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Discovery of a Cutinase-Producing *Pseudomonas* sp. Cohabiting with an Apparently Nitrogen-Fixing *Corynebacterium* sp. in the Phyllosphere†

JOSEPH SEBASTIAN, A. K. CHANDRA, AND P. E. KOLATTUKUDY‡*

Institute of Biological Chemistry and Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164-6340

Received 14 July 1986/Accepted 15 October 1986

A phyllospheric bacterial culture, previously reported to partially replace nitrogen fertilizer (B. R. Patti and A. K. Chandra, Plant Soil 61:419–427, 1981) was found to contain a fluorescent pseudomonas which was identified as *Pseudomonas putida* and a *Corynebacterium* sp. The *P. putida* isolate was found to produce an extracellular cutinase when grown in a medium containing cutin, the polyester structural component of plant cuticle. The *Corynebacterium* sp. grew on nitrogen-free medium but could not produce cutinase under any induction conditions tested, whereas *P. putida* could not grow on nitrogen-free medium. When cocultured with the nitrogen-fixing *Corynebacterium* sp., the *P. putida* isolate grew in a nitrogen-free medium, suggesting that the former provided fixed N_2 for the latter. These results suggest that the two species coexist on the plant surface, with one providing carbon and the other providing reduced nitrogen for their growth. The presence of cutin in the medium induced cutinase production by *P. putida*. However, unlike the previously studied fungal systems, cutin hydrolysate did not induce cutinase. Thin-layer chromatographic analysis of the products released from labeled apple fruit cutin showed that the extracellular enzyme released all classes of cutin monomers. This enzyme also catalyzed hydrolysis of the model ester substrates, *p*-nitrophenyl esters of fatty acids, and optimal conditions were determined for a spectrophotometric assay with *p*-nitrophenyl butyrate as the substrate. It did not hydrolyze triacyl glycerols, indicating that the cutinase activity was not due to a nonspecific lipase. It showed a broad pH optimum between 8.0 and 10.5 with ^{3}H-labeled apple cutin as the substrate. Diisopropylfluorophosphate severely inhibited the enzyme, whereas thiol-directed reagents did not inhibit it, suggesting that catalysis by this bacterial cutinase involves active serine.

It has been suggested that a well-established and mixed population of microorganisms in the phyllosphere could make a substantial contribution to the nitrogen requirements of field vegetation (16). Some nitrogen-fixing organisms have been isolated from the leaf surface (8, 13, 18). These nitrogen fixers, when sprayed on the crop plants, increased crop yield when compared with crops which received no nitrogen fertilizers (8). The carbon source for these organisms on the leaf surface is not known. Proposed sources of nutrients include leaf exudates or organic deposits on the leaf surface. Whether these nitrogen fixers are capable of utilizing any of the leaf surface components has not been determined. If the microorganisms are to utilize the insoluble polymer cutin on the leaf surface, these organisms would have to produce an extracellular cutinase. Although cutinases have been purified and characterized from fungi and pollen (2, 3), little is known about bacterial cutinase. In this paper we report that a phyllospheric fluorescent *Pseudomonas putida* strain, coisolated with a nitrogen-fixing bacterium, may be induced to produce cutinase, and preliminary characterization of this bacterial cutinase activity is reported. We also present evidence that in the phyllosphere the *P. putida* strain might provide the carbon source while the *Corynebacterium* sp. provides fixed nitrogen for both organisms to survive together.

* Corresponding author.

† Scientific paper 7499, project 2001, College of Agriculture and Home Economics Research Center.

‡ Present address: Biotechnology Center, Ohio State University, Columbus, OH 43210.

MATERIALS AND METHODS

Bacterial strains. Cultures of a nitrogen-fixing *Corynebacterium* sp. isolated from the leaves of *Phaseolus mungo* (BS_2) and six other bacterial strains isolated from different leaf surfaces designated M_1, M_2, M_4, B_8, S_1, and S_3 (13) were maintained on 2% Noble agar slants prepared in a nitrogen-free medium (azotobacter medium) and kept at 4°C until used.

Cutin. Apple cutin preparations labeled with ^{14}C or ^{3}H were prepared as described earlier (5), and cutin hydrolysate was prepared by refluxing 40-mesh cutin powder with 10% alcoholic KOH for 16 h under a nitrogen atmosphere. The resulting mixture was acidified with concentrated HCl, and the products were thoroughly extracted with chloroform. The combined chloroform extract was evaporated to dryness under vacuum.

