Perforation and Lysis of Hyphae of *Rhizoctonia solani* and Conidia of *Cochliobolus miyabeanus* by Soil Myxobacteria

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ABSTRACT

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Pigmented hyphae of *Rhizoctonia solani* and conidia of *Cochliobolus miyabeanus* were lysed during burial for 4 wk in field soils. Many small perforations (0.1–2.4 μm; average 0.4 μm) and hollows etched to various depths were observed by SEM in cell walls of the propagules. A species of myxobacteria was isolated from hyphae and conidia in various stages of lysis and cultured on conidia of *C. miyabeanus* suspended in 10% soil extract. The dendriform colonies advanced by streaming at 50–70 μm/hr as groups of cells. Rods crowded around the fungal cells, perforated the cell walls, and emptied the cell contents completely within 6–12 hr after contact between a streaming colony tip and the cell wall. The streaming colony left the emptied cell with many perforations and hollows in the walls that matched those observed on fungal material recovered from soil. Based on size and shape of the vegetative rods, fruiting bodies, and myxospores, the bacteria seem to fit systematically into the family Polyangiaceae and the genus *Polyangium*, but species identification cannot be made yet with certainty.

The hyphae of *Rhizoctonia solani* Kühn are relatively resistant to degradation in soil (12,14) because of their melanized cell walls (19). Nevertheless, antagonists with ability to lyse or parasitize resistant propagules play a role in the biological control of some pathogens (4). Recently, vampyrellid amoebae were shown to perforate and destroy melanized propagules (1,15), including hyphae and sclerotia of *R. solani* in soil (9). *Trichoderma* spp. also play a role in the destruction of propagules of *R. solani* in some soils (3,6,11).

During a study of mycophagous amoebae in various field soils of Japan, microorganisms with the morphology and behavior of myxobacteria were observed in association with perforated hyphae of *R. solani* and conidia of *Cochliobolus miyabeanus* (S. Ito et Kuribayashi) Drechsler ex Dastur. Myxobacteria may be lytic to eubacteria, yeasts, and algae (5,13). Hocking and Cook (8) reported that soil myxobacteria exhibited lytic activity to cultures of some soilborne plant pathogens and partially suppressed damping-off of conifer seedlings. Except for these reports, virtually nothing is known of the effect of these bacteria on plant pathogens in soil.

This report deals with isolation of the myxobacteria responsible for lysis of pigmented propagules of *R. solani* and *C. miyabeanus* in soil, with observations of their morphology, stages of the feeding and lysing process when cultured on fungal cells, and with their possible effect on the population of *R. solani* in soil.

MATERIALS AND METHODS

Soils. Soil samples were collected in June 1981 from a field in Maebashi, Gunma Prefecture, where wheat (*Triticum aestivum* L.) and burdock (*Arctium lappa* L.) had been grown each year for more than 5 yr. The soil was a clay loam, pH 6.0. It was blended, passed through a 4.0-mm sieve, and either used immediately for experiments, or stored for about 2 yr in a plastic bag at room temperature before use.

Fungi. Conidia of *C. miyabeanus* were used to isolate the myxobacteria from soil and culture them. The fungus was grown in darkness for 2 wk at 25 C on potato-sucrose agar (PSA) in a petri dish and then under light for 1 wk to promote sporulation. Conidia were collected with a camel's-hair brush, washed in distilled water by centrifugation (1,500 rpm), and then resuspended in 10% soil extract (SE) (1). The activity of myxobacteria and their ability to lyse fungal cells was observed on mycelial mats of *R. solani* (isolate 1272, AG-4) in 10% SE. This fungus was cultured on a sheet of cellophane on either 1/10 PSA or water agar in a petri dish. After 2 wk at 25 C, the cellophane sheet with adhering mycelium was cut into pieces ~ 1 × 1 cm and floated on distilled water. The mycelial mat was separated from the cellophane and then washed in distilled water before use in SE.

