Journal of Microbiology and Biotechnology
Vol. 4, No. 4, 296-304, 1994

Antifungal Mechanism and Properties of Antibiotic Substances produced by *Bacillus subtilis* YB-70 as a Biological Control Agent

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Antibiotic substances were produced by *Bacillus subtilis* YB-70, a potential biocontrol agent found to suppress root-rot of eggplant (*Solanum melonggena* L) caused by *Fusarium solani*, in a dextrose glutamate medium and isolated by isoelectric precipitation. Partial purification was performed by column chromatography on silica gel with two solvent systems: chloroform-methanol and methanol-chloroform-water as eluting solvents. This active fraction YBS-1s contained antifungal activity were soluble in ethanol, methanol, and water, but were not soluble in other solvents including acetone, butanol, ethyl ether, dimethylformamide, propanol, and etc. High performance liquid chromatography and thin layer chromatographic separation of YBS-1s showed that they have been composed of three biological active bands that were named YBS-1A, -1B, and -1C. The substances were stable to heat and resistant to protease. YBS-1s were active against a wide range of plant pathogenic fungi but did not inhibit the growth of bacteria and yeasts. They were not only fungicidal but also fungistatic against chlamydospores of *F. solani*. The ED_{50} values for the chlamydospore germination and the germ-tube growth of *F. solani* were 0.725μg/*ml* and 0.562μg/*ml*, respectively. Microscopic observations proved the substances restricted the growth of phytopathogenic fungus *F. solani* by spore burst followed by dissolving of its germ-tube, and caused abnormal hyphal swelling after application to chlamydospores or growing hyphae. Cultural filtrate of *B. subtilis* YB-70 also suppressed the development of root-rot of eggplant in pot tests.

Soil-borne plant diseases are responsible for important yield loss on many crops (3, 5, 11, 16). Agrichemicals used for crop protection are adversely effecting the quality of the crops production and the environment (2, 5, 16), thus preferential making the development of alternative ways to control disease. Recently, augmentation with biological control agents is recognized as a plausible approach to disease control (10, 15). The native method of using microbial disease control agents is being intensively studied (5, 6, 7, 14), and so recent effects in many countries to find less hazardous disease control agents from microbial sources for replacing synthetic chemicals are remarkable (2, 10, 15). However, until now microbial products have not really been considered as potential biocontrol agents with a few exceptions. If several economic problems can be solved, this approach will be a prosperous commercial area in the future.

In our previous study (12), we originally isolated antagonistic bacterium YB-70 from suppressive cultivated soil, and identified it as a member of *Bacillus subtilis*. The strain exhibited the powerful antifungal activity *in vitro* among the selected bacteria with antifungal activity and the potential ability as an effective biological control agent *in vivo* against root-rotting fungus, *Fusarium solani*. We proved that the suppressive activity of *B. subtilis* YB-70 on root-rot caused by *F. solani* in eggplant (*Solanum melongena* L) may be due mainly to the production of antifungal substances that were heat-stable and low molecular. Similarly, in several biological control studies on the previous use of *B. subtilis* as biocontrol agents against several phytopathogens (1, 2, 4, 7, 13, 15, 17), all work presented that the mechanism by which *B. subtilis* inhibits growth of phytopathogenic fungi is most likely the production of antibiotics such as iturin A (5, 15), mycosubtilin (5), fengymycin (18), and bacilycin (18). Although several antifungal metabolites of *B.subtilis* have been characterized and they were typical peptides with broad spectra of activity against many genera of fungi, the action mode of these substances is not clear except a few reports (1, 7).

The aims of the present study were to (i) isolate the

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Key words: Antifungal Antibiotics, *Bacillus subtilis*, *Fusarium solani*

biologically active fraction from the cultural filtrate of *B.subtilis* YB-70, (ii) investigate antifungal properties and their involvement in the antagonistic action of the strain against *F.solani*, and (iii) prove the suppressive effect against root-rot caused by *F.solani in vivo* bioassay.

MATERIALS AND METHODS

Culture and Growth Medium

Bacillus subtilis YB-70, originally isolated from suppressive soil rhizosphere in our laboratory (12), was stored at -70°C and used in liquid fermentation to produce the antibiotics. Plant pathogenic fungus *Fusarium solani* causing root rot of many important crops was provided by Korea Ginseng and Tabacco Research Institute (KGTRI) and maintained on potato dextrose agar (PDA) at 4°C.

The other plant pathogenic fungi used to show an antifungal spectrum (Table 1) in this study were obtained from the Rural Development Administration, Suwon, Korea and maintained on PDA at 4°C or room temperature, depending on the species. *F. solani* was used as an indicator throughout this study.

Isolation and Partial Purification of Antibiotic Substances

For the production of antibiotic substances, *B. subtilis* YB-70 was grown with a dextrose glutamate (DG) medium (12) in a jar fermentor (working volume: 3*l*). Fermentation was carried out at 200rpm agitation with 2.5*l*/minute aeration at 30°C for 3 days. The pH of the medium was adjusted to 6.5 with 5N NaOH.

