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Zwittermicin A-Producing Strains of *Bacillus cereus* from Diverse Soils†

ERIC V. STABB,[1,2] LYNN M. JACOBSON,[2] AND JO HANDELSMAN[2]*

Department of Bacteriology[1] and Department of Plant Pathology,[2] University of Wisconsin, Madison, Wisconsin 53706

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Bacillus cereus **UW85 produces a novel aminopolyol antibiotic, zwittermicin A, that contributes to the ability of UW85 to suppress damping-off of alfalfa caused by *Phytophthora medicaginis*. UW85 produces a second antibiotic, provisionally designated antibiotic B, which also contributes to suppression of damping-off but has not been structurally defined yet and is less potent than zwittermicin A. The purpose of this study was to isolate genetically diverse strains of *B. cereus* that produce zwittermicin A and suppress disease. We found that most isolates of *B. cereus* that were sensitive to phage P7 or inhibited the growth of *Erwinia herbicola* produced zwittermicin A; therefore, phage typing and *E. herbicola* inhibition provided indirect, but rapid screening tests for identification of zwittermicin A-producing isolates. We used these tests to screen a collection of 4,307 *B. cereus* and *Bacillus thuringiensis* isolates obtained from bacterial stock collections and from diverse soils collected in Honduras, Panama, Australia, The Netherlands, and the United States. A subset of the isolates screened by the P7 sensitivity and *E. herbicola* inhibition tests were assayed directly for production of zwittermicin A, leading to the identification of 57 isolates that produced zwittermicin A; 41 of these isolates also produced antibiotic B. Eight isolates produced antibiotic B but not zwittermicin A. The assay for phage P7 sensitivity was particularly useful because of its simplicity and rapidity and because 22 of the 23 P7-sensitive isolates tested produced zwittermicin A. However, not all zwittermicin A-producing isolates were sensitive to P7, and the more labor-intensive *E. herbicola* inhibition assay identified a larger proportion of the zwittermicin A producers. Preliminary phenotypic characterization of the zwittermicin A-producing isolates obtained from soil and plant roots revealed a minimum of 31 distinct strains. We tested UW85 and 98 isolates from this world-wide collection for the ability to suppress damping-off disease of alfalfa caused by *P. medicaginis*. The isolates that produced zwittermicin A and/or antibiotic B suppressed the disease more effectively than the isolates that produced neither antibiotic. Our results showed that *B. cereus* strains that produce zwittermicin A and antibiotic B are found in diverse soils and that these strains tend to suppress damping-off disease more effectively than *B. cereus* isolates that do not produce these antibiotics. The rapid microbiological assays that we describe provide a way to exploit the genetic diversity of antibiotic-producing *B. cereus* strains for biological control.**

Management of crop health is essential to agricultural productivity and efficiency. Biological control, or the use of microorganisms to suppress plant pests, offers an attractive alternative or supplement to currently used pest control practices, especially as chemical pesticides are deemphasized because of the development of resistance in pest populations and concerns about human health and environmental quality (4). Through screening programs workers have identified many microorganisms that suppress plant diseases in the laboratory (10, 15, 16, 20, 21, 26, 30–33, 44, 53, 67, 69, 72) and in field experiments (22, 34, 38, 43, 52, 70); however, few of these biological control agents have been effective over a broad range of conditions. This may be, in part, because each strain with biological control activity evolved to compete in the environment from which it was obtained and is not as well adapted to other sites (7). Increasing the effectiveness of biological control in diverse environments is essential to incorporating it into standard agricultural practices.

A resource that has potential for making biological control agents more broadly effective is genetic diversity, the variation within a species. The strategies for improving most biological technologies include exploitation of genetic diversity. Genetically diverse strains have been screened for specific traits to improve the use of microorganisms in the production of fermented foods (5, 13, 37, 40, 50, 58, 66) and antibiotics (17, 49, 57) and in the biodegradation of toxic compounds (63). In agriculture, a wealth of genetic diversity is available for many crops; this diversity extends the usefulness of a crop species far beyond that of any single cultivar (27, 61). Similarly, the genetic diversity of species that exhibit biological control activity is a valuable, largely untapped, resource for broadening and improving biological control strategies.

Successful exploitation of genetic diversity for improving biological control is illustrated by the development of biological control agents for insects. Entomologists have identified and utilized diverse strains of entomopathogenic species that have potential for biological control (1, 4, 9, 11, 12, 14, 18, 23, 35, 46–48, 51, 55, 64, 65, 73). The most notable example is the development of *Bacillus thuringiensis*. Initially, *B. thuringiensis* isolates were recognized because of their ability to kill insects, but early field applications produced limited and variable results (8). The development of methods for identification of new *B. thuringiensis* strains led to a vast array of inoculum strains which have extended the target range and increased the effectiveness of *B. thuringiensis* (1, 4, 11, 14, 18, 65). Concentrating research efforts on *B. thuringiensis* strains rather than

* Corresponding author. Phone: (608) 263-8783. Fax: (608) 263-2626. Electronic mail address: joh@plantpath.wisc.edu.

† This paper is dedicated to the memory of Marsha J. Betley.