Detection and isolation of myxobacteria. The occurrence of myxobacteria in soil samples was determined by use of the "sandwich" method of Old (15). Either a mycelial mat of *R. solani* or conidia of *C. miyabeanus* were placed on either a Millipore filter (10 mm in diameter, 1.2-μm pore size, Millipore Corp., Bedford, MA) or a Toyo membrane filter (13 mm in diameter, 1.0-μm pore size, Toyo Roshi Co., Ltd., Nihonbashi, Tokyo, 103 Japan). Each filter with adhering fungus material was then sandwiched between two Nuclepore filters (25 mm in diameter, 5.0-μm pore size, Nuclepore Corp., Pleasanton, CA) and the margins of the filters were sealed with vacuum grease. The sandwiches were buried in moist soil contained in a plastic petri dish (90 mm in diameter) with the lid perforated for aeration. The sandwiches were removed from the soil after 4 wk at 25 C, and the fungal materials were used for SEM observation and isolation of myxobacteria. The fungal material recovered from soil was placed in a conidial suspension of *C. miyabeanus* in 10% SE to obtain myxobacteria. Nearly pure cultures of the myxobacteria were obtained by transplanting repeatedly to new conidial suspensions with a micropipet.

Observation of morphology and feeding behavior of myxobacteria. Observations on the morphology of colonies, cells, and fruiting bodies, and of the feeding process, were made directly in the cultures of conidial suspension in plastic petri dishes (35 mm in diameter) under either an interference phase contrast microscope

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or an inverted phase contrast microscope. Time sequence observations were made and photographs were taken to clarify the stages of lysis of hyphae of *R. solani* and conidia of *C. miyabeanus*.

SEM procedures. Hyphal mats of *R. solani* or conidia of *C. miyabeanus*, recovered from soil or from aquatic cultures, were immediately fixed in 5.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for more than 2 hr. The materials were dehydrated in a graded ethanol and iso-amylacetate series, critical-point dried, coated with a gold layer by an ion sputter-coating apparatus (Eiko IB-3), and then viewed in a SEM (JEOL JSM 35CF-II).

RESULTS

Isolation of myxobacteria from soil. Many small perforations and hollows were observed on the cell walls of hyphae of *R. solani* (Figs. 1 and 2) and conidia of *C. miyabeanus* recovered from soil after incubation in sandwiches for 4 wk. Average diameter of these structures was 0.4 μm but ranged between 0.1 and 2.4 μm. The perforations occurred in groups on the surface of a cell wall and sometimes coalesced into large holes. The hollows were etched to various depths in the cell walls (Fig. 2). Large perforations and annular depressions (1–6 μm in diameter) typical of those caused by vampyrellid amoebae (1,9,15) were also observed in the fungal walls in this experiment.

Among the many filaments, cocci, and rods of microorganisms observed in association with fungal propagules recovered in the sandwiches from soil, dendriform colonies with rod-shaped bacteria lying end-to-end and longitudinally in rows or columns were frequently observed on and near the hyphae of *R. solani* and conidia of *C. miyabeanus* (Fig. 3). The rods were coated with a slimy substance (Fig. 4). The perforations and hollows in the fungal walls and the presence of these unusual bacterial colonies occurred in both fresh soil and soil that had been stored for 2 yr. The colonies observed are typical of myxobacteria (13).

Colonies and cells of myxobacteria. Dendriform colonies of myxobacteria were observed in aquatic cultures on the bottom of petri dishes during several days after incubation of Millipore filters with the adhering fungal materials from soils with fresh conidia in 10% SE. The strandlike colonies were dense with bacterial cells at the middle and more dispersed (fan-shaped) at the tip (Fig. 5). The vegetative cells advanced slowly as groups on the bottom of the dish, forming streams 10–30 μm in width and spreading radially more than 10 mm. Cells frequently occurred as pairs in a stream. Streaming colonies sometimes branched and then the individual branches joined each other again. The rate of movement depended on cultural conditions, and was recorded at 50–70 μm/hr in a conidial suspension of 10% SE at 25 C. A track of slime was left behind each streaming colony on the bottom of the petri dish. Vegetative cells were flexible, uniformly cylindrical, and nonflagellate with blunt rounded ends. The rods were 0.5–0.8 × 2.7–5.2 μm and surrounded by mucilaginous material.

