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Proteases and Their Involvement in the Infection and Immobilization of Nematodes by the Nematophagous Fungus *Arthrobotrys oligospora*

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The nematophagous fungus *Arthrobotrys oligospora* produced extracellular proteases when grown in a liquid culture, as revealed by measuring the hydrolysis of the chromogenic substrate Azocoll. The extracellular protease activity was inhibited by phenylmethylsulfonyl fluoride (PMSF) and other serine protease inhibitors and partly inhibited by the aspartate protease inhibitor pepstatin and by a cysteine protease inhibitor [L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane, or E-64]. Substrate gel electrophoresis showed that the fungus produced several different proteases, including multiple serine proteases. The function of proteases in the infection of nematodes was examined by treating the fungus with various protease inhibitors. None of the inhibitors tested affected the adhesion of nematodes to the traps, but incubating trap-bearing mycelium with a serine protease inhibitor, PMSF, antipain, or chymostatin, or the metalloprotease inhibitor phenanthroline significantly decreased the immobilization of nematodes captured by the fungus. Inhibitors of cysteine or aspartic proteases did not affect the immobilization of captured nematodes. The effects of PMSF on the immobilization of nematodes were probably due to serine proteases produced by the fungus, since the effects were observed when unbound inhibitor was washed away from the fungus before the nematodes were added to the system. No effects were observed when the nematodes only were pretreated with PMSF.

Nematode-trapping fungi possess the unique ability to capture and infect nematodes. During the infection process, the cuticle is penetrated, the nematode is immobilized, and the prey is finally invaded and digested by the fungus. This sequence of events seems to be present in most nematophagous fungi, but the molecular background is not well understood (13). However, several nematophagous fungi have been reported to produce nematotoxins that immobilize or kill nematodes, and ultrastructural and histochemical studies suggest that the penetration of the nematode cuticle involves the activity of hydrolytic enzymes (13).

Studies of other parasitic fungi, including plant and insect pathogens, have shown that the chemical composition of the surface of the host is important for the hydrolytic enzymes involved in infection. Extracellular enzymes corresponding to the main chemical constituents of the insect cuticle, i.e., protein, chitin, and lipids, have been detected in various entomopathogenic fungi (e.g., references 3, 24, and 25). More detailed studies of the fungus *Metarhizium anisopliae* have shown that proteases are produced more rapidly and in higher concentrations than other cuticle-degrading enzymes (27). Furthermore, it was shown recently by gold-labelled antibodies that a cuticle-degrading protease is secreted by this fungus during penetration of the host cuticle (9).

The nematode cuticle consists mainly of proteins, including collagens (6). Microbial collagenases include both serine proteases and metalloproteases which cleave collagens in their helical parts but also have a broad specificity to other proteins (14). It can therefore be assumed that the activity of proteases is important for the infection of nematodes by nematophagous fungi. Extracellular proteases have been detected and partly characterized from a few nematode-trapping fungi (23), as well as from endoparasites of cyst nematodes (7, 15). However, the importance and function of extracellular proteases in the infection process of nematophagous fungi are not known.

The nematode-trapping fungus *Arthrobotrys oligospora* captures nematodes by using special hyphae which form a three-dimensional network. Previous studies have shown that the adhesion, penetration, and immobilization of nematodes take place within an hour after the nematodes are added to the fungus (20, 28). In this paper, we show that *A. oligospora* produces extracellular proteases when grown in liquid cultures. The function of such proteases during the infection of nematodes was examined by using various protease inhibitors.

MATERIALS AND METHODS

Culture of organisms. *A. oligospora* Fres. (ATCC 24927) was maintained on cornmeal agar (Difco) supplemented with 2 g of K_2HPO_4 per liter. Liquid cultures of *A. oligospora* were obtained by inoculating 4,000 ml of media with a water suspension of conidia prepared from a 1- to 2-week-old culture grown on cornmeal agar. Trap-containing [T(+)] mycelium was grown in a medium containing 0.01% soya peptone (neutralized; Oxoid) supplemented with 0.05 g of phenylalanine and 0.05 g of valine per liter (8). The cultures were grown for 7 days at room temperature; the final pH in the medium was 7.5 to 7.7. For production of hyphae without traps [T(−)], the fungus was grown in the same medium but supplemented with a phosphate buffer to a final concentration of 12 mM (pH 7.0). After 7 days of growth, the pH was 7.2. For the bioassay, a conidial suspension of *A. oligospora* was inoculated onto dialysis membranes, which were placed on agar plates with low-nutrient medium and phenylalanyl-valine (17).