A crude preparation of antifungal antibiotics was isolated from the cultural filtrate of *B. subtilis* YB-70 according to the method of McKeens *et al* (15) with modifications and the procedure of partial purification was shown in Fig. 1. The antifungal substances of the filtered broth were precipitated by acidification (pH 2.0) with HCl and the active components extracted with methanol three times to yield 3.0 to 3.5g/*l* from the precipitates. The methanol extract was then evaporated at 50°C under reduced pressure. The resulting residues containing the antifungal substances were dissolved in water, and freeze-dried to give a dark brown powder. The yield of the active material obtained by this process was about 85 to 90mg/*l*. Crude antifungal substances were applied to a column filled with silica gel (Merck 7734, Kieselgel 60). The gel was washed with 2 volumes of chloroform, and the retained material was further eluted with the following step gradient of chloroform and methanol as shown in Fig. 1. The elute was lyophilized and resuspended in water. Aliquots were applied to a 2nd silica gel column, washed with 2 volumes of methanol-chloroform-water (65:30:5), and then eluted with the same solvent. The separative fraction collected was lyophilized, and resuspended in water. The final fraction was designated as YBS-1s. Antifungal activity against *F.solani* as a test fungus in the procedure of partial purification was

Table 1. Antifungal spectrum of *Bacillus subtilis* YB-70 and its antifungal substances YBS-1s.

Plant pathogenic fungi and other organisms	Inhibition zone (mm)[a]	
	Cell[b]	YBS-1s[c]
Alternaria kikuchiana Tanaka	9	16
Alternaria mali Roberts	9	18
Botrtyis cinerea Person et Fries	15	18
Colletotrichum gloeosporioides	11	19
Collectrichum sp.	12	19
Fusarium oxysporum Schlecht	17	15
Fusarium oxysporum f.sp. *cucumerinum* Owen	8	14
Fusarium solani	16	14
Gaeumannomyces graminis	30	36
Penicillium expansum Link	29	32
Phytophthora capsici Leonian	22	22
Pyricularia oryzae Cavara	18	25
Pythium ultimum Trow	4	No[e]
Rhizoctonia solani Kühn	9	4
Baterial species[d]	No	No
Yeast species[d]	No	No

[a] Plates were incubated at 24°C or 28°C, depending on the species, and scored after 5 days by measuring the distance between the edges of the bacterial colony/well and fungal mycelium. [b] Mycelial plugs (about 6mm square) of the actively growing culture plate of the fungi were placed 2cm from the center of the PDA plates. Each 50*μl* of cell suspension (1×10^7/*ml*) from ovemight culture of *B. subtilis* YB-70 was inoculated at 4cm from the mycelial plugs.[c] YBS-1s, dark brown powder, were dissolved in water (20μg/*ml*), and filled in a well (6mm diameter) made in the center of the PDA plates which were seeded with fungal spore suspensions (approximately 1×10^5 conidia/*ml*). [d] The bacteria and yeasts were *Bacillus pasteruii, B. sphaericus* Meyer and Neide, *B. subtilis* BR151 GMR, *Escherichia coli* W3110, *Pseudomonas fluorescence* KCCM 1751, *P. putida, Candida albicans* (Robin) Berkhout, *Pyricularia grisea* (Cooke) Saccardo, *Saccharomyces rosei, Torulopsis veriabilis* [e] No inhibitory zone Each value represents the mean of three plates.

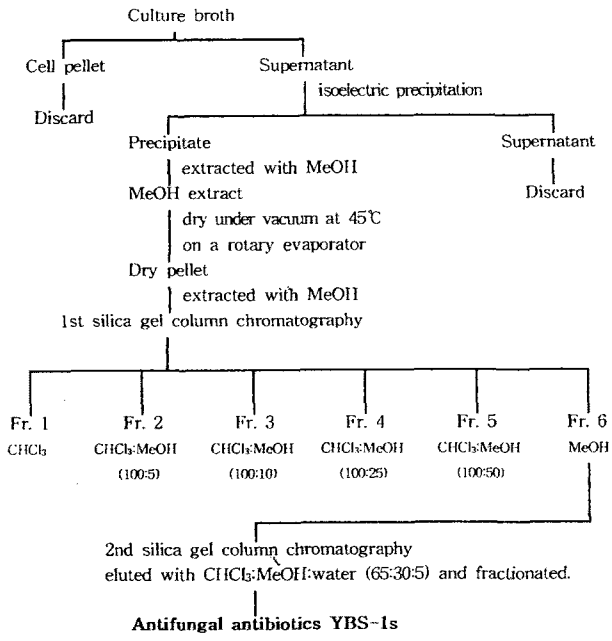

Fig. 1. Purification procedure of antifungal antibiotics YBS-1s produced by *Bacillus subtilis* YB-70.

monitored by a conventional agar hole method (12).

