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Characterization of Antibiotic Substance Produced by *Serratia plymuthica* A21-4 and the Biological Control Activity against Pepper Phytophthora Blight

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The biocontrol agent, *Serratia plymuthica* A21-4, has been developed for controlling pepper Phytophthora blight. *Serratia plymuthica* A21-4 strongly inhibits the mycelial growth, zoospore formation, and cyst germination of *Phytophthora capsici in vitro*. The application of a cell suspension of strain A21-4 to pepper plants in pot experiments and in greenhouse successfully controlled the disease. The bacteria produced a potent antifungal substance which was a key factor in the suppression of *Phytophthora capsici*. The most active chemical compound was isolated and purified by antifungal activity-guided fractionation. The chemical structure was identified as a chlorinated macrolide ($C_{23}H_{31}O_8Cl$) by spectroscopic (UV, IR, MS, and NMR) data, and was named macrocyclic lactone A21-4. The active compound significantly inhibited the formation of zoosporangia and zoospore and germination of cyst of *P. capsici* at concentrations lower than 0.0625 μg/ml. The effective concentrations of the macrocyclic lactone A21-4 for ED_{50} of mycelial growth inhibition were 0.25 μg/ml, 0.25 μg/ml, 0.30 μg/ml and 0.75 μg/ml against *P. capsici*, *Pythium ultimum*, *Sclerotinia sclerotiorum* and *Botrytis cinerea*, respectively.

Keywords : Biocontrol, Macrocyclic lactone A21-4, *Serratia plymuthica* A21-4, Phytophthora blight

Phytophthora blight of pepper is frequently destructive and is considered the principal limiting factor in pepper production in Korea (Hwang and Kim, 1995). Fungicide treatment is the main control means. However sometimes chemical treatments are not satisfactory and have undesirable effects on the environment (Jee et al., 1988). Although numerous attempts have been made to control the disease by biological means, most of them were not practically feasible. A new promising biocontrol agent has been selected and identified as *Serratia plymuthica* (Shen et al., 2002). In previous investigations, the bacterium was effective against *Phytophthora* and *Pythium* spp. and produced antifungal substances (Shen et al., 2002; 2005). Strobel et al. (1999) reported that a strain of *Serratia marcescens* produced Oocydin A which was effective only against Oomycetes. Thaning et al. (2001) selected a soil bacterium, *S. plymuthica* that effectively suppressed apothecial formation of *Sclerotinia sclerotiorum* and identified the chemical structure of the active compound responsible for suppression of the fungus. The both principal compounds are chlorinated macrolides and are known to be almost identical in chemical structure but their active spectrum is quite different.

In this investigation, a potential biocontrol agent, *Serratia plymuthica* A21-4, was evaluated *in vitro* and *in vivo* for the control of Phytophthora blight of pepper. The role of the key antifungal compound of the bacterium in disease suppression was confirmed by an antibiotic deficient mutant. The active chemical compound responsible for inhibition of *Phytophthora* spp. was identified and the chemical structure of active compound was determined.

Materials and Methods

Preparation of antagonistic bacterium and pathogen Cultures and growth media. *Serratia plymuthica* A21-4 was isolated from the root of onion (*Allium cepa* L.). Cultures of the strain were stored at −70°C in tryptic soy broth (TSB) containing 20% glycerol. Strain A21-4 was grown at 28°C in TSB. *Phytophthora capsici* Pa-61 (KACC 40476 NIAST) was grown on V8 juice agar (100 ml V8 juice, 900 ml distilled water, 1 g $CaCO_3$, 15 g agar).

Preparation of zoospores. Four to five ml of sterile distilled water was added to mycelial mats of *P. capsici* Pa-61 which were grown on V8 juice agar for 3 days, at 20-25°C under blue fluorescent light (16 hrs/day). The zoosporangia formed on the mycelia were collected and placed at 4°C for 10-30 min, and then placed at room

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temperature for the zoospores to form the zoosporangia. Liberated zoospores were collected and the concentration was adjusted to 10^4 zoospore/ml for the inoculum.

