Targeted Secretion of Cutinase in *Fusarium solani* f. sp. *pisi* and *Colletotrichum gloeosporioides*

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This work was supported by grants DCB 8819008 and IBN9318544 from the National Science Foundation.
We thank P. Pack for her assistance in preparing this manuscript.
Accepted for publication 3 November 1994.

ABSTRACT

Podila, G. K., Rosen, E., San Francisco, M. J. D., and Kolattukudy, P. E. 1995. Targeted secretion of cutinase in *Fusarium solani* f. sp. *pisi* and *Colletotrichum gloeosporioides*. Phytopathology 85:238-242.

Immunofluorescence studies of cutinase secretion by phytopathogenic fungi *Fusarium solani* f. sp. *pisi* and *Colletotrichum gloeosporioides* were made to determine targeting of the enzyme in germinating spores. Our results indicate that the secretion of cutinase was directed towards the region that penetrates the host. In *F. s. pisi,* which penetrates plant tissue without appressorium formation, cutinase was targeted to the growing tip of germinating spores. In *C. gloeosporioides,* cutinase secretion was directed to the infection peg that arises from the appressorium. Monensin, the carboxylic ionophore, inhibited secretion of cutinase and caused intracellular accumulation of cutinase in *F. s. pisi.* Subcellular fractionation of monensin-treated cells indicated involvement of the Golgi in the secretion and targeting of cutinase by germinating spores of *F. s. pisi.*

Cutinase has been shown to be important for penetration of many fungal pathogens through the cuticle into the plant (6–8,17, 18,24). It is an extracellular enzyme that hydrolyzes the biopolyester, cutin, which is the structural polymer of the plant cuticle (20). In some cases, such as that of *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F.R. Jones) W.C. Snyder & H.N. Hans., the germ tube from the spore directly penetrates the host cuticle; while in others, such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., spore germination results in the formation of an appressorium from which emerges an infection peg that penetrates the cuticle. If cuticular penetration by the fungus is assisted by cutinase, targeted secretion of the enzyme should occur from the structures that are involved in penetration of the cuticle. Using immunofluorescence, we tested whether cutinase is indeed secreted in a targeted manner from the tip of the germ tube in *F. s. pisi* and from the infection peg originating from the appressorium in *C. gloeosporioides.* The mechanism of secretion of cutinase in *F. s. pisi* was also studied using the carboxylic ionophore monensin. The results presented demonstrate that cutinase secretion by both fungi is directed to the structures involved in cuticle penetration and suggest that the secretion of cutinase by the phytopathogen *F. s. pisi* is probably a Golgi-mediated process that is adversely affected by monensin, a carboxylite ionophore known to inhibit Golgi-mediated secretion. Some of the preliminary results were presented at an EMBO Workshop (21).