Fruiting bodies. In cultures older than 1 wk, the colony frequently ceased to advance and the rods crowded into clumps and then into solitary rounded masses after 5–7 hr. After one to several days, each rounded mass, enclosed in an envelope (Fig. 6), became pale brown and optically refractile. These fruiting bodies occurred singly or sometimes in groups of two to ten, and either in the suspension or inside emptied fungal cells. SEM observations revealed that the fruiting body consists of a dense clump of rods surrounded by a mucilaginous envelope (Fig. 7). The mature fruiting body is spherical to elliptical, pale brown to red brown, and 17.1 μm (12.5–22.5 μm) in diameter. The rods making up the fruiting body are often termed myxospores (13); they resemble vegetative cells but are slightly shorter (0.5–0.8 × 1.6–4.5 μm). When fresh conidia were added, the rods dispersed immediately from immature fruiting bodies and again began to stream. However, germination of pigmented, mature fruiting bodies was not observed.

Feeding behavior and perforations. Hyphae of *R. solani* were placed in 10% SE in a small plastic petri dish and a drop of myxobacterial culture was introduced with a micropipet. Colonies of myxobacteria with characteristic streaming developed in association with hyphae. When the tip of a colony contacted the surface of a hyphal wall, it surrounded the cell as a sheath of rods. The sheath became progressively thicker and advanced along the hypha (Fig. 8). The bacterial cells invaded hyphae of *R. solani* extensively, but during the feeding process it was difficult to observe the hyphal cell through the myxobacterial sheath. The colony streamed away from the emptied hyphae at 6–12 hr after initial contact between the bacteria and the hypha (Fig. 9). Many perforations were evident on the cell walls of the emptied hyphae even under the light microscope.

The feeding process was observed more clearly with conidia of *C. miyabeanus* than with hyphae of *R. solani*. The rods gradually crowded around a conidium after contact, forming clumps of cells, especially at both sides of a conidium (Fig. 10). The stream of bacteria sometimes destroyed the wall at the end of a conidium (arrow) within one to several hours of contact and then invaded the conidium through this hole (Fig. 11). The 10–20 cells in each conidium were lysed and gradually disappeared, beginning at the tip, after the invasion of bacteria into the conidium. The bacteria reproduced to make a large colony around the conidium by 4–6 hr, with little movement of the colony to another target during this process (Fig. 12). By 6–12 hr, the contents of the conidium were destroyed and it was filled with rods of the myxobacterium. The colony of myxobacteria began to stream again at 12–24 hr, moving to another place and leaving the emptied conidium with many wall perforations behind (Fig. 13).

SEM observations of the emptied hyphae of *R. solani* or conidia of *C. miyabeanus* revealed that the perforations and hollows on the cell walls were identical in size and shape to those observed on the same fungal materials recovered from soil.

