Production and Partial Characterization of Antifungal Substances Antagonistic to *Monilinia fructicola* from *Bacillus subtilis*

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ABSTRACT

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Antibiotic substances produced by a strain of *Bacillus subtilis* previously found to suppress development of brown rot of stone fruit were produced in a synthetic medium and isolated from the cell-free medium by precipitation by acidification to less than pH 2.5. Impurities were removed by extraction with ethyl acetate and acetone. The active fraction was extracted from the precipitate with 80% ethanol. This extract contained antibiotics that were soluble in ethanol, methanol, isopropanol, and water above pH 7.5; but not soluble in ethyl acetate, acetone, ether, or methylene chloride. Thin-layer chromatographic separation of the extract indicated four biologically active bands. The extract was active against a wide range of plant pathogenic fungi and was fungistatic, not fungicidal, against spores of *Monilinia fructicola*. When tested for activity against *M. fructicola* on peach fruit, the extract showed almost complete suppression of brown rot at 1 mg/ml.

Additional key words: antibiotic, biological control, brown rot, peach, *Prunus*, stone fruit.

Monilinia fructicola (Wint.) Honey causes brown rot disease of several economically important stone fruit crops. The disease has been controlled primarily by chemicals, but recently, fungicide-resistant strains of *Monilinia* have appeared (21). Biological control may be an alternative to chemicals for the control of brown rot. Secondary metabolites of *Bacillus subtilis* (Cohen) have proven to be inhibitory to several plant pathogenic fungi (1,2,5,7,10). Michener and Snell (11) reported two factors, toximycin and mycosubtilin (produced by a strain of *B. subtilis*), which were active in vitro against *M. fructicola*. These broad-spectrum antifungal agents inhibited *M. fructicola* at low concentrations in vitro (15,19).

In biological control studies (3), culture filtrates of *B. subtilis* have proven effective against the agent of bean rust, *Uromyces phaseoli* (Reben.) Wint., and against the incitant of apple leaf scar canker, *Nectria galligena* Bres. (16). Culture filtrates of *B. subtilis* have been reported to control the early blight caused in potatoes by *Alternaria solani* (Ell. et G. Martin) Sor. (18) and to suppress the agents of potato charcoal rot, *Macrophomina phaseolina* (Maubl.) Ashby and *Botryodiplodia solani-tuberosi* Thirum. et O'Brien (17).

Pusey and Wilson (14) isolated a strain of *B. subtilis* (B-3) from soil which inhibited the growth of *M. fructicola*. Cell suspensions of *B. subtilis* sprayed on peaches, nectarines, apricots, or plums significantly reduced brown rot when the fruits were challenged with spores of *M. fructicola*. It was also noted that cell-free culture filtrates had antifungal activity against *M. fructicola*.

The purposes of the present study were to isolate the biologically active fraction of the culture filtrate and to determine the chemical and antifungal properties of the antagonistic substances produced by the B-3 strain of *B. subtilis*.

MATERIALS AND METHODS

Bacterial culture and preparation of spore suspensions. The B-3 strain of *B. subtilis* was originally isolated from the rhizosphere of

an apple tree (14) at the USDA Appalachian Fruit Research Station, Kearneysville, WV. It was identified by specialists at the American Type Culture Collection and is maintained in the Agricultural Research Culture Collection (NRRL B-15813).

The B-3 isolate was stored on silica gel at -20 C. When needed, it was transferred to nutrient agar (NA) plates and grown overnight at 30 C. Isolated colonies were streaked onto a medium containing 6 g of Difco yeast extract, 1.5 g of Difco-Bacto dextrose, 3.6 mg of $MnCl_2 \cdot 4H_2O$, and 25 g of agar per liter of deionized water. The inoculated medium was incubated for 24 hr at 20 C. For spore production, a 1-L Erlenmeyer flask containing 100 ml of the above medium (excluding agar) was inoculated with a loopful of the 24-hr culture and incubated on a rotary shaker at 170 rpm and 30 C for 48 hr. Cells were collected by centrifugation for 20 min at 4,110 g, washed by a second centrifugation in sterile water, and finally resuspended in approximately 20 ml of sterile water. The cells and spores were heated for 1 hr at 60 C and diluted with 125 ml of sterile water. This suspension, which contained about 8×10^6 colony-forming units (cfu) per milliliter was kept refrigerated at 10 C.

