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Biological Activities of Two Fungistatic Antibiotics Produced by *Bacillus cereus* UW85

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Cultures and culture filtrates of *Bacillus cereus* UW85 suppress damping-off of alfalfa caused by *Phytophthora medicaginis*. We studied the role in disease suppression of two antibiotics from culture filtrates of UW85 that reversibly inhibited growth of *P. medicaginis*. We purified the two antibiotics by cation-exchange chromatography and high-voltage paper electrophoresis and showed that one of them, designated zwittermicin A, was an aminopolyol of 396 Da that was cationic at pH 7.0; the second, designated antibiotic B, appeared to be an aminoglycoside containing a disaccharide. Both antibiotics prevented disease of alfalfa seedlings caused by *P. medicaginis*. Purified zwittermicin A reversibly reduced elongation of germ tubes derived from cysts of *P. medicaginis*, and antibiotic B caused swelling of the germ tubes. Mutants generated with Tn*917* or mitomycin C treatment were screened either for antibiotic accumulation in an agar plate diffusion assay or for the ability to suppress damping-off disease of alfalfa. Of 2,682 mutants screened for antibiotic accumulation, 5 mutants were substantially reduced in antibiotic accumulation and disease-suppressive activity. Of the 1,700 mutants screened for disease-suppressive activity, 3 mutants had reduced activity and they accumulated less of both antibiotics than did the parent strain. The amount of antibiotic accumulated by the mutants was significantly correlated with the level of disease suppression. Addition of either zwittermicin A or antibiotic B to alfalfa plants inoculated with a culture of a nonsuppressive mutant resulted in disease suppression. These results demonstrate that *B. cereus* UW85 produces two fungistatic antibiotics that contribute to suppression of damping-off disease of alfalfa.

Biological control, or the use of microorganisms to prevent disease, offers an attractive alternative or supplement to pesticides and genetic resistance for the management of plant disease. Numerous microorganisms with biological control activity have been identified (5, 9, 14, 16, 17, 20, 26, 27, 38, 40, 42, 44), and many have been effective in field experiments (21, 23, 25), but few have suppressed disease consistently under a wide range of conditions. Successful use of existing microorganisms or construction of improved variants for control of plant disease will be enhanced by understanding the mechanisms of biological control at the molecular and biochemical levels and by determining the basis for variability in biological control in the agroecosystem.

The majority of organisms investigated as biological control agents have been gram-negative bacteria such as *Agrobacterium* (3, 21), *Pseudomonas* (16, 17, 27, 35, 38, 42, 44), and *Erwinia* (20, 23, 26, 37) species. Although several *Bacillus* strains (18) and actinomycetes (5) have been reported to have biological control activity, in general the gram-positive bacteria have received far less intensive study than the gram-negative organisms. *Bacillus* spp. and actinomycetes share several features that make them attractive candidates for biological control agents, including their abundance in soil, the production of various biologically active metabolites, and the ability to form stable heat-resistant spores.

We previously described *Bacillus cereus* UW85, which has a wide range of biological effects on plants. It protects alfalfa seedlings from damping-off caused by *Phytophthora medicaginis* (14), tobacco seedlings from *Phytophthora nicotianae* (12), cucumber fruits from rot caused by *Pythium aphanidermatum* (33), and peanuts from *Sclerotinia minor* (29). It also enhances nodulation of soybeans (11) and causes substantial changes in bacterial communities on soybean roots (8). Suppression of alfalfa damping-off in the laboratory is associated with the extracellular fraction from fully sporulated cultures of UW85 (14). In this paper, we describe the biological and chemical properties of two antibiotics from filtrates of UW85 cultures. We present biochemical evidence and a mutant analysis that demonstrate that the antibiotics contribute to disease suppression by UW85.

MATERIALS AND METHODS

Bacterial strains and culture media. *B. cereus* was grown with vigorous shaking in half-strength Trypticase soy broth (TSB; BBL, Cockeysville, Md.). Trypticase soy agar (TSA) was made by adding 15.0 g of agar per liter to TSB before autoclaving. L broth was made according to the method of Maniatis et al. (24). Water agar contained 15.0 g of agar per liter of distilled water. Potato dextrose agar contained 250 g of autoclaved strained potatoes, 20.0 g of dextrose, and 10.0 g of agar in 1 liter of water.

The strains used in this study are described in Table 1. *Erwinia herbicola* LS005 was isolated from an actively growing culture of *Pythium aphanidermatum*, which was a gift from M. J. Havey, University of Wisconsin, Madison. The bacterium was colony purified on TSA and identified by standard bacteriological tests (22) and fatty acid analysis (Five Star Labs, Branford, Conn.) as *E. herbicola*.