Growth medium. The complete medium used in this study was composed of 0.2% yeast extract and 0.8% nutrient broth (Difco Laboratories) and was supplemented with apple cutin powder where indicated. The azotobacter medium was prepared as described in the 1976 American Type Culture Collection manual (p. 276). These media were solidified by the addition of 1.5% agar for preparation of slants or plates.

Identification of bacterial strains. The bacterial isolate designated BS_2 was transferred to the plates made in nutrient broth-yeast extract (NBY) medium. Two morphologically distinct colonies were isolated and streaked on the same plates. These bacterial strains were identified by using the published procedure (17).

Coculture experiments. Erlenmeyer flasks containing 30 ml of azotobacter medium in which sucrose was substituted by cutin was inoculated with 10^3 cells of *P. putida* and 10^3, 2×10^3, or 4×10^3 cells of *Corynebacterium* inoculum. In another set of flasks containing the same media and the same number of *P. putida* bacterial cells, the *Corynebacterium* sp. was substituted by 50, 100, or 200 mg of ammonium sulfate. Boiled *Corynebacterium* inoculum was used for a control. In another control neither ammonium sulfate nor *Corynebacterium* sp. was added to the medium. Incubation was carried out at 30°C in a shaker-incubator (Lab-Line Inc.) at 200 rpm. Aliquots (0.5 ml) were withdrawn from these cultures after different time intervals, and the number of cells of each strain was determined by serial dilution and plating on NBY extract plates. The colonies were counted after 16 h of incubation at 30°C.

Cutinase induction. Different amounts of purified papaya cutin or apple cutin were added to 30 ml of NBY medium, the azotobacter medium supplemented with ammonium sulfate, or the minimal mineral medium usually used to grow fungi on cutin (14), and the cultures were incubated at 30°C. A suspension of cutin hydrolysate (200 mg in 20 ml of water) was subjected to ultrasonic treatment with a Biosonik III equipped with a Biosonik 0.75-in. (1.91-cm) probe; after each 30-s sonication the mixture was cooled in ice, and the process was repeated 15 times. Aliquots of the resulting hydrolysate dispersion were added to 100-ml cultures of NBY medium in Erlenmeyer flasks. For comparison, cutin-containing medium and a control containing neither cutin hydrolysate nor cutin were also used as growth media. Aliquots were assayed for cutinase activity by both spectrophotometric assay and radioassay.

Enzyme preparation. Six liters of NBY medium in which *P. putida* was grown in the presence of cutin for 4 days was centrifuged at $10,000 \times g$, and the supernatant was freeze-dried. The residue was dissolved in 450 ml of 20 mM Tris hydrochloride buffer (pH 8), the residual insoluble material was removed by centrifugation at $10,000 \times g$, and the dark viscous solution was dialyzed overnight against 8 liters of 20 mM Tris hydrochloride, pH 8. To this enzyme solution 3 volumes of cold (−20°C) acetone were added, and the mixture was stirred for 1 h in an ice bath. The precipitate collected by centrifugation at $10,000 \times g$ was dissolved in the same buffer and used as the enzyme source for further experiments.

Enzyme assay. Hydrolysis of *p*-nitrophenyl esters of fatty acids was measured spectrophotometrically at 405 nm as described earlier (5) after optimizing assay conditions for the present enzyme. The reaction was run in 3 ml of 0.1 M sodium phosphate buffer, pH 8, containing 1.6 mM *p*-nitrophenyl butyrate, 0.4% Triton X-100, and appropriate amounts of enzyme at 23°C for 3 to 4 min, during which time linear increases in A_{405} were observed. This initial velocity was used for all activity measurements.

Cutin hydrolase activity was measured by using a radioassay, incubating appropriate amounts of the enzyme in 1 ml of 0.1 M sodium phosphate buffer, pH 8, containing 4 mg of tritiated cutin at 30°C. The reaction was stopped by chilling the tubes on ice and acidifying with 0.1 ml of 1 M HCl followed by filtering the reaction mixture through a glass-wool plug placed in a Pasteur pipette. An aliquot of the filtrate was assayed for radioactivity by liquid scintillation spectrometry. Protein concentration was routinely determined by the method of Lowry et al. (11).

