig. 9B), the middle band contained the vegetative cells (Fig. 9C), and the lower band contained the cyst stage (Fig. 9D).

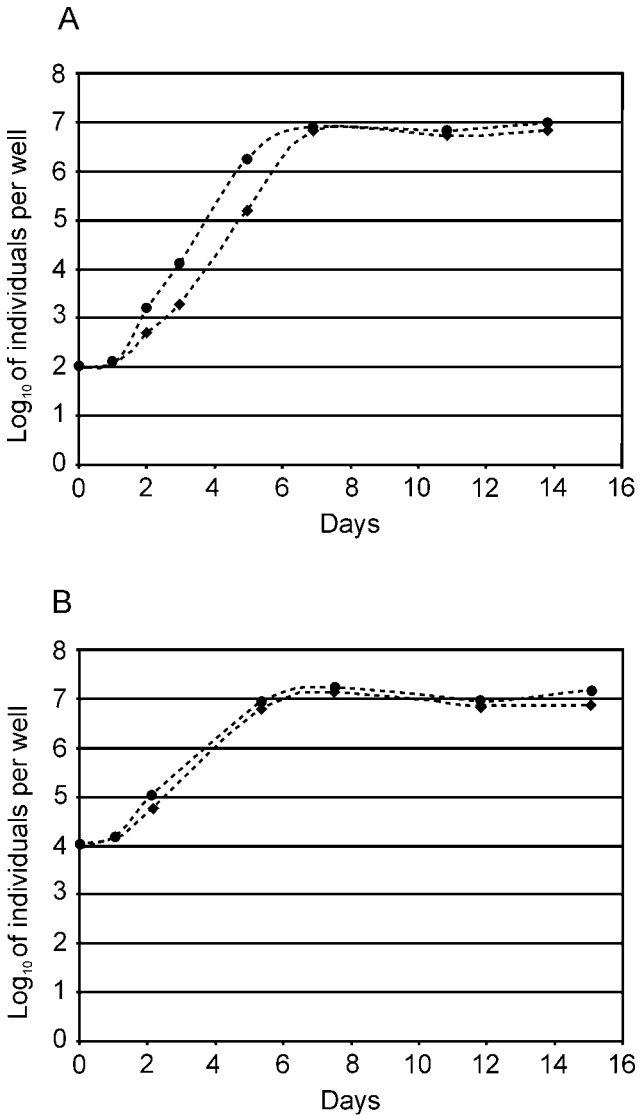

Fig. 7. Growth rate of *Helicosporidium* cultivated at 26 °C. Flasks containing TC100 + FCS media with (●) and without (♦) SF-9 insect cells were seeded with 100 **(A)** or 10^4 **(B)** helicosporidial cells. At intervals the total number of cells in the wells was estimated by hemacytometer counts. Note that after 6-8 d incubation a maximum of 10^7 cells was produced in all four treatments.

All of the tested cell-free media, except CD (minimal salts + glucose media), supported the vegetative growth of this *Helicosporidium* sp. In stationary cultures 12–15 d post-inoculation, this organism produced a continous film over the bottom that coated the surface with mucilaginous cell aggregates that extended into the media. However, less than 10% of the in vitro cells differentiated into mature cysts. These results suggest that the component(s) that signals the in vivo late-stage cyst maturation event, is lacking in the in vitro media. *Helicosporidium*