TABLE 1. Characteristics of soils used in this study

Soil or site	Country	pH	% Organic matter[a]	% Sand[b]	% Silt[b]	% Clay[b]	Most recent vegetation
Lutz	Panama	ND[c]	5.5	ND	ND	ND	Forest
Snyder-Molino	Panama	ND	6.2	ND	ND	ND	Forest
Barbour-Lathrop	Panama	ND	7.1	ND	ND	ND	Forest
Moroceli	Honduras	ND	1.7	ND	ND	ND	Maize and beans
San Matias	Honduras	5.8	1.5	ND	ND	ND	Maize and beans
La Vega1	Honduras	6.3	2.3	ND	ND	ND	Beans
La Vega5	Honduras	5.9	2.5	ND	ND	ND	Sorghum and maize
Arlington, Wis.	United States	6.9	4.2	23	68	9	Alfalfa
Hancock, Wis.	United States	6.6	0.8	87	8	5	Alfalfa and oats
Marshfield, Wis.	United States	6.5	3.1	23	72	5	Alfalfa
Lancaster, Wis.	United States	7.1	2.3	19	68	13	Alfalfa
Madison, Wis.	United States	7.0	3.2	27	56	17	Alfalfa
Taos, N.M.	United States	8.1	4.3	ND	ND	ND	Pasture
Tifton, Ga.	United States	6.3	1.0	87	12	1	Tobacco
Douglas Gully	Australia	6.1	2.2	ND	ND	ND	Vineyard
Lelystad	The Netherlands	7.5	1.8	51	36	13	Potatoes

[a] The organic matter content was determined by measuring the loss of weight after ignition.
[b] The sand, silt, and clay contents were determined by hydrometer measurements.
[c] ND, not determined.

on many diverse species had the advantage that knowledge concerning *B. thuringiensis* was largely transferable among strains, since the toxins required for biological control and the methods used to prepare inocula were similar in all *B. thuringiensis* strains (29, 71). Identification of thousands of *B. thuringiensis* strains and an understanding of the mechanism of insect control have contributed to the success of *B. thuringiensis*-based products, which currently claim more than 90% of the biopesticide market with sales exceeding $\$125 \times 10^6$ annually (4, 14). The use of genetic diversity for overcoming the early limitations of *B. thuringiensis* may serve as a model for the successful development of other biological control systems.

We have previously described *Bacillus cereus* UW85 (= ATCC 53522), which protects alfalfa seedlings from damping-off caused by *Phytophthora medicaginis* (26), tobacco seedlings from *Phytophthora nicotianae* (25), cucumber fruits from rot caused by *Pythium aphanidermatum* (62), and peanuts from *Sclerotinia minor* (54). UW85 produces two antifungal compounds that contribute to suppression of damping-off (60). The more potent of these compounds, zwittermicin A, is a novel aminopolyol (28), and the second compound, provisionally designated antibiotic B, has not been well characterized chemically yet. One goal of this study was to explore the use of genetically diverse zwittermicin A-producing *B. cereus* strains for controlling plant pathogens. In this paper we describe the use of simple tests that rapidly identify zwittermicin A-producing *B. cereus* strains isolated from diverse soils.

MATERIALS AND METHODS

Soils. The geographic origins and physical and chemical characteristics of the soil samples used in this study are shown in Table 1. All of the samples were taken from the surface horizon. Measurements of soil pH, organic matter content, and particle size were made by workers at the University of Wisconsin Soil & Plant Analysis Laboratory, Madison.

Isolation and identification of *B. cereus* isolates. The bacterial strains that we used and their origins are shown in Table 2. Bacteria collected from soybean roots were isolated as previously described from plants grown in a field plot in Madison, Wis. (24). The remaining bacteria collected in this study were isolated by placing either an entire alfalfa root or 1 g of soil in a test tube containing 9 ml of water and sonicating the preparation for 30 s in a model 2200 bath sonicator (Branson Ultrasonics Corp., Danbury, Conn.). Serial 10-fold dilutions of the suspensions were made in water, and 0.1-ml portions from dilutions ranging from 10^{-1} to 10^{-5} were spread onto plates containing either 0.1× Trypticase soy agar (TSA) (Becton Dickinson Microbiology Systems, Cockeysville, Md.) or MinIC medium (24). The plates were incubated at room temperature or 28°C for 1 to 3 days, and plates containing isolated colonies were used for further study. Colonies that had the morphology typical of *B. cereus* colonies (flat, broad, and cream colored) were removed and streaked to obtain isolated colonies. In order to partially select for *B. cereus*, during either the initial plating or subsequent streak plating, the medium was supplemented with polymyxin (25 μg/ml), cycloheximide (100 μg/ml), and ampicillin (50 μg/ml). All isolates were tested for hemolysis of blood agar, which is diagnostic of *B. cereus* (6), and those that were nonhemolytic were removed from the collection. Blood agar was obtained from the Wisconsin State Hygiene Laboratory, Madison. Isolates were stored on 0.5× TSA slants. Alfalfa plants were grown from seeds in soil obtained from Arlington, Wis., for 21 days in a growth chamber at 24°C with a 12-h photoperiod and a light intensity of 244 microeinsteins/m^2/s. Soybean plants were grown from seeds in a field plot in Madison, Wis.

