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Molecular Cloning of *Serratia marcescens* Chitinase Gene into *Escherichia coli*

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Serratia marcescens Chitinase 유전자의 대장균에로의 클로닝

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Abstract — A chitinase gene of *Serratia marcescens* ATCC 27117 was cloned and expressed in *Escherichia coli*. A genomic library of *S. marcescens* was constructed with pUC 19 and screened using the swollen chitin agar plate for chitinolytic clones. A positive clone showing chitin-clearance contains a recombinant pCHI 89, composed of 8.9 Kb chromosomal DNA fragment and pUC 19. Plasmid pCHI 89 produced 58 KD chitinase in *E. coli*, which was coincided with one of five extracellular chitinases produced by *S. marcescens*. Restriction endonuclease cleavage sites of the 8.9 Kb insert DNA fragment were mapped. *E. coli* JM109 harboring pCHI 89 inhibits the growth of a plant pathogenic fungus, *Fusarium oxysporum*.

Chitin, β-1,4-linked polymer of N-acetylglucosamine(NAG) is a major structural component of many agronomically important pests including insects, fungi, and nematodes (1, 2). Chitin hydrolyzing activities(chitinase, EC 3.2.1.14) have been found in a great variety of organisms that do not contain the chitin as their structural component, such as vertebrates, higher plants, and bacteria (3-7). These chitinases were seemed to play an important role in the degradation of chitin waste accumulated in environment and a natural defense mechanism in plants by hydrolyzing the insect exoskeleton and cell wall of fungi (2, 4, 8, 9).

For the past decade, applications of chitin and chitinase have been tried for the reduction of the incidence of plant diseases caused by soil fungi (10-13). It was concluded that chitinases are partly responsible for the antifungal activities of soil bacteria and plants. Although many reports strongly suggested their close correlations, direct evidences for the antifungal activity of chitinase have not appeared. Fuchs *et al.* (14) cloned 57 KD chitinase gene from *S. marcescens* QMB 1466 and expressed in *E. coli* and *Pseudomonas fluorescens*, a soybean root-colonizing pseudomonad, for investigating the role of chitinase in antifungal activity and evaluating *in vivo* efficacy of chitinase. At nearly the same time, Jones *et al.* (11) isolated two chitinase genes, *chi* A and *chi* B, encoding 58 KD and 52 KD chitinases, from *S. marcescens* QMB 1466 and analyzed the complete sequence of *chi* A gene. When *chi* A gene was mutated in the parent strain, the mutant derivative showed a reduced capacity to inhibit the growth of fungal plant pathogen, *Fusarium oxysporum* f.sp. pisi(Fop), which suggested that *S. marcescens* chitinase acts as an antifungal agent. Recently, genes of chitin utilization regulon from *Serratia liquefa-*

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ciens were cloned and their regulation in chitin metabolism was described and suggested in a model (15). In *S. liquefaciens*, chitinase genes, *chi* A and *chi* B are separately located on the chromosome and *chi* B is closely linked with chitobiase gene (*chi* C), common repressor gene (*chi* D), and inducer-related gene (*chi* E). Besides chitinases described above, several other chitinase genes have been cloned from *Vibrio harveyi* (16), *V. vulnificus* (17), *Streptomyces plicatus* (18), and *Bacillus circulans* WL-12 (19), and chitobiase genes have been cloned from *V. harveyi* (16) and *V. vulnificus* (17).

Although the number of reports dealing with microbial chitinases is increasing, 1) regulaton of chitinase genes, 2) secretion mechanism of chitinases through the cell membrane, 3) characteristics and hydrolysis mechanism of chitinases, and 4) direct evidences for antifungal activity of chitinases, are still not clear. For the investigation of chitinase, we chose *S. marcescens* as a chitinase producer. *S. marcescens* is an enteric bacterium closely related to *E. coli* and secretes several enzymes through the membrane into culture broth, including nucleases (20, 21), a lipase (22), protease (23, 24), and chitinases (3, 25). The fact that many of tools and manipulations of *E. coli* are applicable to *S. marcescens* is advantageous for studying *S. marcescens* chitinase (26, 27).

