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Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*

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Many strains of *Bacillus cereus* produce zwittermicin A, a novel antibiotic that contributes to the ability of *B. cereus* to suppress certain plant diseases. The purpose of this study was to identify molecular indicators of zwittermicin A production in *B. cereus* strains, contribute to an understanding of the ecology and evolution of this group of bacteria, and identify potential agents for control of plant disease. The fatty acid composition of 20 strains known to be zwittermicin A producers and 20 strains known to be non-producers was determined. Cluster analysis of the fatty acid methyl ester (FAME) profiles revealed that zwittermicin A producers grouped together in two clusters, apart from most non-producers. Discriminant analysis of the FAME profiles generated models that correctly predicted the zwittermicin A-production phenotype in 17 of 20 zwittermicin A producers and 17 of 20 non-producers. Sixteen random oligonucleotide primers were tested in PCR, and one primer was identified that generated a fragment of 0·48 kb or 0·49 kb from total DNA from 26 of 28 strains known to produce zwittermicin A, whereas PCR with this primer did not generate bands of that size from 16 of 20 non-producing strains. PCR with primers designed to amplify *zmaR*, a gene from *B. cereus* that confers resistance to zwittermicin A, generated DNA fragments of 1·1 kb and 1·0 kb in all 29 zwittermicin A-producing strains tested, amplified a fragment of 0·3 kb in some of the zwittermicin A-producing strains, and amplified no fragments in 20 of 23 non-producing strains in a stock collection of *B. cereus* strains. The *zmaR* primers were tested for their ability to identify new zwittermicin A-producing isolates of *B. cereus* from two soils. All 12 of the isolates that produced the banding pattern characteristic of this primer pair produced zwittermicin A, and none of the 12 isolates that did not have the banding pattern produced detectable zwittermicin A. Seven of the 12 isolates initially identified as zwittermicin A producers with the *zmaR* primers significantly suppressed damping-off of alfalfa, whereas only one of the non-producers suppressed this disease. The results show that FAME and PCR analyses distinguish *B. cereus* strains that produce zwittermicin A from other *B. cereus* strains, that PCR with the primers designed to amplify *zmaR* is the most reliable method of those tested for identification of zwittermicin A producers, and that this method can be used to identify new strains with disease-suppressive activity.

Keywords: antibiotics, antibiotic resistance, biological control, biocontrol, plant disease

INTRODUCTION

The *Bacillus cereus* group of bacteria, which consists of *B. cereus*, *B. thuringiensis*, *B. mycoides* and *B. anthracis*, contains species that live in diverse habitats and are associated with a variety of hosts. Members of the group can be isolated from soil, plant surfaces, insect guts, food products, and diseased animals (Goodlow *et al.*, 1947; Brand *et al.*, 1975;

Abbreviations: FAME, fatty acid methyl ester; *zmaR*, gene for resistance to zwittermicin A; RAPD, randomly amplified polymorphic DNA.

Fravel & Spurr, 1977; Schiemann, 1978; Mikesell *et al.*, 1983; Hallaksela *et al.*, 1991; Väisänen *et al.*, 1991; Grewal & Hand, 1992; Stabb *et al.*, 1994). A defining characteristic of this group is that many members produce biologically active molecules that may harm or protect a host. *B. thuringiensis* produces crystal proteins that are toxic to insects and therefore can be used to protect plants from insect predation (Höfte & Whiteley, 1989). *B. anthracis* produces a potent toxin and is a pathogen of animals (Mikesell *et al.*, 1983; Thorne, 1993). Some strains of *B. cereus* produce an enterotoxin (Turnbull *et al.*, 1979; Turnbull, 1981), some produce antibiotics (Wakayama *et al.*, 1984; Kamogashira *et al.*, 1988; Tschen & Tseng, 1989; Silo-Suh *et al.*, 1994) and some antibiotic-producing strains can suppress plant disease (Tschen & Lee, 1988; Silo-Suh *et al.*, 1994). Understanding the evolution of the *B. cereus* group of bacteria and the toxic molecules they produce will be aided by the study of genetically diverse strains, which will provide the basis for population analyses of bacterial phylogeny and ecology.

