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Fusarium Polycaprolactone Depolymerase Is Cutinase

CATHERINE A. MURPHY,* J. A. CAMERON, SAMUEL J. HUANG, AND ROBERT T. VINOPAL

Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3044

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Polycaprolactone (PCL), a synthetic polyester, is degraded by a variety of microorganisms, including some phytopathogens. Many phytopathogens secrete cutinase, a serine hydrolase that degrades cutin, the structural polymer of the plant cuticle. We compared wild-type strains and a cutinase-negative gene replacement mutant strain of *Fusarium solani* f. sp. *pisi* (D. J. Stahl and W. Schäfer, Plant Cell 4:621–629, 1992) and a wild-type strain of *Fusarium moniliforme* to show that *Fusarium* cutinase is a PCL depolymerase. The wild-type strains, but not the mutant strain, (i) degraded PCL and used it as a source of carbon and energy, (ii) showed induction of secreted PCL depolymerase and an esterase activity of cutinase when grown in the presence of cutin, and (iii) showed induction of PCL depolymerase and an esterase activity of cutinase when grown in the presence of a hydrolysate of PCL, which contains PCL oligomers that are structurally similar to the natural inducers of cutinase. These results together with other details of regulation and conditions for optimal enzyme activity indicate that the *Fusarium* PCL depolymerase, required for degradation and utilization of PCL, is cutinase.

The increasing volume of plastic waste and decreasing landfill capacity for disposing of it has stimulated interest in biodegradable plastics. The major commercial plastics, such as polypropylene, polystyrene, and polyvinyl chloride, show very limited biodegradability (9, 21). Plastics based on polyhydroxyalkanoates, natural microbial polyesters, are biodegradable but very expensive to produce (17). Polycaprolactone (PCL), a synthetic polyester, is degraded by a variety of microorganisms (3–5, 19), but its physical properties make it unsuitable for many applications (9). Similar problems limit the use of the few other available synthetics that are readily biodegradable (9, 17). There is clearly a need for new biodegradable polymers. An understanding of the mechanisms by which natural and synthetic polymers are degraded by microbial enzymes would be useful in the design of new biodegradable plastics. Study of hydrolytic enzymes evolved to attack highly stable natural polymers, e.g. cellulose, chitin, and cutin, might be especially useful.

Microorganisms that degrade PCL are widely distributed in nature (19). PCL degraders, including phytopathogens such as fungi of the genus *Fusarium*, secrete enzymes (depolymerases) that hydrolyze the insoluble polyester to water-soluble products that are used for carbon and energy (3, 18). A barrier to the understanding and application of these enzymes has been the lack of knowledge of their natural substrate and biological function. Two major classes of secreted esterases, lipases and cutinases, are obvious possible identities for PCL depolymerases. Our preliminary observations with column chromatography showed a molecular mass of roughly 25 kDa for a *Fusarium* PCL depolymerase, making cutinase an attractive possibility; cutinases have molecular masses of 22 to 25 kDa (2, 14, 22), while lipases are larger, 30 kDa and above (11, 23, 26). Many phytopathogenic fungi secrete cutinases, which degrade cutin, the polyester structural component of the plant cuticle. The cuticle protects plants from dehydration and invasion by microorganisms, and cutin plays a key role in its barrier properties (22). Cutinases are serine hydrolases specific for primary alcohol esters, the dominant linkage in cutin (12). The hydrolysis products of cutin include 16-hydroxyhexadecanoic acid, 10,16-dihydroxyhexadecanoic acid, and 9,10,18-trihydroxyoctadecanoic acid, which are effective inducers of cutinase synthesis; the hydroxyl group at the omega position is the most important factor for inducing activity (15).

To test the possibility that the natural substrate of *Fusarium* PCL depolymerase is cutin, we compared wild-type and cutinase-negative strains of *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*) for the ability to grow on PCL, to form zones of clearing on agar containing PCL, and to produce PCL depolymerase in liquid culture. Because cutinase-mediated growth on PCL would require induction, we also tested the ability of PCL hydrolysis products to induce cutinase.