MATERIALS AND METHODS

Fungal growth conditions. *F. s. pisi* strain T-8 was obtained from H. D. Van Etten (University of Arizona) and was maintained on V8 juice agar. The fungus was grown in a mineral salts medium at 25 C in unshaken Roux bottles essentially as described by Crawford and Kolattukudy (3). Glucose at 0.2% was the carbon source and cutin was added as an inducer at a concentration of 0.2%. Monensin (Calbiochem-Novabiochem Corp., La Jolla, CA) was dissolved in 95% ethanol and added at final concentrations of 10, 20, 30, and 60 μM to the growth medium. *C. gloeosporioides* was a laboratory isolate (6) and was maintained on potato-dextrose agar plates. Novozyme was obtained from Novo Biolabs (Bagsvaerd, Denmark); driselase, β-glucuronidase, and fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG antibody were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of protoplasts and fractionation of intracellular components. Cultures of *F. s. pisi* were harvested by filtration through Whatman No. 1 filter paper, washed in 0.6 M $MgSO_4$, weighed, and incubated in spheroplasting solution for a period of 2 h at 30 C with gentle shaking. The spheroplasting solution contained 1.2 M $MgSO_4$; 10 mM $NaPO_4$, pH 5.8; with Novozyme at 25 mg/gm of tissue; driselase at 10 mg/gm of tissue; and β-glucuronidase at 0.2 ml/gm of tissue. Protoplasts were filtered through cheesecloth and layered over with trapping buffer (0.6 M sorbitol in 0.1 M Tris HCl, pH 7.0). Centrifugation at 7,000 *g* for 15 min collected the protoplasts at the interface between the two different density solutions. The protoplast layer was transferred to another centrifuge tube, and the protoplasts were washed three times with 1.2 M sorbitol and 10 mM $CaCl_2$ in 10 mM Tris HCl, pH 7.5, by careful resuspension and centrifugation at 7,000 *g* for 5 min. The washed protoplasts were resuspended in 10 ml of 1.8 M sorbitol in 50 mM Tris HCl, pH 7.5, and centrifuged at 3,000 *g* for 5 min with 2 g of glass beads. The supernatant was discarded, and lysis of spheroplasts was carried out by vortexing them in the presence of the glass beads for 1 min essentially as described by Emr et al (10). The beads were washed several times with small volumes (2 ml) of 0.5 M mannitol-50 mM Tris HCl, pH 7.5; and the washes were pooled and centrifuged at 800 *g* for 10 min to remove unlysed protoplasts. The cell-free lysate was centrifuged (12,000 *g*, 10 min) through 0.8 M mannitol onto an equal volume of 30% Percoll-0.5 M mannitol to isolate cellular organelles and membranes. To the material collected at the mannitol-Percoll interface, two volumes of 60% sucrose in 10 mM Tris-0.5 mM dithioerythritol (DTE) were added, overlayed with 40, 35, 30, 25, and 20% sucrose (w/v) in Tris-DTE, and centrifuged for 15 h in an SW 28.1 rotor at 90,000 *g*. One-milliliter fractions collected from the top of the gradient were diluted with 4 ml of Tris-DTE and centrifuged for 90 min in a Ti 50 rotor at 150,000 *g*. The supernatants were discarded and the pellets were resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.2. All centrifugations and manipulations were carried out at 4 C.

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Enzyme assays. Cutinase enzyme activity was measured as previously described by monitoring *p*-nitrophenyl butyrate hydrolysis spectrophotometrically at 405 nm (21). Fumarase (mitochondria), NADPH cytochrome C reductase (endoplasmic reticulum), and α-mannosidase (Golgi apparatus) were assayed spectrophotometrically as described previously (9,12). All assays were conducted at least three separate times, and values presented represent an average.

Immunoblot analysis. For immunoblot analysis, equivalent aliquots of extracellular fluid and mycelial homogenates were used. After the culture fluid was recovered, the mycelia were ground in liquid nitrogen using a mortar and pestle, and the mycelial powder was transferred with 50 mM potassium phosphate buffer, pH 7.0, into a glass homogenizer, and the mixture was homogenized. The supernatant collected by centrifugation was made up to a volume equal to that of the extracellular fluid. From the extracellular fluid and the cell homogenate, 100-μl aliquots were applied for the slot blot analysis.

Samples were applied to nitrocellulose filters by use of a slot blot (Schleicher and Schuell, Inc., Keene, NH), blocked, and washed in 5% nonfat dry milk in TBS (10 mM Tris HCl, pH 7.2, 0.9% saline). The filters were then incubated for 45 min at room temperature with rabbit antibodies prepared against cutinase purified from cutin-grown *F. s. pisi* (24). Excess antibody was removed by three washes in nonfat dry milk in TBS-Tween (0.1% Tween 20). The filters were then incubated for 45 min at room temperature with ^{125}I-Protein A (Dupont NEN, Boston, MA) in nonfat dry milk-TBS to detect the presence of antibody. After three washes in nonfat dry milk in TBS-Tween to remove excess unbound radioactivity, the filters were dried and subjected to autoradiography. Culture filtrates were concentrated by lyophilization.

