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Chitinolytic *Enterobacter agglomerans* Antagonistic to Fungal Plant Pathogens

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Three *Enterobacter agglomerans* strains which produce and excrete proteins with chitinolytic activity were found while screening soil-borne bacteria antagonistic to fungal plant pathogens. The chitinolytic activity was induced when the strains were grown in the presence of colloidal chitin as the sole carbon source. It was quantitated by using assays with chromogenic *p*-nitrophenyl analogs of disaccharide, trisaccharide, and tetrasaccharide derivatives of *N*-acetylglucosamine. A set of three fluorescent substrates with a 4-methylumbelliferyl group linked by β-1,4 linkage to *N*-acetylglucosamine mono- or oligosaccharides were used to identify the chitinolytic activities of proteins which had been renatured following their separation by electrophoresis. This study provides the most complete evidence for the presence of a complex of chitinolytic enzymes in *Enterobacter* strains. Four enzymes were detected: two *N*-acetyl-β-D-glucosaminidases of 89 and 67 kDa, an endochitinase with an apparent molecular mass of 59 kDa, and a chitobiosidase of 50 kDa. The biocontrol ability of the chitinolytic strains was demonstrated under greenhouse conditions. The bacteria decreased the incidence of disease caused by *Rhizoctonia solani* in cotton by 64 to 86%. Two Tn*5* mutants of one of the isolates, which were deficient in chitinolytic activity, were unable to protect plants against the disease.

Various strains of *Enterobacter* spp. have been described as being effective biological control agents antagonistic to many fungal phytopathogens. Several isolates of *Enterobacter cloacae* are known to be biocontrol agents for different rots and preemergence damping-off of pea, beet, cotton, and cucumber plants incited by *Pythium* spp., as well as of *Fusarium* wilt of cucumber and some other plant diseases caused by fungal pathogens (7, 22, 35). *Enterobacter aerogenes* B8 significantly reduced infections of apple crown and root rot caused by *Phytophtora cactorum* (38). Some strains of *Enterobacter agglomerans* were shown to be efficient in the control of plant diseases caused by different bacterial and fungal pathogens (10, 22, 27). Various traits expressed synchronously or in a controlled sequence are considered responsible for the action of these strains as biological control agents (24). The ability to produce an antibiotic-like substance was found in strain *E. aerogenes* B8 (39). Strains of *E. cloacae* and *E. agglomerans* were found to produce hydroxamate siderophores (2, 16) and different volatile and nonvolatile antifungal metabolites (11, 37). Competition for nutrients and rhizosphere colonization ability were considered possible mechanisms of antifungal activity in *E. cloacae* (40) and *E. agglomerans* (10), respectively.

Although some strains of *Enterobacter* spp. suppress many fungal plant pathogens, data pertaining to the chitinolytic activity of these bacteria are still restricted to only one strain of *E. cloacae*, and these data are rather inconsistent (17, 29). It is well known, however, that chitin, an insoluble linear polymer of *N*-acetylglucosamine (GlcNAc) in a β-1,4 linkage, is a major structural component of most fungal cell walls, and that many species of bacteria, streptomycetes, actinomycetes, fungi, and plants produce chitinolytic enzymes (21, 32). Bacteria from the genera *Aeromonas* (12) and *Serratia* (26) and fungi from the genera *Gliocladium* (4) and *Trichoderma* (5), all of which produce chitinolytic enzymes, have been shown to be potential agents for the biological control of plant diseases caused by various phytopathogenic fungi. Evidence that these enzymes are responsible for that effect has been presented (18, 33). Attachment to fungal hyphae was considered an important mechanism in the biocontrol activity of *E. cloacae* strains against *Pythium ultimum* (23), and the stimulation of this binding by chitinolytic enzymes from *Trichoderma harzianum* was demonstrated (17).

In the present work, we found that three soil-borne strains of *E. agglomerans*, which are antagonists of many phytopathogenic fungi, possess strong chitinolytic activity. The strains were isolated in the course of a screening program for rhizospheric bacteria exhibiting a broad range of antagonism toward microbial phytopathogens (3). These strains were found to produce and excrete a complex of chitinolytic enzymes, which were resolved and partially characterized. The role of the chitinolytic enzymes in suppressing fungal phytopathogens was studied by using *Rhizoctonia solani* in cotton as a model, comparing natural isolates with mutants deficient in chitinolytic activity.