The nematode *Panagrellus redivivus* (Goodey) was grown

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axenically in a soya peptone-liver extract medium (18). The nematodes were washed extensively with distilled water (sterile) before being used in the adhesion assays.

Protease inhibitors. The following inhibitors were used: phenylmethylsulfonyl fluoride (PMSF), antipain, chymostatin, 3,4-dichloroisocoumarin (3,4-DCI), L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), pepstatin A, 1,10-phenanthroline, and EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], all from Sigma Chemical Co., St. Louis, Mo., and EDTA from Merck, Darmstadt, Germany. They were all prepared in stock solutions and applied in concentrations within their effective ranges, as given by Beynon and Salvesen (2).

Protease activity. Samples from the liquid cultures were filtered through a nylon mesh. The filtrates were passed through a 0.22-μm-pore-size filter (Millipore Corp.) and concentrated by ultrafiltration (YM-10 membrane; Amicon).

Protease activity was measured in the extracts by using the chromogenic substrate Azocoll (Sigma) by the method of Chavira et al. (5). Portions of 100 to 200 μl of the enzyme extracts were added to a 2-ml suspension of Azocoll (5 mg/ml in a 0.05 M Tris HCl buffer, pH 7.8), and the mixture was incubated at 37°C for 60 to 90 min. After incubation, the samples were cooled, mixed, and set for about 15 min (at 8°C) until undigested substrate had settled at the bottom of the test tubes. The A_{520} of the peptides released in the supernatants was measured, using the Tris buffer as a blank. The activities were expressed in proteolytic units, defined as an absorbance increase of A_{525} per milliliter per minute.

The effects of various inhibitors on the enzyme activity in the extracts were examined by adding various protease inhibitors to the Azocoll-culture filtrate mixture.

The effects of pH on protease activity were investigated by mixing the crude enzyme extracts from T(+) and T(−) cultures with the Britton-Robinson universal buffer system at pHs of between 4 and 10, followed by a protease assay with Azocoll.

Substrate gel electrophoresis. The patterns of extracellular proteases produced by *A. oligospora* were visualized in polyacrylamide gels by incorporating 0.1% gelatin into a sodium dodecyl sulfate (SDS)-polyacrylamide gel (12% acrylamide) (11). The Protean II (Bio-Rad, South Richmond, Calif.) or Midget (Pharmacia LKB, Uppsala, Sweden) system was used for electrophoresis. The analysis was performed in accordance with the manufacturer's instructions, except that the amount of SDS was increased in the sampling buffers from 2 to 4% (to avoid the formation of protein aggregates) and the samples were not boiled in this buffer before being applied to the gels. The amount of protein applied was 0.01 to 0.1 μg. After electrophoresis was complete, the gels were washed with 2.5% Triton X-100 for 1 h to remove SDS. The gels were then incubated at 37°C for 16 to 18 h in either 50 mM Tris HCl buffer (pH 8.0) or 50 mM Tricin-NaOH buffer (pH 7.8). No differences in protease activity were observed regardless of the buffer system used. In some experiments, either PMSF (0.1 mM) or phenathroline (5 mM) was included in these buffers. After incubation, the gels were fixed and stained in 0.2% naphthol blue black (amido black 10B; Sigma) in 7% acetic acid for 1 h at room temperature. The gels were destained with several volumes of methanol-acetic acid-distilled water (30:10:60, vol/vol/vol). Protein molecular weight standards included phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme.

The protein content was determined by the method of Bradford (4), using bovine serum albumin as a standard.

