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The Biocontrol Activity of *Chromobacterium* sp. Strain C-61 against *Rhizoctonia solani* Depends on the Productive Ability of Chitinase

Seur Kee Park[1]*, Myung-Chul Lee[2] and Gary E. Harman[3]

[1]*Department of Agricultural Biology, Suncheon National University, Sunchon 540-070, Korea*

[2]*Division of Cytogenetics, National Institute of Agricultural Science and Technology, RDA, Suwon 441-707, Korea*

[3]*Department of Horticultural Science and Plant Pathology, Cornell University, Geneva, NY 14456, USA*

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A chitinolytic bacterium, *Chromobacterium* sp. strain C-61, was found strongly antagonistic to *Rhizoctonia solani*, a causal agent of damping-off of eggplant. In this study, the biocontrol activity and enzymatic characteristics of strain C-61 were compared with its four Tn*5* insertion mutants (C61-A, -B, -C, and -D) that had lower chitinolytic ability. The chitinase activity of a 2-day old culture was about 76%, 49% and 6% level in C61-A, C61-B and in C61-C, respectively, compared with that of strain C-61. The β-*N*-acetylhexosaminidase (Nahase) activity was little detected in strain C-61 but increased largely in C-61A, C61-B and C61-C. Activities of chitinase and Nahase appeared to be negatively correlated in these strains. Another mutant, C-61D, produced no detectable extracellular chitinase and Nahase. The *in vitro* and *in vivo* biocontrol activities of strain C-61 and its mutants were closely related to their ability to produce chitinase but not Nahase. No significant differences in population densities between strain C-61 and its mutants were observed in soil around eggplant roots. The results of SDS-PAGE and isoelectrofocusing showed that a major chitinase of strain C-61 is 54-kDa with pI of approximately 8.5. This study provides evidence that the biocontrol activity of *Chromobacterium* sp. strain C-61 against *Rhizoctonia solani* depends on the ability to produce chitinase with molecular weight of 54-kDa and pI of 8.5.

Keywords : biocontrol activity, chitinase, *Chromobacterium* sp., damping-off, eggplant, *Rhizoctonia solani*, Tn*5* insertion mutants

Many studies on biological control of soil-borne plant pathogens have been conducted in Korea (Jee et al., 1988; Kim and Hwang, 1992; Park and Kim, 1989; Sen et al., 2002). The mechanisms of biological control are generally classified as antibiosis, parasitism/predation, induced resistance and competition. The parasitism is primarily occurred by cell wall degrading enzymes from antagonists. The chitin is a major structural component of many fungal cell walls. Thus, chitinolytic enzymes, chitinases, are believed to be involved in the biological control of plant pathogens by other microbes including bacteria in the genera *Aeromonas* (Inbar and Chet, 1991), *Serratia* (Ordentlich et al., 1988), *Enterobacter* (Chernin et al., 1995), *Paenibacillus* and *Streptomyces* (Singh et al., 1999) as well as the fungi in the genus *Trichoderma* (Lorito et al., 1993). Chitinases produced by higher plants are considered to have a role in the defense of plants against attack by fungal pathogens (Sahai and Manocha, 1993).

A chitinolytic bacterium, the genus *Chromobacterium* was isolated from soil around eggplants in Korea and has designated it as strain C-61 (Park and Yoo, 1995). Strain C-61 was strongly antagonistic to *Rhizoctonia solani*, a causal agent of damping-off of eggplant. Its antagonistic ability and chitinase activity were much greater than those of *Chromobacterium* sp. strain C-72, *Serratia marcescens, Aeromonas caviae* or *Aeromonas hydrophila* (Park et al., 1995). The chitinase of strain C-61 was considered to play an important role in the control of *R. solani.*

The role of chitinase in biological control of plant pathogens was mainly supported from studies that chitinolytic bacteria or their purified enzymes inhibit fungal growth *in vitro* and *in vivo* (Chet et al., 1990; Lorito et al., 1994). However, their correlations may be more precisely elucidated by the mutants that were reduced in the productive ability of chitinase. In this study, mutants deficient in chitinolytic ability were selected by Tn*5* insertion mutation of strain C-61 and compared to the wild type strain for their ability to produce chitinolytic enzymes and to suppress damping-off of eggplant. The chitinase of strain C-61 was also characterized by comparison with those of each mutant.

Materials and Methods

Organisms and culture conditions. Strain C-61 isolated from soils around eggplants near Suncheon, Korea, was

*Corresponding author.
Phone) +82-61-750-3864, FAX) +82-61-750-3208
E-mail) parksk@sunchon.ac.kr

more similar in its physiological and biochemical properties to *Chromobacterium violaceum* than to any other microbial species (Park and Yoo, 1995). However, in its 16S rRNA gene sequence, strain C-61 had an identity of 93% to *C. violaceum,* which was inadequate to assign this strain definitively to the species *C. vioalaceum* (MIDI Labs, USA). Therefore, we describe strain 61 as a *Chromobacterium* species.