Identification and purification of antifungal antibiotics. We used two cation-exchange resins for the initial purification of the antibiotics. With the first, a 1-liter, 3-day-old, fully sporu-

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TABLE 1. Bacterial strains used in this study

Strain	Parent	Mutagen	Phenotype[a]	Reference
UW85	Wild type	NA[b]	Ant^+ Bic^+	14
UW85n1[c]	UW85	None	Ant^+ Bic^+	10
UW85n1-t[d]	UW85n1	NA	Ant^+ Bic^+	10
BC569	Wild type	NA	Ant^- Bic^-	1
UW030	UW85n1-t	Mitomycin C	Ant^- Bic^-	This study
UW831	UW85n1	Mitomycin C	Ant^- Bic^-	This study
UW167	UW85	Tn*917*	Ant^- Bic^-	This study
UW226	UW85	Tn*917*	Ant^- Bic^-	This study
UW325	UW85	Tn*917*	Ant^- Bic^-	This study
UW419	UW85	Tn*917*	Ant^- Bic^-	This study
UW452	UW85	Tn*917*	Ant^- Bic^-	This study
UW457	UW85	Tn*917*	Ant^- Bic^-	This study

[a] Ant indicates antibiosis phenotype determined by the *E. herbicola* plate assay, and Bic indicates biological control phenotype determined with the test tube damping-off assay.
[b] NA, not applicable.
[c] UW85n1 is a spontaneous mutant selected for resistance to 5 μg of neomycin per ml.
[d] UW85n1-t contains plasmid pBC16.

lated culture of UW85 grown in half-strength TSB was centrifuged to remove spores, and the supernatant was adjusted to pH 7.0 with concentrated HCl. The entire neutralized preparation was applied to a column (2.0 by 35.0 cm) containing carboxymethyl-Sephadex cation-exchange matrix (Sigma Chemical Co., St. Louis, Mo.) in the ammonium form. The column was then washed with 5.0 mM NH_4HPO_4-NH_3 (pH 7.0) and eluted with 0.1 M NH_4OH (pH 10.3). Fractions (10.0 ml) were collected and assayed for inhibition of *P. medicaginis*. Active fractions were dried in a rotary evaporator at 45°C and then resuspended in water. The resuspended material was applied to the origin of a Whatman no. 1 filter paper (15 by 28.5 cm) and subjected to preparative high-voltage paper electrophoresis at pH 9.2 and 2,000 V for 1 h (34). One 1-cm section was cut lengthwise and saved for staining. The rest of the electrophoretogram was cut crosswise into 1-cm strips from which material was eluted with water. The eluants from each section of paper were pooled and assayed for inhibition of *P. medicaginis* to identify the fractions with biological activity. The strip of the electrophoretogram that was saved was stained with ninhydrin (0.25% in acetone) or silver nitrate (36) or heated without staining at 110°C. The fractions that inhibited fungal growth were dried at 45°C, applied to filter paper, and subjected to high-voltage paper electrophoresis at pH 1.7 and 3,000 V for 15 min. The electrophoretogram was cut into 1-cm strips, and the purified material was eluted with water.

A second purification method was used for the isolation of the antibiotics from the mutant cultures and for large-scale purification of the antibiotics. Culture supernatants were obtained as described above but were adjusted to pH 7.0 with 2.0 M NaH_2PO_4 and then applied to a column (2.2 by 30.0 cm) containing Amberlite IRC-50 (Sigma). The column was washed with 5.0 mM NH_4HPO_4-NH_3 (pH 7.0) and eluted with 1.0 M NH_3 (pH 11.2). The material in approximately 30.0 ml was collected from the column after the pH was raised above 10 and dried in a rotary evaporator at 45°C, and the antibiotics were quantified.

Quantification of the antibiotics. An endpoint dilution analysis was used to quantify the antibiotics in cation-exchange fractions of UW85 and mutant cultures. Purified antibiotic standards were applied to filter paper in twofold dilutions next to twofold dilutions of material from the strain to be tested. After paper electrophoresis at pH 9.2, the paper was dried and stained with silver nitrate and inspected for the presence of spots characteristic of each antibiotic. To determine the amount of antibiotic in a sample, the final dilution at which antibiotic was visible was compared with the minimum amount of purified antibiotic required for a visible spot in the standards. The minimum levels of detection were 0.25 μg/ml for zwittermicin A and 0.12 μg/ml for antibiotic B.

Assay for inhibition of fungal growth. Zoospores of *P. medicaginis* were produced from strain M2019 according to the method in the modified bioassay described previously (14) or as follows: approximately 1×10^5 to 2×10^5 zoospores were spread onto V8 agar plates containing chloramphenicol (5.0 μg/ml) and erythromycin (2.0 μg/ml). The plates were incubated at 28°C for 6 days, flooded with 20 to 25 ml of sterile distilled water, and incubated for 16 h at 16°C. Approximately 5.0 to 10.0 ml containing 10^6 zoospores per ml was recovered from these plates. A suspension (0.2 ml) containing 5×10^5 to 1×10^6 *P. medicaginis* zoospores per ml was spread on potato dextrose agar plates. Wells 8 mm in diameter were produced with a flame-sterilized cork borer, 50 to 100 μl of filter-sterilized culture supernatant or purified antibiotic sample was placed in each well, and the plates were incubated at 24°C for 48 h. Zones of inhibition were measured from the well to the perimeter of the visible growth of *P. medicaginis*.

Assays for zoospore lysis and inhibition of germ tube elongation. Zoospore lysis was assayed as described previously (6). Antibiotics to be tested for inhibition of germ tube elongation were suspended in 500 μl of half-strength TSB, and 500 μl of a suspension containing 10^5 zoospores per ml was added. The mixture was agitated briefly with a Vortex mixer and incubated at room temperature for 2, 4, or 24 h. The lengths of germ tubes from germinated cysts were measured under a Zeiss inverted microscope with a micrometer.