Recently, we reported the identification of five chitinase isozymes of *S. marcescens* (25). Here we described the gene cloning of 58 KD chitinase from *S. marcescens* and their expression in *E. coli*.

Materials and Methods

Chemicals and enzymes

Chitin of crab shell was purchased from Sigma Chemical Co. (St. Louis. Mo., USA), restriction enzymes, T_4 DNA ligase, and calf intestinal phosphatase (CIP) from Promega Biotech (Madison, Wi., USA), standard protein size marker and SDS-PAGE reagents from Bio-Rad (Richmond, Calif., USA). Other chemicals and reagents were purchased from Sigma Chemical Co. and were of reagent grade.

Bacterial strains and plasmid

S. marcescens ATCC 27117 was obtained from the Korean Collection for Type Cultures (KCTC, Korea) and used as a gene donor strain of extracellular chitinases. *E. coli* JM 109 was used as a host for transformation and plasmid pUC 19 as a cloning vehicle for all cloning experiments. *Fusarium oxysporum* used in this experiment was isolated from cucumber wilt disease in Korea and used as a test microorganism for antifungal activity of chitinase.

Media and culture conditions

S. marcescens and *E. coli* were routinely grown in Luria Bertani (LB) medium at 30℃ and 37℃, respectively. In order to screen *E. coli* transformants showing chitinolytic activity, the transformants were tooth-picked on LB agar plate supplemented with ampicillin (50 μg/m*l*), IPTG (0.1 mM), and swollen chitin (1%, w/v), and incubated for two weeks at 37℃. The swollen chitin was prepared by the method of Monreal and Reese (3).

Construction of a genomic library

Unless stated otherwise, all recombinant DNA techniques were performed as described by Sambrook *et al*. (28). Chromosomal DNA of *S. marcescens* was extracted by the method of Marmur (29). Plasmid DNA was extracted by the alkaline denaturation method of Ish-Horowitz and Burke (30) and purified by cesium chloride-ethidium bromide ultracentrifugation. Genomic DNA of *S. marcescens* was partially digested with *Eco*RI and electroeluted into three fractions of 3~5 Kb, 5~8 Kb, and 8~11 Kb with Biotrap 1000 (Schleicher & Schuell, Germany). Intact circular plasmid pUC 19 was completely digested with *Eco*RI, dephosphorylated with CIP, and the linear pUC 19 was electroeluted with Biotrap 1000. The chromosomal DNA fractions were ligated with linearized, dephosphorylated plasmid pUC 19 and the ligation mixture was used for transformation of *E. coli* JM109 by the method of Cohen *et al*. (31).

Preparation of chitinase enzyme

E. coli and *S. marcescens* were grown in 200 m*l* LB media with or without ampicillin and the culture supernatants were collected by centrifugation at

5000 rpm (Beckman, rotor JA-10) for 10 min. Proteins in culture supernatant were precipitated by 80% ammonium sulfate saturation and collected by centrifugation at 15,000 rpm (Beckman, rotor JA-20) for 30 min. The protein precipitate was dissolved and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). From 200 m*l* of culture supernatant, final 10 m*l* of extracellular enzyme fraction was obtained. For the preparation of intracellular enzyme, the washed cell suspension was disrupted by sonication (Vibra-cell, Sonics & Materials Inc., Conn., USA) for 1.5 min, and centrifuged at 10,000 rpm for 10 min, and then the supernatant was collected. These enzyme fractions were used for the chitinase assay and SDS-PAGE.

Chitinase assay

For the colorimetric assay of chitinase, the reaction mixture contained 700 μ*l* of 0.5% (w/v) swollen chitin in 50 mM potassium phosphate buffer (pH 6.6) and 700 μ*l* of enzyme solution. The enzyme reactions were conducted in shaking incubator at 30℃, 150 rpm for 2 or 4 hr. Reducing sugars produced from chitin were measured by Somogyi method (32) using a standard curve for N-acetylglucosamine (NAG). One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μmole of NAG per min under the described conditions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and active staining of chitinase

SDS-PAGE was conducted according to the method of Laemmli (33) with SE600 electrophoresis system (Hoeffer Scientific Ins., Calif., USA). A slightly modified method of Trudel and Asselin (34) was used for the active staining of chitinase. 12% (w/v) polyacrylamide gel containing 0.01% (w/v) glycol chitin and 0.1% (w/v) SDS was used for electrophoresis. After electrophoresis, gel was incubated at 37℃ for 24 hr in 0.1 M sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100, washed with distilled water, and incubated for further 12 hr at 37℃. Gel was then stained with 0.01% (w/v) Calcofluor white M2R, a fluorescent dye, in 0.5 M Tris-HCl (pH 8.9) for 2 hr and destained in distilled water for 2 hr at room temperature. The chitinase-active bands appeared as dark under the UV-transilluminator.