MATERIALS AND METHODS

Cultures and growth media. The three strains of bacteria used in this work (IC960, IC993, and IC1270) were independently isolated from the rhizosphere of different grape bushes in vineyards in the Samarkand region (Republic of Uzbekistan). The isolates were identified as *Enterobacter* (*Pantoea*) *agglomerans* by microbiological and biochemical tests and by gas chromatographic analysis of the whole-cell fatty acid methyl esters. The identification was performed independently in the Bacterial Division of the All-Russia Collection of Microorganisms (Puschino, Moscow Region, Russia) and in the Department of Plant Pathology, Auburn University. The strains were found to produce as yet unidentified antibiotic-like substances and to exhibit proteolytic activity (our unpublished observation). *Escherichia coli* S17-1 [*pro thi hsdR hsdM*$^+$ *recA* (RP4-2 Tc::Mu-Km::Tn*7*) inserted into the chromosome, Sm^r Rif^s] carrying the pSUP2021 plasmid [Ap^r Cm^r Tc^r Km^r (Tn*5*) ColE1 replicon *tra mob*$^+$], a suicide vector suitable for the introduction of transposon Tn*5* into most gram-negative bacteria (34), was used for transposon mutagenesis. Phytopathogenic fungi were from our and from the Moscow University (Russia) collections. Plasmid pMNU4, which is a derivative of plasmid pUC18 carrying the Tn*5* insertion in a

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fragment of *Rhizobium* DNA, was a gift from H. K. Mahanty (University of Canterbury, Canterbury, New Zealand). For bacterial growth, liquid or solid (1.5% [wt/vol] agar) Luria broth and agar, nutrient broth and agar (Difco), and minimal 925 medium (15) supplemented with 0.2% (wt/vol) glucose were used. To induce chitinolytic activity, bacteria were grown in liquid synthetic medium (SM) (20) with 0.2% (wt/vol) colloidal chitin prepared by the method of Rodriguez-Kabana et al. (31) by partial hydrolysis with 10 N HCl for 1.5 h at room temperature. The colloidal chitin was then washed several times with large volumes of tap water and then washed with distilled water for 5 to 7 days to adjust the pH. The mutants deficient in chitinolytic activity were grown on SM with chitin supplemented with 0.4% (wt/vol) casein enzymatic hydrolysate (Sigma). Potato dextrose agar (PDA; Difco) was used for cultivation of fungi.

Assay for antagonism to fungi. Bacteria were grown for 48 h in Luria or nutrient broth at 30°C with aeration. The suspension of cells was streaked in a line at the center of a PDA plate and incubated at 30°C for 24 to 48 h. Two 5-mm-diameter agar disks from an actively growing fungal culture were placed 3 to 4 cm away from each side of the bacterial growth area, and the plates were incubated at 30°C for 3 to 20 days, until mycelium growing from the two sides on a control plate (with a stretch of sterile water instead of bacteria down the middle) came into contact.

Detection of chitinolytic activity on plates. To test for chitinolytic activity on the plates, cells were streaked on semiminimal medium, i.e., a mixture of SM and nutrient broth (3:1) supplemented with colloidal chitin (0.2%) and solidified with 1.5% agar. The plates were incubated at 30°C for 72 to 96 h until zones of chitin clearing could be seen around the colonies.

Preparation of extracellular and intracellular proteins. Cells were grown in SM with 0.2% colloidal chitin as the sole carbon source or in minimal 925 medium with 0.2% glucose for up to 120 h at 30°C with aeration. Aliquots were removed at the indicated intervals. The cells were centrifuged, and the supernatant was filtered through 0.45-μm filters (Schleicher & Schuell). Intracellular proteins were extracted from cells with a French pressure cell press (Aminco) at 1,500 lb/in^2. Debris was separated by centrifugation, and the extracts were filtered as described above. In some experiments, leupeptin (final concentration, 2 μg/ml) and phenylmethylsulfonyl fluoride (PMSF; final concentration, 0.2 mM) (both from Sigma) were added as protease inhibitors. Filtrates containing extracellular or intracellular proteins were used for solution assays of chitinolytic enzymes. For analysis by gel electrophoresis, the filtrates were first dialyzed and concentrated in Micro-ProDiCon membranes (molecular weight cutoff, 25,000) against distilled water at 4°C in a Micro-P negative-pressure microprotein dialysis/concentrator (Bio-Molecular Dynamics).

Assays of chitinolytic activity in solutions. A chromogenic assay procedure was performed by the method of Roberts and Selitrennikoff (30) with modifications described previously (9, 12). The following chromogenic oligomers of *N*-acetyl-β-D-glucosamine (GlcNAc) were used as substrates: *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-GlcNAc), *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose [pNP-(GlcNAc)$_2$], and *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose [pNP-(GlcNAc)$_3$] (Sigma). The standard reaction mixture contained ca. 8 μg of the proteins tested in 0.1 M phosphate buffer, pH 6.5, and 25 μl of stock solution (1 to 2 mg/ml) of one of the three above-mentioned substrates. The reaction mixture was incubated at 40°C in a waterbath until a slight yellow-green color appeared. The reaction was terminated by adding an equal volume of 0.2 M Na_2CO_3. The release of the chromophore *p*-nitrophenol from the substrates was measured at 410 nm, and 1 U of enzymatic activity was defined as 1 μmol of pNP/μg of protein/h. Chitinolytic activity was also determined by the release of GlcNAc from chitin by the method of Reissig et al. (28). In this case, 1 U of chitinolytic activity was expressed as 1 μmol of GlcNAc/mg of protein/h. The production of GlcNAc was monitored at 544 nm. Endochitinase activity in culture filtrates was measured by the reduction of turbidity of a suspension of 1% colloidal chitin by a procedure described elsewhere (9). One unit of endochitinase activity was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5% (9).