Assay for inhibition of growth of *E. herbicola*. *E. herbicola* LS005 was inoculated into 5.0 ml of half-strength TSB, and the mixture was incubated overnight at 28°C with vigorous agitation. The culture was diluted in sterile distilled water, and 10^5 cells were spread on a water agar plate. Wells were made in the agar by removing a 1-cm plug of agar with a flame-sterilized cork borer. Bacterial cultures to be tested (100 μl) were placed in the wells, and the plates were incubated for 2 days at 21 to 24°C. Plates were scored for the presence or absence of a zone of inhibition of growth around the wells.

Test tube assay for suppression of damping-off in vermiculite. To screen mutants for disease-suppressive activity, they were grown for 3 days in TSB, and each mutant was tested on three or six plants in the modified assay for biological control of alfalfa damping-off caused by *P. medicaginis* described previously (14). Mutants that did not reduce seedling mortality compared with controls treated with TSB were rescreened on a total of nine plants. Those mutants that did not reduce seedling mortality in the second screen were tested on 54 plants. The SAS statistical computer program was used for statistical analyses (31).

Plate assay for disease of alfalfa seedlings. Alfalfa seeds were germinated as described previously (14) and placed on water agar plates at a density of 10 seedlings per plate. A 10.0-μl mixture of approximately 25 zoospores of *P. medicaginis* and purified antibiotic or water was applied to each seedling. The plates were incubated at room temperature, and the seedlings were scored for symptoms of disease after 4 or 5 days. Diseased seedlings were characterized by browning of the stems and visible growth of the *P. medicaginis* on the surface of the seedling.

Transformation of UW85 by electroporation. *B. cereus* UW85 was grown overnight in 5 ml of L broth with vigorous

agitation. The culture was diluted to 500 ml in fresh L broth and incubated at 28°C with vigorous agitation. When the culture reached an optical density of 0.3 (600 nm), it was centrifuged at 10,000 × *g* and resuspended in 80 ml of EP buffer at 4°C. EP buffer contained 0.5 mM K_2HPO_4-KH_2PO_4, 0.5 mM $MgCl_2$, and 272 mM sucrose. The cells were centrifuged again, resuspended in 0.5 ml of EP buffer, and stored on ice. Plasmid DNA was isolated from *B. subtilis* by the method described by Bron (2), and 0.1 to 1.0 μg of DNA in 1 to 10 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA) was mixed with 0.1 ml of the cell suspension in a cold, sterile microcentrifuge tube; the tubes were incubated on ice for 5 to 10 min. The suspension of cells and DNA was transferred to a chilled cuvette with a 0.2-cm interelectrode gap (Bio-Rad, San Francisco, Calif.) and electroporated at 25 μF, 200 Ω, and 1.2 kV in a Gene Pulser electroporation apparatus (Bio-Rad). Immediately following pulse delivery, 1.5 ml of L broth was transferred to the cuvette. The cell suspension was transferred to a 16-mm test tube, which was incubated at 37°C with shaking for 5 h, and then spread on L agar plates containing 10.0 μg of chloramphenicol (Sigma); the plates were incubated at 28°C overnight.

Transposon mutagenesis of UW85. Mutagenesis with Tn*917* was conducted with pTV32 as the transposon vector as described by Youngman et al. (45), with the following modifications. pTV32 was introduced by electroporation as described above. It was cured at 44°C because UW85 did not grow at higher temperatures. Following growth at 44°C for 48 h, the colonies were replica plated onto fresh medium containing lincomycin (25.0 μg/ml) and erythromycin (1.0 μg/ml) or erythromycin alone (10.0 μg/ml) and incubated overnight at 28°C. The lincomycin- and erythromycin-resistant colonies were patched onto fresh medium containing chloramphenicol (5 μg/ml). The chloramphenicol-sensitive, lincomycin- and erythromycin-resistant colonies were studied further.

Treatment of UW85 derivatives with mitomycin C. Mitomycin C treatment was conducted with two strains of UW85 that carry selectable markers. UW85n1 is a spontaneous mutant of UW85 that is resistant to 5.0 μg of neomycin per ml, and UW85n1-t was produced by transferring plasmid pBC16 from *B. cereus* 569UM-1 into UW85n1 by conjugation (1). pBC16 confers resistance to tetracycline, which was used to select for transconjugants. For each batch of cells to be mutagenized, a single colony was inoculated into 1.0 ml of TSB amended with 5.0 μg of neomycin per ml. When UW85n1-t was treated, the medium was amended with 5.0 μg of neomycin and 10.0 μg of tetracycline per ml. After 12 to 16 h of incubation at 28°C with vigorous shaking, 5.0 μl of each culture was transferred to a 250-ml sidearm flask containing 50.0 ml of TSB, which was incubated at 28°C with vigorous shaking. The optical density of the culture at 600 nm was determined, and when the culture was growing exponentially, 1.0 μg of mitomycin C per ml was added. After 9 h at 28°C, the survivors in the culture were spread on TSA plates, which were incubated for 3 days at 28°C; 100 survivors from each treated batch were picked and colony purified on TSA plates amended with 5.0 μg of neomycin per ml. The colonies derived from UW85n1-t were transferred to plates containing 10.0 μg of tetracycline per ml to screen for loss of the plasmid, and isolates that were resistant to both neomycin and tetracycline were saved for further study.