In vitro experiments for the suppression of *Phytophthora capsici*

Inhibition of zoosporangia and cyst germination. A loopful of bacterial cells of the strain A21-4 grown on TSA medium for 48 h was suspended in 0.1 M $MgSO_4$ and adjusted to a concentration of 10^8 cell/ml. Inhibition of cyst and zoosporangia germination of *P. capsici* Pa-61 by strain A21-4 *in vitro* was assayed on a glass slide. A suspension of cysts and zoosporangia of *P. capsici* (10^4 spores/ml) was mixed with an equal volume of bacterial suspension of A21-4 (10^8 cfu/ml), and the mixture was placed on a glass slide. The slides were kept in Petri plate at 30°C and the number of germinated cysts and zoosporangia were examined every 2 hours until 8 hours.

Inhibition of zoosporangia formation. Mycelial disks (8 mm in diameter) of *P. capsici* Pa-61 grown on V8 agar were transferred to a new Petri plate, covered with 3 ml of the bacterial suspension (10^8 cfu/ml), and illuminated with blue fluorescent light at 20-25°C, After 16 hours of incubation the number of zoosporangia was counted. The disks in the control plates were covered with 3 ml of sterilized water.

Experiments on the suppression of Phytophthora blight

Pot experiment. The roots of 50-day-old pepper seedlings (cv. Nok-Kwang) that were obtained from a commercial plug nursery were soaked into the cell suspension of strain A21-4 (10^9 cfu/ml) for one hour and transplanted to pots (10 cm in diameter, 9 cm depth, soilless: Tosilee Sinangro Co, Korea). Pepper seedlings were inoculated by drenching 5 ml of zoospore suspension of *P. capsici* (10^4 cell/ml) on each pot and kept on a greenhouse bench until 3 weeks after transplanting. The plants showing wilting and dark brown discoloration on the stems near the soil line were counted as infected plants.

Greenhouse experiment. The greenhouse was located in Daegok-Myon, Jinju-Si, where pepper has been continuously cultivated and Phytophthora blight occurred severely in previous years. The roots of 50-day-old pepper seedlings (cv. Nok-Kwang) which were grown in a commercial plug nursery were soaked in the bacterial suspension (10^8 cfu/ml) for one hour and transplanted in the vinyl house. Seven days after transplanting, strain A21-4 suspension (10^8 cfu/ml) was added again by drenching onto the soil around the pepper plants. The disease suppression by A21-4 was compared to chemical fungicide treatment (ethaboxam: Guardian Bayer Crop Science Co. Korea), applied four times at intervals of 10 days. The numbers of infected plants were examined afterward. The plants showing typical Phytophthora blight symptom were counted up to 150 days. The method and conditions of pepper cultivation in the vinyl house were based on ordinary farming practices in the Jinju area.

Identification of antifungal compounds produced by A21-4 culture of antagonistic bacterium A21-4 for antifungal compound production. A single colony of the A21-4 was streaked onto modified minimal agar medium (glycerol 1.0%, K_2HPO_4 0.7%, KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.01%, pH 7.0). The inoculated Petri plates were incubated at 28°C for 96 hours.

The zoospores of *P. capsici* (10^5 spore/ml) were plated onto PDK (Potato Dextrose Broth 10 g, Bacto peptone 5 g, Agar 15 g) agar plate (diameter 15 mm), and a paper disk which contained the antifungal compounds was placed in the center of the Petri plate. The plates were incubated at 28°C and the diameters of clear zone were examined after 24 hours.

The bacterial cells were grown on modified minimal agar medium. Cells were removed from the culture plates and the chemical compounds produced by strain A21-4 were extracted with chloroform. The chloroform extracts were combined and evaporated to dryness to give a brown extract. The crude antibiotic extracts were purified by silica-gel column chromatography. The crude extracts were applied to a silica-gel (silica-gel G60, 70-230 mesh ASTM, Merck) column chromatography (40×400 mm), and eluted with stepwise gradients of chloroform/methyl alcohol (1/0, 60/1, 40/1, 30/1, 20/1, 10/1, 5/1, 3/1, 1/1, to 0/1, v/v). Each fraction of the elutant was concentrated in a vacuum evaporator and its antifungal activity was examined. The fraction which exhibited biological activity was further purified by silica-gel column chromatography. The column (20×300 mm) was eluted with stepwise gradients of chloroform/methyl alcohol (1/0, 40/1, 30/1, 20/1, 10/1, 8/1, 6/1, 4/1, 2/1, 1/1 to 0/1, v/v). Each fraction was concentrated in vacuum evaporator and its antifungal activity was examined. The fraction which exhibited biological activity was further purified once more with silica-gel column chromatography. The column (10×150 mm) was eluted with stepwise gradients of chloroform/acetone (1/0, 20/1, 15/1, 10/1, 8/1, 6/1, 4/1, 2/1, 1/1, to 0/1, v/v). The antifungal compound was loaded onto preparative TLC ($60GF_{254}$, 20×20 cm, 0.25 mm, Merck) and developed with chloroform/methyl alcohol (8/1, v/v). The band showing antifungal activity was purified by preparative TLC, and the active fraction was scraped-off and eluted with methyl alcohol.