Antifungal activity

Fusarium oxysporum was spotted on the center of potato dextrose agar plate and *E. coli* JM109(pUC19), *E. coli* JM109(pCHI 89), and *S. marcescens* were inoculated at its periphery. After the incubation at 30℃ for 5 days, the fungal growth inhibition was examined.

Results and Discussion

Cloning of chitinase gene

S. marcescens ATCC 27117 secretes five chitinase isozymes as reported in our previous paper (19). As shown in Fig. 1, *S. marcescens* ATCC 27117 hydrolyzed the swollen chitin on agar plate and exhibited chitin-clearance around colony when incubated for 2 days at 37℃. But, *E. coli* JM109 could not hydrolyze the swollen chitin and chitin-clearance was not found during the prolonged incubation

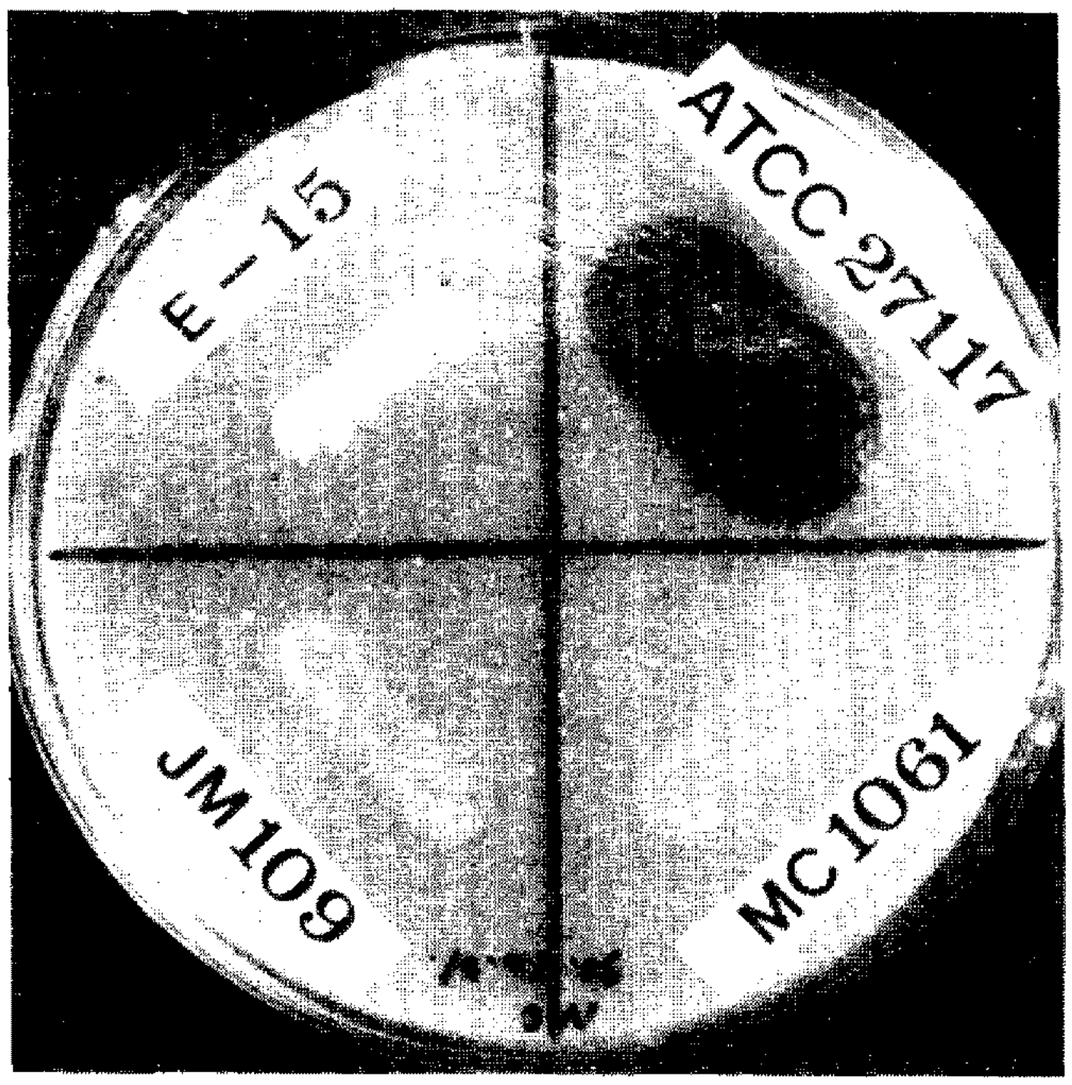

Fig. 1. Chitinolytic activities of *S. marcescens* and *E. coli* on swollen chitin agar plate.
E-15 and ATCC 27117 are *S. marcescens* strains. JM109 and MC1061 are *E. coli* strains.

up to 2 weeks. We chose *S. marcescens* ATCC 27117 as a gene donor, *E. coli* JM109 as a host for transformation, and the swollen chitin agar plate as a chitinase screening plate.

Chromosomal DNA of *S. marcescens* ATCC 27117 was partially digested with *Eco*RI and fractionated into 3~5 Kb, 5~8 Kb, and 8~11 Kb DNA fragments. The chromosomal DNA fractions were ligated with the dephosphorylated pUC 19 linearized by *Eco*RI digestion and the ligate was used to transform *E. coli* JM109. The transformants grown on LB plate containing 50 μg/m*l* of ampicillin were tooth-picked onto the swollen chitin agar plate to screen the chitinolytic clones. Of about 1×10^4 transformants, one chitinase-positive clone showing the chitin-clearance around the colony was detected against turbid swollen chitin. This clone contains about 8.9 Kb insert DNA in pUC 19 plasmid. To confirm the presence of chitinase gene in this insert chromosomal DNA, the recombinant plasmid DNA was purified from the chitinolytic clone and retransformed into *E. coli* JM109. As shown in Fig. 2, all of the retransformants examined showed the chitin-clearance around colonies by hydrolyzing the swollen chitin and contained the same plasmid DNA that was used for retransformation. These results indicated that a chitinase gene is present in the insert chromosomal DNA fragment of pUC 19. We called the chimeric plasmid of pUC 19 and 8.9 Kb chromosomal DNA fragment as pCHI 89.