Antibiotic production. Potato-dextrose agar (PDA) slants were streaked with loopfuls of the spore suspension and incubated for 24 hr at 30 C. Five milliliters of sterile 0.15 M NaCl was pipetted onto each slant tube. The tubes were gently shaken to wash the cells from the agar surface. One milliliter of the cell suspension was used to inoculate each flask of antibiotic production medium. The medium contained 20 g of Difco-Bacto dextrose, 5 g of DL-glutamic acid, 1.02 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of K_2HPO_4, 0.5 g of KCl, and 1 ml of trace element solution (0.5 g of $MnSO_4 \cdot H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$, and 0.015 g of $FeSO_4 \cdot 7H_2O$ in 100 ml of water) per liter. The pH of the media was adjusted to 6.0-6.2 with 5N NaOH and 500 ml aliquots were dispensed into 2-L Erlenmeyer flasks and autoclaved at 120 C and one atmosphere pressure for 15 min. The inoculated flasks were incubated on a shaker at 170 rpm and 30 C for 3 days.

Antibiotic assay. The medium used to assay the antibiotic contained 5 g of Difco-Bacto dextrose, 1 g of DL-asparagine, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.59 g of $Ca(NO_3)_2 \cdot 4H_2O$, 15 g of agar, and 1 ml of trace element solution (0.04 g of $FeSO_4 \cdot 7H_2O$, 0.04 g of $ZnSO_4 \cdot 7H_2O$, 0.36 g of $MnCl_2 \cdot 4H_2O$, 0.01 g of thiamine, and 0.5 mg of biotin in 100 ml of water) per liter. The medium was autoclaved at 120 C and one atmosphere pressure for 15 min and dispensed into 15×100-mm petri plates. Conidia of *M. fructicola*

were harvested with 4–5 ml sterile water containing 100 ppm of Tergitol from 7- to 21-day cultures grown in the dark on Difco malt extract agar. The spore suspension (0.1 ml) was spread over the surface of each assay plate with a sterile, bent glass rod. After 15–30 min, wells were made in the center of each plate with an 8-mm-diameter cork borer. Aliquots (0.1 ml) of the material to be assayed were pipetted into the wells and the plates were incubated in the dark at 25 C for 2–3 days. The size of the inhibition zone indicated relative activity of the antibiotics.

Extraction of the antibiotics. The production medium was centrifuged for 20 min at 16,500 g to remove bacterial cells. The antibiotics were precipitated from the supernatant by adjusting the pH to 2.5 with concentrated HCl. This material was centrifuged for 10 min at 16,500 g. The pellet containing the active fraction was extracted three times with 80% ethanol. The ethanol extract was taken to dryness under vacuum at 55 C on a rotary evaporator. Inactive substances were removed by sequential extraction with ethyl acetate and acetone. The resulting residue containing the antibiotic activity was dissolved in 80% ethanol, designated as crude antibiotic extract, and stored in capped bottles at 4 C. The yield of active material obtained by this process was approximately 800 mg/ L of production medium.

Fruit assay. The effectiveness of various concentrations of crude antibiotic extract against infections of peaches [*Prunus persica* (L.) Batsch] by *M. fructicola* was determined by the method of Pusey and Wilson (14) with the following modifications: peaches were washed in 0.01% Tergitol and 300 ppm (a.i.) Botran (75% WP) to reduce *Rhizopus* infections. (There was no significant difference [$P > 0.05$] in diameters of lesions caused by *M. fructicola* on peaches washed with Tergitol plus Botran and those washed with Tergitol only.) A single wound, 3 mm in diameter and 3-mm deep, was made on each fruit, then one drop (approximately 50 μl) of the crude ethanolic extract was placed into each wound, followed after drying by one drop of spore suspension containing 10^5 spores of *M. fructicola* per milliliter. Treated fruits were incubated at 25 C for 72 hr. Fruits of peach cultivars Regina, Harbrite, and Norman were used in these tests.

