How cellular slime molds evade nematodes

(predator/prey/amoeba/soil ecology/chemotaxis)

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ABSTRACT We have found a predator–prey association between the social amoeba *Dictyostelium discoideum* and the free soil living nematode *Caenorhabditis elegans*. *C. elegans* feeds on the amoebae and multiplies indefinitely when amoebae are the sole food source. In an environment created from soil, *D. discoideum* grows and develops, but not in the presence of *C. elegans*. During development, *C. elegans* feeds on amoebae until they aggregate and synthesize an extracellular matrix called the slime sheath. After the sheath forms, the aggregate and slug are protected. Adult nematodes ingest *Dictyostelium* spores, which pass through the gut of the worm without loss of structure and remain viable. Nematodes kill the amoebae but disperse the spores. The sheath that is constructed when the social amoebae aggregate and the spore coats of the individual cells may protect against this predator. Individual amoebae may also protect themselves by secreting compounds that repel nematodes.

Cellular slime molds are soil protozoans that feed on bacteria. When confronted with starvation, the amoebae aggregate and undertake an elaborate developmental program. The motility of the amoebae and the development of the organism have provoked the interest of biologists for many years (1). Single amoebae aggregate and form slugs of 100,000 cells; each slug culminates to create a ball of spores on a slender stalk. About 20% of the cells die in the formation of the stalk. The positioning of spores supported on top of a stalk may aid in dispersal of the spores by soil arthropods or annelids (2, 3). The life cycle, which has never been seen in the wild, is usually imagined to occur without interference, and no protective value against predators has been ascribed to early stages in development, such as the aggregate or the slug.

Less attention has been paid to the evolution and ecology of the organism than to its motility and development, yet the elaborate mechanisms that these soil amoebae use to aggregate and form fruiting bodies did not evolve in isolation from predators. A variety of amoebae are commonly found in soil (4, 5), but we do not know what percentage of these have multicellular capability. Other multicellular soil denizens include fungi, nematodes, microarthropods including mites and other arachnids, and annelids (6). For reasons of scale and abundance, we think that the most likely to feed directly on individual amoebae are the nematodes. In this report we show that *Caenorhabditis elegans* feeds on slime mold amoebae, but that multicellular development provides protection from nematode predation. The putative relationship between the slime molds and the nematodes has additional features: dauer larvae climb into the spore masses and disrupt them. Adult worms disperse the spores. Using fresh isolates, we found that amoebae of *Dictyostelium purpureum* repel a species of nematode found in the same soil. Several aspects of the life cycle may

enhance survival in an environment that also supports a ubiquitous predator.

MATERIALS AND METHODS

Culture Conditions: Coculture of Cellular Slime Molds and Nematodes. Cellular slime molds and nematodes were grown on lawns of *Klebsiella aerogenes* on SM or SM/5 plates as described by Sussman (7). The plates were spread with a suspension of the bacteria and then appropriate numbers of amoebae were deposited. *C. elegans* adult hermaphrodites were deposited with a pick. For growth on a stable lawn of amoebae, 200 μ l of a log phase axenic culture ($\approx 5 \times 10^6$ cells/ml) of the mutant UK7 was spread on a Petri dish containing 10 ml of HL/5 medium (8) solidified with 1.5% agarose. The initial density of amoebae was 5×10^4 – 10^5 /cm². UK7 amoebae grow to form an even lawn but do not aggregate as wild-type cells do. *C. elegans* adult hermaphrodites were deposited on the amoebal lawns. For growth in soil, the methods of Singh (9) were used. Briefly, potting soil was autoclaved and 60 g were deposited in a 150 mm diameter Petri dish. A stationary phase suspension of *K. aerogenes* was diluted 1:20 in water and 20 ml were added to the soil. Cellular slime molds and nematodes were deposited in the soil as described above. Fresh isolates of several cellular slime mold species and several nematode species were recovered from North Carolina soils by plating soil samples on LP agar supplemented with bacteria as described by Cavender and Raper (10). Chemotaxis of nematodes was analyzed as described by Ward (11) using Phytigel (Sigma) as a transparent substratum.