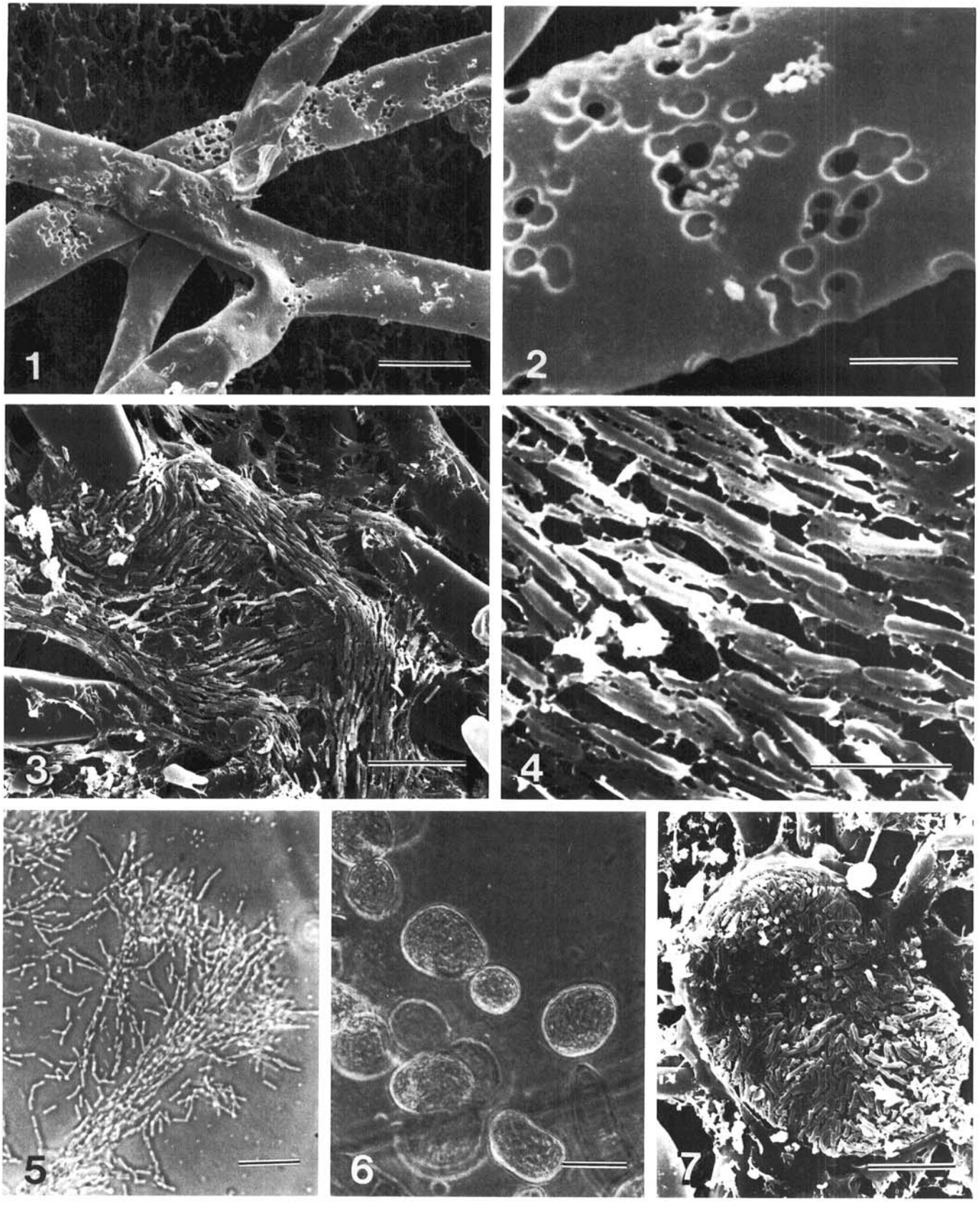
Life cycle of the myxobacterium in aquatic culture. Some of the stages in the life cycle of the myxobacterium observed in aquatic culture are illustrated schematically in Fig. 14. At least 12 hr and up to 36 hr were required for a complete cycle under the cultural conditions described above, ie, a cycle starting from the introduction of a new conidial suspension until the formation of the mature fruiting body.

