

Antifungal, Synergistic Interaction Between Chitinolytic Enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*

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ABSTRACT

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Biocontrol strains from the genera *Enterobacter* and *Pseudomonas* and two chitinolytic enzymes from *Trichoderma harzianum* isolate P1 were combined and tested for antifungal activity in bioassays. Inhibitory effects on spore germination and germ tube elongation of *Botrytis cinerea*, *Fusarium solani*, and *Uncinula necator* were synergistically increased by mixing fungal enzymes and cells of *Enterobacter cloacae* but not of *Pseudomonas* spp. Culture filtrate of *E. cloacae* contained antifungal compounds and produced moderate levels of inhibition, either in plate assays or in bioassays conducted in potato-dextrose broth. However, the combination

of bacterial culture filtrate with fungal chitinolytic enzymes generated only an additive response, indicating that the presence of bacterial cells was required for a synergistic effect. Chitinolytic enzyme activity in the presence of chitinous substrates enhanced the growth of *E. cloacae* and readily restored the ability of bacterial cells to bind to hyphae of the pathogens despite high concentrations of D-glucose or sucrose in the medium. The results of this study suggest that transgenic bacteria, capable of binding to fungal cell walls and expressing fungal genes encoding cell wall-degrading enzymes, may be powerful biocontrol agents.

Additional keywords: bacterial adhesion, fungitoxic compounds.

Efficacy in biocontrol of plant pathogenic fungi may be increased by combining different organisms, metabolites, or genes to provide more effective control than individual components can provide. *Enterobacter cloacae* is a frequent organism in the spermosphere and rhizosphere of plants. It is rhizosphere competent and binds to fungal cell walls (4,7,13,15,18,21,27). Bacteria in the species *Pseudomonas putida* and *P. fluorescens* include strains able to promote plant growth and suppress plant pathogens through a variety of mechanisms, including production of siderophores, antibiotics, and hydrogen cyanide (1,2,26). Imperfect fungi in the genus *Trichoderma* have a substantial ability to suppress a wide range of plant pathogenic fungi by various mechanisms, including the production of cell wall-degrading enzymes (5). Chitinolytic enzymes from *T. harzianum* Rifai and the closely related fungus *Gliocladium virens* J.H. Miller, J.E. Giddens, & A.A. Foster act synergistically to inhibit the growth of a variety of plant pathogenic fungi at low protein concentrations (16).

It is conceivable that bacterial biocontrol strains may act synergistically with chitinolytic enzymes to inhibit plant pathogenic fungi. Chitinolytic enzymes may release nutrients from hyphae of target fungi. Bacterial biocontrol agents could utilize these nutrients for proliferation, and the subsequent increase in bacterial populations should enhance the ability of these bacteria to act as biocontrol agents. Synergism would be most likely to occur if the biocontrol bacteria bind to the target hyphae, because the bacteria would be in a perfect position to utilize nutrients released by the chitinolytic enzymes.

The purpose of this study was to determine whether combinations of chitinolytic enzymes and bacterial biocontrol agents

act synergistically to inhibit plant pathogenic fungi. We demonstrate that there is strong synergism between *E. cloacae* and chitinolytic enzymes and suggest strategies for using this combination for effective suppression of plant disease. Effects of chitinase enzyme activity on both bacterial growth and attachment of bacterial cells to the hyphal walls of target fungi also are described.

MATERIALS AND METHODS

Strains and media. Antifungal activities of chitinolytic enzymes or bacterial strains were assayed with an isolate of *Botrytis cinerea* Pers.:Fr. isolated from grapes, an isolate of *Fusarium solani* (Mart.) Sacc. isolated from peas, and an isolate of *Uncinula necator* (Schwein.) Burrill isolated from grapes. In addition, *Pythium ultimum* Trow isolate P4, described elsewhere (9), and *Rhizoctonia solani* Kühn isolate R2, isolated from soil, also were used in inhibition assays on agar plates to detect fungitoxic metabolites in filtrates from bacterial cultures. Conidia of *B. cinerea* and *F. solani* were produced from cultures grown at 20–25 C on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI), whereas spores of *U. necator* were produced on grapes grown aseptically in tissue culture. Conidia were suspended in water (0.4 osmol mannitol), filtered through sterile Kimwipes (Kimberly-Clark, Roswell, GA) to remove hyphal fragments, and adjusted to 10^5 – 10^6 propagules ml^{-1} for use in bioassays.

T. harzianum isolate P1 (ATCC 74058), which suppresses *B. cinerea* (23) and other fungi in vivo (G. E. Harman and G. Nash, unpublished data), was used to produce chitinolytic enzymes. Conidia from this isolate were obtained from cultures grown on PDA and harvested after 7 days by washing the agar surface with sterile water.

E. cloacae strain E6 (ATCC 39978), *P. fluorescens* strain TL-1, and *P. putida* strain BK-1 (3) were used as biocontrol bacteria;

all suppress a number of plant diseases or increase plant growth (3,7,18,21,27). Bacterial cells were grown in either potato-dextrose broth (PDB; Difco Laboratories) for *E. cloacae* or King's Medium B broth (3) for *Pseudomonas* spp., at 28–30 C on a rotary shaker at 200 rpm to mid-log phase ($OD_{600} = 0.4-0.6$), harvested by centrifugation, washed in 0.85% NaCl solution for *E. cloacae* or sterile water for *Pseudomonas* spp., and resuspended in sterile water before use in bioassays.

