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NANNOCYSTIS EXEDENS AS POTENTIAL BIOCOMPETITIVE AGENT AGAINST TOXIGENIC *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Willie James Taylor

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DEDICATION

This thesis is dedicated to my wife, Helen, for her love and the support she gave me to help me accomplish my goals. Being a role model for my son, little Willie Jr., allowed me to keep focused on completing this thesis.

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ABSTRACT

Aflatoxins, secondary metabolites produced by toxigenic strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus*, are potent hepatocarcinogens found in foods and feeds. Since the discovery of aflatoxins in 1960, no effective approach has been found to eliminate or decrease the population of the molds responsible for aflatoxin contamination at the field level. The objective of this study was to identify a biocompetitive agent that would parasitize toxigenic strains of *A. flavus* and *A. parasiticus*. *Nannocystis exedens* was selected as the biocompetitive agent because it is a predator bacterium which lyse other bacteria and yeasts. In addition, the bacterium is found commonly in soils and dung of herbivorous animals and it is nonpathogenic.

Aspergillus flavus ATCC 1687, *Aspergillus flavus* ATCC 26946 and *Aspergillus parasiticus* NRRL 3145 were grown in the presence of *Nannocystis exedens* ATCC 25963, and growth and production of aflatoxin B_1 and G_1 were determined after 14 days of incubation at 28°C. *N. exedens* inhibited growth and aflatoxin B_1 and G_1 production by all three aflatoxigenic molds. When *N. exedens* was grown in close proximity with each of the molds on 0.3% trypticase-peptone yeast extract agar, zones of inhibition developed between the bacterium and each mold colony. Flattening of the mold colony on the sides nearest *N. exedens*, growth of mold colony away from the bacterial colony, and general stunting of growth of the mold colony were observed. When *N. exedens* was added to the center of the cross streak of a mold colony, lysis of the mold colony by the

bacterium was observed after 24 hours. Bacterial growth was only observed in the center of each mold colony. Microscopic observations revealed that *N. exedens* grew parasitically on spores, germinating spores, hyphae and sclerotia of the aflatoxigenic molds. *N. exedens* developed as streaming colonies which surrounded the propagules of each mold as a sheath of tightly packed rods, clumps of rods, or as individual rods.

These results indicate that *Nannocystis exedens* ATCC 25963 may be effective in interfering with and/or inhibiting growth and aflatoxin production by toxigenic strains of *A. flavus* and *A. parasiticus* in the field.

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CHAPTER I

INTRODUCTION

Nannocystis exedens Reichenbach is a Gram-negative, aerobic, rod-shaped, gliding bacterium commonly found in soils and dung of herbivorous animals (80). *N. exedens* is a micropredator that obtains its nutrients by lysing living and dead cells of other bacteria and yeasts (80). *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 (66,80). Several members of the *Myxococcales* have been reported to antagonize soilborne plant pathogens (33,51,52,99); however, there is virtually nothing known about the effects of the *Myxococcales* on aflatoxigenic molds.

Aflatoxigenic molds produce aflatoxins (85). Aflatoxins are toxic secondary metabolites which cause illness or death in animals and humans when feeds or foods that contain them are consumed (10). Only toxigenic strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus* are known to produce aflatoxins (70,85,115). These yellow-green molds grow saprophytically and parasitically on agricultural products in the field and in storage (115). The field is where aflatoxigenic molds first become associated with crops and where the aflatoxin contamination process may begin (10). The contamination of agricultural commodities with aflatoxin is considered an unavoidable process (71). Economic losses associated with aflatoxin contamination are losses of crops, reduced value of crops as feed or export commodities, and decreases in livestock and

poultry production (10). The best approach to preventing aflatoxin contamination of foods and feeds is to prevent or limit the growth of aflatoxigenic molds in the field. However, current cultural practices are often insufficient when environmental factors favor or facilitate invasion by aflatoxigenic molds (58,101,115). Use of biocompetitive bacteria, molds or yeasts to control aflatoxigenic molds has been suggested as a supplement or alternative to current cultural practices (10). The overall objective of this investigation was to identify a biocompetitive microorganism that would parasitize toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Specifically, this investigation was conducted to: (a) determine the antagonizing effect of *Nannocystis exedens* on toxigenic strains of *A. flavus* and *A. parasiticus*; (b) to determine the form of *N. exedens* antagonism and (c) to determine the affects of *N. exedens* cell suspensions on aflatoxin B_1 and G_1 biosynthesis.

CHAPTER II

LITERATURE REVIEW

The Discovery of Aflatoxins

The discovery of aflatoxins resulted from the death of more than 100,000 turkey poults in the south and east of England in 1960 from an apparently new disease that was called "turkey X disease" (38). It was discovered that Brazilian peanut meal contaminated with highly toxic compounds was responsible for the deaths (38). Under further investigation, the meal was found to be infested with molds which were identified as *Aspergillus flavus* and *Aspergillus parasiticus*. These molds produced toxic compounds which were called aflatoxins that were responsible for "turkey X disease." (38).

Molds that Produce Aflatoxins

Molds that produce aflatoxins are called aflatoxigenic molds (85). There are only three molds known to be aflatoxigenic: *Aspergillus flavus* Link (85), *Aspergillus parasiticus* Spear (85) and *Aspergillus nominus* Kurtzman, Horn and Hesseltine (70). Aflatoxigenic molds are members of the genus *Aspergillus* which was first named in 1729 by Antonio Micheli (92). Micheli noticed that the spore chains of certain mold isolates rose radially (Fig. 1). He thought the spore chain's resembled a holy water sprinkler which was called an aspergillum. Thus, he begin using the latin word *Aspergillus* to describe molds with radiate spore chains. The taxonomy of the genus *Aspergillus*

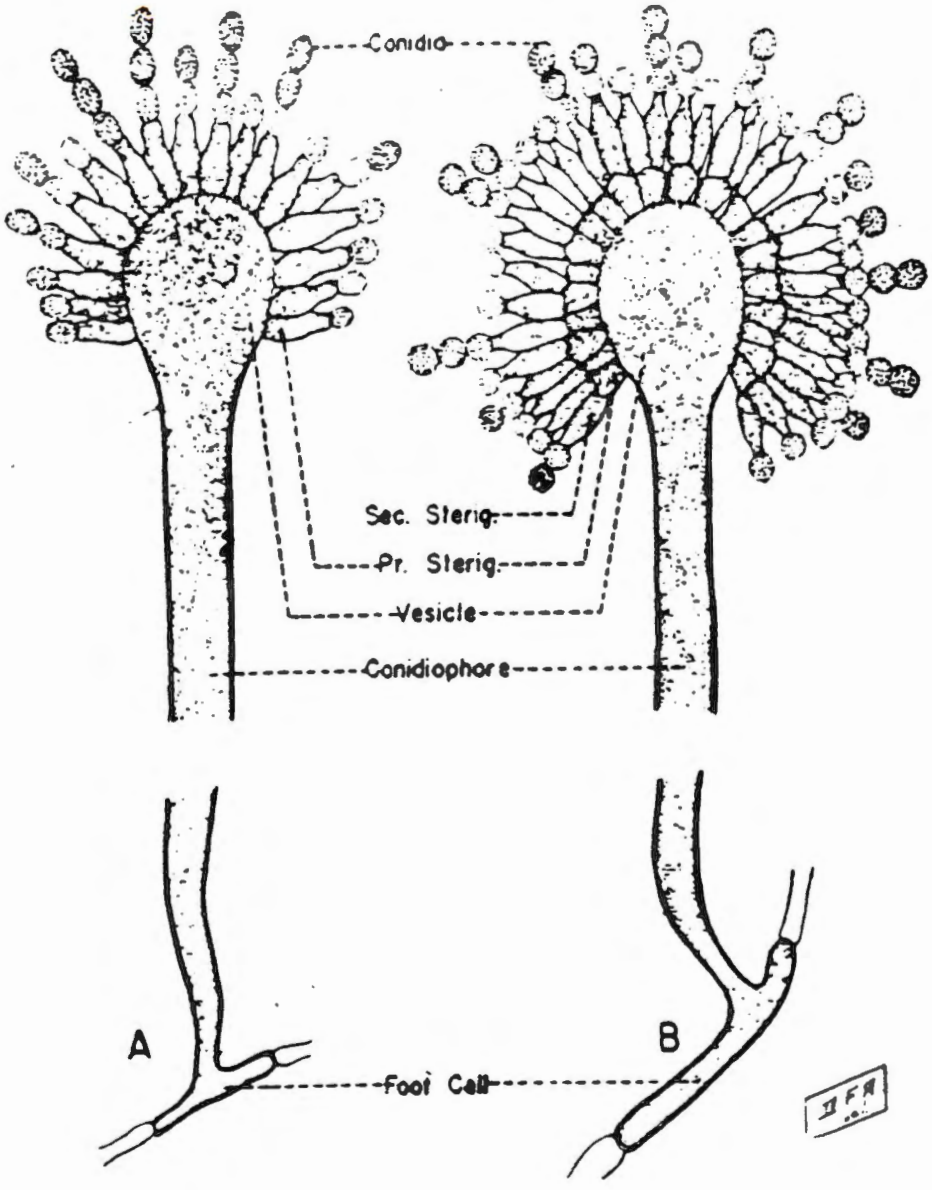

Figure 1. Morphology of aflatoxigenic molds. A) *Aspergillus parasiticus* Spear; B) *Aspergillus flavus* Link and *Aspergillus nominus* Kurtzman, Horn and Hesseltine (107).

is based primarily upon color and morphology characteristics (67,91,92,106,107). Keys published by Raper and Fennell (106) have been used universally for the last 29 years to identify the *Aspergillus* species. They divided the genus *Aspergillus* into 18 groups and 132 species with 18 varieties. Aflatoxigenic molds are in the *Aspergillus flavus* group. The key characteristic which separates the *A. flavus* group from the other groups is on in which young colonies (2-3 days) on Czapek's agar containing 3% sucrose are persistently yellow green at 25°C. The important characteristics of aflatoxigenic molds are summarized in Table 1 (70,92).

Aflatoxigenic molds also may be identified by plating on *Aspergillus flavus* and *parasiticus* agar, AFPA (91). AFPA is composed of peptone 10 g; yeast extract 20 g; ferric ammonium citrate 0.5 g; chloramphenicol 100 mg; agar 15 g; distilled water 1 L; and dichlorane 2 mg. The final pH of this medium is 6.2. AFPA is incubated at 30°C for 42-48 hours. Dichloran inhibits spreading fungi, while chloramphenicol inhibits bacteria. Aflatoxigenic molds develop yellow green spores and a bright orange reverse. The orange color is due to the reaction of ferric ammonium citrate with kojic acid and aspergillic acid (7).

Habitat of Aflatoxigenic Molds

Aspergillus flavus and *A. parasiticus* species are ubiquitous in their distribution. *A. parasiticus* species are found mainly in cultivated soils of tropical and subtropical regions (27,29). *A. flavus* species are found generally in cultivated

TABLE 1. Important characteristics of aflatoxigenic molds on Czapek's agar containing 3% sucrose incubated at 25°C (70,92).

Characteristic	*A. flavus*	*A. nominus*	*A. parasiticus*
Conidial heads (color)	yellow green	yellow green	yellow green
Conidial heads (shape)	radiate	radiate	radiate
Conidiophores (color)	hyaline	hyaline	hylaine
Conidiophores (surface)	rough	rough	rough or smooth
Sterigmata	secondary or primary	secondary or primary	primary
Conidia	globose, subglobose, echinulate	globose, subglobose, echinulate	globose, echinulate
Vesicle	elongated, globose, subglobose	globose, subglobose	flask- shaped, subglobose
Sclerotia	globobe, subglobose	vertically elongated with white tip	absent
Growth at 45°C	yes	no	yes
Hulle Cells	absent	absent	absent
Cleistothecia	absent	absent	absent

soils of temperateregions of the world (27,29). *A. flavus* and *A. parasiticus* species are common contaminants of a wide variety of commodities in the field and storage areas.

Foods in which aflatoxins have been found include cereal grains (corn, sorghum, wheat, rice and barley), peanuts, treenuts (Brazil nuts, hazel nuts, pecans, almonds, walnuts and pistachio nuts), oil seeds (cottonseed and copra), cassava, cocoa beans, copra, beans and cowpeas (4,100,114). Animal feeds formulated from cereal grains, peanuts, treenuts and oilseeds also can become infested with *A. flavus* and *A. parasiticus* species (98,105,120). Stored agricultural products infested with *A. flavus* and *A. parasiticus* species generally are associated with poor quality products, inadequate drying, improper storage conditions and/or insect infestation. *A. flavus* and *A. parasiticus* species grow saprophytically and parasitically on agricultural products in the field and storage (81,115). The organisms can grow on seeds, kernels, decaying plant materials and insects (28,114). Aflatoxigenic molds may overwinter as sclerotia or mycelia (114,115). Sclerotia are compact masses of hyphae capable of surviving unfavorable environmental conditions (114,115). Sclerotia are able to survive in soils; whereas, spores and mycelia are unable to survive in soils in the absence of plant debris (73). When spores germinate and hyphae extend into the soil, the hyphae is lysed by other soil microorganisms; thus, spores or mycelia in the soil remain dormant until an available food source is present (73,114,115). *A. parasiticus* species appear to be more adapted to soil environment; whereas, *A. flavus* species seems adapted to aerial and foliar environment (28).

Spores are the major infective and dispersal agents responsible for infestation and contamination of standing crops in the field (115). Sclerotia and mycelia are primary sources of spores. Spores are borne directly upon these structures. The secondary source of spores are field soils that leave crop residues, sclerotia and mycelia on or near the soil surface. Insects, floral parts and leaves that become contaminated with spores are also a secondary source of spores (28,115). Spores may be transmitted to standing crops in the field: (a) by insects which become contaminated with spores while feeding upon the fungus and/or moldy substrates (115); (b) air movement (28); (c) wind-driven soil containing spores followed by rain (28); (d) combine harvest of crops (114); (e) cultivation of fields next to standing crops which contains sclerotia and (f) crop debris infested with airborne spores (114).