Immunofluorescence microscopy. To monitor cutinase targeting in germinating spores and the effect of monensin on cutinase secretion, FITC-conjugated goat antibody (Sigma) directed against the rabbit anti-cutinase antibody (11,24) was used. Spores of *F. s. pisi* or *C. gloeosporioides* were germinated on a coverslip coated with 10,16-dihydroxypalmitic acid isolated from cutin hydrolysate (30) in the presence or absence of 30 μM monensin for 4–6 h at room temperature. Control spores were incubated on a coverslip for the same time period without the hydroxy-fatty acid or monensin. The germinated spores were incubated with the rabbit antibody prepared against cutinase purified from either *F. s. pisi* (25) or *Colletotrichum capsici* (11) overnight at 4 C, washed with 1% nonfat dry milk in 10 mM potassium phosphate buffer, pH 7.6, and incubated with the FITC-conjugated secondary antibody for 2 h at room temperature and washed as above. Microscopy was carried out with a Nikon Optiphot Microscope with an epifluorescent lamp and a blue filter (480 nm). The rabbit antibodies used were shown to be specific for cutinase from *F. s. pisi* and closely related species (24) or specific for cutinase from *C. capsici* and closely related *C. gloeosporioides*. Western blot analysis showed only one band with both antibodies (W. F. Ettinger, C. L. Soliday, and P. E. Kolattukudy, *unpublished*).

RESULTS

Targeting of secretion of cutinase. Germinating conidia of *F. s. pisi* were immunocytochemically examined using FITC-conjugated secondary anti-rabbit IgG directed against anti-cutinase antibody. In quiescent, ungerminated spores treated with cutin hydrolysate to induce cutinase production, fluorescent antibody staining was detected over the entire spore surface (Fig. 1A). When germination was initiated, fluorescence was confined to