Antifungal spectrum. The antibiotic extract was tested for activity against the fungi listed in Table 1 by the method described previously for *M. fructicola*. Spore suspensions, or when necessary, fragmented mycelial suspensions were spread on the agar surface prior to removing an 8-mm-diameter center well from the agar and the addition of 100 μl of 1 mg/ml antibiotic extract. All fungi were tested in triplicate. Control plates contained 100 μl of 80% ethanol in the 8-mm-diameter center well. Plates were incubated in the light at 24 C. Inhibition zones of the treated plates were measured after clearly visible fungal lawns had covered the control plates.

Antifungal properties. To determine the fungicidal or fungistatic properties of the extract, the 80% ethanol solution designated as crude extract was taken to dryness and the residue was dissolved in 0.1 M sodium phosphate buffer, pH 8.0, to give 1,000 μg/ml, 500 μg/ml, 250 μg/ml, and 100 μg/ml. These solutions and Benlate (50% WP) at 100 μg/ml were tested by using the cellophane-disk transfer method (13). A direct-soak method was also used which consisted of collecting 2×10^6 spores of *M. fructicola* in centrifuge tubes and suspending them in 1 ml of the buffered extract described in the cellophane-disk method. The control consisted of spores suspended in buffer alone. After 2 hr, 0.1 ml of spore suspension was removed, mixed with 0.9 ml of malt extract broth, and incubated for an additional 4 hr. The remainder of the spores were collected by centrifugation at 15,000 g for 15 min, washed twice with sterile water, and suspended in malt extract broth. After 4 hr, 0.1 ml of each suspension was spread on malt extract agar plates. All treatments were conducted in triplicate.

Thin-layer chromatography (TLC). The 80% ethanol fraction containing antibiotic activity was spotted onto 20 × 20-cm silica gel plates (Silica Gel 60 F-254 [fluorescing indicator]; Curtin-Matheson Scientific, Inc., Houston, TX). The plates were developed with ethanol:water (2:1, v/v) and the bands were visualized with UV light or iodine vapor. All detected bands and areas between bands were scraped from the plates, eluted with 80% ethanol, and 200 μl was bioassayed for antibiotic activity. Similar

plates were sprayed with 0.2 g of ninhydrin per 100 ml of 95% ethanol, and heated at 110 C for 5–10 min to detect ninhydrin-positive materials.

RESULTS

Chemical properties of the antibiotic. The active substances were in the cell-free culture medium. Below pH 5, the antibiotic activity was precipitated from solution and was stable when autoclaved for 15 min at one atmosphere gauge pressure. Above pH 7, however, activity was not detected after autoclaving. At room temperature and at 4 C, it was stable in acidic, neutral, and alkaline solutions for several days. The crude ethanol extract exhibited no loss of activity when stored at 4 C for over 6 mo.

The biologically active fraction was insoluble in anhydrous diethyl ether, ethyl acetate, acetone, and methylene chloride. After evaporation of the solvents, the residues had no inhibitory effect against *M. fructicola*. It was slightly soluble in *n*-butanol and soluble in ethanol, methanol, isopropanol, and water above pH 7.5.

When the crude extract was developed on TLC, it separated into four biologically active bands which inhibited *M. fructicola*. The bands with R_f values of 0.48 and 0.55 were ninhydrin positive, whereas the 0.60 and 0.67 R_f bands were not. Analysis of the crude extract by high-performance liquid chromatography showed four active cyclic polypeptide peaks with similar properties (*unpublished*).