Once the spores have been disseminated onto standing crops, infestation and aflatoxin contamination are not inevitable. Unless suitable conditions are present, the spores may remain dormant on the surface of the kernels. Factors favoring or facilitating infestation and aflatoxin contamination in the field include: (a) crops (corn, peanuts, cotton and tree nuts) high in sugars (glucose and fructose), amino acids (glutamic and aspartic acid), trace metals (zinc and magnesium) and lipids; (b) lack of microbial competitors such as *Penicillium*, *Aspergillus* and *Fusarium* species which compete for the same substrates as aflatoxigenic molds; (c) favorable environmental conditions such as ambient temperatures (35-37°C), high soil temperature (28-30°C), high moistures levels (18-18.5%) and high relative humidities (80-90%); (d) harvesting practices, such as

mechanical damage to crops, dense plant population and minimal-tillage; (e) insects, birds and rodent damage to crops; (f) the use of agricultural pesticides which result in weed resistance, insect resistance and death of competitors (resistant weeds compete with standing crops for water and nutrients, resistant insects transmit spores and absence of competitors allow aflatoxigenic molds to grow abundantly), and (g) toxigenic strains are genetically capable of producing aflatoxins (10,28,58,77,89,101).

Aflatoxins

Aflatoxins are toxic secondary metabolites and are produced by *Aspergillus flavus* Link (85), *Aspergillus parasiticus* Spear (85) and *Aspergillus nominus* Kurtzman, Horn and Hesseltine (70). There are six aflatoxins of concern, aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2 (Fig. 2) (18). The B and G aflatoxins occur naturally; whereas, the M aflatoxins are produced in the milk of lactating animals fed aflatoxin B_1-contaminated feed (76). Aflatoxin B_1, which represents the most toxic of the aflatoxins, is produced in the greatest quantities, followed by G_1, B_2, and G_2 (18). Aflatoxin B_1 is the most potent naturally-occurring carcinogen known at present (18). *A. flavus* and *A. nominus* produce only the B aflatoxins; whereas, *A. parasiticus* produces both B and G aflatoxins (18,70). The B aflatoxins are bifurano coumarins fused to cyclopentanone, and the G aflatoxins are bifurano cumarins fused to a lactone (82,103).

The subscripts 1 and 2 designate the chromatographic mobility (Rf values) pattern of the compounds on thin layer silica gel chromatography plates (82). The properties of

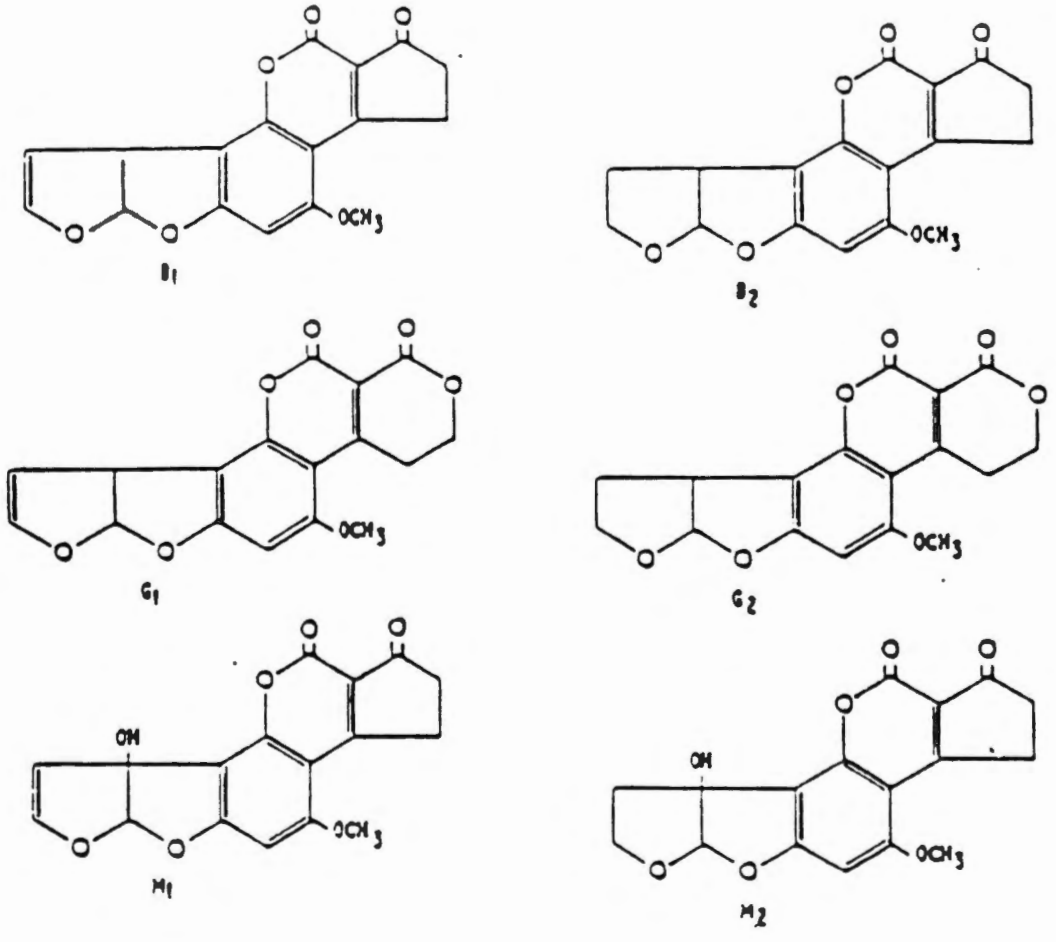

Figure 2. Chemical structures of the aflatoxins.

aflatoxins are listed in Table 2.

Biosynthesis of Aflatoxins

The biosynthesis of aflatoxin B_1 have been investigated extensively (32,25,77,82,103,110) (Fig. 3). Aflatoxins are secondary fungal metabolites which are defined as substances formed during the end of the exponential growth phase of the fungus (77). Aflatoxins have no apparent significance in the fungal growth or physiology of fungi (77). The sequence of events leading to aflatoxin B_1 synthesis has been discussed by Fitzell et al. (32), Detroy and Hesseltine (25), Maggon et al. (77), Mirocha et al. (82), Steyn et al. (103) and Venkitasubraminan et al. (110). Aflatoxin formation is initiated as a result of exhaustion of nutrients such as phosphate, nitrogen and some trace metals. Primary precursors, such as pyruvate, acetate, malonate and methionine accumulate to toxic levels which causes the fungus to cease growing. Tricarboxylic acid cycle (TCA) is triggered by the accumulation of pyruvate to provide intermediates such as acetate, acetyl CoA and malonate for the biosynthesis of aflatoxin B_1. Fatty acids which were previously synthesized via the TCA cycle are no longer synthesized via the TCA cycle, and acetate, acetyl CoA, citrate and other TCA intermediates accumulate. The accumulation of acetyl CoA and malonate inhibit the TCA cycle. The accumulation of pyruvate and acetate inhibits the biosynthesis of fatty acids. The pathway that was used to synthesize fatty acids then is used to synthesize acetate polyketides (101).

TABLE 2. Physical and chemical properties of selected aflatoxins (82).

Aflatoxin	Molecular formula	Melting point (C°)	Fluorescence[1] emission wavelenght (nm)
B_1	$C_{17}H_{12}O_6$	268-269	425, V
B_2	$C_{17}H_{14}O_6$	286-289	425, V
G_1	$C_{17}H_{12}O_7$	244-246	450, SB
G_2	$C_{17}H_{14}O_7$	237-240	450, SB
M_1	$C_{17}H_{12}O_7$	299	425, V
M_2	$C_{17}H_{14}O_7$	293	425, V

[1]V indicates Violet, SB indicates sky blue when viewed under ultraviolet radiation after development in chloroform:acetone (88:12).

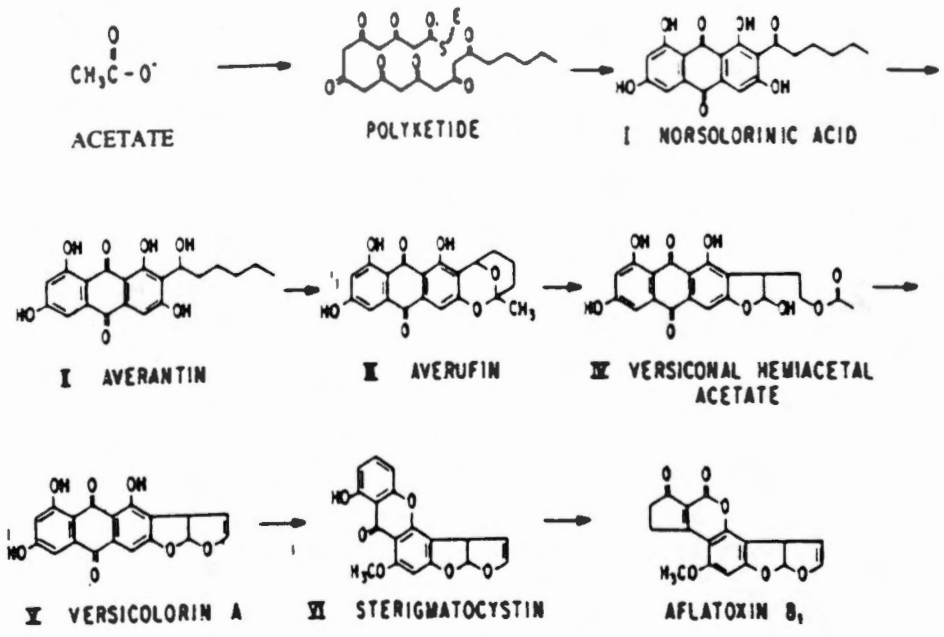

Figure 3. Biosynthesis of aflatoxin B_1 (77).

Why Aflatoxins are synthesized

The reason why aflatoxins are produced is not well understood, but aflatoxins are not required for growth because they are produced only after growth has terminated (77,101,113). At present, the detoxification theory is the most commonly accepted postulate as why aflatoxins are produced (77). The hypothesis is based on known biosynthetic pathways of aflatoxin. According to this theory, aflatoxigenic molds produce aflatoxins when large amounts of primary metabolic precursors such as acetyl CoA, TCA intermediates, pyruvate, acetate, malonate and amino acids accumulate during rapid growth. During the stationary phase, the precursors were no longer needed for the synthesis of proteins, sugars, lipids and amino acids that were essential for growth. Because these primary precursors may become toxic to the mold, the molds get rid of them by diverting them toward the biosynthesis of aflatoxins which are released into the surrounding environment (20,21,77,113).

Role of Aflatoxins

Actual role of aflatoxins in the environment is not known; however, several authors have suggested that aflatoxins function as waste products (101,113), as phytoxins (81), as antimicrobial agents (2,9), as antifeedents (57), as antibiotics (1) and as insect toxins (57).

Effects of Aflatoxins

Aflatoxins are teratogenic, carcinogenic and mutagenic. When animals ingest feeds containing aflatoxins, poisoning resulting from the consumption of the toxic compounds is termed aflatoxicoses (85). The toxicity of aflatoxins is due to the biotransformation of aflatoxin B_1 to 2,3-epoxyaflatoxin B_1 which is the toxic form (82). The activation of aflatoxin B_1 involves the mixed-function oxidase enzyme systems in the liver (10,82). These enzyme reactions result in the activation of aflatoxin B_1 into its toxic form (10,82). The 2,3-epoxyaflatoxin B_1 is the carcinogenic, teratogenic and mutagenic forms of aflatoxins which react with DNA (82,85). When aflatoxin B_1 binds with DNA, polymerases responsible for DNA and RNA synthesis are inhibited. Thus, DNA, RNA and protein synthesis are inhibited (50,82,85,108).

The liver of animals is the primary target organ of aflatoxins (10). The organ decolorizes, increases in size and exhibits hemorrhagic and necrotic zones (85). The first visible symptoms of aflatoxin poisoning are loss of appetite and reduction of growth (85). A few days before death, the animals may become melancholy, stagger, show nervous symptoms, develop muscular spasms and display incoordination of movement (Moreau and Moss, 1979). Dairy and beef cattle, pigs, poultry and trouts are susceptible to aflatoxin poisoning, while sheep are relatively resistant (10,85,88). A variety of other animals such as dogs, rabbits, guinea-pigs, monkeys and rats are susceptible also to aflatoxin poisoning (85).

Aflatoxicosis in humans in the United States where the food supply is well regulated has not been documented; however, acute aflatoxicosis in humans has been reported from Africa, Southeast Asia and India (10,104,119). In all reported cases, aflatoxin poisoning resulted from the consumption of moldy food containing relatively high levels of aflatoxins (10); furthermore, liver cancer was reported in all cases.

Economic losses associated with aflatoxin contamination include: crop loss, reduced value of crops as feed or export commodities, disposal of useless crops, decreases in livestock and poultry production and death of livestock (10). Surveillance, sampling and testing of crops, monitoring and testing livestock, regulatory programs, research and extension are additional costs associated with aflatoxin contamination (10). Aflatoxin contamination costs the nation's peanut growers an estimated $25 million annually according to the Peanut Advisory Board in Atlanta (65).

Regulation of Aflatoxins

Because aflatoxins are potent carcinogens that are considered unavoidable natural contaminants, the U.S. Food and Drug Administration (FDA) established regulatory guidelines for acceptable levels in foods and feeds. The action level for foods is 20 ppb total aflatoxins. However, milk has an action level of 0.5 ppb because milk containing aflatoxin M_1 presents a risk to infants and young children. The action level for feeds for all species of animals is 20 ppb. However, there are exceptions for cottonseed meal and corn used as feed. The action level for cottonseed meal used as feed is 300 ppb. The

action level for corn used as feed for finishing beef cattle is 300 ppb, while corn used for finishing swine (> 100 lbs) is 200 ppb. For breeding cattle, breeding swine and mature poultry the action level is 100 ppb (10,18).