Enzyme assay and production. Enzyme nomenclature and assays used in this study have been described elsewhere (8). The endochitinase and 40-kDa chitin 1,4- β -chitobiosidase (hereafter designated chitobiosidase) from *T. harzianum* isolate P1 tested in this study are potent inhibitors of spore germination and germ tube elongation of several chitin-containing fungi, and their combined biological activities are strongly synergistic (16). The two enzymes were produced and purified as reported by Harman et al (8). Briefly, *T. harzianum* isolate P1 was grown for 5 days in Richard's medium modified as previously described (8). The culture filtrate obtained by centrifugation and filtration was fractionated by gel filtration chromatography, followed by chromatofocusing and, if necessary, isoelectricfocusing in a Rotofor cell (BioRad, Richmond, CA) to provide electrophoretically pure proteins (8). Protein concentration in the enzyme preparations was determined by the Micro BCA protein assay (Pierce, Rockford, IL) with trypsin inhibitor from soybean (Sigma Chemical Company, St. Louis, MO) as the standard protein. Enzyme solutions were kept at 4 C and were utilized for bioassays within 2 wk. Otherwise they were concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY) and stored at -20 C until use.

The culture filtrate of *E. cloacae* was tested for endochitinase, chitobiosidase, *N*-acetyl- β -glucosaminidase (hereafter designated NAGase; EC 3.2.1.30), and glucan 1,3- β -glucosidase (hereafter designated glucosidase; EC 3.2.1.58) activity, because the antifungal activity of *E. cloacae* was enhanced by the addition of chitinolytic enzymes from *T. harzianum*. The bacteria were grown for 3 days at 28–30 C until mid- to late-log phase ($OD_{600} = 0.7-1.0$) in 250-ml Erlenmeyer flasks containing 100 ml of a synthetic medium (SMCS) with colloidal chitin and sucrose or only sucrose (SMS) as carbon sources. SMCS contained 680 mg of KH_2PO_4 , 870 mg of K_2HPO_4 , 200 mg of KCl, 1 g of NH_4NO_3 , 200 mg of $CaCl_2$, 200 mg of $MgSO_4 \cdot 7 H_2O$, 2 mg of $FeSO_4$, 2 mg of $ZnSO_4$, 2 mg of $MnSO_4$, 42 g of moist, colloidal chitin purified as described by Vessey and Pegg (25), 5 g of sucrose, and 1 L of distilled water (final pH 6.0). The culture filtrate was harvested by centrifugation and filtered through a 0.45- μ m filter. It was dialyzed against distilled water overnight at 4 C, concentrated by dialysis (6- to 8-kDa cutoff) about 20- to 25-fold (6) with polyethylene glycol (35,000 MW; Fluka Chemika-Biochemika, Buchs, Switzerland), and assayed for enzyme activity (8).

Antifungal bioassay and effect of chitinolytic activity on bacterial populations. The *in vitro* antifungal bioassay described by Lorito et al (16) was used, with some variations, with *B. cinerea*, *F. solani*, and *U. necator* as the test fungi. A solution or suspension to be tested was prepared that contained either 1) a single pure enzyme, 2) a 1:1 mixture of the two chitinases, 3) bacterial cells from a single strain, or 4) any combination of chitinases with a single bacterial strain. The solutions or suspensions that contained enzymes were made at 3 \times the desired concentration of the protein in the bioassay, whereas the solutions or suspensions that contained bacteria had $1.5-2.5 \times 10^4$ bacterial cells ml^{-1} . In addition, a suspension of 10^6-10^7 conidia ml^{-1} of the test fungus and a 3 \times PDB solution were prepared. Finally, 20 μ l of the solution or suspension to be tested (sterile water for the control), 20 μ l of the conidia suspension, and 20 μ l of broth were mixed in a sterile Eppendorf tube. The final reaction mixture had a pH of 5.0–5.5. To determine the effect of chitinolytic enzyme activity on bacterial growth, additional control samples containing PDB, bacteria, enzymes (singly or in combination), and water instead of the test fungus or containing only water, medium, and bacteria also were included. Tubes were incubated at 25 C and

after 24–30 h the percentage of germinating conidia was determined (percentage of the first 100 spores seen on a microscope slide), and the length of 20 germ tubes was measured and averaged. Data were transformed into values representing the treatments as a percentage of the control (in which % inhibition of control = 0) by the following equation: $\%I = (1 - \%S_t/\%S_c) \times 100$, in which %I represented the percent inhibition, $\%S_t$ represented the percentage of spores germinating (or average of the length of 20 germ tubes) in the treatment of interest, and $\%S_c$ represented the percentage of spores germinating (or average of the length of 20 germ tubes) in the control. Density of bacterial cells not associated in clusters was quantified for each test sample by a Petroff-Hausser counting chamber (Thomas Scientific, Philadelphia, PA) and compared with controls. Cell counts per milliliter were transformed to a logarithmic scale for statistical analyses.

Formation of bacterial aggregates around the hyphae of the pathogen also was monitored and photographed. These observations were performed at the end of the incubation period as well as during the bioassay, with small aliquots of each reaction mixture for microscopic examination. In some experiments, sucrose at a final concentration of 10 or 100 mM was added to inhibit bacterial attachment (18).