On the basis of fatty acid profiles determined by Five Star Laboratories, Branford, Conn., and Microbial ID, Newark, Del., 47 isolates were classified as members of the *B. cereus* group, which includes *Bacillus mycoides*, *Bacillus anthracis*, and *B. thuringiensis*. The unique rhizoidal morphology of *B. mycoides* strains differentiates them from *B. cereus* (56), and none of the isolates in this collection exhibited *B. mycoides*-like morphology. *B. anthracis* is not hemolytic and is usually sensitive to ampicillin (56); therefore, this collection probably did not contain any *B. anthracis* strains. Differentiation between *B. cereus* and *B. thuringiensis* is difficult with standard methods (2, 39, 68, 74). Therefore, we followed current recommendations (19, 41, 56) and considered all of the isolates obtained in this study *B. cereus* isolates. Strains BGSC4A9, BGSC4B1, BGSC4C3, HD1, BGSC4E1, BGSC4F1, BGSC4G1, BGSC4H1, BGSC4I1, BGSC4J1, and BGSC4S2 were previ-

TABLE 2. Strains used in this study

Strain(s)	Origin	Reference
ATCC 7064, ATCC 27877, ATCC 12826	American Type Culture Collection	
BGSC6A3, BGSC6E1, BGSC6E2, BGSC4A9, BGSC4B1, BGSC4C3, HD1, BGSC4E1, BGSC4F1, BGSC4G1, BGSC4H1, BGSC4I1, BGSC4J1, BGSC4S2	*Bacillus* Genetic Stock Center	
T	University of Wisconsin Department of Bacteriology Collection	
UW85	Alfalfa root, Arlington, Wis.	26
SOY130	Soybean root, Walnut St. Farm, Madison, Wis.	This study
ALF1, ALF9, ALF10, ALF13, ALF19, ALF23, ALF52, ALF53, ALF79, ALF83, ALF85, ALF94, ALF95, ALF98, ALF99, ALF108, ALF109, ALF115, ALF117, ALF133, ALF137, ALF144, ALF154, ALF157, ALF161, ALF166, ALF167, ALF173	Roots of alfalfa plants planted in soil from Arlington, Wis., and grown in a growth chamber	This study
LUTZ21, LUTZ58, LUTZ128	Lutz soil	This study
SNY14, SNY42, SNY44, SNY45, SNY73	Snyder-Molino soil	This study
BAR78, BAR145, BAR177	Barbour-Lathrop soil	This study
MOR1, MOR28, MOR37	Moroceli soil	This study
SM32, SM43, SM44	San Matias soil	This study
VGA19, VGA118, VGA137	La Vega1 soil	This study
VGA562, VGA577, VGA598	La Vega5 soil	This study
AS7-4, AS8-4, AS8-13, AS4-12, ARL8	Arlington soil	This study
HS1-3, HS23-11, HS24-8, HS24-9	Hancock soil	This study
MS1-9, MS3-2, MS8-2	Marshfield soil	This study
LS2-2, LS2-12, LS33-2	Lancaster soil	This study
WS4-12, WS8-8, WS10-15, WS16-4, WS22-12	Madison soil	This study
TNM68, TNM155, TNM243	Taos soil	This study
TG38, TG42, TG126	Tifton soil	This study
DGA34, DGA37, DGA84, DGA94	Douglas Gully soil	This study
LN24, LN75, LN100	Lelystad soil	This study

ously classified by other workers as *B. thuringiensis*, and we retained that species designation for these strains in this study.

Assays for sensitivity to phage P7. Isolation and characterization of phages P7 (= φATCC75237) and PB will be described elsewhere. To propagate these phages, we spread 3 ml of melted soft agar (4 g of agar per liter) containing approximately 10^6 PFU of phage and an excess of *B. cereus* UW85 cells on 0.5× TSA plates. The plates were incubated overnight at 28°C, and then the soft agar was scraped off the plates and suspended in 0.5× Trypticase soy broth (1 ml per plate). The agar and cells were removed by centrifugation, and the supernatant was passed through a 0.2-μm-pore-size filter. The phage titers were typically 10^{10} PFU/ml.

To screen large numbers of isolates for P7 sensitivity, grids of 48 isolates were grown on 0.1× TSA, and then cells were transferred with a metal replicator (3) onto 0.1× TSA plates that had been inoculated with dilutions of P7 so that they contained approximately 10^8, 10^4, and 10^3 PFU per plate. A 0.1× TSA plate containing no phage was used as the control. Isolates that, when replicated onto plates containing P7, showed uneven growth, growth containing plaques, or less growth than on plates without P7, were tested by the soft-agar overlay assay (described below) to determine if they were sensitive to P7. Most isolates that were resistant to P7 as determined by the primary screening test were tested by the soft-agar method.