Inhibitors and chelators. The enzyme preparation was incubated with the test compounds in 0.1 M sodium phosphate buffer, pH 8, containing 0.4% Triton X-100 for 30 min at room temperature, prior to cutinase assay.

Identification of monomers released from cutin by cutinase. ^{14}C-labeled apple fruit cutin (6), 0.64 mg, was finely minced with a razor blade and incubated with 6 mg of crude enzyme preparation in 1 ml of 0.1 M sodium phosphate buffer, pH 8, for 2 h at 30°C. After the incubation the reaction mixture was acidified, the products were thoroughly extracted with chloroform, and the solvent was evaporated off with a stream of N_2. The residue was subjected to thin-layer chromatography on Silica Gel G (Merck & Co., Inc.) with ethyl ether-hexane-formic acid (80:20:1) as the developing solvent. The radioactive products were identified by comparison of their R_f values with those of previously identified cutin hydrolysis products.

Temperature stability. Aliquots (1 ml) of the extracellular fluid were incubated at different temperatures from 30 to 80°C for various periods, 100-μl aliquots were withdrawn, and the enzyme activity was determined spectrophotometrically. Similar experiments were carried out with four different fungal cutinases produced by *Fusarium solani pisi*, *F. roseum culmorum*, *Colletotrichum gloeosporioides*, and *F. roseum sambucinum* (3) for comparison. Freeze-dried extracellular fluid dissolved in 10 mM sodium phosphate buffer, pH 8, was used in this experiment.

RESULTS

Detection and identification of a nitrogen-fixing bacterium and a cutinase-producing bacterium which cohabits with it. To test whether the apparently nitrogen-fixing phyllospheric bacteria could use the plant cuticular polyester as a carbon source for growth, the different bacterial strains isolated from plant leaves were tested for their ability to grow on a nitrogen-free medium with cutin as the sole carbon source. Of the seven bacterial isolates used in this study, two did not grow on slants made in azotobacter medium after 7 days of incubation. The five isolates which showed growth were transferred to 25 ml of the same medium in which sucrose was substituted by cutin isolated from the fruits of apple or papaya. In such a medium only one isolate, BS_2, survived with cutin as the sole source of carbon. When this bacterial isolate was transferred and grown on agar plates made in NBY medium, two morphologically distinct colonies were visible. One was a gram-negative bacterium and the other was gram positive. The gram-negative isolate tested positive for cytochrome *c* oxidase, catalase, arginine dihydrolase, acid from glucose (oxidation-fermentation test), growth on citrate agar, growth at 4°C, and fluorescein on King's B. It tested negative for gelatin hydrolysis, starch hydrolysis, and denitrification. On the basis of these results, this isolate was identified as fluorescent *P. putida*. This species, however, was found to grow at 41°C, which is not one of the common characteristics of *P. putida*. Therefore, the present isolate is considered to be a temperature-resistant isolate of *P. putida*. The gram-positive bacterium was not fully identified, but it showed the common characteristics of a *Corynebacterium* sp. It formed sporeless, yellowish orange colonies on NBY medium and grew on the azotobacter medium, suggesting that it fixes N_2.

The cultures containing the two coisolated bacteria could be maintained on slants made in nitrogen-free mineral medium for several months. In our initial studies we were growing the BS_2 isolate containing both bacteria on mineral medium with cutin as the sole source of carbon. Thus, both bacterial species grew together in both media. To determine

TABLE 1. Effect of coculturing with *Corynebacterium* sp. on growth of *P. putida* in a nitrogen-free medium[a]

Culture no.	Azotobacter medium (carbon-free) with 100 mg of apple fruit cutin plus:	No. of cells	
		12 h	24 h
1	No addition	0	0
2	50 mg of $(NH_4)_2SO_4$	2×10^4	3.3×10^5
3	100 mg of $(NH_4)_2SO_4$	3.5×10^4	9.4×10^6
4	200 mg of $(NH_4)_2SO_4$	4.8×10^5	9.4×10^6
5	10^3 *Corynebacterium* cells	2×10^5 (2×10^5)[b]	3.5×10^5 (9×10^5)
6	2×10^3 *Corynebacterium* cells	2×10^5 (3×10^5)	2.7×10^6 (1.9×10^6)
7	3×10^6 *Corynebacterium* cells	2.3×10^5 (6×10^5)	4.3×10^6 (8.3×10^6)