The possibility that chitinolytic activity of the enzymes from *T. harzianum* may stimulate the growth of *E. cloacae* also was investigated in a different experiment. Two kinds of substrates were used: conidia of test fungi or moist, purified colloidal chitin (25). Sterile Eppendorf tubes containing 200 μ l of PDB cultures inoculated with 2×10^6 conidia of test fungi or 200 μ l of a 2% chitin suspension in PDB were incubated at 25 C. After 24 h, 10 μ l of endochitinase or chitobiosidase was added to the tubes at a final concentration of 50–100 μ g ml^{-1} . Reaction mixtures had a pH of 5.0–5.5. Control samples contained PDB, substrate, and sterile water instead of enzyme or PDB and enzyme and sterile water instead of substrate or only PDB. Tubes were reincubated at 25 C. After 24 h, all samples were centrifuged, and the supernatant was recovered and filtered through a 0.45- μ m polysulfone filter (Whatman, Maidstone, England) to remove fungal tissues or chitin in suspension (where present). One hundred microliters of the resulting filtrate was placed in a well of a sterile ELISA (enzyme immunosorbent assay) plate containing approximately 500 bacterial cells suspended in 10 μ l of sterile water. Samples were mixed, absorbance at 560 nm was determined, and the plate was incubated in a humid chamber at 30 C over moistened towels. Bacterial growth was monitored after 24, 29, and 48 h by measuring the absorbance at 560 nm and subtracting the initial value from the timed reading. Differences in bacterial cell density among samples also were evaluated microscopically by transferring small aliquots of each sample to a Petroff-Hausser counting chamber (Thomas Scientific).

Determination of production of fungitoxic compounds by *E. cloacae*, *P. fluorescens*, and *P. putida*. Inhibition assays on plates were carried out against *Pythium ultimum*, *R. solani*, *B. cinerea*, and *F. solani*. Bacterial strains were grown for 5 days at 28–30 C in 25 ml of PDB (King's B medium for *Pseudomonas* spp.) on a rotary shaker at 200 rpm. The media were adjusted to pH 6.8 for *E. cloacae* and to pH 7.4 for *Pseudomonas* spp. by adding 0.1 N HCl or 0.1 N NaOH. The culture filtrate was collected by centrifugation, sterilized by filtration through a 0.45- μ m filter, mixed with PDA at 50 C (1:4 ratio), and poured into petri dishes. Control plates contained culture filtrate that had not been inoculated with bacteria. The medium was allowed to solidify, was inoculated in the center with a plug of mycelium from different test fungi, and was incubated at 25 C, and radial growth was recorded daily. Results were expressed as percent inhibition of radial growth of the test fungus compared to the control plates.

To determine if the synergistic interaction observed between *E. cloacae* and fungal enzymes required the presence of bacterial cells in the bioassay described above, crude culture filtrate from *E. cloacae* also was tested for its ability to inhibit spore germination of *B. cinerea*, *F. solani*, and *U. necator*, either alone or in combination with chitinases.

Analysis of data. Each experiment was repeated on two separate

days and contained three treatment repetitions each time. Separate analyses of variance (ANOVA) for each experiment indicated the experimental errors did not differ significantly; therefore, the results were combined and analyzed by standard procedures for a factorial design. The statistical significance of differences between means was determined by the *t* test (LSD) for pairwise comparisons. Data are presented as means \pm SE (standard error). Limpel's formula as described by Richer (19) was used to determine antifungal synergistic interactions between chitinolytic enzymes and bacteria or enzymes and culture filtrate from *E. cloacae*. Limpel's formula is $E_e = X + Y - (XY/100)$, in which E_e is the expected effect from additive responses of two inhibitory agents and X and Y are the percentages of inhibition relative to each agent used alone. Thus, if the combination of the two agents produces any value of inhibition greater than E_e , then synergism exists. To determine the presence of synergism, either enzyme, initial inoculum of bacteria, or culture filtrate from *E. cloacae* was used at ED₂₀-ED₃₀ levels of inhibition of spore germination. For this purpose, different amounts of bacterial inoculum or culture filtrate were previously tested, and ED₂₀ values for the enzymes were obtained by polynomial regression analysis of dosage response curves (16). To obtain ED₂₀-ED₃₀ levels of

inhibition, different protein concentrations were used. Ten to 45, 78-50, and 2.5-11 $\mu\text{g ml}^{-1}$, were used for endochitinase, chitobiosidase, and a 1:1 mixture of endochitinase and chitobiosidase, respectively. The treatments were performed simultaneously, and each was considered a single experiment.

RESULTS

Antifungal bioassay in liquid and solid medium. Cells of *E. cloacae* strain E6 inhibited spore germination and germ tube elongation of *B. cinerea*, *F. solani*, and *U. necator* (Fig. 1A and B). In bioassays, the addition of 250-500 bacterial cells produced approximately 30% inhibition of spore germination and 40-45% inhibition of germ tube elongation of test fungi. Moreover, increasing or reducing the initial concentration of *E. cloacae* proportionally affected the level of inhibition, although it was never higher than 65% even if 1×10^6 cells ml^{-1} were added to reaction mixtures (data not shown). Chitinolytic enzymes from *T. harzianum* had ED₂₀-ED₃₀ values varying between 25 and 75 $\mu\text{g ml}^{-1}$ for single enzymes and between 8 and 16 $\mu\text{g ml}^{-1}$ for a 1:1 mixture of endochitinase and chitobiosidase, depending on the test fungus. When a single chitinolytic enzyme or a mixture