MATERIALS AND METHODS

Fungal strains and media. *F. solani* f. sp. *pisi* (*N. haematococca*) 77-2-3 and 77-102 were the gift of Wilhelm Schäfer, Institut für Genbiologische Forschung, Berlin, Germany. Strain 77-102 is a cutinase-negative mutant constructed by transformation-mediated gene disruption of the single cutinase gene of the wild-type strain 77-2-3 (25). A second wild-type strain of *F. solani* f. sp. *pisi* (ATCC 38136) was obtained from the American Type Culture Collection, Rockville, Md. *Fusarium moniliforme* was from the University of Connecticut culture collection.

Cultures were maintained on potato dextrose agar (Difco Laboratories) or V8 juice agar (V8 juice, 200 ml; $CaCO_3$, 3 g; agar, 25 g per liter) at 22°C. Mineral medium (MM) for substrate utilization and induction experiments contained the following (per liter): $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $MgSO_4$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 1 mg; $CaCl_2$, 1 mg; H_3BO_3, 10 μg; $MnSO_4$, 10 μg; $ZnSO_4$, 70 μg; $CuSO_4$, 50 μg; MoO_3, 10 μg (15).

PCL plate clearing assay. PCL (Cellomer Associates, Inc., Webster, N.Y.) with a molecular weight of approximately 10,000 was used. A 1% suspension of PCL crystals was prepared by precipitating the polymer from an acetone solution with water and filtering the resulting suspension to remove large aggregates, as described by Jarrett et al. (10). After autoclaving and cooling of MM containing 10 g of agar per liter to 50°C, the PCL suspension was added to a final concentration of 500 mg/liter, resulting in a cloudy appearance. Triplicate plates of MM agar with PCL (MM-PCL) were inoculated with test cultures by taking small agar plugs from the edge of advancing hyphae grown on V8 juice agar. The cultures were incubated at 22°C and examined daily for growth and zones of clearing.

Cutin isolation. Cutin was isolated from apples as described by Walton and Kolattukudy (28), with modifications. Peels from Cortland and McIntosh apples were boiled in oxalate buffer (4 g of oxalic acid and 16 g of ammonium oxalate per liter) for 2 to 4 h. Cuticle was collected by filtration through cheesecloth, washed several times with distilled water, dried, and ground in a Wiley mill (60 mesh followed by 100 mesh; Arthur H. Thomas). Powdered cuticle was extracted with chloroform-methanol (2:1, vol/vol) overnight with mild stirring. A second extraction was done with chloroform in a Soxhlet apparatus for 24 h. The cuticle was dried at 80°C and then washed several times with distilled water. Pectin and

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, University of Connecticut, 75 N. Eagleville Rd., U-44, Storrs, CT 06269-3044. Phone: (203) 486-4252. Fax: (203) 486-1784.

cellulose were removed by treatment with *Rhizopus* sp. pectinase (1 g/liter) and *Aspergillus niger* cellulase (5 g/liter; Sigma Chemical Co., St. Louis, Mo.) in pH 4.0 acetate buffer (50 mM) at 22°C for 48 h. Cutin was recovered by filtration, washed with distilled water, and dried at 105°C.

Assay of PCL depolymerase and esterase production. Roux flasks containing 100 ml of MM supplemented with 0.2% cutin were inoculated with 2 to 4 ml of 24- to 48-h *Fusarium* cultures grown in MM with 0.1% glucose. The flasks were incubated at 22°C without shaking. Because the cutinase-negative mutant does not grow on cutin, cultures to compare wild-type and mutant strains were supplemented with 0.1% sodium acetate to eliminate the possibility that the absence of depolymerase activity in mutant cultures was due to lack of growth. The production of cutinase is subject to glucose repression; acetate represses cutinase production much less (7). Control cultures contained 0.1% sodium acetate as a sole source of carbon and energy. Supernatants of experimental and control cultures, clarified by centrifugation in a Microfuge (Beckman), were assayed for esterase and PCL depolymerase activity daily.