In the second test used to determine the sensitivity of bacterial isolates to P7, each isolate was grown on 0.5× TSA, and cells were scraped off the plates and mixed in soft-agar overlays so that lawns were formed on fresh 0.5× TSA plates. Tenfold dilutions of P7 were placed in 5-μl drops on the plates, which were then incubated at 28°C. If plaques appeared, the strain was considered P7 sensitive ($P7^s$). Lawns of two isolates, ARL8 and HS23-11, were cleared by undiluted drops of P7, but P7 did not form isolated plaques on these isolates at lower concentrations. The clearing due to high-titer drops appeared to be due to P7 rather than to a chemical present in UW85 lysates, since high-titer drops of lysates of PB, which produces turbid plaques on UW85, did not cause clearing on lawns of ARL8 and HS23-11. Therefore, these strains were also considered $P7^s$. Isolates whose lawns appeared to be unaffected by P7 were considered P7 resistant ($P7^r$).

Assay for inhibition of *Erwinia herbicola*. Inhibition of *E. herbicola* LS005 was assayed as described previously (60), with the following modifications. Three-day-old cultures of each *B. cereus* isolate grown in 0.5× Trypticase soy broth were tested to determine whether they inhibited *E. herbicola* on 0.001× TSA plates. Isolates that produced visible zones of inhibition of *E. herbicola* were tested again. Isolates that produced visible zones of inhibition in both tests were considered Eh^+. Isolates that did not detectably inhibit *E. herbicola* in each of two initial tests were considered Eh^-. Some *B. cereus* isolates did not inhibit *E. herbicola* during the initial test but did inhibit this organism after storage at −20°C. Other isolates (ALF115, HD1, and BGSC4S2) had variable phenotypes; these isolates produced either small zones of inhibition or no zones in subsequent tests and were classified Eh^-.

Assay for zwittermicin A and antibiotic B. Zwittermicin A and antibiotic B were identified in culture supernatants by cation-exchange chromatography, using CM SEP-PAK cartridges (Millipore Corp., Millford, Mass.), followed by high-voltage paper electrophoresis (HVPE) as described elsewhere (45). The cationic fraction obtained from the equivalent of 4 ml of culture supernatant was applied to the paper, which was stained with silver nitrate after electrophoresis as described previously (60). Isolates that produced material indistinguishable from authentic zwittermicin A and authentic antibiotic B

after HVPE were designated zwittermicin A producers and antibiotic B producers, respectively. To verify the structural identity of the zwittermicin A produced by nine representative isolates, putative zwittermicin A was purified from these isolates as described previously (60) and subjected to proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and fast atom bombardment mass spectrometry (28).

Assay for suppression of alfalfa damping-off. Bacterial isolates were grown for 3 days in 0.5× Trypticase soy broth and were tested by a damping-off assay described previously (26). Each isolate was tested on 15 plants in each of seven independent experiments, with the following exceptions: strain ATCC 12826 was omitted from experiments 1 and 2; strain BAR78 was omitted from experiment 4; and strains WS8-8 and LS2-12 were omitted from experiment 5. Statistical analyses (analysis of variance, Dunnet's comparison test, standard error of least squared mean) were performed by using the SAS computer program (59). The data from the seven experiments were pooled and were analyzed as if they were data from a single experiment with seven blocks. The data in Table 3 did not deviate from normality and therefore were not transformed. In Table 3 the standard error based on least squared means is shown rather than the standard error to account for significant block effects. Figure 1 shows data that were not transformed; however, arcsin square root transformation of these data had no effect on groupings.

Testing diversity of strains. To estimate the diversity of zwittermicin A and antibiotic B producers, we tried to determine the minimum number of unique zwittermicin A- and/or antibiotic B-producing strains in our collection. We considered two isolates to be distinct only if phenotypic differences between them could be demonstrated. Therefore, isolates were subjected to a series of phenotypic tests. All characterization tests were performed with isolates that had been colony purified on 0.5× TSA. To test for antibiotic resistance, isolates were streaked onto 0.5× TSA containing tetracycline (5 μg/ml), neomycin (5 μg/ml), or chloramphenicol (1 μg/ml) and were incubated at 28°C overnight. Isolates that grew similarly when they were streaked in the presence and in the absence of antibiotic were classified as antibiotic resistant. To test isolates for pigment production, they were grown on 2-(*N*-morpholino)ethanesulfonic acid (MES) minimal medium at 28°C for 7 days and then scored visually. MES minimal medium contained 9.75 g of MES per liter, 2 g of $(NH_4)_2SO_4$ per liter, 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter, 0.25 mg of $MnSO_4 \cdot 7\ H_2O$ per liter, 1.25 g of $K_2HPO_4 \cdot 3H_2O$ per liter, 2 g of L-glutamic acid per liter, 10 mg of thiamine per liter, 15 g of agar per liter, 40 mg of $FeCl_3 \cdot 6H_2O$ per liter, 5 g of sucrose per liter, 1 mM threonine, 1 mM serine, 1 mM leucine, 1 mM valine, and 1 mM alanine; the pH of this medium was adjusted to 6.1 (45). MES-Thr medium was MES minimal medium lacking threonine. We determined the ability of isolates to grow on MES-Thr medium by streaking the isolates onto MES-Thr plates, incubating them at 28°C for 4 days, and recording the appearance of colonies for each strain. Phages φATCC7064 and φATCC27877 were obtained from the American Type Culture Collection and were propagated on bacterial strains ATCC 7064 and ATCC 27877, respectively. Phage φ63 was propagated on strain Bt-1, and both φ63 and Bt-1 were obtained from R. Landen (36). The sensitivities of isolates to phages φ63, φATCC7064, and φATCC27877 were determined by the soft-agar overlay method described above for P7; plaque formation was used as the indicator of sensitivity.

