Nannocystis exedens: A Potential Biocompetitive Agent against *Aspergillus flavus* and *Aspergillus parasiticus*

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ABSTRACT

This study examined the potential for controlling toxigenic *Aspergillus flavus* and *Aspergillus parasiticus* by biological means using a myxobacterium commonly found in soil. The ability of *Nannocystis exedens* to antagonize *A. flavus* ATCC 16875, *A. flavus* ATCC 26946, and *A. parasiticus* NRRL 3145 was discovered. Cultures of aflatoxigenic fungi were grown on 0.3% Trypticase peptone yeast extract agar for 14 days at 28°C. When *N. exedens* was grown in close proximity with an aflatoxigenic mold, zones of inhibition (10 to 20 mm) developed between the bacterium and mold colony. A flattening of the mold colony on the sides nearest *N. exedens* and general stunting of growth of the mold colony were also observed. When *N. exedens* was added to the center of the cross-streak of a mold colony, lysis of the colony by the bacterium was observed after 24 h. Microscopic observations revealed that *N. exedens* grew on spores, germinating spores, hyphae, and sclerotia of the molds. These results indicate that *N. exedens* may be a potential biocontrol agent against *A. flavus* and *A. parasiticus*.

Aspergillus flavus Link and *Aspergillus parasiticus* Spear are aflatoxin-producing fungi that grow on agricultural products in the field and in storage *(21, 34)*. Aflatoxigenic molds pose a serious threat to humans and domestic animals, because aflatoxins are very toxic compounds that cause illness or death when feeds or foods contaminated by them are consumed *(3)*. The best approach to preventing aflatoxin contamination of foods and feeds is to prevent the growth of aflatoxigenic molds in the field where the fungi and their spores first become associated with agricultural commodities. A combination of cultural practices, pesticides, and resistant plants is used to control preharvest aflatoxin contamination. However, these practices often are insufficient when environmental factors, such as temperature, moisture, and insect and bird damage, favor or facilitate aflatoxigenic molds invasion *(2, 14, 30, 33)*.

The development of biocompetitive microorganisms to control toxigenic *A. flavus* and *A. parasiticus* has been investigated as an alternative approach for controlling aflatoxin contamination. It has been reported that nontoxigenic strains of *A. flavus* and *A. parasiticus (2, 5, 7)*, lactic acid bacteria *(4, 16)*, soilborne bacteria *(17)*, molds *(19, 24)*, and yeasts *(23)* decrease or inhibit aflatoxin production of toxigenic strains of *A. flavus* and *A. parasiticus* on artificial media, in sterile substrates, and in the field. Competition for space, nutrients, and synthesis of antifungal compounds by the biocompetitive agents are attributed to the effects on aflatoxin synthesis.

The antagonistic effects of mycoparasitic fungi and predatory bacteria on toxigenic *A. flavus* and *A. parasiticus* have not been investigated adequately. *Paecilomyces lilacinus, Paecilomyces varioti, Chaetomium* species, and *Gliocladium* species have been reported to parasitize sclerotia of *A. flavus* in soils *(10, 31, 34)*.

Nannocystis exedens Reichenbach is a gram-negative, aerobic, rod-shaped, gliding bacterium commonly found in soils and dung of herbivorous animals *(26)*. *N. exedens* is a micropredator that obtains its nutrients by lysing living and dead cells of other bacteria and yeasts *(26)*. *N. exedens* is a member of *Myxococcales,* which are believed to play a major role in control of the population of many plant disease bacteria and fungi in aerated soils *(11, 26)*. Several members of the *Myxococcales* have been reported to antagonize soilborne plant pathogens *(11, 22, 27, 28)*; however, there is virtually nothing known about the effects of the *Myxococcales* on aflatoxigenic molds. Out of curiosity, we conducted a brief preliminary study and found that *N. exedens* was a strong inhibitor of growth of *A. flavus* and *A. parasiticus.*

The objectives of this investigation were to evaluate the effects of *N. exedens,* a microorganism commonly found in soil, on growth and survival of aflatoxigenic fungi and to characterize the inhibition that results from contact of aflatoxigenic fungi with *N. exedens.*

MATERIALS AND METHODS

Microorganisms: aflatoxigenic molds. *A. flavus* ATCC 16875 and *A. flavus* ATCC 26946, aflatoxin- and sclerotia-producing molds, were obtained from the culture collection of the American Type Culture Collection in Rockville, Md. *A. parasiticus* NRRL 3145, an aflatoxin-producing mold, was obtained

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from the culture collection of the Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, in Peoria, Ill. Stock cultures were grown and maintained on potato dextrose agar (Difco Laboratories, Detroit, Mich.) slants and stored at 4°C.