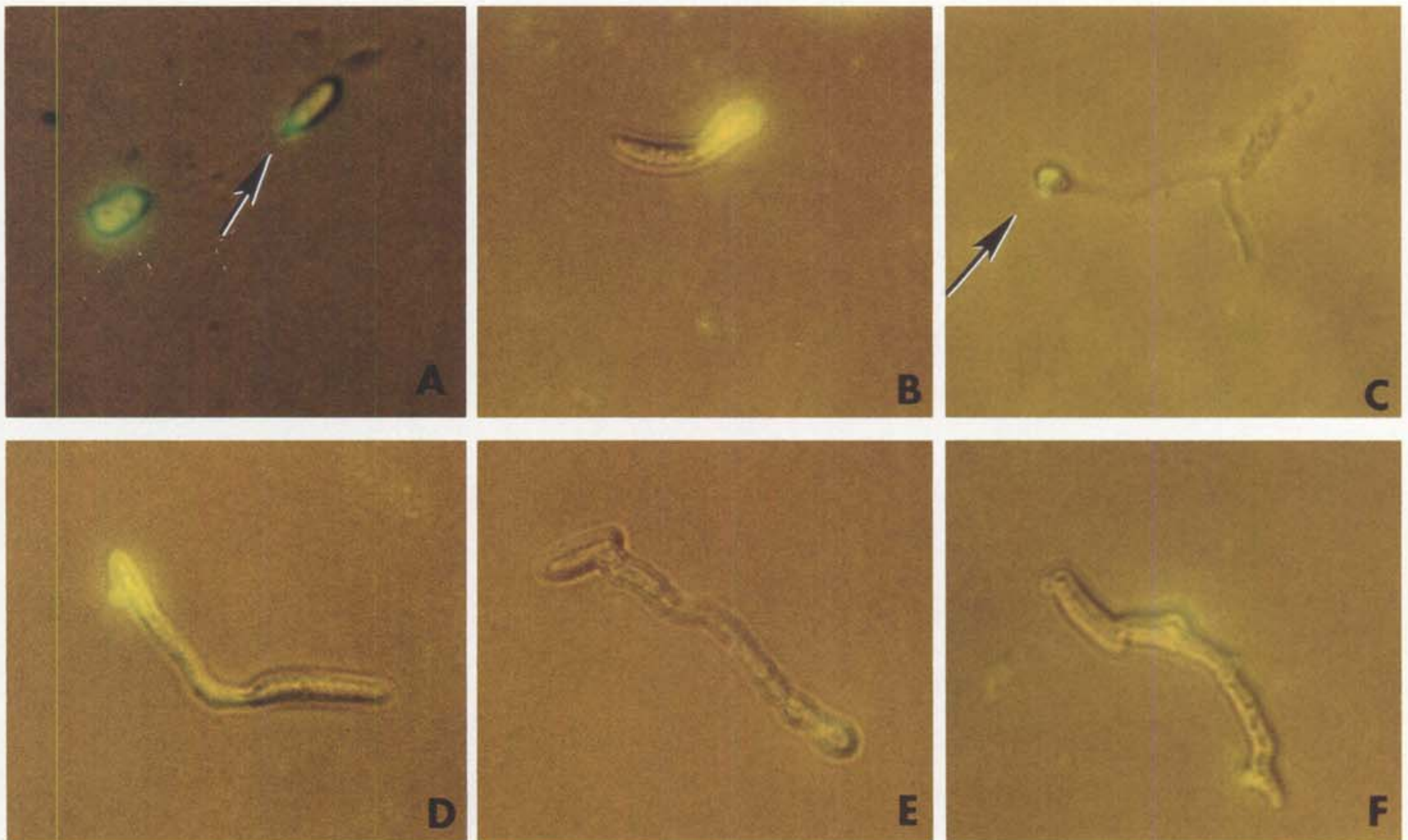

Fig. 1. Immunofluorescence localization of cutinase secretion in fungal spores in the presence of 10,16-dihydroxypalmitic acid and the effect of monensin on the targeting of cutinase secretion. Fluorescent regions represent the presence of cutinase. **A,** Distribution of cutinase on the surface of an ungerminated spore as well as the targeting of secretion of the enzyme to the point of germination initiation (arrow) in *Fusarium solani* f. sp. *pisi*. **B,** Targeting of secretion of cutinase to the germ tube in *F. s. pisi*. **C,** Localization of cutinase at the infection peg arising from the appressorium of *Colletotrichum gloeosporioides* (arrow). **D, E, and F,** Effects of 5, 10, and 20 μM monensin, respectively. Changes in the morphology of the germ tube are noticeable in **E** and **F**.

a specific area where the beginnings of the germ tube could be observed (Fig. 1A, arrow). This targeting of the secretion of cutinase to the germ tube originating from the spore was apparent even several hours after spore germination (Fig. 1B). When *C. gloeosporioides* conidia were similarly treated with rabbit antiserum prepared against *C. capsici* (this antibody cross-reacts with *C. gloeosporioides* cutinase) and FITC-conjugated secondary antibody, fluorescence indicating cutinase secretion was found to be localized in the infection peg germinating from the appressorium rather than in the primary germ tube (Fig. 1C). With both fungi, experiments with preimmune serum showed no fluorescence. Omission of the cutin monomer, which would be expected to preclude cutinase induction, also gave no immunofluorescence with either fungus. These experiments were repeated several times with observations of several hundred spores for each sample. Virtually all of them showed the same pattern of fluorescence except for a few percent that did not germinate.

Effect of monensin on secretion of cutinase. In an initial attempt to test whether the ionophore monensin interfered with the secretion of cutinase, monensin was added at different concentrations to *F. s. pisi* cultures in a mineral medium with cutin at the time of inoculation. Cutinase activity in the culture medium was monitored for 4 days. The level of the enzyme found in the medium was only about 30% of that in the controls by the second day (Fig. 2). The enzyme level began to recover after this time. All concentrations of monensin tested from 10 to 60 μM were effective in suppressing cutinase secretion by the fungus (Fig. 2). Cutinase activity in the medium increased markedly on days three and four in cultures to which 10 and 20 μM monensin had been added. Cutinase activity remained low in cultures to which 30 or 60 μM of monensin had been added. These results suggested that the monensin interfered with the secretion of cutinase.