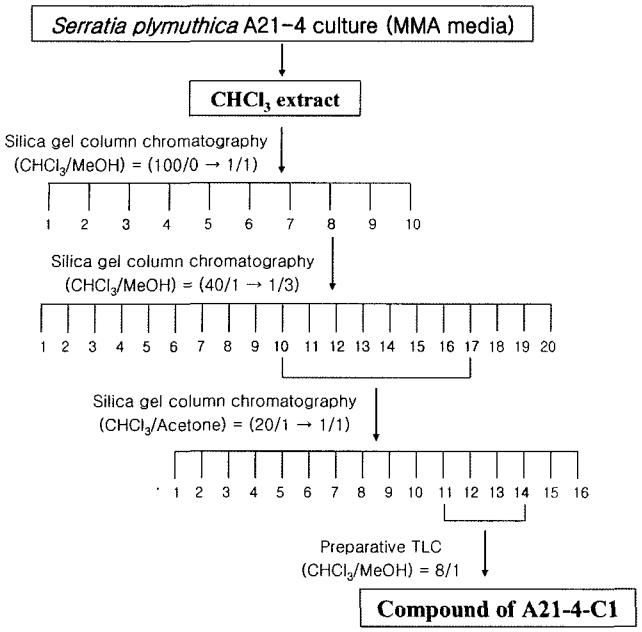

Fig. 1. Schematic diagram for isolation of antifungal compound produced by *S. plymuthica* A21-4.

Determination of chemical structural of the isolated compound. The UV spectrum of the purified antibiotic was determined in chloroform solution with a Beckman DU-600 spectrophotometer. IR spectrum of the purified antibiotic was obtained with a Bruker IFS 66 spectrophotometer for samples as thin films on a KBr window. ^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectra were obtained with a Bruker DRX-500 superconducting FT-NMR spectrometer and NOESY spectra (500 MHz). The sample was prepared in $CDCl_3$. The mass spectrum was obtained on a JEOL JMS-700 spectrometer by the direct probe method, with electrospray ionization (ESI mass) and fast atom bombardment ionization (FABI mass).

Antifungal activities of isolated antifungal compound

Inhibition of mycelial growth of plant pathogenic fungi. The serially diluted antibiotic was amended in 10% V8A or PDA plates at 0 to 1 μg/ml, and a 8-mm agar plug of plant pathogenic fungi was placed onto the center of the plate. The following fungi were tested: *Pythium ultimum*, *Phytophthora capsici*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*. The diameters of mycelial growth were measured from 3 replicates of each treatment. The ED_{50} values of the mycelial growth inhibition were calculated by a probit analysis (Probit analysis ver. 0.9).

Inhibition of cyst germination of *P. capsici*. Inhibition of cyst germination of *P. capsici* Pa-61 by isolated antifungal compounds was assayed on a glass slide. A suspension of cysts of *P. capsici* (10^4 spores/ml) was mixed with an equal volume of serially diluted antibiotic (0 to 1 μg/ml), and a drop of the mixture was placed on a glass slide. The slides were kept in Petri plate at 30°C, and after incubation for 2 hours, the number of germinated cysts were examined.

Inhibition of zoosporangia formation of *P. capsici*. Mycelial disks (8 mm in diameter) of *P. capsici* Pa-61 which were grown on V8 agar were transferred to a new empty Petri plate, covered with 3 ml of the serially diluted antibiotic (0 to 1 μg/ml), and illuminated with blue fluorescent light at 20-25°C. After 16 hours of incubation, the number of zoosporangia was counted. Plugs in the control plates were covered with the same amount of sterilized water.

