

Isolation and Characterization of *Bacillus* Strains for Biological Control

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The object of this study was to characterize *Bacillus* strains GB-017 and GB-0356, which produce antifungal substances, especially for plant pathogens. In addition, this study was undertaken to characterize the culture conditions required for the production of antifungal substances and to document some of the properties of the antifungal substance produced by these soil-isolated strains. Strains GB-0365 and GB-017 were found to be bacillus-shaped, gram-positive and motile, and to inhibit *Botrytis cineria*, *Fusarium* sp., *Pythium* sp., and *Rhizoctonia solani*. Antagonistic activity was maintained up to pH 9.0, and the antifungal activity was stable to heat at 80°C for 1 h. Antifungal substances were separated and purified using ion exchange and adsorption columns including WK-10(H⁺) (pH 7.0), HP-20 column (pH 3.0) and IPA (pH 3.0). Its UV absorption spectrum showed major peaks at 231 and 259 nm, corresponding to polyene and lactone. A fast atom bombardment mass spectrum (FAB MS) showed a highest peak at 441 m/z and major peaks at 192, 205, and 370 m/z.

Key words: *Bacillus*, antagonistics, antifungal substance, biocontrol

Biological control, based on microorganisms to suppress plant disease, offers a powerful alternative to synthetic chemicals. The abuse of chemical pesticides or fungicides to cure or prevent plant diseases has caused soil pollution and detrimental effects in humans. It is desirable to replace chemical pesticides with materials that possess the following three criteria: high specificity against the targeted plant pathogens; easy degradability after effective usage; and low mass production cost. Current practices for controlling plant disease are based largely on genetic resistance in the host plant, management of the plant and its environment, and synthetic pesticides (Strange, 1993). There is a demand for new methods to supplement existing disease control strategies, and to achieve better disease control. Moreover, alternatives to many of the synthetic pesticides currently in use are needed, because they may lose their usefulness; due to revised safety regulations (Duke *et al.*, 1993; Benbrook *et al.*, 1996); concern over non-target effects (Dernoeden and McIntosh, 1991; Elmholt, 1991); or due to the development of resistance in pathogen populations (Russell, 1995). Thus, there is a need for new solutions to plant disease problems that provide effective control, while minimizing the negative con-

sequences for human health and upon the environment (Cook *et al.*, 1996).

Under these circumstances, the biocontrol of some soil-borne diseases has been attracting attention. As most of the soil-borne plant pathogens are fungi, biocontrol by bacteria has been intensively investigated (Lewis and Papaizas, 1984). The use of bacteria has also been investigated mainly because the genetic and biochemical analysis of bacteria and the mass production of bacterial products are more straightforward than those of fungi, and thus the issue of bacterial control is expected to have great potential. *Agrobacterium*, *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Streptomyces*, and others have been reported to be bacterial control agents (Liang *et al.*, 1982; Weller, 1988; Gutterson, 1990; Dowling and OGara, 1994).

The use of a gram-positive *Bacillus* species as a biocontrol agent is relatively rare, and has received less intensive study than the use of gram-negative bacteria. The antagonists studied have been mainly *Bacillus subtilis* and occasionally *B. megaterium*, *B. cereus*, *B. pumilus*, and *B. polymyxa* (Utkhede, 1984; Silo *et al.*, 1994). As *Bacillus* spp. have the characteristics of, being widely distributed in soils, having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. Moreover, they are considered safe biological agents, and their potential as biocontrol agents is considered to be high. However, the evaluation of bacteria has focused pri-

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marily on disease suppression (Siala and Gray, 1974; Acen *et al.*, 1988; Silo-suh *et al.*, 1994), but the population dynamics and mechanisms of suppressing plant pathogens in soil by *Bacillus* spp. have not been extensively investigated.

The object of this study was to characterize *Bacillus* strains GB-017 and GB-0356, which produce antifungal substances for plant pathogens. We also investigated the culture conditions required for the production of antifungals and the properties of the antifungal substance produced by *Bacillus* spp. isolated from soil.

Materials and Methods

Antagonist isolation and identification

Putative antagonists were isolated from the soil in 1996. Soils were collected and washed in 200 ml of sterile 0.05 M phosphate buffer [0.2 M KH_2PO_4 , 70 ml; 0.2 M K_2HPO_4 , 30 ml; and deionized water, 300 ml; pH 6.5] on a rotary shaker for 10 min at 150 rpm. The washings were discarded, and the soils were washed a second time for 10 min in an ultrasonic bath. Washings from sonicated samples (0.1 ml) were plated on nutrient yeast dextrose agar (NYDA) and incubated for 24–48 h at 25°C. Colonies were isolated on the basis of their different visual characteristics. After isolation, all colonies were purified by single colony isolation after triple re-streaking on NYDA medium. Putative antagonists were identified using the general bacterial identification method (Hyun *et al.*, 1999).