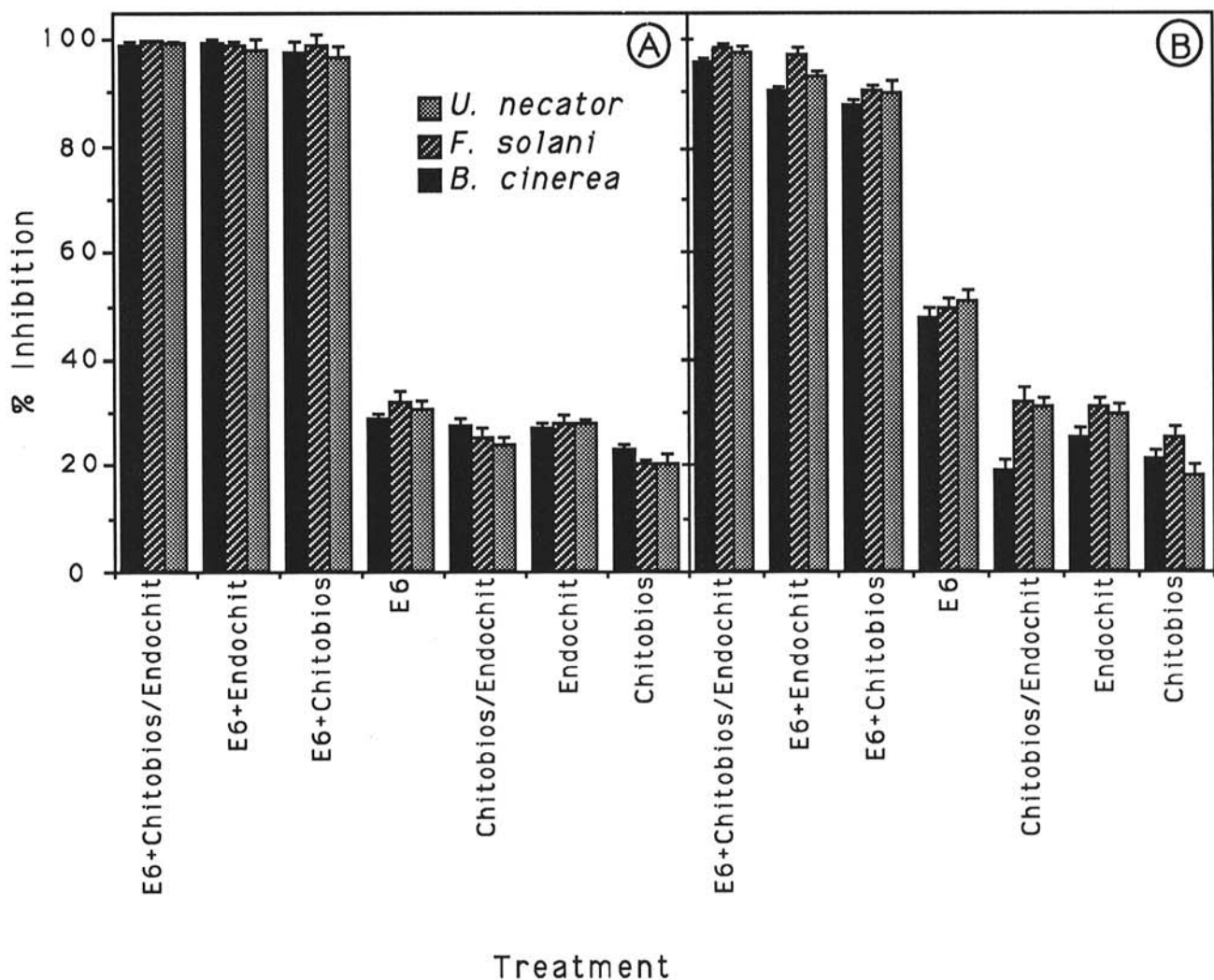


Fig. 1. Effect of *Enterobacter cloacae* strain E6 and chitinases from *Trichoderma harzianum* on **A**, conidial germination and **B**, germ tube elongation of *Botrytis cinerea*, *Fusarium solani*, and *Uncinula necator*. Chitobios = chitobiosidase; Endochit = endochitinase; Chitobios/Endochit = 1:1 mixture of endochitinase and chitobiosidase; E6 = *E. cloacae* strain E6. The enzyme solutions were used at concentrations producing 20-30% inhibition of spore germination. Enzyme concentrations for *U. necator*, *F. solani*, and *B. cinerea*, respectively, were chitobiosidase: 50, 75, and 50 $\mu\text{g ml}^{-1}$; endochitinase: 20, 50, and 25 $\mu\text{g ml}^{-1}$; and 1:1 mixture of chitobiosidase and endochitinase: 9, 25, and 8 $\mu\text{g ml}^{-1}$. Initial bacterial inoculum was 250-500 cells. The bioassay, performed as described by Lorito et al (21), data transformation, and treatments are described in the text. The treatments indicated in each figure were performed simultaneously, and each was considered a single experiment. Separate analyses of variance (ANOVA) of each experiment indicated that the experimental errors did not differ significantly; therefore, the results were combined and analyzed by standard procedures for a factorial design. The statistical significance of differences between means was determined by the *t* test (LSD) for pairwise comparisons. Data are presented as means \pm SE.

of enzymes was combined with cells of *E. cloacae*, levels of inhibition close to 100% were detected for both spore germination and germ tube elongation of all the fungi tested (Fig. 1A and B). After statistical analyses of the data, significant differences were observed in percent inhibition of spore germination with the presence or absence of *E. cloacae*, the four enzyme treatments, as well as the bacteria-enzyme interaction ($P < 0.001$). However, no differences were found among the three fungal isolates, the fungi-bacteria, fungi-enzyme, or the fungi-bacteria-enzyme interactions ($P > 0.05$). In the enzyme treatment, endochitinase and the endochitinase plus chitobiosidase combination were significantly different in comparison to the chitobiosidase alone and to the control treatment ($P > 0.05$). In contrast, the percent inhibition of hyphal elongation showed significant differences among all three fungal isolates, the presence or absence of *E. cloacae*, the four enzyme treatments, as well as the fungi-enzyme, bacteria-enzyme, and fungi-bacteria-enzyme interactions ($P < 0.001$). The fungi that showed inhibition of hyphal growth, from the greatest to the least, were *Fusarium* > *Uncinula* > *Botrytis*. In the enzyme treatment, endochitinase and the endochitinase plus chitobiosidase combination were significantly different in comparison to the chitobiosidase alone and the control treatment ($P > 0.05$). The antifungal interaction detected when a single chitinolytic enzyme or a mixture of enzymes was combined with cells of *E. cloacae* was synergistic in nature. In all cases, the combination of enzymes and *E. cloacae* produced values of inhibition much higher than the expected additive response (E_e), determined by calculating the E_e values of Limpel's formula. For instance, the E_e value for percent inhibition of spore germination of *U. necator* in the presence of *E. cloacae* and the mixture of enzymes was $30.5 + 24 - [(30.5 \times 24)/100] = 47.18$, whereas the observed value of inhibition was 99%.