Control of Aflatoxin Contamination

The best approach to preventing aflatoxin contamination of foods and feeds is to prevent or limit the growth of aflatoxigenic molds in the field. A combination of cultural practices, use of pesticides and use of resistant plants is used to control toxigenic molds. However, these practices often are insufficient when environmental factors such as temperature, moisture and insect and bird damage favor or facilitate aflatoxigenic molds invasion (10,58,101,115). Since aflatoxins are considered an unavoidable naturally occurring contaminant, the greatest effort has been expended on detoxification methods to remove or inactivate commodities contaminated with aflatoxins (10,37). Detoxification may be accomplished by physical, chemical or biological treatments (10). Physical methods which degrade or detoxify aflatoxins include roasting (12), solvent extractions (37) and use of absorbents such as activated carbon, bentonite, clays and alumina silicates (10).

Many chemicals have been investigated for their ability to degrade or inactivate aflatoxins. Ammonia has received the most attention for treatment of aflatoxin contaminated feed (10). Other chemicals which have been reported to degrade or detoxify aflatoxins include strong acids and bases, oxidizing agents and bisulfites (31,79).

Biological Control of Aflatoxins

Biological control is defined as, " the reduction of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (5). There is good evidence that biological control strategies can be devised successfully to control toxigenic strains of *A. flavus* and *A. parasiticus*. Biological control of aflatoxin could be based on microbial degradation, competition, antibiosis and/or parasitism (56). There is a search for bacteria, yeasts and molds as biocompetitive agents against aflatoxigenic molds. Most of the studies have dealt mainly with the interaction between aflatoxigenic molds and other fungi, occurring naturally on grains and grown in competition. Only a limited number of fungi, yeasts and bacteria have been found to degrade, detoxify or inhibit aflatoxins. It is a well documented fact that toxigenic cultures of *A. flavus* and *A. parasiticus* are capable of degrading aflatoxins (6,79). Huynh and Lloyd (52) postulated that aflatoxin-degrading substances are synthesized in young mycelia and as the mycelia age these substances degrade aflatoxins. Huynh et al. (53) identified the aflatoxin-degrading substances as peroxidase enzymes. They also suggested that at least one other enzyme was involved in the degradation of aflatoxins; however, they were unable to isolate the enzyme. Microorganisms other than *A. flavus* and *A. parasiticus* are capable of degrading aflatoxins. *Aspergillus niger, A.terreus NRRL 1967, A.luchuensis NRRL 2053, Penicillium raistricki* NRRL 2038 (13), *Corynebacterium rubrum, Trichoderma viride* and *Mucor ambiguus* (78) were reported to partially degrade aflatoxin B_1 to the less toxic compounds, aflatoxicol. Other microorganisms such as

Nocardia asteroides (2), *Scopulariopsis brevicaulis* (58), *Dactylium dendroides* NRRL 2575, *Mucor griscocyanus* NRRL 3359, *Helminthosporium sativum* NRRL 3356, *Absidia repens* NRRL 1336 and *Mucor alternans* NRRL 3358 (22,23,24) reportedly degrade aflatoxin to aflatoxicol. *Rhizopus oryzae NRRL 395, R. arrhizus NRRL 2582 and R.stolonifer NRRL 1477* reportedly degrade aflatoxin B_1 to aflatoxicol and aflatoxin G_1 to parasiticol (15). Only one bacterium, *Flavobacterium aurantiacum* NRRL 2038 has been found to remove aflatoxin B_1 without producing toxic by-products (13,75). Recently, Kasim Fara et al.(62) reported that natural contaminants of Thailand maize, *A. niger, R. oryzae* and *Bacillus sterothermophlis* also were able to degrade aflatoxin B_1. Coallier-Ascah and Idziak (14) reported that *Streptococcus lactis* was able to degrade preformed aflatoxin when inoculated into a growing *A. flavus* culture. They also reported that *S. lactis* was destroyed by aflatoxin and compounds produced by *A. flavus*. Biodegradation of aflatoxins to non-toxic derivatives is being considered realistically as an supplement to physical and chemical detoxification methods (10). However, prior to applying microorganisms to detoxify aflatoxins, much research is required to isolate and characterize the enzymes responsible for degradations (6).

Biocompetitive bacteria, yeasts and molds reportedly inhibit or interfere with growth and aflatoxin production of toxigenic cultures 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 effect on growth and aflatoxin synthesis (11). Weckbach and Morth (112) reported that

Rhizopus nigricans and *Saccharomyces cerevisiae* inhibited *A. parasiticus* growth and toxin production in mixed cultures. It was suggested that *R. nigricans* metabolized aflatoxins. Kimura and Hirano (64) reported that *Bacillus subtilis* strains isolated from soils inhibited growth and aflatoxin production by *A. parasiticus* and *A. flavus* in maize and groundnuts under laboratory conditions. These authors suggested that *B. subtilis* may synthesize a compound that inhibits fungal growth and aflatoxin production. Toxigenic *Fusarium moniliforme* also inhibited aflatoxin production of *A. flavus* when co-inoculated onto sterile maize kernels (11,121). Inhibition was suggested as being due to physical competition for nutrients (11). Wicklow et al. (117) reported that when *A. flavus* was paired with *A. niger* or *Trichoderma viride* on sterilized maize kernels, no aflatoxin production was detected. Mixon et al. (83) reduced aflatoxin production in groundnuts by applying *Trichoderma harzianum* preparation to soil amended with gypsum. The effectiveness of some *Trichoderma* species as biocontrol agents has been attributed to their enzymes, antibiotics production or mycoparasitism (74). Cuero et al. (19) demonstrated that growth and aflatoxin production of *A. flavus* were significantly reduced when inoculated with *Bacillus subtilis* or *T. harzianum* in preharvest maize. They suggested that *B. subtilis* and *T. harizanum* out competed *A. flavus* for trace elements such as zinc, copper and manganese which are essential for toxigenic mold growth and aflatoxin production. Table 3 summarizes other microorganisms that have been screened as potential biocompetitive agents against aflatoxigenic molds.

TABLE 3. Microorganisms screened as potential biocompetitive agents against toxigenic *Aspergillus flavus* and *Aspergillus parasiticus*.

Microorganism	Aflatoxin Production	Growth	Form of Antagonism	Reference
Brevibacterium lines	PI	PI	C	(112)
Atoxigenic *A. flavus*	PI	PI	C	(8,17)
L. acidophilus, *L. bulgaricus*, *L. plantarum*	CI	CI	C	(61)
Acremomium strictum	PI	PI	C	(116)
Atoxigenic *A. parasiticus*	PI	PI	C	(30)
Streptococcus lactis	PI to CI	NI	A	(14)
Pichia guilliermondii	PI	PI	?	(87)
Rhizopus arrhizus	PI	PI	A	(72)

CI = complete inhibition; PI = partial inhibition; NI = no inhibition; A = antiobiosis; C = competition; ? = not mentioned

Most of the biological control studies have focused on the biological approach using bacteria, molds, yeasts and nontoxigenic strains of *A. flavus* and *A. parasiticus* which occurr naturally on grains. The rationale was that the biocompetitive agent would compete with aflatoxigenic molds and prevent aflatoxin contamination. However, these biocompetitive agents are also undesirable microorganisms. Another approach for controlling aflatoxin contamination is the employment of predatory bacteria and molds found in the soil microflora with aflatoxigenic molds. There have been relatively few reports on the effects of predatory bacteria and molds on growth and aflatoxin production. *Paecilomyces lilacinus*, a mycoparasitic fungus, was reported to colonize and degrade sclerotia of *A. flavus* (40,118 1990). *Paecilomyces* species are known to produce degradative enzymes which allow them to parasitize other fungi (34). *Chaetomium* species, *Paecilomyces varioti* and *Gliocladium* species also have been reported to suppress sclerotia germination of *A. flavus* in soils (102). Parasitism and predation are common mechanisms for fungal biological control agents such as *Trichoderma species* and *Gliocladium virens* (56). *Trichoderma species* and *Gliocladium virens* are two biological control products registered by the U.S. Environmental Protection Agency (EPA) for control of plant diseases. The antagonistic effects of mycoparasitic fungi and parasitic bacteria on toxigenic *A. flavus* and *A. parasiticus* have not been investigated adequately. A clear understanding of the ecology of aflatoxigenic molds and the potential biocompetitive agent is necessary in order to control aflatoxin contamination biologically. The effectiveness of potential biocompetitive agents must be assessed in vitro before being

put into practice although it is always difficult to try to stimulate natural habitats in the laboratory.

The Genus *Nannocystis*

Nannocystis is found in the Order *Myxococcales* and the Family *Polyangiaceae* (80). Bacteria of the *Myxococcales* are Gram negative, aerobic, non-endosporeforming, unicellular rods which move by gliding motility. The *Myxococcales* produce fruiting bodies and myxospores within the fruiting bodies. In additional, all bacteria within the *Myxococcales* typically produce extracellular enzymes which hydrolyze proteins, nucleic acids, fatty acids esters and various polysaccharides. Most are capable of lysing live and dead cells of other bacteria, yeasts and molds. Thus, the *Myxococcales* are micropredators and microscavengers which obtain their nutrients by lysing living and dead cells of other bacteria, yeasts and molds. Genera of *Polyangiaceae* have vegetative cells with blunt, rounded ends, and the myxospores resemble the vegetative cells (80). Only one species, *Nannocystis exedens*, is distinguished within the genera *Nannocystis* (80). However, 9 strains of *N. exedens* (Table 4) have been isolated from soils (94). No strain of *N. exedens* have been shown to be pathogenic. Morphological and physiological characteristics of *N. exedens* are summarized in Table 5. *N. exedens* is a Gram-negative, stout rod with blunt ends (80). Vegetative cells are 1.5-5 μm long and 1.1-2 μm wide (Fig. 4). Cells of *N. exedens* tend to become ovoid, ellipsoid or club-shaped in old cultures (94). At this stage, they look like yeast cells. Vegetative cells of *N. exedens* are

TABLE 4. Strains of *N. exedens* (94).

Species	Strain	Source	Date of Isolation
Nannocystis exedens	*Na e1*[1]	red, sandy desert soil, Navajo, Arizona	Oct. 1968
	Na e2	black forest soil, Cimarron, New Mexico	Jan. 1969
	Na e3	black prairie soil, Columbus, Nebraska	Febr. 1969
	Na e4	black prairie soil, Columbus, Nebraska	Febr. 1969
	Na e5	black prairie soil, Columbus, Nebraska	Febr. 1969
	Na e6	sandy soil, Steriling, Colorado	Dec. 1966
	Na e7	soil from jungle, Tutuila, Samoa	Dec. 1966
	Na e8	soil from coconut grove, Upolu, Samoa	Dec. 1966
	Na e9	soil from the banks of Mississippi river in Minneapolis, MN	Oct. 1966

[1]*Nannocystis exedens* ATCC 25963.

TABLE 5. Morphological and physiological characteristics of *Nannocystis* (80,94).

Characteristic	Strain *Na e1*
Shape	Rod
Gram stain	-
Growth in air	+
Growth anaerobic	-
Bacteriolytic	+
Attacks yeast cells	+
OF test	Oxidative
Catalase	+
Nitrate reduced	+
Hydrolysis of	
Starch	-
Gelatin	+
Casein	+
Peptone from casein	+[1]
Agar	+
Growth at	
23°C	+
30°C	+
36°C	+
Growth at approximately pH 7	Optimum
Carbohydrate acid from	
Glucose	+[3]
Galactose	+[3]
Sucrose	+[3]
Cellulose	-
Vitamin B_{12}	Enhances growth
Cultures have earthy smell	+[2]
Squalene and sterols	Present

[1] 0.3% inhibits growth.
[2] Due to geosmin.
[3] Does not stimulate growth.

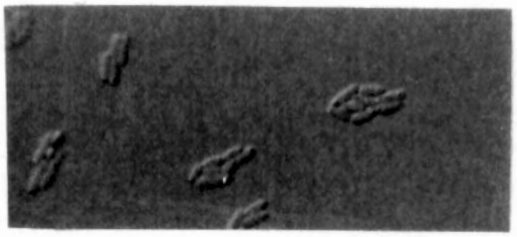

Figure 4. *Nannocystis exedens* ATCC 25963 vegetative cells on 0.3% TPYA slide culture.

ivory or brightly colored, usually orange to tomato-red. When in contact with a solid surface, *N. exedens* moves by gliding, leaving behind polysaccharide slime tracks. The cells within the slime occur as clumps or lines resembling strings of beads (Fig. 5). Under conditions of nutrient deprivation, vegetative cells aggregate to form fruiting bodies. The fruiting bodies are composed of slime and tightly packed clumps of cells. Within the fruiting bodies, the vegetative cells are converted to resting cells called myxospores. The myxospores are ovoid or spherical and measure 0.75 and 1.5 μm in diameter. (80,94). Fruiting bodies containing myxospores are called sporangia (80). Sporangia of *N. exedens* are solitary and may be embedded within the substrate or partially raised above the surface of the substrate. When embedded within the agar, the sporangia are slender and ovoid to spherical and may be 6 x 3.5 μm to 30 x 15 μm. When sporangia are raised slightly above the surface, they look like tiny knobs or heads (80,94). Sporangia are 110 x 40 μm, bean- or sausage-shaped and may be branched. The sporangia have a tough wall that confers some degree of resistance to heat, ultraviolet light and desiccation. The sporangia of *N. exedens* may be colorless, pale yellow-brown to dark red-brown (80,94). When favorable environmental conditions are present, the sporangia release the myxospores which germinate and elongate into rod-shaped cells. *N. exedens* is commonly found in soils, decaying plant debris and dung of herbivorous animals in warm and temperate climate zones all over the world. It is a very common myxobacterium found in every pinch of aerated soils with pH ranges near neutral. The bacterium does not grow in cold climate zones and soils with extreme pH values (80).