RESULTS

Association of zwittermicin A production with P7^s and Eh$^+$ isolates. We have shown previously that *B. cereus* UW85 produces two antibiotics, the novel aminopolyol zwittermicin A (28) and antibiotic B, which contribute to the suppression of alfalfa seedling damping-off (60). UW85 was originally identified in labor-intensive screening experiment in which biological control activity was examined (26). The purposes of this study were (i) to devise simple assays that could detect other (although not necessarily all) strains that produce zwittermicin A, (ii) to determine whether zwittermicin A producers also produce antibiotic B, and (iii) to determine whether production of zwittermicin A and antibiotic B is associated with biological control activity in *B. cereus* strains other than UW85. The bases for the approach used were as follows: (i) preliminary characterization of phage P7, which indicated that the host range of this phage might be limited to strains that produce zwittermicin A, and (ii) evidence that purified zwittermicin A inhibits *E. herbicola* (60). Therefore, we investigated whether sensitivity to P7 (the P7^s phenotype) and the ability to inhibit *E. herbicola* (the Eh$^+$ phenotype) were characteristics that could be used to identify zwittermicin A producers.

We screened 4,307 *B. cereus* and *B. thuringiensis* isolates for sensitivity to phage P7. Because the P7 sensitivity assay can be performed with a large number of strains more rapidly than the *E. herbicola* inhibition assay, only the isolates identified as P7^s and 1,876 of the remaining isolates were tested for the ability to inhibit *E. herbicola*. The isolates were obtained from geographically diverse soil samples collected at 16 locations in five countries (Table 1), from alfalfa and soybean roots, and from stock culture collections (Table 2). The numbers of P7^s and Eh$^+$ isolates obtained from each source and the numbers of isolates tested are shown in Table 4. P7^s isolates were identified in samples obtained from 14 of the 16 soils examined, as well as from alfalfa and soybean roots (Table 4). Most of the 87 P7^s isolates were Eh$^+$; the only exceptions were SNY73 and LN100. P7^r Eh$^+$ isolates were identified in samples obtained from each of the soils, as well as from alfalfa roots (Table 4). Approximately 2% of the isolates examined (85 of 4,307 isolates) were P7^s Eh$^+$, and 7% (132 of 1,876 isolates) were P7^r Eh$^+$.

We examined representative P7^s, P7^r, Eh$^+$, and Eh$^-$ isolates to determine whether they accumulated zwittermicin A and antibiotic B. To determine what isolates would be tested for antibiotic production, we chose a total of 90 isolates, each of which was a random representative of a particular P7^s-P7^r Eh$^+$-Eh$^-$ phenotype from a particular sample, and then we selected 14 isolates nonrandomly, including previously described biological control strain UW85. Of the 23 new P7^s isolates tested, 22 produced zwittermicin A (Table 3). Of the 22 zwittermicin A producers, 21 produced antibiotic B as well. One P7^s isolate produced neither antibiotic (Table 3). These data showed that phage typing with P7 was a good, if indirect, predictor of zwittermicin A production.

All 49 new Eh$^+$ isolates tested (21 P7^s isolates and 28 P7^r isolates) produced zwittermicin A, and 41 of these isolates (21 P7^s isolates and 20 P7^r isolates) also produced antibiotic B (Table 3). Thus, although it is a more labor-intensive and time-consuming phenotype to test, the Eh$^+$ phenotype was also a good predictor of zwittermicin A production and identified a greater proportion of the zwittermicin A producers than the P7^s phenotype did. We also examined 34 P7^r Eh$^-$ isolates obtained from roots and soil, as well as 18 P7^r Eh$^-$ *B. cereus* and *B. thuringiensis* strains obtained from stock collections. Of

TABLE 3. Antibiotic production and suppression of alfalfa damping-off by *B. cereus* isolates