Esterase activity was determined spectrophotometrically with *p*-nitrophenyl caproate (PNPC) as the substrate by a procedure adapted from Purdy and Kolattukudy (22). PNPC was used as a model substrate to detect cutinase activity; PNP esters are good substrates for purified cutinase (15). The assay mixture (1-ml total volume) contained 5 mM PNPC (100 μl), 50 mM potassium phosphate buffer (pH 6.8; 700 to 875 μl), and culture supernatant (25 to 200 μl). The A_{405} was recorded for 3 min. Activity was expressed as the increase in A_{405} minute^{-1} milliliter of supernatant^{-1}.

PCL depolymerase activity was measured by a densitometric assay using a suspension of PCL crystals, as described by Cameron and Costa (6). The assay mixture (1.5-ml total volume) contained 100 μl of a 1% PCL suspension (as described for the plate clearing assay), 50 mM potassium phosphate buffer (pH 6.8; 0.4 to 1.15 ml), and culture supernatant (0.25 to 1 ml). The optical density at 600 nm was recorded for 3 min, during which active preparations showed a linear decrease. Activity was expressed in units of decrease in optical density at 600 nm minute^{-1} milliliter of supernatant^{-1} × 10^3.

Induction of PCL depolymerase by PCL hydrolysate. PCL was hydrolyzed with filter-sterilized supernatant from a culture of the wild-type strain 77-2-3 grown on cutin. Supernatant (3 ml), containing 328 U of PCL depolymerase activity, was added to 10 ml of a 1% PCL suspension (described above), and the mixture was incubated at 22°C for 2 days. After incubation, the turbid, milky PCL suspension had been transformed into a clear solution, indicating hydrolysis of PCL. The cutin-grown culture supernatant might have contained low levels of cutin hydrolysis products, which are inducers of cutinase and thus perhaps of PCL depolymerase. (16-Hydroxyhexadecanoic acid [Sigma] does induce PCL depolymerase [data not shown].) Therefore, a control mixture containing 3 ml of supernatant and 10 ml of 50 mM phosphate buffer (pH 6.8) was prepared along with the PCL hydrolysate. The PCL hydrolysate and the control mixture were then heated at 75°C for 30 min to inactivate PCL depolymerase. No depolymerase activity was detected in the PCL hydrolysate or the control after the heat treatment.

For induction, the *Fusarium* strains were grown in MM supplemented with 0.075% glucose at 22°C for 65 h (15); the PCL hydrolysate (2 ml; final concentration, approximately 100 μg/ml) was then added to one set of cultures, while the second set received 2 ml of the control mixture. Lin and Kolattukudy determined that 100 μg of cutin hydrolysate per ml was enough to induce cutinase synthesis (15). The PCL depolymerase activity of experimental and control cultures was measured at least daily.

Effect of pH on depolymerase activity. The *Fusarium* strains were grown on MM containing 0.2% cutin and 0.1% acetate for about 2 weeks. The supernatant from these cultures was analyzed for depolymerase activity at several different pH values. Potassium phosphate buffer (50 mM) at pH 5.5, 6, 7, and 8 and 50 mM glycine-NaOH buffer at pH 9 and 10 were used in the depolymerase assay.

RESULTS

PCL degradation. *F. solani* wild-type strains 77-2-3 and ATCC 38136 and *F. moniliforme* were able to degrade PCL, as indicated by a zone of clearing on MM-PCL agar plates. Zones of clearing could be easily observed after 48 h (Fig. 1A). Two of these strains, 77-2-3 and ATCC 38136, are known cutinase producers (13, 25). The cutinase-negative mutant strain 77-102 grew to a limited extent on MM-PCL agar but did not produce a zone of clearing (Fig. 1B). Growth was probably supported by carryover of nutrients from the inoculum. Neither mutant nor wild-type strains produced zones of clearing on MM-PCL agar containing 0.5% glucose (data not shown). Induction of cutinase in *Fusarium* spp. is repressed by glucose (15).