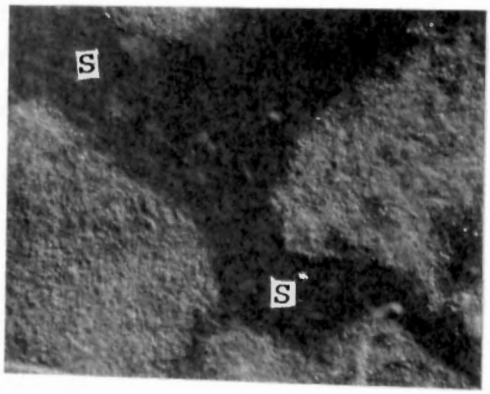

Figure 5. Swarm (S) of *Nannocystis exedens* ATCC 25963 vegetative cells on 0.3% TPYA slide culture.

All strains of *N. exedens* are strict aerobes and chemoorganotrophs; that is, they derive their energy from oxidative degradation of organic compounds. They are not fastidious and may be cultivated on ordinary bacteria media. *N. exedens* may be enriched and isolated by inoculating streaks of living *Escherichia coli* or *Sarcina lutea* on water agar containing cycloheximide with small amounts of soil, decaying plant debris or dung. The crude cultures are incubated at 28-30°C for 4-14 days. Pure cultures may be maintained on streaks of autoclaved *E. coli* or yeast cells on 0.3-1.5% trypticase peptone yeast extract agar. *N. exedens* also grows well in liquid media containing 0.3-1.5% trypticase peptone yeast extract. On solid media containing 0.05-3% peptone, *N. exedens* produces swarm colonies that are round or irregular flat sheets or mounds. Agar corrosion is usually moderate or absent. On water agar, the swarms produce corrosion of the agar in the form of radiating furrows, channels, tunnels, bubbles and holes. The swarm may be yellow-orange to brick-red, purple or brown to almost black, depending on the strain and the age of the culture. *N. exedens* grows in liquid media as coarse flakes.

CHAPTER III

MATERIALS AND METHODS

Test Organisms

Aspergillus flavus ATCC 16875 and 26946 were obtained from the culture collection of the American Type Culture Collection (ATCC) in Rockville, MD. *Aspergillus parasiticus* NRRL 3145 was obtained from the culture collection of the Northern Regional Research Center, ARS-USDA in Peoria, IL. *A. flavus* ATCC 16975 and 26946 produce aflatoxin B_1,B_2, and sclerotia. *A. parasiticus* NRRL 3145 produces aflatoxin B_1,B_2,G_1, and G_2. Stock cultures were grown at 28°C for 7 days on potato dextrose agar (Difco) (PDA) slants, stored at 4°C and transferred every 8 weeks.

Nannocystis exedens ATCC 25963 (strain *Na e* 1) was obtained from the culture collection of ATCC in Rockville, MD. *N. exedens* is a known micropredator. The bacterium was grown on trypticase peptone-yeast extract agar (TPYA) and broth (TPYB) containing 1.5% trypticase peptone (Difco), 0.1% yeast extract (Difco), 0.1% calcium chloride dihydrate, and/or 1.5% agar. The pH of TPYA and TPYB was adjusted to 7.2 with 1N NaOH and autoclaved at 121°C for 15 min. *N. exedens* was maintained on 1.5% TPYA and TPYB at 28°C and transferred every 3 weeks.

Inoculum Preparation

Mold suspensions were prepared by collecting spores from 7-day old PDA slant cultures incubated at 28°C. Slants were washed with five mL of sterile distilled water. The spores were loosened from the slants by brushing with a sterile inoculating loop. One mL of the suspension was then pipetted into a 13 x 100 mm test tube containing three mL of sterile distilled water to give an inoculum with a concentration approximately 10^1 spores and mycelia/mL. The total count of the four mL suspension was determined using the spread plate method on PDA (Table 6). Prior to preparation of mold inoculum, stock cultures were allowed to come to room temperature overnight.

N. exedens cell suspensions were prepared by cultivating the bacterium in 50 ml of 1.5% TPYB for three days at 28°C. Then, culture was centrifuged at 10,000 rpm for 15 min at 5°C. The supernatant was decanted and 50 mL of 0.3% TPYB broth was added to the cell pellet. Then, culture was incubated at 28°C for 48 hours. The 48-hours culture was used as the bacterium inoculum for this investigation. The total cell count of the suspension was determined using the spread plate method on 1.5% TPYA plates (Table 6).

Test Media

To detect antagonism and to determine the effects of *N. exedens* culture suspensions on aflatoxin B_1 and G_1 synthesis, 0.3% TPYA and 0.3% TPYSA were used as the test media, respectively. TPYA at 0.3% was prepared as previously described.

TABLE 6. Colony forming units/mL of mold and bacteria inoculum.

Inoculum	volume of inoculum(mL)	CFU/mL
Aspergillus parasiticus	4	7.6×10^8
A. flavus[1]	4	4.4×10^8
A. flavus[2]	4	2.7×10^8
N. exedens	50	6.1×10^7

[1]*A. flavus* ATCC 16875.
[2]*A. flavus* ATCC 26946.

TPYSA contained 2% yeast extract, 2% sucrose, 0.3% trypticase peptone, 0.1% calcium chloride dihydrate, and 1.5% agar. The pH of TPYSA was adjusted to 7.2 with 1N NaOH and autoclaved at 121°C for 15 min.

In *Vitro* Screening for Biological control

Several experiments were conducted to determine the effects of *N. exedens* ATCC 25963 on *A. flavus* (ATCC 16975 and 26946) and *A. parasiticus* NRRL 3145 growth and aflatoxin production.

Screening of *N. exedens* for antifungal activity. To determine if *N. exedens* released diffusible antifungal compounds into the medium, 0.2 mL of the bacterium suspension was streaked down the center of a 100 x 15 mm petri plate containing 15 mL of 0.3% TPYA, and 0.2 mL of the mold suspension was streaked next to the bacterium streak on the same day. On another petri plate, 0.2 mL of the mold suspension was streaked down the center of the plate, and 0.2 mL of the bacterial suspension was streaked on each side the mold streak on the same day. All plates were incubated at 28°C for 14 days.

Screening of *N. exedens* for lytic activity. To determine if *N. exedens* would attack aflatoxigenic molds, three drops of the spore suspension was 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.

Direct observation of *N. exedens* predator 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 prior to inoculation. The mold suspension (15 μL) was spot inoculated onto the center of the slide and 15 μL of the bacterium suspension was spot-inoculated onto the mold suspension on the same day. A sterile 22 x 50 mm No. 1 1/2 cover slip was placed over the slide following inoculation. All slide cultures were placed into a petri plate moist-chamber which consisted of a petri plate lined with moist Whitman's No. 4 filter paper and two sterilie Durham tubes taped to the filter paper. Cultures were incubated at 28 $^{\circ}$C for 36 hours. Slides were examined under a microscope equipped for photography at 20x and 100x (oil).

Effects of *N. exedens* on aflatoxin B_1 and G_1 synthesis. The objective of this experiment was to determine the effects of *N. exedens* culture suspensions on aflatoxin B_1 and G_1 synthesis of aflatoxigenic molds. The mold suspension (0.1 mL) and the bacterium suspension (0.1 mL) were added to a 15 mL screw cap test tube and mixed thoroughly. The mixture was then pipetted onto the surface of a TPYSA plate (25 mL/plate) and spread over the surface of the plate with a sterile glass hockey stick. All

plates were incubated at 28°C for 14 days.

Aflatoxin analysis. The entire content of each plate was placed into a 250 mL Erlenmeyer flask and extracted twice with 50 mL of chloroform on a mechanical shaker for 30 min. The chloroform phase (bottom layer) was filtered through anhydrous sodium sulfate, collected in a 100 mL round bottom flask, and evaporated to dryness at 60°C on a rotary evaporator. The extracts were reconstituted with 0.5 mL of acetonitrile and filtered through a 0.45 μm filter into a dark vial.

Aflatoxin B_1 and G_1 contents of each sample were determined at the end of the experiment on Redi-Plate Silica Gel G-precoated TLC plates (20 x 20 cm coated with a 0.25 mm thick layer of silica gel G; Fisher Scientific Company, Pittsburgh, PA). Each plate was heat activated at 90°C for 30 min and allowed to cool at room temperature for 15 min. Activated plates were spot inoculated with 10 μL of sample, and the TLC plate was developed for 30-45 min in a saturated chromatographic tank containing chloroform-acetone (88:12, v/v). After development, TLC plates were dried at room temperature and exposed to longwave ultra-violet light (425 nm) for visual estimation. Sample spots were compared with aflatoxin B_1 and G_1 standards (Sigma Chemical Company, St. Louis, MO, Table 7).

TABLE 7. Aflatoxin standards for standard TLC plates.

Standard[1]	Solvent	Concentration (μg/mL)
Aflatoxin B_1	Chloroform	1000
Aflatoxin G_1	Chloroform	1000

[1]5 mg of standard was dissolved in 5 mL of chloroform.

Aflatoxin standards were prepared by adding five mL of chloroform to five mg crystallized B_1 or G_1 standard to give concentration of five mg/mL. Standard TLC plates were prepared by spotting 0.1-10 μg of each standard on the TLC plates. Aflatoxins B_1 and G_1 levels were determined by comparing sample spots with aflatoxin standards spots and dividing the observed μg of B_1 and/or G_1 sample spot by mL of sample spotted on TLC plate.

Experimental Design

In determining the effect of *N. exedens* on mold growth, experiments were designed with one temperature: 28°C; one mold inoculum level: 10^1 spores and mycelia/mL; three aflatoxigenic molds: *Aspergillus flavus* ATCC 16875, *A. flavus* ATCC 26946, and *A. parasiticus* NRRL 3145; one test media: 0.3% TPYA; one biocompetitive agent: *Nannocystis exedens* ATCC 25963; and one inoculation procedure: mold plus bacterium. Controls consisted of mold suspensions without bacteriun.

In determining the effect of *N. exedens* on aflatoxin production, the experiment was designed as previously mentioned; however, 0.3% TYPSA was used as the test medium. The randomized block experimental design was used with a total of one block for each mold. Each block consisted of one treatments. Each treatment consisted of one amount of each of the following: temperature, inoculum level, test medium and inoculation procedure. There was one treatment per mold. The experiment was done in triplicate and repeated twice. Controls consisted of mold without bacterium.

CHAPTER IV

RESULTS AND DISCUSSION

Screening of *N. exedens* for antifungal activity

Aspergillus flavus ATCC 16875, *A. flavus* ATCC 26946, or *A. parasiticus* NRRL 3145 were streaked next to *Nannocystis exedens* ATCC 25963 streaks on 0.3% TPYA plates. All plates were incubated at 28°C for 14 days. Fig. 6 and 7 show the effects of *N. exedens* on *A. flavus* (ATCC 16875 and 26946) when the mold was streaked down the center of the plate. Similary results were observed when *N. exedens* streaked next to *A. parasiticus*. Figures 8 shows the effects of *N. exedens* on *A. parasiticus* when the bacterium was streaked the down the center of the plate. Similary results were observed when *A. flavus* ATCC 16875 and *A. flavus* ATCC 26946 were next to *N. exedens*. There was a general restriction of growth of each mold colony when *N. exedens* was streaked on the side of each mold (Fig. 6 and 7). A noticeable zone of inhibition was observed between the bacterial and mold colonies when the mold was streaked next to *N. exedens* (Fig. 8). The reaction was not a mutual inhibition at a distance type reaction because there was a distinct flattening of the colony of each mold on the sides nearest *N. exedens* which continued to grow. The colony of each mold was growing away from the colony of *N. exedens*. The rate of mold growth and sporulation was not altered by *N. exedens*; however, the extent of growth and sporulation was decreased markedly in the presence of *N. exedens* as compared with mold alone.

