Control of Plant Diseases by Chitinase Expressed from Cloned DNA in *Escherichia coli*

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ABSTRACT

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A DNA fragment carrying the *chi*A gene from *Serratia marcescens* was subcloned into the plasmid pBR322. The resulting plasmid, pCHIA, includes a 2-kilobase-pair segment upstream of the *chi*A gene and presumably carries the gene regulatory elements. To obtain high levels of chitinase expression, we introduced the leftward operator promoter of bacteriophage λ, oLpL, upstream of the *chi*A gene. The resulting plasmid, pLCHIA was introduced into cells of *Escherichia coli*. High levels of chitinase were produced and secreted following induction, and the enzyme was partially purified. When *Sclerotium rolfsii* was sprayed with partially purified chitinase produced by the cloned gene described above, rapid and extensive bursting of the hyphal tips was observed. This chitinase preparation was found to be effective in reduction of disease incidence caused by *S. rolfsii* in beans and *Rhizoctonia solani* in cotton under greenhouse conditions (62% disease reduction in both diseases). A similar effect was obtained when we used viable cells of *E. coli* containing the plasmid pLCHIA. However, *E. coli* carrying the plasmid lacking the pL promotor did not have any effect. These results suggest a role for chitinase in biological control of plant pathogenic fungi.

The protection of plants against fungal infection is mainly dependent upon the introduction of hazardous pesticides into the environment. Biological control provides an alternate means of reducing the incidence of plant disease without the negative aspects of pesticide application (20). Interactions between biocontrol agents and plant pathogens have been studied extensively, and the application of biocontrol agents to protect some commercially important crops is promising (4). Parasitism via degradation of the cell walls of pathogenic fungi is an important mechanism in biological control (7,19). A major structural component of many fungal cell walls is chitin, which is uniquely absent from higher plants and mammals. Chitinases capable of hydrolyzing this polymer to chitobiose dimers are produced by various organisms and have been implicated in the biocontrol process (5). *Serratia marcescens*, a gram-negative bacterium, was found to be an effective biocontrol agent of *Sclerotium rolfsii* Sacc. under greenhouse conditions (up to 75% disease reduction). Several application methods were tested; drench and drip applications of suspensions of *S. marcescens* were more effective in controlling *S. rolfsii* than spraying, mixing in soil, or seed coating. This bacterium significantly reduced damping-off incidence of bean, caused by *Rhizoctonia solani* Kühn, by 50% but was not effective against *Pythium aphanidermatum* (Edson) Fitzp. in cucumber (18).

S. marcescens is capable of secreting several chitinolytic enzymes (9). The gene *chi*A, coding for the major chitinase produced by *S. marcescens*, recently was cloned and sequenced (10). Prolonged incubation was found to be required for secretion of chitinase by cells of *Escherichia coli* harboring a plasmid carrying the *chi*A gene (10).

In this report we describe the construction of a system for the production of chitinase in *E. coli*. We demonstrate the effectiveness of partially purified enzyme, applied in the irrigating water, in protecting bean seedlings against the plant pathogens *S. rolfsii* and *R. solani*. Moreover, *E. coli* harboring a plasmid expressing the chitinase gene of *S. marcescens* can act as a model biocontrol system.

MATERIALS AND METHODS

Bacterial and fungal strains. *S. marcescens* isolated from a plant rhizosphere (18) was grown on LB medium (14). Strain A2097 of *E. coli* carrying the defective prophage λ cI857 ΔHI ΔBamHI (13) was grown on LB medium. *S. rolfsii* and *R. solani* isolated from diseased plants were grown on synthetic medium (17).