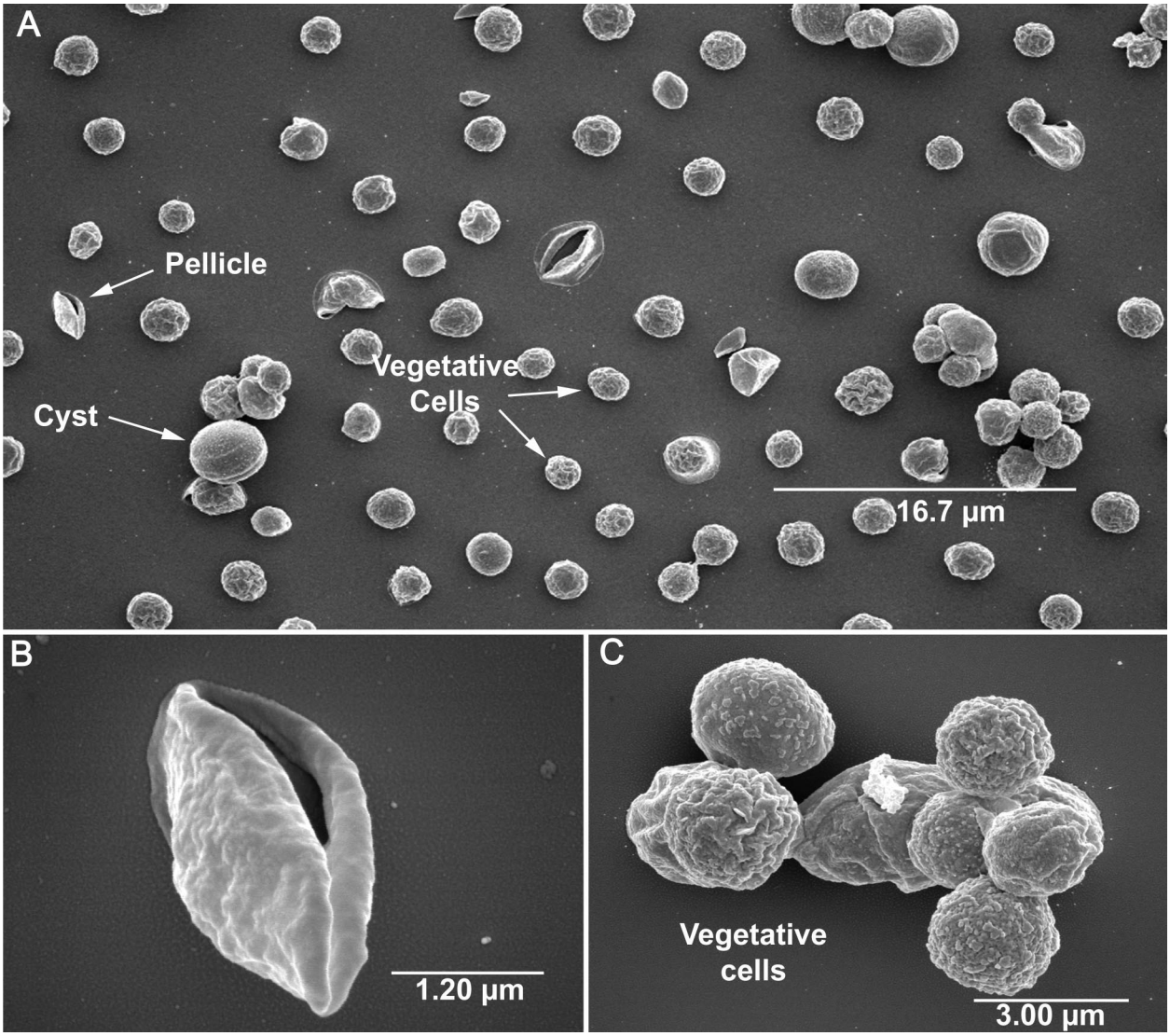

Fig. **8**. SEM of helicosporidial cells produced in vitro. **(A)** Developmental stages of the *Helicosporidium* sp. after 48 h of growth. Note the presence of empty pellicles that have released vegetative cells. **(B)** A pellicle that has split open, like a bivalve mollusc shell, releasing vegetative cells into the medium. **(C)** Vegetative cells, demonstrating a range of different sizes with a wrinkled surface topography that is distinct from that observed with the cyst stage.

sp. did not replicate at 35 °C, and cells exposed to 35 °C for 4 d failed to replicate when transferred to the 25 °C permissive temperature.

RFLP analysis. The genomic DNA of both the in vitro and in vivo cells was easily extracted using the Masterpure™ Yeast DNA kit. Microscopic examination revealed the presence of numerous, highly refractile cysts before treatment; after incubation in the lysis buffer at 50 °C the cells appeared to dehisce. The cyst were split open, releasing the filamentous cells. No massive disruption of the ovoid cells or elongate cells was observed in these preparations. The DNA preparations, electrophoresed on an 0.8% agarose gel and stained with ethidium bromide, produced a single, discrete ~ 20-kb band that could be directly PCR-amplified with the various pairs of rDNA primers. RFLP analysis using both *Eco*RI and *Nde*I to digest the amplified ITS-5.8S region of DNA extracted from both in vivo and in vitro cell preparations produced identical digest patterns (Fig. 10). Similarly the amplified 18S region of both DNA preparations digested with *Hind*III produced identical fragments of approximately 650- and 450 bp.