To determine whether the observed effect of monensin was, in fact, due to a suppression of secretion of the cutinase rather than to inhibition of the biosynthesis or activity of the enzymes, the culture filtrate as well as the crude fungal extracts were tested for the presence of cutinase. Figure 3 shows an autoradiograph of a slot blot of cutinase detected with ^{125}I-labeled Protein A from the culture filtrate and cytoplasm of monensin-treated (30 μM) and untreated cultures. There was also a corresponding decrease in the *p*-nitrophenylbutyrate hydrolase activity (data not shown). Immunoreactive cutinase level in the culture filtrate of the monensin-treated sample was much lower than that found in the control. On the other hand, cutinase was found in the cytoplasm of the monensin-treated sample, while there was little or no cutinase detected in the cytoplasm of the untreated culture.

Subcellular localization of cutinase in monensin-treated and untreated cultures. In order to determine the particular subcellular fraction or fractions in which cutinase was localized in monensin-treated and untreated samples, cellular organelles and membranes isolated at a Percoll-mannitol interface were fractionated on sucrose gradients essentially as described previously (9,10). These gradients allowed the separation of various intracellular components such as the endoplasmic reticulum, Golgi apparatus, and mitochondria. The identity of each component was determined by assaying for the presence of marker enzymes for each component. All gradient fractions were also assayed for the presence of cutinase as described earlier (22). In monensin-treated samples, peak cutinase activity was associated with the lighter density fractions, which also contained the peak of the α-mannosidase activity, the marker enzyme for the Golgi apparatus (9) (Fig. 4).

Effect of monensin on targeting secretion of cutinase. Treatment with low concentrations of monensin (5 μM) (Fig. 1D) had a negligible effect on targeting of cutinase to the hyphal tip in the germinating *F. s. pisi*; fluorescence was still visible. Treatment of *F. s. pisi* spores with 10 μM monensin resulted in less well defined targeting of cutinase to the tip of the germ tube (Fig. 1E). When 20 μM monensin was applied, besides the loss of cutinase targeting, the hyphae were malformed, appearing bulbous and irregular (Fig. 1F).

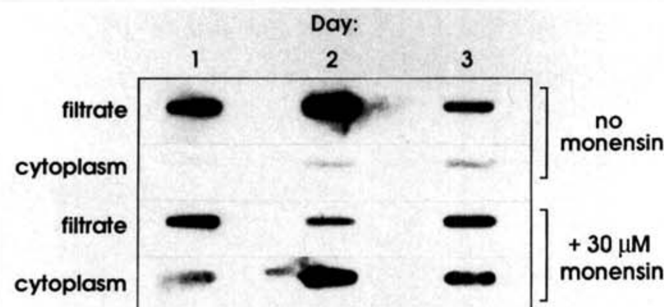

Fig. 3. Immunoblot using anticutinase antibody and ^{125}I-Protein A to detect the presence of intracellular and secreted cutinase in monensin-treated and untreated cultures of *Fusarium solani* f. sp. *pisi*. Culture filtrate and cytoplasmic fractions from monensin-treated (30 μM) and monensin-free 1-, 2-, and 3-day-old cultures were used.