Results

Inhibition of cyst and zoosporangia germination of *P. capsici*. Strain A21-4 inhibited the germination of cysts and zoosporangia of *P. capsici*. In sterilized distilled water, the germination rate of cyst and zoosporangia was about 56% and 79% at 2 hours after incubation, and reached 85% and 98% at 8 hours after incubation, but in the treatment with strain A21-4, the germination rate of cysts and zoosporangia was only 7% and 11% at 8 hours after incubation (Table 1).

Inhibition of zoosporangia formation of *P. capsici*. Zoosporangia and zoospores were not formed in the plate treated with strain A21-4 suspension, but many zoosporangia were formed in sterilized distilled water (Fig.

Table 1. Effect of *S. plymuthica* A21-4 on the germination of cysts and zoosporangia of *Phytophthora capsici*

Treatment	Germination (%) of *P. capsici*							
	cyst				zoosporangia			
	2 hr	4	6	8	2 hr	4	6	8
A21-4	0.0 a[a]	3.6 a	4.6 a	7.4 a	7.8 a	8.1 a	9.8 a	10.8 a
D.W (control)	56.4 b	60.3 b	72.7 b	84.8 b	79.1 b	89.4 b	97.8 b	98.2 b

[a]Means in the column followed by different letters are significantly different at 5% probability level (Turkey's studentized range test).

Fig. 2. Effect of *S. plymuthica* A21-4 on the formation of zoosporangia of *P. capsici* on V8 juice Agar. A: Treatment of sterilized distilled water; B: Treatment of A21-4 cell suspension (10^8 cfu/ml).

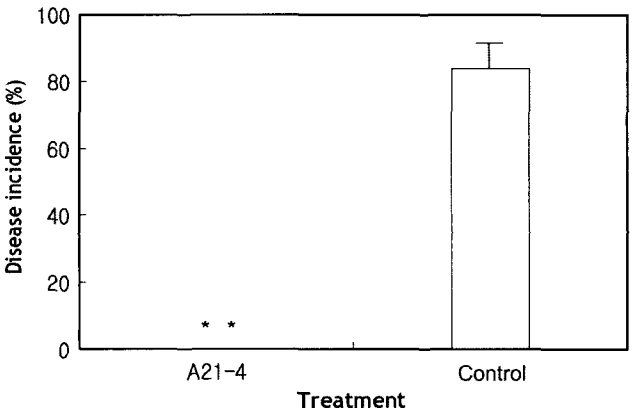

Fig. 3. Suppression of Phytophthora blight of pepper treated with *Serratia plymuthica* A21-4 10 days after treatment. Asterisks on the top of bars indicate significant difference by Duncan's multiple range test at $P=0.01$.

2). A21-4 strain inhibited successfully the formation of zoosporangia and zoospores of *P. capsici*.

Disease suppression in the pot experiment. Disease symptoms began to appear 6 days after inoculation. The disease incidence of pepper plant in non-treated control was 83.7%. None of the diseased plants were observed in the pepper plants treated with strain A21-4 (Fig. 3).

Disease suppression in the greenhouse. One month after transplanting, the infection rate of pepper treated with a chemical fungicide, Ethaboxam was 5%, on the other hand none of diseased plants were observed in the A21-4 treatment. Five months after transplanting, the infection rate of pepper treated with the fungicide was 27.3%, however,

Table 2. Effect of *S. plymuthica* A21-4 on suppression of Phytophthora blight in a farmer's vinyl house compared with That of a fungicide Ethaboxam

Treatment	Disease incidence (%)		
	30 days	60 days	150 days
A21-4	0.0 a[a]	2.3 a	4.7 a
Ethaboxam	5.0 b	13.7 b	27.3 b

[a]Means in the column followed by different letters are significantly different at 5% probability level (Turkey's studentized range test).