HPLC Analysis of Antifungal Antibiotics

Antifungal substances YBS-1s were dissolved in methanol, filtered, concentrated, and re-filtered through a 0.45-μm filter (Tosoh W-13-5) before injection onto a C_{18} analytic high performance liquid chromatography (HPLC) column. The final preparations were then subjected to HPLC (Tosoh PX-8010) on TSKgel ODS-120T (8μm, 4.6 × 150mm) maintaining a flow rate of 1m*l* per minute. The column was eluted with water-acetonitrile mixture using a gradient of acetonitrile in water. YBS-1s were eluted out with 30 to 50% of acetonitrile in water, and was collected in 1m*l* fractions that were monitored by UV detection (Tosoh UV8010) at 254nm and their bioactivity against *F. solani*.

Antifungal Bioassay

Antifungal substances YBS-1s were tested for the antifungal activity against *F. solani* by the method of Kim *et al* (12).

Chemical Properties of Antibiotic Substances

Active substances YBS-1s were partially characterized by thin-layer chromatography (TLC). YBS-1s were spotted onto 20cm^2 silica gel plates containing a fluorescent indicator (Merck 5735, Merck & Rahway, NJ), and then developed in equilibration tanks containing ethanol:water (2:1 v/v). The bands were visualized with UV light or iodine vapor. All detected bands and areas between the bands were scraped off separately, and eluted with methanol. Eluted substances were tested for inhibition of the mycelial growth of *F. solani*.

To assess the influence of temperature, pH, and protease on the activity of antifungal antibiotics YBS-1s, they (20μg/m*l*) were incubated at various temperatures and pHs, and with enzymes before performing the antifungal assay against *F.solani*. All of the enzymes were purchased from Sigma chemical Co.

Spore Germination Assay and Antifungal Properties

Spore germination bioassay according to the method of De Cal et al (5) was used. A 50μ*l* of antibiotic compound YBS-1s was mixed with an equal volume of each spore suspension (1×10^5/m*l*) in the wells of acid-cleaned depression slides, and the slides were incubated for 24hrs in a petri dish containing filter paper (Whatman filter paper No.2) moistened with distilled water. Germination rate of the chlamydospores and the length of germ-tubes was examined under a light microscope at 400× magnification.

The results of the relative activity of different concentration levels of YBS-1s on *F.solani* were processed by the probit-analysis method (8).

Antifungal Spectrum of Antibiotic substances

The antifungal substances YBS-1s were tested for the antifungal activity against the fungi listed in Table 1 by the method described above.

Pot Bioassay

Pot bioassay was performed by the method of Kim *et al* (12). The cultural supernatants from *B. subtilis* YB-70 were prepared by the aseptic filtration using a membrane filter (Whatman membrane filter, pore size 0.45μm). The membrane filtrate was added to sterilized soil infested with *F.solani*.

RESULTS

Characterization of Antifungal Antibiotics

Antifungal antibiotics YBS-1s from a potentially powerful antagonistic YB-70 were readily precipitated at an acid condition up to pH 5.0, and were soluble in ethanol, methanol, and water, but insoluble in other nonpolar solvents including acetone, *n*-butanol, ethyl ether, dimethylformamide, *n*-propanol, *iso*-propanol, ethyl acetate, methylene chloride, chloroform, and benzen. TLC on silica gel plates developed in a solvent system (ethanol:water = 2:1, v/v) revealed the presence of R_f 0.67, 0.86, and 0.96, respectively. All bands stained brown in iodine vapors and tested positively with ninhydrin reaction. Other biochemical tests including Benedict, Ehrlich, Fehling, Molish, and xantoprotein test were negative. In other solvent systems used by Loeffler *et al* (13) and Besson *et al* (4), R_f values of YBS-1s were shown in Table 2. HPLC analysis suggested YBS-1s have three biological active substances (Fig. 2). When YBS-1s were

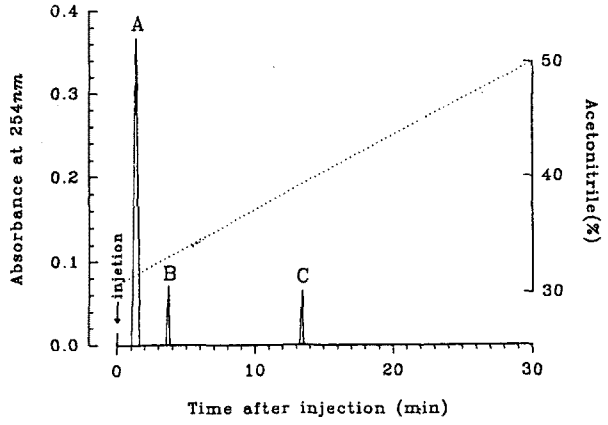

Fig. 2. HPLC chromatogram of antifungal substances YBS-1s.
Antifungal antibiotics YBS-1s produced from *Bacillus subtilis* YB-70 were analyzed in Tosoh PX-8010 HPLC equipped with diode-array (*Tosoh UV8010*), using a column (4.6×150mm) packed with TSKgel ODS-120T. Sample (20μl) were eluted with a linear acetonitile gradient from 30 to 50% (0~30 min). The flow rate was 1ml/min. There were detected by UV-absorption at 254nm.