Genetically diverse collections of strains can also have practical importance in the deployment of these organisms for agricultural uses. In most industrial applications of microbiology, genetic diversity has been the major tool in developing economically feasible processes. In the application of *B. thuringiensis* and other *Bacillus* species for insect biological control, substantial effort has been directed toward identifying genetically diverse strains that have novel toxic activities or are adapted to particular environments (Angus & Norris, 1968; Dulmage, 1970; Goldberg & Margalit, 1977; de Barjac, 1985; Frachon *et al.*, 1991; Feitelson *et al.*, 1992; Tailor *et al.*, 1992). Large collections containing thousands of *B. thuringiensis* strains have become the basis for development of this organism for commercial uses in agriculture (Carlton, 1993). Despite the demonstrated usefulness of genetic diversity in insect biological control, genetic diversity has not been a major focus in biological control of plant disease. Such an approach deserves attention.

Many *B. thuringiensis* and *B. cereus* strains isolated from plant roots or soil produce an unusual antibiotic, zwittermicin A (He *et al.*, 1994; Silo-Suh *et al.*, 1994; Stabb *et al.*, 1994), an aminopolyol that represents a new class of antibiotic. We previously described *B. cereus* strain UW85 (ATCC 53522), which suppresses diseases of plants caused by oomycete pathogens in the laboratory and field (Handelsman *et al.*, 1990, 1991; Smith *et al.*, 1993; Osburn *et al.*, 1995), an activity that is attributable, in part, to production of zwittermicin A by UW85 (Silo-Suh *et al.*, 1994). Since zwittermicin A has interesting biological activities and its production is a widespread trait among strains of *B. cereus* from diverse geographical origins (Stabb *et al.*, 1994), it was a logical trait for which to screen in a search for diverse strains with biological control activity. The only accurate method for identifying zwittermicin A production is the test for the antibiotic itself. Rapid methods based on indirect indicators of zwittermicin A producers are needed to provide insight into the genetics, phylogeny and evolution of zwittermicin A producers (Stabb *et al.*, 1994). Furthermore, methods are needed for detection of zwittermicin A producers in their native habitats as has been done in ecological studies of other organisms with 'phylogenetic stains', which are based on fluorescently labelled oligonucleotides (DeLong *et al.*, 1989; Hahn *et al.*, 1992; Assmus *et al.*, 1995).

The goal of this study was to conduct phenotypic and genotypic analysis to identify traits that would provide more effective ways to screen for strains of *B. cereus* that produce the antibiotic zwittermicin A and control plant disease. Others have shown that analyses of fatty acid methyl ester (FAME) profiles (Abel *et al.*, 1962; Kaneda, 1968; Lambert *et al.*, 1983; DeBoer & Sasser, 1986; Väisänen *et al.*, 1991; Chase *et al.*, 1992; Rainey *et al.*, 1994), DNA patterns in pulsed-field gel electrophoresis (Carlson *et al.*, 1994; Rainey *et al.*, 1994), RAPDs (Welsh & McClelland, 1990; Mazurier *et al.*, 1992; Wang *et al.*, 1993; Andersen *et al.*, 1996), 16S rRNA gene sequences (Stackebrandt & Charfreitag, 1990; Ash *et al.*, 1991a, b; Stackebrandt *et al.*, 1991; Aquino de Muro *et al.*, 1992), and PCR products with primers based on characterized genes (Gustafson, 1992; Darrasse *et al.*, 1994; Leite *et al.*, 1994; Schraft & Griffiths, 1995) have all provided phylogenetic information about members of the *B. cereus* group or other bacteria. We focused on the methods that we expected to have the greatest power to distinguish bacterial strains within a species. Here we describe the use of fatty acid analysis, RAPD analysis, and PCR amplification of a zwittermicin A-resistance gene to distinguish strains that produce zwittermicin A from other *B. cereus* strains.

METHODS

Bacterial strains. *B. cereus* strains isolated from soils from the USA, Panama, Australia, the Netherlands and Honduras were from a collection described by Stabb *et al.* (1994). *B. cereus* isolates were gathered as previously described (Halverson *et al.*, 1993; Stabb *et al.*, 1994). Briefly, after dilution plating soil samples on semi-selective medium (Halverson *et al.*, 1993), colonies with morphology typical of *B. cereus* (flat, broad and cream coloured) were picked and streaked for isolated colonies. Colonies were tested for haemolysis and those that were negative were discarded. Two species similar to *B. cereus* – *B. mycoides* and *B. anthracis* – were presumably excluded from the collection by removing isolates that displayed the rhizoid colony morphology of *B. mycoides* and the non-haemolytic, ampicillin-sensitive phenotypes characteristic of *B. anthracis* (Claus & Berkeley, 1986; Priest, 1993). It is difficult to differentiate between *B. cereus* and *B. thuringiensis* with standard methods (Logan & Berkeley, 1984; Zahner *et al.*, 1989; Ash *et al.*, 1991a; Väisänen *et al.*, 1991); thus, in accordance with recent taxonomic recommendations (Gordon, 1975; Lysenko, 1983; Priest, 1993), we considered all isolates gathered in this study as *B. cereus*. *B. cereus* strains 6464, 33018, F0837/76 and F4810/72 were obtained from A.-B. Kolstø (Carlson & Kolstø, 1994). Since not all of the Wisconsin and Honduras isolates were distinguished from each other by phenotypic typing as was done by Stabb *et al.* (1994), we will refer to them as isolates and not as strains.