The strain was naturally resistant to 200 μg ampicillin (Ap) ml^{-1} (Park and Yoo, 1995), and was grown on nutrient broth (Difco, USA) containing 50 μg Ap ml^{-1} at 28°C. *Escherichia coli* WA 803 containing the suicide vector pGS*9*::Tn*5* was grown at 37°C on Luria-Bertani (LB) broth or LB plates containing 50 μg kanamycin (Km) ml^{-1}. *Rhizoctonia solani* was grown on potato dextrose agar (PDA, Difco, USA) at 28°C. Strain C-61 and its mutants were stored at -70°C in NB containing 30% glycerol.

Transposon mutagenesis and screening of Chia$^-$ mutants. Tn*5*, which codes for kanamycin resistance (De Bruijin and Lupski, 1984) was contained in the plasmid vector pGS*9*. The plasmid was transferred into strain C-61 using the following procedure. *E. coli* donor strain WA 803 was grown overnight at 37°C on a rotary shaking incubator in LB broth containing Km. Strain C-61 recipient cells were grown at 28°C on a rotary shaking incubator in NB containing Ap for 24 hrs. Approximately 10^9 donor cells and 10^9 recipient cells were precipitated and suspended in 500 μl of sterilized H_20. The mixtures of donor and recipient cells suspension (1:1) were transferred to nutrient agar (NA) plates and incubated for 12 hrs at 28°C. Transconfugants were selected on NA plates containing Ap and Km, and then were transferred to chitin agar plates (Sneh, 1981). Mutants reduced in ability to degrade chitin (Chia$^-$ mutants) were selected on the basis of reduction in size of clear zones.

Preparation of crude enzyme. Cells of strain C-61 or its mutants were grown in NB for 1 day and 150 μl of these cultures were inoculated in Erlenmeyer flasks (500 ml) containing 150 ml of the chitin medium, and then incubated on a rotary shaker (180 rpm) at 28°C. Aliquots of the culture solution (30 ml) were collected every day. Culture supernatants were obtained by centrifugation at 10,000 g for 20 min. Solid ammonium sulfate was slowly added to the culture supernatant at 4°C to give 80% saturation. After incubation overnight with continuous stirring, the precipitate was collected by centrifugation at 10,000 g for 30 min, and dissolved in 300 μl of 20 mM sodium acetate buffer, pH 5.0. The solutions were used as crude enzymes after dialysis, and their protein concentrations were determined by protein assay kit (Sigma, USA) using bovine serum albumin as a reference protein.

Enzyme assays. Chitinase activity was determined by amount of *N*-acetyl-β-D-glucosamine (GlcNAc) that released from colloidal chitin or from glycol chitin. Colloidal chitin was prepared from chitin (Sigma, USA) by the method described in basic plant pathology methods (Dhingra and Sinclair, 1987). Glycol chitin was obtained by acetylation of glycol chitosan (Sigma, G7753) using the method of Trudel and Asselin (Trudel and Asselin, 1989). A reaction mixture (1.5 ml) containing 0.5% (w/v) colloidal or glycol chitin and an enzyme solution in 20 mM sodium acetate buffer, pH 5.0, was incubated for 30 min at 37°C. After boiling for 5 min, pellets were removed by centrifugation at 10,000 g for 5 min. A mixture of 1.3 ml color reagent solution (0.05% potassium ferricyanide in 0.5 M sodium carbonate) and 1.0 ml supernatant solution were boiled for 15 min and were measured at 420 nm. The OD values were calculated as amount of reducing sugar using a standard curve for *N*-acetyl-β-D-glucosamine (Sigma, USA). A unit of enzyme activity is defined as the amount of enzyme required to release 1 μmole of GlcNAc in the 30 minutes per μl of enzyme.

Various synthetic substrates were also used in order to obtain information on the substrate specificity and possible modes of action of enzymes from the *Chromobacterium* strain. The substrates used included using *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (*p*NP-GlcNAc). This substrate is specific for the Nahase (Tronsmo and Harman, 1993). Two other substrates tested were *p*-nitrophenyl-β-D-*N,N*-diacetylchitobiose (*p*NP-(GlcNAc)2) and *p*-NP-β-D-*N,N,N*-triacetylchitotriose (*p*NP-(GlcNAc) 3) (all from Sigma). With all of these nitrophenyl substrates, the reaction mixture (0.1 ml) contained 10 mM of the substrate, and an enzyme solution in 20 mM sodium acetate buffer, pH 5.0. After incubation for 30 min at 37°C, the reaction was stopped by addition of 0.1 ml of 0.2M Na_2CO_3 and measured at 405 nm. The OD values were calculated as amount of *p*-nitrophenol and a unit of enzyme activity is defined as the amount of enzyme, which released 1 μmole of *p*-nitrophenol per min per μl of enzyme.

Gel electrophoresis. Crude enzymes were partially purified using Rotofor cell (Bio-Rad, USA). The culture solutions (300 ml) in the maximum optimum period of each strain were precipitated with solid ammonium sulfate as described above. The precipitates were dissolved in 6 ml of 20 mM sodium acetate buffer (pH 5.0) and were desalted by dialysis. The proteins were mixed with 1.2 ml of 40% ampholines with pH ranges of 3-10 and with 10.8 ml of deionized H_2O. The samples were loaded in Mini Rotofor Chamber and run at 12W constant power for 4 hours with

0.1 M H_3PO_4 for the anode and 0.1 M NaOH for the cathode. After harvesting, the fractions containing chitinases were determined using SDS-PAGE analysis and activity assay. The fractions were pooled and dialyzed to remove ampoholytes.