Protein content was determined with the Bio-Rad protein assay reagent and bovine serum albumin as a standard.

Detection of chitinolytic enzymes after gel electrophoresis. Proteins concentrated in the Micro-ProDiCon system were prepared in Laemmli sample buffer (13) without 2-mercaptoethanol (except when specifically indicated) and incubated for 10 min at room temperature or for 3 min at 55°C prior to loading. Each lane contained about 8 μg of protein. The proteins were separated by sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE). Enzymes were reactivated in the gels by removing SDS by the casein-EDTA procedure (19) as modified by Haran et al. (8). Enzyme activity was detected on gels by using fluorescent substrates as described by Tronsmo and Harman (36). The chitinolytic enzymes appeared as fluorescent bands under UV light because of enzymatic hydrolysis of the fluorescent substance 4-methylumbelliferone from the *N*-acetylglucosamine mono- and oligosaccharides. The following substrates were used: 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc); 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside [4-MU-(GlcNAc)$_2$]; and 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose [4-MU-(GlcNAc)$_3$] (Sigma). These compounds served as analogs of disaccharide, trisaccharide, and tetrasaccharide chitin derivatives, respectively, with the 4-methylumbelliferyl group linked by β-1,4 linkage to the *N*-acetylglucosamine monosaccharide (in the case of 4-MU-GlcNAc) or oligosaccharides [in the case of 4-MU-(GlcNAc)$_2$ and 4-MU-(GlcNAc)$_3$]. The molecular weights of the renatured chitinases were estimated by using low-range prestained standards (Bio-Rad Laboratories), which included the following proteins: phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue G-250 prepared as described by Neuhoff et al. (25).

Tn*5* mutagenesis. A spontaneous mutant of strain IC1270, resistant to rifampicin, was used as a recipient in mating on membrane filters with *E. coli* S17-1 carrying the pSUP2021 plasmid by a procedure described previously (34). Kanamycin-resistant transconjugants occurred at a frequency of 10^{-5} per recipient cell. Among 3,750 clones of the transconjugants screened for deficiency in suppression of various phytopathogenic fungi in vitro, 6 were found to be unable to inhibit fungal growth. One of these, designated IC1270-E1, failed to hydrolyze colloidal chitin in the plate test. This mutant was used for further studies. Another 3,000 clones of the transconjugants were tested directly for deficiency in chitinolytic activity on plates. Four mutants were unable to form clearing zones on agar medium with colloidal chitin. Mutant IC1270-2h, which was deficient in chitinolytic activity but was still able to suppress growth of the fungi on plates, was chosen for further studies.

DNA manipulations. Extraction of plasmid pMNU4 DNA from *E. coli* by the alkaline lysis method, isolation of genomic DNA from strain IC1270 and its Tn*5* mutant, digestion of the DNA by restriction endonucleases, electrophoretic separation of the restriction fragments, isolation of the fragments from the agarose gel, and Southern hybridization were performed by standard procedures (1). The 3.3-kb *Hin*dIII fragment of pMNU4 was used as a DNA probe. Labeling of the fragment with α-^{32}P-labeled CTP (3,000 Ci/mmol; Amersham) was carried out by using the random prime DNA labeling kit (Boehringer, Mannheim, Germany). Restriction enzymes were purchased from Fermentas MBI and used under the conditions recommended by the manufacturer.

Greenhouse assays. *R. solani* damping-off of cotton (*Gossypium barbardense* L. "Pima") seedlings was chosen as a model to test the efficacy of *E. agglomerans* isolates as biocontrol agents, using a previously described procedure (5, 12). Polypropylene boxes were two-thirds filled with sandy loam soil (pH 7.2), and 10 seeds were placed in each box. A seed cover layer (one-third of the pot's depth) was infested with a preparation of *R. solani* and mixed with a water suspension of the bacteria being tested (ca. 10^8 cells per kg). Soil infested with the pathogen but not treated with bacteria served as a control. Each strain was tested in six replicates, and experiments carried out at 28 to 30°C were repeated five times. Disease incidence was determined after 12 to 14 days as the percentage of seedlings with root rot.

RESULTS

Antifungal activity. Isolates IC960, IC993, and IC1270 were found to suppress the growth of various fungal phytopathogens in vitro. The inhibition zones between pathogenic fungi and

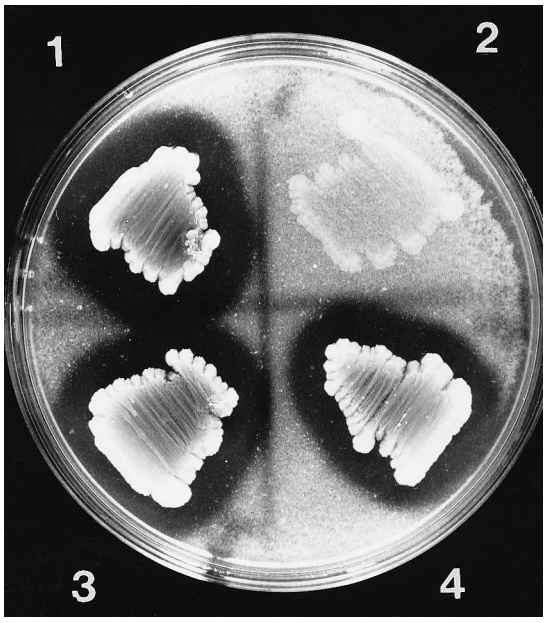

FIG. 1. Clearing zones of colloidal chitin formed by chitinases produced by *E. agglomerans*. Quadrants: 1, strain IC1270; 2, mutant IC1270-E1; 3, strain IC960; 4, strain IC993.