DISCUSSION

Morphologically, the cyst stage of *Helicosporidium* sp. isolated from *S. jonesi* is similar to that described from helicosporidia detected in other invertebrate hosts. Purrini (1984) conducted an EM examination of *Helicosporidium*-infected orbatid mites and described the cysts as spores containing three uninucleate germ cells and a single filamentous cell. Enclosing the spore was a dense bilayer exosporium and a more transparent inner endosporium. Pekkarinen (1993) identified a hyperparasite of a trematode infecting the mussel *Mytilus edulis* as helicosporidia by the presence of discoidal spores containing three distinct sporoplasms and a coiled filament cell.

Enclosing both the cyst stage and the vegetative stages is a unique pellicle (Lindegren and Hoffman 1976). These cells divide within the pellicle until reaching a four or eight cell stage,

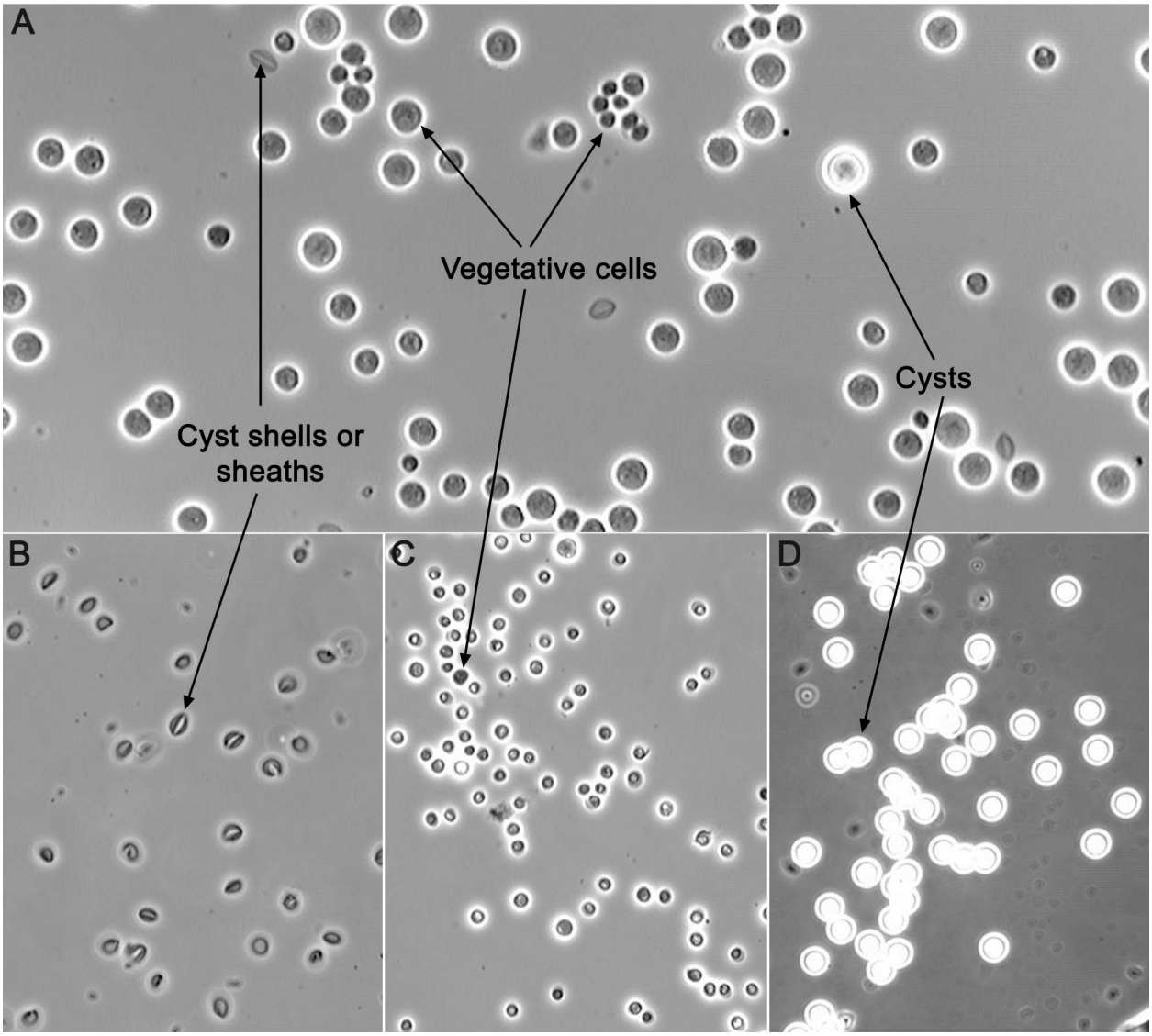

Fig. **9**. Light micrographs of helicosporidial cells produced in vitro. **(A)** Actively growing culture of the *Helicosporidium* sp. demonstrating stages that could be partitioned on a linear Ludox gradient. This produced three distinct bands: a top band containing empty pellicles **(B),** a middle band containing vegetative cells **(C),** and the lower band containing mature cysts **(D).**

after which point they mature into the cyst or spore stage. A similar morphogenesis was observed during both the in vitro and in vivo development of *Helicosporidium* sp. from *S. jonesi.* Wheat germ, concanavalin A, and peanut lectin probes, which bind to sugars containing n-acetyl glucosamine, mannose, and galactose residues, respectively, failed to bind to the surface of either cyst or vegetative cell preparations (Boucias, D. G., unpubl. data). Furthermore, the pellicle layers surrounding the vegetative cells failed to elicit the ''non-self'' recognition defense response of circulating hemocytes. Alternatively, the pellicle layer associated with cysts was recognized as non-self and was rapidly phagocytosed by *G. mellonella* hemocytes.