DISCUSSION

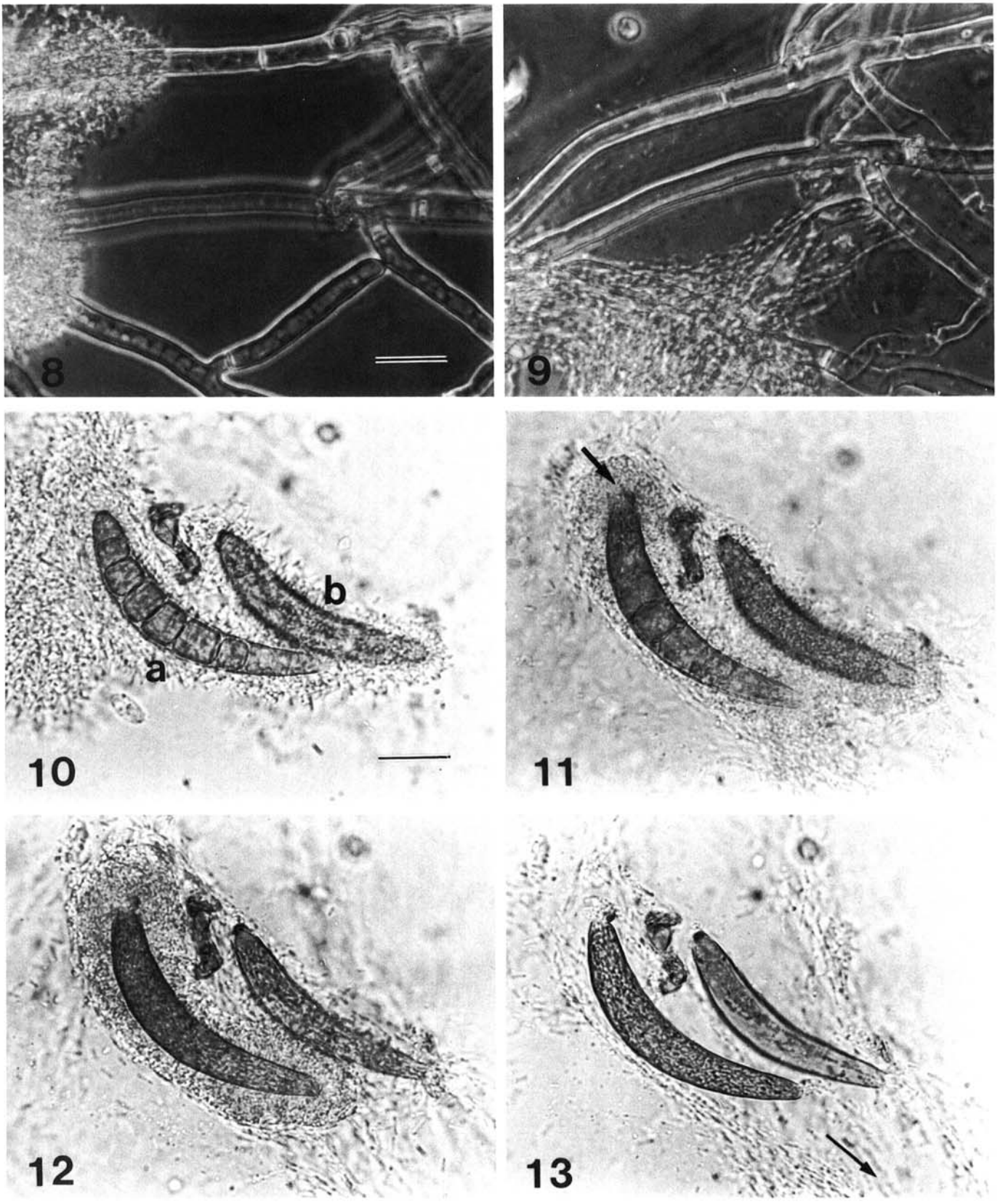
The small perforations and etched hollows observed on the cell walls of hyphae of *R. solani* and conidia of *C. miyabeanus* recovered from soils apparently are caused by one or more species of myxobacteria because colonies typical of myxobacteria (13) were constantly observed in association with perforated and etched fungal materials recovered from soil, and myxobacteria obtained from lysed fungal material from soil and cultured on fresh hyphae and conidia in water multiplied as a sheath around hyphae and conidia and advanced as colonies of mobile cells over and through hyphae and conidia, leaving fungal propagules without cross walls and cell contents, and leaving the outer walls with perforations and etched hollows identical to those observed on material from soil. Evidence from SEM observations of the perforations and hollows, and from sequential observation of the feeding process indicated that the myxobacteria caused erosion and perforation of cell walls by enzymatic action, then invaded the cells through the perforations and fed on the cell contents.

The etched hollows were the same size as perforations and hence were apparently incomplete perforations. The hyphal walls of *R. solani* consist of an electron-transparent outer layer and a more electron-dense inner layer (2,20). These walls developed many more hollows than complete perforations (Fig. 2), possibly because the outer layer is more susceptible than the inner layer to erosion by myxobacteria.