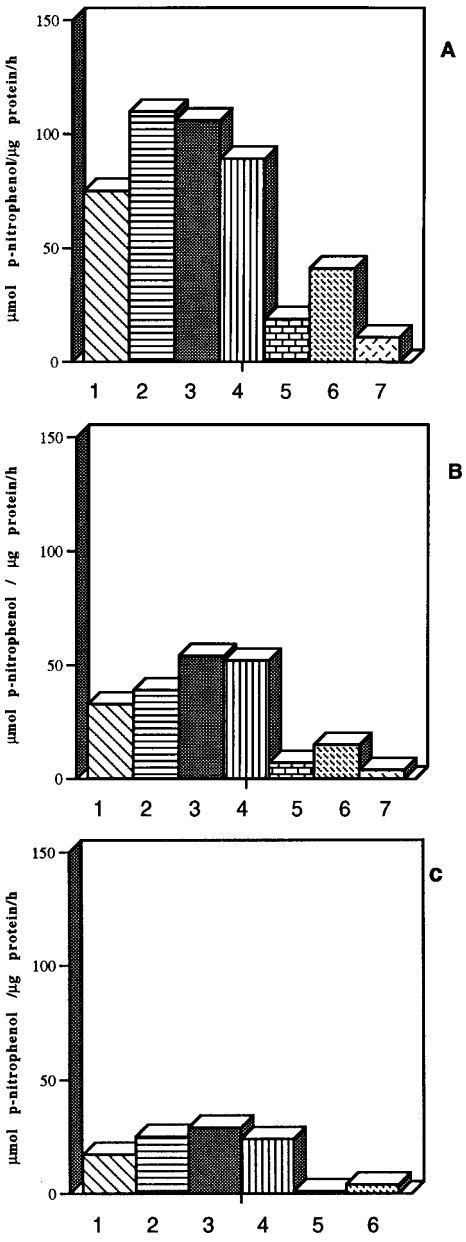

FIG. 2. Chitinolytic activity in extracellular and intracellular proteins of *E. agglomerans* strains grown for 60 h with chitin as the sole carbon source. Assays were done with (A) *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, (B) *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose, and (C) *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose. Columns 1 to 3, 5, and 7, chitinolytic activity in extracellular proteins of strains IC1270, IC960, IC993, and mutants IC1270-E1 and IC1270-2h, respectively. Columns 4 and 6, chitinolytic activity in intracellular proteins of strain IC1270 and its mutant IC1270-E1, respectively. Averages of 4 to 12 independent determinations are presented.

the strain tested were up to 20 mm for *Bipolaris sorokiniana*, *Botrytis cinerea*, *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. moniliforme* f. sp. *lactis*, *Sclerotium rolfsii*, and *Verticillium dahliae* and up to 30 mm for *F. oxysporum* f. *lycopersicum*, *F. oxysporum* f. sp. *meloni*, *F. oxysporum* f. sp. *vasinfectum*, *F. roseum*, and *R. solani*. No significant difference in the antifungal activity of the isolates was observed, but strain IC960 was unable to inhibit the growth of *B. sorokiniana* and only strain IC1270 was found to be suppressive for *Pythium* spp. (data not shown).

Chitinolytic activity. All three *E. agglomerans* strains hydrolyzed colloidal chitin after 72 to 96 h of growth on semiminimal agar (SM plus nutrient broth at a 3:1 ratio) supplemented with colloidal chitin as the sole carbon source. Large zones of clearing around the growing bacteria were observed. The Tn*5* mutant IC1270-E1 did not exhibit this activity (Fig. 1). Mutant IC1270-2h was also unable to form clearing zones (not shown).

These results suggested that chitinolytic enzymes may be secreted by the strains into the culture medium. To investigate this, bacteria were grown in SM with chitin for up to 120 h. At 24-h intervals, aliquots of cell cultures were taken, and the presence of chitinolytic enzymes in extra- and intracellular proteins was examined in reaction mixtures with colloidal chitin or chromogenic oligomers of GlcNAc. The chitinolytic activity in the culture fluid reached its maximum at about 60 h of cultivation (data not shown), and this time period was chosen for all later experiments presented here. The chromophore *p*-nitrophenol was released from all three substrates, but the level of activity was highest in the reaction with the pNP monosaccharide derivative (pNP-GlcNAc) (Fig. 2). As shown for strain IC1270 (Fig. 2, column 4), the activity was found not only extracellularly, but also in intracellular proteins of the strains, and it was even higher than in the secreted proteins. Similar results were obtained with the intracellular proteins of strains IC960 and IC993 (data not shown). In the colloidal chitin reaction, specific chitinolytic activity in the extracellular proteins varied between 2 and 5 U for all three strains.