Cloning of *chi*A gene in *E. coli*. DNA of the mentioned isolate (17) of *S. marcescens* was subjected to partial digestion with the restriction enzyme *Sau*3a, and DNA fragments (5–10 kilobase pairs [kbp]) were isolated. A gene library was prepared in the λ D69 phage cloning vector (15), and two oligonucleotides, designated A and B, were synthesized with an Applied Biosystems Model 380A synthesizer (Applied Biosystems, Foster City, CA). The phage library was screened for *chi*A with these oligonucleotides. Oligonucleotide A (5'-CCATCGCCTGGGGGCAACACCAAGTTCGCCATCG-3') is identical to residues 89 to 121 covering the region coding for the amino terminus of the *chi*A gene product (10). Oligonucleotide B (5'-CGCCGGCGCTGTTGCCCAGGCTGGCGTTCATGC-3') is identical to the antisense strand of residues 1646 to 1678 coding for the carboxyl terminal end of chitinase (10). These oligonucleotides were labeled at the 5' terminus with $(\gamma^{-32}P)ATP$ and T4 polynucleotide kinase (14). A phage carrying a 9-kbp DNA insert was found to hybridize to both oligonucleotides and was selected for further analysis. From this clone, a 4.7-kbp *Bam*HI/ *Eco*RI DNA fragment carrying the *chi*A gene was subcloned into the vector pBR322 (3), producing the plasmid pCHIA. This chimeric plasmid then was cleaved with the enzyme *Bam*HI and ligated with a 250-bp *Bgl*II/ *Bam*HI fragment carrying the λ operator promotor (oLoL) region isolated from the plasmid pSA100 (1). The ligation mixture was used to transform strain A2097 of *E. coli* carrying the defective prophage λ cI857 ΔHI ΔBamHI (13). One colony, designated A5187, was found to contain the desired plasmid (see below). The same strain of *E. coli* (A2079) transformed by the plasmid pCHIA and designated A5186 was retained for use as a control. The λ temperature-sensitive repressor carried by the defective prophage binds to the pLoL region and thus prevents transcription at 30 C. Upon heat induction at 42 C, the repressor is inactivated and the oLpL

becomes an effective promoter for the transcription of downstream gene(s) (14).

Secretion of chitinase from *E. coli* after heat induction. A culture of *E. coli* (A5187) carrying the pLCHIA plasmid was grown in LB medium containing ampicillin (50 μg/ml) at 30 C to an absorbance of 0.4 at 600 nm and transferred to 42 C for 3 hr. The cells were removed by centrifugation with an Eppendorf centrifuge. An aliquot of the supernatant was precipitated in 7% trichloroacetic acid at 0 C, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophresis (SDS-PAGE) and stained with Coomassie blue (12).

A parallel aliquot was precipitated with $(NH_4)_2SO_4$ (40–80%). The precipitate was resuspended in 50 μl of Tris-HCl, pH 6.8, and the proteins were separated on the same gel. A strip of the gel containing the $(NH_4)_2SO_4$-precipitated proteins was removed before the staining step and was cut into eight horizontal slices with a scalpel. Each slice was placed into a dialysis bag containing 50 mM Tris-glycine, pH 8.3, and 0.05% SDS. The sealed bags were placed in a horizontal gel electrophoresis apparatus filled with the same buffer until the bags were just covered. Proteins were electroeluted at a constant voltage of 140 V for 4 hr at 4 C. SDS was removed by extensive dialysis of the bags against 20 L of 20 mM Tris-HCl, pH 6.8. Buffer from each bag was freeze-dried and resuspended in water 1/10 of the original volume. An aliquot of 50 μl representing each segment was spotted on a water agar plate containing 0.3% (w/v) chitin and further incubated at 30 C for 24 hr. A clearing halo around the spot indicated chitinase activity.

Large-scale production of chitinase. A large-scale (10 L of fermentor) preparation of chitinase was made from a culture of strain A5187 grown at 30 C in LB medium containing 50 μg of ampicillin/ml. The culture was grown to an absorbance of 0.4 and heated to 42 C for 30 min for the induction of chitinase production. The temperature was reduced to 40 C, and the culture was grown for an additional 3 hr before harvesting. A control preparation was made from strain A5186. The cells were removed and the supernatant containing the enzyme was further purified by precipitation with $(NH_4)_2SO_4$ (40–80%). The precipitate was resuspended in Tris-HCl buffer, pH 7.0, at a concentration of 12.0 units/ml. Chitinase was determined by the release of N-acetyl-D-glucosamine after application of purified chitobiase (E.C. 3.2.1.29) (11). One chitinase unit was defined as the amount of enzyme that liberates 1 μmol of N-acetyl-D-glucosamine from chitin per minute (6).