RESULTS

Purification and chemical properties of UW85 antibiotics. Filtrates of cultures of UW85 suppressed alfalfa disease caused by *P. medicaginis* (14) and inhibited growth of the pathogen in an agar plate assay (Fig. 1). To determine whether the same compounds were responsible for disease suppression and growth inhibition, we first purified two antibiotics that inhibited growth of *P. medicaginis* in the plate assay. Both antibiotics bound to carboxymethyl-Sephadex or Amberlite IRC-50 at pH 7.0 and were eluted when the pH was raised above 10.0. The active fractions were pooled and further purified by high-voltage paper electrophoresis at pH 9.2. We identified two spots on the paper electrophoretograms associated with inhibitory activity in the agar plate diffusion assay. The first was designated zwittermicin A and had a relative mobility (R_f) of 0.30 compared with Orange G. The second antibiotic was given the provisional designation antibiotic B and had an R_f of 0.032. The spots on the electrophoretogram at pH 9.2 that coincided with biological activity were eluted and subjected to high-voltage paper electrophoresis at pH 1.7, in which zwittermicin A and antibiotic B had mobilities similar to a dication (R_f = −1.042 and −0.909, respectively). The active materials coincided on the electrophoretograms with spots that stained purple with ninhydrin, stained negatively with silver nitrate, and turned brown when the paper was heated at or above 110°C for 20 min. Fast atom bombardment mass spectroscopy indicated that zwittermicin A has a molecular mass of 396 Da. ^1H, ^{13}C, and two-dimensional nuclear magnetic resonance analyses and elucidation of the structures of the antibiotics will be presented elsewhere. The proposed structure of zwittermicin A, which is an aminopolyol, is shown in Fig. 2. The staining properties and nuclear magnetic resonance profile of antibiotic B are consistent with those of an aminoglycoside.

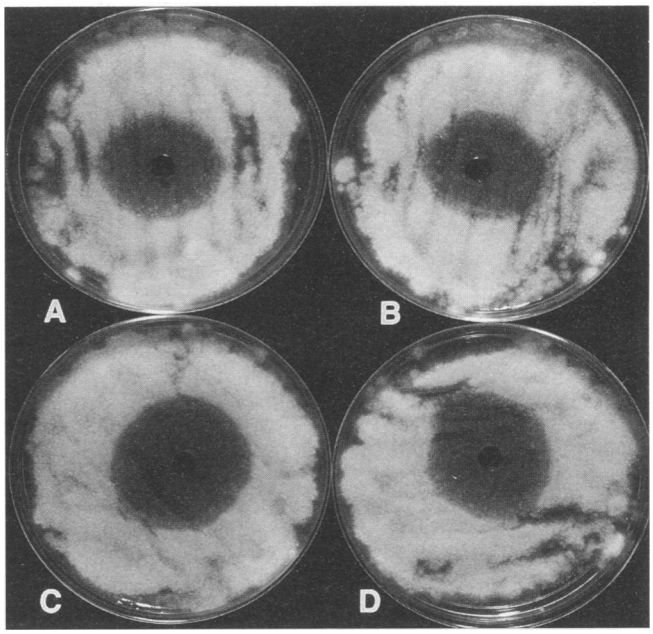

FIG. 1. Inhibition of growth of *P. medicaginis* on agar plates by (A) 100 μl of UW85 culture filtrate, (B) the cationic fraction from 250 μl of UW85 culture filtrate, (C) 4 μg of purified zwittermicin A, or (D) 40 μg of purified antibiotic B.

Biological properties of the antibiotics. To determine whether the antibiotics interfered with germination or germ tube elongation, we treated cysts of *P. medicaginis* with purified antibiotics from cultures of UW85. After 2 h in rich medium, more than 90% of the cysts had germinated in all samples (Fig. 3). Germ tubes in those samples treated for 4 h with medium

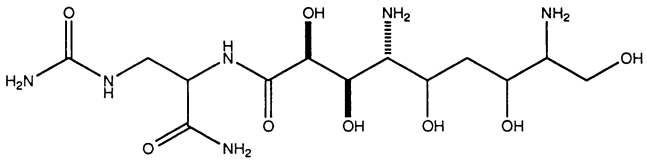

FIG. 2. Proposed structure of zwittermicin A.

alone averaged 276 μm, whereas those in samples treated with 100 μg of purified zwittermicin A per ml averaged 130 μm (Fig. 4). Germ tube length was inversely proportional to the concentration of zwittermicin A. High concentrations of antibiotic B reduced germ tube elongation during the 4-h incubation (Fig. 4), and 24 h after treatment with antibiotic B, the tips of the germ tubes were swollen and deformed. The antibiotics had no visible effect on swimming behavior of zoospores of *P. medicaginis* as determined by direct microscopic observation. Viable *P. medicaginis* was recovered after a 24-h incubation with either zwittermicin A or antibiotic B by plating the germinated cysts onto V8 agar. Therefore, the antibiotics appeared to reversibly block normal development of germ tubes derived from cysts of *P. medicaginis*, although they did not affect zoospore behavior or cyst germination.