PCL depolymerase and esterase production by *Fusarium* strains grown on cutin. All wild-type *Fusarium* strains grown in MM with cutin and acetate showed PCL depolymerase activity in the culture supernatants, but no depolymerase activity was detected in cultures of the cutinase-negative mutant (Fig. 2). The absence of depolymerase activity in cultures of the mutant was not due to lack of growth; sodium acetate supported extensive growth, as indicated by hyphal mass production. Control cultures of all strains, with sodium acetate as the sole source of carbon, showed no detectable depolymerase activity. Several repetitions of this experiment gave similar results, although the time course of enzyme production varied slightly.

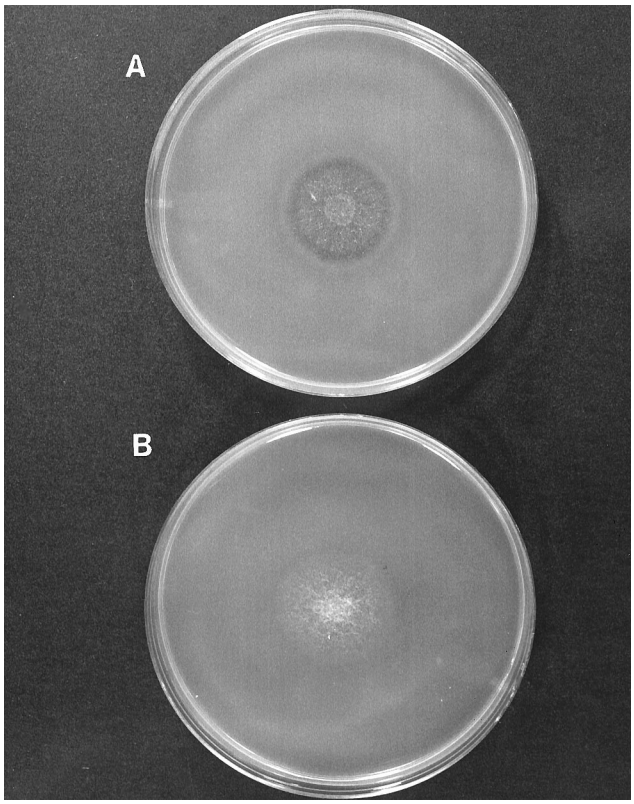

FIG. 1. PCL degradation by *Fusarium* strains. Minimal agar medium containing PCL was inoculated with fungal strains. A zone of clearing, indicative of PCL degradation, was observed for wild-type strains, e.g., 77-2-3 (A), but not for the cutinase-negative mutant strain 77-102 (B).

High levels of esterase activity were detected in the supernatant of cultures grown in the presence of cutin, except for cultures of the mutant strain, which showed less than 5% of wild-type activity (Fig. 3). All strains grown on acetate alone showed less than 5% of wild-type esterase activity in cultures grown on acetate and cutin (data not shown). The depolymerase data in Fig. 2 and the esterase data in Fig. 3 are from the same experiment.

Stahl and Schäfer constructed the cutinase-negative mutant strain 77-102 by transformation-mediated replacement of the single cutinase gene of strain 77-2-3 with a cutinase gene disrupted by an insertion (25). With a denaturing polyacrylamide gel visualized with a ^{14}C-labeled serine hydrolase inhibitor and fluorography, they showed that culture fluids of the two strains grown in the presence of cutin differed in a single band, a major 24-kDa protein, as expected, present in the parental strain but missing from the mutant (25). We grew *Fusarium* cultures in the presence of cutin and separated the proteins present in the supernatant on a sodium dodecyl sulfate-polyacrylamide gel, visualized with a protein stain. Concentrated culture filtrates from the wild-type strains contained a major protein with a molecular mass of about 24 kDa that was miss-