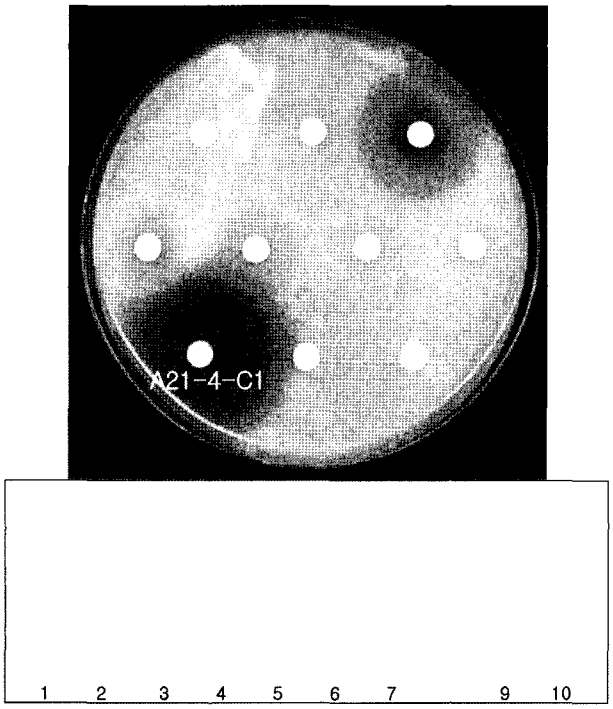

Fig. 4. Direct inhibition on plated zoospores of *P. capsici* on agar plate and TLC patterns of 1st column of antibiotic extracts from *S. plymuthica* A21-4.

that of A21-4 treated pepper was only 4.7% (Table 2).

Isolation and structural determination of antifungal compound

Isolation and purification of antifungal compound. Through a series of column chromatography and TLS separation, we obtained antibiotic extract (A21-4-C1) which showed strong antifungal activity to *P. capsici* (Fig. 4). Further separation and purifying works the most active chemical compound was isolated. The single spot of active chemical compound developed on TLC plate is shown in Fig. 5, that large clear inhibition zone induced by active compound.

Structural determination. Physical-chemical properties of compound A21-4-C1, the active chemical compound

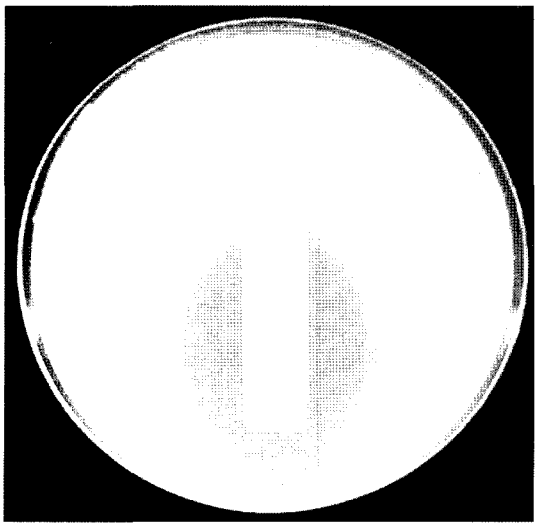

Fig. 5. Direct inhibition of of mycelial growth of *P. capsici* on TLC by the purified antifungal compound produced by *S. plymuthica* A21-4. TLC strip was placed on the surface of agar medium where the zoospores of *P. capsici* were inoculated.

Table 3. Physical-chemical properties of antifungal compound

Appearance	Solid
Molecular formula	$C_{23}H_{31}O_8Cl$
Molecular weight	470.1707
Melting point	101-103°C
UV λ_{max} nm, (log ε) in $CHCl_3$	276 (2.87)
IR ν_{max} (KBr) cm^{-1}	3433(OH), 1737(C=O), 756(Cl)
Rf value[a]	0.38

[a]silica gel TLC (kieselgel 60F254, Merck) developed with chloroform : methanol, 8 : 1

produced by A21-4, are shown in Table 3. The compound was isolated as a colorless solid. The molecular formula of compound A21-4-C1 was established as $C_{23}H_{31}ClO_8$ on the basis of HRFAB-MS (*m/z* 493.1603, calculated for $C_{23}H_{31}Cl\ NaO_8$ $[M+Na]^+$ 493.1605) and corresponds to eight degrees of unsaturation. The IR spectrum showed absorption band for a hydroxyl group (3433 cm^{-1}), carbonyl group (1737 cm^{-1}) and chlorine group (756 cm^{-1}). ^1H and ^{13}C NMR including DEPT experiment revealed signals for three angular methyl groups [δ_H 1.79, 1.89, 2.04 (each 3H, s)], six methylenes, eight methines (three olefinic and five oxygenated methines), three non-protonated olefinics and three carbonyl groups. The COSY and HMBC experiments of the compound A21-4-C1 showed that its structure has the same skeleton of haterumalide A and oocydin A. We named the compound macrocyclic lactone A21-4 (Fig. 6).