A decreased level of chitinolytic activity was exhibited by proteins secreted by the IC1270-E1 and IC1270-2h mutants compared with their parent strain (Fig. 2, columns 5 and 7). Chitinolytic activity was also significantly lower in intracellular proteins of the E1 (Fig. 2, column 6) and 2h (data not shown) mutants. These results indicate that both mutants are deficient in chitinolytic enzyme production. The low level of chitinolytic activity in the secreted proteins could be a consequence of this deficiency.

When the chitin in the medium was replaced with glucose or sucrose, only one of the strains (IC960) showed constitutive chitinolytic activity, but this activity was very low, less than 10% of that exhibited in the presence of chitin (data not shown).

Extracellular proteins in culture filtrates of strains IC960, IC993, and IC1270 grown with chitin as the sole carbon source reduced the turbidity of colloidal chitin by about 50% (ca. 10 U of endochitinase activity).

Identification of chitinolytic enzymes. The chitinolytic activity of extracellular and intracellular proteins which had been renatured following their separation by SDS-PAGE was determined with a set of three fluorescent chitin derivatives. The chitinolytic enzymes appeared as fluorescent bands under UV light as a result of hydrolysis of the fluorescent substance 4-methylumbelliferone from the GlcNAc mono- and oligosaccharides (Fig. 3). Four distinct enzymes, designated according to their apparent molecular masses as CHIT89, CHIT67, CHIT59, and CHIT50, were detected in the culture medium of strains IC960 and IC993 growing on chitin. In strain IC1270, only CHIT89, CHIT67, and CHIT59 were found, with the activity of the 59-kDa enzyme being apparently lower than in the two other strains. This difference is probably not due to proteolysis, as preparations with and without protease inhibitors (leupeptin and PMSF) showed the same level of chitinolytic activity and similar chitinase profiles (data not shown). As shown in Fig. 3, CHIT89 and CHIT67 activities were detected by all three substrates, CHIT59 activity was detected with analogs of trimeric [4-MU-$(GlcNAc)_2$] and tetrameric [4-MU-

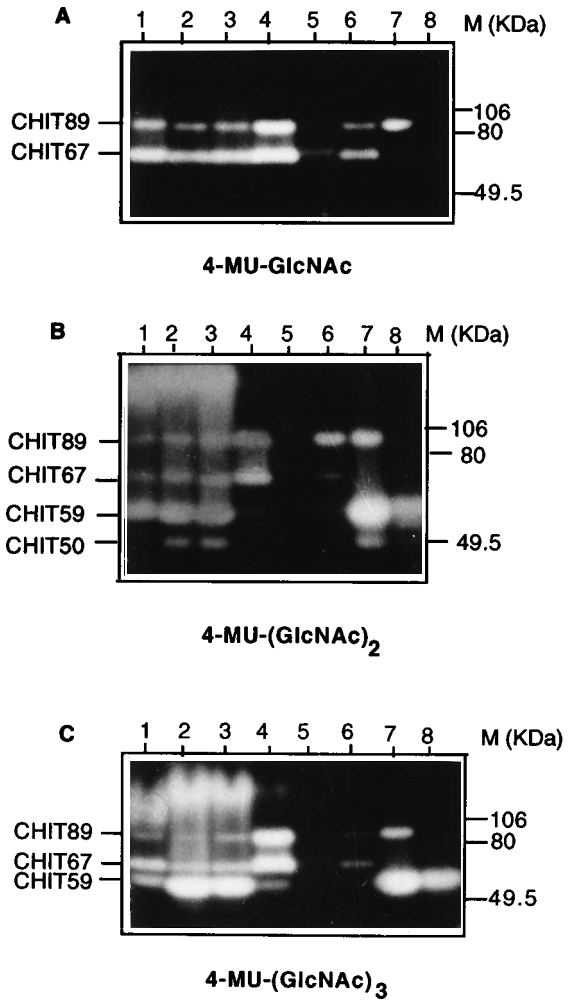

FIG. 3. Detection of chitinolytic activity of extracellular and intracellular proteins produced by *E. agglomerans* strains grown on SM with chitin after separation by SDS-PAGE. Chitinolytic activity was detected with the substrates (A) 4-MU-GlcNAc, (B) 4-MU-$(GlcNAc)_2$, and (C) 4-MU-$(GlcNAc)_3$. Lanes 1 to 3 and 5, extracellular proteins from strains IC1270, IC960, IC993, and mutant IC1270-E1, respectively. Lanes 4 and 6, intracellular proteins from strain IC1270 and its Tn*5* mutant IC1270-E1, respectively. Lanes 7 and 8, extracellular proteins of strain IC993 heated at 55°C in sample buffer, without and with 2-mercaptoethanol, respectively. Low-range prestained SDS-PAGE standards (Bio-Rad) were used as size markers (M).