Antifungal properties. The crude extract was not fungicidal. After 2 hr of exposure to the test solutions, spores of *M. fructicola* on cellophane disks were transferred to malt extract agar. Within 4 days, growth was observed on all disks treated with the extract but not on the disks treated with Benlate. When spores of *M. fructicola*

TABLE 1. Antifungal spectrum of an antibiotic extract obtained from a culture of *Bacillus subtilis* isolated from apple roots[a]

Fungi	Inhibition zone (mm)[b]	
	Extract	Control
Armillaria mellea (Vahl ex Fr.) Krumm.[c,d]	0	0
Botryosphaeria dothidea (Moug. ex Fr.) Ces. et de Not.[e]	34	0
B. obtusa (Schw.) Cooke[d]	35	0
Botrytis cinerea Pers. ex Fr.	26	0
Ceratocystis ulmi (Buis.) C. Moreau	26	0
Coniothyrium olivaceum Bon.	22	0
Cytospora sp. Tul.	21	0
Endothia parasitica (Murr.) P. J. et H. W. Anderson	37	0
Epicoccum purpurascens Ehr. ex Wallr.	27	0
Fusarium sp. Link[e]	18	0
Geotrichum candidum Link ex Pers.	13	11
Glomerella cingulata (Ston.) Spauld. et Schrenk	27	0
Monilinia fructicola (Wint.) Honey	31	0
Penicillium expansum Lk. ex Thom	23	12
Phytophthora cactorum (Leb. et Cohn) Schroet.	12	0
Pythium aphanidermatum (Edson) Fitzp.[c]	0	0
Pythium irregulare Buis.[c]	0	0
Rhizopus sp. Ehr. ex Cda.	16	0
Scelerotium rolfsii Sacc.[c,d]	0	0

[a] Crude extract (100 μl) dissolved in 80% ethanol was assayed. The assay medium was potato-dextrose agar except: malt-dextrose-peptone agar for *S. rolfsii* and *A. mellea*; cornmeal agar for *P. aphanidermatum* and *P. irregulare*; and peach bark extract agar for *Cytospora* sp. Control treatment was 100 μl of 80% ethanol. An 8-mm-diameter well was removed from the agar and the test solution was placed in it.
[b] Each value represents the mean of three plates.
[c] Hyphae grown in broth and fragmented in a blender were used for the assay. To produce a lawn on the assay plate, 0.1 ml of a 10^5–10^6 spores per milliliter suspension was used for all other assays or fragmented hyphae were used as inoculum.
[d] Culture obtained from F. F. Hendrix, Department of Plant Pathology, University of Georgia, Athens.
[e] Culture obtained from C. C. Reilly, USDA/ARS, S. E. Fruit & Tree Nut Research Laboratory, Byron, GA.

were soaked directly in the same concentrations of extract or Benlate for 2 hr and 0.1 ml was transferred to malt extract agar, growth was observed on all plates within 4 days (Table 2).

The crude extract was fungistatic at concentrations of 500 and 1,000 $\mu g/ml$. After 22 hr of exposure to the extracts or to Benlate, using the cellophane disk and the direct soak methods, less than 1% germination was observed at extract concentrations of 1,000 and 500 $\mu g/ml$. At 100 $\mu g/ml$, 1.8% spores germinated on the cellophane disks and 4.5% germinated in the extract solution. Spores in Benlate at 100 $\mu g/ml$ germinated and formed clumps, but developed no further. Due to the clumping, it was not possible to determine the percentage of germinated spores. Fungicidal and fungistatic test results are summarized in Table 2.

The crude extract inhibited growth of all sporulating fungi tested (Table 1). It did not prevent growth of the fragmented hyphae of the fungi which could not be induced to sporulate.

Regression analysis of the fruit assay results showed that lesion diameter decreased with increasing concentrations of extract. The linear and quadratic effects were significant at $P < 0.05$. At 1,000 $\mu g/ml$ concentration the extract suppressed lesion development by 98% (Table 3).