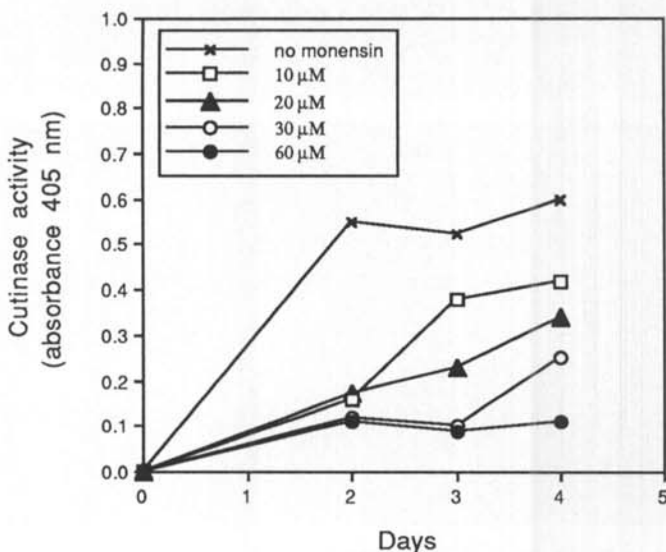

Fig. 2. Effect of different concentrations of monensin on secreted cutinase activity. Concentrations of monensin used in the growth medium were: 0, 10, 20, 30, and 60 μM. Monensin was added with the fungal inoculum. The control (no monensin) growth medium contained 95% ethanol in an amount equivalent to that added with the highest monensin concentration.

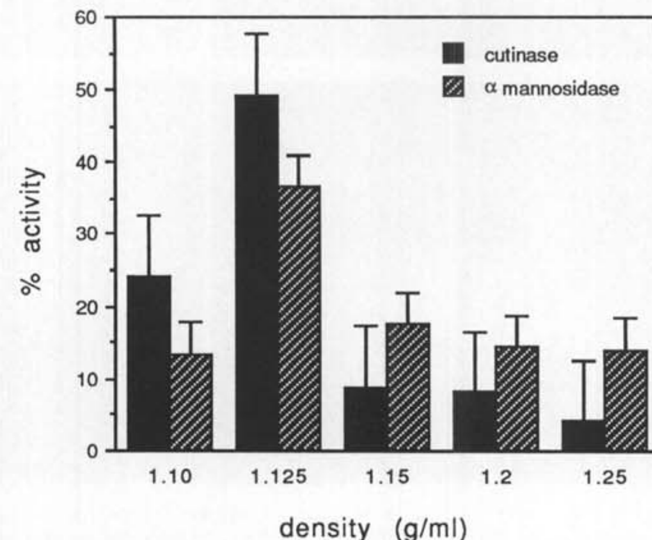

Fig. 4. Distribution of cutinase (solid bars) and α-mannosidase (hatched bars) activity over a sucrose gradient used to separate intracellular organelles from monensin-treated cultures of *Fusarium solani* f. sp. *pisi*. Bars represent the standard error.

DISCUSSION

Many lines of evidence suggest that cuticular penetration of some fungi into their hosts is assisted by extracellular cutinase produced by the fungus (13,15). Even though immunoelectron microscopic evidence showed that *F. s. pisi* infecting its host produces extracellular cutinase (23), targeting of the secretion to the penetration structure has not been previously demonstrated. In other fungi, such as *C. gloeosporioides,* the germ tube differentiates into an appressorium, and the infection peg emerges from this infection structure to actually penetrate into the host. If cutinase plays an important role in assisting the penetration by such organisms, as previously suggested by other approaches (5–7), cutinase secretion should be targeted to this infection peg. The immunofluorescence labeling studies presented in this paper demonstrate such a targeting of cutinase secretion both in the direct penetrant (*F. s. pisi*) and in *C. gloeosporioides,* which requires appressorium formation before the infection peg penetrates. These results are consistent with the hypothesis that cutinase assists in the fungal penetration of the host cuticle.

The secretion pathway for cutinase has not been studied previously. The observed targeting implies that the cytoskeleton might be involved. The results presented in this paper show that monensin, an agent known to disrupt Golgi-type vesicle-mediated secretion (27,29), inhibits secretion of cutinase. When monensin was added to the liquid cultures of *F. s. pisi,* there was a significant decrease in the cutinase activity in the medium. The inhibitory effect was most pronounced 2 days after the addition of monensin to the medium. Cutinase activity began to recover 3–4 days after the addition of monensin, especially with the lower concentrations of the drug. With higher concentrations, the recovery took longer. Degradation of the drug was probably the reason for the recovery.