Hyphae of *R. solani* are relatively resistant to degradation in soil (12,14), possibly because of melaninlike materials in the walls (19). Nevertheless, hyphae of this fungus are lysed in soil (10,12,14,16,17). Lytic microorganisms or mycoparasites (eg, *Trichoderma* spp.) are known to attack melanized propagules and to affect the biology of soilborne plant pathogens in soil (4). Recently, mycophagous soil amoebae were found to perforate and destroy fungal propagules with melanized or pigmented cell walls,



Figs. 1–7. Light and scanning electron micrographs of etched hollows and perforations in cell walls of *Rhizoctonia solani* and *Cochliobolus miyabeanus*, and of the morphology of myxobacteria associated with etched and perforated fungal material. **1,** Perforations and hollows in cell walls of hyphae of *R. solani* recovered from soil after 4 wk of incubation (bar = 10 μm). **2,** Larger magnification of Fig. 1 (bar = 2 μm). **3,** Colonies with cells of myxobacteria lying end-to-end as columns among conidia of *C. miyabeanus* recovered from soil after 4 wk of incubation (bar = 20 μm). **4,** The vegetative and apparently mobile rods of myxobacteria contained in slime (bar = 5 μm). **5,** Portion of a colony showing a typical arrangement of rods in columns of two and with a fan-shaped terminus (bar = 20 μm). **6,** Fruiting bodies of the myxobacteria (bar = 20 μm). **7,** Larger magnification of a fruiting body, showing the dense arrangement of myxospores and a mucilaginous envelope surrounding the clump of myxospores (bar = 10 μm).



Figs. 8–13. Optical micrographs showing stages of lysis of hyphae of *Rhizoctonia solani* (Figs. 8–9) and conidia of *Cochliobolus miyabeanus* (Figs. 10–13) by the myxobacteria. **8,** Myxobacteria as a sheath around and streaming over the hyphal cells of *R. solani*. Note that the cell contents can still be observed (bar = 20 μm). **9,** A later stage of Fig. 8, showing emptied hyphal cells after 9 hr. **10,** Conidia of *C. miyabeanus* (a) just attacked by the myxobacteria, and (b) already emptied (time 0, bar = 20 μm). **11–13,** At time 2, 4, and 6 hr after Fig. 10, showing gradual disappearance of conidial cells, a broken tip on one conidium (arrow) surrounded by a clump of myxobacteria, the gradual entry of bacteria into the conidium, and finally **13,** the myxobacteria moving in streams away (direction indicated by arrow) from the emptied conidia (time, 10 hr).