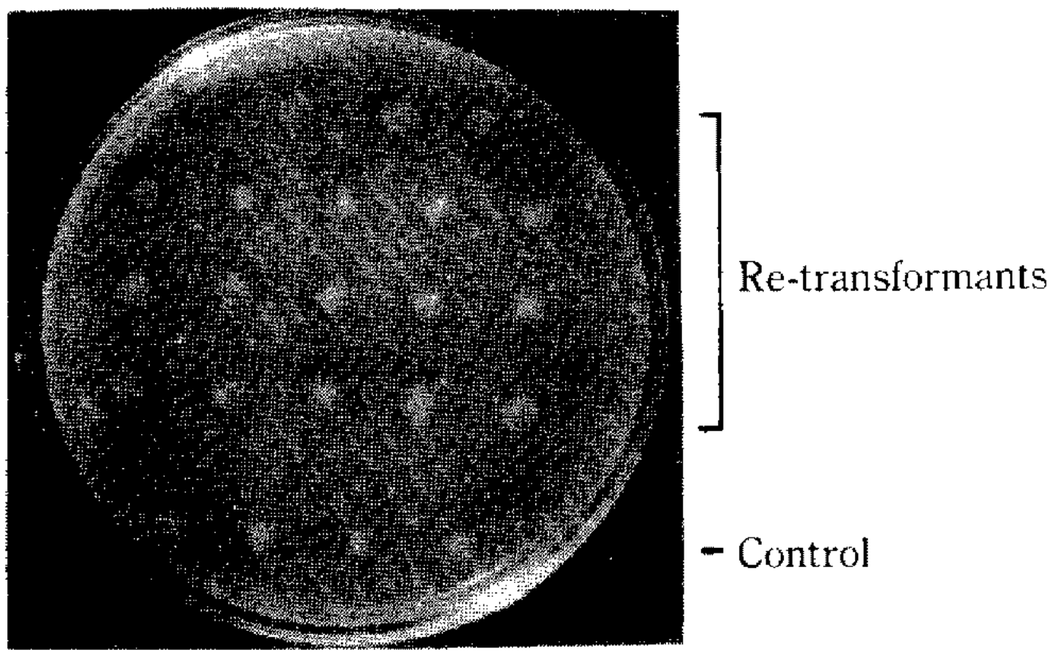

Fig. 2. Chitin-clearance of retransformants harboring a recombinant plasmid pCHI 89.
The recombinant plasmid pCHI 89 containing 8.9 Kb chromosomal DNA fragment in pUC 19 was isolated from a positive clone and used to retransform *E. coli* JM109. Retransformants and *E. coli* JM109(pUC 19) as a control were grown on swollen chitin agar plate for 7 days at 37℃.