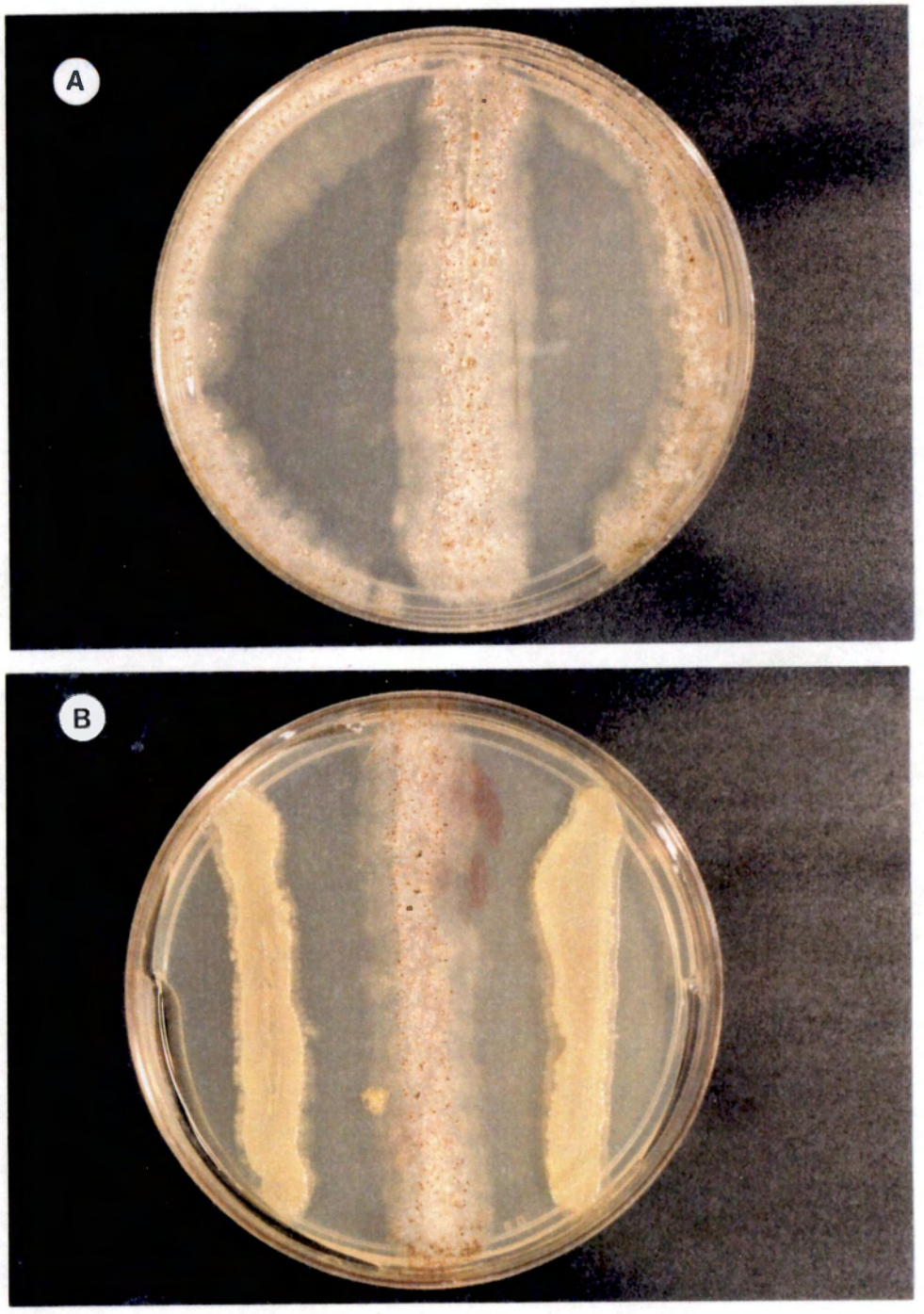

Figure 6. Inhibition of growth of *Aspergillus flavus* ATCC 16875 by *Nannocystis exedens ATCC* 25963 on TPYA after 14 days at 28°C. (A) *A. flavus* ATCC 16875 alone. (B) *N. exedens ATCC* 25963 streaked next to *A. flavus* ATCC 16875.

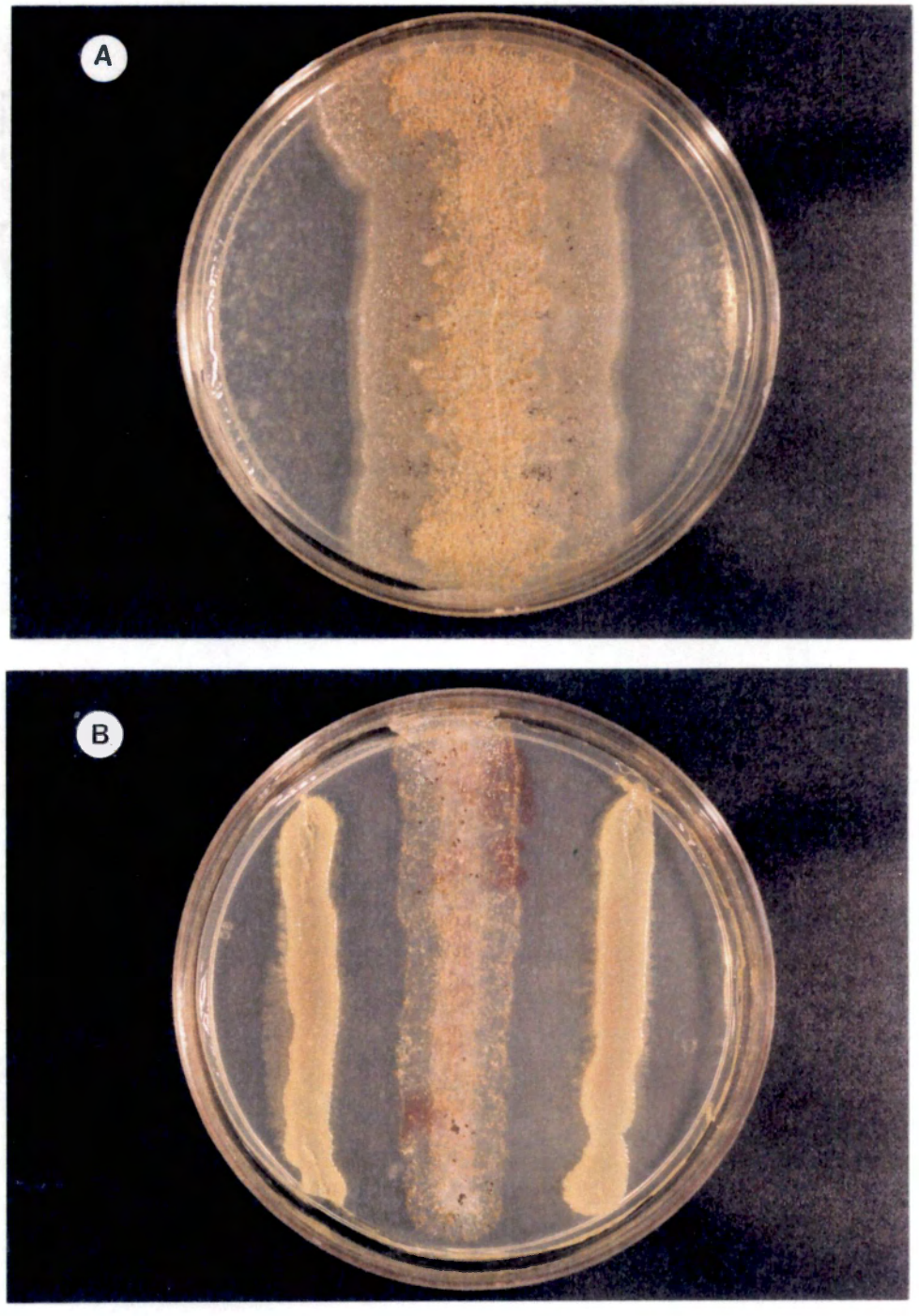

Figure 7. Inhibition of growth of *Aspergillus flavus* ATCC 26946 by *Nannocystis exedens ATCC* 25963 on TPYA after 14 days at 28°C. (A) *A. flavus* ATCC 26946 alone. (B) *N. exedens ATCC* 25963 streaked next to *A. flavus* ATCC 26946.

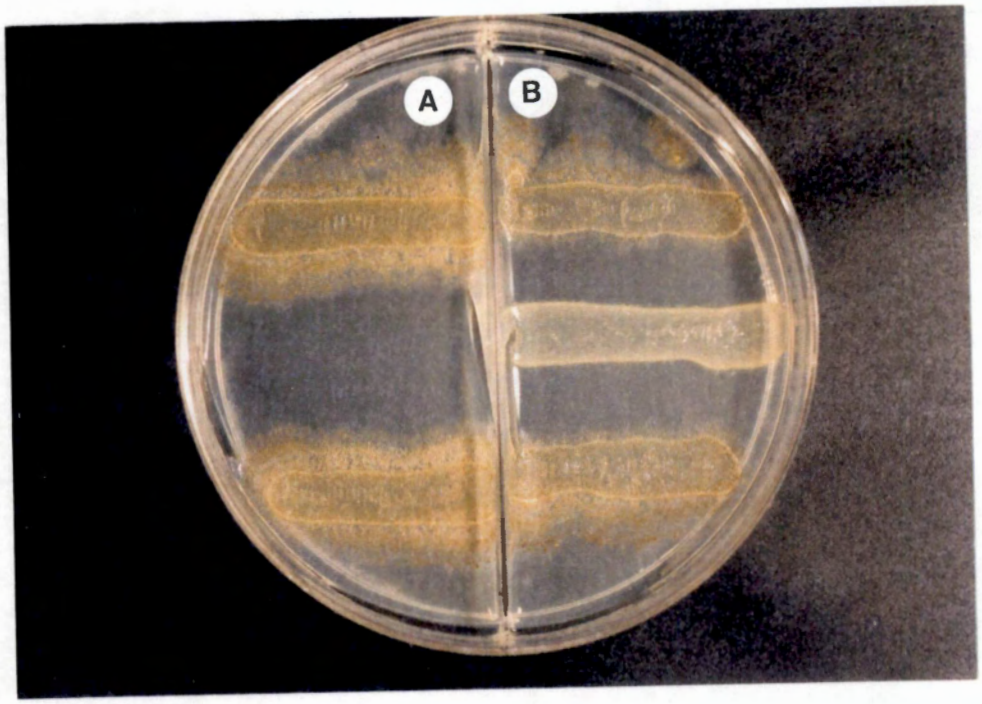

Figure 8. Inhibition of growth of *Aspergillus parasiticus* NRRL 3145 by *Nannocystis exedens ATCC* 25963 on TPYA after 14 days at 28°C. (A) *A. parasiticus* NRRL 3145 alone. (B) *A. parasiticus* NRRL 3145 streaked next to *N. exedens ATCC* 2596.

Mycelia growth did not extend into the medium and there was a decrease in the extent of sporulation as compared with that of the control plates.

These results represent the first direct evidence demonstrating that *N. exedens* ATCC 25963 will antagonize toxigenic *A. flavus* ATCC 16875, *A. flavus* ATCC 26946 and *A. parasiticus* NRRL 3145. Several of the criteria for antagonism described by Johnson et al. (60) were observed when *N. exedens* was grown in close proximity with each mold on 0.3% TPYA. The zones of inhibition which developed between *N. exedens* and each mold colony, flattening of the mold colony on the sides nearest *N. exedens*, mold colony growing away from the bacteria colony and a general stunting of the mold colony are criteria for antagonism described by Johnson et al. (60). The form of antagonism expressed by *N. exedens* during this investigation appears to be due to the release of one or more diffusible, antifungal compounds into the surrounding medium. One of the characteristic features of the *Myxococcales* is that they produce a wide variety of antimicrobial agents (80,94,96). However, relatively few of these agents have been characterized as antibiotics. The following myxobacterial antibiotics have been characterized: antibacterial antibiotics from *Myxococcus coralloides* (3), *M. xanthus* (36), *M. fulvus* (55), *M. virescens* (36), *Sorangium* species (90), and *Cystobacter fuscus* (69). Antifungal antibiotics have been described from *Polygangium cellulosum* (16,95), *M. xanthus* (86) and *M. fulvus* (35). Results from this study indicate that *N. exedens* produces inhibitory compounds that are responsible for inhibition of growth of *A. flavus* (ATCC 16875 and 26946) and *A. parasiticus* NRRL 3145. The fact that almost 80% of soil

myxobacteria produce antibiotics which allow them to compete favorably with other members of the soil microflora suggests that *N. exedens* also produces antibiotics, particularly since *N. exedens* is the most common myxobacteria found in soils. However, at present no investigation has reported on isolation and characterization of any antimicrobial agents from *N. exedens*. Foster et al. (33) isolated 164 strains of myxobacteria from the soil and found that 77.4% excreted antibiotics into the medium and 29% had antifungal activity. However, he failed to isolate *N. exedens* because he added nystatin to the isolation media. Nystatin is an antifungal compound which acts by binding to steroids in the cytoplasmic membrane (63). *N. exedens* has steroids present (68). Although no attempt to isolate the antifungal compound in this investigation, the present results suggest that antifungal compounds were released into the medium, and further research should include isolation and characterization of these antifungal compounds produced by *N. exedens*.

Screening of *N. exedens* for lytic 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, lyses of each mold by *N. exedens* was observed only on the inoculated area (Fig. 9-11). 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.

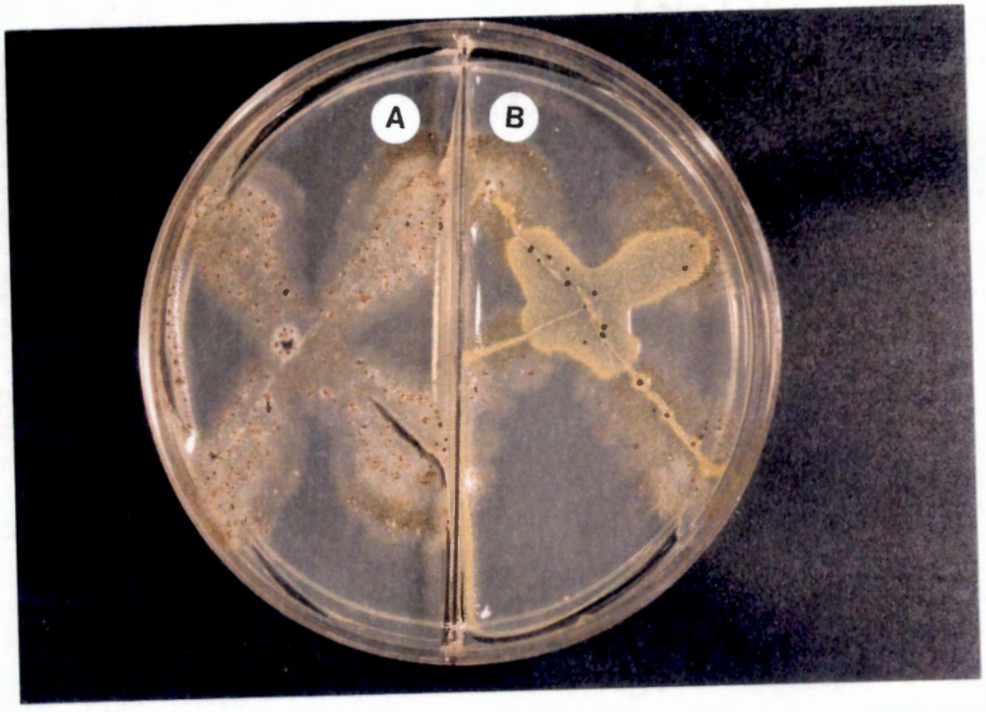

Figure 9. Lysis of *Aspergillus flavus* ATCC 16875 by *Nannocystis exedens ATCC* 25963 on TPYA after 7 days at 28°C. (A) *A. flavus* alone. (B) *N. exedens* colony in the center of an *A. flavus* cross-streak.