Fatty acid analysis. Prior to fatty acid extraction and analysis, cells were spread on plates made from full-strength trypticase soy broth (TSB, Difco) amended with 15 g agar l^{-1}, and the

plates were incubated at 28 °C for 18 h. The cells were scraped off the plate with an autoclaved stick, transferred to an autoclaved 1·5 ml microfuge tube and washed once with 0·75 ml $NH_4H_2PO_4$ buffer (5 mM, pH 7·0). The cell pellets were immediately frozen and stored at −80 °C. Fatty acids were analysed at the Kellogg Biological Station, Michigan State University, using the microbial identification system (MIDI), which is based on gas-liquid chromatography of FAMEs. Three separate FAME profiles were generated for each strain tested, starting from three independent colonies, and the three analyses were combined to form a mean 'library' profile for each strain. Twenty-seven fatty acids were included in the FAME library profile. Statistical analyses of the library profiles were conducted using the SAS computer program (SAS Institute, 1988). For discriminant analysis of fatty acid profiles, the 'DISCRIM' procedure was used with the 'method = normal' option to compute a linear discriminant function. This was combined with the 'crossvalidate' and 'crosslist' functions, so that the fatty acid and zwittermicin A production profiles of 39 strains were used to generate a linear discriminant function that grouped strains as zwittermicin A producers or non-producers based on fatty acid profiles. The zwittermicin A production or non-production phenotype of the remaining (fortieth) strain in the data set was predicted based on its fatty acid profile and the discriminant function generated from the other 39 strains. This process was iterated so that each of the 40 strains was classified as a zwittermicin A producer or non-producer based on the profiles of the remaining 39 strains in the set. For cluster analysis, the 'AVERAGE' method was used.

DNA isolation. DNA was prepared from the bacteria by either large-scale purification based on the method of Marmur (1961) or a rapid cell-lysis procedure. For the large-scale purification, 3·0 ml of an overnight culture grown in brain heart infusion medium (BHI, Difco) was added to 500 ml BHI in a 2800 ml Fernbach flask and incubated with shaking at 28 °C for 5 h. The cells were centrifuged (10000 ***g***, 10 min) and resuspended in 80 ml TSE (10 mM Tris pH 8·0, 10 mM EDTA, 300 mM NaCl). The cells were centrifuged again (10000 ***g***, 10 min) and the supernatant discarded. The cell pellets were frozen at −20 °C overnight to break the cell walls. The pellets were thawed at 37 °C, resuspended in 10 ml solution A (10 mM Tris pH 8·1, 10 mM EDTA, 50 mM NaCl, 20%, w/v, sucrose) containing 5·0 mg lysozyme ml^{-1}, incubated at 37 °C for 30 min, and vortexed briefly at 10 min intervals. An additional 5·0 mg lysozyme was added to the solution, which was incubated for 30 min, and 2·5 ml 20% SDS was added, followed by incubation at 65 °C for 10 min. Five millilitres of 5 M sodium perchlorate (NaHClO) was added and the tubes were inverted repeatedly for 5 min, and then 15 ml of chloroform/isoamyl alcohol (50:1, v/v) was added, and the preparations were mixed gently for 20 min. The tubes were centrifuged (12000 ***g***, 15 min), and the aqueous layer containing the DNA was transferred to a clean tube. After the addition of 2–3 vols 100% ethanol, the tubes were incubated at −20 °C overnight to precipitate the DNA. The pellets were centrifuged (12000 ***g***, 15 min) and the supernatants discarded. The pellets were dried under vacuum in a Speed Vac and resuspended in 5·0 ml sterile distilled water. The DNA was incubated at 37 °C for 2 h with 40 units RNase followed by 100 µl of 20 mg proteinase K ml^{-1} at 65 °C for 1 h. Then 5 ml water and 10 ml phenol were added, the tubes were inverted several times and incubated at 65 °C for 10 min, and inverted again. The tubes were centrifuged (10000 ***g***, 10 min), and the aqueous layer was transferred to a new tube. One millilitre of 3 M sodium acetate, pH 5·4, and 30 ml 100% ethanol were then added, and the DNA was precipitated at −20 °C overnight. The DNA was pelleted by centrifugation and the supernatant discarded. The DNA pellet was washed twice with 15 ml 70% ethanol and dried under vacuum. The purified DNA was resuspended in 500 µl TE (10 mM Tris/HCl, 1 mM EDTA, pH 8·0).