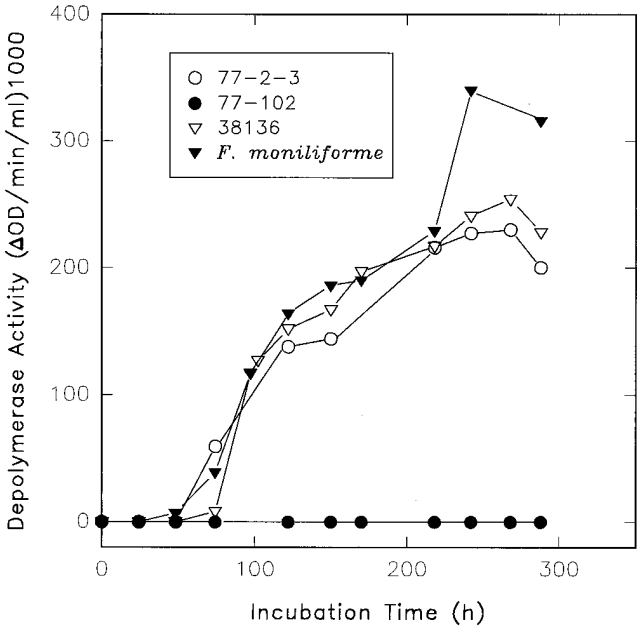

FIG. 2. PCL depolymerase activity of *Fusarium* strains grown in medium with cutin. Wild-type *F. solani* f. sp. *pisi* 77-2-3 and ATCC 38136, wild-type *F. moniliforme*, and the *F. solani* f. sp. *pisi* cutinase-negative mutant strain 77-102 were grown in MM with 0.2% cutin and 0.1% acetate. No depolymerase activity was detected in the supernatant of cultures grown on acetate alone (data not shown). OD, optical density.

ing from the supernatant of strain 77-102 (data not shown), in agreement with the results of Stahl and Schäfer. We attempted to show directly that the 24-kDa protein had PCL depolymerase activity by separation of filtrates on nondenaturing gels

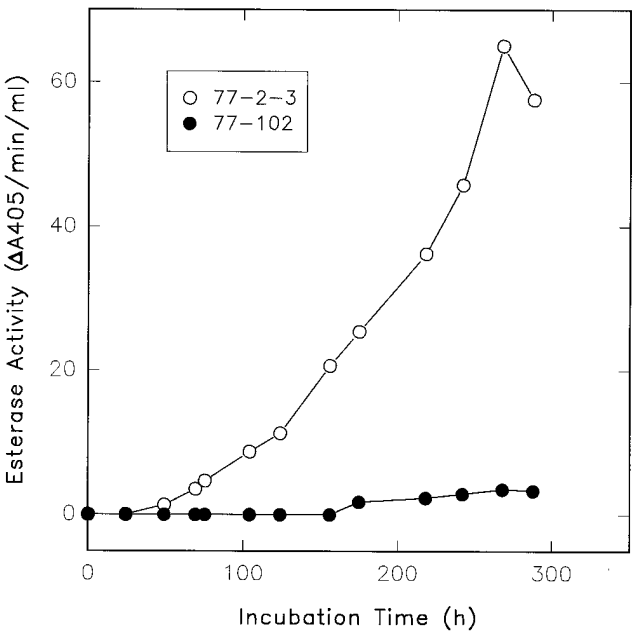

FIG. 3. PNPC esterase activity of *Fusarium* strains grown in medium with cutin. Wild-type *F. solani* f. sp. *pisi* 77-2-3 and the cutinase-negative mutant strain 77-102 were grown as described in the legend to Fig. 2. All strains grown on acetate alone showed less than 5% of wild-type esterase activity in cultures grown on acetate and cutin (data not shown). OD, optical density.

by the procedure used by Purdy and Kolattukudy for analysis of purified *F. solani* cutinase (23), followed by activity staining and elution for assay. PCL depolymerase activity remained in the stacking gel (data not shown), probably the result of aggregation of cutinase by phenolics in crude cutin-grown culture fluids, as noted by Purdy and Kolattukudy (23).