Screening of antagonists by the disc diffusion method

To screen potential antagonists, microorganisms were prepared with cultures grown for 24–48 h on NYDA in 4.5 ml of 0.05 M phosphate buffer. For further experiments, antagonist suspensions were prepared by growing cultures in nutrient yeast dextrose broth (NYDB) (NYDA without agar) for 24–48 h at $28 \pm 1^\circ\text{C}$ with shaking at 150 rpm. The medium was centrifuged at $8315 \times g$ for 10 min and cells were resuspended in deionized water. Desired concentrations of cells were obtained by adjusting the suspension according to a standard curve using a spectrophotometer by measuring the optical density at 420 nm.

Sixteen plant pathogens were tested in this experiment: *Botrytis cineria*, *Fusarium* sp., *Pythium* sp., *Phytophthora capsici*, *Alternaria citri*, *Cladosporium cucumerium*, *Colletotrichum gloeosporioides*, *Pleospora* sp., *Rhizoctonia solani* MAFF 511103, *Rhizoctonia solani* MAFF 305245, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Saccharomyces cerevisiae*. These pathogens were sampled from late June through to the first week in August, 1996 at the National Horticultural Research Institute in Suwon and grown on potato dextrose agar (PDA) medium. A haematocytometer was used to determine the concentrations of the conidial suspensions, which were prepared from 10,

14 and 7-day-old cultures of sixteen plant pathogens.

To examine the effects of growth parameters on the production of antifungal, the active strain was incubated under various conditions: temperatures at 4–50°C; pH 3.0–11.0; and 0.5–20% NaCl. After incubating on a rotary shaker (85 rpm), the antifungal activity of culture supernatants was assayed by the disc diffusion method.

Characterization of antifungal substance

The culture supernatant was tested for stability to heat and pH, and its activity was measured by the disc diffusion method. To estimate the effective purifying method of the antifungal substances produced by the active strain, the culture supernatant was fractionated by ion exchange and adsorption columns including WK-10(H^+), HP-20 column and IPA (isopropanol).

Results and Discussion

Antagonist isolation and identification

Among more than 250 microorganisms isolated, 100 were tested by primary screening against plant pathogens. More than 60% of these showed some antagonist activity (a >15% reduction in colony diameter) but only 2% of the tested strains reduced the incidence of infected wounds by more than 50%, and reduced lesion diameters by more than 75% (data not shown). The most effective microorganism was the strain GB-0365.

GB-0365 strain was bacillus-shaped under the electron microscope, gram-positive, and motile. The strain was characterized as *Bacillus subtilis*, and called *Bacillus subtilis* GB-0365. In the case of GB-017 strain, it was also bacillus-shaped, gram-positive, and motile. This strain was also characterized as *Bacillus* sp., and called *Bacillus* sp. GB-017 (Table 1). Most biocontrol agents have been selected *in vitro* or in sterile soil based on disease suppression after artificial inoculation. Isolates of *Bacillus subtilis* (commonly, strains GBO3 and MBI600) have been shown to possess *in vitro* inhibitory activity against nine different soil-borne pathogens, and to control damping-off and improve plant establishment and seedling vigor in the field. Many strains of *B. subtilis* have been shown to be potential biocontrol agents against fungal pathogens. Evidence to date suggests that the principal mechanism of this antifungal action involves the production of antibiotics, especially within soil microsites (Fravel, 1988). However, it is likely that several mechanisms act in concert to achieve control, including the production of volatiles, which have a significant effect on soil microbiology (Linderman and Gilbert, 1975). *B. subtilis* strains also produce volatiles that antagonise a range of organisms including the soil-borne plant pathogens *Rhizoctonia solani* and *Pythium ultimum* (Wright and Thompson, 1985; Fiddaman and Rossal, 1993).