[a] Growth is indicated by the number of cells found at each time point. Initial inoculum of *P. putida* was 10^3 cells in each assay.
[b] Number in parentheses is number of corynebacterial cells.

whether both species fixed N_2 and degraded cutin, pure cultures of each were grown alone on nitrogen-free medium and in cutin-containing medium. The *P. putida* strain alone could not grow on a nitrogen-free mineral medium. Addition of increasing concentrations of ammonium sulfate gave progressively higher rates of growth. To test whether the *Corynebacterium* sp. could provide fixed nitrogen for the *P. putida* strain, ammonium sulfate was replaced with the *Corynebacterium* strain in the nitrogen-free medium and *P. putida* showed growth. In such coculturing experiments the growth of *P. putida* depended on the inoculum size of the *Corynebacterium* culture. With 10^3 cells of *Corynebacterium* sp. the growth of *P. putida* was about the same as that obtained with 50 mg of $(NH_4)_2SO_4$ (Table 1), and with 3×10^3 cells of *Corynebacterium* sp. the growth of *P. putida* was about half the maximal growth observed with optimal levels of ammonium sulfate. Since *P. putida* could not grow on complete azotobacter medium (containing 0.2% sucrose), the observed growth of *P. putida* could not have been due to the small amount of sucrose contained in the *Corynebacterium* inoculum used in these experiments. Furthermore, boiled inoculum of corynebacteria did not support growth of *P. putida*. Thus, it is concluded that the growing corynebacteria provided fixed nitrogen for the growth of the *P. putida* (Table 1). The *Corynebacterium* sp. showed no growth in azotobacter medium in which sucrose was replaced by cutin. However, when such a medium was inoculated with *P. putida*, the *Corynebacterium* sp. grew quite well (Table 1). Since cutin was the only carbon source provided in such cultures, it is clear that the *Corynebacterium* sp., which is unable to degrade cutin, obtained carbon released from the polymer by *P. putida*.

TABLE 2. Effect of composition of media on cutinase induction in *P. putida* strain

Composition of medium	^3H liberated (cpm)
Azotobacter medium (alone)	320
Azotobacter medium + ammonium sulfate	480
Azotobacter medium + ammonium sulfate + apple cutin	5,390
Azotobacter medium + papaya cutin (5 mg/ml)	2,180
Mineral medium + papaya cutin (5 mg/ml)	830
Mineral medium + apple cutin (5 mg/ml)	2,520
Nutrient broth, 0.8%	300
Nutrient broth, 0.8%, + 0.2% yeast extract + apple cutin (1 mg ml)	9,520
Nutrient broth, 0.8%, + 0.2% yeast extract + apple cutin (2 mg/ml)	11,000
Nutrient broth, 0.8%, + 0.2% yeast extract + apple cutin (3 mg/ml)	12,300
Nutrient broth, 0.8%, + 0.2% yeast extract + apple cutin (4 mg/ml)	13,100
Nutrient broth, 0.8%, + 0.2% yeast extract + apple cutin (5 mg/ml)	13,100
Nutrient broth, 0.8%, + 0.2% yeast extract + papaya cutin (5 mg/ml)	4,200

Optimum conditions for cutinase production by the *P. putida* strain. Since the *P. putida* strain showed ability to grow on cutin as the sole source of carbon, it was suspected that this bacterium produced an extracellular cutin-degrading enzyme. The cutin-degrading activity of the extracellular fluid was measured by the release of soluble radio-