Relatively few reports have provided details on the in vivo development of *Helicosporidium* spp. Weiser (1970) examined *Helicosporidium* sp.-infected hepialid larva. He observed cells adjacent to a wound in the cuticle and speculated that this organism, like entomopathogenic fungi, gained ingress through the cuticle and then infected all tissues. Our studies show that helicosporidia are transmitted per os. Light microscopic examination of midgut preparations demonstrated that the cyst stage binds to the peritrophic matrix and dehisced. Similarly, the addition of midgut fluid to a cyst preparation activates dehiscence. Upon activation the pellicles rupture and release filamentous cells. Incubated in the midgut luminal fluid the ovoid cells lysed, whereas the released filamentous cell became uncoiled and remained intact. Significantly, direct observations demonstrate that the invasive cell is probably the filamentous cell and not the ovoid cells that have previously been believed to represent sporoplasms. Adhesion of intact cysts to the peritrophic matrix suggests that mechanical pressure expressed during cyst dehiscence may mediate ingress though the midgut barriers (peritrophic matrix and/or the microvillar membranes). The released filamentous cells are long enough (37 ± 4.3 μm) to breach the peritrophic matrix (1–5 μm) and the ectoperitrophic space, then gain entry into the midgut cell. Under in vitro conditions the filamentous cells will thicken and divided twice, forming a linked four-cell complex. Morphologically, these thickened cells were similar to the elongate cell phenotype