We tested the purified antibiotics for the ability to suppress disease of alfalfa caused by *P. medicaginis* on agar plates. Alfalfa seedling survival was directly proportional to the concentration of each antibiotic, although zwittermicin A provided better disease suppression than antibiotic B at lower concentrations (Fig. 5). For example, when 4 μg of zwittermicin A was added to each seedling, 100% of the seedlings survived, whereas only 60% of the seedlings survived when treated with 100 μg of antibiotic B per seedling.

Isolation of Ant$^-$ and Bic$^-$ mutants of UW85. To determine whether the antibiotics were associated with disease suppression by UW85, we generated mutants with Tn*917* or mitomycin C and screened them for antibiosis in an *E. herbicola* plate assay and for the ability to suppress plant disease in the test tube assay. Mutants were designated Ant$^-$ for reduced antibiosis and Bic$^-$ for reduced biological control activity. The mutants and their parents are listed in Table 1. Of 2,682 Tn*917* mutants screened for antibiosis, UW167, UW226, UW325, UW452, and UW457 did not produce zones of inhibition in the *E. herbicola* assay and were designated Ant$^-$ (Table 2). We screened an additional 1,500 mutants for disease-suppressive activity, and one, UW419, did not suppress disease and was designated Bic$^-$. Derivatives of UW85 marked with antibiotic resistance were subjected to mitomycin C treatment. Antibiotic resistance was used only as a marker to differentiate mutants derived from UW85 from contaminants, although the neomycin-resistant mutant UW85n1 was less effective than UW85 in disease suppression in some experiments (Table 2). A total of 200 survivors of mitomycin C treatment were screened in the alfalfa damping-off assay, and two independent mutants, UW030 and UW831, that were deficient in disease-suppressive activity were identified (Table 1). The Tn-*917* insertions mapped on a 14-kb cryptic plasmid, and preliminary data suggest that the Tn insertions are not causal to the mutant phenotypes (data not shown).

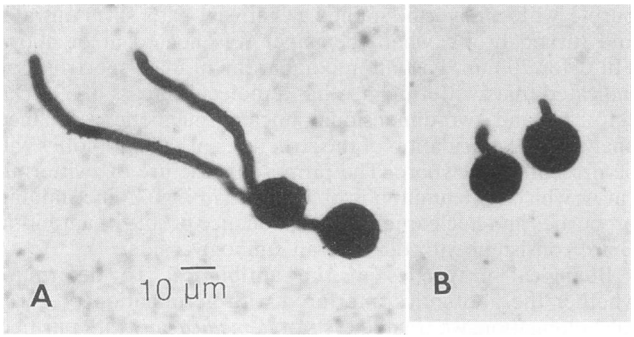

FIG. 3. Effect of zwittermicin A on germinating cysts of *P. medicaginis* 2 h after transfer to rich medium. (A) No treatment; (B) 100 μg of zwittermicin A per ml.

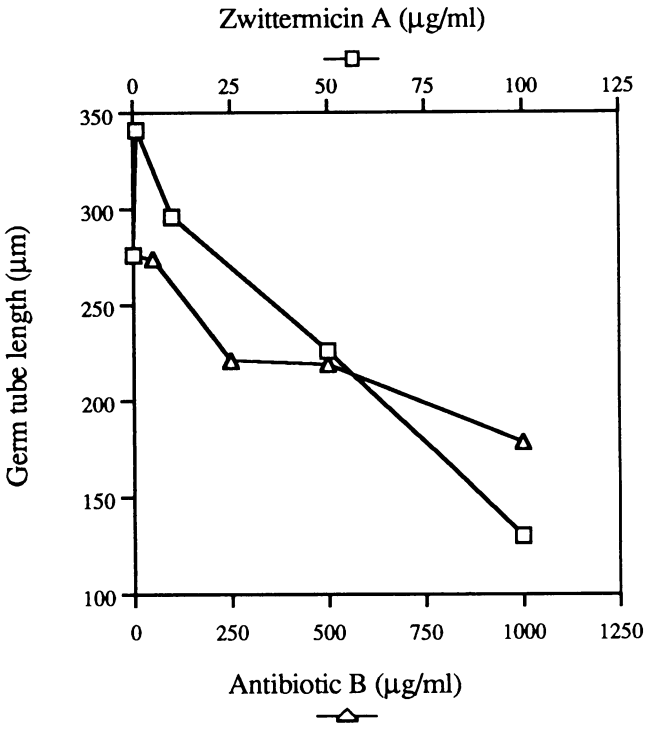

FIG. 4. Inhibition of germ tube elongation by zwittermicin A and antibiotic B. *P. medicaginis* zoospores were treated for 4 h with either zwittermicin A or antibiotic B. Each value represents the mean of 15 germ tubes, and the data are representative of two independent experiments. Log length = 1.5 − 0.0037 [zwittermicin A] and r = 0.958; log length = 1.4 − 0.00018 [antibiotic B] and r = 0.980.