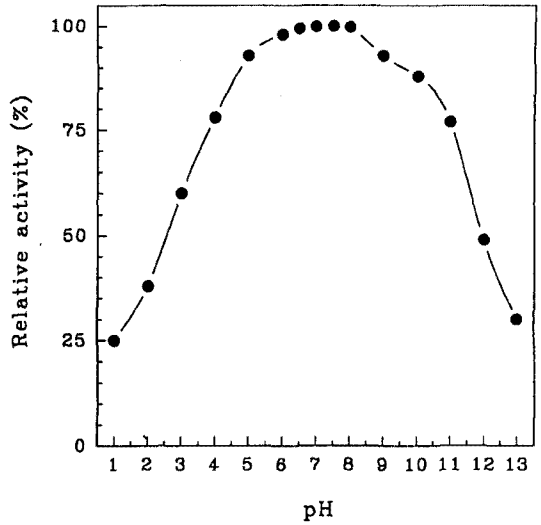

Fig. 3. pH stability of antifungal antibiotics YBS-1s.
The pH of the YBS-1s (20μg/ml) were adjusted from 1.0 to 13.0 with 0.5N HCl and 0.5N NaOH. After standing for 24hours at room temperature, the pH was readjusted to 7.0. The remaining antifungal activities were assayed against *F.solani*.

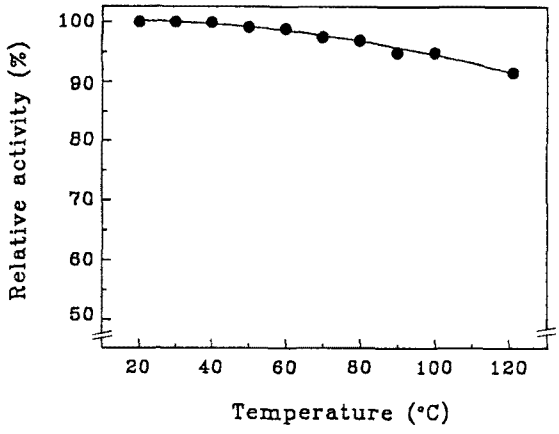

Fig. 4. Heat stability of antifungal antibiotics YBS-1s.
YBS-1s (20μg/ml) was placed in test tubes and treated at various temperature for 30min. The remainding antifungal activity was determined against *F.solani*.

subjected to an analytical HPLC column, each of them eluted as a single peak of activity, and in each case the elution position coincided with that of the UV light absorbance peak. The three compounds differed in retention time. Compound YBS-1A was more hydrophobic and more abundant than the other two compounds.

Stability of Antifungal Antibiotics

As shown Fig. 3 and 4, the antifungal antibiotics YBS-1s retained over 90% of their activity after being heated at 100°C for 30min at pH 7.0. About 82% of the activity was retained after autoclaving for 20min at 121°C. They were also stable at room temperature in the wide range of pH, but lost about 70% of their activity at an extremely acidic (pH 1.0) and alkaline (pH 13.0) condition for 24 hrs. YBS-1s were resistant to the enzymes such as trypsin, pronase, β-glucosidase, lysozyme, α-chymotrypsin, papain, pepsin, prteinase K, and alkaline phosphatase. None of enzymes metioned above had any visible effect on antifungal activity of YBS-1s at the highest concentrations used in our experiments. YBS-1s exhibited no loss of activity after storage for over 12 months at 4°C.

Antifungal Spectrum

Antifungal spectrum of antibiotic substances YBS-1s was wide, since they inhibited the growth of 12 out of 14 phytopathogenic fungi tested (Table 1). Strong inhibition was observed against *Alternaria kikuchiana, A. mali, Botrtyis cinerea, Colletoctrichum* sp., *C. gloeosporioides, F. oxysporum, F. oxysporum* f.sp. *cucumerinum, Gaeumanomyces graminis, Penicillium expansum, Phytophthora capsici,* and *Pyricularia oryzae*. However, *Rhizoctonia solani* was only partially inhibited, and *Pythium ultimum* was almost or totally unaffected (Table 1). Interestingly, the antifungal substances YBS-1s did not inhibit the growth of some yeast at the same concentration (Table 1).

Antifungal Mechanism of the Antibiotic Substances YBS-1s

The effect of the concentration and exposure time to YBS-1s on chlamydospore germination and germ-tube development of *F. solani* was shown in Table 2. After 24hr of incubation at 28°C, the germination rate of chlamydospores treated with YBS-1s was reduced to 0.3% compared to 79.6% of untreated cells. After exposure for 3 and 5 days, the germination of chlamydospores

was not observed, but less than 10^{-3} to 10^{-2} of germination was, but not all, observed in some test tubes treated with YBS-1s. Germination of chlamydospores in the concentration of 25μg/ml, and 5μg/ml, of YBS-1s was 14.0 and 25.3%, respectively, whereas germination did not occur above 50μg/ml,. The rate of spore germination was severely reduced for 5 days, and this effect is accompanied with a decrease of the number of spore. Fig. 5 shows the probit-log concentration lines and the effective dose (ED_{50}) values for YBS-1s to the chlamydospore germination and the germ-tube growth of *F. solani*. The ED_{50} calculated for the germination and the germ-tube growth of YBS-1s, were 0.725μg/ml, and 0.562μg/ml, respectively.