P. putida strain BK-1 and *P. fluorescens* strain TL-3 did not produce more than 10% inhibition of the spore germination or reduce germ tube elongation for any of the fungi tested, even if the concentration of inoculum was as high as 10^5 cells ml^{-1} (data not shown). Moreover, the addition of cells of *Pseudomonas* spp. to samples containing any combination of enzymes from *T. harzianum* did not increase the level of inhibition for any fungus tested (data not shown).

E. cloacae strain E6 accumulated fungitoxic compounds in liquid medium when grown for 5 days in PDB. The culture filtrate from this bacterium produced moderate levels of inhibition against different fungi, either when incorporated into solid medium or when mixed with PDB (Table 1). However, no synergistic effect occurred with a combination of culture filtrate from *E. cloacae* and any chitinase from *T. harzianum*. For example, 20 μl of culture filtrate and 25 μg of endochitinase ml^{-1} resulted in values of inhibition (for spore germination of *B. cinerea*) of 21 and 28%

when used alone and of 38% when used in combination. In this case, according to Limpel's formula (19), the association of these components produced no synergism, only an additive response. The culture filtrate of *E. cloacae* contained a low level of endochitinase activity and no detectable chitobiosidase, NAGase, or glucosidase activity. Five-hundred microliters of dialyzed and concentrated culture filtrate reduced the turbidity of a suspension of colloidal chitin (8) by 28 or 14% if the bacteria were grown on inducing (SMCS) or noninducing (SMS) medium, respectively.

Crude culture filtrates of *P. fluorescens* or *P. putida* produced levels of fungal inhibition on agar plates, ranging between 5 and 15% (data not shown).

Effect of chitinolytic activity on bacterial cell density. After 24–30 h of incubation in the presence of any test fungus and any solution of chitinolytic enzymes, the cell densities of *P. putida* and *P. fluorescens* were similar to the controls. In addition, no cell clusters or adhesion of bacteria to the hyphae or the spores of test fungi was observed during the bioassay.

Cells of *E. cloacae* formed large aggregates around the hyphae (Fig. 2B, C, and D) and the spores of test fungi in the presence but not in the absence of chitinolytic enzymes (Fig. 2A), sometimes covering large areas of the hyphal surface (Fig. 2C). Adhesion of bacteria to test fungi was visible 4–5 h after the bioassay tests began and after 24 h, resulted in extensive destruction of the test fungus (Fig. 2D). Adhesion of bacteria to test fungi also occurred when sucrose was added to the mixture. After 24–30 h, the density of cells of *E. cloacae* not associated in clusters was approximately 10-fold higher in samples containing any test fungus in the presence of chitinolytic enzymes (from 1.2 to 1.4×10^6) than in controls without enzymes, in controls without fungus, or in control medium without enzyme and fungus (from 1.2 to 0.9×10^5). Statistical analysis showed that the proliferation of bacterial cells was not significantly enhanced in the absence of either the test fungus or the enzyme(s) added singly relative to the control, but the combination of enzyme(s) and fungus resulted in a significant increase in bacterial cell density compared to the control ($P < 0.001$). There was no significant difference in bacterial cell density among the three fungal isolates used. Observed results with the other fungi were similar.

Chitinolytic activity of enzymes produced by *T. harzianum* in the presence of a chitinous substrate stimulated the growth of *E. cloacae* (Fig. 3). Reaction mixtures containing moist, purified colloidal chitin or conidia of *B. cinerea*, *F. solani*, or *U. necator* were incubated in the presence or absence of endochitinase or chitobiosidase, and the resulting culture filtrates were inoculated with *E. cloacae*. After 29 h, the absorbance at 560 nm was much higher in samples containing a chitinous substrate incubated in the presence of chitinase compared to a control medium or to controls containing a substrate incubated without enzyme or an

TABLE 1. Inhibition of spore germination, germ tube elongation, and colony extension of different phytopathogenic fungi by culture filtrate^a from *Enterobacter cloacae* strain E6

Fungi	% Inhibition of colony extension ^b	% Inhibition of spore germination ^c	% Inhibition of germ tube elongation ^c
<i>Fusarium solani</i>	25.5 ± 1.5 ^d	22 ± 2.8	15.5 ± 4.2
<i>Botrytis cinerea</i>	22.5 ± 2	20 ± 3.5	19 ± 3.8
<i>Pythium ultimum</i>	30.5 ± 3.5	nd ^e	nd
<i>Rhizoctonia solani</i>	40 ± 3.5	nd	nd

^a *E. cloacae* strain E6 was grown for 5 days at 30 C in 25 ml of potato-dextrose broth (PDB), and the culture filtrate was collected by centrifugation and sterilized by filtration.

^b Inhibition assays for colony extension were conducted on potato-dextrose agar (PDA). Culture filtrate was mixed with PDA at 50 C (at 1:4 ratio) and was poured into petri dishes. Control plates contained culture medium that had not been inoculated with *E. cloacae*. Medium was allowed to solidify, was inoculated in the center with test fungi, and was incubated at 25 C. Radial growth was determined after 2 days, and the results were expressed as percent growth inhibition of the test fungus compared to the control plates.