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (Laemmli, 1970) in 10% polyacrylamide gels containing 0.01% glycol chitin. Samples were mixed with an equal volume of 30% (W/V) sucrose, 5.0% (W/V) SDS in 250 mM Tris-HCL (pH 6.8) and 0.02% (W/V) bromophenol blue without 2-mercaptoethanol but were not boiled so that enzyme activity could be detected. After electrophoresis, a gel was stained with silver nitrate (Hames and Rickwood, 1990), and their molecular weights were determined by comparison with Silver Stain SDS-PAGE standards, low range (Bio-Rad).

Detection of bands of chitinase activity was conducted by method of Trudel and Asselin (Trudel and Asselin, 1989). The SDS-PAGE gels contained 0.01% glycol chitin and bands of chitinases were visualized using the following procedure. The gels were incubated at 37°C for 3 hours with slow shaking in 100 mM sodium acetate buffer, pH 5.0, containing 1% (W/V) Triton X-100, and then stained with 0.01% (W/V) Calcoflour White M2R (Sigma, USA) in 500 mM Tris-HCl, pH 8.9 for 5 min. After destaining by soaking for at least one hour at room temperature in distilled water, lytic zones were observed under the UV-transilluminator.

Isoelectrofocusing (IEF) was performed on 10% agarose gel containing ampholine of pH 3.5-10.0 using Multiphor II system (Pharmacia Biotech, USA). After isoelectrofocusing, the gel was stained with silver nitrate and isoelectric points were determined by comparison with IEF standards (Bio-Rad, USA).

***In vitro* fungal inhibition assay.** Inhibition of *R. solani* growth by bacterial strains *in vitro* was assayed on PDA plates. Samples from overnight cultures of strain C-61 and its mutants in NB were spotted on the edge of the plates and dried. An agar disks (0.5 cm in diameter) of the pathogen grown on PDA for 3 days at 28°C were placed in the center of the plate. After incubation at 28°C for 3 or 4 days, the inhibition zone between the edges of the bacterial colony and the fungal mycelium was observed.

Damping-off suppression assay. Suppression of damping-off of eggplant by bacterial strains was assayed in rectangular plastic pots (15 × 6 × 10 cm). Cultures of *R. solani* were grown for 7 days on PDA and ground in a Waring blender, and then were inoculated into autoclaved oatmeal-soil (2:8 w/w) in 500 ml flasks. After incubation for 15 days at 28°C, the colonized oatmeal-soils were blended and sieved through a seize with openings of 0.25 mm. Inoculum from the oatmeal-soil mixtures were added to the autoclaved soils from eggplant fields (sandy loam) at the rate of 1% (w/w) in the plastic pots. Four weeks old seedlings of eggplant were planted in two rows with 5 plants per row in the plastic pots filled with the infested soil and cultivated in the green house. On the day of planting, 50 ml of bacterial suspensions (ca. 10^9 cells/ml), which were grown in NB containing Ap for 24 hrs at 28°C, was drenched onto the surface of soil in each pot. Sterile distilled water was added to different pots as a control. Damping-off incidence was measured daily until 25 days after treatment. Two experiments were conducted in a greenhouse with three replicates per an experiment. In addition, the bacterial population inoculated in soil of eggplants was determined using a dilution plate method on the chitin agar plates containing 50 μg Ap ml^{-1} for strain C-61, and 50 μg Ap ml^{-1} and 50 μg Km ml^{-1} for mutants.

Southern blot hybridization. Genomic DNA was isolated by a modification of the process of Ausbel et al. (Ausbel et al., 1992). A pellet from bacterial cultures (1.5 ml) was resuspended in 0.33 ml of 50 mM glucose, 25 mM Tris (pH 8.0) and 10 mM EDTA with 2 mg/ml lysozyme and incubated for 20 min at room temperature. It was incubated for 10 min at 50°C after adding 8.4 μl of 10% SDS, for 1 hour at 37°C after adding 13.4 μl of 2.5 mg/ml RNAse A, for 10 min at 50°C after adding 17 μl of 0.5 M EDTA, and then overnight at 37°C after adding 5 μl of 10 mg/ml proteinase K. The DNA was extracted once with phenol, once with phenol:chloroform, then with chloroform and finally spooled out after adding isopropanol. The DNA was washed, dried and respended in 100 μl TE buffer. pGS*9*::Tn*5* was isolated by the alkaline lysate procedure (Crosa and Falkow, 1981), and then purified following addition of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (Maniatis et al., 1982). The purified plasmid was restricted with *Hpa*I and electrophoresed. Bands of Tn*5* with about 5.4 kb were excised and extracted by treatment with Agarase (BMB, Germany). The fragments were labeled with dioxigenin according to the manufacturers directions (DIG DNA Labeling and Detection Kit, BMB, Germany) for use as a probe. Genomic DNA for southern analyses was digested with *Eco*RI, and then was run in 0.8% agarose gels with Tris-borate buffer. The agarose gel was acid-depurinated in 0.25 M HCl for 20 min to facilitate transfer of large fragments. DNA fragments then transferred to nitrocellulose (HybondTM-N, Amersham, USA) by the method of Southern (Maniatis et al., 1982). The blotted nitrocellulose filters were prehybridized, hybridized, washed and detected according to the instructions of the

manufacturers.