$(GlcNAc)_3$] chito-oligosaccharides, and CHIT50 activity was revealed only with the trimeric analog.

The chitinolytic enzymes of strain IC993 were found to differ in their ability to be renatured by the casein-EDTA procedure following heat treatment. CHIT89 retained its activity after treatment at 55°C for 3 min but was completely or significantly inactivated (depending on the substrate) by heating in the presence of 2-mercaptoethanol. CHIT67 was irreversibly inactivated by heating with or without 2-mercaptoethanol. CHIT59 activity could be renatured regardless of the presence of 2-mercaptoethanol in the buffer, and this was observed even after boiling for 30 min. The activity of CHIT50 was rather resistant to heating (55°C, 3 min) with or without 2-mercaptoethanol (Fig. 3, lanes 7 and 8). Similar results were obtained with the corresponding enzymes from strains IC1270 and IC960 (data not shown).

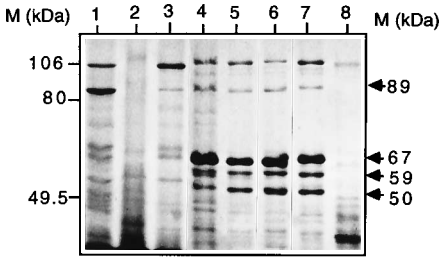

FIG. 4. Coomassie blue staining of extra- and intracellular proteins produced by *E. agglomerans* strains grown on SM with chitin (lanes 1 to 7) or glucose (lane 8) after separation by SDS-PAGE. All samples were heated for 3 min at 55°C in sample buffer without (lanes 1 to 6 and 8) or with (lane 7) 2-mercaptoethanol. Lanes 1 and 3, intracellular proteins of strain IC1270 and its Tn*5* mutant IC1270-E1, respectively. Lanes 2, 4, and 5, extracellular proteins of strains IC1270-E1, IC1270, and IC960, respectively. Lanes 6 to 8, extracellular proteins of strain IC993. The molecular masses of the marker proteins are shown on the left.

Some additional chitinolytic activity, with a molecular mass higher than 106 kDa, was observed in secreted proteins when the trimeric and tetrameric, but not the dimeric, chitin derivatives were used as substrates. However, the activity could not be found in intracellular proteins, and it disappeared following heating (Fig. 3B and C). It can therefore be assumed that this activity stemmed from the unresolved complex of the lower-molecular-mass chitinolytic enzymes.

The intracellular proteins of the Tn*5* mutant IC1270-E1 exhibited significantly less chitinolytic activity than those of the parent wild-type strain (Fig. 3, lanes 6 versus lanes 4), and little activity was found in the secreted proteins of the mutant (lane 5).

Bands corresponding to the above chitinolytic enzymes could be observed by SDS-PAGE after Coomassie blue staining of the proteins excreted by the strains grown in the presence of colloidal chitin. The 67-, 59-, and 50-kDa enzymes were the most abundant proteins secreted by strains IC960 and IC993, with an additional, weaker band corresponding to the 89-kDa protein (Fig. 4, lanes 5 and 6). These proteins were not visible when strain IC993 was grown on glucose instead of chitin (Fig. 4, lane 8). The same results were obtained for strains IC1270 and IC960 despite the ability of the latter strain to produce minor chitinolytic activity constitutively (data not shown). The 89-, 67-, and 59-kDa proteins were found in secreted fractions of strain IC1270 (Fig. 4, lane 4). These proteins correspond to the chitinolytic enzymes of the strain, but an additional abundant protein secreted by IC1270 (apparently 53 kDa) is probably different from CHIT50, as no activity of the enzyme was found in this strain (Fig. 3B, lane 1). Among the intracellular proteins of strain IC1270, a band coinciding with CHIT89 was among the most abundant, and CHIT67 could also be distinguished, whereas the 59-kDa band was only slightly visible (Fig. 4, lane 1). This result agrees with the data shown in Fig. 3B and C, lanes 4. None of these bands were seen in the secreted proteins of mutant IC1270-E1 (Fig. 4, lane 2), but intracellular proteins with molecular masses of 89 and 67 kDa were still visible as weak bands (Fig. 4, lane 3). The CHIT59 and CHIT50 enzymes, which retained some activity when heated in the presence of 2-mercaptoethanol (Fig. 3B and C, lanes 8), could be detected under these conditions by Coomassie staining (Fig. 4, lane 7). However, protein bands corresponding to CHIT89 and CHIT67, despite their inactivation by heating in the presence or absence of 2-mercaptoethanol, respectively (Fig. 3, lanes 7 and 8), could also be seen following staining (Fig. 4, lanes 4 to 8).