For a rapid cell-lysis method of DNA extraction, the isolate was inoculated from a colony into 2·0 ml BHI medium and incubated at 28 °C with shaking for 16 h. One millilitre of the culture was transferred to a 1·5 ml microfuge tube and centrifuged (14000 ***g***, 10 min). The supernatant was discarded, and the pellet was resuspended in 500 µl sterile distilled water. The cells were heated in a 98 °C water bath for 15 min. The cell debris was removed by centrifugation (14000 ***g***, 5 min), and the DNA solution was transferred to a new microfuge tube.

DNA amplification. DNA prepared by the large-scale purification method from several strains was initially used for screening random primers and testing the *zmaR* primers. Amplification with DNA prepared by the cell-lysis method was then compared with the purified DNA method and was found to produce identical results. Therefore, the cell-lysis method was used in testing further strains.

DNA was amplified in 50 µl reaction mixtures containing 10 mM Tris pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin (Perkin Elmer Cetus 10× buffer), 200 µM of each dNTP, 1·25 units native *Taq* polymerase (Perkin Elmer Cetus), 10–20 ng template DNA, and 2 µM primer. The reaction mixture was overlaid with 30 µl mineral oil (Sigma). PCR with random primers was carried out in a thermocycler (Robocycler, Stratagene) as follows: one cycle at 94 °C for 5 min; 45 cycles of 94 °C for 1 min, 32 °C for 1 min, 72 °C for 2 min; and a final extension of 72 °C for 5 min. Conditions for PCR with the resistance gene primers were the same except the annealing temperature was 56 °C instead of 32 °C. A 15 µl sample of the PCR reaction mixture was subjected to electrophoresis in a gel containing 0·75% Synergel (Diversified Biotech) and 0·5% Seakem agarose (FMC) in TBE (0·089 M Tris base, 0·089 M boric acid, 0·002 M EDTA pH 8·0). Bands were visualized on a UV transilluminator by staining with ethidium bromide, and data were digitally recorded with a digital imager (Alpha Innotech). Analysis of each strain was repeated at least twice and the results were highly reproducible.

Primer design. Random primers were from a kit of 60% GC decamers (Genosys). Primers for *zmaR* (see footnote, Table 2) were based on the full sequence of the gene (GenBank accession no. U57065) (Milner *et al.*, 1996b). Primer A0678 was based on the sequence from nucleotides 191 to 210, and primer A0677 was based on the sequence from nucleotides 1142 to 1123. The primers were synthesized by the University of Wisconsin Biotechnology Center with an Applied Biosystems DNA Synthesizer.

Detection and quantification of zwittermicin A. Zwittermicin A was detected in bacterial cultures by the method previously reported (Milner *et al.*, 1995). Briefly, culture supernatants were passed through a CM SEP-PAK cartridge (Millipore) and zwittermicin A was eluted and separated from other compounds by high-voltage paper electrophoresis at pH 9·2. The papers were stained with silver nitrate to detect zwittermicin A. The concentration of zwittermicin A was determined by an endpoint dilution assay (Silo-Suh *et al.*, 1994) in which the minimum detection was generally 0·25 µg ml^{-1}, although the exact limit of detection varied with the experiment; therefore a standard of known concentration was included in every experiment.

Assay for sensitivity of bacteria to zwittermicin A. A single colony was inoculated into 1·0 ml of half-strength TSB and incubated with shaking at 28 °C for 6 h. Cultures were streaked

radially from the centre to the edge of a plate containing Mueller–Hinton agar amended with 38 mM MOPS and 41 mM Tris and adjusted to pH 8·1. Zwittermicin A (300 μg) was then added to a 6 mm well cut in the middle of the agar, and the plate was incubated at room temperature. After 16 h, plates were scored for presence or absence of a zone of inhibition of growth around the well.