DISCUSSION

Most of the known antifungal agents produced by *B. subtilis* are polypeptides (2,4,5,8,9,12,15,19,20). Besson et al (4) described

TABLE 2. The effects of the antibiotic extract from *Bacillus subtilis* as a fungicidal and fungistatic agent against spores of *Monilinia fructicola*

Antibiotic extract concentration ($\mu g/ml$)	Fungicidal test[a] (+/− growth)		Fungistatic test[b] (% germination)	
	Cellophane disk[c]	Direct soak[d]	Cellophane disk[c]	Direct soak[d]
1,000	+	+	0.5	0.3
500	+	+	0.5	0.6
250	+	+	0.6	−
100	+	+	1.8	4.5
0 + 100 $\mu g/ml$ Benlate	−	+	3.5	(clumping)
0	+	+	97.2	>90.0

[a] Spores were transferred to malt extract agar after 2 hr of exposure to the crude extract. Growth was determined after 4 days of incubation at 25 C in the dark. The experiment was repeated twice in triplicate.

[b] Spores were exposed to the antibiotics for 22 hr. For each treatment, crude extract containing spores was checked for percent germination by microscopic examination.

[c] Spores were contained on cellophane disks in contact with filter paper saturated with the test substance plus 0.5% sucrose, according to the method of Neely (13).

[d] Soaked in test tubes containing 10^6 spores and 0.4 ml of antibiotic extract supplemented with 0.5% sucrose.

TABLE 3. Reduction in brown rot decay of fruit that had been wounded, treated with antibiotic extract from cultures of *Bacillus subtilis*, challenged with *Monilinia fructicola* spores, and incubated for 3 days at 25 C in a moist chamber

Antibiotic extract concentration ($\mu g/ml$)	Brown rot lesions (mm[a])				
	Test 1	Test 2	Test 3	x̄	Inhibition (%)[b]
1,000	0	2	0	1	98
500	5	23	17	15	69
250	19	39	27	28	42
100	43	40	46	43	10
0 (control)	51	41	51	48	0

[a] Each value per test represents the mean of 10 fruit. Peaches were wounded, treated with extract, and inoculated with spores of *M. fructicola*, and lesion diameter was determined after 3 days.

[b] Percent inhibition = [1 − (extract lesion conc. lesion/control lesion)] × 100.

iturin A, an antifungal cyclic polypeptide which was produced by several strains of *B. subtilis* and has solubilities similar to our crude extract. When chromatographed on TLC, iturin A yields a single band. Another cyclic polypeptide, mycosubtilin, is partially soluble in 70% ethanol. *M. fructicola* is completely inhibited by purified mycosubtilin at 2.5 $\mu g/ml$ (19). Hosono and Suzuki (6) described an acylpeptide which inhibited cyclic AMP phosphodiesterase and was soluble in ethyl acetate. Eumycin (7) possessed solubility characteristics similar to the crude extract described here, but it is not active against *Monilinia*. The aspergillus and rhizoctonia factors found by Michener and Snell (11) were active against *M. fructicola* but soluble in water. Asante and Neal (1) reported three low-molecular-weight acids produced by a bacterium belonging to the genus *Bacillus*. The acids were identified as acetic acid, isobutyric acid, and α-methyl butyric acid. Biological activity of the acids remained when the culture was autoclaved at neutral pH; in contrast, all activity was lost from our substance when it was autoclaved at pH 7. The four active cyclic polypeptides we obtained are similar to iturin A (*unpublished*).

The crude extract was fungistatic at 500 $\mu g/ml$, but not at 100 $\mu g/ml$. Lesions resulting from infection of peaches by *M. fructicola* were almost totally controlled at 1,000 $\mu g/ml$ (approximately 50 μg per wound). Since the crude extract contained a large amount of inactive material, the purified antibiotic should be active at very low concentrations.

The crude extract was active against 15 of the 19 fungal species tested for sensitivity. Of the fungi that were sensitive to the compound, spores and not hyphae were assayed. The extract showed no activity against the fragmented hyphae of the four fungi which could not be induced to sporulate. No comparison was made between inhibition of spore germination and hyphal growth of the same species. Pusey and Wilson (14) theorized that the primary antibiotic action was inhibition of spore germination rather than hyphal growth. However, zones of inhibition on our assay plates remained after weeks of incubation. Apparently, the antifungal materials act against spore germination and hyphal growth, although the mode of suppression is not known.