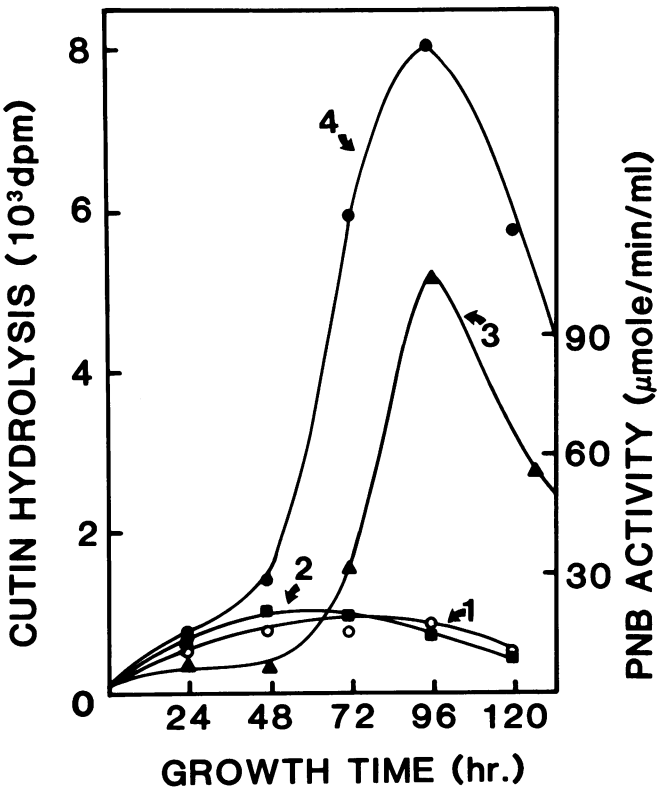

FIG. 1. Time course of induction of cutinase: in NBY medium alone (1), or the same medium supplemented with cutin hydrolysate (2), or cutin (3), measured spectrophotometrically. The induction of enzyme was also measured with tritiated apple cutin as shown in the case of cutin-supplemented medium (4). The assays were done as described in the text. PNB, *p*-Nitrophenyl butyrate.

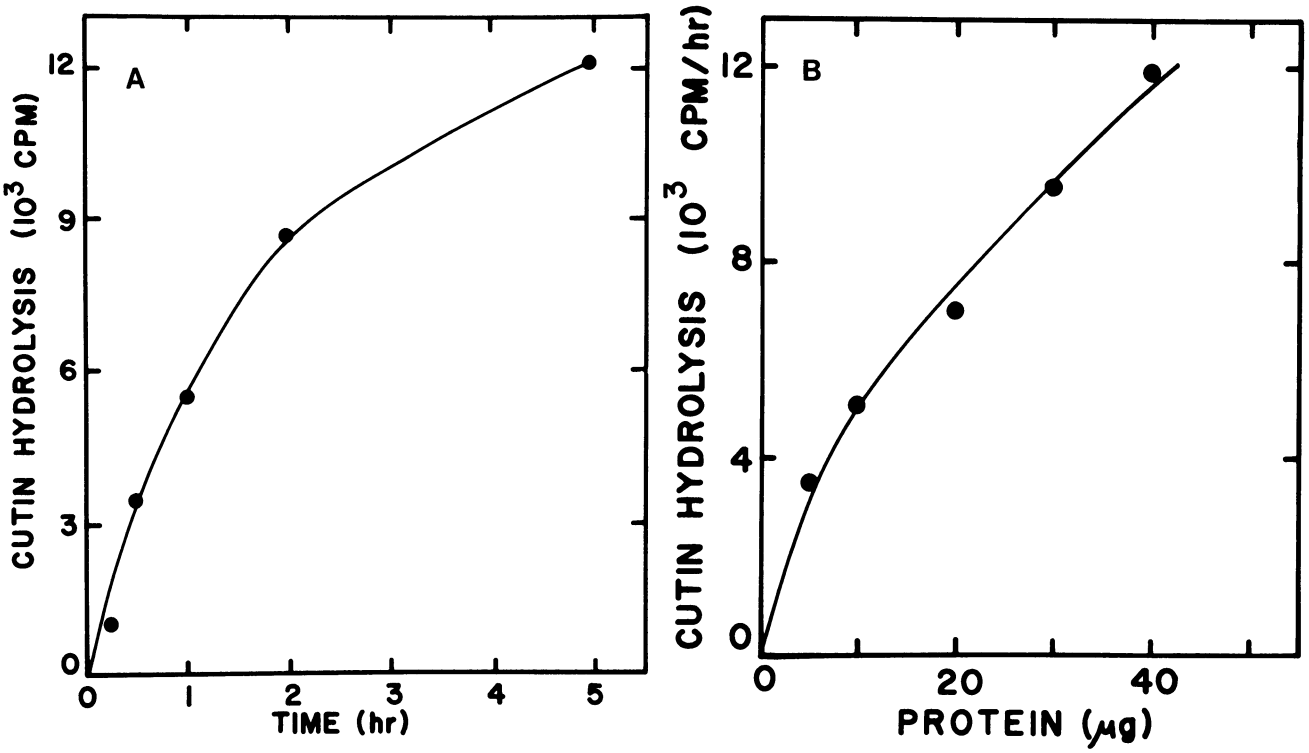

FIG. 2. Effect of time (A) and protein concentration (B) on rate of release of water-soluble radioactive products from ^{14}C-labeled cutin by the extracellular enzyme from a *P. putida* strain. The enzyme assays were done as described in the text.

