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Antifungal Activity of *Serratia marcescens* Culture Extracts against Phytopathogenic Fungi: Possibility for the Chitinases Role

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Serratia marcescens co-cultured with various phytopathogenic fungi, including _Rhizopus stolonifer, Helminthosporium allii, Pyricularia oryzae, Fusarium oxysporium_ and _Collectothricom cassiicola_, in an LB- agar medium containing 1.5% swollen chitin, significantly inhibitied fungal growth. Fungal hyphae grew rapidly outward from the culture dish center, but the hyphal extensions of the pathogenic fungi were significantly inhibited in a perimetric contact area with _S. marcescens_. This was especially evident in pathogenic fungi which have a high chitin content in their cell walls. The extracellular chitinase activities of _S. marcescens_ were increased seven fold by the addition of 1.5% swollen chitin to the LB-broth, compared to chitinase activities in a culture medium without chitin. The type of induction was dependent on the various forms of chitin used. When the culture supernatant of _S. marcescens_ or the chitinases of _Streptomyces griceus_ purchased from Sigma Chemical Co., were incubated with the mycelium of _F. oxysporium_, the mycelium gradually burst as cultivation time progressed and completely lysed after incubation for 2 days. On the other hand, _E. coli_ extract did not hydrolyze the _F. oxysporium_ mycelium at all. These data showed that the chitinolytic activities of _S. marcescens_ play important roles in the biochemical control of phytopathogenic fungi.

Biological control mechanisms against phytopathogenic fungi (4, 5) can provide an important method for reducing the incidence of plant disease (6, 10) without the negative aspects of hazardous pesticides. Much attention has been paid to chitinolytic enzyme systems as a natural defense mechanism against plant pathogens (25, 26). They degrade chitin, the main structural component of fungal cell walls, and produce glycosidic fragments acting as elicitors for generating pathogenesis related second messengers in plant cells (11, 12, 16). As the chitinases are active determinants in plant disease systems (20, 23, 31), extensive studies have been focused on plant chitinases. The role of plant chitinases in the possible defense mechanisms against parasitic fungi (17, 27, 32), induction of enzymes by ethylene (18, 21), and distribution of enzymes after the infection processes (2, 15, 19, 35) have been well studied. Chitinases are also produced by a variety of living organisms other than plants, including bacteria, molds, fungi, and some vertebrates (24). *S. marcescens,* which is a gram negative bacterium, is a good candidate for an effective biological control agent (7) because it gives rise to higher extracellular chitinase activities, which are greatly enhanced by several inducing materials (9).

We describe the antifungal activity of *s. marcescens* against various phytopathogenic fungi and the possible roles of chitinases in the degradation of fungal cell walls.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The microooganism used in this study was *S. marcescens* KCTC 2172. It was capable of lysing the hyphae

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of phytopathogenic fungi. It was maintained on an LB agar medium at 4℃ and was subcultured every month. The culture medium contained 5 g of yeast extract, 10 g of tryptone, 10 g of NaCl, and 15 g of swollen chitin per liter. Swollen chitin was prepared according to the method of Monreal and Reese (24), Fungal mycelia developed after 3 days culture of *Rhizopus stolonifer, Helminthosporium allii, Pyricularia oryzae, Fusarium oxysporium* and *Collectothricom cassiicola* were used as substrates for the chitinolytic enzyme systems. For the induction of chitinases, *S. marcescens* was grown on an LB-medium at 30℃ containing 1.5% swollen chitin, with vigorous shaking.