The development of germ-tubes was reduced apparently when YBS-1s were added to the medium. After 5 days of incubation at 28°C with the concentration of 25μg/ml of YBS-1s, the length of germ-tubes of *F. solani* was less than 10μm comparing with the length, an 800μm of the culture without substances. However, the growth of chlamydospores was not seen above a concentration of 25μg/ml. Additionally, the elongation of germ-tubes also decreased in proportion to increases in the exposed time. Thus, antifungal substance YBS-1s were involved in the inhibition of the chlamydospore germination as well as the germ-tube growth of *F. solani*. Both are connected with a decrease of the number of chlamy-

Table 2. R_f values of the antifungal antibiotics YBS-1s partially purfied from the cultural filtrate of *Bacillus subtilis* YB-70.

Solvent system[a]	R_f value[b]		
	YBS-1A	YBS-1B	YBS-1C
A	0.33	0.20	0.08
B	0.54	0.49	0.28
C	0.28	0.14	0.09
D	0.03	0.07	0.03
E	0.03	0.09	0.03
F	0.38	0.29	0.23
G	0.84	0.79	0.52
H	0.96	0.86	0.67

[a] Solvent system A ; *n*-BuOH:acetic acid:water (65:10:5), B ; *n*-BuOH: acetic acid:water (3:2:2), C ; *n*-BuOH:acetic acid:water (4:3:3), D ; *n*-BuOH:acetone:water (16:24:4), E ; $CHCl_3$:MeOH:water (65:25:4), F : MeOH:$CHCl_3$:water (65:35:5), G ; *n*-PrOH:Pyridine:acetic acid:water (15 :10:3:12), H ; EtOH:water (2:1). [b] Estimation of R_f value was carried out with silica gel TLC (Merck No. 7734, $60GF_{254}$). YBS-1A, -1B, and -1C were separated from the preparative TLC of YBS-1s under UV light (solvent : ethanol-water, 2:1 v/v).

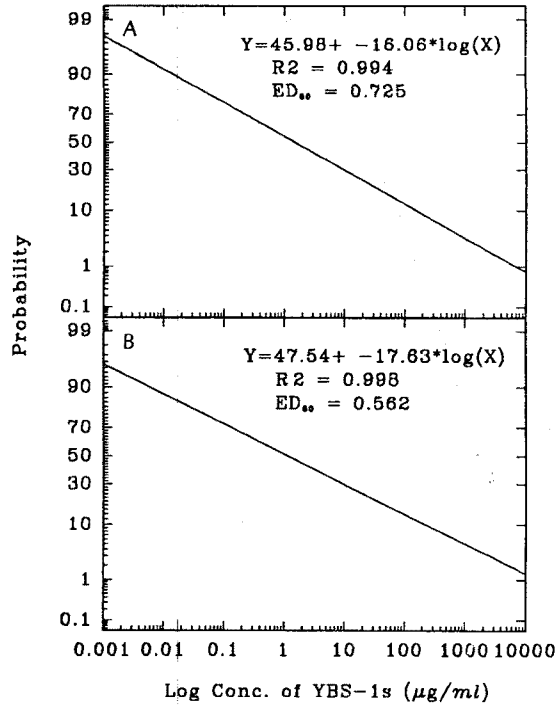

Fig. 5. Antifungal activity of antibiotic substances YBS-1s against *F. solani* represented as probit-log concentration lines and ED_{50} (μg/ml).
A, Toxicity of YBS-1s to the chlamydospore germination of *F. solani*. B, Toxicity of YBS-1s to the germ-tube growth of *F. solani*

Table 3. Effect of the antifungal substances YBS-1s of *Bacillus subtilis* YB-70 on the chlamydospore germination and the germ-tube growth of *Fusarium solani*.

YBS-1s Conc.	Spore germination(%)[b]			Germ tube length(μm)[c]		
(μg/ml)[a] Days[d]	1	3	5	1	3	5
Control[e]	79.6	97.5	99.3	18 ±2	〉200	〉〉〉[f]
300	0.3	—[g]	—	—	—	—
200	11.3	—	—	〈3	—	—
100	28.4	—	—	〈6.2	—	—
50	30.3	10.2	—	〈12	〈10	—
25	38.7	15.8	14.0	〈20	〈15	〈10
5	51.6	39.8	25.3	42 ±6	30 ±8	25 ±8

Chlamydospore (1×10^5/ml) germination and germ-tube growth were observed in the PDB with antifungal substances YBS-1s of *B. subtilis* YB-70 grown in dextrose glutamate (DG) medium at 30°C for 84hrs. [a] Concentration of antifungal substances YBS-1 partially purified from 3 day cultural broth (DG) of *B. subtilis* YB-70. [b] Spore germination (%) - relative germination ratio of *F. solani* chlamydospores treated with YBS-1s to those of total spores treated with water. [c] Germ-tube length (μm) was measured with a heamatormeter under a light microscope at 400× magnification. [d] Culture days [e] Control - spores were cultured in PDB with water instead of YBS-1s. [f] Over growth. [g] The chlamydospore germination and germ-tube growth were not observed. All the data was obtained as a mean value from the measurment of 40~50 chlamydospores or 20~25 germ-tubes in triplicates.