Induction of PCL depolymerase (cutinase) by PCL hydrolysate. PCL dimers and trimers, products of enzymatic PCL degradation (3, 18), are structurally similar to cutin degradation products (C_{16} and C_{18} omega hydroxy fatty acids) that are known inducers of cutinase (Fig. 4). The addition of the cutinase inducer 16-hydroxyhexadecanoic acid to culture medium, as described previously (15), induced PCL depolymerase production by *F. solani* ATCC 38136 and *F. moniliforme* (data not shown). To test whether PCL hydrolysis products induce PCL depolymerase, *Fusarium* strains were grown in preinduction medium containing 0.075% glucose as the carbon source for 65 h, by which time glucose was exhausted (15). No depolymerase activity was detected in the experimental or control cultures during this time (Fig. 5). Enzymatically prepared PCL hydrolysate was then added to the experimental cultures, while control cultures received a heat-treated cutin-grown culture supernatant mixture without PCL. Culture supernatants were assayed for PCL depolymerase activity at intervals over the next 55 h. One hour after the addition of PCL hydrolysate, depolymerase activity was detected in the culture supernatant of strains 77-2-3, ATCC 38136, and *F. moniliforme*. Depolymerase activity increased in these three cultures for the next 24 h, after which there was a decline in activity. No depolymerase activity was detected in the supernatant of the cutinase-negative mutant culture or in any of the control cultures at any time. Lack of activity in the control cultures confirms that induction was due to PCL products, not to carryover of cutin degradation products in the strain 77-2-3 supernatant.

pH optimum of PCL depolymerase. The effect of pH on PCL depolymerase activity from the wild-type *Fusarium* strains is shown in Fig. 6. The highest activity was observed at pH 9 and 10, which is the region of pH optimum of *Fusarium* cutinases (13). We were unable to determine the pH optimum of the cutin- and PCL-induced esterase activity because the esterase substrate is unstable at alkaline pH (23).

DISCUSSION

Although PCLs have been shown to be degraded by enzymes secreted by a number of bacteria (3, 4) and fungi (5, 6, 10), the natural substrates for these depolymerases have not been identified. Lipases have activity against PCL (27), and thus some PCL depolymerases are probably lipases (3, 6) with various lipids as the biologically relevant substrates. Another possibility for a natural substrate is cutin, a polyester found in the plant cuticle. Many phytopathogens, including fungi (2, 14, 22) and bacteria (8), secrete cutinase, a serine hydrolase that breaks ester bonds in cutin. Nishida and Tokiwa showed that some fungal phytopathogens degrade PCL and suggested an involvement of cutinase (20), but they did not determine whether the enzymes active against PCL were cutinases or, instead, lipases or other hydrolases. We have presented genetic, regulatory, and enzymatic evidence that the *Fusarium* PCL depolymerase required for utilization of PCL for growth is cutinase.

Our genetic evidence was made possible by the availability of a well-studied cutinase producer, *F. solani* f. sp. *pisi* (the causal agent of foot rot of pea plants), and an isogenic cutinase-negative mutant of it constructed by Stahl and Schäfer, a recombinant strain in which the single endogenous cutinase gene is replaced by a disrupted functionless copy, resulting in the