Product analysis of pCHI 89

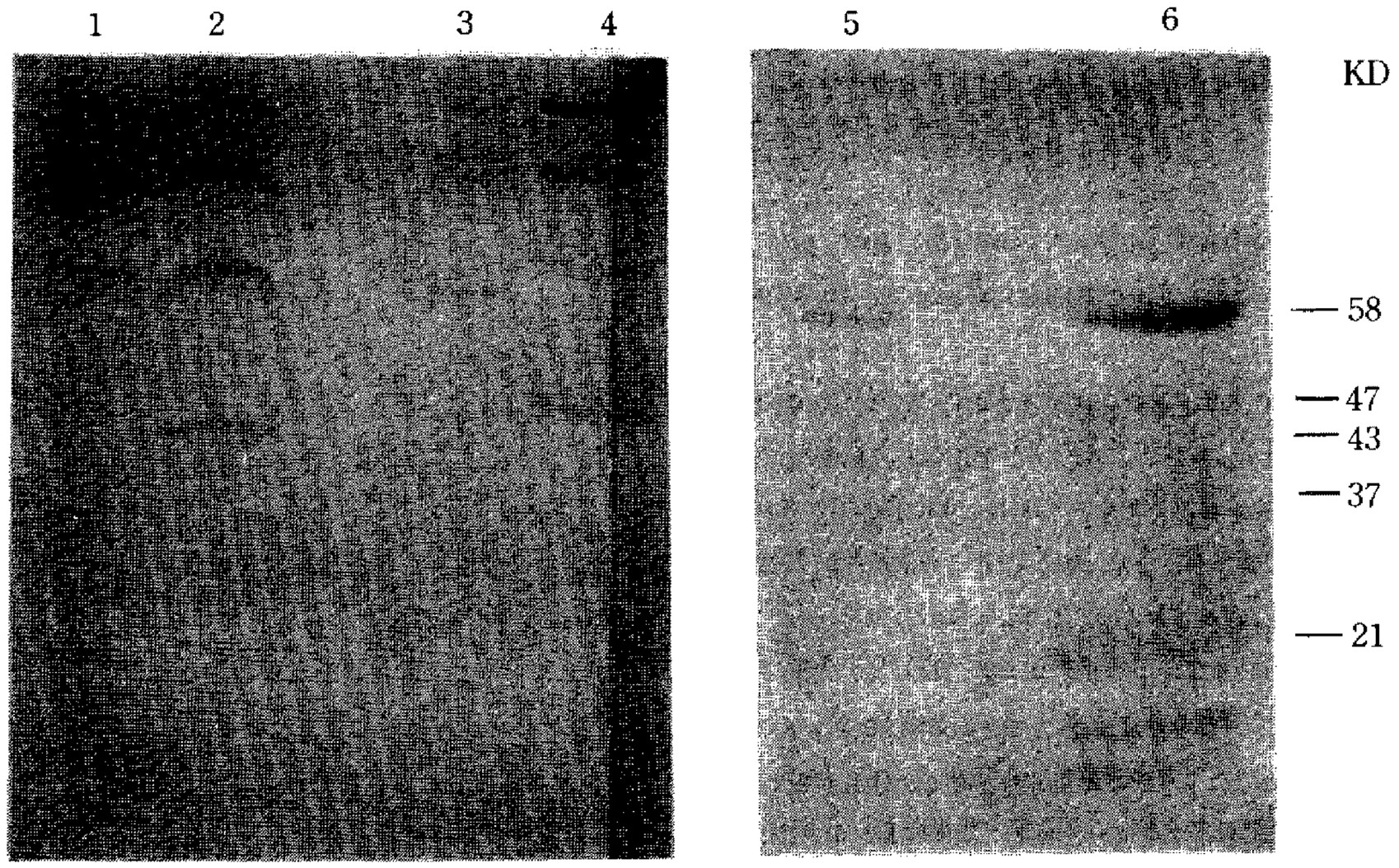

Fig. 3. SDS-PAGE and active staining of chitinase produced by *E. coli* JM109(pCHI 89).
E. coli JM109(pUC 19) and *E. coli* JM109(pCHI 89) were grown in LB medium containing 50 μg/m*l* ampicillin and 0.1 mM IPTG for 24 hr at 37℃. Intracellular (lanes 1, 2, and 5) and extracellular (lanes 3 and 4) enzymes were prepared by the methods described in Materials and Methods. Lanes: 1 and 3; *E. coli* JM109(pUC 19), 2, 4, and 5; *E. coli* JM109 (pCHI 89), 6; *S. marcescens* chitinases purified by chitin affinity chromatography.

To identify the gene product of pCHI 89, extracellular and intracellular enzyme fractions were prepared from *E. coli* JM109(pCHI 89) grown in LB broth containing 50 μg/m*l* of ampicillin and 0.1 mM IPTG for 24 hr. The enzyme fractions were analyzed by SDS-PAGE and active-staining method. As shown in Fig. 3, *E. coli* JM109(pUC 19) used as a control showed three faint bands having higher molecular weight than 58 KD chitinase band. We could not find out what kinds of enzymes they are. By judging from the fact that the active-staining method used in this experiment can stain not only chitinase but also lysozyme, the three faint bands might be related with the cell wall-hydrolyzing enzyme of *E. coli*. One additional thick band was found in *E. coli* JM109(pCHI 89) when compared with those of *E. coli* JM109(pUC 19). This thick band of *E. coli* JM109(pCHI 89) was exactly coincided with 58 KD chitinase band of *S. marcescens* used as a gene donor. This result suggested that plasmid pCHI 89 encodes the 58 KD chitinase gene of *S. marcescens*. We tried to measure the chitinase activity of *E. coli* JM109(pCHI 89) by colorimetric method. But we could not measure reliable chitinase activity, perhaps due to the low-level expression of chitinase gene in *E. coli*. 58 KD chitinase band was also found in the intracellular enzyme fraction as well as in the extracellular enzyme fraction of *E. coli* JM109(pCHI 89). The intensity of chitinase bands of two enzyme fractions was nearly the same, suggesting that about half of the chitinase is contained in the cell and the half is secreted into culture broth. In our previous paper (19), most of the 58 KD chitinase of *S. marcescens* was found to be secreted into culture broth.

Restriction enzyme map of pCHI 89

Restriction enzyme mapping of pCHI 89 was carried out by single and double restriction endonuclease digestions. 8.9 Kb insert DNA of pCHI 89 has no *Eco*RI site, two *Bam*HI sites, three *Hin*dIII sites, three *Sal*I sites, and four *Sac*II sites. Relative distances of cleavage sites of the restriction enzymes were decided by the total or partial digestion. The result is shown in Fig. 4. Insert DNA fragment of pCHI 89 showed somewhat different restriction enzyme map when compared with that of *chi* A gene

