The Journal of Microbiology, September 2003, p.196-201 Vol. 41, No. 3
Copyright © 2003, The Microbiological Society of Korea

# Isolation and Characterization of *Bacillus* Strains for Biological Control

**Han-Soo Kim[1], Jiyong Park[1], Sung-Won Choi[2], Kee-Hyun Choi[2], Gung Pyo Lee[3], Soo Jung Ban[4], Chang Hoo Lee[4], and Chung Sun Kim[4,*]**

*[1]Department of Biotechnology, Yonsei University, Seoul 120-749, Korea*
*[2]Institute of Life Science, Greenbiotech Co., Kyonggi Province, 413-835, Korea*
*[3]Institute of Natural Science, Seoul Women's University, Seoul 139-240, Korea*
*[4]Division of Bioindustrial Science, College of Life and Environmental Science, Korea University, Seoul 136-701, Korea*

(Received March 28, 2003 / Accepted May 28, 2003)

**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). and IPA. 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 consequences 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-

* To whom correspondence should be addressed.
(Tel) 82-2-3290-3484; (Fax) 82-2-921-2891
(E-mail) chungsk@korea.ac.kr

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±1°C with shaking at 150 rpm. The medium was centrifuged at 8315×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.*, *Phytophtora capsici*, *Alternaria citri*, *Cladosporium cucumerium*, *Colletotrichum gloeosporiodes*, *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 | | form 5°C to 50°C | form 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 | Glucose | + | - |
| carbohydrate sources | 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) | |
|---|---|---|
| | *B. subtilis* GB-0365 | *Bacillus* sp. GB-017 |
| *Bacillus subtilis* | 0 | 0 |
| *Staphylococcus aureus* | 0 | 0 |
| *Escherichia coli* | 0 | 0 |
| *Pseudomonas auruginosa* | 0 | 0 |
| *Candida albicans* | 0 | 0 |
| *Saccharomyces cerevisiae* | 0 | 0 |
| *Botrytis cinera* | 21 | 17 |
| *Fusarium* sp. | 18 | 15 |
| *Pythium* sp. | 17 | 18 |
| *Phytophtora capsici* | 14 | 11 |
| *Alternaria citri* | 14 | 15 |
| *Cladosporium cucumerium* | 0 | 0 |
| *Colletotrichum gloeosporiodes* | 15 | 15 |
| *Pleospora* sp. | 17 | 14 |
| *Rhizoctonia solani* MAFF5 11103 | 14 | 17 |
| *R. solani* 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

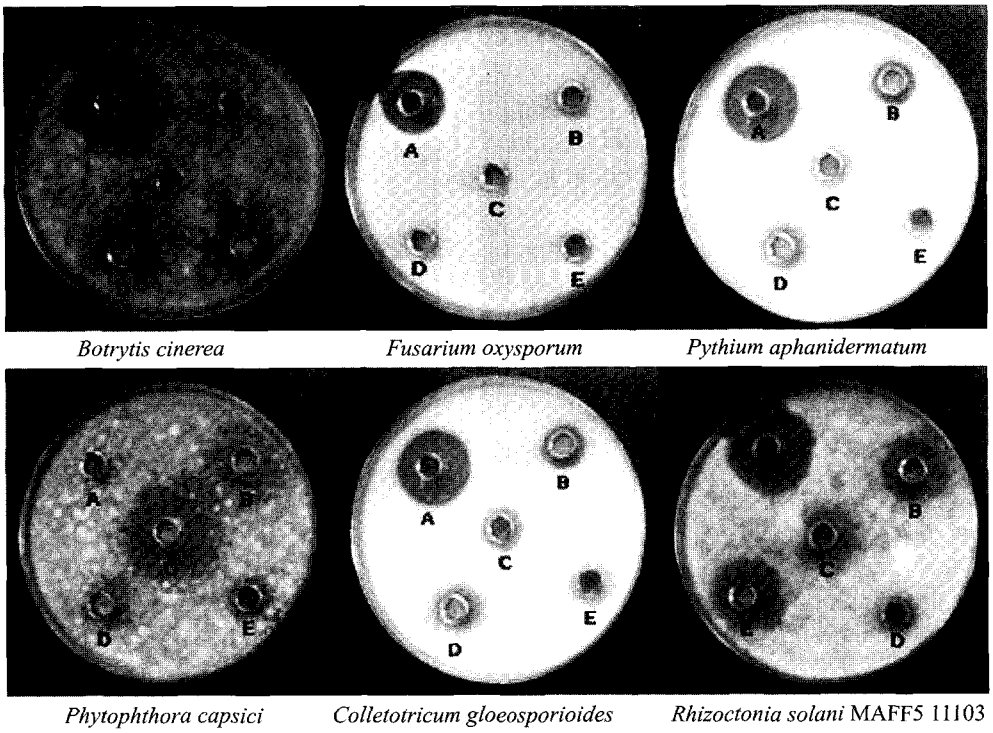


**Fig. 1.** Results of *in vitro* antagonism testing after 5 days for bacterial strain GB-0365. (A) $6.3\times10^6$, (B) $3.15\times10^6$, (C) $6.3\times10^5$, (D) $3.15\times10^5$, (E) $6.3\times10^4$ cells 1 ml.