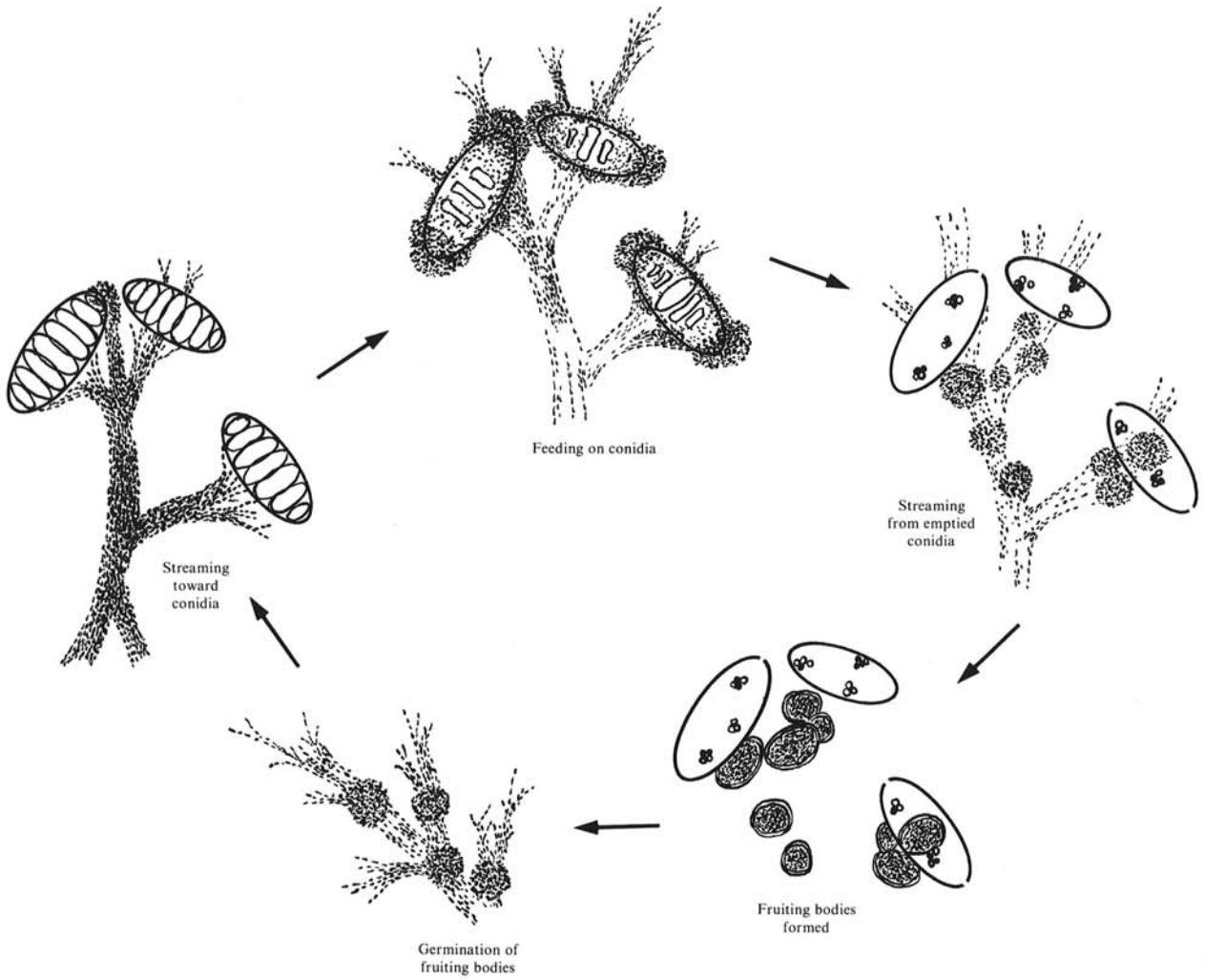


Fig. 14. Schematic representation of the various stages in the life cycle of the mycophagous myxobacterium in aquatic culture.

including hyphae and sclerotia of *R. solani* (1,9,15). Cook and Baker (4) suggested that amoebae may be important mainly in destruction of pathogen inoculum during dormant or saprophytic existence of the pathogen in soil. Similarly, the myxobacteria are likely to affect dormant or saprophytic survival of *R. solani*, and perhaps other pathogens that exist in soil as pigmented propagules, hastening the death of propagules and thereby causing the pathogen population to decrease more rapidly than if the myxobacteria were not present. Additionally, these bacteria may also have potential to guard the infection court against a pathogen; Hocking and Cook (8) reported that myxobacteria experimentally introduced into soil actively colonized the rhizosphere of conifer seedlings without affecting seedling development and gave partial control of damping-off of the seedlings. They also stated that the myxobacteria identified as *Cytophaga johnsoniae* and *Sorangium* (*Polyangium*) sp. exhibited lytic activity to cultures of *Pythium intermedium, R. solani, Fusarium oxysporum,* and *F. solani.*

Peterson (18) suggested that although myxobacteria are cosmopolitan and occur in large numbers in a wide variety of habits, they generally go unrecognized because of their slow growth, poor ability as competitors, and lack of proper isolation techniques. In this experiment, myxobacteria were detected and isolated by the "sandwich" method devised by Old (15) for baiting mycophagous amoebae from soil. Presumably the myxobacteria entered the "sandwich" through pores in the Nuclepore filters and then reproduced on the fungal materials sandwiched between the filters during incubation in soil, in the same way described for the vampyrellid amoebae (15).

Myxobacteria are characterized by production of a polysaccharide slime, which results in distinctive slimy colonies; ability to glide on solid surfaces; and production of resistant fruiting bodies (5,13,18). The family Polyangiaceae is distinguished from other families of myxobacteria by the occurrence of vegetative cells of uniform diameter with blunt, rounded ends and with myxospores that resemble vegetative cells. The genus *Polyangium* is characterized by sessile sporangia and cells that are rod-shaped and never coccoid (13). According to these criteria, the mycophagous bacterium described herein seems to belong in the family Polyangiaceae and the genus *Polyangium.* Based on dimensions of the vegetative rods, fruiting bodies, and myxospores, and based on the nature of necrotic parasitism, the bacterium is similar to *P. parasiticum* Geitler (7,13). However, identification of the species cannot be made with certainty. The myxobacterium observed in the present study have not been cultured on agar media.

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