Assay for alfalfa damping-off. Biological control activity of bacterial isolates from soils from Madison (Wisconsin) and Zamorano (Honduras) was determined by a method developed in our laboratory (D. W. Johnson and others, unpublished data). Ten surface-sterilized alfalfa seeds of cultivar Webfoot MPR were placed into a cell of a 96-cell bedding plant container (Hummert International) containing autoclaved vermiculite. The containers were placed in flats of water raised to the level at which the seeds were sown and were watered from the bottom with distilled water. A 1·0 ml suspension of 250–500 zoospores of *Pythium torulosum* was added to each well followed by 1·0 ml of culture of the *B. cereus* isolate that had been grown in half-strength TSB for 4 d at 28 °C. The seeds were then covered with a 1·0 cm layer of autoclaved vermiculite.

We tested 24 *B. cereus* isolates identified in this study, strains UW85 (Handelsman *et al.*, 1990), and UW030 (a mutant of UW85 that does not produce detectable zwittermicin A; Silo-Suh *et al.*, 1994), half-strength TSB and water, all with zoospores; and UW030, UW85 and water without zoospores, for a total of 31 treatments. Emergence was scored 12 d after planting. The treatments were arranged in a randomized complete block design with six blocks. Each treatment in each block consisted of three cells, containing a total of 30 seeds. The experiment was repeated three times. The error was homogeneous among the three experiments; thus the data from the three experiments were pooled (Snedecor & Cochran, 1980). Each isolate was compared with the control containing water plus zoospores by the Dunnett's one-tailed *t*-test at $P = 0{\cdot}05$ (Dunnett, 1955).

RESULTS

We conducted a phenotypic and genotypic analysis of *B. cereus* strains to identify traits that are reliable predictors of zwittermicin A production. FAME analysis, RAPD analysis and PCR-amplification of a zwittermicin A-resistance gene (*zmaR*) were applied to our collection of *B. cereus* strains, which have known zwittermicin A-producing phenotypes. The genotypic analysis of *zmaR* was also applied to newly identified *B. cereus* isolates for which the ability to produce zwittermicin A had not previously been determined.

Fatty acid analysis

The FAME profiles of each of 20 zwittermicin A-producing and 20 non-producing strains were determined and compared by cluster analysis (Fig. 1) and discriminant analysis to determine their relatedness. By cluster analysis, zwittermicin A producers grouped in two clusters. One cluster contained seven zwittermicin A producers and seven non-producers; a second cluster contained the remaining 13 zwittermicin A producers and no non-producers (Fig. 1). Thirteen non-producers were grouped in clusters separate from the two clusters containing zwittermicin A producers. Thus, if inclusion in a cluster is used as a predictor of zwittermicin A synthesis, seven of the 40 strains would be misidentified.

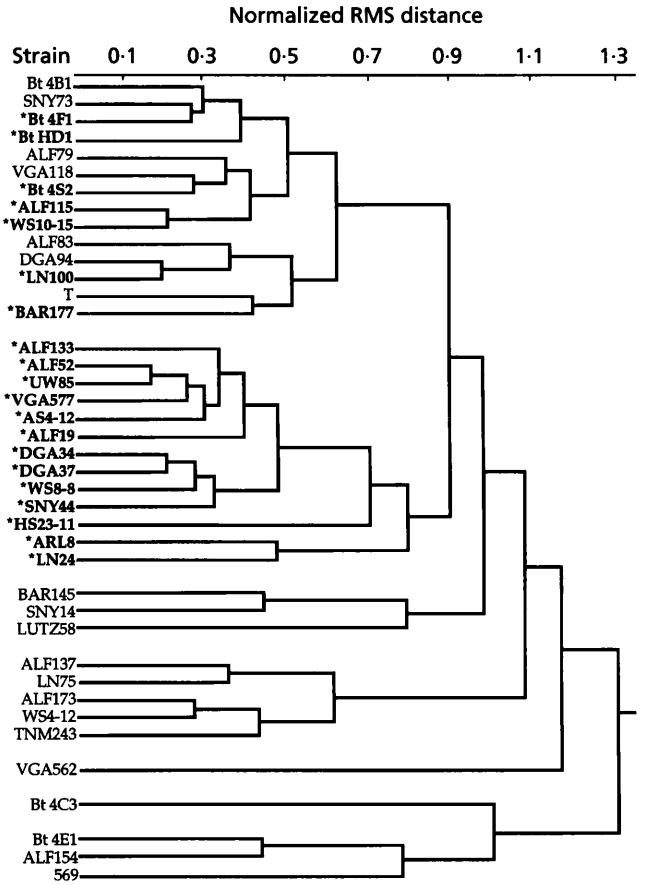

Fig. 1. **Cluster analysis of FAME profiles. FAMEs were analysed from 40 different strains (20 zwittermicin A producers and 20 non-producers) using the Microbial Identification System. Three FAME profiles were generated for each strain tested, and the three analyses were combined to form a library profile for each strain. Cluster analysis was conducted using the SAS Computer Program using the 'AVERAGE' method. Strain names in bold-face type and indicated with an asterisk represent zwittermicin A producers.**