Strain	Zwittermicin A production[a]	Antibiotic B production[b]	% of healthy seedlings[c]
P7^s Eh$^+$ isolates			
MOR28	+	+	82
DGA34	+	+	80
AS4-12[d]	+	+	78
SOY130	+	+	77
ALF23	+	+	77
HS1-3	+	+	76
MS1-9	+	+	76
WS22-12	+	+	76
UW85[d]	+	+	73
VGA598	+	+	72
AS8-13	+	+	72
SM32	+	+	70
VGA19	+	+	70
ALF108	+	+	69
HS23-11[d]	+	+	69
LUTZ128	+	+	68
ALF94	+	+	68
ARL8	+	+	66
SNY45	+	+	63
SNY44[d]	+	+	63
BAR78	+	+	61
TG42	+	+	ND
P7^s Eh$^-$ isolates			
LN100	+	−	59
SNY73	−	−	35
P7^r Eh$^+$ isolates			
WS10-15[d]	+	+	78
ALF133	+	+	75
ALF95	+	+	74
DGA37	+	+	74
ALF9	+	+	73
TNM155[d]	+	+	72
WS8-8[d]	+	+	72
ALF53	+	+	70
ALF19	+	+	69
LS33-2	+	+	68
ALF52	+	+	67
LS2-12[d]	+	+	67
WS16-4	+	+	66
MS3-2	+	+	66
VGA577	+	−	66
LUTZ21	+	+	65
AS8-4	+	+	64
ALF167	+	+	63
ALF161	+	+	61
SNY42	+	−	61
BAR177	+	−	60
MOR37	+	−	52
HS24-8	+	+	50
VGA137	+	−	50
LN24	+	−	49
SM43	+	−	47
TNM68	+	−	ND
TG38	+	+	ND
P7^r Eh$^-$ isolates			
WS4-12	−	−	70
DGA84[d]	+	−	65
MOR1	+	−	57
BGSC4S2[e]	+	−	56
DGA94	−	+	51
SM44	−	+	49
ALF83[d]	−	−	49
VGA562	−	−	48
ALF85[d]	−	−	47
ALF99[d]	−	−	47
ALF115	+	−	47
ALF117	−	−	46
ALF173	−	+	46
MS8-2	−	−	45
ALF79	−	+	45
ALF154	−	+	44
BGSC6E1	−	−	43
BGSC4E1[e]	−	−	43
ALF144[d]	−	−	43
ALF13	−	+	43
ALF98	−	−	42
ALF109[d]	−	−	42
ALF157	−	−	41
ALF166	−	−	41
HD1[e]	+	−	41
AS7-4	−	−	40
BGSC6A3	−	−	40
ATCC 7064	−	−	39
T	−	−	39
LUTZ58	−	−	39
ALF10	−	+	38
BGSC4I1[e]	−	−	38
ATCC 27877	−	−	36
VGA118	−	−	36
LS2-2	−	−	36
HS24-9	−	−	36
BGSC6E2	−	−	36
BGSC4H1[e]	−	−	36
ALF1	−	−	34
SNY14	−	−	34
BAR145	−	−	33
BGSC4J1[e]	−	−	32
ATCC 12826	−	−	31
LN75	−	−	31
BGSC4B1[e]	−	−	30
BGSC4C3[e]	−	−	27
ALF137	−	−	22
BGSC4F1[e]	+	−	13
BGSC4G1[e]	−	−	11
BGSC4A9[e]	−	−	9
TNM243	−	+	ND
TG126	+	−	ND

[a] Zwittermicin A accumulation was determined by HVPE and staining.
[b] Antibiotic B accumulation was determined by HVPE and staining.
[c] Disease suppression was measured by determining the percentage of healthy plants 7 days after coinoculation with a *B. cereus* or *B. thuringiensis* culture and *Phytophthora medicaginis*. Fifteen plants were used for each treatment in seven separate experiments; thus, the total number of plants used for each treatment was 105. Treatment with *Phytophthora medicaginis* only and no inoculation resulted in 0 and 100% healthy plants, respectively. We calculated the standard errors based on the least squared mean to compensate for significant block effects. The standard errors based on the least squared mean were 7% for all isolates included in all seven experiments and 8% for isolates WS8-8, LS2-12, BAR78, and ATCC 12826. ND, not determined.
[d] Isolate that was selected nonrandomly.
[e] *B. thuringiensis* strain obtained from a stock collection.

the 34 isolates obtained from roots and soil, 4 produced zwittermicin A, and 8 produced antibiotic B (Table 3). Three *B. thuringiensis* strains obtained from stock collections produced zwittermicin A (Table 3). These results showed that neither the P7^s phenotype nor the Eh$^+$ phenotype could be used to identify all zwittermicin A producers, but that either test could be used to identify antibiotic-producing *B. cereus* isolates.

We determined whether the compounds identified as zwittermicin A by HVPE were identical to authentic zwittermicin A by examining putative zwittermicin A obtained from certain

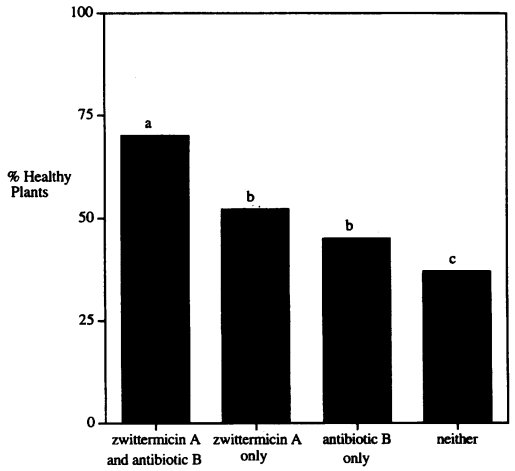

FIG. 1. Disease suppression by isolates grouped on the basis of their zwittermicin A and antibiotic B production profiles. The mean percentages of healthy alfalfa plants are shown for 39 isolates that produced both zwittermicin A and antibiotic B, 14 isolates that produced only zwittermicin A, 7 isolates that produced only antibiotic B, and 38 isolates that accumulated neither antibiotic. Bars labeled with the same letter do not differ significantly at $P < 0.05$ as determined by analysis of variance.

strains by ^{1}H-NMR and mass spectrometry. Putative zwittermicin A was purified from isolates HD1, ARL8, SNY44, LS2-12, DGA37, VGA577, LN100, HS23-11, and AS4-12 and subjected to ^{1}H-NMR analysis. The ^{1}H-NMR spectra of all nine of these compounds exhibited the distinguishing characteristics of the authentic zwittermicin A spectrum (28). The putative zwittermicin A samples purified from the first seven of these isolates were examined by mass spectrometry, and each had a mass of 396, which is identical to the mass of authentic zwittermicin A (28).