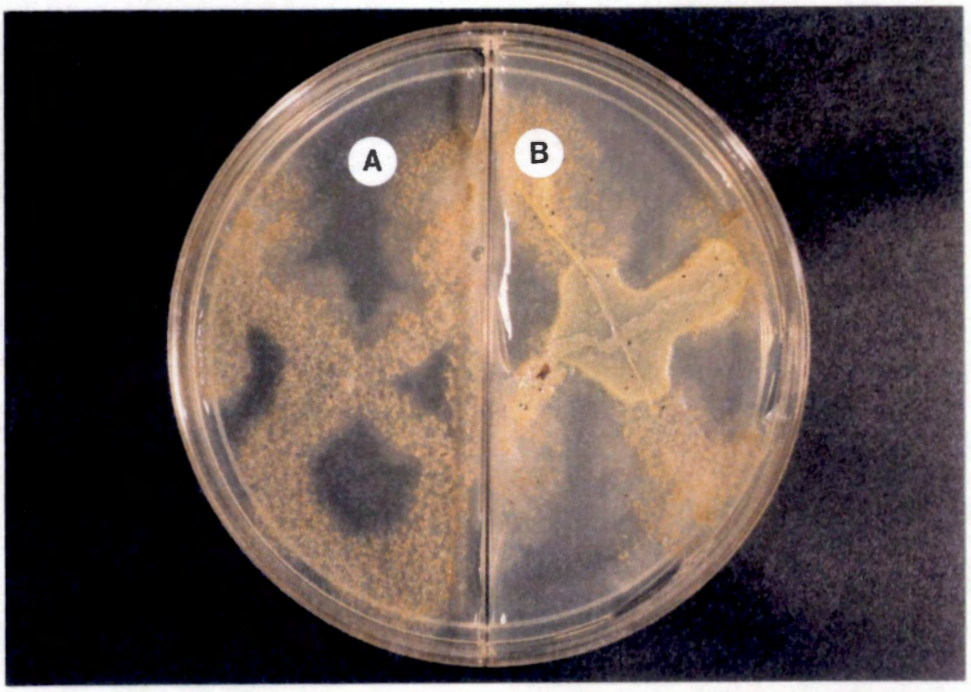

Figure 10. Lysis of *Aspergillus flavus* ATCC 26946 by *Nannocystis exedens ATCC* 25963 on TPYA after 7 days at 28°C. (A) *A. flavus* alone. (B) *N. exedens* colony in the center of an *A. flavus* cross-streak.

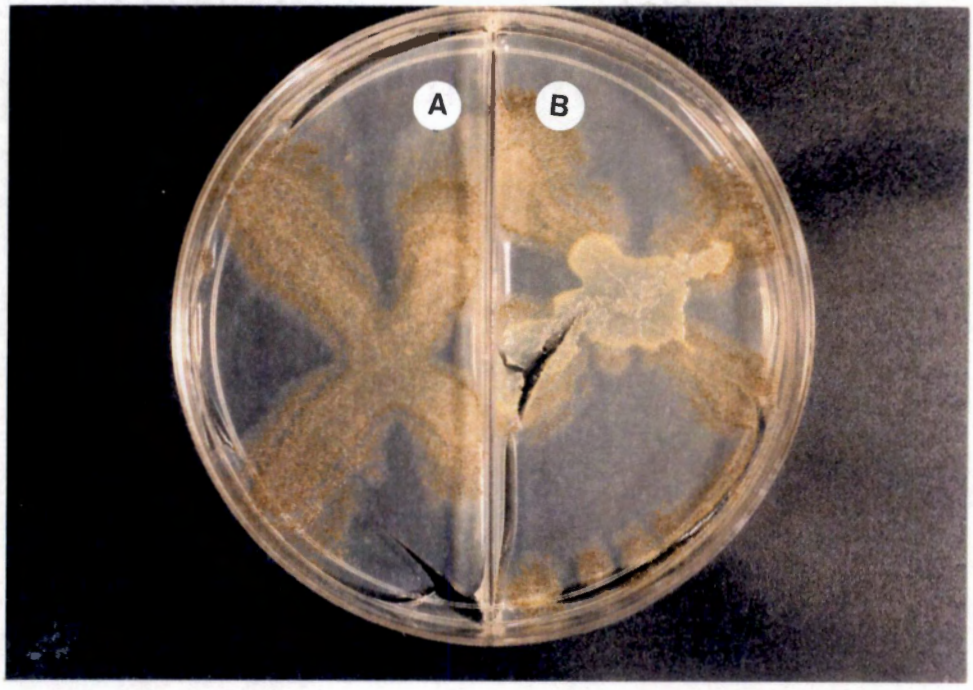

Figure 11. Lysis of *Aspergillus parasiticus* NRRL 3145 by *Nannocystis exedens ATCC* 25963 on TPYA after 7 days at 28°C. (A) *A. parasiticus* alone. (B) *N. exedens* colony in the center of an *A. parasiticus* cross-streak.

Outside of the zone of lyse, mycelia growth, spore and sclerotia germination were observed.

The present results represent the first evidence that *N. exedens* ATCC 25963 will grow parasitically on aflatoxigenic molds. The growth of *N. exedens* on the mold colonies indicates lytic activity (60,26,111). The agar-plate lysing test indicates that *N. exedens* actively destroyed some of the mold colony in the inoculated area. *Myxococcales* are known to lyse prey organism by the excretion of extracellular enzymes (80). Reichenbach (94) proved that *N. exedens* will lyse both living and heat killed cells of *Sarcina lutea*, *Escherichia coli* and yeasts. Although Reichenbach (94) did not isolate or characterize any lytic enzymes of *N. exedens*, his results suggest that *N. exedens* possessses a variety of lytic enzymes which allow the bacterium to attack bacteria and yeasts. Likewise, the present results from this investigation also suggest that *N. exedens* possesses lytic enzymes which allowed the bacterium to lyse *A. flavus* (ATCC 16875 and 26946) and *A. parasiticus* NRRL 3145 although these enzymes were not isolated during this investigation. Nevertheless, this study demonstrated that *N. exedens* will parasitize aflatoxigenic molds as *N. exedens* parasitized cells of *Sarcina lutea*, *Escherichia coli* and yeasts in Reichenbach's investigation.

Direct observation of *N. exedens* predator 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 hours (Fig. 12-16). *N. exedens* developed as streaming colonies which surrounded spores, germinating spores, hyphae and sclerotia as a sheath of tightly packed rods, clumps of rods, or as individual rods. Sclerotia were surrounded by a sheath of streaming rods which became progressively thicker on one area of the sclerotia. Strands of hyphae were generally aligned with one or more rows of *N. exedens*. The bacteria formed large spherical fruiting bodies around the propagules of each mold also.

The present results represent the first evidence of actual contact between *N. exedens* ATCC 25963 and its host during the feeding process. It was shown that *N. exedens* will parasitize spores, germinating spores, hyphae and sclerotia of aflatoxigenic molds. The bacteria preyed upon the mold as streaming colonies of tightly packed rods, clumps of rods, or as individual rods. There was direct cell to cell contact between the bacterium and the molds (Fig. 12-16). The *Myxococcales* comprise the only group of bacteria known to aggregate to form a swarm during feeding. Rosenberg and Varon (96) suggested that a higher concentration of extracellular lytic enzymes produced by the swarm allow more efficient feeding. Shimket (97) described the social behavior of myxobacteria during the feeding process. Thousands of individual cells aggregate together to form a swarm which is embedded in an extracellular polysaccharide-protein-lipid slime layer (Fig. 5). The swarms serve as a means of acquiring nutrients by binding, denaturing, and hydrolyzing cell walls and proteins. Extracellular proteases and bacteriolytic enzymes are excreted into the sticky slime layer which allows the myxobacter to attach to its prey.

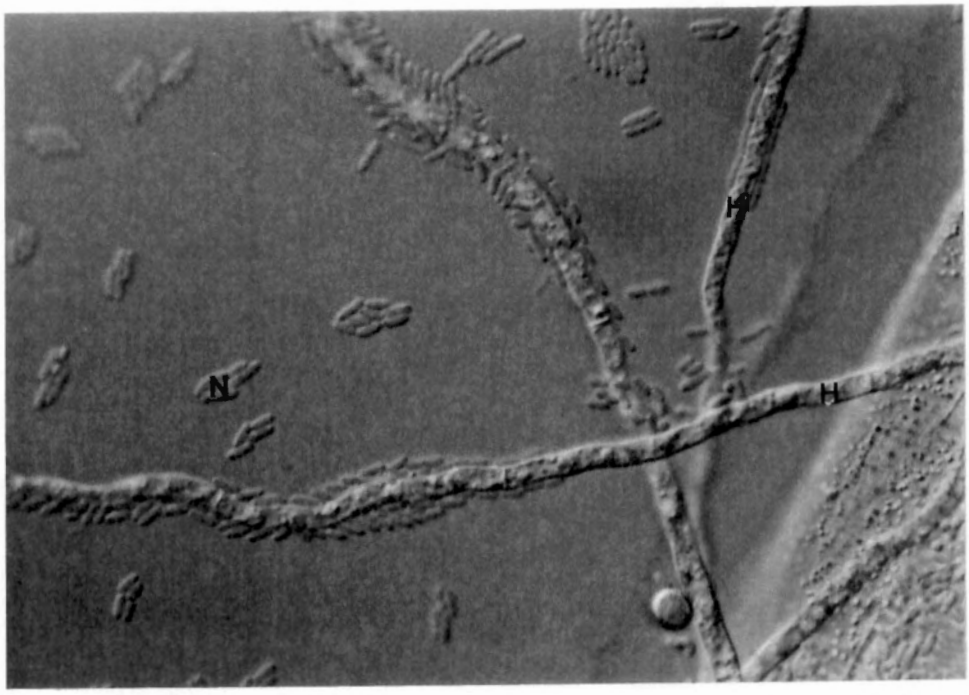

Figure 12. Lysis of hyphae (H) of *Aspergillus flavus* ATCC 16875 by *Nannocystis exedens ATCC* 25963 (N) at 36 hours.

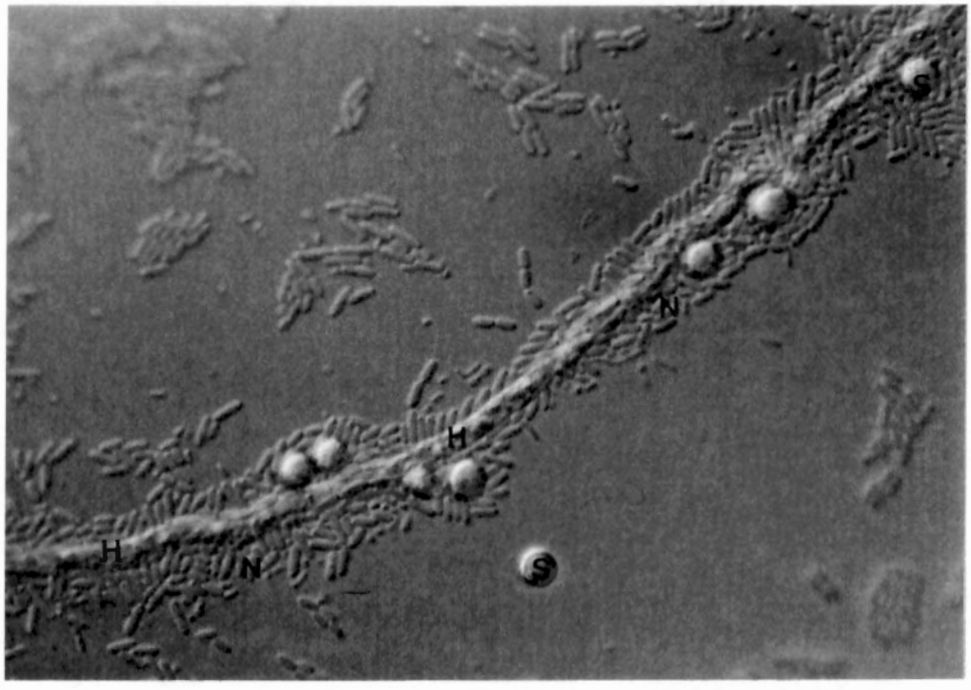

Figure 13. Lysis of hyphae (H) and spores (S) of *Aspergillus flavus* ATCC 26946 by *Nannocystis exedens ATCC* 25963 (N) at 36 hours.

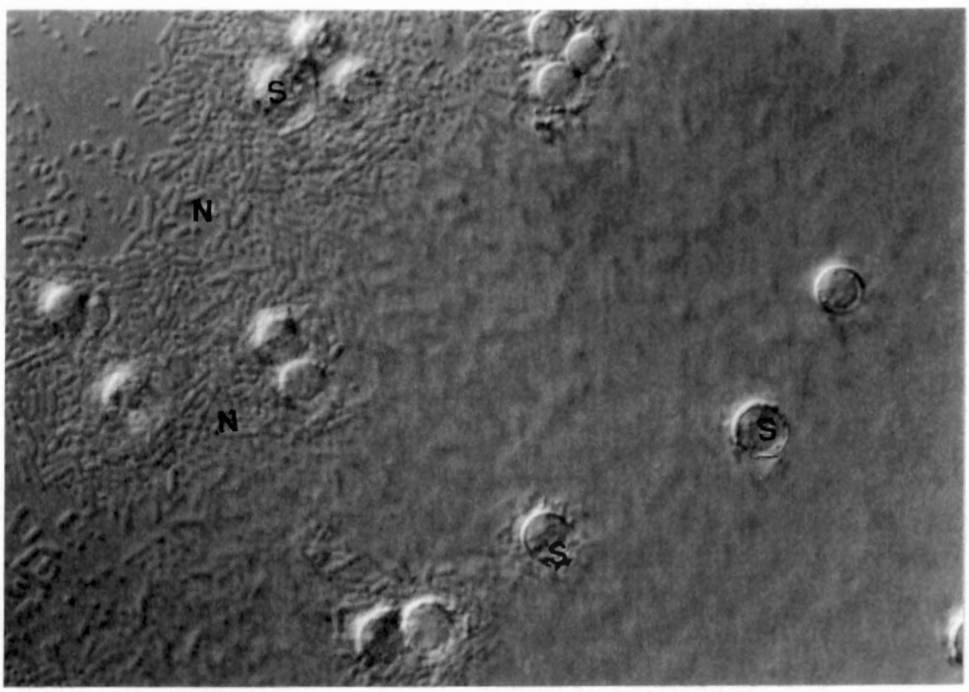

Figure 14. Lysis of spores (S) of *Aspergillus parasiticus* NRRL 3145 by *Nannocystis exedens ATCC* 25963 (N) at 36 hours.