Biocompetitive agent. *N. exedens* ATCC 25963 was obtained from the culture collection of American Type Culture Collection in Rockville, Md. The bacterium was grown and maintained on 1.5% Trypticase peptone yeast extract agar (TPYA) at 28°C.

Inoculum preparation: aflatoxigenic molds. Mold inoculum was prepared by collecting spores from 7-day-old potato dextrose agar slant cultures incubated at 28°C. Slants were washed with 5 ml of sterile distilled water. The spores were loosened from the slants by brushing with a sterile inoculating loop. One milliliter of the suspension was then pipetted into a 13 by 100-mm test tube containing 3 ml of sterile distilled water to give an inoculum with a concentration of approximately 10^7 fungal spores per ml. The concentration of the suspension was determined using direct microscopic counting and confirmed by the spread plate method on potato dextrose agar.

Biocompetitive agent. *N. exedens* cell suspensions were prepared by cultivating the bacterium in 50 ml of 1.5% TPYA for 3 days at 28°C. The culture was centrifuged at $10,000 \times g$ for 15 min at 5°C. The supernatant was decanted, and 50 ml of 0.3% TPYA was added to the cell pellet *(8)*. The total cell count of the suspension was determined using the spread plate method on 1.5% TPYA plates.

Test medium. To determine the effects of *N. exedens* culture suspensions on the growth of *A. flavus* and *A. parasiticus,* 0.3% TPYA was used as the test media, which contained 0.3% Trypticase peptone (Difco), 0.1% yeast extract (Difco), 0.1% calcium chloride dihydrate, and 1.5% agar. The pH was adjusted to 7.2 with 1 N NaOH and autoclaved at 121°C for 15 min.

In vitro screening for biological control: screening for antifungal activity. To determine whether *N. exedens* released diffusible compounds into the medium, a 100 by 15-mm petri plate containing 15 ml of 0.3% TPYA was streaked down the center with 0.2 ml of mold inoculum and 0.2 ml of bacterial suspension, which was streaked on each side of the mold streak on the same day. All plates were incubated at 28°C for 14 days.

Screening for lytic activity. To determine if *N. exedens* would lyse aflatoxigenic molds, three drops of the mold inoculum were cross-streaked on the surface of a 0.3% TPYA plate and allowed to dry for 15 min. After 15 min, one drop of the bacterial suspension was added to the center of the streak on the same day. All plates were incubated at 28°C and observed daily for growth of *N. exedens* on the mold.

Observation of *N. exedens* predatory behavior. To observe the predatory behavior of *N. exedens* microscopically, observations were made directly from slide cultures. A thin film of 0.3% TPYA was obtained on a glass slide by dipping the slide into molten 0.3% TPYA several times followed by cooling. Medium along the edges of the slide was removed with a sterile scalpel before inoculation. The mold inoculum (15 μl) was spot inoculated onto the center of the slide, and 15 μl of the bacterium suspension was spot inoculated onto the mold inoculum on the same day. A sterile 22 by 50-mm no. 1½ coverslip was placed over the slide following inoculation. All slide cultures were placed into a petri plate moist chamber, which consisted of a petri plate
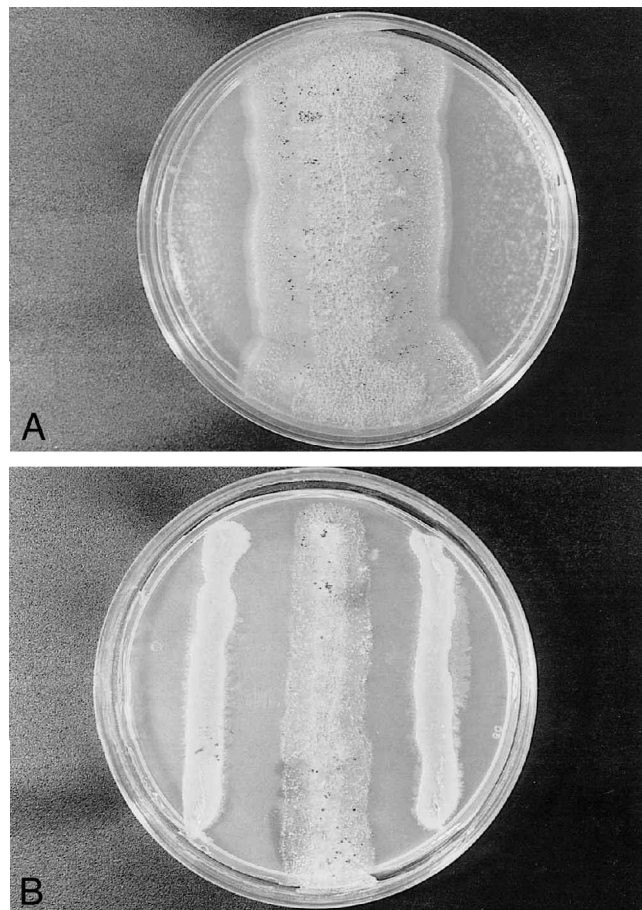