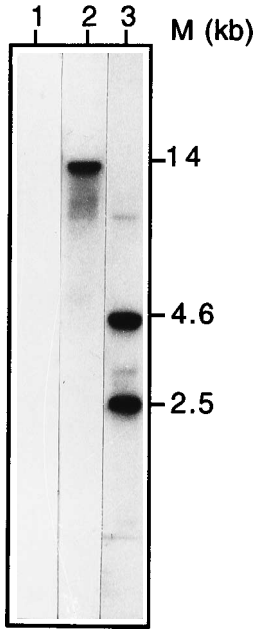

FIG. 5. Southern hybridization of Tn*5* insertions in a mutant of strain IC1270. Genomic DNA was digested with *Eco*RI (lanes 1 and 2) or *Bam*HI (lane 3) and probed with a 3.3-kb *Hin*dIII internal Tn*5* fragment of plasmid pMNU4. Lanes: 1, IC1270; 2 and 3, IC1270-E1. The molecular sizes of fragments are shown in kilobases.

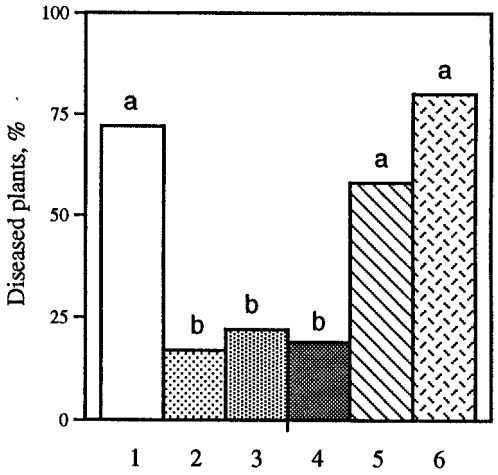

FIG. 6. Biocontrol activity of the *E. agglomerans* isolates against *R. solani* on cotton. Columns 1 to 6, control, isolates IC1270, IC960, and IC993, and the Tn*5* mutants IC1270-E1 and IC1270-2h, respectively. Data from five independent determinations were analyzed. The difference between columns headed by the same letter is not significant.

Analysis of the Tn*5* insertion. Southern hybridization of *Eco*RI- or *Bam*HI-digested DNA from strain IC1270 and its mutants demonstrated that in the mutant IC1270-E1, Tn*5* was inserted at only one site, since only one *Eco*RI fragment hybridized with the Tn*5* probe and only two bands of hybridization appeared after digestion of the mutant genomic DNA with *Bam*HI (Fig. 5). Tn*5* was inserted into the IC1270-2h mutant's genome at two sites, different from IC1270-E1 (data not shown).

Biocontrol activity. Use of the *E. agglomerans* strains to reduce the incidence of disease caused by *R. solani* in cotton revealed a suppressive effect (Fig. 6). When the bacteria were applied as a bacterial suspension mixed with the seed cover layer, the number of seedlings with root rot symptoms caused by the fungus decreased, and disease reduction varied between 64 and 86%, depending on the strain. The disease level was significantly lower than that of untreated plants ($P < 0.05$). IC1270-E1 and IC1270-2h, deficient in chitinolytic activity, were unable to serve as biocontrol agents. When mutant IC1270-E1, which lost both the antibiotic-like and proteolytic activities of the parent strain IC1270, was used, only 20% disease reduction was observed (Fig. 6, column 5), which was not significantly different ($P > 0.05$) from the untreated control (Fig. 6, column 1). In spite of the fact that IC1270-2h inhibited the growth of *R. solani* on plates, it did not protect cotton against this fungus in the greenhouse (Fig. 6, column 6).

DISCUSSION

Several strains of *Enterobacter* spp. which can suppress the growth of different pathogenic fungi have been described previously (7, 22, 27, 35, 38). With one exception, strain E6 of *E. cloacae* (17, 29), none of these strains were described as producing chitinolytic enzymes. However, there is a wealth of data supporting the important role of these hydrolytic enzymes, which degrade chitin, a major structural component of the cell wall of almost all fungi, in bacterium-fungus and fungus-fungus antagonisms (for reviews, see references 14 and 32).

The chitinolytic strains described here were selected primarily for their ability to suppress the growth of several phytopathogenic fungi in vitro, and no enrichment techniques were used to specifically select chitinolytic bacteria. Nevertheless, we found that three isolates, identified as *E. agglomerans*, exhibited strong chitinolytic activity, as determined by the formation of clearing zones on chitin agar, by the release of *p*-nitrophenol from the chromogenic chito-oligosaccharide analogs, and by the release of reducing GlcNAc from colloidal chitin. The level of specific chitinolytic activity in all the *Enterobacter* strains tested by the latter assay was very similar to that found for chitinolytic *Serratia marcescens* and *Aeromonas caviae* (12).

We used a set of fluorescent 4-MU glucosides of GlcNAc mono- and oligosaccharides as substrates to identify the chitinolytic activity of proteins renatured following their separation by SDS-PAGE. Two strains (IC960 and IC993) produced at least four chitinolytic enzymes, designated according to their apparent molecular masses as CHIT89, CHIT67, CHIT59, and CHIT50. The last enzyme was not detected in any preparations of the third strain, IC1270, and this strain's CHIT59 activity was apparently weaker than that of the other strains (Fig. 3). The enzymes differed in substrate specificity. CHIT89 and CHIT67 released fluorescent 4-MU from all three substrates. CHIT59 released 4-MU from trimeric [4-MU-$(GlcNAc)_2$] and tetrameric [4-MU-$(GlcNAc)_3$] chitin analogs, but not from the monosaccharide derivative 4-MU-GlcNAc. CHIT50 was detected only when [4-MU-$(GlcNAc)_2$] was used.