Table 4. The minimal fungicidal and fungistatic concentration of antifungal substances YBS-1s against *Fusarium solani*.

Fungicidal Conc. (μg/ml)[a]	Fungistatic Conc. (μg/ml)[b]
2,000	500

[a] Spore were transferred to PDA after 2hrs of exposure to YBS-1s. Growth was determined after 4 days of incubation at 28°C in the dark. The value was expressed as a concentration of YBS-1s with which no growth were obseved on the PDA plate. [b] Spore were exposed to the antibiotics for 22hrs. For each treatment, YBS-1s containing spores was checked for percent germination by microscopic observation. The value was a concentration of YBS-1s at which the chlamydospore germination of the fungus was completely inhibited. All experiments were repeated twice in triplicate.

Table 5. Survival ratio of eggplant seedlings treated with cultural filtrate of *B. subtilis* YB-70 on *F. solani*-infested pots.

Treatment	Survival ratio[a] (%)
Untreated in infested soil	19± 5[b]
With cultural filtrate in noninfested soil	97± 2
With the cultural filtrate in infested soil[c]	92± 3
With the bacterial cells in infested soil	93± 2

[a] Values were expressed as a percentage of the total number of seedlings transplanted. [b] Each value was the average of three replicates of 25 seedlings each: the maximum deviation from that the mean value of the indiviual replicates is also shown. [c] The cultural filtrate from the bacterium were prepared by the method described in materials and methods

dospores.

YBS-1s were not only fungicidal but also fungistatic to the chlamydospores of *F.solani* (Table 4). When the chlamydospores were transferred to PDA after 2hrs of exposure to the test solutions with YBS-1s, no growth was observed on all plates treated with a 2000μg/ml of YBS-1s for 4 days. When the chlamydospores were soaked directly at or below 1000μg/ml of YBS-1s for 2 hrs, growth was observed on plates within 4 days. The YBS-1s were fungistatic to *F. solani* at a concentration of 0.5μg/ml. After 22hrs of exposure, using direct soak method, less than 10% germination was seen at YBS-1s concentrations of 100 and 250μg/ml. At 50μg/ml, 11% of the chlamydospores germinated on the PDA.

Light microscopic observation showed that chlamydospores of *F. solani* exposed to YBS-1s were not germinated after 24hrs of the treatment (Fig. 6 B), whereas untreated chlamydospores were normally germinated (Fig. 6 A). The hyphae had abnormal structure in the presence of YBS-1s. Antagonized mycelium of the fungus became more gross and irregular (Fig. 6 D) than untreated *F. solani* that exhibited normal hyphae development including smooth cell walls of uniform thickness and straight hyphal growth (Fig. 6 C). However, there was no swelling of hyphal tips and bursting of hyphal strands at any stage. A decrease in the number of chlamydospores, in our spore germination test, proved that some germinated chlamydospores in the presence of YBS-1s were bursting and then died (Fig. 7). It was probably suggested that YBS-1s permeated in growing germ-tubes of *F. solani* chlamydospores (Fig. 7 A), and then caused a rupture of immature germ-tube (Fig. 7 B and C). Finally, the chlamydospores were dissolved and dead (Fig. 7 D).

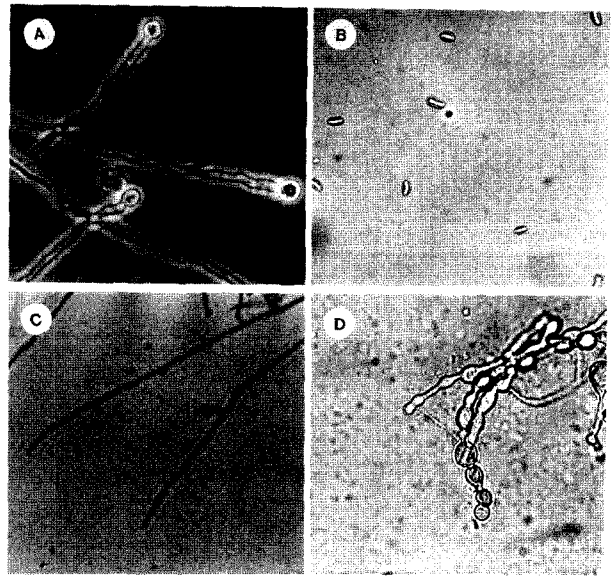

Fig. 6. Morphological effects of antifungal antibiotics YBS-1s on the chlamydospore germination (B) and hyphal growth of *Fusarium solani* (D).
A, Normal germ-tube elongation after spore germination of *F. solani* in a potato dextrose broth (PDB) after 24hrs. B, No germination of *F. solani* chlamydospores treated with YBS-1s (100μg/ml) C, Normal hyphal growth of *F. solani* after incubation for 48hrs at 28°C in PDB. D, The abnomal hyphal growth of *F. solani* in PDB treated with YBS-1s (10 0μg/ml).