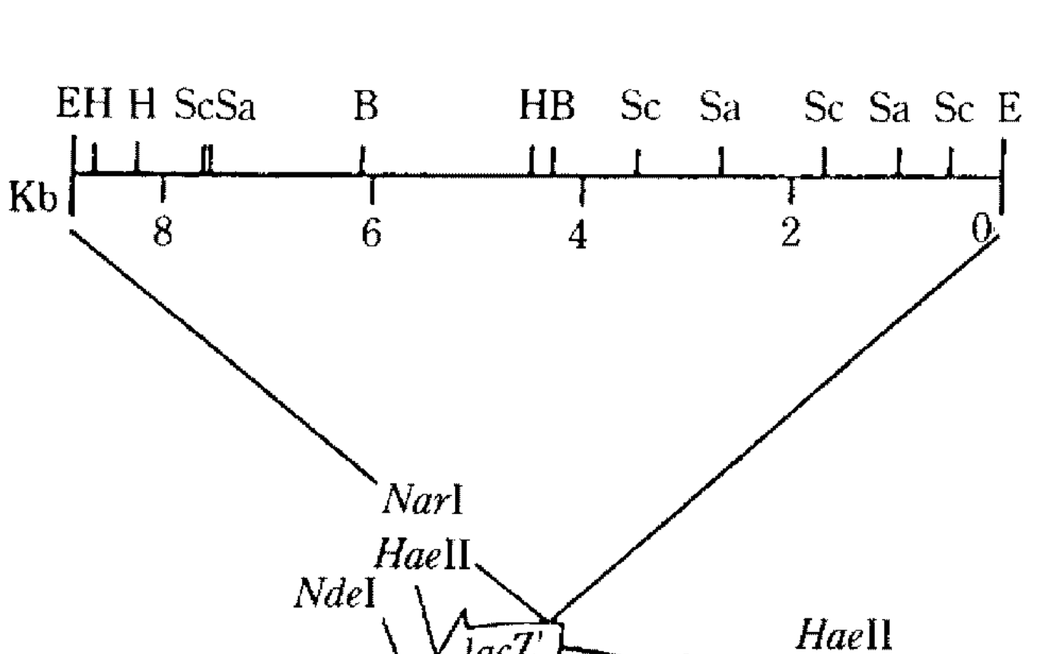

Fig. 4. Restriction endonuclease map of pCHI 89. Restriction endonucleases: E; *Eco*RI, H; *Hin*dIII, B; *Bam*HI, Sa; *Sal*I, and Sc; *Sac*II.