Correlation of disease suppression with production of putative zwittermicin A and antibiotic B by *B. cereus* isolates. We tested 99 isolates for their ability to suppress damping-off disease of alfalfa seedlings caused by *Phytophthora medicaginis*. We grouped these isolates on the basis of their zwittermicin A and antibiotic B production profiles, and the biological control activities of the groups were compared (Fig. 1). The isolates that produced both zwittermicin A and antibiotic B were more disease suppressive than the other isolates ($P < 0.05$, as determined by analysis of variance). The isolates that produced either zwittermicin A or antibiotic B, but not both, were more disease suppressive than the isolates that produced neither ($P < 0.05$, as determined by analysis of variance). The data in Fig. 1 reflect the results of seven separate experiments that were pooled. The level of homogeneity in these experiments was sufficient to warrant combining the experimental data for analysis. Whether the data were considered data from seven separate experiments or were combined, the isolates that produced both zwittermicin A and antibiotic B always exhibited the best disease suppression, and the isolates that produced neither of these antibiotics always exhibited the poorest disease suppression. The disease suppressiveness ranks of the two groups of isolates that produced only one of the antibiotics were not the same in all seven experiments. The values for all 99 isolates tested are shown in Table 3 to illustrate the range of levels of disease suppressiveness of the isolates used in the composite analysis described above. Of the 99 isolates tested, 79 increased the percentage of healthy seedlings compared with plants treated with only *Phytophthora medicaginis* ($\alpha = 0.01$, as determined by Dunnet's comparison test). None of the isolates provided significantly greater seedling protection than previously described strain UW85 ($\alpha = 0.01$, as determined by Dunnet's comparison test).

TABLE 4. Frequency of P7^s and P7^r Eh$^+$ isolates obtained from soils and plant roots

Source of isolates	No. of P7^s isolates/ total no. tested[a]	No. of P7^r Eh$^+$ isolates/ total no. tested
Stock collections	0/18	0/18
Soybean roots	1/85	0/5
Alfalfa roots	3/179	8/179
Lutz soil	1/189	4/96
Snyder-Molino soil	5/96	4/96
Barbour-Lathrop soil	3/217	2/217
Moroceli soil	3/83	3/60
San Matias soil	1/48	2/48
La Vega1 soil	1/48	2/48
La Vega5 soil	1/143	4/95
Arlington soil	4/637	2/50
Hancock soil	3/297	7/37
Marshfield soil	1/585	8/81
Lancaster soil	0/556	11/95
Madison soil	51/426	8/51
Taos soil	0/334	20/334
Tifton soil	1/139	11/139
Douglas Gully soil	6/112	33/112
Lelystad soil	2/115	3/115
Total	87/4,307 (2.0)[b]	132/1,876 (7.0)

[a] The actual numbers of P7^s isolates may be higher since most isolates that were not identified as P7^s isolates in the initial screening test were not retested. Most P7^s isolates were also Eh$^+$; the only exceptions were LN100 and SNY73.
[b] The values in parentheses are percentages.

Diversity among zwittermicin A- and antibiotic B-producing isolates. To determine whether we had isolated strains that were diverse, or at least distinguishable, we tested the zwittermicin A- and antibiotic B-producing isolates to identify phenotypic differences that defined a minimum number of distinct classes of strains. The phenotypes used to distinguish strains included production of pigments on MES minimal medium, the rate of growth on MES-Thr medium, sensitivity to phages P7, ϕATCC7064, ϕATCC27877, and ϕ63, plaque morphology, colony morphology, and resistance to neomycin, tetracycline, and chloramphenicol. On the basis of phenotypic differences we distinguished at least five distinct strain classes among the eight isolates listed in Table 3 that produced antibiotic B but did not accumulate zwittermicin A. There were at least 10 distinct strain classes among the 13 isolates (excluding previously isolated *B. thuringiensis* strains) that produced zwittermicin A but not antibiotic B, and seven distinct P7^s strain classes and 14 distinct P7^r strain classes among the 42 isolates that produced both zwittermicin A and antibiotic B (data not shown). The phenotypic differences among the strains suggest that genetically diverse *B. cereus* strains produce zwittermicin A and antibiotic B.

DISCUSSION

We found that phenotypically diverse strains of *B. cereus* and *B. thuringiensis* isolated from a variety of soils produce the novel antibiotic zwittermicin A, which, as we showed previously, contributes to biological control by *B. cereus* UW85 (60). Zwittermicin A-producing strains were found in soils that are geographically, physically, and biologically diverse (Table 1).