If monensin inhibited secretion of cutinase rather than inhibiting the biosynthesis or activity of the enzyme, accumulation of cutinase in the cells might be expected. To test this possibility, the levels of cutinase in the extracellular medium and the cell extracts were measured immunologically. In the controls with no monensin, virtually all of the enzyme was found in the medium and very little within the cells. These results showed that cutinase was efficiently secreted. In the monensin-treated cells, substantial amounts of cutinase were found in the cells, and the level of the extracellular enzyme was much less than that found in the control. These results demonstrate that monensin inhibited the secretion of cutinase.

Monensin treatment not only inhibited secretion of cutinase but also caused a diffused distribution of the immunofluorescence in the treated spores, indicating disrupted targeting. In addition, monensin caused malformation of hyphae. The bulbous outgrowths on the hyphae from monensin-treated samples probably reflects defective targeting of cell wall components needed for normal development of the germ tube. Such abnormalities are consistent with the current concepts concerning the involvement of cytoskeleton in the deposition of the walls in the developing germ tube.

To test for the involvement of a Golgi-mediated secretion process in the extracellular cutinase production, subcellular organelles were fractionated using sucrose density gradients. By assaying the known marker enzymes, organelle distribution was determined. When secretion of cutinase was inhibited by monensin, cutinase activity was associated with the lighter density fractions, which also contained α-mannosidase, a marker for Golgi. Thus, it appears that in *F. s. pisi,* secretion of cutinase is a Golgi-mediated process.

Even though many lines of evidence suggest that cuticular penetration of the host is assisted by extracellular cutinase (13,15), when and where the enzyme is produced has not been elucidated. The results presented here demonstrate that cutinase secretion induced by cutin monomers is targeted to the penetrating structure, whether it is the initial germ tube or the infection peg originating from the appressorium. These results are consistent with the postulated role for cutinase in pathogenesis. Immunoelectron microscopic evidence that cutinase is produced during an actual infection of the host (23), and prevention of infection by inhibition of cutinase by chemicals including suicide inhibitors, polyclonal antibodies (6,16,17), and monoclonal antibodies (1), strongly suggested that fungal infection by such fungi is assisted by cutinase. Cutinase-deficient mutants of certain fungi were found to be less virulent, but the virulence could be restored by the addition of exogenous cutinase (4,7). Transfer of a cutinase gene into a pathogen that normally requires a wound for infection enabled the pathogens to infect intact organs (8). Even though several lines of evidence strongly suggest that cutinase assists the infection process, two gene-disruption studies have been presented as evidence against any significant role for cutinase in virulence. When a cutinase gene was disrupted in *F. s. pisi* and the transformant was assayed at one high spore concentration, the pathogen infected the host (26). Since expression of virulence depends on the inoculum potential (i.e., spore concentration), difference in virulence may not be detected at a single concentration of spores. Furthermore, it is becoming increasingly clear that organisms that successfully retain the ability to infect their host organisms have evolved ways to compensate for the loss of expression of a single gene. In the extensively studied case of *Erwinia* pectate lyases, certain genes are expressed only in the host (2). Animal pathogens are known to express only certain genes while they are in the host (19). We found multiple cutinase genes in the highly virulent strains of *F. s. pisi* even by normal Southern hybridization (14), and there may be other genes that have not been detected. Thus, caution should be exercised not to over-interpret results obtained from a single gene-disruption study. In the other gene-disruption study, a cutinase gene was disrupted in *Magnaporthe grisea,* and this transformant did not show significantly lower virulence on rice plants than did the wild type (28). Since the transformant retained the bulk of its ability to produce a cutinolytic enzyme(s), this experiment cannot be used to assess the role of cutinase in virulence. However, since rice has a thin cuticle and *M. grisea* (which produces very little cutinase when compared to other pathogens [P. E. Kolattukudy, *unpublished*]) has a highly melanized appressorium, it is possible that cutinase plays little role in this host-pathogen system. The relative importance of physical force and assistance by cutinase probably varies a great deal in the different host-pathogen systems; thus, the role cutinase plays in virulence is likely to range from essential to insignificant, depending on the host-pathogen system.

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