Lysis of hyphal tips. Agar disks bearing mycelium of *S. rolfsii* (10 mm in diameter) were sprayed with the above chitinase preparation, incubated for 3 min at 30 C, and then prepared for scanning electron microscopy as previously described (18). Hyphal tips were treated with the partially purified chitinase excreted by strain A5187 or with the protein excreted by strain A5186.

Greenhouse experiments. Soil samples (sandy loam, pH 7.2) were infested by either *R. solani* (8) or *S. rolfsii* (40 μg of sclerotia per 1 kg of soil). Polypropylene boxes (7 × 19 × 14 cm) were filled with infested soil and planted either with 10 bean seeds (*Phaseolus vulgaris* L. 'Brittle Wax') or 10 cotton seeds (*Gossypium barbadense* L. 'Pima') each. All experiments were conducted under greenhouse conditions at a temperature of 28–32 C. Each treatment contained six replicates, and the experiments were repeated three times.

The chitinase solution was applied daily after dilution in tap water (50 ml containing 0.04 units of chitinase per box). The boxes were divided into three groups. The first was irrigated daily with the chitinase preparation extracted from strain A5187. The two control groups included boxes that were irrigated with tap water or boxes irrigated with excreted protein preparation from strain A5186 carrying the plasmid without the pL promotor.

Plant protection by *E. coli* carrying the chitinase gene. Bacterial cultures were grown in LB medium containing 50 μg of ampicillin/ml to mid-log phase at 30 C. The cultures were harvested by centrifugation, washed, and resuspended in TM buffer (10 mM Tris-HCl, pH 7.4; 10 mM $MgSO_4$) at 1/10 of the original volume,

and stored for 16 days at 4 C. Plants were irrigated with tap water, tap water containing strain A5186 of *E. coli* as a control, or tap water containing strain A5187 of *E. coli*. The bacterial cells were added daily in the irrigation water to a final concentration of 10^8 per milliliter. The experiment was carried out as described above in a growth chamber at 39–42 C, a temperature at which the repressor is destroyed and the promoter is active in transcription. All experiments were repeated at least three times, and data were analyzed with Duncan's multiple range test with a significance level of $P = 0.05$.

RESULTS

Expression of chitinase in *E. coli*. A DNA fragment carrying the *chi*A gene from *S. marcescens* was subcloned into the plasmid pBR322 to generate pCHIA. Cleavage by various restriction enzymes established the similarity of this DNA fragment to the published chitinase sequence (10). Very low levels of chitinase were observed in an *E. coli* culture carrying this plasmid. This is probably due to inefficient transcription of *chi*A in the heterologous bacteria. To obtain high levels of chitinase expression, the leftward operator promoter of bacteriophage λ, oLpL, was inserted upstream of the *chi*A gene. The resulting plasmid, pLCHIA (Fig. 1A), was introduced into *E. coli* cells expressing a thermolabile λ repressor. At 42 C, the repressor is inactivated and oLpL is no longer repressed. When the cells were grown at this temperature on chitin-containing plates containing 0.3% chitin, a clearing halo was produced, indicating that chitinase was synthesized and secreted from the cells to the surrounding medium.

Fermentation conditions for the production of high levels of chitinase were established. Cells carrying the plasmid pLCHIA (strain A5187) were grown at 30 C and then shifted to an elevated temperature for the induction of chitinase synthesis. Intact cells were removed by centrifugation and the supernatant was found to contain chitinase activity. After 3 hr of induction, the level of chitinase reached 0.228 units/ml. A culture of the same bacterial strain carrying the plasmid pCHIA (strain A5186), which lacks the pL promoter, produces only 4% as much chitinase (0.0085 units/ml). Analysis of the supernatant of the induced cells (A5187) by SDS-PAGE showed a major protein band of 58 kDa (Fig. 1B).