In an effort to improve on the reliability of the cluster analysis of FAME profiles to predict zwittermicin A production, discriminant analysis was tested. In this iterative analysis, the FAME profiles of 39 strains were used to construct a predictive model that correlates elements of the FAME profile with zwittermicin A production. The FAME profile of the remaining strain (of the 40-strain data set) was tested against the model. This process was conducted 40 times, such that each strain was used once as the test strain. Seventeen of 20 zwittermicin A producers and 17 of 20 non-producers were correctly identified in the discriminant analysis. The six strains that were misidentified were Bt 4C3, ALF83, LUTZ58, ALF115, ARL8 and BAR177.

To determine whether a single fatty acid or multiple fatty acids were responsible for distinguishing zwittermicin A producers in the above analyses, and to identify this fatty

Table 1. Correlation between particular fatty acids and zwittermicin A production in *B. cereus* strains

Fatty acid*	Percentage of total fatty acid content of zwittermicin A producers†	Percentage of total fatty acid content of non-producers†	Pearson correlation coefficient‡
16:1 IsoI or 14:0 3OH§	4·9±1·0	3·0±0·9	0·70 (0·0001)
14:0 Iso	7·2±1·0	5·5±1·1	0·64 (0·0001)
17:0 Iso	5·2±0·8	7·0±1·3	−0·64 (0·0001)
15:0 Iso	27·5±2·1	31·0±2·7	−0·58 (0·0001)
15:0 Anteiso	4·4±0·4	3·7±0·6	0·55 (0·0002)
17:1 Anteiso A	0·8±0·3	0·5±0·2	0·50 (0·0010)
15:0 2OH	0·8±0·2	0·5±0·3	0·48 (0·0015)
16:0	3·63±0·7	4·71±1·3	−0·46 (0·0026)
Iso 17:1 ω5c	4·48±0·9	3·61±1·0	0·42 (0·0063)
15:1 ω5c	0·12±0·2	0·0±0·0	0·41 (0·0076)

* Fatty acids were assigned by comparison to standards using the MIDI system (see Methods).

† The percentage of total fatty acid content represented by the particular fatty acid was calculated by integration of fatty acid peaks. Numbers represent the means for 20 zwittermicin A producers or 20 non-producers plus or minus the standard deviation.

‡ Correlation between fatty acid and zwittermicin A production; numbers in parentheses represent the *P* value.

§ 16:1 IsoI is indistinguishable from 14:0 3OH; however, the former is typical of Gram-positive bacteria and the latter is typical of Gram-negative bacteria.

acid(s), correlation analyses were performed (SAS, 1988). Of the 27 fatty acids included in the FAME libraries, 10 showed a significant ($P < 0·01$) positive or negative correlation with zwittermicin A production (Table 1).

RAPD analysis

Sixteen random 10-nucleotide primers were screened for the ability to distinguish zwittermicin A-producing strains from non-producers in a PCR assay. PCR with primer 4-60-32 (see sixth footnote, Table 2) produced either a 0·48 kb or a 0·49 kb fragment in 26 of the 28 zwittermicin A-producing strains tested and in four of the 20 non-producing strains tested (Table 2, Fig. 2). PCR with the remaining 15 random primers did not distinguish zwittermicin A-producing strains from non-producing strains. The 0·49 kb fragment from UW85 and the 0·48 kb fragment from strain WS10-15 hybridized only with fragments of 0·49 kb and 0·48 kb, respectively, in all strains tested (data not shown).

PCR amplification of a zwittermicin A-resistance gene

We recently identified a gene from *B. cereus* UW85, designated *zma*R, that confers resistance to zwittermicin A upon *Escherichia coli* and *B. cereus* (Milner *et al.*, 1996b). Two primers, A0677 and A0678 (fifth footnote, Table 2), were designed based on the DNA sequence of the resistance gene. These primers amplified DNA fragments of 1·1 kb and 1·0 kb, and from some strains, a fragment of 0·30 kb, from all the zwittermicin A producers and from three of the 23 non-producers (Table 2, Fig. 3). All strains from which these fragments were amplified were also resistant to zwittermicin A except ALF83 and 33018 (Table 2). A probe generated from cloned *zma*R DNA hybridized to all PCR-amplification products (1·1, 1·0 and 0·3 kb; data not shown) in all strains tested. When each primer was used separately in the PCR reaction or DNA template was omitted, no amplified products were detected (data not shown). The same pattern of three bands was generated when the template was *zma*R cloned on a 1·1 kb fragment in the *E. coli* sequencing vector pGEM, or in the shuttle vector pHT304 (Arantes & Lereclus, 1991). Therefore, all of the bands generated by this PCR reaction appear to be from within the *zma*R sequence.