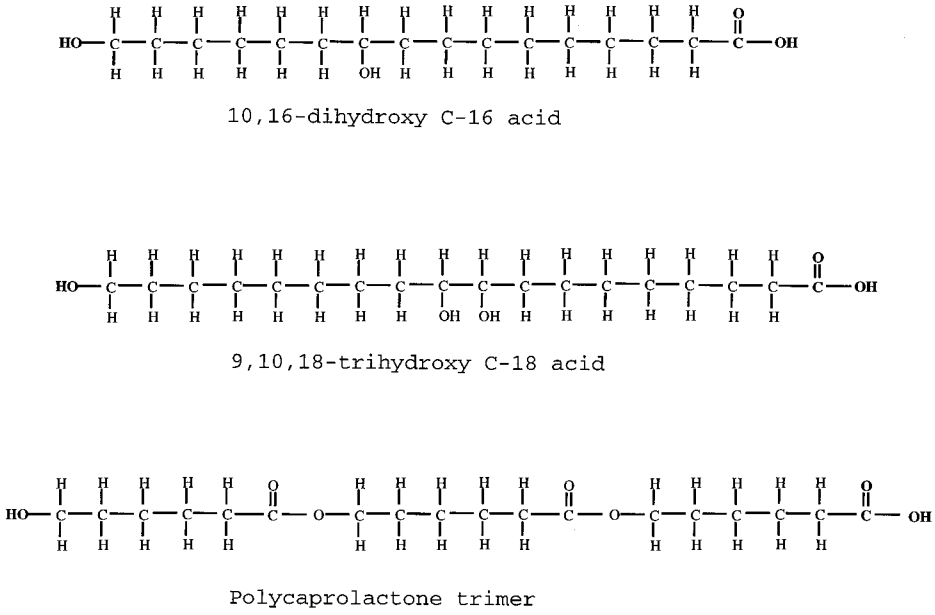

FIG. 4. Comparison of the chemical structures of two cutin monomers that are inducers of cutinase with the PCL trimer.

absence of a single serine hydrolase of the known molecular weight of cutinase (25). Stahl and Schäfer confirmed that their isogenic cutinase-positive and structural gene-negative strains had identical germination efficiencies, growth rates, and colony morphologies when grown on complete medium, supporting the conclusion that any difference in phenotype between the wild-type and mutant strains is due to the absence of cutinase in the mutant. We showed that wild-type *F. solani* f. sp. *pisi* strains and wild-type *F. moniliforme* (a maize pathogen) degraded PCL, while the cutinase-negative *F. solani* f. sp. *pisi* strain did not (Fig. 1).

Our regulatory evidence that PCL depolymerase is cutinase is based on studies of induction of PCL depolymerase and an associated esterase activity. Cutinase activity is induced in the supernatant of *F. solani* f. sp. *pisi* and other fungal phytopathogens grown in medium containing cutin as a carbon source (2, 14, 15), and we found PCL depolymerase activity in the culture supernatant of wild-type *Fusarium* strains grown on cutin or on PCL but not on other carbon sources. Cutinase induction is

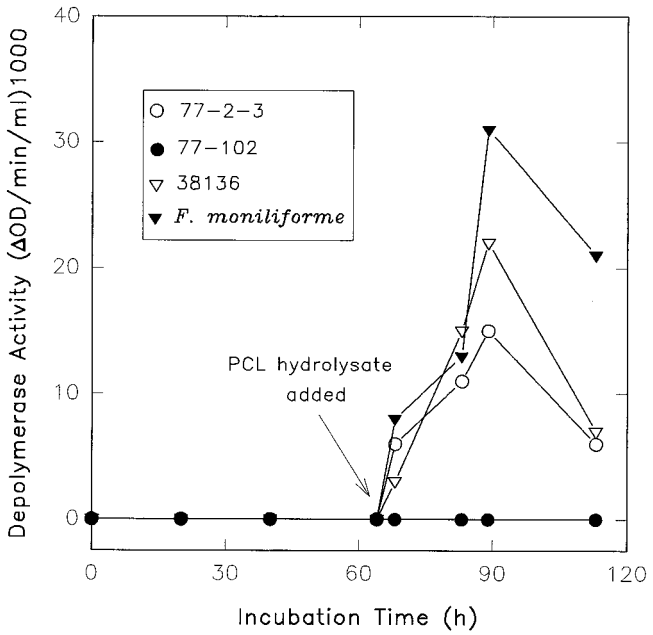

FIG. 5. Induction of PCL depolymerase by PCL hydrolysate. Wild-type *F. solani* f. sp. *pisi* 77-2-3 and ATCC 38136, wild-type *F. moniliforme*, and the *F. solani* f. sp. *pisi* cutinase-negative mutant strain 77-102 were grown in MM with 0.075% glucose. At 65 h (arrow), PCL hydrolysate was added to the cultures. OD, optical density.