Co-cultivation of *S. marcescens* with Various Phytopathogenic Fungi

Antifungal activity was estimated using the hyphal extension inhibition test described by Roberts and Selitrennikoff (29), with some modification. An agar disc (5 mm dia.) of fungal conidia grown on an LB medium for 3 days was transferred to the center of a new LB-agar plate (86 mm dia.) containing 1.5% swollen chitin and further incubated for 3 days at 30℃. At one side of fungal conidia grown annularly, a loop of *S. marcescens* or *E. coli* was streaked aseptically and co-cultured for 2 days. The fungal hyphae grew outward from the center, unless effective antifungal agents were present around the growth perimeter. The growth inhibtion of fungal hyphae in the contact area between the fungi and *S. marcescens* was examined. The phytopathogenic fungi used in this study were *Rhizopus stolonifer, Helminthosperium allii, Pyricularia oryzae, Fusarium oxysporium* and *Collectothricom cassiicola*. These species are very harmful patogens to many important agricultural crops (1).

Lysis of Fungal Mycelium

Fungal mycelium was prepared from the pathogenic fungi, *F. oxysporium*, which causes serious wilting of various host plants. It was inoculated onto an LB-agar medium, onto which nitrocellulose paper was overlayed and incubated for 3 days at 25℃. After removal of the nitrocellulose paper, the nitrocellulose paper was slowly introduced into 10 ml of 50 mM sodium acetate pH 6.8 buffer and the solubilized mycelium was transferred to a slide glass having a single depression. The culture supernatant of *S. marcescens*, grown on an LB-medium with and without 1.5% swollen chitin at 30℃ for 7 days, was concentrated 20 fold with 80% ammonium sulfate precipitation. After the precipitate was dialyzed against M-9 minimal medium, 100 μl of this solution was added to the suspension of fungal mycelium on the slide glass. Partially purified chitinases of *Streptomyces griceus*, purchased from Sigma, and the culture supernatant of *E. coli* were also tested by the same method as positive and negative controls. The lysis of fungal mycelium was observed under an inverted microscope (Diaphot Mea 112 DB, Nikon, Japan) at 30℃ during the incubation period of the mixture.

RESULTS

Production of Extracellular Chitinases from *S. marcescens*

Since the production of chitinases was induced by the addition of chitin into the culture medium (9), *S. marcescens* was cultured in LB broth containing 1.5% swollen chitin at 30℃ with vigorous shaking. The extracellular chitinase activities were monitored during cell growth of *S. marcescens*. As the chitin particle interferes with the determination of absorbance, an aliquot of broth was passed through a coarse sintered glass filter, which retained the chitin particles but not the bacteria. *S. marcescens* grew rapidly and reached a stationary phase of growth within two days, however, the chitinases were maximally produced during the seventh day of culture. Extracellular chitinase activities were enhanced seven fold by the addition of 1.5% swollen chitin to the LB-medium. Pretreatment of chitin affected the production of chitinases from *S. marcescens*. Acid treatment of powdered chitin that lead to swelling or size reduction of chitin was very effective for chitinase induction as shown in Fig. 1. Longer periods of incubation resulted in the decrease both in cell density and chitinase activities.

Antifugal Activity of *S. marcescens*

Chitinases play important defensive roles against phytopathogenic fungi in plants (25, 26), microorganisms, and the serum of vertebrates (34), such as ruminants and fishes. Since *S. marcescens* produced higher level of extracellular chitinases, it was tested for antifungal activity by its ability to inhibit the hyphal extention of *F. oxysporium*, which is a harmful pathogen causing wilting, especially in cucumber and melon. The hyphae of *F. oxysporium* grew rapidly outward from the center until effective antifungal agents produced from *S. marcescens* were contacted. Hyphal growth of *F. oxysporium* was significantly invaded by *S. marcescens* that was cocultured with the fungi in an LB-agar medium containing 1.5% swollen chitin. As *S. marcescens* grew simultaneously with pathogenic fungi, the hyphae of *F. oxysporium* were intruded at the zones of contact between *S. marcescens* and the pathogenic fungi. As shown in Fig. 2B, the invaded zone of pathogenic fungi by the colony of *S. marcescens* was clearly demarcated as red pigment of *S. marcescens* intruded into the fungal mycelia. On the other hand, *E. coli*, a similar gram negative enteric bacterium tested as a control, did not inhibit the hyphal growth of *F. oxysporium*. Rather, the hyphae of *F. oxys-*