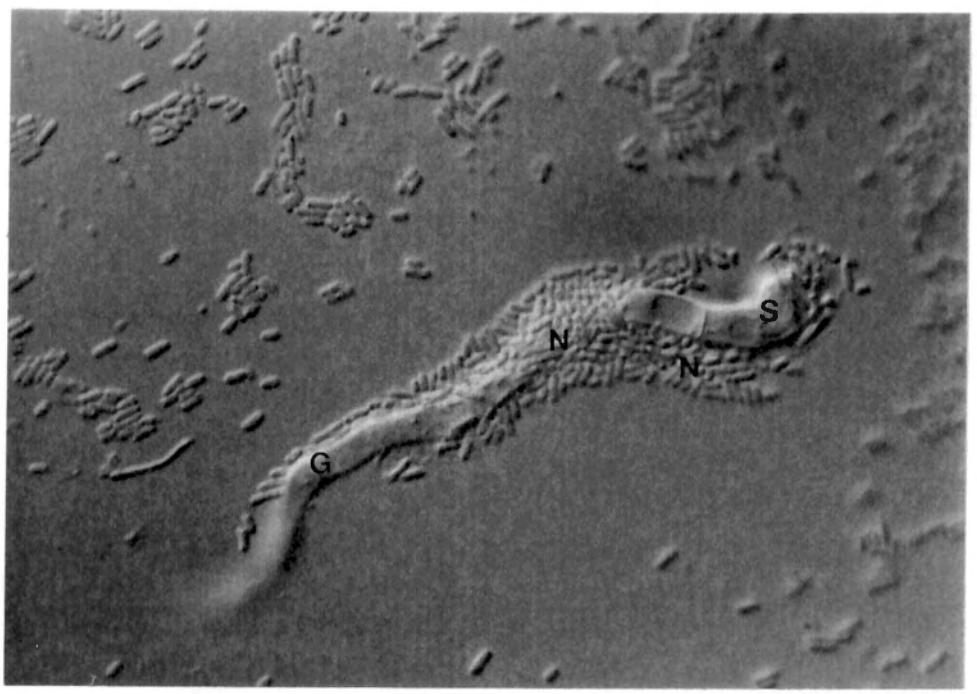

Figure 15. Lysis of a germinating spore (S) and germ tube (G) of *Aspergillus parasiticus* NRRL 3145 by *Nannocystis exedens ATCC* 25963 (N) at 36 hours.

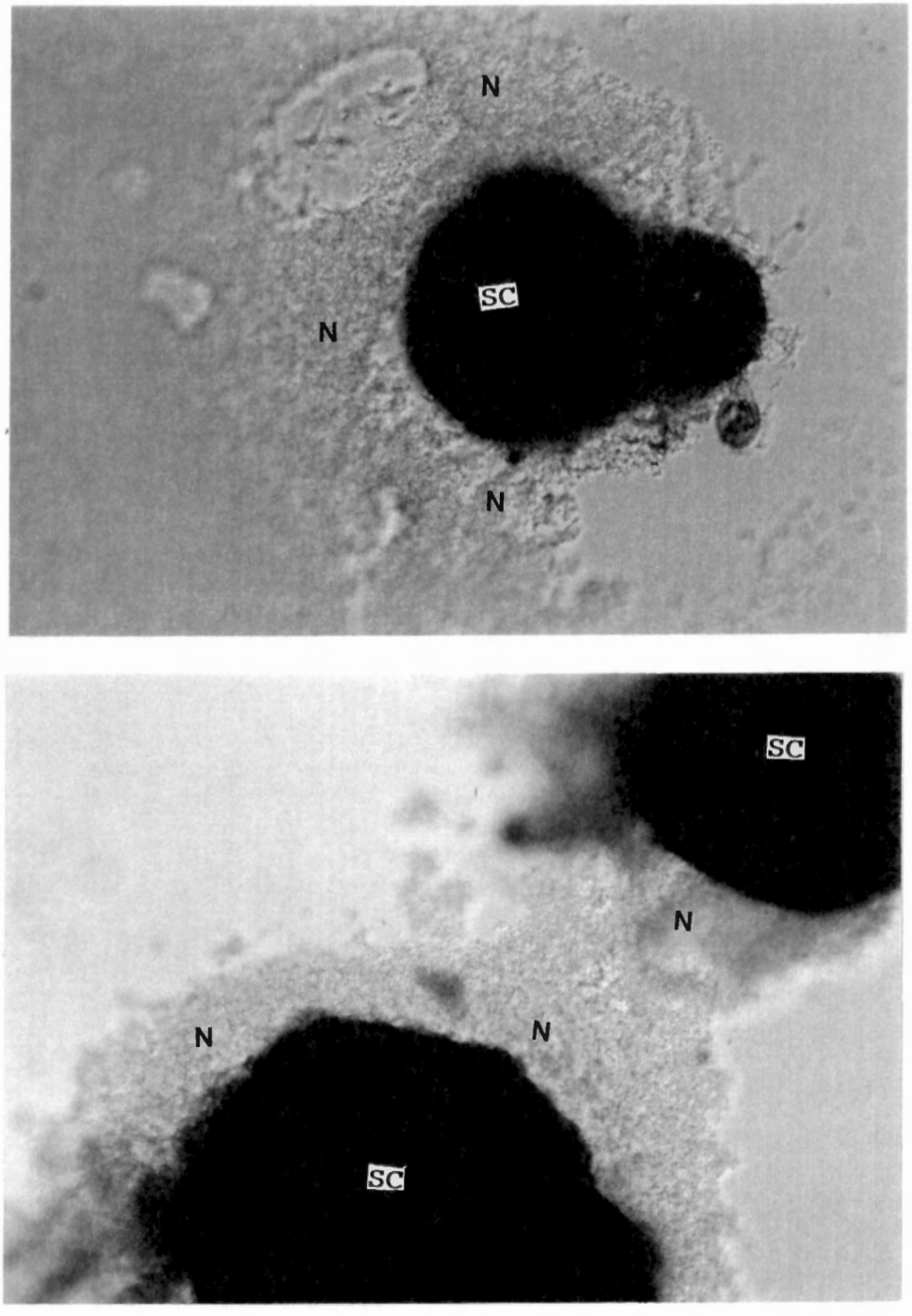

Figure 16. Lysis of sclerotia (SC) of *Aspergillus flavus* ATCC 26946 by *Nannocystis exedens ATCC* 25963 (N) at 36 hours.

When the prey becomes attached to the myxobacter, bacteriolytic enzymes dissolve the host's cell walls. The host is lysed and proteases dissolve the host's cytoplasmic membranes. Also, proteins are hydrolyzied into amino acids and peptides by proteases. The intracellular content of the cells is then dissolved by other extracellular enzymes released into the slime layer. Observations of the feeding behavior of *N. exedens* on aflatoxigenic molds suggests a correlation to the feeding behavior as described by Shimket (97). The formation of swarms and the binding of *N. exedens* to spores, hyphae, germ tubes, and sclerotia were observed during this investigation. Predation and parasitism appears to be another mechanism of survival of *N. exedens*. Future studies might lead to the examination of the enzymes involved in the lysis of aflatoxigenic molds.

Effects of *N. exedens* on aflatoxin B_1 and G_1 synthesis

A. flavus ATCC 16875, *A. flavus* ATCC 26946, or *A. parasiticus* NRRL 3145 were mixed with *N. exedens*, spreaded onto the surface of 0.3% TPYSA plate with sterile hockey stick and incubated at 28°C for 14 days. Aflatoxin B_1 and G_1 content of each sample were determined on Redi-Plate Silica Gel G-precoated TLC plates which were developed in chloroform-acetone (88:12). Aflatoxin B_1 and G_1 levels were calculated as μg/mL of 0.3% TPYSA. Figures 17-19 present the effects of *N. exedens* on the growth of *A. flavus* ATCC 16875, *A. flavus* ATCC 26946 and *A. parasiticus* NRRL 3145 respectively. Extensive mycelial growth, spore formation and sclerotia formation were

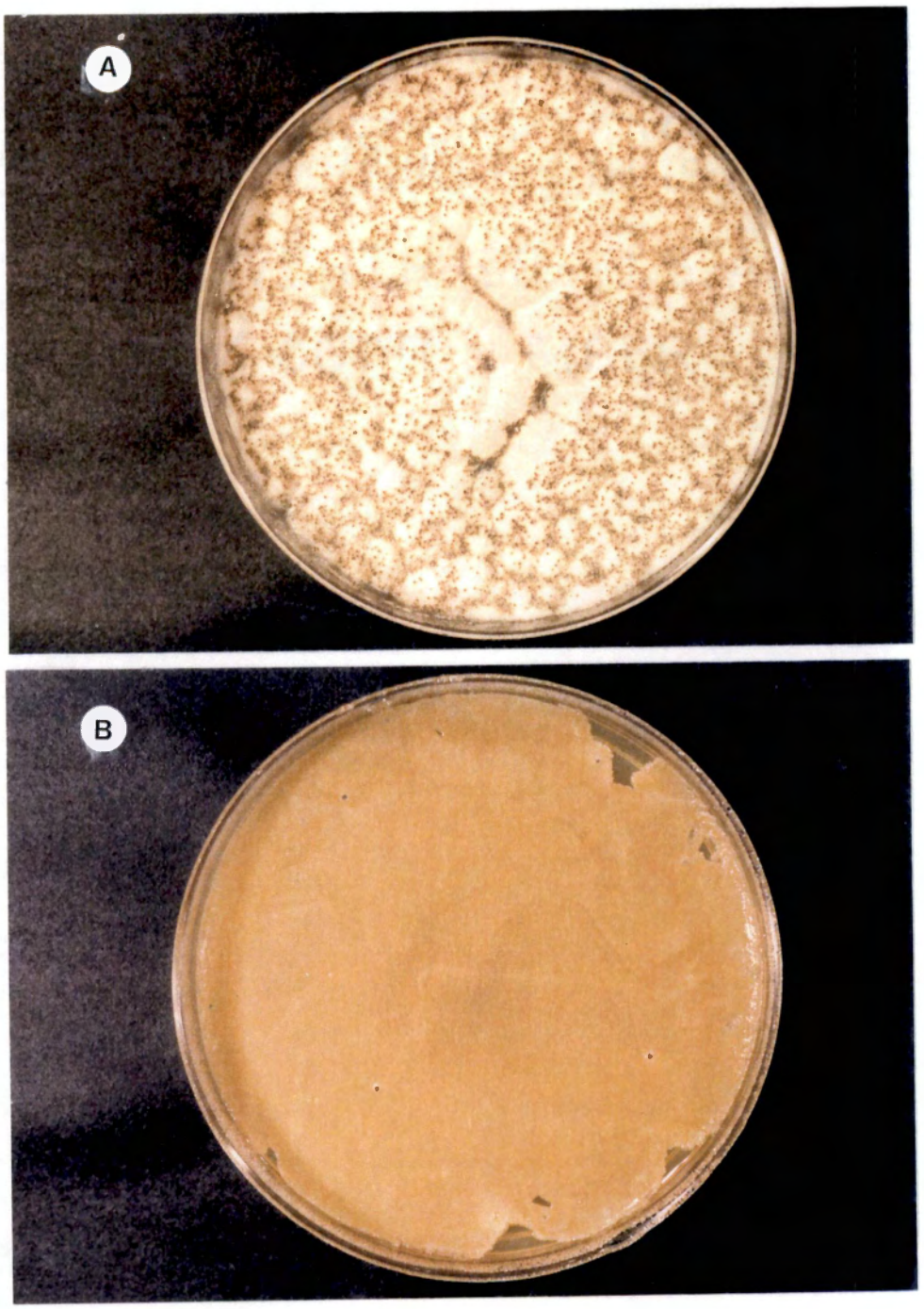

Figure 17. Inhibition of *Aspergillus flavus* ATCC 16875 growth on TPYSA by *Nannocystis exedens ATCC* 25963 after 14 days at 28°C. (A) *A. flavus* alone. *A. flavus* plus *N. exedens*.