Strain Bt 4E1 was sensitive to zwittermicin A, and PCR with the primers designed to amplify *zma*R generated a band of 0·7 kb, but this band did not contain sequences that hybridized with the probe to the cloned *zma*R (Table 2). These data suggest that Bt 4E1 contains sequences that hybridize with the primers, but does not contain an authentic homologue of *zma*R.

Identification of new zwittermicin A-producing isolates of *B. cereus* by PCR

The utility of the *zma*R primers to screen for new zwittermicin A-producing *B. cereus* isolates from soil was tested with 58 *B. cereus* isolates from a soil in Madison, Wisconsin, and 59 *B. cereus* isolates from a soil in Zamorano, Honduras. Amplification products with primers A0677 and A0678 were detected in reactions from 27% of the isolates collected. The quantity of

Table 2. Phenotypic and genotypic analysis of *B. cereus* and *B. thuringiensis* strains

Strain*	Origin†	ZmA production‡	ZmA resistance§	ZmA resistance gene‖	Random primer PCR products¶
UW85	USA	+	R	+	+
ALF19	USA	+	R	+	+
ALF52	USA	+	R	+	+
ALF53	USA	+	R	+	+
ALF95	USA	+	R	+	+
ALF115	USA	+	R	+	+
ALF133	USA	+	R	+	+
ALF161	USA	+	R	+	+
ARL8	USA	+	R	+	+
AS4-12	USA	+	R	+	+
BAR177	Panama	+	R	+	+
DGA34	Australia	+	R	+	+
DGA37	Australia	+	R	+	+
DGA84	Australia	+	R	+	+
HS23-11	USA	+	R	+	+
LN24	Netherlands	+	R	+	−
LN100	Netherlands	+	R	+	−
MS3-2	USA	+	R	+	+
MOR28	Honduras	+	R	+	+
MOR37	Honduras	+	R	+	+
SNY44	Panama	+	R	+	+
SOY130	USA	+	R	+	+
VGA577	Honduras	+	R	+	+
WS8-8	USA	+	R	+	+
WS10-15	USA	+	R	+	+
6464	ATCC	+	R	+	ND
Bt HD1	BGSC	+	R	+	+
Bt 4F1	BGSC	+	R	+	+
Bt 4S2	BGSC	+	R	+	+
569	BGSC	−	R	+	−
Bt 4B1	BGSC	−	S	−	−
Bt 4C3	BGSC	−	S	−	−
Bt 4E1	BGSC	−	S	(−)	+
33018	ATCC	−	S	+	ND
F0837/76	PHLS	−	S	−	ND
F4810/72	PHLS	−	S	−	ND
ALF10	USA	−	S	−	−
ALF79	USA	−	S	−	−
ALF83	USA	−	S	+	−
ALF137	USA	−	S	−	−
ALF154	USA	−	S	−	−
ALF173	USA	−	S	−	−
BAR145	Panama	−	S	−	−
DGA94	Australia	−	S	−	−
LN75	Netherlands	−	S	−	−
LUTZ58	Panama	−	S	−	+
SNY14	Panama	−	S	−	−
SNY73	Panama	−	S	−	+
TNM243	USA	−	S	−	−
VGA118	Honduras	−	S	−	+
VGA562	Honduras	−	S	−	−
WS4-12	USA	−	S	−	−

* Bt indicates *B. thuringiensis*.

† BGSC, Bacillus Genetic Stock Center; ATCC, American Type Culture Collection; PHLS, Public Health Laboratory Services.

‡ Production of zwittermicin A (ZmA) by all strains was determined by Stabb *et al.* (1994), except for 6464, 33018, F4810/72 and F0837/76 (determined in this study).

§ Resistance of strains to zwittermicin A: R, resistant; S, sensitive.

‖ PCR products with the resistance gene primers A0677 (TAAAGCTCGTCCCTCTTCAG) and A0678 (ATGTGCACTTGTATGGGCAG). + indicates a 1000 bp fragment; − indicates no 1000 bp fragment. (−) indicates that a fragment of 700 bp is produced and a 1000 bp fragment is not produced. The 700 bp fragment does not hybridize with *zmaR* from *B. cereus* UW85.