Labeling Cells and Fluorescence Microscopy. A 1 mM solution of di-di-octadecyl-3',3'',3-tetraethyl-indocarbocyanine perchlorate (DiI; Molecular Probes) in 1% bovine serum albumin was diluted 1:500 in a log phase culture of amoebae (AX3) growing in HL/5 medium. Mutant amoebae (aggregation defective, UK7) were grown overnight in the dark and then washed three times in cold sterile 17 mM Na/K phosphate buffer (pH 6.0). About 10^7 amoebae were placed on a 60-mm Petri dish containing 10 ml of HL/5 medium solidified with 1.5% agar. A number of adult and larval nematodes (N2 strain, wild type) were placed on the lawn of amoebae and allowed to feed for 3 h. Nematodes were then removed to 10 mM Na-azide (to stop movement) and viewed with a Nikon epifluorescence microscope equipped with appropriate filters. Images were acquired with a silicon intensified target (SIT) camera (32–64 frame average) and digitized with an Image 1 image processing package (Universal Imaging, West Chester, PA). Phase and fluorescence images were combined with Image 1 and then printed with a dye sublimation printer. To stain spores, 1% calcofluor white M2R (ref. 12; Sigma, fluorescent brightener 28) was used. Calcofluor binds cellulose in the spore wall. Spores were incubated for 30 min in a 1%

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Abbreviation: DiI, di-di-octadecyl-3',3'',3-tetraethyl-indocarbocyanine perchlorate.

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solution in water, washed, and viewed as above except that a filter set for blue fluorescence was used.

Scanning Electron Microscopy. Mixed cultures of *Dictyostelium discoideum* and *C. elegans* were developed on Millipore filters and then fixed and prepared for scanning electron microscopy by placing the filter on a solid brass cylinder that had been cooled in liquid nitrogen. The filter and cylinder were placed in a Kinney vacuum evaporator and left at high vacuum for 48 h to dry the fruiting bodies and nematodes by freeze sublimation. The dried filters were coated with gold-palladium and examined with a Hitachi S-570 scanning electron microscope.

RESULTS

We first asked whether nematode larvae and adults feed on *Dictyostelium* amoebae. Single larvae were plated on the HL/5 axenic medium of *Dictyostelium* solidified with agar. When no amoebae were provided, the nematodes did not increase in number and died. When *Dictyostelium* amoebae were supplied, the nematodes matured and laid eggs that hatched. The resultant larvae developed into adults and cleared the dish of amoebae. The nematodes grew with a generation time of 3–4 days, approximately the same as on lawns of *Escherichia coli*. The nematodes have been cultured for more than 10 generations on lawns of *D. discoideum*. In these experiments we used mutant amoebae (R. Sugang, personal communication) that did not form aggregates or fruiting bodies and thus remained accessible to the nematodes (see below).

For a direct view of nematodes feeding, we grew *Dictyostelium* amoebae with DiI, a lipophilic fluorescent dye that amoebae take up into intracellular vesicles. After the amoebae had been labeled for several hours they were fed to *C. elegans*. Fig. 1 shows individual labeled amoebae and the labeled gut of an adult worm. Every worm that was incubated with labeled amoebae had a fluorescent gut. As expected from the growth studies, larvae also ingested amoebae (not shown). We confirmed these observations by differential interference contrast microscopy of nematodes feeding on amoebae. Individual labeled amoebae are larger than the buccal cavity of the nematode. *C. elegans* can ingest whole cells, but often the nematodes rupture an individual amoeba.

Do nematodes prey on amoebae or prevent their growth in an environment that mimics that of the soil? Singh (9, 13, 14) worked out conditions that permit the growth of *Dictyostelium* in sterilized soil. Two large Petri dishes of soil containing *K. aerogenes* were prepared. One dish received an inoculum of soil that contained nematodes, while a control dish received no nematodes. The next day, the center of each dish was inoculated with 10^7 spores of *D. discoideum*. Fig. 2 shows a trace of the appearance of slugs and fruiting bodies in dishes with and without nematodes. The central hatched circle marks the spot of inoculation. Only a few fruiting bodies appeared in the nematode-infected culture, and these were very close to the point of introduction. The control culture spread outward, as shown, with slugs moving efficiently through the uneven substratum and fruiting bodies forming on a variety of surfaces. Formation of slugs in the nematode infected culture was severely inhibited.