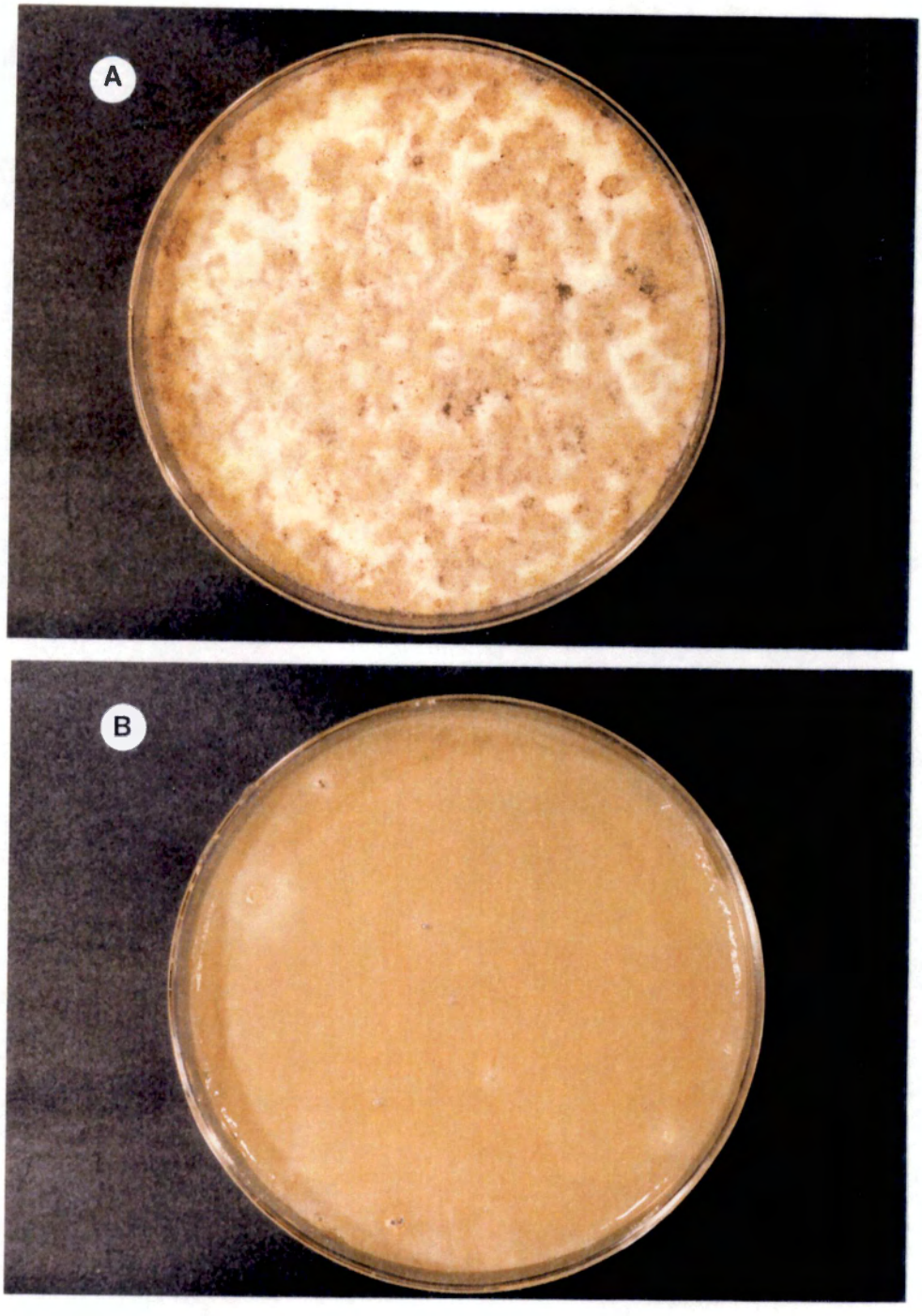

Figure 18. Inhibition of *Aspergillus flavus* ATCC 26946 growth on TPYSA by *Nannocystis exedens ATCC* 25963 after 14 days at 28°C. (A) *A. flavus* alone. (B) *A. flavus* plus *N. exedens.*

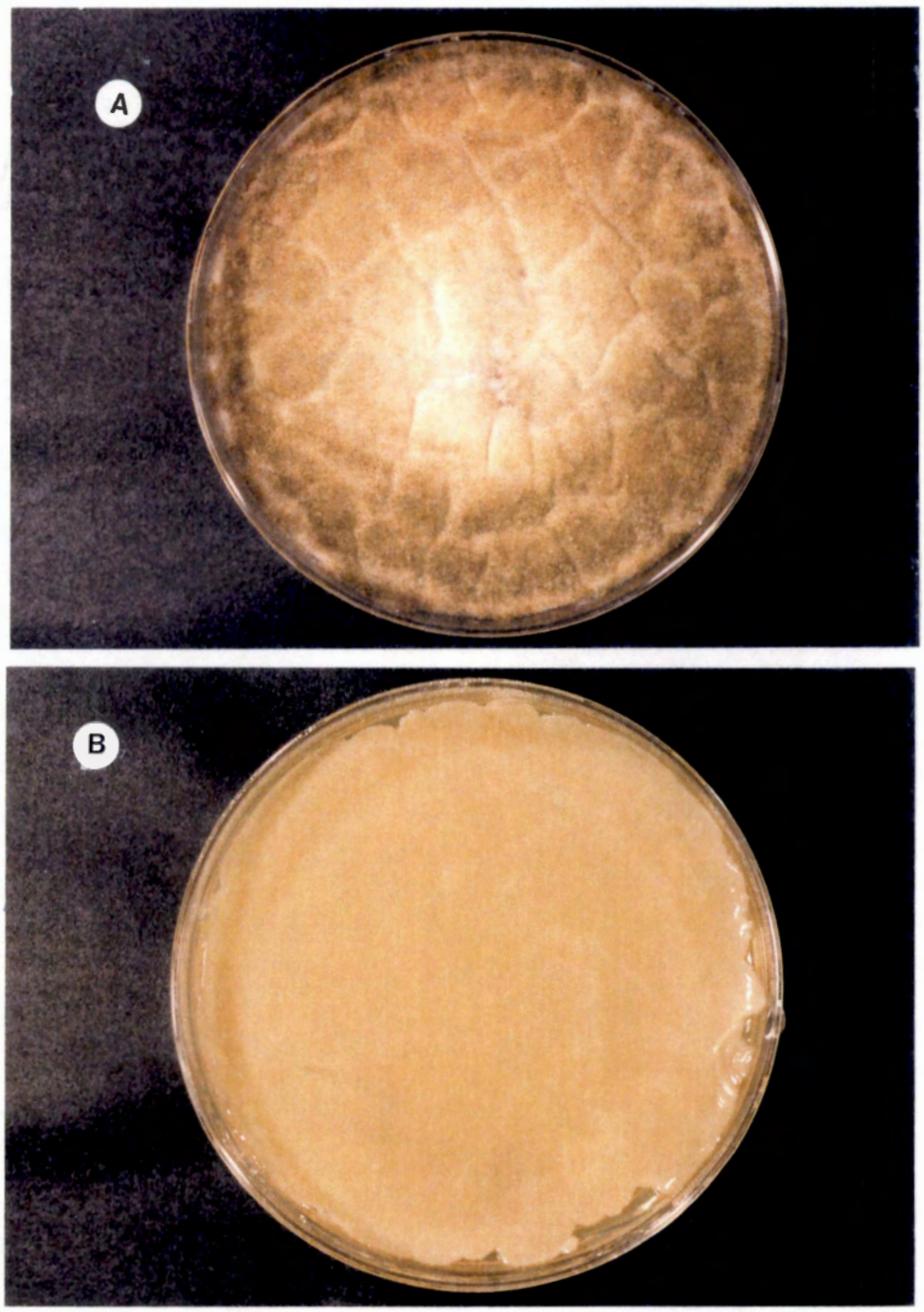

Figure 19. Inhibition of *Aspergillus parasiticus* NRRL 3145 growth on TPYSA by *Nannocystis exedens ATCC* 25963 after 14 days at 28°C. (A) *A. parasiticus* alone. (B) *A. parasiticus* plus *N. exedens*.

observed at day five when molds were inoculated along on 0.3% TPYSA. Mycelia growth, spore formation, and sclerotia germination were not observed when the molds were inoculated with *N. exedens* on 0.3% TPYSA. Only the growth of *N. exedens* was observed after 14 days when plates were inoculated with the bacteria plus the molds. Table 8 shows the effects of *N. exedens* on aflatoxin B_1 and G_1 production of *A. flavus* ATCC 16875, *A. flavus* ATCC 26946 and *A. parasiticus* NRRL 3145. When *A. flavus* ATCC 16875 was inoculated alone, aflatoxin B_1 (40 μg/mL) was produced by the mold. When *A. flavus* ATCC 26946 was inoculated alone, aflatoxin B_1 (500 μg/mL) was produced by the mold. When *A. parasiticus* NRRL 3145 was inoculated alone, aflatoxin B_1 (50 μg/mL) and G_1 (200 μg/mL) were produced by the mold. However, when each mold was inoculated with *N. exedens*, no aflatoxin was detected on TLC plates.

The present results represent the first evidence that *N. exedens* ATCC 25963 culture suspensions inhibit growth and aflatoxin production of *A. flavus* (ATCC 16875 and 26946) and *A. parasiticus* NRRL 3145. The inhibition of growth and production of aflatoxins in this study was due probably to parasitism of the propagules of *A. flavus* ATCC 16875, *A. flavus* ATCC 26946 and *A. parasiticus* NRRL 3145 by *N. exedens*. It was also shown that *N. exedens* will parasitize spores, germinating spores, hyphae and sclerotia of aflatoxigenic molds. The inhibition of aflatoxin production by *Lactobacillus* species (39,61), *Bacillus subtilis* (64,19), *Fusarium moniliforme* (11), *Rhizopus nigricans* and *Saccharomyces cerevisiae* (112), *Tricoderma viride* (118) and *Aspergillus niger* (118) have been reported;

TABLE 8. Effect of the *Nannocystis exedens* ATCC 25963 on aflatoxin production by *Aspergillus flavus* ATCC 16875, *A. flavus* ATCC 26946 and *A. parasiticus* NRRL 3145 in 0.3% TPYSA inoculated for 14 days at 28°C.

	Aflatoxin (μg/ml)	
	B_1	G_1
A. flavus[1]	40	
A. flavus[2]	500	
A. parasiticus	50	200
A. flavus[1] + *N. exedens*	ND	
A. flavus[2] + *N. exedens*	ND	
A. parasiticus + *N. exedens*	ND	ND

[1]*A. flavus* ATCC 16875.

[2]*A. flavus* ATCC 26946.

ND= Not Detected.

however, in these studies, inhibition of aflatoxin production was due to low pH, competition for nutrients and/or the release of inhibitory compounds. Although aflatoxin production was inhibited, growth of toxigenic *Aspergillus flavus* or *A. parasiticus* was not inhibitied completely; furthermore, many of the biocompetitive agents that inhibited growth or aflatoxin production were themselves undesirable contaminants. Until this investigation of *Nannocystis exedens*, no biocompetitive agent had been shown to inhibit aflatoxin production as a result of parasitism of the aflatoxigenic molds. Myxobacteria have been reported to parasitize soilborne pathogenic fungi. Homma (52) reported that a mycophagous bacterium isolated from soil lysed *Rhizoctonia solani* and conidia of *Cochliobolus miyabeanus*. The bacterium was a member of the family *Polyangiaceae* and genus *Polyangium* and was similar to *P. parasiticum* Geitler. Hocking and Cook (51) reported that *Cytophaga johnsonae* and three myxobacteria of the group *Sorangium* were able to lyse *Pythimum intermedium*, *Rhizoctonia solani*, *Fusarium oxysporum* and *F. solani*. Hocking and Cook (51) suggested that the myxobacteria could provide a useful degree of protection to tree seedlings in the presence of root-pathogens. The results of our investigation suggest that *N. exedens* is also a mycophagous bacterium which could provide a useful degree of protection to field crops in the presence of aflatoxigenic mold.

CHAPTER V

CONCLUSION

The effects of *Nannocystis exedens* ATCC 25963 cell suspensions on growth and aflatoxin B_1 and G_1 production by *Aspergillus flavus* ATCC 16975, *A. flavus* ATCC 26946 and *A. parasiticus* NRRL 3145 were investigated by simultaneously inoculating *N. exedens* with each mold on 0.3% TPYA and 0.3% TPYSA. This investigation indicated that *Nannocystis exedens* ATCC 25963 may be a potential biocompetitive agent against toxigenic strains of *A. flavus* and *A. parasiticus*. *N. exedens* inhibited the growth of *A. flavus* ATCC 16875, *A. flavus* ATCC 26946, and *A. parasiticus* NRRL 3145 on 0.3% TPYA. Likewise, production of aflatoxin B_1 and G_1 by all three aflatoxigenic molds was completely inhibited by *N. exedens* on 0.3% TPYSA. The results study showed that inhibition of growth and production of aflatoxin were probably due to the release of diffusible antifungal compounds into the medium and/or parasitism of spores, hyphae, germinating spores and sclerotia by *N. exedens*. Currently, the only reliable and practical approach to limiting aflatoxigenic mold growth in the field is cultural practices. However, sound management of contamination in the field prior to harvest often is insufficient due to environmental factors which predispose the crop to mold invasion. Detoxification methods often are ineffective and expensive and produce toxic residues. Screening microorganisms as potential biocompetitive agents against toxigenic strains of *Aspergillus flavus* and *A. parasiticus* has been investigated; however, no biocompetitive agent has

been reported to inhibit growth or aflatoxin production as a result of parasitism. This study has demonstrated that *N. exedens* will parasitize spores, germinating spores, hyphae and sclerotia of aflatoxigenic molds. Thus, *N. exedens* as a potential natural antagonist to control the population of toxigenic *Aspergillus* species in the field is suggested in this investigation.

These results justify further investigations to identify the best methods to utilize *N. exedens* as a biological control agent. Future studies might lead to the isolation and characterization of antifungal compounds and lytic enzymes produced by *N. exedens* and determination of the effects of antifungal compounds and lytic enzymes of *N. exedens* on aflatoxigenic molds. In addition, future studies also may include determining the the following: (a) optimum conditions for production of antifungal compounds and parasitism; (b) the effects of aflatoxin on *N. exedens*; (c) the effects of *N. exedens* on aflatoxin production in crops susceptible to infestation with aflatoxigenic molds; (d) screening other members of *Myxococcales* as potential biocompetitive agents against aflatoxigenic molds; (e) and determining the affects of co-inoculation of substrates with aflatoxigenic molds plus *N. exedens* plus another biocompetitive agent on aflatoxin production. No single method is currently available to completely eliminate aflatoxin contamination. However, coupling potential biocompetitive agents with current control methods will be one step closer to achieving the goal of eliminating aflatoxin from susceptible commodities.

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