Antifungal activity of macrocyclic lactone A21-4. This

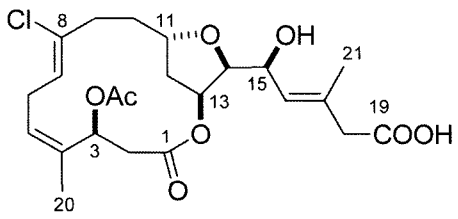

Fig. 6. The structure of macrocyclic lactone A21-4 produced by *Serratia plymutica* A21-4 with relative stereochemistry.

macrocyclic lactone A21-4 showed very strong activities against tested phytopathogenic fungal species. ED_{50} values of the macrocyclic lactone A21-4 measured on the basis of mycelial growth inhibition on *P. ultimum*, *P. capsici*, *S. sclerotiorum* and *B. cinerea* were 0.25, 0.25, 0.30, and 0.75 μg/ml, respectively.

Inhibition of zoosporangia formation by macrocyclic lactone A21-4. Inhibition of zoosporangia formation of *P. capsici* by the active compound obtained from A21-4 in vitro was assayed on a petri plate. Zoosporangia and zoospores were not formed at the lowest concentration (0.0625 μg/ml) of the macrocyclic lactone A21-4 on V8 juice agar or PDA plate, but abundant zoosporangia were formed on the plates treated with sterilized distilled water. Macrocyclic lactone A21-4 also inhibited successfully the germination of cysts and germ tube elongation of *P. capsici*. All of the concentrations of the macrocyclic lactone A21-4 tested in this experiment inhibited the cyst germination of *P. capsici*. In the sterilized distilled water the cyst germination rate was 92.6%, and the length of germination tube was 0.13 mm at 2 hours after incubation, but the treatment of 0.0625 μg/ml of macrocyclic lactone A21-4, the cyst germination rate was 10.8%, and its length of germination tube was only 0.04 mm (Table 4, Fig. 7).

Table 4. Effect of macrocyclic lactone A21-4 on the formation of zoosporangia and the germination of cyst of *P. capsici*

Concentration of macrocyclic lactone A21-4 (μg/ml)	Formation rate of zoosporangia (%)	Cysts of *P. capsici*	
		Germination rate (%)	Length of germination tube (mm)
1.00	0.00 a[a]	0.00 a	–
0.50	0.00 a	0.00 a	–
0.25	0.00 a	6.18 b	0.006 a
0.125	0.00 a	8.71 bc	0.014 b
0.0625	0.00 a	10.81 c	0.045 c
D.W	100 b	92.57 d	0.130 d

[a]Means in the column followed by different letters are significantly different at 5% probability level (Turkey's studentized range test).

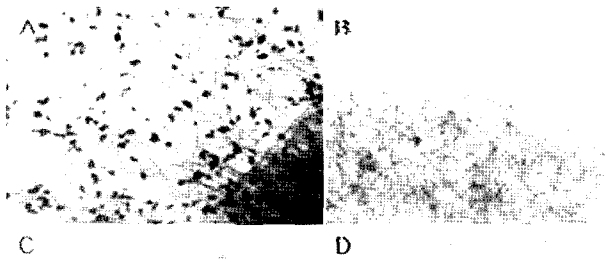

Fig. 7. Inhibition of zoosporangia formation and cyst germination of *P. capsici* by macrocyclic lactone A21-4 produced by *S. plymuthica* A21-4. (A: Normal formation of zoosporangia; B: Suppressed formation of zoosporangia by macrocyclic lactone A21-4; C: Normal germination of cysts; D: Suppressed germination of cysts by macrocyclic lactone A21-4.)