Results

Isolation and identification of Tn*5* insertion mutants. Ap^r and Km^r transconjugants of strain C-61 were recovered at a frequency of 8.4×10^{-8} per initial recipient cell. Eleven mutants, which produced smaller clear zones on chitin agar plates, were obtained from 2,560 transconjugants and classified as 4 groups based on sizes of the clear zones and on results of Southern blot hybridization. One representative strain from each group was selected for further experiments and designated as C61-A, C61-B, C61-C or C61-D. When incubated for 6 days at 28°C, a clear zone of strain C-61 was largest and followed by order of C61-A, -B and C. Strain and C61-D did not clear chitin within 6 days (Fig. 1A).

To identify Tn*5* insertion in the mutants, *Eco*RI-digested total DNAs of strain C-61 and its mutants were hybridized with a labeled 5.4 kb *Hpa*I fragment of Tn*5*. Tn*5* has no *Eco*RI sites and numbers of hybridized fragments indicate numbers of Tn*5* elements inserted in the DNAs. Strain C-61 did not contain any Tn*5* element, but its mutants were contained one or more Tn*5* elements in different sizes of DNA fragments. The Tn*5* elements were detected in a single band of about 15-kb fragment in C61-A, 2 bands of >23- and 13.0-kb in C61-B, a band of about 9.6 kb in C61-C, and a band of about 12.4 kb in C61-D (Fig. 2).

Suppression of *Rhizoctonia in vitro* and *in vivo*. Inhibition of hyphal growth of *R.* solani on PDA was greatest with strain C-61. C61-A was somewhat inhibitiory but no inhibition was detected with strains C61-B or C61-C (Fig. 1B). Strain C61-D also lacked ability to inhibit growth of the pathogen (data not shown).

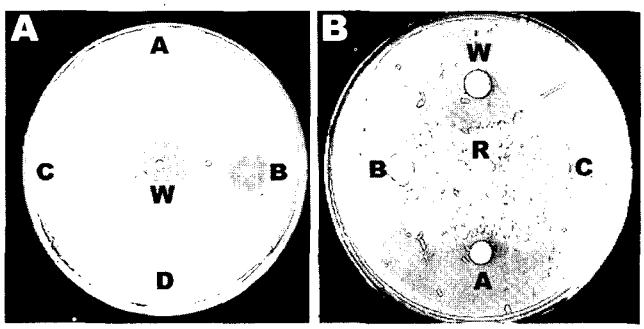

Fig. 1. Clearing of colloidal chitin (A) and inhibition zones against *Rhizoctonia solani* (B) induced by growth of colonies of *Chromobacterium* strain C-61 and its mutants. Clearing zones were observed 6 days after inoculation of the bacterium on medium containing colloidal chitin and inhibition of *R. solani* was observed 4 days after inoculation of PDA with the bacteria and the pathogen. Within each figure W represents strain C-61, A represents strain C61-A, B represents C61-B, C represents C61-C and D represents C61-D, R represents *R. solani*. Strain C61-D had no ability to control *R. solani* in this assay (data not shown).

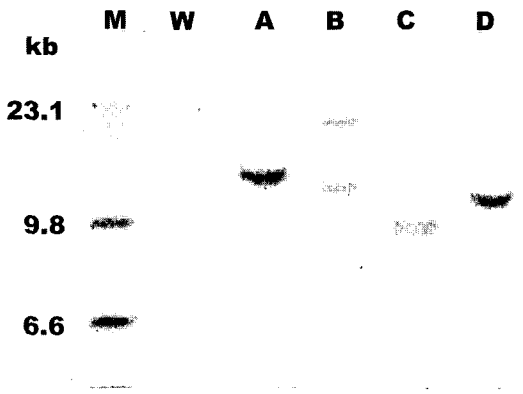

Fig. 2. Southern blot analysis of *Eco*Ri-digested total DNA from *Chromobacterium* strain C-61 (W) and its mutants; C61-A (A), C61-B (B), C61-C (C) and C61-D (D), and DIG-labeled *Hin*dIII DNA (M). Blots of digested DNA were hybridized with digoxigenin-labeled 5.4 kb *Hpa*I fragments of Tn*5*.

The suppression of damping-off in eggplant soil infested with *R. solani* was also highest in treatment of strain C-61, and followed by C61-A and C61-B. However, strains C61-C or C61-D did not significantly suppress damping-off relative to the nontreated check. To determine whether the reduced suppression of the mutants was related to lesser abilities of the strains to colonize roots, the density of strains in the rhizosphere was investigated. Numbers of both strain C-61 and its mutants continuously decreased until 20 days after treatment, and maintained approximately ranges of 5×10^4 to 6×10^5 CFUg^{-1} soil. No significant differences were observed in population densities between strain C-61 and its mutants (Table 1).

Table 1. Effect of *Chromobacterium* strain C-61 and its mutants on suppression of damping-off of eggplant caused by *Rhizoctonia solani* and of population densities of the bacteria in soil around roots twenty days after planting

Strain	Diseased plants (%)[x,y]	Bacterial numbers (log cfu/g soil)[x,y]
None	63 a	Not done
C-61	14 c	4.9 a
C-61A	33 b	5.6 a
C-61B	50 ab	4.5 a
C-61C	63 a	5.0 a
C-61D	61 a	4.8 a

[x] Bacterial cell concentrations used to treat soil ranged from 5.9×10^7 to 2.5×10^8 cfu/g soil.