**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

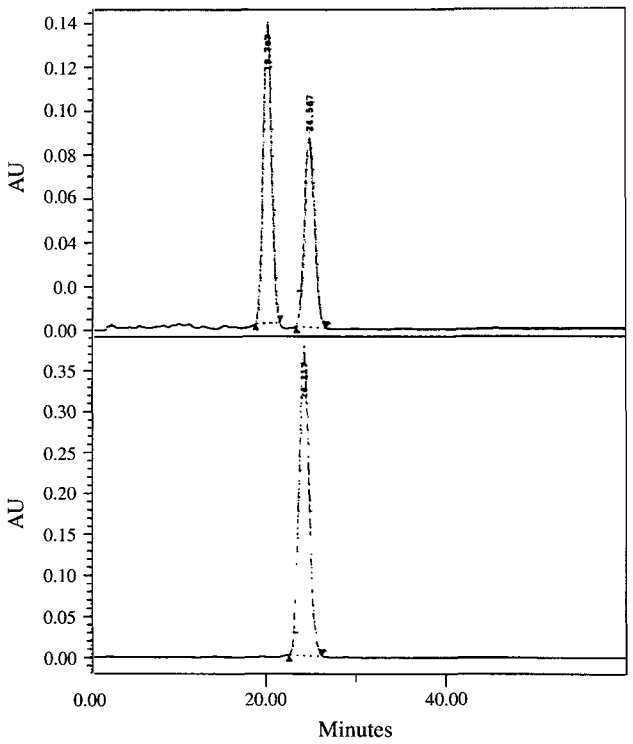


**Fig. 2.** HPLC profile of the antibiotics produced by *Bacillus subtilis* GB-0365.

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

| Reagents | Detected compound | Results[z] |
|---|---|---|
| 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 | + |

[z]+, detected; –, not detected

green and ninhydrin, failed to produce color responses. The antifungal substance dissolved in chloroform but not in $H_2O$ 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

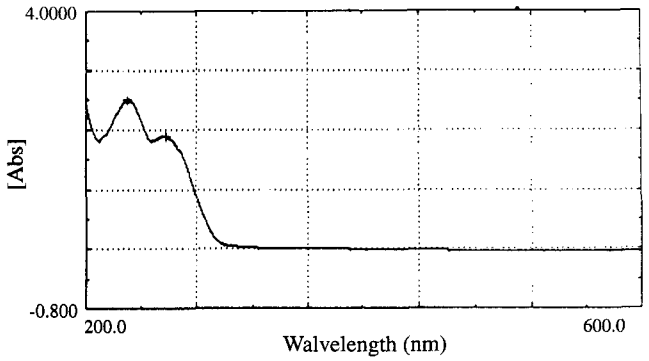


**Fig. 3.** UV spectrum of the antibiotics produced by *Bacillus subtilis* GB-0365.

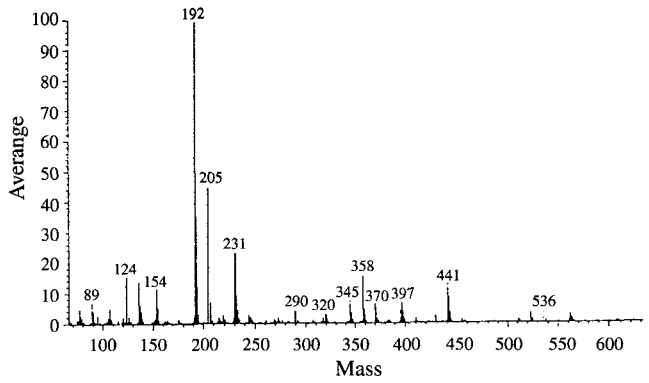


**Fig. 4.** Mass spectrum of the antibiotics produced by *Bacillus subtilis* GB-0365.

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).

## Acknowledgment

This study was supported by a grant from the Agricultural R&D Promotion Center (19980091) to CHL and KHC.

## References

Acen, M.J., C.R. Moore, and M. Alexander. 1988. Survival and growth of bacteria introduced into soil. *Soil Biol. Biochem.* 20,

509-515.

Asaka, O. and M. Shoda. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.* 62, 4081-4085.

Benbrook, C.M., E. Groth, J.M. Halloran, M.K. Hansen, and S. Marquardt. 1996. Pest management at the crossroads, p. 272. Consumers Union, Yonkers.

Brannen, P.M. 1995. Potential modes of action for suppression of root diseases and yield enhancement when using *Bacillus subtilis* seed inoculants on cotton, p. 205-208. *In* Proceedings of the Beltwide Cotton Production Research Conferences. National Cotton Council, Memphis, TN.