^c Inhibition assays for spore germination and germ tube elongation were conducted in PDB as described by Lorito et al (21). Twenty microliters of culture filtrate from *E. cloacae* was mixed with 20 μl of conidia suspension of the test fungus (water for control) and 20 μl of 3× PDB. The reaction mixture was incubated at 25 C. After 24–30 h, percent spore germination and germ tube elongation were microscopically determined and compared with the control to obtain percentages of inhibition.

^d Each value represents the mean across two experiments with three replicates per experiment. Values are followed by standard errors.

^e Not determined.

enzyme without substrate (Fig. 3). Differences in bacterial cell density between samples also were confirmed microscopically (data not shown). There was a significant difference in bacterial growth (Δ absorbance values) with the enzyme, substrate, and enzyme-substrate interaction ($P < 0.001$) as a result of chitinolytic

enzyme activity. All substrates differed significantly from one another ($P < 0.05$), except for *Botrytis* and *Uncinula*, which did not differ from each other ($P > 0.05$). The three enzyme treatments differed significantly from one another ($P < 0.05$) in their effect on bacterial growth: endochitinase $>$ chitobiosidase $>$ no enzyme.

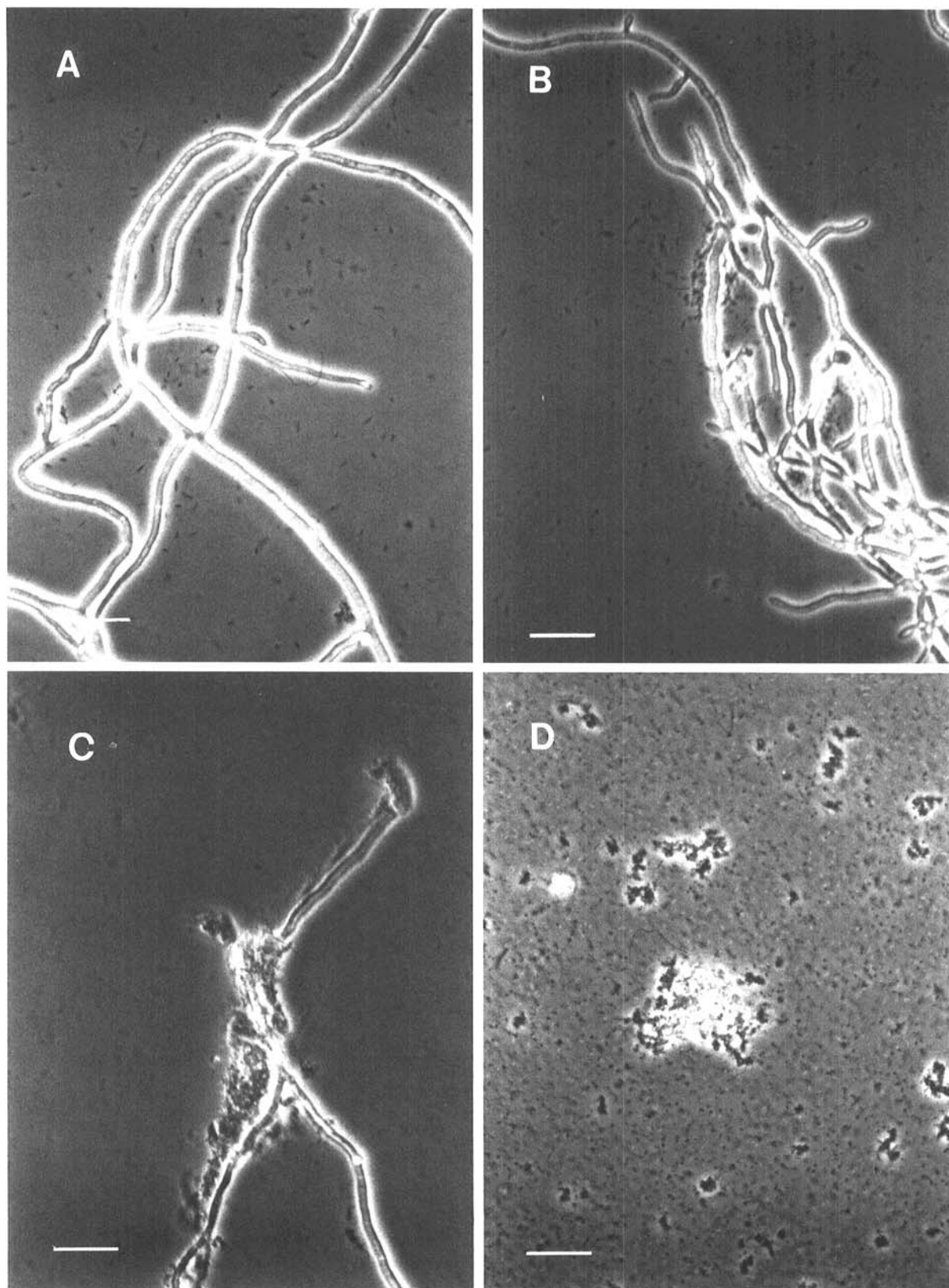


Fig. 2. Effect of $50 \mu\text{g ml}^{-1}$ of endochitinase from *Trichoderma harzianum* isolate P1 on the ability of cells of *Enterobacter cloacae* strain E6 to bind to the hyphal walls of *Botrytis cinerea*. Young hyphae of *B. cinerea* in potato-dextrose broth (PDB) + 100 mM sucrose inoculated with cells of *E. cloacae* in the absence of **A**, the enzyme and **B**, 3–4 h, **C**, 6 h, and **D**, 24 h after the addition of the endochitinase. Bars = $8 \mu\text{m}$.