To demonstrate that the 58-kDa protein band corresponds to the chitinase enzyme, the SDS-PAGE gel was sliced and the proteins were electroeluted and assayed for chitinase activity as described in the Materials and Methods section. Chitinase activity was concentrated in the region containing the 58-kDa protein band (data not shown). Further proof that the 58-kDa protein is identical to the chitinase of *S. marcescens* was obtained by
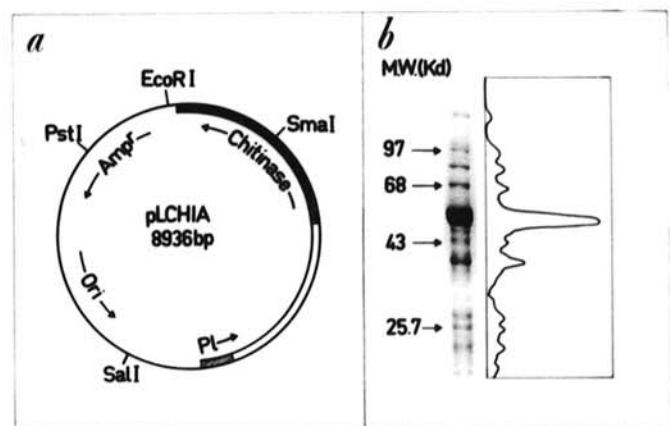
Fig. 1. Restriction map of the plasmid pLCHIA. **A,** The *Sma*I restriction site was used to help determine the orientation of the clonal gene. **B,** Separation by sodium dodecyl sulfate-polyacrylamide gel electrophresis of the protein extracted from cells of *Escherichia coli* that harbor the plasmid pLCHIA.

determining the amino terminus of the 58-kDa protein band by Edman degradation. These results demonstrate that the bacterial strain we have constructed can provide a convenient source for chitinase.

Effect of chitinase on hyphal tips. We tested whether the chitinase produced by *E. coli* can degrade hyphal tips of *S. rolfsii*. When *S. rolfsii* was sprayed with partially purified chitinase produced by the cloned gene described above, rapid and extensive bursting of the hyphal tips was observed by scanning electron microscopy (Fig. 2B). When these tips were sprayed by proteins excreted by strain A5186 of *E. coli*, no effect was observed (Fig. 2A).

Greenhouse experiments. Bean seeds were sown in soil artificially infested with *S. rolfsii* and grown under greenhouse conditions. The boxes were irrigated daily with tap water containing a diluted chitinase preparation. Control boxes were irrigated with tap water or with tap water containing a protein preparation obtained from equivalent amounts of culture medium of strain A5186 of *E. coli*. We found that chitinase can effectively reduce the number of diseased plants (Fig. 3). During the course of these experiments, more than 50% of the plants irrigated with tap water showed severe disease symptoms. The addition of chitinase to the irrigation water significantly reduced the percentage of plants showing symptoms. The protein preparation isolated from *E. coli* carrying the plasmid pCHIA contained low levels of chitinase activity but did not significantly reduce levels of disease as compared to the control.

To test the effectiveness of the chitinase preparation in reducing inoculum potential, the plants used in the first experiments, that is, the first growth cycle, were uprooted and the soil was mixed and divided again into six boxes. Bean seeds again were sown and irrigated daily with tap water only. In the second growth cycle, the disease rate in the soil previously treated with chitinase was significantly lower than in the two control soils. Disease incidence after 14 days was 28% in the chitinase treatment, 52% in water control, and 47% in soil previously treated with extracellular proteins prepared from *E. coli* that did not produce chitinase.