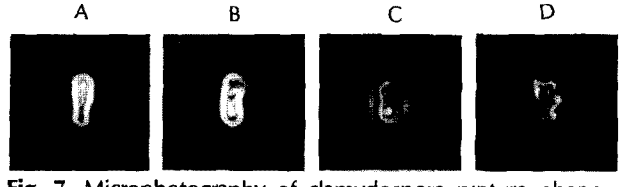

Fig. 7. Microphotography of clamydospore rupture phenomena of *F. solani* treated with YBS-1s.
A 50μl drop of antifungal antibiotics YBS-1s (100μg/ml) was mixed with an equal volume of each spore suspension (1×10^5/ml) prepared by the materials and methods in the wells of acid-cleaned depression slides, and slides were incubated for 12hrs (A), 24hrs (B), 36hrs (C), and 48hrs (D), respectively in a sterile petridish containing filter paper (Whatman No. 2) moistened with distilled water. Morphological change of chlamydospore was observed under light microscope(×400).

In vivo Biocontrol Test

Ratio of surviving eggplant from seedlings treated with *B. subtilis* YB-70 cells or the cell-free medium of the strain in pots infested with *F. solani* were shown in Table 5. Treatment of seedlings with the bacterial cells or with

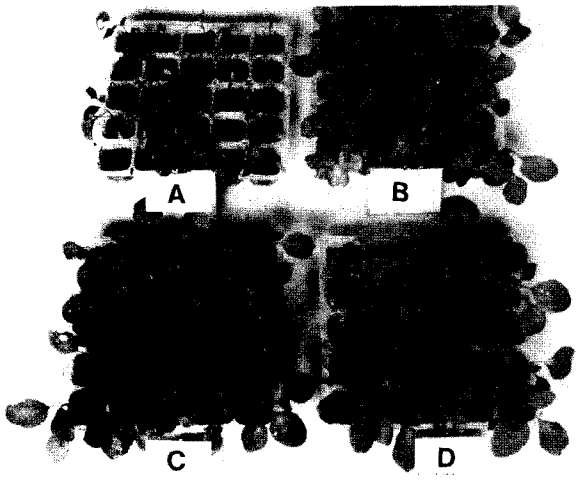

Fig. 8. Suppressive effect of the cultural filtrate of *Bacillus subtilis* YB-70 on root-rot development by *Fusarium solani* on eggplant (*Solanum melogena* L).

Eggplant in infested or non-infested soil was treated with water, cultural filtrate or cells for 30 days and the development of root-rot was observed. A, Untreated eggplant in infested soil. B, Eggplant treated with cultural filtrate in noninfested soil. C, Eggplant treated with cultral filtrate in infested soil. D, Eggplant treated with the bacterial cells in infested soil.

the cultural filtrate exhibited an increase in the number of surviving eggplants to 93% and 92%, respectively, while non-treatment of infested soil resulted in a decrease to 19%. Application of cultural filtrate containing antibiotic substances YBS-ls in soil infested with *F. solani* resulted in 73% disease suppression of seedling root-rot on eggplant after 30 days. None of the treatments was phytotoxic to the seedlings in the presence of *F. solani* (Fig. 8 A). The number of surviving eggplants treated with the bacterial cells or the cultural filtrate was about 5 times higher compared to plants grown in the presence of the fungus (Fig. 8 C and D). In the present of the fungus, eggplant that had been treated with either the bacterial cell or the cultural filtrate had the same number of survivers as lacking the fungus.

DISCUSSION

In a previous report (12), we had been able to isolate the biocontrol agent *Bacillus subtilis* YB-70 that produced the dialysable heat-stable antifungal substances and the main antifungal mechanism involving the suppression against root-rot caused by *Fusarium solani* has been attributed to its antibiotics production. Therefore, the purpose of this study was to isolate the antifungal active fraction that mediated the suppression of root-rot disease from the culture filtrate of *B. subtilis* YB-70 (Fig. 1), and investigate their antifungal and chemical properties. Accordingly, in this paper, we reported that antifungal substances YBS-1s (YBS-1A, -1B, and -1C), isolated from the culture filtrate of *B. subtilis* YB-70 by HPLC analysis (Fig. 2), have a fatal wound inflicted on the germ-tube growth and chlamydospore germination of the phytopathogenic fungus in microscopic observations (Fig. 6 and 7).