active materials from the insoluble ^3H-labeled cutin (5). When a mineral medium, previously used to grow fungi with cutin as the sole source of carbon, was used with the present bacterium, very little cutinase production was detected. In the azotobacter medium the presence of either papaya or apple cutin caused induction of extracellular cutinase activity (Table 2). When reduced nitrogen was provided in the form of ammonium sulfate together with cutin, an enhanced induction was obtained with either cutin preparation in the azotobacter medium. Nutrient broth supplemented with yeast extract provided a much better medium in which cutinase could be induced by the presence of cutin (Table 2). The degree of induction of cutinase depended on the amount of cutin in the medium, and 0.4% (wt/vol) cutin gave maximal induction. Apple cutin was much more effective than papaya cutin in inducing cutinase. Induction of the enzyme was very slow up to 72 h of growth, and there was a dramatic increase in the activity during the next 24 h with a subsequent decline (Fig. 1). Since cutinases from fungi and pollen are known to catalyze hydrolysis of *p*-nitrophenyl esters of short-chain fatty acids (2), the extracellular fluid was assayed with both labeled cutin and *p*-nitrophenyl butyrate as substrates; both assays gave similar patterns of induction (Fig. 1). Since the rate of growth of the organism in the medium containing a suspension of the insoluble cutin powder could not be readily measured, the time course of induction could not be compared with rate of growth. In any case, the extracellular fluid of 96-h-old cultures, which showed the maximal enzyme activity, was used for all subsequent experiments.

It has been suggested that small amounts of hydrolysate that would be generated by the trace amounts of polymer hydrolase released by starving fungal cells might be the real inducers of polymer hydrolases in fungi (9). To test whether such a mechanism might also operate in bacteria, cutin hydrolysate was included in the medium at 0.2 or 1.0 mg/ml. Under these conditions cutin hydrolysate failed to induce cutinase in the present bacterial cultures (Fig. 1).

Properties of cutinase generated by *P. putida*. Since the extracellular fluid was intensely colored and viscous but was low in protein concentration, a preliminary fractionation was done to obtain an enriched enzyme preparation. The enzyme activity was completely stable to lyophilization. The freeze-dried residue was dissolved in water (10% of the volume of the original extracellular fluid) and dialyzed against 0.02 M Tris hydrochloride, pH 8. The protein contained in the

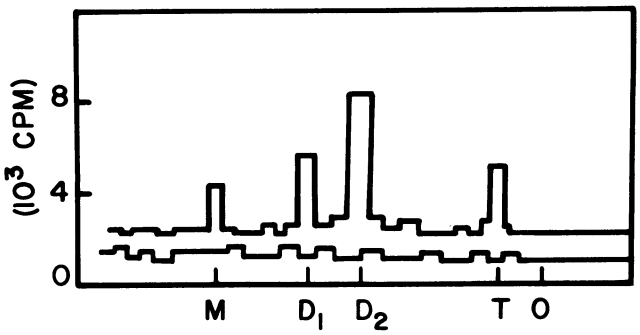

FIG. 3. Radio-thin-layer chromatography of reduced cutin monomers obtained by $LiAlH_4$ reduction of the soluble components enzymatically released from the labeled cutin (top) and a corresponding fraction obtained with a boiled enzyme control (bottom). Chromatography was done on 0.5-mm silica gel with ethyl ether-hexane-formic acid (8:2:1) as the developing solvent. O, Origin; T, 1,9,10,18-tetrahydroxyoctadecane; D_2, 1,7,16-trihydroxyhexadecane; D_1, 9,18-trihydroxyoctadecane; M, alkane-α,ω-diol.

viscous solution was precipitated by different concentrations of acetone, and cutinase activity of the recovered protein fractions was assayed. Excellent recovery (80%) of the cutinase activity was noted when the acetone content was 75%. The recovered protein was used as the source of cutinase activity in all subsequent experiments. The amount of radioactivity released by the enzyme preparation from labeled apple cutin increased with time of incubation (Fig. 2A). The rate was nearly rectilinear for up to 2 h and subsequently decreased. The rate of release of radioactivity increased with increasing protein concentration (Fig. 2B). The rate of cutin hydrolysis was very low and did not significantly change as the pH increased up to 7.0. Further increases up to pH 8 caused a dramatic increase in the rate of hydrolysis and further increases were obtained up to pH 9.0; higher pH values up to 11 showed only small additional changes in the rate.