At early stages of development, when cells stream into aggregates, passing nematodes disrupt the streams of cells and feed on the amoebae. Once aggregates have formed, nematode larvae sometimes ring the base of an aggregate, but are excluded from the mound of adherent cells. *D. discoideum* secretes cAMP, which it uses for chemotaxis. *C. elegans* is chemotactic toward cAMP (11), and this observation may account for the attraction.

Aggregated cells synthesize a mucopolysaccharide coat called the sheath that can be seen as a glistening cover to the collected cells. *C. elegans* does not penetrate aggregates after

the tight aggregate stage when the sheath has formed. Slugs containing 100,000 cells migrate in the presence of nematodes that pass under, or less frequently, over them. In hundreds of observed encounters we have not detected any disruption. Fig. 3 shows photographs of such contacts. Nematode larvae have passed under the slug, and there is no distortion of the slug surface.

Adult worms do not digest spores. Nematodes destroy amoebae, but spores survive the worm's gut. Only adult *C. elegans* ingest the spores; larvae of *C. elegans* do not. *Dictyostelium* spores contain cellulose, which binds the fluorescent dye calcofluor white M2R (12). We incubated labeled spores with adult nematodes, and after several hours the worms were recovered, washed, and viewed by fluorescence microscopy. Fluorescent spores were detected along the length of the gut, as shown in Fig. 4. The spores retained their shape while in the gut and were excreted intact (not shown). Videomicroscopy of worms feeding on unlabeled spores was also used to detect spores along the entire gut and during excretion (data not shown).

Because the spores were not visibly altered by passage through the *C. elegans* gut, we asked whether the spores were viable after excretion. We fed them to the adult nematodes and then separated uneaten spores by three successive washes of individual nematodes. A 100 μ l aliquot of the last wash was plated on a lawn of *K. aerogenes* to determine whether any spores were carried over in the wash. A single washed nema-

FIG. 1. *C. elegans* feeds on *Dictyostelium* amoebae. *Dictyostelium* amoebae were labeled with DiI as described. Red shows the fluorescence of the amoebae; green is due to transmitted phase light. Most amoebae appear yellow because of overlapping red fluorescence and refracted green phase light. After 3 h of feeding on a solid substratum the worms were removed and examined. Panels show combined fluorescence and phase images of two adult worms. (Bar = 150 μ m).

FIG. 2. Nematodes limit spread of amoebae in soil. One plate was inoculated with 2–3 g of soil from a soil culture infected with *C. elegans*. The next day 10^7 spores were added at the points marked by the hatched circles. After 66 h there were slugs or fruiting bodies at the positions marked by open circles, after 86 h there were slugs or fruiting bodies as marked by triangles, and after 110 h there were slugs or fruiting bodies at the places marked by closed circles.

tode in about 10 μ l of water was placed on the edge of a Petri dish of SM/5 agar spread with *K. aerogenes*. Nematodes moved rapidly from the point of origin onto the rest of the dish, and after 3 days plaques of *Dictyostelium* appeared in the lawn of bacteria (data not shown). These were often in a trail 5–6 cm from the point of deposition. The average number of colonies formed per nematode is shown in Table 1. A much larger volume (100 μ l) was tested for spore carryover than was required to deposit a single nematode, but in the majority of cases there were no uningested spores in the second wash. Microscopy did not reveal any tendency for spores to stick to the surface of the worms.

Nematodes may affect *Dictyostelium* in another way. *C. elegans* dauer larvae form under conditions of starvation after the second larval molt and are specialized for survival and dispersal. They are motile but do not feed. In the soil conditions described above, nematodes multiply rapidly, but after 6–7 days, dauer larvae appear. Dauer larvae climb the stalks of slime mold fruiting bodies and become enmeshed in the spore

FIG. 3. Nematodes do not disrupt *Dictyostelium* slugs. Axenically growing amoebae were washed, plated on agar prepared with distilled water, and allowed to form slugs. Nematodes were removed from a culture growing on *K. aerogenes* and mixed with the slugs. Two examples from among several hundred are shown. Slugs are the large bodies running from top to bottom. Nematode larvae are 0.5–1 mm long.