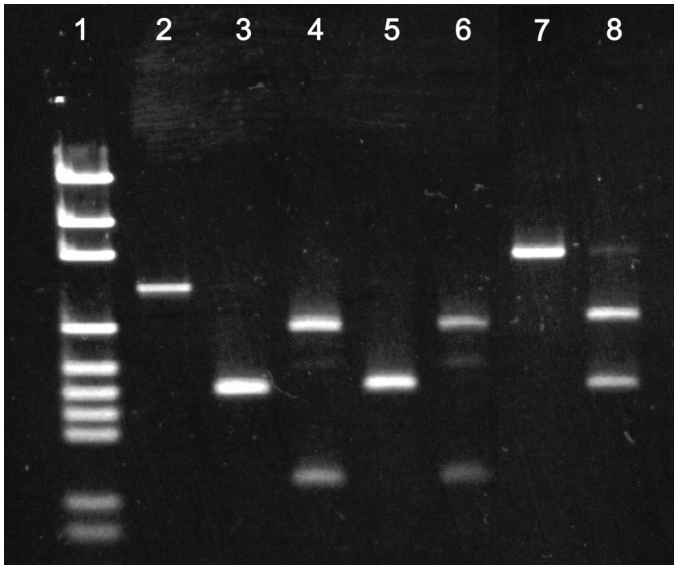

Fig. **10**. RFLP of PCR-amplified DNA from *Helicosporidium* sp. produced in vitro and in vivo. **Lane 1:** pGEM standards. **Lane 2:** uncut 950 bp PCR-amplified ITS1-5.8S-ITS2 region. **Lanes 3-6:** The ITS1-5.8S-ITS2 fragment amplified from genomic DNA extracted from either in vivo (lanes 3, 4) or in vitro (lane 5,6) produced identical digests when cut with EcoRI (lanes 3, 5) and NdeI (lanes 4,6). **Lane 7:** uncut 1100 bp PCR amplified 18S fragment. Lane 8: HindIII digest of fragment 7.

described by Kellen and Lindegren (1974). Potentially, the filamentous cells upon penetration of the host columnar epithelium differentiate into an intermediate stage that gains ingress through this tissue and enters the hemocoel. Within the hemocoel, vegetative cells replicated in an extracellular fashion without stimulating a host defense reaction. The insect hemocytes at this stage produced pseudopodia and displayed a normal spreading behavior, suggesting that *Helicosporidium* sp. can successfully evade the functional cellular defense response. These observations suggest that the pellicle of these vegetative stages may mimic host surface epitopes. A similar scenario has been found with the insect mycopathogen *Nomuraea rileyi*, which has an outer surface that mimics the insect basement membrane (Pendland and Boucias 1998). However, in the injected waxmoth larvae, mature cysts were recognized as ''nonself'' and elicited a rapid host-cell defense response.

Over the years, Helicosporidia have been associated with various taxonomic groups. Weiser (1964) concluded that the vegetative stages and cyst stage were unlike any known protozoan's and proposed that *Helicosporidium* be clustered with the primitive Ascomycetes in the subfamily Nematosporideae. However, other investigators have been unable to associate this organism with either protozoan or fungal taxa. In several respects, we have found that the vegetative cycle of *Helicosporidium* sp. from *S. jonesi* is similar to that reported for unicellular, achorophyllic algae belonging to the genus *Prototheca* (Pore 1985). Members within this genus are characterized by production of spherical or oval cells within which are contained anywhere from two to 16 smaller cells. Similar to Helicosporidia, the vegetative cells of *Prototheca* split open or dehisce, releasing daughter cells (~3.0 μm diam.) from the parent cell wall or pellicle. Interestingly, protothecans, affiliated with aquatic environments, are known to be pathogenic to a variety of vertebrates including humans (Galan et al. 1997; Mohabeer et al. 1997). The anuran gut parasite, originally named *Prototheca richardsii*, also produces cells that are morphologically similar to the helicosporidial vegetative cycle. *Prototheca richardsii* has been reclassified recently into the genus *Anurofeca* and placed within the Ichthyosporea, a clade of protists having a phylogenetic position at the fungal-animal divergence (Baker et al. 1999). It is important to note that none of the *Prototheca* species is known to produce the filament cell-ovoid cell cyst that is the diagnostic characteristic of the *Helicosporidium* spp.

Helicosporidium sp., capable of in vitro development in various media, possesses growth requirements much simpler than the typical entomopathogenic protozoa. The minimal nutritional requirements for growth and development suggest that this *Helicosporidium* sp. may have a free-living lifestyle in the aquatic environment until cysts are ingested by a host organism. Survival of this *Helicosporidium* is enhanced further by having a broad host range and by producing large numbers of cysts in the nutrient-rich host environment. Potentially, the parasitic portion of the life cycle would also provide a mechanism for spread to new habitats. This type of life history is similar to *Prototheca* species that have both free-living and parasitic developmental sequences. The association between *Helicosporidium* and the algae is supported by recent comparative analysis of DNA sequence data: actin and tubulin genes have been shown to be clustered with the clade of green algae (Tartar, A., pers. commun.).

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