Biological and chemical properties of UW85 mutants. To determine whether antibiosis and antibiotic accumulation were associated with disease suppressiveness, we tested all of the mutants that were initially identified as either Ant$^-$ or Bic$^-$ for inhibition of *E. herbicola*, inhibition of *P. medicaginis*, biological control activity in the alfalfa damping-off assay, and accumulation of materials that had properties identical to those of zwittermicin A and antibiotic B (Table 2). All of the mutants that were identified as Ant$^-$ in the *E. herbicola* inhibition assay were significantly reduced ($P \leq 0.05$) in the ability to inhibit growth of *P. medicaginis* and suppress disease. The three mutants, UW419, UW030, and UW831, that were initially identified for their inability to suppress disease were significantly reduced ($P \leq 0.05$) in antibiosis. Two mutants accumulated no detectable antibiotics, one mutant accumulated a trace of antibiotic B, and five mutants accumulated traces of zwittermicin A and antibiotic B (Table 2). The mutant characterization showed that antibiotic accumulation was con-

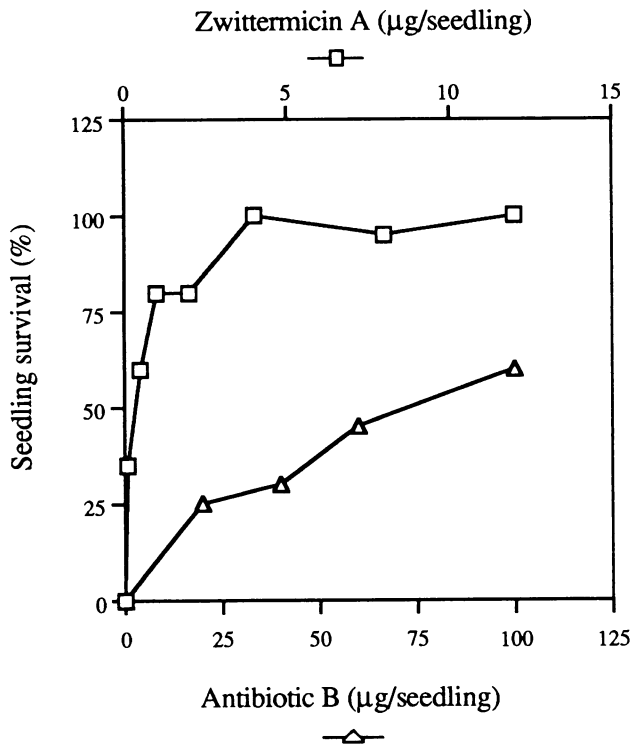

FIG. 5. Effect of zwittermicin A and antibiotic B on damping-off of alfalfa seedlings caused by *P. medicaginis*. Each value represents the percent survival of 20 seedlings on agar plates, and the data are representative of two independent experiments. Each seedling was inoculated with 25 zoospores of *P. medicaginis* and scored 5 days later. % Survival = 71.1 + 32.5 log [zwittermicin A] and r = 0.984; % survival = 9.66 + 0.53 [antibiotic B] and r = 0.981.

sistently associated with disease suppression. Furthermore, the amount of antibiotic accumulated by the mutants was significantly correlated with the level of disease suppression (Table 2).

We showed previously that filtrates of UW85 cultures lyse zoospores of *P. medicaginis*, although zoospore lysis does not appear to be sufficient for disease suppression because other strains of *B. cereus* lyse zoospores and do not suppress disease (6). Zoospore lysis activity is associated with the ion concentrations in the culture medium that result from growth of UW85 or other sporulating bacteria (6); therefore, zoospore lysis would not be expected to be altered in the Bic$^-$ mutants, which sporulated normally. Under the conditions described previously, we tested mutants UW030 and UW831 and found that they lysed 69 and 60% of the zoospores, respectively, and UW85 lysed 57% of the zoospores. These mutants were therefore unaffected in zoospore lysis activity.

Antibiotics suppressed disease when mixed with Bic$^-$ mutant cultures. To determine if the purified antibiotics could suppress disease in the test tube bioassay in vermiculite, we applied mutant UW030 to alfalfa plants with purified zwittermicin A or antibiotic B. The survival of alfalfa seedlings inoculated with *P. medicaginis* improved with the addition of increasing concentrations of zwittermicin A or antibiotic B (Table 3). Whereas 9% of the plants survived after they were treated with *P. medicaginis* and 300 μl of UW030 culture, 98% of the seedlings survived when 100 μg of zwittermicin A was added to the culture. The addition of 120 μg of antibiotic B to a UW030 culture increased seedling survival to 35%. These data show that either antibiotic reduced disease when applied to a mutant culture that lacked the ability to accumulate either antibiotic, although zwittermicin A suppressed disease better than antibiotic B at lower concentrations. In three independent experiments, zwittermicin A at 30 to 60 μg per tube or antibiotic B at 60 to 120 μg per tube was added to mutants UW167, UW419, and UW452 in the test tube assay. The

TABLE 2. Antibiotic accumulation and disease suppression by mutants and parental strains of *B. cereus*