Bioassay. A biological assay using a dialysis membrane technique (19) was used to examine the effects of protease inhibitor addition on capture and infection of nematodes. Pieces of dialysis membrane (1 to 2 cm^2) containing T(+) mycelium (50 to 100 traps per cm^2) were transferred to petri dishes and incubated with a solution of protease inhibitor, dissolved in 0.5% (vol/vol) methanol. After 15 min, the nematodes (10 to 20 per membrane) were added. After 3 to 4 and 20 to 22 h of interaction, captured nematodes, including mobile and immobile (i.e., with arrested movement) nematodes, and free nematodes were counted under a light microscope. The experiments were performed with five parallels and repeated at least twice. Controls were incubated with 0.5% (vol/vol) methanol, since some of the inhibitors were not soluble in water buffers. In some experiments the inhibitor solutions were washed away from the fungus by rinsing the pieces of membrane containing the treated mycelium five times with 2.0 ml of 0.5% methanol before the nematodes were added. Finally, in one experiment the nematodes were incubated with the inhibitor solution for 15 min, washed by centrifugation with three 5-ml washes of 0.5% methanol, and then added to the fungus grown on the dialysis membranes. The statistical significance of the differences in frequencies of mobile and immobile captured nematodes in treated versus control samples was tested by χ^2 analysis.

The viability of *A. oligospora* after treatment of the mycelium with the inhibitors was examined by transferring one piece of membrane culture incubated with the inhibitor solutions for 15 min to an agar plate with low-nutrient medium. The mycelia were incubated, and the radial growth of the colonies was observed after 1 to 3 days.

RESULTS

Extracellular proteases. T(+) and T(−) mycelia of *A. oligospora* secreted very low levels of proteins into the growth media (<0.5 mg/liter), but extracellular protease activity was detected in concentrated culture filtrates by measuring the hydrolysis of the protein-dye conjugate Azocoll. The specific activities in the crude enzyme extracts were 0.4 and 2.0 proteolytic units per mg of protein in the T(+) and T(−) extracts, respectively. Enzyme activities in extracts from both T(+) and T(−) cultures were inhibited by various serine protease inhibitors (PMSF, antipain, chymostatin, and 3,4-DCI) (Table 1). The cysteine protease inhibitor E-64 and the aspartic protease inhibitor pepstatin partly inhibited extracellular protease activity. Minor effects on protease activity were observed when a metal chelator, EDTA, EGTA, or phenanthroline, was added to the filtrates.

pH. Analysis of the protease activity at various pH values, with Azocoll as the substrate, showed that extracts from both T(+) and T(−) cultures had optimal activity at alkaline pH (8.0 to 8.6) (Fig. 1). However, both types of extract had a second, somewhat lower optimal activity at acidic pH (5.0 to 6.3).

Electrophoresis. Substrate gel electrophoresis of culture filtrates from *A. oligospora* showed the presence of several bands (or regions) with protease activity (Fig. 2, lanes A and B). At least three of these bands seemed to be identical in the T(+) and T(−) filtrates, as revealed by their electrophoretic mobility on SDS-polyacrylamide gel electrophoresis. Samples from T(−) cultures contained at least two protease bands that were not detected in the samples from the T(+) cultures. PMSF inhibited the activity of all protease bands except that of a high-molecular-weight band at 97 kDa

TABLE 1. Effects of various inhibitors on protease activity in culture filtrates from *A. oligospora*[a]

Inhibitor	% of control (SD)	
	T(+)	T(−)
PMSF (1.0 mM)	2.0 (0.4)	2.6 (2.8)
PMSF (1.0 mM) + DTT (5 mM)	16.4 (7.8)	11.8 (1.8)
DTT (5 mM)	89.9 (8.5)	116.7 (19.8)
Antipain (10 μM)	14.3 (7.3)	17.1 (6.51)
Chymostatin (10 μM)	3.7 (0.9)	2.1 (2.1)
3,4-DCI (50 μM)	78.2 (18.1)	48.8 (10.2)
E-64 (10 μM)	70.6 (17.3)	95.4 (24.5)
Pepstatin (10 μM)	79.7 (14.0)	73.0 (9.3)
Phenanthroline (10 mM)	93.8 (11.1)	119.0 (26.9)
EDTA (10 mM)	98.5 (10.9)	82.1 (18.0)
EGTA (10 mM)	85.4 (18.7)	90.7 (16.7)
$CaCl_2$ (1.0 mM)	86.6 (16.5)	96.7 (2.1)

[a] The fungus was grown in liquid media, with T(+) and T(−) designating mycelium with and without nematode-trapping networks, respectively. Protease activity was determined at pH 7.8, using the chromogenic substrate Azocoll. The proteolytic activities in the controls were between 1.1×10^{-3} and 2.9×10^{-3} proteolytic units. DTT, dithiothreitol; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane. No inhibitors were added to controls. Means of three samples are given.

present in all samples and that of a 20-kDa band present in the T(−) cultures (Fig. 2, lanes C and D). Treating the gels with phenanthroline did not remove any of the protease bands in these samples.