[y] Values are means of two experiments with three replicates per treatment. Values followed by similar letters are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Activity of chitinolytic enzymes. There was a correlation between activity of the enzymes on different substrates when the various strains were compared across time between strains, with the exception of when *p*NP-GlcNAc. For example, in 2-day old cultures, the correlation between activity as measured on collodial chitin and glycol chitin had an r2 value of 0.95, that between colloidal chitin and *p*NP-(GlcNAc)2 had a r2 value of 0.96, and between collidal chitin and *p*NP-(GlcNAc)3had an r2 value of 0.73. In culture filtrates from 5 days old cultures, a similar relationship was evident although r2 values were lower, 0.25, 0.84 and 0.56. In all cases described above except the comparison of activity of 5 days old culture filtrates when activity of glycol chitin and colloidal chitin, slopes and positions of linear regressions were also very similar. Therefore, in most cases, all of the substrates gave similar comparisons between strains and we therefore will present data obtained only from colloidal chitin as representative of all substrates except *p*NP-GlcNAc.

With *p*NP-GlcNAc as the substrate (β-N-acetylhexosaminidases; Nahases activity), almost no activity could be detected in culture filtrates from strain C-61 at any time. However, with strains C61-A, C61-B and C61-C, Nahase activity increased as time of culture increased. At 5 days of culture, Nahase activities of C61-A, C61-B and C61-C increased 18-fold, 24-fold and 43-fold, respectively, compared with those of strain C-61 (Fig 3A). Strain C-61D produced no detectable Nahases even after 7 days of incubation (data not shown).

With colloidal chitin (chitinase activity), culture filtrates of strain C-61 showed the greatest levels of chitinase activity and activity was highest after 2 days of culture. At this time, chitinase activity of C61-A, C61-B and C61-C was about 76%, 49% and 6% level, respectively, compared with those of strain C-61. Subsequently, chitinase activity in culture filtrates of strain C-61 and C61-A decreased while activity with C61-B remained essentially constant over time and the chitinase activity of C61-C increased. At day 5, the chitinase levels in culture filtrates from all strains were similar (Fig. 3B). Chitinase activity was not detected in C61-D even after 7 days of culture.

The levels of protein secreted into culture filtrates generally followed the pattern of the chitinase activity. At two days, protein levels were greatest with strain C-61. Subsequent patterns of increase or decrease were very similar to that described for chitinase (Fig. 3C). Almost no protein could be detected in culture filtrates of C61-D; only 0.1 mg/ml was detected even after 7 days of culture.

Characteristics of chitinases. The extracellular proteins from strain C-61 and its mutants were separated into alkaline and acidic proteins using isolectric focusing over a range of pH 3 to 10. All of the chitinase activity was in the alkaline fractions; these contained about 75% of the total protein produced by strain C-61. These alkaline fractions were pooled and analyzed on SDS-PAGE gels.

Nearly all of the alkaline proteins present in the various fractions could be accounted for chitinase bands. With strain C-61, four major and two minor protein bands were visible; the four major bands detected with silver staining all corresponded to activity bands as detected with glycol chitin. Their molecular weights were estimated to be 54-, 53-, 52-, and 51-kDa. Strain C61-A had no chitinase activity at 51-kDa, while both the 51- and 52-kDa bands were absent in C61-B, and the 51-, 52-, and 53-kDa bands were absent in strain C61-C. The 54-kDa band was present in all of these strains, but not in C-61D (Fig. 4A, B).

These proteins were analyzed further on isoelectrofocusing gel. The major bands had pI values of 8.5 to greater than 9.6. We could not find the 54-kDa band in

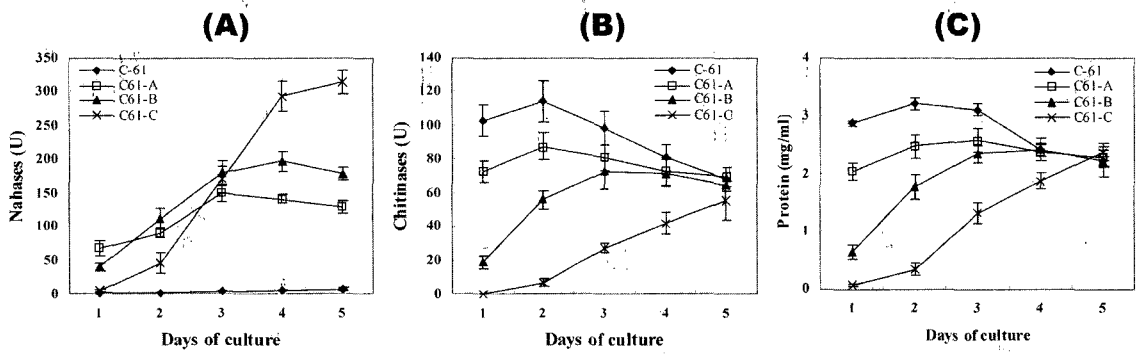

Fig. 3. Activity of β-*N*-acetylhexosaminidases (Nahases) (A) and chitinases (B), and protein concentration (C) of extracellular proteins of *Chromobacterium* strain C-61(C-61) and its mutants (C61-A, -B, and -C) according to culture periods. A unit of Nahases activity was defined as the amount of enzyme, which released 1 μmole of *p*-nitrophenol per min per μl of enzyme using *p*-nitrophenyl-β-D-*N*-acetylglucosaminide as substrate. A unit of chitinases was defined as the amount of enzyme required to release 1 μmole of GlcNAc in the 30 minutes per μl of enzyme using colloidal chitin.