According to current practice (21, 32), the chitinolytic enzymes were divided into three principal types. Endochitinase (EC 3.2.1.14) is defined as an enzyme catalyzing the random hydrolysis of 1,4-β linkages of GlcNAc at internal sites over the entire length of the chitin microfibrils. Exochitinase, or chitibiosidase (9), catalyzes the release of diacetylchitobiose units from the chitin chains as the sole product. *N*-Acetyl-β-1,4-D-glucosaminidase (EC 3.2.1.30) is a chitinolytic enzyme which also acts in exo-splitting mode on diacetylchitobiose and higher analogs of chitin. The GlcNAc monomers are formed in

the course of the reaction. Based on this system of nomenclature, CHIT89 and CHIT67, which were able to produce 4-MU from dimeric, trimeric, and tetrameric chitin derivatives, are considered *N*-acetyl-β-1,4-D-glucosaminidases. CHIT59 produced 4-MU from the trimeric and tetrameric but not from the dimeric chito-oligosaccharide analogs, and thus we conclude that it is an endochitinase. The presence of endochitinase activity was further confirmed by the reduction in chitin turbidity caused by the culture filtrates. The level of this activity was found to be close to the level of an endochitinase from *Trichoderma harzianum* (9). CHIT50 only produced 4-MU from the trisaccharide analog 4-MU-$(GlcNAc)_2$, and consequently we defined it as a chitobiosidase. Coomassie blue staining of the proteins secreted into the culture medium supported our conclusion that these chitinolytic activities represent four different enzymes. The inactivation of CHIT89 by heating in the presence of 2-mercaptoethanol suggests the presence of sulfhydryl groups in the enzyme's active center.

Since this complex of chitinolytic enzymes has not yet been purified, it is difficult to consider other characteristics of the individual enzymes. However, the total chitinolytic activity was characterized by a relatively wide pH optimum (4.5 to 8.5) and stability within this pH range (data not shown). These characteristics can be advantageous for these strains for use as antifungal agents. Despite the very similar profiles of chitinolytic enzymes shown by isolates IC960 and IC993, they differed in their ability to produce the enzymes constitutively. The constitutive chitinolytic activity of strain IC960 was, however, much lower than the induced activity.

The Tn*5* mutant of strain IC1270 did not express any chitinolytic activity when grown on agar medium with chitin. Some activity was found in its extra- and intracellular proteins, but it was significantly lower than that of the parent strain. It could be suggested, therefore, that mutant IC1270-E1 is defective in chitinolytic enzyme production. The inability of the mutant to form clearing zones on chitin agar could be considered a consequence of the significant decrease in chitinolytic enzyme production, leading to a decrease in their amount in the culture medium. As was already mentioned, in addition to the deficiency in chitinolytic activity, the mutant was deficient in the antibiotic-like and proteolytic activities of the parent strain IC1270. However, according to the Southern analysis data, Tn*5* was inserted into only one site of the mutant genome. It could be suggested, therefore, that the insertion led to a pleiotropic mutant phenotype. The existence of global regulation of the expression of several traits which relate to antifungal activity was recently described in a biological control strain of *Pseudomonas fluorescens* (6). It is still unclear whether a similar regulatory pathway is present in our *E. agglomerans* strains and if the Tn*5* insertion in the mutant IC1270-E1 indeed altered the expression of a set of genes involved in the antagonistic activity of the parent strain. In spite of the fact that Tn*5* was inserted into two sites of the IC1270-2h mutant's genome, this mutant lost the chitinolytic but not the antibiotic or proteolytic activities of its parent strain. We still did not know, however, if only one or both of these insertions affected chitinolytic activity.

The data presented here constitute the most complete evidence yet for the existence of a complex of chitinolytic enzymes in bacteria of the *Enterobacter* genus. The only known exception is *E. cloacae* E6, although observations made regarding this strain have been inconsistent (17, 29). Contrary to *E. cloacae* E6, our *Enterobacter* isolates grew when colloidal chitin was supplied as the sole carbon source, and significant levels of *N*-acetyl-β-glucosaminidase, endochitinase, and chitobiosidase activities were found in the culture supernatants as well as in intracellular proteins.

Greenhouse experiments revealed biocontrol activity of the *E. agglomerans* strains with *R. solani* in cotton as the model. The biocontrol effect disappeared when the plants were treated with the Tn*5* mutant E1 or 2h instead of the parent strain IC1270. These data demonstrate the importance of the ability to produce and excrete chitinolytic enzymes for biocontrol; mutant 2h, which lost only chitinolytic activity but not antibiotic or proteolytic activities, was no more efficient as a biocontrol agent than mutant E1, which lost all three activities. The complexity and diversity of the chitinolytic enzyme system, with its complementary modes of hydrolyzing chitin, may contribute significantly to the antagonistic activity of the strains described.

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