Bioassay. None of the tested protease inhibitors affected the adhesion of nematodes to the traps of *A. oligospora*, since the ratios of total captured nematodes (mobile and immobile) to free-living nematodes were not significantly different in the samples treated with the inhibitors compared with those in the controls (χ^2 tests; df = 1; $P \geq 0.05$). However, treating T(+) mycelium of *A. oligospora* with the serine protease inhibitor PMSF or the metalloprotease inhibitor phenanthroline affected the immobilization of captured nematodes, since this treatment significantly decreased the proportion of immobilized nematodes of the total number of captured nematodes (Table 2). In contrast, the immobilization of nematodes did not decrease when the specific cysteine protease inhibitor E-64 or the aspartic protease inhibitor pepstatin was used.

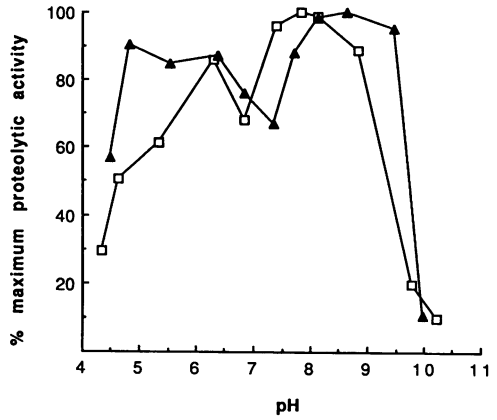

FIG. 1. Activity of extracellular proteases in culture filtrates from *A. oligospora* at various pHs. □, samples from T(+) mycelium; ▲, T(−) mycelium. The maximum proteolytic activity was 2.4×10^{-3} proteolytic units.

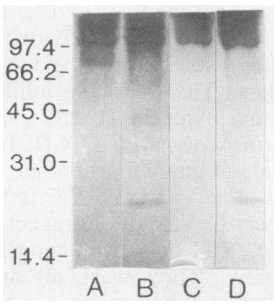

FIG. 2. Substrate gel electrophoresis of extracellular proteases in culture filtrates from *A. oligospora*. Negative copy is shown; protease activities are visualized as dark bands (clearing zones). Lane A, filtrate from T(+) mycelium; lane B, filtrate from T(−) mycelium; lane C, T(+) filtrate incubated in the presence of the serine protease inhibitor PMSF; lane D, T(−) filtrate incubated in the presence of PMSF. Molecular weights ($\times 10^3$) are indicated on the left.

The effects of PMSF on the immobilization of captured nematodes decreased during incubation of the system. After 20 to 22 h of interaction, the proportions of captured nematodes immobilized were 53.5% (21.4% after 3 to 4 h; cf. Table 2) in samples treated with 1.0 mM PMSF and 86.7% (49% after 3 to 4 h) in samples treated with 0.1 mM PMSF. The corresponding values in controls were 89.7% after 20 to 22 h and 78.9% after 3 to 4 h of incubation. Since PMSF is degraded quite rapidly in water solutions (12), the effects of treating the fungus with a solution of PMSF (0.1 mM in 0.5% methanol) incubated at room temperature for 20 h before addition to the fungus were tested. The proportion of immobilized nematodes in samples after using this inhibitor solution was not significantly different from that in controls (χ^2 = 1.70; not significant; 3 to 4 h of interaction). The effects of phenanthroline on the immobilization of nematodes were similar after 20 to 22 and 3 to 4 h of interaction. After 20 to 22 h, the proportion of immobilized nematodes in samples

TABLE 2. Capture of nematodes by *A. oligospora* after treatment of the mycelium with various protease inhibitors[a]