Cook, R.J., W.L. Bruckart, J.R. Coulson, M.S. Goettel, R.A. Humber, R.D. Lumsden, J.V. Maddox, M.L. McManus, L. Moore, S.F. Meyer, P.C. Jr. Quimby, J.P. Stack, and J.L. Vaughn. 1996. Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biol. Control.* 7, 333-351.

Dernoeden, P.H. and M.S. McIntosh. 1991. Disease enhancement and Kentucky bluegrass quality as influenced by fungicides. *Agron. J.* 83, 322-326.

Dowling, D.N. and F. OGara. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends Biotechnol.* 12, 133-141.

Duke, S.O., J.J. Menn, and J.R. Plimmer. *1993*. Challenges of pest control with enhanced toxicological and environmental safety, p. 113. *In* Duke, S.O., J.J. Menn, and J.R. Plimmer (eds.), Pest Control with Enhanced Environmental Safety. American Chemical Society, Washington, DC.

Effler, W.T., R.D. Tanner, and G.W. Malaney. 1989. Dynamic *in situ* fractional of extracelluar proteins produced in a bakers yeast cultivation process, p. 235-255. *In* Fiechter, A., H. Okada, and R.D. Tanner (eds.), Bioproducts and bioprocesses. Springer-Verlag Berlin, Heidelberg, New York, London, Paris, Tokyo.

Elmholt, S. 1991. Side effects of propiconazole (Tilt 250 EC) on non-target soil fungi in a field trial compared with natural stress effects. *Microb. Ecol.* 22, 99-108.

Fiddaman, P.J. and S. Rossal. 1993. The production of antifungal volatiles by *Bacillus subtilis*. *J. Bacteriol.* 74, 119-126.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of diseases. *Ann. Rev. Phytopathol.* 26, 75-91.

Gutterson, N. 1990. Microbial fungicides: recent approaches to elucidating mechanisms. *Crit. Rev. Biotechnol.* 10, 69-91.

Hyun, M., B. Kim, I. Chang, H. Park, H. Kim, G. Kim, M. Kim, and D. Park. *1999*. Isolation and identification of an anaerobic dissimilatory Fe(III)-reducing bacterium, *Shewanella putrefaciens* IR-1. *J. Microbiol.* 37, 206-212.

Kenney, D.S., C.R. Howell, and E.B. Minton. 1992. Studies on the mode of action of *Bacillus subtilis* as a biocontrol agent in cotton, p. 198. *In* Proceedings of the Beltwide Cotton Production Research Conferences. National Cotton Council, Memphis, TN.

Klich, M.A., A.R. Lax, and J.M. Bland. 1991. Inhibition of some mycotoxigenic fungi by iturin A, a peptidolipid produced by *Bacillus subtilis*. *Mycopathologia* 116, 77-80.

Lewis, J.A. and G.C. Papaizas. 1984. An approach to stimulate population proliferation *Trichoderma* species and other potential biocontrol fungi introduced into natural soil. *Phytopathology* 74, 1240-1244.

Liang, L.N., J.L. Sinclair, L.M. Mallory, and M. Alexander. 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. *Appl. Environ. Microbiol.* 44, 708-714.

Linderman, R.G. and R.G. Gilbert. 1975. Influence of volatiles of plant origin on soil borne plant pathogens, p. 90-99. *In* Bruehl, G.W. (Ed.), Biology and Control of Soil-Borne Plant Pathogens. American Phytopathological Society, St Paul, MN.

Rubin, A.J., E.A. Cassel, O. Henderson, J.D. Johnson, and J.C. Lamb . 1966. Microfloatation: new low gas-flow rate foam separation technique for bacteria and algae. *Biotechnol. Bioeng.* 8, 135-151.

Russell, P.E. 1995. Fungicide resistance: occurrence and management. *J. Agric. Sci.* 124, 317-323.

Siala, A. and T.R. Gray. 1974. Growth of *Bacillus substilis* and spore germination in soil observed by a fluorescent-antibody technique. *J. Gen. Microbiol.* 81, 191-198.

Silo-suh, L.A., B.J. Lethbridge, S.J. Raffel, H. He, J. Clardy, and J. Handesman. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl. Environ. Microbiol.* 60, 2023-2030.

Strange, R.N. 1993. Plant disease control: towards environmentally acceptable methods, p. 354. Chapman and Hall, New York.

Utkhede, R.S. 1984. Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Can. Bot.* 62, 1032-1035.

Weller, D M. 1988. Biological control of soilborne plant pathogens in the shizosphere with bacteria. *Annu. Rev. Phytopathol.* 26, 379-407.

Wright, S.J.L. and R.J. Thompson. 1985. *Bacillus* volatiles antagonize cyanobacteria. *FEMS Microbiol. Lett.* 30, 263-267.