Discussion

In recent years, *Serratia plymuthica* has been promising candidates for the biocontrol agent of Phytophthora diseases. *Serratia plymuthica* has been isolated from the rhizosphere or as endophytes of sugarbeet, oilseed rape, melon, and tomato. Haefele et al. (1991) applied for a patent with biological control of corn seed rot and seeding blight caused by *Pythium* spp. and *Fusarium* spp. Some isolates of the genus *Serratia* have been shown to suppress several fungal plant pathogens, including *Verticillium dahliae* (Berg et al., 2002; Frankowski et al., 1998; Kurze et al., 2001), *Sclerotinia sclerotiorum* (Thaning et al., 2001), *Rhizoctonia solani* (Frommel et al., 1991), *Botrytis cinerea* (Kamensky et al., 2003), *Fusarium* sp. (Frommel et al., 1991), and *Pythium* sp. (Benhamou et al., 2000; Frommel et al., 1991). In addition, *S. plymuthica* strains may secrete the plant growth hormone indole acetic acid (IAA), which can directly promote root growth (Kalbe et al., 1996).

Many workers reported that the mechanisms of fungal suppression by *S. plymuthica* may be related to production of cell wall degrading enzymes like chitinases and β-1,3-glucanases (Berg et al., 2002; Frankowski et al., 1998; Kamensky et al., 2003; Kurze et al., 2001) and siderophores (Frankowski et al., 1998; Kamensky et al., 2003). The major structural component of most fungal cell walls is chitin. However, the cell wall of genera *Pythium* and *Phytophthora* contains mostly cellulose mixed with β-glucan. Therefore chitinase and β-1,3-glucanases is hypothetically not effective against those fungi. Although strain *S. plymuthica* A21-4 also produced chitinolytic enzymes and siderophores (data not shown), there were no inhibitory effects on the *Rhizoctonia* and *Fusarium*. However, *S. plymuthica* A21-4 strongly inhibited the growth of the *Pythium* and *Phytophthora* species. It is suggested that suppression of *Phytophthora* and *Pythium* by *S. plymuthica* A21-4 is not due to the production of chitinolytic enzymes or siderophores.

Thaning et al. (2001) isolated an antibiotic from *Serratia plymuthica* and proved other possible mechanisms of suppression of fungi. In this study, *S. plymuthica* A21-4 produced powerful antifungal substance and was the key factor to suppress the growth of *Phytophthora*. We selected antibiotic defective mutant SSM1 through transposon mutagensis and confirmed the mutant lost antagonistic activity against *P. capsici* and *P. ultimum*. Through the column chromatography and TLC separation, the mutant lacked the most active compound responsible for inhibition of the fungus.

The macrocyclic lactone A21-4, purified from the cultured agar medium of *S. plymuthica* A21-4, was identified as chlorinated macrolide ($C_{23}H_{31}O_8Cl$), with a molecular weight, 470.1707. Similar chlorinated macrolide compounds, reported by other research groups as compounds inhibiting cell division of fertilized sea urchin eggs (Takada et al., 1999), showed inhibition of the growth of several oomycetes fungi such as *Phytophthora* spp. and *P. ultimum* but no inhibition or minimal effect on Ascomycetes including *Sclerotinia sclerotiorum* (Strobel et al., 1999). Thaning et al. (2001) reported that a chlorinated macrolide isolated from *S. plymuthica* inhibited apothecial formation and ascospore germination of *S. sclerotiorum*. Although the molecular formula and structural skeleton of the active compound obtained from strain A21-4 is same as haterumalide A (Takada et al., 1999) and oocydin A (Strobel et al., 1999), macrocyclic lactone A21-4 isolated in this study showed significant inhibition not only to *Phytophthora* spp., *Pythium* spp., but also *S. sclerotiorum* and *B. cinerea*. Further work might be required to develop the antibiotic compound macrocyclic lactone A21-4 as a biofungicide and to apply to Sclerotinia rot and gray mold diseases.

In conclusion, the root colonizing bacterium *S. plymuthica* A21-4 is promising and applicable biocontrol agent against Phytophthora blight of pepper. Because the bacterium A21-4 colonized the rhizosphere of pepper and other vegetable crops, it is capable of protecting pepper roots from *Phytophthora capsici* in the field soil. In addition, strain A21-4 produces a powerful antifungal compound, macrocyclic lactone A21-4. The antibiotics shows much stronger activity than a commercial fungicide against Phytophthora blight of pepper.

Acknowledgments

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