Inhibitor	Captured nematodes		Statistics[b]	
	Mobile	Immobile (%)[c]	χ^2	*P*
PMSF, 1.0 mM (ser)[d]	33	9 (21.4)	50.9	<0.001
PMSF, 0.1 mM (ser)	47	37 (49.0)	22.9	<0.001
E-64, 10 μM (cys)	38	108 (74.0)	1.12	NS
Pepstatin, 1.0 μM (asp)	4	40 (90.9)	3.4	NS
Phenanthroline, 1.0 mM (metal)	127	95 (52.9)	56.0	<0.001
EDTA, 1.0 mM (metal)	39	100 (71.9)	2.13	NS
Control	41	153 (78.9)		

[a] A bioassay using a dialysis membrane technique was used. Pieces of dialysis membrane containing T(+) mycelium were incubated with the inhibitors for 15 min before the nematodes were added. Captured (including mobile plus immobilized) nematodes and free nematodes were counted under a light microscope after 3 to 4 h of incubation. At this time, 40 to 70% of the added nematodes were captured. The experiments were performed with five parallels and repeated at least twice. E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane.

[b] χ^2 tests (df = 1) comparing the frequencies of mobilized and immobilized nematodes in treated-versus-control samples. NS, not significant.

[c] Percentage of captured nematodes which were immobilized.

[d] In parentheses is the class of proteases affected by the inhibitor: ser, serine; cys, cysteine; asp, aspartic; metal, metalloproteases.

TABLE 3. Capture of nematodes by *A. oligospora* after treatment of the mycelium or the nematodes with serine protease inhibitors[a]

Inhibitor	Captured nematodes		Statistics[b]	
	Mobile	Immobile (%)[c]	χ^2	*P*
PMSF, 0.1 mM (fungus)[d]	194	168 (46.4)	35.4	<0.001
PMSF, 0.1 mM (nematode)[d]	58	156 (72.9)	0.04	NS
Antipain, 10 μM[e]	30	40 (57.1)	5.94	<0.05
Chymostatin, 10 μM[e]	32	33 (50.8)	9.76	<0.01
Control	45	127 (73.8)		

[a] The dialysis membrane bioassay described in Table 1, footnote *a*, was used.

[b] χ^2 tests (df = 1) comparing the frequencies of mobilized and immobilized nematodes in treated-versus-control samples. NS, not significant.

[c] Percentage of captured nematodes which were immobilized.

[d] The fungus or the nematode was incubated with the inhibitors for 15 min. Unbound inhibitors were washed away before the nematode or fungus was added.

[e] The fungus was incubated with the inhibitor for 15 min before the nematodes were added.

treated with this inhibitor was 46.7% (52.9% after 3 to 4 h). Phenanthroline also seemed to affect the degradation of the captured nematodes. Two days after the fungus and nematodes were incubated with this inhibitor, the fungus did not seem to have started to colonize the nematodes and there were still some mobile captured nematodes present. In contrast, nematodes in the control samples, as well as in samples treated with the other inhibitors, were heavily colonized by the fungus.

Further experiments showed that the effects of PMSF on the immobilization of nematodes were also present when unbound PMSF was washed away from the treated fungus, before the nematodes were added to the system (Table 3). However, when the nematodes were treated with PMSF prior to the interaction with the fungus, the proportion of immobilized nematodes was not significantly different from that in the control samples. The effects of treating the fungus with two other serine protease inhibitors, antipain and chymostatin, were also tested. Both inhibitors decreased the proportion of immobilized nematodes, but to a lesser extent than PMSF (Table 3).

Viability. The viability of the fungus treated with 1.0 or 0.1 mM PMSF did not seem to be affected because the growth rate of mycelium treated with these inhibitor solutions was not different from that of the controls. The radial growth rates in these samples were 1 to 2 (day 1), 6 to 7 (day 2), and 8 to 10 (day 3) mm. However, no growth was observed during the first 2 days of incubation in samples treated with phenanthroline, and at day 3, growth was only 1 to 2 mm.