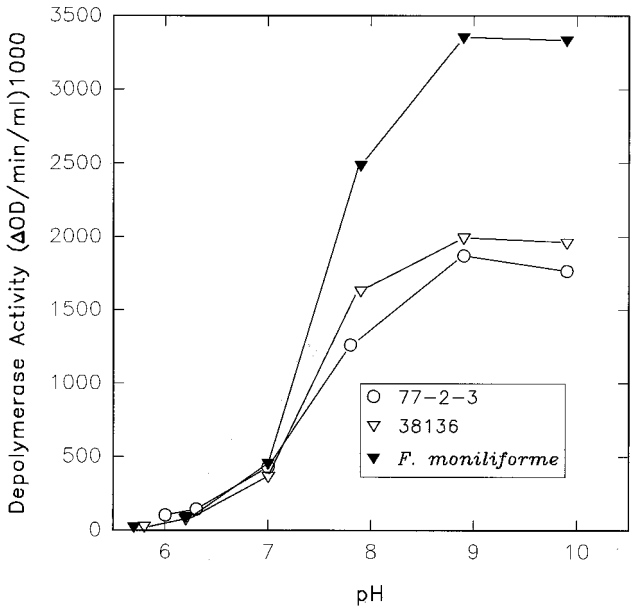

FIG. 6. Effect of pH on *Fusarium* PCL depolymerase activity. OD, optical density.

subject to glucose repression (15), and we found that the presence of glucose in PCL agar prevented clearing of PCL, even by wild-type strains. We detected no depolymerase activity in supernatants of the cutinase-negative mutant, even when cutin or PCL was present in the growth medium. Stahl and Schäfer reported that the mutant grew very poorly in medium containing cutin as a sole source of carbon and lacked cutinolytic activity (25). We eliminated the possibility that the absence of activity in the mutant cultures was due to lack of growth on cutin by including acetate as an alternate carbon source. Wild-type cultures grown on acetate and cutin, including *F. solani* ATCC 38136 and *F. moniliforme*, showed high PCL depolymerase activity but lacked activity when grown on acetate alone. Cutinase can be assayed by its activity against esterase substrates (13, 22), and we found in our induction experiments that PNPC esterase activity always paralleled PCL depolymerase activity, except for the low levels of esterase activity observed in mutant cultures and in wild-type cultures grown without cutin or PCL, probably reflecting esterases other than cutinase. Although PNPC esterase activity is a convenient way to monitor cutinase, PNP esters are not specific substrates for cutinase (22). Specific assay of cutinase has required radiolabeled cutin (13); we are trying to develop a colorimetric assay.

Cutinase synthesis is induced naturally by a variety of C_{16} and C_{18} omega hydroxy fatty acids that are released from cutin by cutinase (15). One of these, 16-hydroxyhexadecanoic acid, induces PCL depolymerase in wild-type *Fusarium* strains. The products of PCL hydrolysis by several fungal PCL depolymerases have been shown to be a mixture of monomers, dimers, and trimers (3, 18). PCL dimers and trimers are structurally similar to natural inducers of cutinase (Fig. 4). We showed that PCL hydrolysis products induce PCL depolymerase synthesis in wild-type *Fusarium* strains but not in the cutinase-negative mutant.

Finally, our demonstration that the pH optimum of *Fusarium* PCL depolymerases is in the region of pH 9 to 10 is in accord with published reports for *Fusarium* and other fungal cutinases (14, 22). Fungal lipases typically have neutral or acidic optima (1).

Taken together, genetic, regulatory, and enzymatic evidence strongly support the conclusion that the *Fusarium* PCL depolymerase required for utilization of PCL is cutinase. Other enzymes with PCL depolymerase activity, for example, lipases or other hydrolases, might be produced by *Fusarium* spp. under conditions we have not investigated.

The regulation and enzymology of cutinases have been well studied (14, 15, 22). The cutinase gene from *F. solani* f. sp. *pisi* has been cloned and sequenced (24), and the molecular structure of the product enzyme has been solved by X-ray diffraction studies (16). The chemical nature of cutin is also quite well understood (28). With the demonstration that *Fusarium* cutinases are PCL depolymerases, this knowledge may be applied to the development of new biodegradable polymers.

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