Strain	Inhibition zone (mm)		Alfalfa seedling survival (%)[a,b]	Antibiotic detected (μg/ml)[c]	
	E. herbicola[d]	*P. medicaginis*[b,e]		Zwittermicin A	Antibiotic B
Expt 1					
UW85	9	14.4 ± 0.75 A	94 ± 3.0 A	15.0	36.0
UW167	NZ[f]	7.4 ± 0.24 B	17 ± 8.0 C	1.5	1.0
UW226	NZ	8.4 ± 0.68 B	64 ± 5.8 B	4.0	8.0
UW325	NZ	6.8 ± 0.48 BCD	53 ± 14.4 B	1.5	0.56
UW419	NZ	5.2 ± 0.97 DE	56 ± 6.0 B	<0.25	<0.12
UW452	NZ	5.0 ± 0.32 E	21 ± 11.5 C	<0.25	0.16
UW457	NZ	7.0 ± 0.41 BC	63 ± 12.6 B	1.6	0.12
UW030	NZ	5.3 ± 0.63 DCE	16 ± 0.85 C	<0.25	<0.12
Expt 2					
UW85	ND[g]	14.3 ± 0.33 A	82 ± 4.7 A	ND	ND
UW85n1-t	ND	12.6 ± 0.33 A	63 ± 2.7 A	ND	ND
UW85n1	ND	10.6 ± 0.33 B	29 ± 16.7 B	ND	ND
UW831	NZ	8.3 ± 0.33 C	14 ± 10.3 BC	1.0	4.0
UW030	NZ	7.0 ± 0 C	3 ± 2.8 C	ND	ND
Bc569	ND	7.3 ± 0.33 C	0 ± 0 C	ND	ND

[a] Each value represents the percent survival (± standard error) of 144 alfalfa seedlings in test tubes containing vermiculite and inoculated with zoospores of *P. medicaginis*. The data are representative of two independent experiments. For experiment 1, (% survival)^3 = 58,488 + 51,431 [zwittermicin A], r = 0.952, and $P \leq 0.001$; (% survival)^3 = 93,482 + 20,445 [antibiotic B], r = 0.940, and $P \leq 0.001$.

[b] Values in the same column followed by the same capital letter do not differ significantly in an analysis of variance ($P \leq 0.05$).

[c] Each value represents the amount of antibiotic isolated from 1 ml of culture.

[d] Inhibition of *E. herbicola* on water agar plates.

[e] Inhibition of *P. medicaginis* on potato dextrose agar plates.

[f] NZ, no zone of inhibition detected.

[g] ND, not determined.

TABLE 3. Effect of zwittermicin A and antibiotic B on disease suppression by mutant UW030

Treatment	Antibiotic added (μg/test tube)[a]	Plant survival (%)[b]
50% TSB + Pm[c]	None	7 ± 1.8 D[d]
UW85 + Pm	None	76 ± 6.6 B
UW030 + Pm	None	9 ± 4.8 D
UW030 + Pm + ZmA[e]	20	43 ± 13.3 C
UW030 + Pm + ZmA	100	98 ± 1.8 A
UW030 + Pm + antibiotic B	60	17 ± 6.4 CD
UW030 + Pm + antibiotic B	120	35 ± 4.9 C

[a] Concentration of antibiotic added to each test tube containing three seedlings.

[b] Each value represents the percent survival (± standard error) of 54 seedlings grown in test tubes filled with vermiculite. The data are representative of two independent experiments. All of the untreated alfalfa seedlings survived.

[c] Pm indicates that 3×10^3 zoospores of *P. medicaginis* were added to each test tube.

[d] Values followed by the same capital letters do not differ significantly at $P \leq 0.05$ in an analysis of variance.

[e] ZmA, zwittermicin A.

purified antibiotics improved seedling survival when added to each mutant in all three experiments, although the increase in disease suppression was statistically significant ($P \leq 0.05$) in only one of the experiments with the addition of zwittermicin A to mutants UW419 and UW452.

DISCUSSION

The activity of the antibiotics and mutant analysis suggest that the fungistatic antibiotics produced by UW85 contribute to its ability to suppress damping-off of alfalfa. The two antibiotics inhibited growth of *E. herbicola* and *P. medicaginis* on agar plates. Zwittermicin A substantially inhibited elongation of germ tubes derived from *P. medicaginis* cysts, and antibiotic B slightly inhibited elongation and also caused swelling of the tips of germ tubes. Both antibiotics protected alfalfa seedlings from disease caused by *P. medicaginis.*

The aminopolyol antibiotic, zwittermicin A, may represent a new class of *Bacillus* antibiotics. *Bacillus* spp. produce antibacterial and antifungal peptides (32, 39), lipopeptides (19, 28), and aminoglycosides (4, 15) whose chemical properties differ from those of the antibiotic we describe here. To our knowledge, this is the first report of a linear aminopolyol antibiotic produced by *Bacillus* spp. The wide target range of activity for zwittermicin A is intriguing and warrants further investigation to determine whether it inhibits prokaryotes and eukaryotes by similar mechanisms.