FIGURE 1. *Inhibition of growth of* A. flavus *ATCC 29469 by* N. exedens *ATCC 25963 on 0.3% TPYA after 14 days at 28°C. (A)* A. flavus *ATCC 16875 (control). (B)* N. exedens *ATCC 25963 streaked next to* A. flavus *ATCC 25963. N,* N. exedens*; H, hyphae; S, spore.*

lined with moist Whatman's no. 4 filter paper and two sterile Durham tubes taped to the filter paper. Cultures were incubated at 28°C for 36 h. Slides were examined under a phase-contrast microscope equipped for photography.

All of the experiments described above were repeated in triplicate with six replications. Data presented are the means of those experiments.

RESULTS AND DISCUSSION

Screening for antifungal activity. *N. exedens* ATCC 25963 was streaked parallel to *A. flavus* ATCC 16875, *A. flavus* ATCC 26946, or *A. parasiticus* NRRL 3145 on 0.3% TPYA plates. All plates were incubated at 28°C for 14 days. Several of the criteria for antagonism described by Johnson et al. *(15)* were observed when *N. exedens* was grown in close proximity with each mold (Fig. 1). First, a noticeable zone of inhibition was observed between the bacterial and mold colony. Second, mycelia growth did not spread through spore dispersal throughout the medium on the petri plate compared with the control plate. Third, there was a general restriction of growth of each mold colony when *N. exedens* was streaked next to the mold. The rate of mold growth and sporulation was not altered by *N. exedens* (i.e., growth was observed at approximately the same time); however, the extent of growth and sporulation was de-

TABLE 1. *Growth of* A. flavus *and* A. parasiticus *in proximity to* N. exedens *to determine occurrence and degree of antagonism of* N. exedens *against* A. flavus *and/or* A. parasiticus

Organism[a]	Growth zone diameter (mm)
A. flavus ATCC 16875 (A)	77
A. flavus ATCC 26946 (B)	54
A. parasiticus NRRL 3145	54
A. flavus (A) and *N. exedens*	37
A. flavus (B) and *N. exedens*	22
A. parasiticus and *N. exedens*	27

[a] Each fungus was spot inoculated in the center of an agar plate and *N. exedens* spot inoculated 20 mm from each side of fungal inoculum.

creased markedly in the presence of *N. exedens* compared with mold alone (Table 1). Growth margins of the aflatoxigenic fungi on plates not treated with *N. exedens* ranged from 54 to 77 mm. In the presence of *N. exedens,* growth margins were reduced by greater than 50%. In addition, sporulation of toxigenic fungi was almost totally inhibited by the presence of *N. exedens.*

To our knowledge, these results represent the first direct evidence demonstrating that a soilborne predatory bacterium will antagonize aflatoxigenic molds. The form of antagonism expressed by *N. exedens* during this investigation may be due to the release of diffusible, antifungal metabolites into the culture medium; however, further studies are needed to confirm the cause of inhibition. Myxobacteria, such as *Myxococcus coralloides (1), Myxococcus xanthus (22), Myxococcus fulvus (13), Myxococcus virescens (9), Sorangium* species *(25), Cystobacter fuscus (18),* and *Polygangium cellulosum (27),* have reportedly produced a number of antibacterial and/or antifungal antibiotics. Foster et al. *(8)* isolated 164 strains of myxobacteria from the soil and found that 77.4% excreted antibiotics into the medium and 29% had antifungal activity.