Substrate specificity. The enzyme preparation did not catalyze hydrolysis of trioleylglycerol at detectable rates, suggesting that the observed hydrolysis of cutin was not catalyzed by a nonspecific lipase which could have been present in the extracellular fluid. The commonly used protease substrates benzoyl-L-tyrosine ethyl ester (for chymotrypsin), orcein-impregnated elastin (for elastase), and *p*-toluenesulfonyl L-arginine methyl ester (for trypsin) were not hydrolyzed, showing that the cutinase activity is not due to a proteolytic enzyme.

To determine whether the bacterial cutinase releases all types of cutin monomers or a selected class of monomers, the enzyme preparation was incubated with ^{14}C-labeled apple cutin in which all classes of monomers were known to be labeled (6). Thin-layer chromatographic analysis of the products showed that all classes of apple cutin monomers were released (Fig. 3), and the distribution of ^{14}C among the monomers released by the enzyme was indistinguishable from that observed in the chemical hydrolysate.

Chelators and inhibitors. The enzyme activity was not affected by either thiol-directed reagents or chelators. Active serine-directed reagents such as diisopropylfluorophosphate and *O,O*-diethyl-*O*-(3,5,6-trichloro)-2-pyridyl-phosphorothioate, an agent known to be a potent inhibitor of fungal cutinase (7), severely inhibited this bacterial enzyme at low concentrations (Table 3). Phenyl boronic acid, the transition-state analog inhibitor of active serine-containing enzymes, also severely inhibited the bacterial cutinase.

Temperature stability. The stability of cutinase to heat was measured by incubating the extracellular fluid at different temperatures for different periods, and the remaining enzymatic activity was measured at 30°C. The enzyme was completely stable at 60°C for 1 h and retained 85% of the activity after 1 h at 70°C. On the other hand, the four fungal cutinases were unstable above 45°C and lost more than 80% of the activity in 1 h at 60°C (Fig. 4).

TABLE 3. Effect of various inhibitors on cutinase produced by *P. putida*

Inhibitor(s)	Concn (mM)	Inhibition (%)
Hydroxymercuribenzoate, *N*-ethylmaleimide, iodoacetamide	5	0
Diisopropylfluorophosphate	0.025	100
	0.050	100
Mercuric chloride	0.50	0
Chelators (EDTA or 8-hydroxy quinoline)	5.0	0
Divalent metal ions	2.0	0
Phenyl boronic acid	5	63
	10	83
Sodium dodecyl sulfate	2.0	100
O,O-Diethyl-*O*-(3,5,6-trichloro)-2-pyridyl-phosphorothioate	1	92

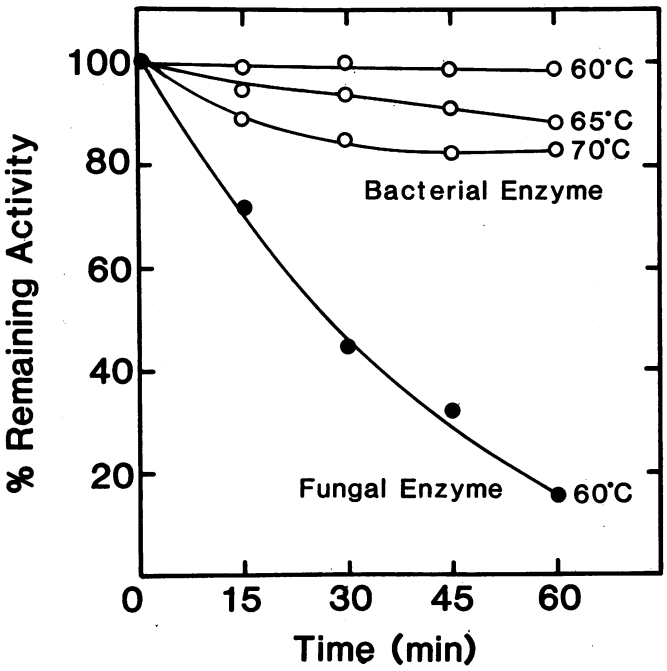

FIG. 4. Effect of temperature on cutinase stability. Aliquots (1 ml) of the extracellular fluid were incubated at different temperatures from 30 to 80°C for various times. Enzyme activity was determined as described in the text.