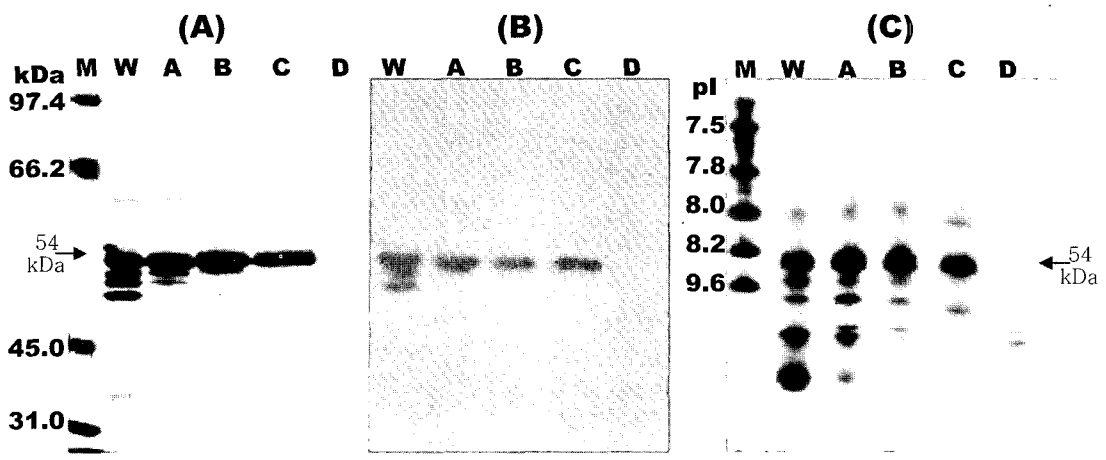

Fig. 4. Bands of protein (A) and chitinase activity (B) after SDS-PAGE and bands of protein after isoelectrofousing (C) with partial purified proteins of *Chromobacterium* strain C-61(W) and its mutants; C61-A (A), C61-B (B), C61-C (C) and C61-D (D). After SDS-PAGE on gels containing 0.01% glycol chitin, protein bands were detected by silver nitrate staining and chitinase activities were detected under UV illuminator after staining with Calcofulor White M2R. After isoelectrofocusing on starch gel containing ampholine of pI 3.5-10, protein bands were detected by silver nitrate staining. Molecular weight markers (M) are shown on the left.

proteins from strain C-61 and C61-A because of several bands. However, the 54-kDa protein was a principal band in C61-C and not present in C61-D as indicated by the SDS-PAGE gel. Based on the proteins of these strains, the pI of 54-kDa protein was estimated to be 8.5 (Fig. 4C).

Discussion

Chitinolytic enzymes produced by bacteria and fungi have been postulated to play an important role in the biocontrol of fungal pathogenic fungi (Chernin et al., 1995; Inbar and Chet, 1991; Lorito et al., 1993; Ordentlich et al., 1988). However, these results were mainly supported from studies that chitinolytic bacteria or their purified enzymes inhibit fungal growth *in vitro* and *in vivo* (Chet et al., 1990; Lorito et al., 1994). In this study, we showed relation between chitinolytic ability of *Chromobacterium* sp. strain C-61 and their biocontrol ability against *R.solani* using wild type and its mutants, and also characteristics of major chitinase related to the biocontrol.

The wild type strain C-61 had high level of chitinolytic ability and biocontrol activity *in vitro* and *in vivo* but a mutant C61-D had no chitinolytic ability and biocontrol activity, indicating that chitinolytic enzyme is involved in the biocontrol activity. In the mutants C61-A and C61-B with lower chitinolytic ability than strain C-61, their biocontrol activity decreased as chitinolytic ability was reduced. These phenomena were observed in relation between strain C-61 and *Chromobacterium* sp. strain C-72, *S. marcescens, A. caviae* or *A. hydrophila* in the previous experiment (Park et al., 1995); i.e., their biocontrol activity was proportional to the chitinolytic ability. However, we could not suggest that the biocontrol is associated with chitinolytic enzyme, because it can be affected by other factors. In this study, relation between chitinolytic ability and biocontrol activity was compared between wild type and its mutants. The results suggested that the biocontrol activity of strain C-61 depends on the productive ability of chitinolytic enzyme.

The C61-C, which had lower chitinolytic ability than C61-B, showed no biocontrol activity *in vitro* and *in vivo*. This indicates that the biocontrol effect is not occurred in strains producing a little amount of chitinolytic enzyme; i.e., the biocontrol effect may be occurred only in strains with higher chitinolytic ability than that of C61-C. A number of chitinolytic bacteria present in soil but most of the strain can hardly inhibit *R. solani.* Such phenomena are considered to occur because the strains have low chitinolytic ability like C61-C.