In this study, isolates that produced zwittermicin A were generally superior to isolates that did not produce zwittermicin A or antibiotic B isolates in suppressing the damping-off of alfalfa seedlings caused by *Phytophthora medicaginis* (Fig. 1). Many of the zwittermicin A producers that we identified also produced antibiotic B, which also contributes to biological control by UW85 (60). Isolates that produced both zwittermicin A and antibiotic B were generally superior to isolates that produced one or neither of these antibiotics in suppressing damping-off (Fig. 1).

Discovery and description of new zwittermicin A-producing strains were facilitated by development of the phage P7 sensitivity assay and the *E. herbicola* inhibition assay. Almost all of the $P7^s$ or Eh^+ isolates that we tested produced zwittermicin A; therefore, these assays can be used to rapidly identify new zwittermicin A-producing strains obtained from a variety of soils. Neither assay identified all of the zwittermicin A producers, however, and the number of $P7^s$ or Eh^+ isolates obtained from a given soil probably provides a low estimate of the total population of zwittermicin A-producing *B. cereus* isolates. If the goal is to find new zwittermicin A-producing strains, but not necessarily all of them, the accuracy of these assays is sufficient. Both assays can be performed with many strains simultaneously and are much less labor intensive than tests for inhibition of *Phytophthora medicaginis*, disease suppression on alfalfa seedlings, and chemical detection of antibiotics. The phage P7 sensitivity assay and the *E. herbicola* inhibition assay each has its advantages. Isolates can be processed more quickly in the P7 sensitivity assay, which allows more isolates to be screened. However, a larger proportion of the zwittermicin A-producing isolates in each soil was identified with the *E. herbicola* inhibition assay. The size of the zone of inhibition in the *E. herbicola* assay may be correlated with the amount of antibiotic produced, providing quantitative data on antibiotic production that the phage assay cannot provide. The assays which we describe above provide useful methods for identifying zwittermicin A-producing strains obtained from any soil, although improvements may lead to assays that identify a greater proportion of the zwittermicin A-producing strains in a soil.

We observed striking correlations among the $P7^s$ and Eh^+ phenotypes, disease suppression, and antibiotic production; however, interpretations of these correlations must be treated cautiously given the actual number of strains that have been examined in each assay. It is likely that most Eh^+ isolates produce zwittermicin A, since all of the Eh^+ isolates tested in our HVPE analysis produced zwittermicin A-like material and all zwittermicin A-like compounds tested by ^{1}H-NMR and mass spectrometry were indistinguishable from zwittermicin A. However, most Eh^+ isolates were not examined by ^{1}H-NMR and mass spectrometry, and it is possible that some Eh^+ isolates produce antibiotics other than zwittermicin A or antibiotic B or that in some cases the zwittermicin A and antibiotic B identified by HVPE is structurally distinct from authentic zwittermicin A and antibiotic B. The microbiological assays provide rapid, indirect, initial screening tests for zwittermicin A-producing strains, but antibiotic production should be verified by chemical methods.

The ability to rapidly identify diverse zwittermicin A- and antibiotic B-producing *B. cereus* strains that suppress plant disease should facilitate the testing of new approaches for improving biological control. One limitation on the use of biological control in agriculture has been variability under different environmental conditions. For example, UW85 consistently suppresses disease in Wisconsin, but it is less effective in the southern United States (26a). It is possible that zwittermicin A- and antibiotic B-producing strains obtained from southern soils will be more effective than UW85 in disease suppression in those locations. Similarly, it is possible that zwittermicin A producers obtained from specific crop species or cultivars will be more effective in disease suppression on those crops or cultivars. Moreover, a mixture of genetically diverse disease-suppressive strains might be more effective than a single strain. Screening *B. cereus* strains for either sensitivity to phage P7 or inhibition of *E. herbicola* can be used to identify strains that can be used to test these hypotheses in the field.

Our data suggest that zwittermicin A producers are found in many different soils and comprise roughly 9% of the culturable *B. cereus* soil population, since 9% of the isolates that we examined were Eh^+ and all of these Eh^+ isolates produced zwittermicin A. This estimate of the total zwittermicin A-producing population may be low, since some zwittermicin A producers were Eh^-. The existence of such a high level of zwittermicin A producers suggests that there may be an opportunity to manage native populations of *B. cereus* to achieve plant disease suppression. It may be possible to increase the native populations of zwittermicin A-producing strains in soil and on the roots of plants by cultural practices or by using specific crop cultivars and thus avoid isolation and inoculation of individual strains. Extensive research and testing will be required to determine whether these approaches are feasible or effective for managing diseases in the field.

It is interesting that one strain that produced authentic zwittermicin A was a previously described *B. thuringiensis* strain, HD1, which is widely used in insect control (11). Recently, Manker et al. (42) reported that HD1 produces a molecule that appears to be indistinguishable from zwittermicin A and potentiates the entomocidal activity of *B. thuringiensis* endotoxin.

The genetic diversity of *B. thuringiensis* strains has been exploited for control of insects, but genetic diversity has not been central to the development of strategies for biological control of plant diseases. Use of the diversity within a group of bacteria that share a common mechanism of disease suppression may allow workers to capitalize on existing knowledge concerning mechanisms, physiology, and growth habits, while exploiting the differences among strains to face the challenges of diverse environments.

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