Table 1. Characteristics of the isolated *Bacillus* strains GB-0365 and GB-017

Morphological Characteristics		GB-0365	GB-017
Shape		Bacillus (round)	Bacillus (round)
Gram-staining		Positive	Positive
Endospore form		Ellipsoidal shape	Ellipsoidal shape
Motility		Present	Present
Biological Characteristics			
Range of growth temperature		from 5°C to 50°C	from 5°C to 45°C
Optimal growth temperature		25~35°C	25~35°C
Range of growth pH		5.0~8.0	5.0~8.0
Biochemical oxygen demand		facultative anaerobic	facultative anaerobic
Catalase		+	+
Voges-Proskauer response		-	+
Starch catabolic ability		+	+
Casein catabolic ability		+	+
Gelatin catabolic ability		+	+
Reduction of Nitrate		+	+
Tolerance to NaCl		up to 10.0% (w/v)	up to 10.0% (w/v)
Acid production from carbohydrate sources	Glucose	+	-
	Arabinose	+	+
	Xylose	-	-
	Mannitol	+	-

Table 2. Antimicrobial activity of *Bacillus subtilis* GB-0365 and *Bacillus* sp. GB-017

Name of test organism	Effect of growth repression (mm)	
	<i>B. subtilis</i> GB-0365	<i>Bacillus</i> sp. GB-017
<i>Bacillus subtilis</i>	0	0
<i>Staphylococcus aureus</i>	0	0
<i>Escherichia coli</i>	0	0
<i>Pseudomonas auruginosa</i>	0	0
<i>Candida albicans</i>	0	0
<i>Saccharomyces cerevisiae</i>	0	0
<i>Botrytis cinera</i>	21	17
<i>Fusarium</i> sp.	18	15
<i>Pythium</i> sp.	17	18
<i>Phytophthora capsici</i>	14	11
<i>Alternaria citri</i>	14	15
<i>Cladosporium cucumerium</i>	0	0
<i>Colletotrichum gloeosporioides</i>	15	15
<i>Pleospora</i> sp.	17	14
<i>Rhizoctonia solani</i> MAFF5 11103	14	17
<i>R. solani</i> MAFF 305245	16	15

Screening of antagonist by the disc diffusion method

In terms of antagonism between *Bacillus subtilis* GB-0365 and plant pathogens (Table 2), it was found to inhibit *Botrytis cineria*, *Fusarium* sp., *Pythium* sp., and *R. solani* (Fig. 1). In addition, *Bacillus* sp. GB-017 was found to be inhibitory to *Pythium* sp., *Botrytis cineria*, *Fusarium* sp., and *R. solani*.

The suppression of wilt by *Fusarium* and yield increases in cotton have been reported (Kenney *et al.*, 1992), while inoculation with *B. subtilis* strain GBO3 increased the number of healthy cotton plants by 13.3% versus a standard chemical seed treatment (Brannen, 1995). The antifungal activity of *B. subtilis* is achieved via the production of iturins, which possess broad spectrum of antibiotic