When *N. exedens* was added to the center of the cross-streak of *A. flavus* ATCC 16875, *A. flavus* ATCC 26946, or *A. parasiticus* NRRL 3145, lysis of each mold by *N. exedens* was observed only in the inoculated area (Fig. 2). A yellowish zone of bacteria growth was observed in the center of each mold colony. This area was free of mycelia and spores. In addition, the sclerotia of *A. flavus* ATCC 16875 and 26946 did not germinate in this yellowish area. Outside the zone of lysis, mycelial growth and spore and sclerotia germination were observed. The growth of *N. exedens* on these mold colonies may be due to lytic activity and not competitive inhibition *(5, 15, 32).* Further studies are necessary to confirm the mechanism of inhibition. Myxobacteria are known to lyse prey organism by the excretion of extracellular enzymes *(20).* Reichenbach *(26)* reported that *N. exedens* lysed both living and heat-killed cells of *Sarcina lutea, Escherichia coli,* and yeasts when *N. exedens* was added to streaks of these microorganisms. Results from our study indicated that *N. exedens* had a similar effect on aflatoxigenic molds. This is the first report, to our
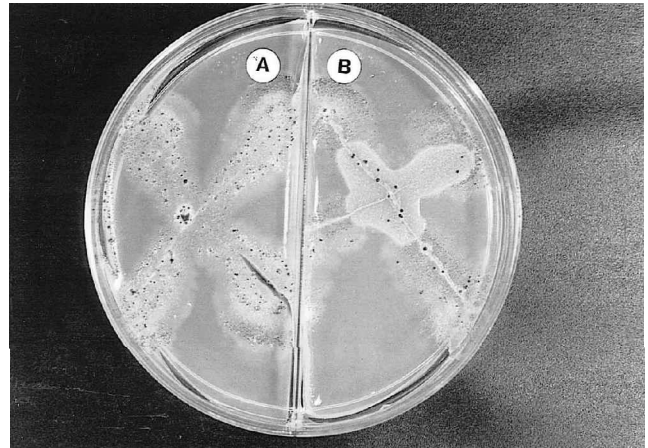

FIGURE 2. *Lysis of* A. flavus *ATCC 16875 by* N. exedens *ATCC 25963 on 0.3% TPYA after 7 days at 28°C. (A)* A. flavus *(AF) control. (B)* N. exedens *(NE) colony in the center of an* A. flavus *(AF) cross-streak.*

knowledge, of a predatory bacterium that may lyse aflatoxigenic fungi.

Observation of *N. exedens* predatory behavior. Parasitism of the propagules of *A. flavus* ATCC 16875, *A. flavus* ATCC 26946, and *A. parasiticus* NRRL 3145 by *N. exedens* was observed after incubation at 28°C for 36 h (Figs. 3 and 4). *N. exedens* developed as streaming colonies that surrounded spores, germinating spores, hyphae, and sclerotia as a sheath of tightly packed rods, clumps of rods, or individual rods. Figure 3 shows the lysis of hyphae and spores of *A. flavus* ATTC 26946 by *N. exedens* after 36 h of contact. Lysis is demonstrated by the collapse of spores and hyphae. Spores and sclerotia would not germinate even when washed in 50 ppm of hypochlorite solution and removed to a fresh medium at a pH that inhibited growth of *N. exedens* in three successive transfers.

Figure 4 shows the lysis of germinating spores and germ tube of *A. parasiticus* by contact with *N. exedens.* The germ tube was almost totally collapsed in fungi treated with *N. exedens* compared with the control germ tube that was growing normally.
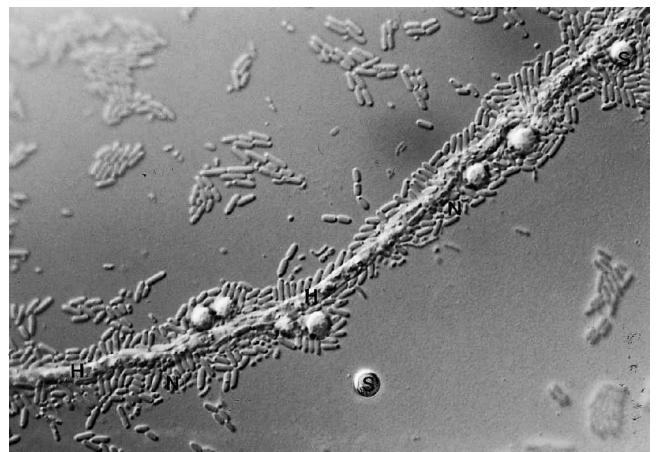