The chemical and physical properties of the antibiotics introduced significant difficulties into their purification and detection. Small water-soluble molecules are abundant in bacterial cultures, causing separation on the basis of molecular size or solubility to be ineffective. We capitalized on their ionic properties for purification by ion-exchange chromatography and electrophoresis. Although zwittermicin A is a very weak acid, it migrated as an anion at pH 9.2, as did other amide-containing compounds such as allantoin (data not shown). The amount of sample that can be applied to a paper electrophoretogram is small (typically not more than 1.0 mg), thereby preventing large-scale purification of the antibiotic by this method. We depended on biological activity as the detection method during purification because the antibiotics do not contain chromophores that could be detected spectrophotometrically. This added substantially to the effort required to test fractions, and it reduced overall yield, since a portion of each fraction was sacrificed for the biological detection assay. We are developing improved methods for antibiotic purification.

All of the mutants deficient in antibiotic accumulation lacked disease-suppressive activity, suggesting a role for the antibiotics in biological control. The mutant analysis must be interpreted with caution since the genetic lesions in the mutants have not been characterized, and therefore we cannot eliminate the possibility that each mutant contains more than one mutation or a pleiotropic mutation. However, three lines of evidence suggest that the lack of antibiotics in the mutants affected their disease suppressiveness. First, we isolated eight independent mutants that were affected in both antibiotic accumulation and disease suppressiveness. It is improbable, although not impossible, that all of the mutants would be affected in both antibiotic accumulation and disease suppressiveness if these phenotypes were not causally associated. Second, we found a strong quantitative relationship between the amount of antibiotic accumulated by the mutants and their level of disease suppressiveness (Table 2). Third, the purified antibiotic suppressed disease as effectively as the culture filtrate of the parent strain. Definitive genetic evidence for the roles of zwittermicin A and antibiotic B in disease suppression by UW85 will be derived from mapping, complementing, and cloning the mutations from the mutants and from transferring the genes for antibiotic biosynthesis to other bacteria. However, routine genetic analysis of the mutants is limited by the lack of established molecular techniques for *B. cereus*. In future studies, we intend to develop the tools to conduct a detailed genetic analysis of these strains.

The residual disease-suppressive activity in some of the mutants that lack detectable antibiotics may be due to zoospore lysis activity or to another factor. It is interesting that many of the most effective biological control agents, such as *Agrobacterium tumefaciens* K84 and *Pseudomonas fluorescens* 279, reduce infection by the pathogen through more than one mechanism, including antibiosis (16, 17, 21, 35, 37), siderophore production (43), and site exclusion (3). UW85 may also depend on multiple strategies for disease suppression, since the data suggest that the antibiotics are required, but not sufficient, for the full biological control activity of UW85 cultures.

Zwittermicin A had a strong inhibitory effect on the damping-off pathogen in the agar plate assays but inhibited disease only at high concentrations in the test tube bioassay. The ionic environment in which zwittermicin A is present may play a role in the activity of the antibiotic. The plate assays were conducted under relatively nonionic conditions, whereas the vermiculite used in the test tube bioassay has a high cation-exchange capacity. Powell and Prosser (30) showed that concentrations of nitrapyrin required to inhibit *Nitrosomonas europaea* in vermiculite were 10-fold higher than the concentration required to inhibit the same organism in liquid culture. We found that the addition of purified antibiotics from UW85 directly to the test tube assay did not increase seedling survival above control levels, possibly due to binding of the antibiotics by the vermiculite. Passage of the antibiotics through vermiculite reduced the recoverable activity measured in the assay for antibiosis against *P. medicaginis* (data not shown). The antibiotics had strong activity in the test tube assay when added to cultures of UW030, suggesting that the UW030 culture may provide an ionic environment conducive to antibiotic activity in vermiculite.

UW85 has biological control activity against various pathogens in the laboratory and in the field and on a wide range of plant species, including alfalfa, soybeans, tobacco, and cucumbers (12, 13, 14, 33), but we do not know whether the same mechanism is involved in disease suppression under all conditions. UW85 also improves soybean nodulation in the laboratory and in the field (11), and the basis for this effect is unknown. The Bic$^-$ mutants provide a tool to determine whether the same antibiotics are required for biological control in the test tube bioassay for damping-off and in the field and whether the antibiotics that contribute to suppression of alfalfa damping-off are also involved in suppression of other diseases and in enhancement of soybean nodulation.

On the basis of the data we present, it is tempting to suggest that UW85 controls damping-off by inhibiting growth and development of the pathogen. However, it would be premature to conclude that the only activity of the antifungal antibiotics is against the pathogen. It is also possible that the antibiotics incite a host defense response (41) or, in the field, affect the microbial flora on the alfalfa root, providing an environment that is hostile to the pathogen (7). Mutants of the pathogen that are resistant to the antibiotics would contribute to determining whether they act directly on the pathogen or on the plant or the microbial community in the rhizosphere.

Understanding the synthesis, regulation, and activity of the

fungistatic antibiotics will provide insight into the potential of UW85 for biological control and may suggest strategies for improving its efficacy in the field. Coupling an understanding of the mechanism of biological control by UW85 with an understanding of its population dynamics (10) and impact on the microbial communities (8) on roots may lead to the construction of predictive models that describe the complex interactions that result in or detract from biological control in the agroecosystem.

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