The average value for the samples containing fungi without enzyme was significantly lower than the average of the controls containing only medium or only enzyme ($P < 0.001$).

DISCUSSION

In an early study, neither antibiotics, toxins, nor cell wall-degrading enzymes were detected in a culture filtrate of *E. cloacae* strain E6 (18), although more recently volatile and nonvolatile antifungal metabolites were found in cultures grown on a glucose asparagine medium (24). Moreover, Howell et al (11) showed that ammonia was one of the volatile antifungal metabolites produced by *E. cloacae* strain E6-rif (ATCC 39979), a spontaneous rifampicin-resistant mutant of strain E6. The hydromaxate siderophore aerobactin also was produced by six strains of *E. cloacae* (12), although it did not appear to be involved in the inhibition of *Pythium ultimum* (24). In our experiment, the filtrate obtained from PDB cultures of *E. cloacae* strain E6 contained antifungal compounds able to produce moderate levels of inhibition of *B. cinerea*, *F. solani*, and *U. necator* at the quantities tested. However, the nature of the single compounds responsible for the inhibitory effect and their relative amounts in the culture

filtrate were not investigated. We found only a low level of endochitinase activity in a dialyzed, concentrated culture filtrate of *E. cloacae* strain E6, whereas chitobiosidase, NAGase, and glucosidase activities were not detectable. These results contrast the work of Roberts and Sheets (20), who found *N*-acetyl- β -glucosaminidase but not endochitinase or chitobiosidase activities in the culture supernatant of the same strain of *E. cloacae*. This discrepancy may be due to the differences in culture medium and in the enzyme assay.

Chitinolytic enzymes from *T. harzianum* are strong antifungal agents in vitro, especially when used in combination (16). The addition of these enzymes (or a combination of them) to a suspension of cells of *E. cloacae* synergistically increased the inhibitory effect of the mixture against all the fungi tested. This finding supports the results of Kowk et al (14) who presented evidence of synergism in vivo between *T. hamatum* and *E. cloacae*. Chitinolytic enzymes may have been involved in this interaction, although the nature and details of the synergistic interaction between bacteria and fungal enzymes were not reported. On the other hand, two biocontrol strains of the genus *Pseudomonas* were unable to form aggregates around the hyphae of pathogens or to synergistically interact with the enzymes from *T. harzianum*.