FIG. 4. *Dictyostelium* spores remain intact in the nematode gut. Spores were harvested from mature fruiting bodies of strain NC4 and resuspended in water. The spores were centrifuged and placed on an agar surface with a population of *C. elegans*. After 4 h nematodes were viewed as described for Fig. 1, except that a filter for blue fluorescence was used. The phase image is superimposed.

masses, where they writhe and cause the fruiting bodies to move. Fig. 5 Right shows an example of a dauer larva in a spore mass. Adult nematodes remain at the base of the stalks of the *Dictyostelium* fruiting bodies, as shown in Fig. 5 Left. In cultures of soil inoculated with nematodes and *D. discoideum*, many dauer larvae crawl up the stalk, so that one stalk, stripped of its spores, can support 10–20 dauer larvae. The spore masses are maintained by surface tension and when disturbed by the dauer larvae, they slide down the stalk to the substrate. Spores in these soil cultures are still viable, even though intact fruiting bodies are no longer visible (data not shown).

D. discoideum and *C. elegans* are laboratory strains and it is not clear that the interactions detailed above would be observed in fresh isolates. To answer this question and because other aspects of the relationship of social amoebae and nematodes might be apparent in fresh isolates, we isolated amoebae and nematodes that inhabited a single small sample of soil. Among the slime mold species recovered from North Carolina forest soil were *Dictyostelium purpureum*, *Dictyostelium mucoroides*, *Dictyostelium minutum*, and *Polysphondylium violaceum*. Several rhabditid species of nematode were recovered that differ from *C. elegans* but have not been further identified.

The recovered nematodes all feed on the UK7 tester strain of *D. discoideum*. The most robust newly recovered nematode species feeds on amoebae of *D. purpureum*, a cellular slime mold recovered from the same soil sample. Other aspects of the interaction also occur. Fluorescent labeled amoebae are ingested by the new isolate of nematode, although the preference of the worms is for bacteria. The new nematode is unable to penetrate the aggregates of *D. purpureum*. Spores of *D. purpureum* pass safely through the gut, and the ability of the

Table 1. Spores are transported by nematodes

Exp.	Number of worms	Total colonies	Spores/worm	
			Avg.	Range
1	15	75	5.0	1–15
2	17	45	2.6	1–9

Adult hermaphrodites were fed washed spores for 3–4 h on an agarose plate. Individual nematodes were then removed and washed in 2 ml aliquots of water. Each worm (in ≈ 10 μ l H_2O) was placed on an SM/5 plate prespread with *K. aerogenes* and incubated for several days at 22°C. Colonies of *D. discoideum* appeared after 2 days. Spores were deposited at variable distances from the origin up to a distance of 5 cm, depending on the path of the nematodes. A 100 μ l aliquot of the second wash was plated to assure that there was no carryover of spores in the wash.

FIG. 5. Dauer larvae climb into spore masses. *Dictyostelium* and *C. elegans* were cocultivated on filters and fixed for scanning electron microscopy as described. (Left) Adult nematodes at the base of a fruiting body. (Right) Dauer larva that has climbed into the spore mass. (Left, $\times 50$; Right, $\times 78$.)

dauer larvae to crawl into the spore masses is also apparent in the wild combination. An additional aspect of the relationship was detected. High densities of *D. purpureum* amoebae repel the new rhabditid species. These results are shown in Fig. 6. Ten nematodes were placed in the central 7-mm strip of each plate. Bacteria (*K. aerogenes*) were placed on one side and the new isolate of *D. purpureum* on the other. Control plates with no bacteria or amoebae show nematodes that have moved randomly. Bacteria alone attract the worms. When bacteria and amoebae are used, the preference is again for the bacteria. Even when amoebae alone are the only food, the worms are repelled into the empty region of the plate. *D. purpureum* secretes an agent to which the worms are negatively chemotactic. Labeling experiments show that these worms will eat the amoebae when the entire lawn is composed of *D. purpureum*.