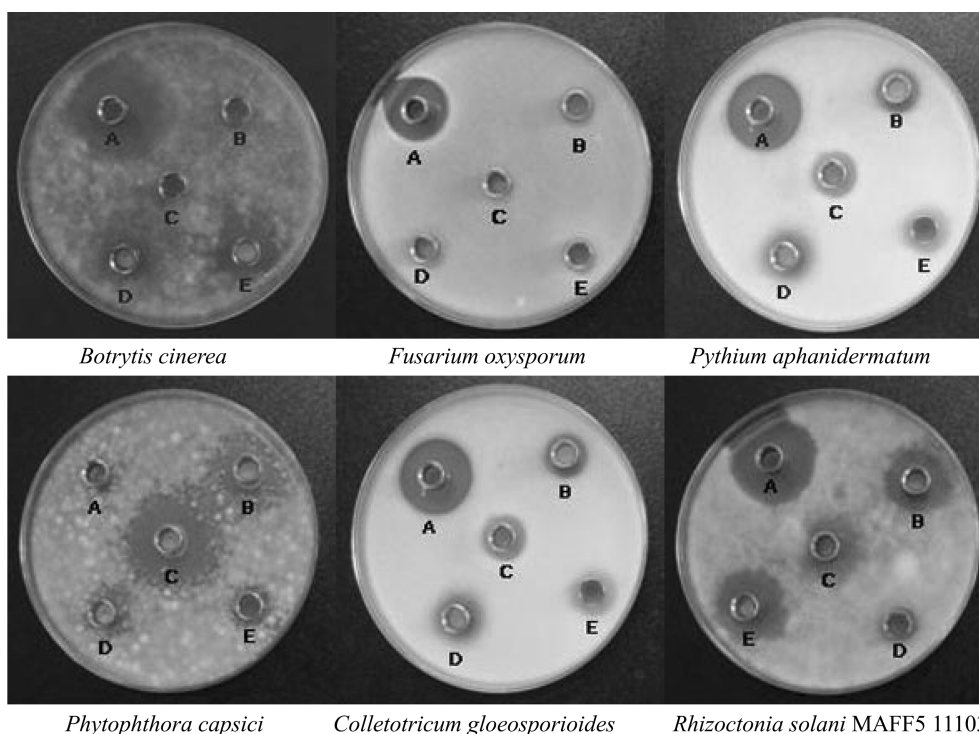


Fig. 1. Results of *in vitro* antagonism testing after 5 days for bacterial strain GB-0365. (A) 6.3×10^6 , (B) 3.15×10^6 , (C) 6.3×10^5 , (D) 3.15×10^5 , (E) 6.3×10^4 cells 1 ml.

Table 3. Result of ion-exchange and adsorption column chromatography of the antibiotics produced by *Bacillus subtilis* GB-0365

Resin	pH	Activity of unbound fraction ^z	Activity of elution fraction ^y
Control			+++
SA20AP OH ⁻	3.0	—	++
	7.0	++	—
	10.0	++	—
SA20AP Cl ⁻	3.0	+	+
	7.0	++	—
	10.0	++	—
Amberlite IRA45 OH ⁻	3.0	—	+
	7.0	—	+
	10.0	+	—
WK-10 H ⁺	7.0	+++	—
	10.0	++	+
WK-10 Na ⁺	7.0	++	—
	10.0	+	+
SKIB H ⁺	3.0	++	+
	7.0	+	+
	10.0	+	+
HP20	3.0	—	+++
	7.0	—	++
	10.0	+	+

^z, ^y—: Inhibition <20%, +: Inhibition 20%~50%
 ++: Inhibition 50%~70%, +++: Inhibition 70%

activity (Klich *et al.*, 1991). *Bacillus* spp. has also been reported to suppress diseases caused by *Pythium* spp., *R. solani* and *Fusarium* spp. (Weller, 1988; Kenney *et al.*, 1992; Brannen, 1995).

Characterization of antifungal substance

In order to characterize the antifungal substances, several physiological and chemical conditions were examined. Antagonistic activity was maintained up to pH 9.0, and the antifungal was stable to heat at 80°C for 1 h (Table 1). The optimum culture condition for *Bacillus* strains GB-0365 and GB-017 was; in YEP medium supplemented with 10% soluble starch (pH 7.0) at 30°C for 36 h.

Various kinds of ion exchange and adsorption column chromatography were used for estimating antifungal activity (Table 3). We successfully separated and refined the antifungal substances using ion exchange and adsorption columns, i.e., WK-10(H⁺) (pH 7.0), HP-20 column (pH 3.0) and IPA (pH 3.0). Fig. 2 shows the antibiotics produced by *Bacillus subtilis* GB-0365 by prep-HPLC. TLC was used to separate the antifungal substances produced by *Bacillus subtilis* GB-0365 (Table 4). Hexane and ethyl acetate (4:6) were used for the development solvent of TLC plate. Hydroxyl or ketone residues were colored using vanillinperchloric acid, anisaldehyde, and phenol sulfuric acid. Therefore, we suggest that the antifungal substances contain sucrose. In addition, we consider that the structure does not contain aliphatic carboxylic acid and amino acid because bromophenol blue, bromophenol

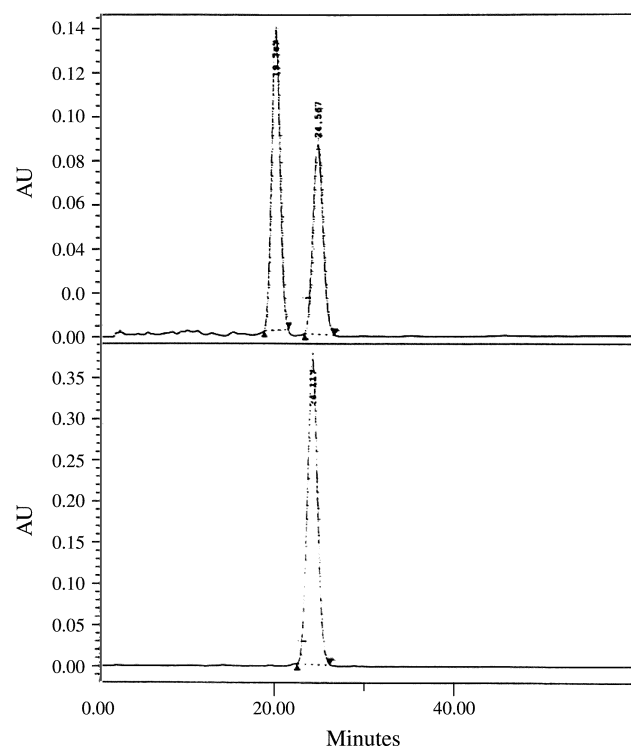


Fig. 2. HPLC profile of the antibiotics produced by *Bacillus subtilis* GB-0365.