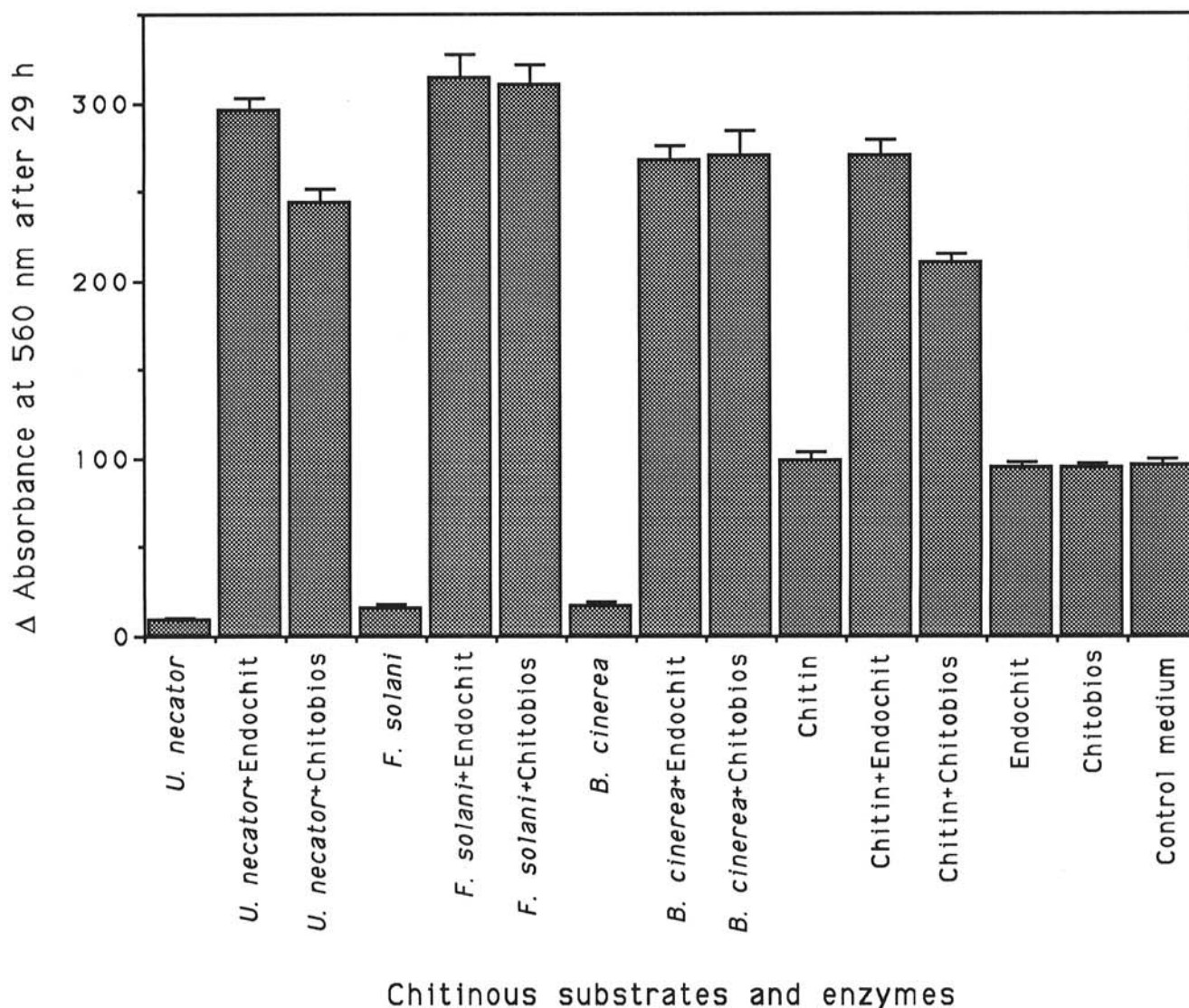


Fig. 3. Effect of chitinase activity on overall growth of *Enterobacter cloacae* strain E6. Chitobios = chitobiosidase $100 \mu\text{g ml}^{-1}$; Endochit = endochitinase $50 \mu\text{g ml}^{-1}$; Chitin = 2% suspension of colloidal chitin in potato-dextrose broth (PDB). Different chitinous substrates, including colloidal chitin or conidia of different fungi, were incubated at 25 C in the presence or absence of endochitinase or chitobiosidase from *Trichoderma harzianum* isolate P1. Treatments are described in the text. The treatments indicated in the figure were performed simultaneously, and each was considered a single experiment. Separate analyses of variance (ANOVA) of each experiment indicated the experimental errors did not differ significantly; therefore, the results were combined and analyzed by standard procedures for a factorial design. The statistical significance of differences between means was determined by the *t* test (LSD) for pairwise comparisons. Data are presented as means \pm SE.

These results may indicate that the ability to bind is required for a successful biocontrol interaction between bacterial cells and chitinolytic enzymes, although the correlation between these events requires further investigation. Moreover, the presence of bacterial cells per se also seemed critical for a synergistic effect, because the combination of culture filtrate from *E. cloacae* and chitinolytic enzymes from *T. harzianum* produced only an additive response.

Stasz and Harman (22) found that cells of *E. cloacae* contain agglutinins capable of binding to various components of fungal cell walls. The ability of *E. cloacae* to adhere to hyphae of *Pythium ultimum* has been associated with its biocontrol efficacy (7,18). Maloney and Nelson (17) suggested a genetic basis for this correlation, whereas other experiments have shown that mutants deficient in binding to carboxymethylcellulose retain their biocontrol ability (E. B. Nelson, *personal communication*). Nelson et al (18) also reported that some sugars such as D-glucose or sucrose inhibit both bacterial binding and antifungal activity, and, consequently, *E. cloacae* is able to protect only plant seeds with low sugar exudation. In our experiments, the presence of chitinolytic enzymes in the reaction mixture affected the population of *E. cloacae*, apparently, in two ways: 1) bacterial growth was enhanced and 2) the ability of bacterial cells to bind to the fungal hyphae was altered substantially. The increment in bacterial growth rate elicited by chitinous substrates incubated in the presence of chitinases suggests that hydrolytic enzyme activity may increase the quantity of nutrients in the reaction mixture. This would be accomplished by release of either chitin oligomers (and/or monomers), fungal cell contents, or both, with consequent proliferation of bacterial cells around the hyphae of test fungi. The adhesion of cells of *E. cloacae* strain E6 to fungal hyphae was, as expected, inhibited in a glucose-rich medium such as PDB. However, the ability to bind was enhanced by adding enzymes from *T. harzianum* even at concentrations as low as 8–16 $\mu\text{g ml}^{-1}$; this coincided with an increase of inhibitory activity. Similarly, the addition of 100 mM sucrose (final concentration) in reaction mixtures containing chitinolytic enzymes did not prevent either bacterial binding or the synergistic effect. These results suggest that cell wall-degrading enzymes may somehow remove sugar inhibition on receptor sites located on bacterial cells and also increase the number of sugar residues available for attachment of bacteria (18).

Secretion of chitinolytic enzymes is common among bacteria (6). However, fungal chitinases (and possibly the genes encoding them) appear to be more effective than are enzymes from other sources in their ability to inhibit pathogenic fungi (16). In addition, a high level of synergism between several systemic fungicides and the same enzymes used in this study and a β -1,3-glucosidase from *T. harzianum* has been observed (M. Lorito and G. E. Harman, *unpublished data*). Therefore, the development of transgenic bacterial strains able to produce fungal hydrolases may become very useful in managing economically important diseases such as *Pythium* damping-off (7,10,18), postharvest fruit spoilage (27), and other turf pathogens (E. B. Nelson, *personal communication*), either by reducing the incidence of the disease or by enhancing the effect of pesticides. For instance, the expression of *T. harzianum* chitinase genes and an efficient mechanism for chitinolytic enzyme secretion in *E. cloacae* strain E6 may increase the effectiveness of the strain and extend the ability of the bacterium to protect plant seeds, or other plant parts, regardless of the level of carbohydrates exuded during seed germination.

The proliferation of *E. cloacae* in a medium previously used for the growth of test fungi was significantly lower than in a control medium or in samples containing enzymes without fungi. This suggests that fungi may have released antibiotic metabolites into the medium or that fungi and bacteria will compete for nutrients. Regardless, the addition of cell wall-degrading enzymes greatly favored bacterial growth. It is conceivable that a similar, competitive situation may occur in the soil, rhizosphere, or sphere. In these environments, a beneficial, transgenic bacterial strain may take advantage of the ability to secrete fungal cell wall-degrading enzymes by both inhibiting the growth of antagonistic

fungi and using fungal cell walls as an additional carbon source.

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