To check the effect of inoculum level on the control of *S. rolfsii*, soil was inoculated with three levels of 40, 80, and 120 mg of sclerotia of *S. rolfsii* per kilogram of soil. The infected soil was irrigated with chitinase preparation or with tap water as a control. At inoculum levels of 40 and 80 mg of sclerotia per kilogram of soil, disease incidence was significantly reduced (42 and 36%, respectively) (Fig. 4). In another experiment, the chitinase preparation reduced the incidence of damping-off of cotton caused by *R. solani* by 62% at 20 days after sowing.

Similar experiments were carried out at 40 C with beans, *S. rolfsii*, and whole viable cells of *E. coli*. These cells also were effective in inhibiting *S. rolfsii*, although to a lower degree (Fig. 5). Disease incidence after 16 days was 35% in water controls, 37% in soils treated with strain A5186 of *E. coli*, and 19% in soils treated with strain A5187 of *E. coli*. Disease incidence in soils treated with strain A5187 of *E. coli* was significantly less than that in the other treatments ($P = 0.05$). When the same experiment was carried out at 30 C, a temperature at which the pL promoter is repressed, no protection was observed.
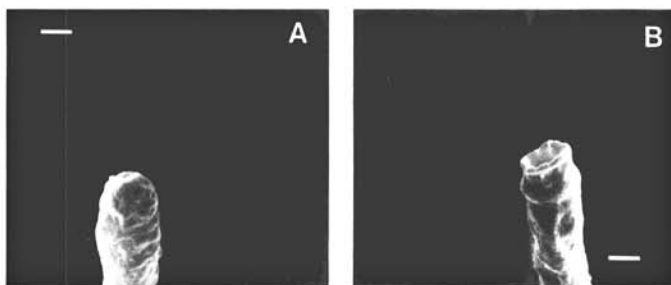

Fig. 2. Scanning electron micrographs showing the effect of the partially purified chitinase (see Materials and Methods section) on hyphal tips of *Sclerotium rolfsii*. **A,** Control tip treated with the extracellular proteins from *Escherichia coli* lacking the pL promotor on the plasmid. **B,** Hyphal tip treated with the partially purified chitinase. Bar = 1 μm.

Fig. 4. Effect of inoculum level on the control of *Sclerotium rolfsii*. Soil irrigated daily with water (solid line) or with chitinase preparation (broken line). Asterisk indicates significant difference ($P = 0.05$) in percent of diseased plants in the same inoculum level.
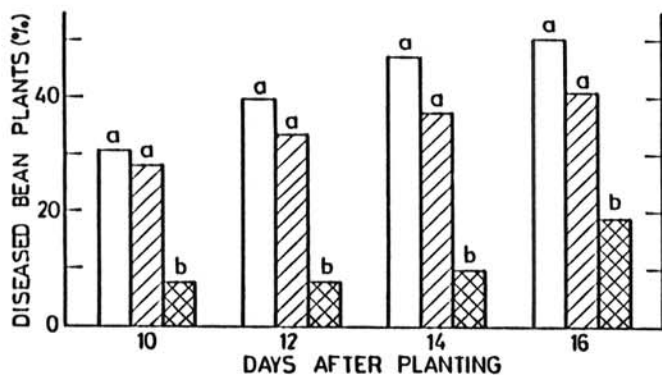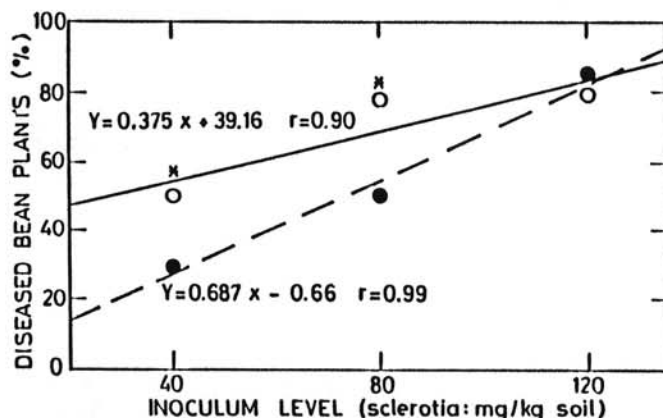

Fig. 3. Effect of chitinase preparation on the control of *Sclerotium rolfsii* in beans. Boxes were irrigated daily with tap water (□), extracellular proteins prepared from strain A5186 of *Escherichia coli* (▨), or chitinase from strain A5187 of *E. coli* (⊠) at 0.04 units per box. Columns followed by the same letter are not significantly different ($P = 0.05$).