The chitinolytic enzymes from strain C-61 and its mutants were assayed to elucidate relation between productive ability of specific chitinolytic enzyme and control activity. Strain C-61 is considered not to produce Nahases because its extracellular proteins are little active on the dimeric analogue *p*-nitrophenyl (pNP)-GlcNAc. It is very interested that strain C-61 do not produce Nahases, because most of chitinolytic bacteria containing *Enterobacter* sp. (Matsuo et al., 1999), *Serratia marcescens* (Tews et al., 1996), *Aeromonas hydrophila* (Lan et al., 2004) and *Alteromonas* sp. (Tsujibo et al., 1995) were reported to produce the enzyme. The proteins of strain C-61 were active on pNP-$(GlcNAc)_2$, pNP-$(GlcNAc)_3$, colloidal chitin and glycol chitin. It is clear that strain C-61 produces endochitinase, because it would not expected to be active on the tetrameric

analogue pNP-$(GlcNAc)_3$ if the proteins contained only a chitin1,4-β-chitobiosidases (chitobiosidase). However, it is difficult to explain whether strain C-61 produces only endochitinase or both of endochitinase and chitobiosidase, because the activity on the trimeric analogue pNP-$(GlcNAc)_2$ can be occurred by either chitobiosidase or endochitinase.

The Nahases activity increased largely in the mutants, and especially as chitinases activity decreased, activity of Nahases increased. In the strain C-61 and its mutants, the biocontrol activity was directly proportional to their chitinases activity but was contradictory to their Nahases activity. Especially, the C-61C that produced copious amounts of Nahases had no biocontrol activity. These results indicate that the biocontrol is not occurred by Nahases but is occurred by chitinases. The Nahase from *T. harzianum* was reported to have strong antifungal activity (Lorito et al., 1994). However, we do not know in this study whether Nahases from the mutants have no antifungal activity or their amounts are not enough for the biocontrol.

The principal extracellular alkaline proteins from strain C-61 are chitinases and further, alkaline proteins constitute about 75% of the total extracellular protein from this microbe. This conclusion was evidenced by the fact that nearly all of the protein bands observed in SDS-PAGE were chitinases and by the fact that the time course of chitinase activity very closely parallels the level of proteins in culture filtrates. Strain C-61 secreted four chitinases of 54-, 53-, 52- and 51-kD. Among the chitinases, a major chitinase was thought to be 54-kDa, because it was detected in all of strains with chitinolytic ability but not detected in the C61-D deficient to chitinolytic ability. Recently we also found that two chitinases of 54- and 53-kDa are detected in the extracellular proteins of *E. coli* containing a chitinase gene of 54-kDa. Therefore, the chitinases of 53-, 52- and 51-kDa is considered to result from cleavage of 54-kDa. The chitinase of 54-kDa was estimated to be approximately pI of 8.5. Molecular weight of chitinase of strain C-61 is quite different from chitinases of *Chromobacterium violaceum* (Chernin et al., 1998) and pI value is very high tendency as compared with those of other chitinolytic microorganisms; i.e., *Aeromonas hydrophila* (Yabuki et al., 1986), *Bacillus circulans* (Watanabe et al., 1990) and *Trichoderma harzianum* (Harman et al., 1993).

In summary, this work provides strong evidence that (a) chitinase from *Chromobacterium* are the primary extracellular proteins produced by this bacterium and that (b) they are responsible for the bacterium's activity against *R.solani* both *in vitro* and *in vivo*. Our results also suggest that the productive ability of chitinase is important in the control of plant disease using chitinolytic organisms. Even if bacteria or plants produce chitinase, the disease suppression may be not occurred if their productive ability is low like C61-B or C61-C.

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References

Ausubel, F. M., Brent, R., Kingston, R. R., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. 1992. Short protocols in molecular biology. Second edition. John Wiley and Sons. USA.

Chet, I., Ordentlich, A., Shapira, R. and Oppenheim, A. 1990. Mechanisms of biocontrol of soil-borne plant pathogens by rhizobacteria. *Plant Soil* 129:85-92.

Chernin, L. S., Ismailov, Z., Haren, S. and Chet, I. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ. Microbiol.* 57:2426-2428.

Chernin, L. S., Fuente, L., Sobolev, V., Haren, S., Vorgias, C., Oppenheim, A. and Chet, I. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.* 63:834-839.

Chernin, L. S., Winson, M. K., Thompson, J. M., Haran, S., Bycroft, B. W., Chet, I., Williams, P. and Stewart, G. S. 1998. Chitinolytic activity in *Chromobacterium violaceum*: substrate analysis and regulation by quorum sensing. *J. Bacteriol.* 180:4435-4441.

Crosa, J. H. and Falkow, S. 1981. Plasmids, PP. 266-282, In: *Manual of methods for general bacteriology*, Gerharadt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R., Phillips, G. B. (eds), American Society for Microbiology, Washing DC.

De Bruijin, F. J. and Lupski, J. R. 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids-a revew. *Gene* 27:131-149.

Dhingra, O. D. and Sinclair, J. B. 1987. Basic plant pathology methods. CRC Press, Inc. Florida.

Hames, B. D. and Rickwood, D. 1990. Gel electrophoresis of proteins. 2 nd ed. Oxford University Press, New York.