Table 4. Responses to various color former of the antibiotics produced by *Bacillus subtilis* GB-0365

Reagents	Detected compound	Results ^a
Aluminum chloride	Flavonoid	+
Anisaldehyde	Sugar	+
Anthrone	Ketose and oligosaccharide	+
Bromophenol blue	Aliphatic carboxylic acid	–
Bromophenol green	Dicarboxylic acid	–
Iodine	Universal	+
Ninhydrin	Amino acid and Amines	–
Orinol	Glycolipid	–
Perchloric acid	Steroid, Thiophosphate ester	+
Phenol sulfuric acid	Carbohydrate	+
Silver Nitrate-Pyrogallol	Carboxylic acid	–
Sulfuric acid	Universal	+
Vallin-Perchloric acid	Higher alcohol and ketone	+

^a+, detected; –, not detected

green and ninhydrin, failed to produce color responses. The antifungal substance dissolved in chloroform but not in H₂O or hexane. Its UV absorption spectrum showed major peaks at 231 and 259 nm, which is typical of polyenes and lactones (Fig. 3). The fast atom bombardment mass spectrum (FAB MS) produced a highest peak at 441 m/z and major peaks at 192, 205, and 370 m/z (Fig. 4).

Some sophisticated separation methods, such as membrane separation or chromatographic procedures, are used successfully in the laboratory but have limitations as large-scale processes. Foam separation is possible and

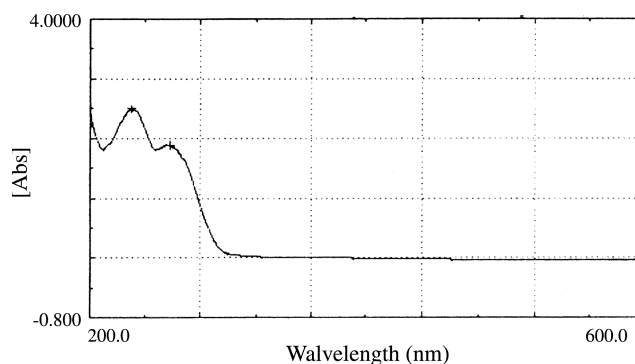


Fig. 3. UV spectrum of the antibiotics produced by *Bacillus subtilis* GB-0365.

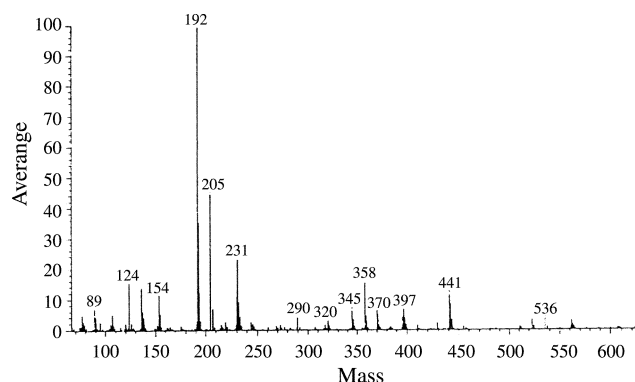


Fig. 4. Mass spectrum of the antibiotics produced by *Bacillus subtilis* GB-0365.

offers an easier separation method. Foam separation has been reported for the removal of several microorganisms from aqueous suspensions (Rubin *et al.*, 1966) and for the separation of the extracellular proteins produced by *Saccharomyces cerevisiae* (Effler *et al.*, 1989). However, foam separation and the concentrations of peptidolipidic substances in jar fermentations is generally cumbersome and the use of antifoam agents is in most cases unavoidable. The advantage of this method is that it is compatible with the chemical nature of the product, which contains both hydrophobic and hydrophilic moieties. The method also utilizes minimal quantities of antifoam agents, which often cause physiologic and economic problems. The sample obtained using this procedure was used for plant testing and its effectiveness was confirmed (Asaka and Shoda, 1996).

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