FIGURE 3. *Photomicrograph showing lysis of hyphae and spores of* A. flavus *ATCC 26946 by* N. exedens *ATCC 25963 at 36 h.*
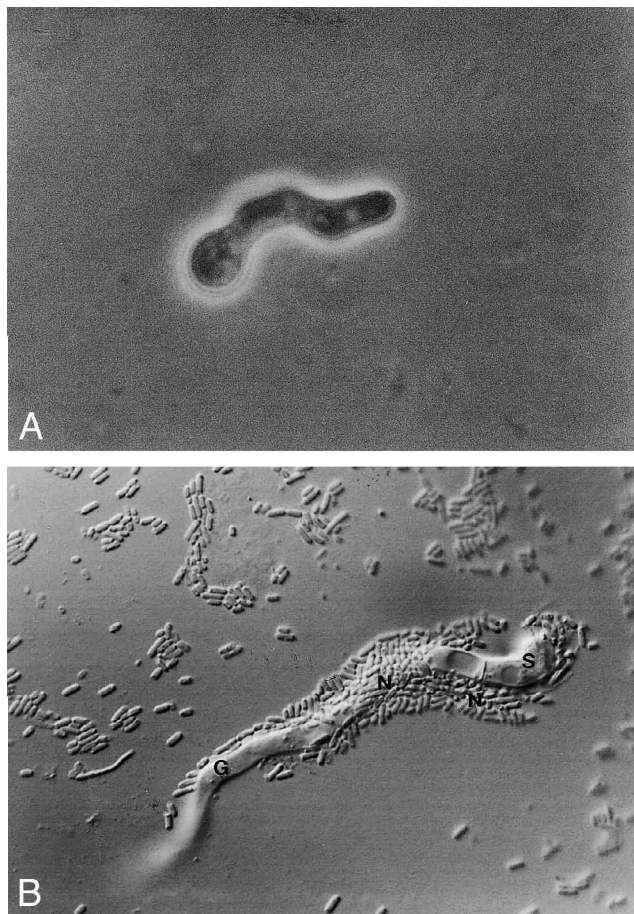
FIGURE 4. *Photomicrographs showing control germ tube (A) and lysis of germinating spore and germ tube (B) of* A. parasiticus *NRRL 3145 by* N. exedens *ATCC 25963 at 36 h. G, germ tube; N,* N. exedens; *S, spore.*

In all photomicroscopic observations, the *N. exedens* appeared to be particularly attracted to germ tubes, and swarms were always present on these delicate structures.

To our knowledge, these results represent the first evidence demonstrating that a soilborne predatory bacterium may parasitize aflatoxigenic molds. These results show that the growth of *N. exedens* on the mold colonies (Fig. 2) was probably due to lytic activity instead of competitive inhibition (according to the definition of Johnson et al. *(15)*). The *Myxobacteriaceae* comprise the only group of bacteria known to aggregate to form a swarm during feeding. Rosenberg and Varon *(28)* suggested that a higher concentration of extracellular lytic enzymes produced by the swarm allows more efficient feeding. Observations of the feeding behavior of *N. exedens* on aflatoxigenic molds suggest a correlation to the feeding behavior as described by Shimket *(29)* on bacteria. The formation of swarms and the binding of *N. exedens* to spores, hyphae, germ tubes, and sclerotia were observed during this investigation. Predatory behavior and parasitism appear to be another mechanism of survival of *N. exedens.* Myxobacteria have been reported to parasitize soilborne pathogenic fungi. Homma *(12)* reported that a mycophagous bacterium similar to *Polyangium parasiticum* Geitler lysed *Rhizoctonia solani* and conidia of *Cochliobolus miyabeanus.* Hocking and Cook *(11)* reported that

three myxobacteria of the group *Sorangium* were able to lyse *Pythimum intermedium, R. solani, Fusarium oxysporum,* and *Fusarium solani.* They suggested that the myxobacteria could provide some protection of tree seedlings in the presence of root pathogens. The results of our study suggest that *N. exedens* is also a mycophagous bacterium.

This investigation demonstrated that *N. exedens* ATCC 25963 may be a potential biocompetitive agent against toxigenic strains of *A. flavus* and *A. parasiticus.* The results of this study suggest that the inhibition of growth of the molds may be due to the production and release of antifungal compounds into the medium and/or parasitism by *N. exedens.* Studies are needed to characterize the cause(s) of inhibition. These results justify further investigations to determine the feasibility of using *N. exedens* as a biological control agent against *A. flavus* and *A. parasiticus* in the soil environment. Future studies should include the effects of *N. exedens* on aflatoxin production, the isolation of antifungal compounds and/or lytic enzymes produced by *N. exedens,* and a determination of the effects of these antifungal microorganisms on growth of aflatoxigenic molds and aflatoxin production.

ACKNOWLEDGMENT

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