Bacillus sp. have previously been reported as promising agents for biocontrol of phytopathogenic fungi (2, 7, 9, 15). All work presented so far is the principal mode of action of these antagonists as the production of antibiotics (1, 2, 7, 9, 10, 15, 20). Most of the known antifungal agents produced by *B. subtilis* are polypeptides with board antimicrobial activities (4, 10, 13, 15). Besson *et al* (4) reported iturin A, an antifungal lipopeptide that was produced by several strains of *B. subtilis* and has solubilities similar to our antifungal antibiotics YBS-1s, but it is soluble in dimethylformamide and ninhydrin-negative. Another antifungal compound, fengymycin (13) possessed solubility characteristics similar to the YBS-1s described here, but it has powerful antagonistic properties against *Rhizoctonia solani* and bacteria such as *B. subtilis* and *Escherichia coli*. YBS-1s showed little activity against *F. solani* and no inhibitory to the bacteria (Table 1). Crude extracts from the *B. subtilis* strain used by Mckeen *et al* (15) separated into four biologically active bands, with R_f values of 0.48, 0.55, 0.60, and 0.67 in the same solvent system, which inhibited *Monilinia fructicola* caused brown-rot disease of several economically important stone fruit crops. The active band with a R_f value of 0.67 is similar to our band of R_f value of 0.67, but it is ninhydrin-negative. Ferreira *et al* (7) reported that crude antifungal extracts from a strain of *B. subtilis* inhibited *Eutypa lata* causing dieback in grapevines showed two biologically active bands, with R_f values of 0.55 and 0.59. All active bands described by these reports above are different from our three biological active bands with R_f values of 0.67, 0.86, and 0.96. Furthermore, YBS-1s had R_f values different from antifungal antibiotics containing bacillomycin, bacilycin, eumycin, iturin A, and mycosubtilin as reported by other similar studies (4, 13).

The action mode of antagonism to soilbome plant pathogens has been elucidated conclusively only in a few cases (1, 7, 17). Light microscope was, in our study, used to investigate the events leading to the inhibition of hyphal growth and spore burst of *F. solani* exposed to YBS-1s. Microscopic observations obviously showed that the action mode of antagonism between YBS-1s and hyphal strands of *F. solani* may be antibiosis that was responsible for restricting the hyphal elongation (Fig. 6). Fungal preparations exposed to YBS-1s exhibited irregular hyphae with bulging (Fig. 6 D), whereas untreated *F.solani* exhibited normal hyphal development including straight growth and branching (Fig. 6 C). It is well known

that strains of *B. subtilis* produce antibiotic substances that can influence the morphology of fungal mycelium of several fungi (1, 2, 7, 17). Ferrira *et al* (7) have reported that hyphal tips of *Eutypa lata* antagonized by *B. subtilis* became malformed, and much swelling occurred at the tips of the hyphal strands. In the fungicidal effect of *B.subtilis*, Backhouse *et al* (1) have suggested that hyphal death of *Sclerotium cepivorum* followed rupture of hyphal walls and leakage of cytoplasm. However, in our study, no swelling of hyphal tips and bursting of hyphal strands at any stage were shown. Thus, our observations suggested that a different antagonistic mechanism contributed the antibiosis of YBS-1s against *F. solani*. It has been reported by several similar studies (2, 7, 15) that the suppression of spore germination and germ-tube growth of several fungi were caused by *B. subtilis*. A conflict result was reported by Swinburne *et al* (17) who found that some fungal spores were not inhibited from culture filtrate of *B. subtilis*, but their germ-tube growth was highly toxic by its cultural filtrate. In our study, antifungal substances YBS-1s were straightly toxic to the germ-tube growth and chlamydospore germination according to the ED_{50} values for them (Fig. 4, 6, and Table 3). Also, YBS-1s had not only fungicidal action but also fungistatic action at the concentration of 2000μg/ml and 500μg/ml, respectively (Table 4). In these results, we may conclude that the structural changes of hyphae and the failure of chlamydospores of *F. solani* to germinate that occurred in this study are considered antibiosis, as defined by Backer and Cook (3), in which YBS-1s may raid into immature germ-tubes growing from chlamydospores and spore death followed the rupture of germ tubes and leakage of cytoplasm (Fig. 7).

In pot bioassay, the cultural filtrate of *B. subtiis* YB-70 protected eggplant against root-rot disease caused by *F. solani* similar to the strain did (Table 4 and Fig. 8). Without the fungus, the cultural filtrate had no influence on the eggplant. Moreover, over 70% disease suppression had been evidence of a potential biocontrol ability of YBS-1s in the cultural filtrate. Since the cultural filtrate contained a very small amount of active materials YBS-1s, each of the purified antibiotics should be active at a very low concentration. Therefore, the use of YBS-1s produced by *B. subtilis* YB-70 may be an economical way to suppress plant disease caused by *F. solani* or other plant pathogenic fungi, and so it is expected that the studies on biological control will contribute greatly to the development of non-pollutional agents against plant pathogens. Accordingly, a problem whether antibiosis from YBS-1s played a significant role in the natural ecosystem lies, because antagonistic phenomena were complex and may involve many kinds of substances. However, successful biocontrol in a regulated environment (Table 4, Fig. 8) encourages research into field applications. In addition, as the antibiotic substances are safer in the changing of environment and have a fungicidal ability, effective induction of the suppression can be possible in soil systems.

Acknowledgement

This research was supported by the grant (93-0500-014-2) from the Korea Sciences and Engineering Foundation.

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(Received March 31, 1994)