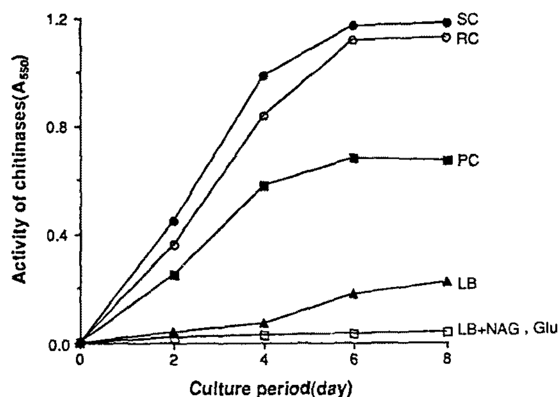

Fig. 1. Change in chitinase activities during the growth of *S. marcescens* cultured at 30°C with vigorous shaking (180 rpm).

Activities of extracellular chitinases were monitered by the formation of a reducing sugar (A_{550}) cultured in LB-broth containing various forms of chitin: SC; swollen chitin, RC; regenerated chitin, PC; powdered chitin, NAG; N-acetyl glucosamine, Glu; glucose.

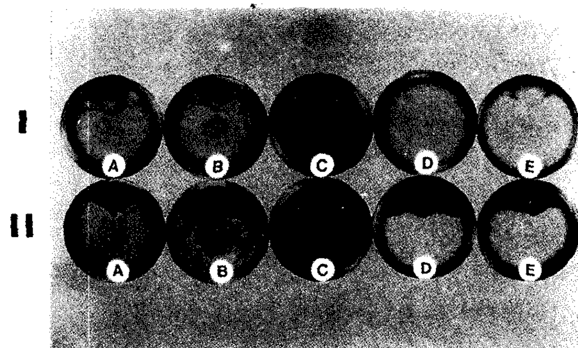

Fig. 3. Inhibition of mycelial growth of various phytopathogenic fungi, including *Rhizopus stolonifer* (A), *Helminthosporium allii* (B), *Phyricularia oryzae* (C), *Fusarium oxysporium* (D) and *Collectothricom cassiicola* (E) by antifungal agents produced by *S. marcescens*.

In panels I and II, a loop of *E. coli* and *S. marcescens* respectively was streaked.

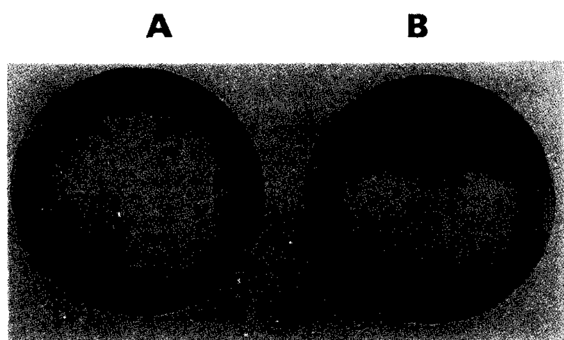

Fig. 2. Inhibition of hyphal extension of *F. oxysporium* by antifugal agents produced by *S. marcescens*.

After growing the fungal mycelium on one side of an LB-agar plate containing 1.5% swollen chitin for 3 days, a loop of *E. coli* (A) and *S. marcescens* (B) was streaked aseptically on the other side and further co-cultured at 30℃ for 2 days.

porium grew over the *E. coli* colony (Fig. 2A). From this result, it was concluded that *S. marcescens* secreted antifungal agents into the medium greatly inhibiting the growth of pathogenic fungi. Similar assays using some representative phytopathogenic fungi for important agricultural crops were also performed. Results are shown in Fig. 3. *S. marcescens* showed strong antifungal activity against a broad spectrum of phytopathogenic fungi, with different degrees of growth inhibition. There might be several factors involved in this fungal inhibiting ability of *S. marcescens*. However, secretion of chitinases is believed to play a critical role in the growth inhibition of fungi because the hyphal growth of *F. oxysporium*, which contains about 40% chitin as a cell wall component (8), was more severely inhibited than the hyphal growth of *P. oryzae*, which contains far less chitin in its cell wall.