DISCUSSION

Bioassays performed with various inhibitors showed that the activity of proteases is not involved in the adhesion of nematodes to the traps. In other parasite-host systems, including the adherence of the protozoan *Trichomonas vaginalis* to epithelial cells, it has been shown recently that protease action is a prerequisite for attachment (1). However, the protease inhibitor experiments demonstrated that the activity of serine proteases is involved in the immobilization of nematodes captured by *A. oligospora*. Treatment with the metalloprotease inhibitor phenanthroline also decreased the proportion of immobilized nematodes, but the growth experiments showed that this inhibitor, in contrast to PMSF, decreased the viability of the T(+) mycelium. The effects of phenanthroline could, therefore, be due to the inactivation of processes not directly involved in the infection of the nematodes.

Besides the serine proteases, PMSF as well as antipain and chymostatin can inhibit some cysteine proteases (2). However, since no effects were observed when the specific cysteine protease inhibitor E-64 was used (2), the effects of the former inhibitors were probably due to the inactivation of serine proteases. Furthermore, it has been reported that PMSF also can inactivate other types of enzymes, including esterases (12), but no such side effects have been described for antipain or chymostatin (2).

The effects of PMSF were probably on serine proteases produced by the fungus, since they were observed when unbound inhibitors were washed away from the traps before the nematodes were added to the system but not when the nematodes only were pretreated with this inhibitor. PMSF reacts irreversibly with serine residues in the active sites of serine proteases (2). Thus, the decreased effects of PMSF, due to degradation of the inhibitor solution, indicate that the treated fungus was able to synthesize and secrete new proteases involved in the immobilization of the captured nematodes during the incubation period.

Analysis of the protease activity in the liquid-grown cultures demonstrated that the major family of extracellular proteases produced by *A. oligospora* was serine proteases. The effect of 3,4-DCI was considerably less than that of the other serine protease inhibitors, even though it has been demonstrated that this inhibitor is effective against a variety of different serine proteases (10). However, the effects of 3,4-DCI can be dramatically decreased by the presence of interfering inhibitors and substrates (10); such compounds might have been present in the crude enzyme extracts from *A. oligospora*. That PMSF mainly affected extracellular serine proteases and not cysteine proteases was indicated by showing that the protease activity was only sensitive to the reducing compound dithiothreitol to a very low extent. In contrast to cysteine proteases, serine proteases are not sensitive to reducing agents (21). Serine proteases have been described in fungi from all major taxonomic groups, including insect pathogens (3, 21, 26). An extracellular serine protease from *Verticillium suchlasporium*, a nematophagous fungus infecting eggs of cyst nematodes, was recently purified and partly characterized (15). These proteases, as well as the crude enzyme extract from *A. oligospora*, have their optimal activity at alkaline pH. The presence of a second pH optimum for the protease activity at acidic pH values, as well as the inhibitory effects of pepstatin, indicate that *A. oligospora* also produces aspartic extracellular proteases (21). Furthermore, substrate gel electrophoresis indicated that there might be some differences in the patterns of proteases produced in T(+) and T(−) hyphae. However, further studies are needed to examine the variety of proteases produced by *A. oligospora* under different growth conditions.

The mechanisms by which the serine protease inhibitors affect the immobilization of nematodes are not known. Olthof and Estey (22) reported that *A. oligospora* produces substances with nematicidal effects during infection of the nematode *Rhabditis* sp. The activity of serine proteases might be important for the release or activation of such toxins during infection. Another possibility is that the applied inhibitors might inactivate serine proteases involved in the penetration of the nematode cuticle. On the basis of

microscopic observations, it has been suggested that the immobilization of nematodes by *A. oligospora* occurs during the penetration of the nematode (20, 28). It is not clear whether the inhibitors used in the bioassay experiments affected extracellular or intracellular enzymes or both. Fungal enzymes that are involved in the degradation of insect cuticles have mainly been considered to be extracellular enzymes (3, 9, 24, 25, 27). However, the proteases involved in the interactions with nematodes can be derived from preexisting intracellular enzymes. It has been shown recently that the main family of intracellular enzymes from *A. oligospora* consists of serine proteases (22a).

The use of inhibitors to examine the function of proteases has its limitations, since there is a risk that processes other than those involving protease activity might be affected (21). A more precise means of analyzing the function of protease activity would be through the isolation of appropriate mutants. Protease-deficient mutants have been isolated from *Candida albicans* to study the importance of proteinase secretion during the infection of mice (16). The use of similar mutants of nematophagous fungi will further clarify the involvement of fungal proteases in the infection of nematodes.

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