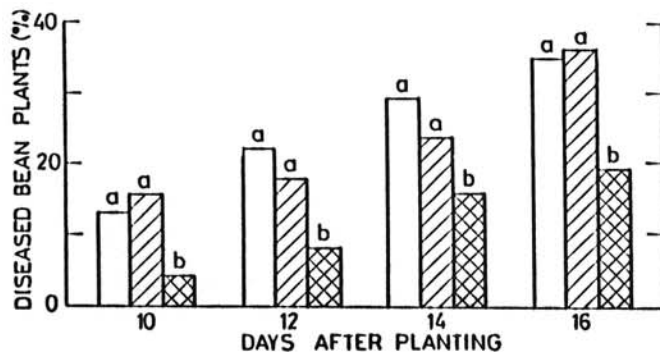Fig. 5. Effect of genetically engineered *Escherichia coli* on control of *Sclerotium rolfsii* in beans. Soil irrigated daily with tap water (□), engineered *E. coli* lacking the pL promotor (▨), or *E. coli* bearing the pLCHIA plamsid (⊠). Columns followed by the same letter are not significantly different ($P = 0.05$).

DISCUSSION

Parasitism is one of the main mechanisms involved in the antagonistic activity of biocontrol agents. This process is apparently a complex of several stages such as chemotropism, recognition, excretion of extracellular enzymes, and lysis of the host (4).

A large number of reports dealing with biological control of soilborne pathogens have proposed that chitinolytic activities play a major role in plant protection (2,4,7,16,18). However, to our knowledge, there is no direct proof demonstrating that purified chitinase can protect growing plants from fungal infection. The availability of a large supply of chitinase produced by genetically engineered bacteria enabled us to test this hypothesis directly. It was previously shown that a nonpurified supernatant from a culture of *S. marcescens*, which is a mixture of various hydrolytic enzymes including several chitinases, is capable of degrading fungal tips (19). The hyphal tips of *S. rolfsii* are the sites of hyphal growth and contain exposed chitin (6). Indeed, we have demonstrated by electron microscopy that the partially purified chitinase is very effective in lysing hyphal tips of *S. rolfsii*. These results encouraged us to take the next step and ask whether the single chitinase produced by a genetically engineered bacteria can protect plants from fungal infection.

The chitinase preparation was found to be effective in reduction of disease incidence caused by *S. rolfsii*. Moreover, even in a second growth cycle, the effectiveness of this treatment was maintained. This is probably due to the reduction of inoculum potential of the pathogen (8). Chitinase preparation also was found to be effective against *R. solani*. Additional experiments will be necessary to determine the fate of chitinase in the soil.

We continued by asking whether a recombinant enterobacterium, *E. coli*, with the ability to produce a single chitinase, can serve as a biocontrol agent. Since the pL promotor directing chitinase synthesis is active only at 42 C, this experiment was run at high temperature, a rather extreme condition for the plants and fungus. Disease development in the plants irrigated daily with *E. coli* lacking the promotor was rapid and similar to the untreated control, whereas disease was significantly reduced upon irrigation with the chitinase-producing strain of *E. coli*.

The genetically engineered *E. coli*, a nonsoil bacterium, served here as a model system to demonstrate the importance of chitinase in controlling a chitin-containing plant pathogen. The ability to protect plants by the application of a lytic enzyme opens the way for the development of chitinase as an alternative to direct control of soilborne pathogenic fungi. It further suggests that the introduction of such engineered genes into soil bacteria will increase control efficacy by combining high expression of a gene coding for a lytic enzyme with rhizosphere competence.

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