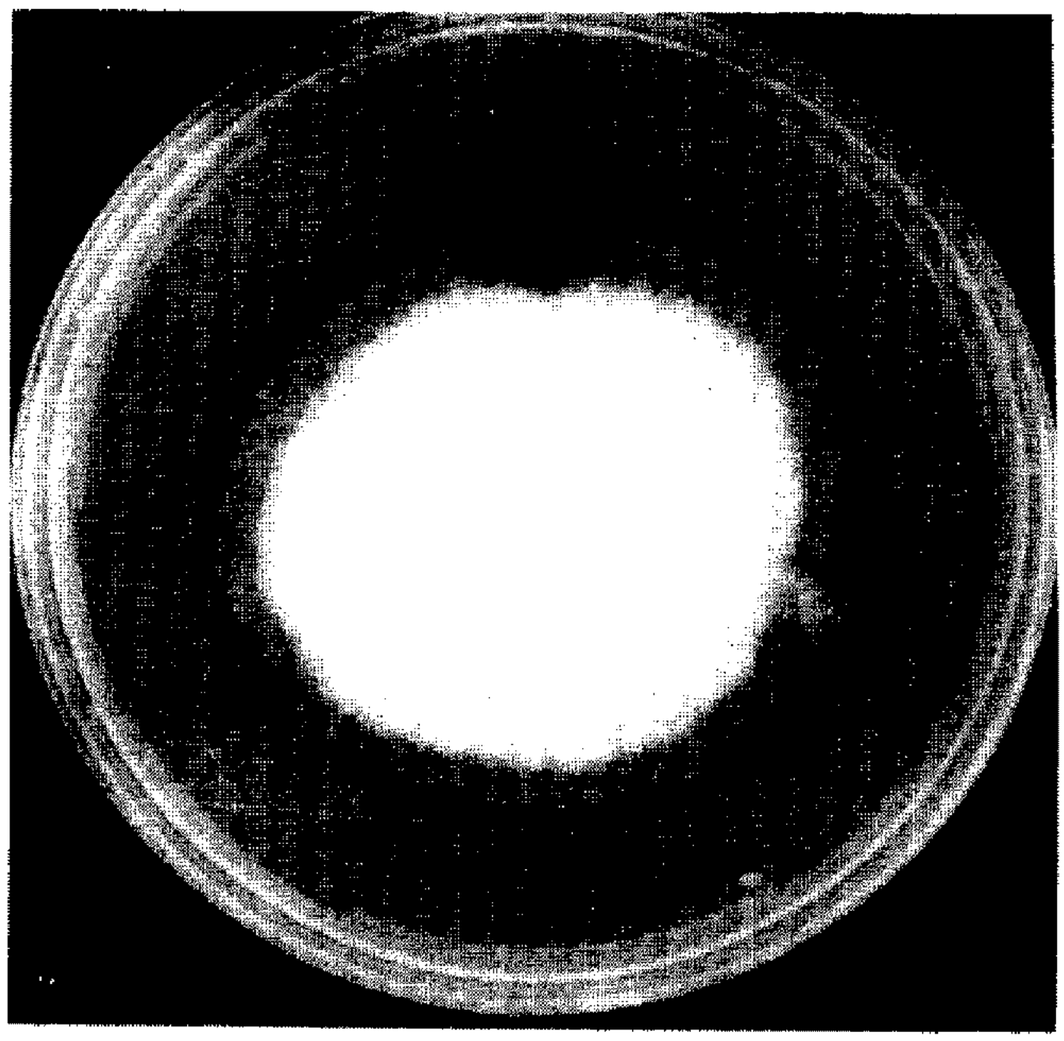

Fig. 5. Antifungal activity of *E. coli* JM109(pCHI 89) against a plant-pathogenic fungus, *Fusarium oxysporum*. A; *S. marcescens*, B; *E. coli* JM109(pUC 19), and C; *E. coli* JM109(pCHI 89).

encoding 58 KD chitinase of *S. marcescens* QMB 1466 (11). In spite of this difference, pCHI A and pCHI 89 encoded the same 58 KD chitinase of *S. marcencens*. We thought that the difference may be due to the different kinds of gene donor. Chitinase gene of pCHI A came from *S. marcescens* QMB 1466 and chitinase gene of pCHI 89 from *S. marcescens* ATCC 27117. DNA sequencing of chitinase gene may elucidate the clear differences between two chitinase genes. For this, we are in progress of subcloning and sequencing of the chitinase gene.

Antifungal activity of *E. coli* JM109(pCHI 89)

Chitinases of *S. marcescens* have been assumed to have antifungal activity for pathogenic fungi. We tested the antifungal activity of *E. coli* JM109(pCHI 89) encoding 58 KD chitinase of *S. marcescens*. As shown in Fig. 5, *E. coli* JM109(pUC 19) used as a control did not inhibit the growth of *Fusarium oxysporum*. But *E. coli* JM109(pCHI89) and *S. marcescens* inhibited the fungal growth. This result suggested that 58 KD chitinase is reponsible for a part of antifungal activities of *S. marcescens*.

요 약

본 연구에서는 *Serratia marcescens* ATCC 27117 균주로부터 키티나아제 유전자를 대장균으로 클로닝하고 발현시켰다. pUC 19 플라스미드를 이용하여 *S. marcescens*의 genomic library를 만들고 팽화된 키틴이 포함된 한천배지에서 키티나아제 활성을 가지는 클론을 선별하였다. 약 1×10^4 transformant들 중에서 키티나아제 활성을 보이는 하나의 클론을 선발하였으며 이것은 pUC 19 플라스미드속에 8.9 Kb 염색체 DNA 삽입단편을 가지고 있었다. 이 키티나아제 유전자는 SDS-PAGE와 활성염색법으로 확인해본 결과 *S. marcescens*의 5가지 키티나아제 아이소자임 중에서 58 KD 키티나아제를 coding하는 것으로 나타났다. 8.9 Kb DNA 삽입단편의 제한효소 지도를 작성하였고, 이 유전자를 함유한 대장균은 식물병원성 곰팡이 *Fusarium oxysporum*의 성장을 저해하는 것으로 나타났다.

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