DISCUSSION

A predator-prey relationship may exist between *C. elegans* and *D. discoideum*. *C. elegans* and *D. discoideum* compete for the same food—bacteria. If the nematodes deplete the bacterial population, the starving amoebae may respond by aggregating and synthesizing the mucopolysaccharide sheath. Such an act would be adaptive since the nematodes would otherwise destroy them. In this view, the aggregates and slugs can be seen as convoys of cells, sequestered from marauding nematodes. The aggregating cells would be vulnerable to nematodes during the first 12 h of their development, before they have assembled and had time to secrete their mucopolysaccharide coating. We speculate that enough sheath material to protect them can only be synthesized by aggregates of cells. The sheath has been subjected to a number of biochemical and electron microscopic analyses (15, 16). It is a trilamellar cellulose and protein rich structure up to 50 nm thick that is confined to the periphery of the aggregate and slug and does not penetrate between the cells (17, 18).

Individual cells may have evolved independent ways to protect themselves from ingestion. *C. elegans* ruptures the amoebae. This method of feeding might be defeated by a microcyst or a macrocyst, which have rigid cellulose walls and are larger than spores. The microcyst is a single cell dormant form of some species of slime mold (19), although not of *D. discoideum*. The macrocyst is a putative sexual stage of *Dictyostelium* and other slime mold species and is composed of many cells surrounded by a trilaminar cellulose wall (20). Many

other soil amoebae which are not multicellular may be prey for nematodes (5). Most of these are capable of forming a single cell cyst, which could be protective. An additional means of protection may be the secretion of substances that repel the worms, as shown in Fig. 6.

Once the slug has moved away from its original environment, culmination would occur as an additional adaptation to aid in dispersal (2, 3), perhaps by microarthropods or annelids. Adult *C. elegans* may participate in dispersal by carrying spores in their digestive tracts. Nematodes also disperse bacteria, the food of the cellular slime molds, and it is possible that the relatively sessile bacteria and slime mold spores are deposited and grow in a favorable spot, while the more motile nematodes move on. Nematodes are not the only means of dispersal among the soil macro fauna. Huss (21) demonstrated that earthworms and pillbugs contained several *Dictyostelium* species and, as in the case of the nematodes, spores were more

FIG. 6. Hepes buffered plates (pH 7.2) were solidified with 2% phytigel and *K. aerogenes* was spread on one side (9×10^8) and *D. purpureum* (5×10^8) was spread on the other in the combinations indicated. The amount of protein on the side containing amoebae is 20–50 times greater than on the bacterial side. Ten adult hermaphrodites were deposited in the center strip, allowed to migrate for 1 h, and then killed with chloroform vapor. Their tracks were recorded using negative contact prints (11).

resistant than amoebae. Dispersal of various slime mold species by birds has also been described in an extensive study (22). Dictyostelid spores may pass up the food chain and be dispersed over wide areas by migratory songbirds.

We postulate that the *Dictyostelium* spore coat is adapted to survive the conditions of the nematode gut. As the information in Fig. 4 shows, the spores pass through the gut without change of shape and in viable form. Mutants that lack spore coat proteins (23, 24) may be vulnerable to the gut of the worm, although the spores that they produce appear normal under laboratory conditions. Such a possibility was envisioned by Loomis and his colleagues (23).

That there may be a close association of free living soil nematodes and the social amoebae has not been appreciated. For the nematodes, the amoebae are a source of food and may also benefit the nematodes by providing a stalk that places the dauer larvae, the stage specialized for dispersal, in a more advantageous position for that dispersal. The long-term advantage for the amoebae, put forward as a hypothesis, may be that through selection, the slime molds have evolved protective, antipredator mechanisms that depend on size increase by assembly of a multicellular organism. This may then lead to even more successful dispersal, both through creation of a migratory slug and a fruiting body that projects into the interstices of the soil, an environment of small arthropods.

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