Degradation of Fungal Cell Walls by Culture Extracts of *S. marcescens*

Extracellular culture extracts of *S. marcescens* were concentrated with 80% ammonium sulfate and dialyzed against an M-9 minimal medium. When the dialized culture supernatant of *S. marcescens* was incubated with a mycelium of *F. oxysporium* as the sole substrate of carbon source in a slide glass, extensive bursting of the fungal mycelium was observed. As shown in Fig. 4, the mycelium of *F. oxysporium* was slightly degraded by the culture extracts of *S. marcescens* incubated in LB-broth without chitin. When the mycelium was incubated with the culture supernatant of *S. marcescens* grown in LB-broth in the presence of 1.5% swollen chitin, it was significantly burst after incubation for 1 day (Fig. 4, A-2). Also, the septum was further separated and the cell wall of the mycelium was completely lysed with incubation of the mixture for 2 days, resulting in the release of the cytosolic materials of the fungal mycelium into the external medium (Fig. 4, A-3). Comparing this result with the chitinase activities produced from *S. marcescens* cultured with and without chitin on an LB-medium, as shown in Fig. 1, the lysis of fungal cell walls was in proportion to the chitinase activities. This was also confirmed by partially purified chitinases of *Streptomyces griceus*, purchased from Sigma. They had nearly the same potency to degrade fungal cell walls (Fig. 4D) as the filtrate