DISCUSSION

It has been suggested that small amounts of hydrolysate that would be generated by the trace amounts of cutinase released by starving cells might be real inducers of the enzyme in fungi (9). In fact, a low level of chemically prepared cutin hydrolysate was shown to induce cutinase production in glucose-grown fungi (9). However, cutin hydrolysate was not an effective inducer of cutinase in the present bacterial system. In the fungal system the induction of cutinase was shown to be repressed by glucose (9). In contrast, the bacterial induction of cutinase was not repressed by the addition of glucose after 72 h of growth. This lack of catabolite repression and lack of induction of the enzyme by cutin hydrolysate suggest that the mechanism of the induction in the bacterial system is different from that observed in the fungal systems.

The properties of the present bacterial enzyme preparation resemble those of fungal cutinases. The pH dependence of cutin hydrolysis is quite similar to that observed with fungal cutinases (15) but quite different from that of pollen cutinase, which showed maximal activity at pH 6.5 (12). Bacterial cutinase was inhibited by active serine-directed reagents such as diisopropylfluorophosphate and other organic phosphates. But it was not inhibited by thiol-directed reagents such as iodoacetamide or *p*-hydroxymercury benzoate. It was also not affected by metal ion chelators or divalent metal ions. In this respect it resembles the fungal

cutinases (3, 15) and differs from pollen cutinase, which has a thiol group at the active site (12).

The function of the present bacterial cutinase is quite different from those of fungal and plant cutinases previously reported. The proposed role of the pollen cutinase (12) in self-incompatibility involves regulation of penetration of stigma cuticle by the pollen tube. Fungal phytopathogens use cutinase to penetrate the cuticular barrier during infection (4). The only previously studied bacterial cutinase was that produced by *Streptomyces scabies* (10). Since it has been shown that cutinase hydrolyzes the polyester domains of suberin (1), it seems likely that the major function of the enzyme from *S. scabies* is to break down the suberized periderm layer that protects potato tuber, a natural host of this pathogen. The function of the present bacterial cutinase represents an interesting example of a mutually beneficial interaction between two bacterial species on the plant surface. According to the experimental results presented, it is clear that the *Corynebacterium* sp. can provide reduced nitrogen required for growth of the *P. putida* strain, and the latter can provide a carbon source from cutin for the growth of the former. Thus, both can survive together with the plant cuticular polymer as the sole source of carbon without any exogenous source for reduced nitrogen. Presumably, the two survive on the leaf surface in this manner, although other nutrients such as plant exudates and waxes might also provide nutrients. The present case is reminiscent of the recently reported associative cellulolysis and dinitrogen fixation by cocultures of *Trichoderma horzianum* and *Clostridium butyricum* (19) and cellulolytic nitrogen-fixing bacterium from the glands of Deshyaes in shipworms (20). An additional feature of the phyllospheric system is that the *Corynebacterium* sp. presumably provides nitrogen to the host plant as well as the associative microbial partner. How much reduced nitrogen is provided by the phyllospheric bacteria to the plant under field conditions is not known. The demonstration that the bacterial culture sprayed on the plants can significantly increase the yield of wheat which received no nitrogen fertilizer (13) suggests that the contribution of reduced nitrogen from the phyllospheric bacteria might be significant under tropical conditions.

One of the unique features of the present bacterial cutinase is its unusual temperature stability. Even at 70°C this bacterial enzyme is quite stable, whereas all fungal enzymes are unstable above 45°C. This stability might be advantageous or even necessary for the function of the enzyme because in a tropical climate the enzyme must function on the plant surface. Thus, the observed temperature stability of the enzyme generated under our experimental conditions supports the hypothesis that this enzyme functions in the field, as suggested above.

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