Harman, G. E., Hayes, C. K., Lorito, M., Broadway, R. M., Di Pietro, A., Peterbauer, C. and Tronsmo, A. 1993. Chitinolytic enzymes of *Trichoderma harzianum*:Purification of chitobiosidase and endochitinase. *Phytopathology* 83:313-318.

Inbar, J. and Chet, I. 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. *Soil. Biol. Biochem.* 23:973-978.

Jee, H. J., Nam, C. G. and Kim, C. H. 1988. Studies on biological control of Phytophthora blight of red-pepper I. isolation of

antagonists and evaluation of antagonistic activity *in vitro* and in greenhouse. *Korean J. Plant Pathol.* 4:305-312.

Kim, B. S. and Hwang, B. K. 1992. Isolation of antibiotic-producing bacteria antagonistic to *Phytophthora capsici* from pepper-growing soils and evaluation of their antibiotic activity. *Korean J. Plant Pathol.* 8:241-248.

Laemmli, UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.

Lan, X., Ozawa, N., Nishiwaki, N., Kodaira, R., Okazaki, M. and Shimosaka, M. 2004. Purification, cloning, and sequence analysis of beta-N-acetylglucosaminidase from the chitinolytic bacterium *Aeromonas hydrophila* strain SUWA-9. *Biosci. Biotechnol. Biochem.* 68:1082-1090.

Lorito, M., Harman, G. E., Hayes, C. K., Broadway, R. M., Tronsmo, A., Woo, S. L. and DiPietro, A. 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* 83:302-307.

Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L. and Harman, G. E. 1994. Purification, characterization, and synergisitic activity ofa glucan 1,3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. *Phytopathology* 84: 398-405.

Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. Molecular cloning:a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Matsuo, Y., Kurita, M., Park, J. K., Tanaka, K., Nakagawa, T., Kawamukai, M. and Matsuda, H. 1999. Purification, characterization and gene analysis of N-acetylglucosaminidase from *Enterobacter* sp. G-1. *Biosci. Biotechnol. Biochem.* 63:1261-1268.

Ordentlich, A., Elad, Y. and Chet, I. 1988. The role of chitinase of *Serratia marceacens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* 78:84-88.

Park, J. H. and Kim, H. K. 1989. Biological control of Phytophthora crown and root rot of greenhouse pepper with *Trichoderma harzianum* and *Enterobacter agglomerans* by improved method of application. *Korean J. Plant Pathol.* 5:1-12.

Park, S. K., Lee, H. Y. and Kim, K. C. 1995a. Antagonistic effect of chitinolytic bacteria on soilborne plant pathogens. *Korean J. Plant Pathol.* 11:47-52.

Park, S. K., Lee, H. Y. and Huh, J. W. 1995b. Production and some properties of chitinolytic enzymes by antagonistic bacteria. *Korean J. Plant Pathol.* 11:258-264.

Park, S. K. and Yoo, J. G. 1995. Isolation and identification of chitinolytic bacteria from soil. *J Agric. Sci. Res. Sunchon Nat'l Univ.* 9:95-102.

Sahai, A. S. and Manocha, M. S. 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. *FEMS Microbiol. Rev.* 11:317-338.

Shen, S. S., Choi, O. H., Lee, S. M. and Park, C. S. 2002. *In vitro* and *in vivo* activities of a biocontrol agent, *Serratia plymuthica* A21-4, against *Phytophthora capsici*. *Korean J. Plant Pathol.* 18:221-224.

Singh, P. P., Shin, Y. C. Park, C. S. and Chung, Y. R. 1999. Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. *Phytopathology* 89:92-99.

Sneh, B. 1981. Use of rhizosphere chitinolytic bacteria for biological control of *Fusarium oxysporum* in carnation. *Phytopath. Z.* 100:251-256.

Tews, I., Vincentelli, R. and Vorgias, C. E. 1996. N-Acetylglucosaminidase (chitobiase) from *Serratia marcescens*: gene sequence, and protein production and purification in *Escherichia coli*. *Gene* 170:63-67.

Tronsmo, A. and Harman, G. E. 1993. Detection and quantification of N-acetyl-β-D-glucosaminidase, chitobiosidase, and endochitinase in solutions and on gels. *Anal. Biochem.* 208:74-79.

Trudel, K. and Asselin, A. 1989. Detection of chitinase activity after polyacrylcmide gel electrophresis. *Anal. Biochem.* 178:362-366.

Tsujibo, H., Fujimoto, K., Kimura, Y., Miyamoto, K., Imada, C., Okami, Y. and Inamori, Y. 1995. Purification and characterization of beta-N-acetylglucosaminidase from *Alteromonas* sp. strain O-7. *Biosci. Biotechnol. Biochem.* 59:1135-1136.

Watanabe, T., Oyanagi, W., Suzuki, K. and Tanaka, H. 1990. Chitinase system of *Bacillus circulans* Wl-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* 172:4017-4022.

Yabuki, M., Mizushima, K., Amatatsu, T., Ando, A., Fujii, T., Shimada, M. and Yamashita, M. 1986. Purification and characterization of chitinasee and chitobiase produced by *Aeromonas hydrophila* subsp. *anaerogenes* A 52. *J. Gen. Appl. Microbiol.* 32:25-38.