The antibiotic compounds we studied exhibited strong antifungal properties against a broad range of plant pathogenic fungi. Once these compounds are identified and the mode of action is elucidated, the compounds have the potential to control not only brown rot but also other plant diseases.

LITERATURE CITED

1. Asante, G. S., and Neal, A. L. 1964. Characterization of fungistatic substances produced by a *Bacillus* antagonistic to *Ceratocystis ulmi*. Phytopathology 54:819-822.
2. Babad, J., Pinsky, A., Turner-Graff, R., and Sharon, N. 1952. An antifungal polypeptide produced by *Bacillus subtilis*. Nature 170:618-619.
3. Baker, C. J., Stavely, J. R., Thomas, C. A., Sasser, M., and MacFall, J. S. 1983. Inhibitory effect of *Bacillus subtilis* on *Uromyces phaseoli* and on development of rust pustules on bean leaves. Phytopathology 73:1148-1152.
4. Besson, F., Peypoux, F., Michel, G., and Delcambe, L. 1976. Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis*. J. Antibiotics 29:1043-1049.
5. Burachik, M., Leardini, N. A., and Paladini, A. C. 1964. Three antifungal polypeptides from *Bacillus subtilis*. Experientia 20:504-505.
6. Hosono, K., and Suzuki, H. 1983. Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. I. Purification, physiochemical properties and structure of fatty acid residues. J. Antibiotics 36:667-673.
7. Johnson, E. A., and Burdon, K. L. 1946. Eumycin—A new antibiotic active against pathogenic fungi and higher bacteria, including bacilli of tuberculosis and diphtheria. J. Bacteriol. 51:591.
8. Katz, E., and Demain, A. C. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. Bacteriol. Rev. 41:449-474.
9. Korzybski, T., Kowszyk-Gindifer, Z., and Kurylowicz, W. 1978. Antibiotics isolated from the genus *Bacillus* (Bacillaceae). Pages 1529-1661 in: Antibiotics—Origin, Nature and Properties. Vol. III. American Society of Microbiology, Washington, DC.

10. Landy, M., Warren, G. H., Rosenman, S. B., and Colio, L. G. 1948. Bacillomycin: An antibiotic from *Bacillus subtilis* active against pathogenic fungi. Proc. Soc. Exp. Biol. Med. 67:539-541.

11. Michener, H. D., and Snell, N. 1949. Two antifungal substances from *Bacillus subtilis* cultures. Arch. Biochem. 22:208-214.

12. Nandi, P., and Sen, G. P. 1953. An antifungal substance from a strain of *B. subtilis*. Nature 172:871-872.

13. Neely, D. 1978. A cellophane-transfer bioassay to detect fungicides. Pages 15-18 in: Methods for Evaluating Plant Fungicides, Nematicides, and Bactericides. American Phytopathological Society, St. Paul, MN. 141 pp.

14. Pusey, P. L., and Wilson, C. L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Dis. 68:753-756.

15. Stessel, G. J., Leben, C., and Keitt, G. W. 1953. Partial purification and properties of the antifungal antibiotic, toximycin. Phytopathology 43:23-26.

16. Swinburne, T. R., Barr, J. G., and Brown, A. E. 1975. Production of antibiotics of *Bacillus subtilis* and their effect on fungal colonists of apple leaf scars. Trans. Br. Mycol. Soc. 65:211-217.

17. Thirumalachar, M. J., and O'Brien, M. J. 1977. Suppression of charcoal rot in potato with a bacterial antagonist. Plant Dis. Rep. 61:543-546.

18. Vasudeva, R. S., and Chakravarthi, B. P. 1954. The antibiotic action of *Bacillus subtilis* in relation to certain parasitic fungi, with special reference to *Alternaria solani*. Ann. Appl. Biol. 41:612-618.

19. Walton, R. B., and Woodruff, H. B. 1949. A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth. J. Clin. Investig. 28:924-926.

20. Yajima, T. 1955. On the classification of antifungal antibiotics. J. Antibiot. (Ser. A) (Tokyo) 8:189-195.

21. Zehr, E. I. 1982. Control of brown rot in peach orchards. Plant Dis. 66:1101-1105.