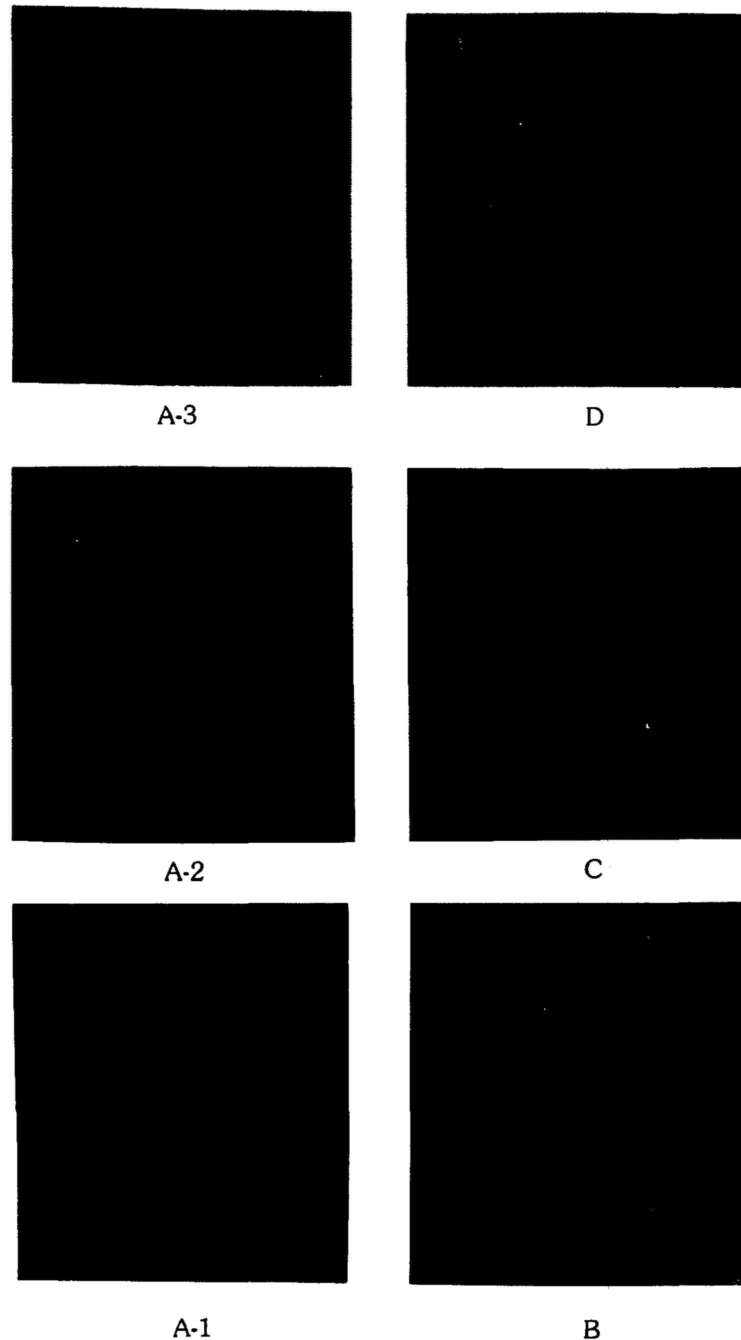

Fig. 4. Mycelial lysis of *F. oxyspolium* by the chitinolytic activities of *S. marcescens*.
Cell supernatant of *S. marcescens* cultured in LB-broth with (A) or without (B) 1.5% swollen chitin was concentrated 20 fold by 80% ammonium sulfate precipitation and dialyzed against M-9 minimal medium. Each of these solutions was co-cultivated with the mycelium of *F. oxyspolium* as a substrate for antifungal agents in a slide glass at 30℃ for 0 hour (A-1), 1 day (A-2), and 2 days (A-3 & B). For positive and negative controls, the cell supernatant of *E. coli* (C), or partially purified chitinases of *S. griceus* purchased from Sigma (D), were incubated for 2 days under the same conditions. Photomicrograph is ×800.

obtained from *S. marcescens* in LB-broth containing 1.5 % swollen chitin. However, when the mycelium of *F. oxyspolium* was incubated with filtrates of *E. coli* as a control, the fungal mycelium was not lysed at all (Fig. 4 C). These data suggest that the chitinases secreted by *S. marcescens* played critical roles in antifungal activity for the various pathogenic fungi tested. Fig. 4 also shows that the sensitive sites of the fungal mycelium are located in the specific regions of tips, septa, and the branches of hyphae, which agrees with the results of Matraux and Boller (21). We therefore conclude that chitinases produced by *S. marcescens* attack these sites first, and then gradually degrade other parts of the cell walls resulting in the complete lysis of the fungal mycelium.

DISCUSSION

A number of papers have proposed that chitinolytic enzyme systems play important roles in antifungal activity against various phytopathogenic fungi (22, 29). Crude extracts prepared by 80% ammonium sulfate precipitation from the culture supernatant of *S. marcescens* lysed the mycelium of various pathogenic fungi (30). They played a role during the formation of the septum, and during cell division by degrading hyphal tips, septa, and branches of hyphae (Fig. 4) where cell growth was occurring and exposed to chitin, as described by Kritzman *et al.* (14). These results agree with those of Polachek and Rogenberger (28) and Skujins *et al.* (33). Even though there may be other mechanisms involved in the biochemical control abilities of *S. marcescens*, it appears that chitinolytic activities play important roles in the control of plant pathogens (3, 13). Hwang *et al.* (1991) (9) showed that there are at least five chitinase isozymes in cell extracts of *S. marcescens*. The efficient lysis of pathogenic fungal cell walls by chitinases from *S. marcescens* provides a new means for plant disease control without the pollution effects of agricultural chemicals. Direct evidence that the antagonistic activity of *S. marcescens* is due to the effect of chitinases should be further confirmed by the use of purified chitinases from *S. marcescens*. This work is now in progress in our laboratory.

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