¶ PCR product from random primer 4-60-32 (GCAGGATACG). + indicates 480 bp or 490 bp fragment; − indicates no 480 bp or 490 bp fragments. ND, Not determined.

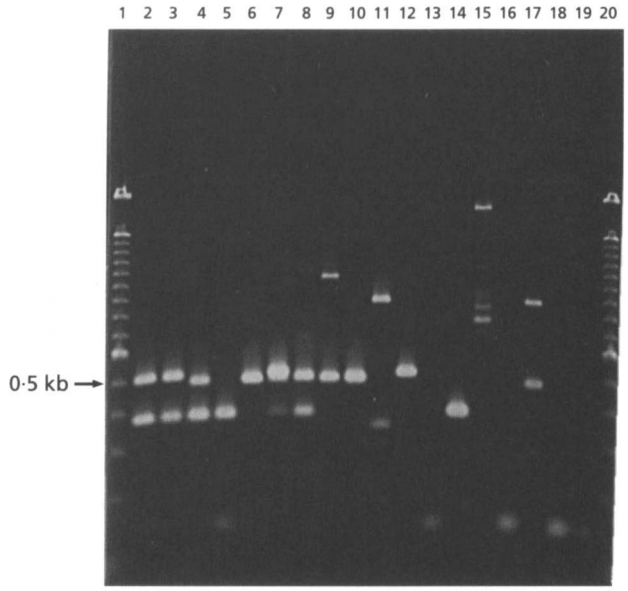

Fig. 2. PCR products amplified with primer 4-60-32 separated by agarose gel electrophoresis. Lanes 1 and 20, 100 bp ladder; lanes 2–10, zwittermicin A-producing strains of *B. cereus* and *B. thuringiensis* – UW85, AS4-12, WS10-15, LN100, ALF133, ARL8, BAR177, DGA37, Bt 4S2; lanes 11–19, zwittermicin A non-producing strains of *B. cereus* and *B. thuringiensis* – Bt 4B1, SNY73, ALF10, ALF79, SNY14, TNM243, DGA94, WS4-12, ALF173.

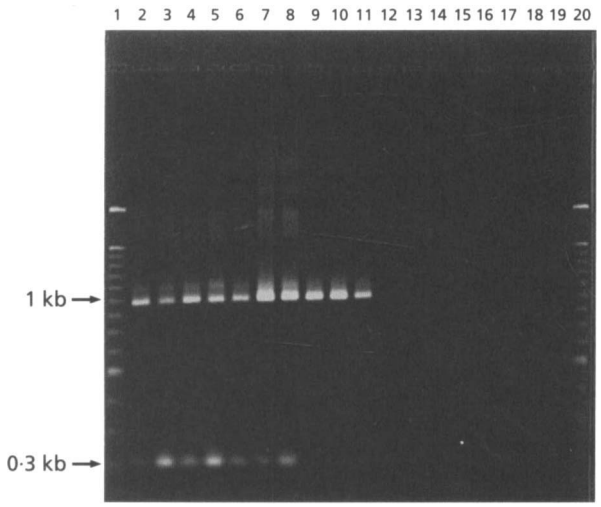

Fig. 3. PCR products amplified with primers A0677 and A0678 separated by agarose gel electrophoresis. Lanes 1 and 20, 100 bp ladder; lane 2, pZmR7, a cosmid containing *zmaR*; lanes 3–10, zwittermicin A-producing strains of *B. cereus* and *B. thuringiensis* – UW85, ALF95, ALF161, SNY44, WS10-15, MOR28, Bt HD1, DGA37; lanes 11–19 – zwittermicin A non-producing strains: ALF83, VGA562, BAR145, ALF173, ALF137, SNY73, WS4-12, DGA94, LN75.

zwittermicin A made by the isolates in culture was determined for seven positive and six negative isolates from Wisconsin, and five positive and six negative isolates from Zamorano, plus strains UW85 (positive) and UW030 (negative) (Table 3). All 12 isolates that generated *zmaR*-characteristic products in the PCR test with the *zmaR* primers produced zwittermicin A, whereas none of the 12 isolates for which no detectable product was amplified in the PCR test produced detectable quantities of zwittermicin A (Table 3).

Table 3. ***zmaR*** **genotype, accumulation of zwittermicin A in culture, and disease suppressiveness of *B. cereus* strains from Madison (Wisconsin) and Zamorano (Honduras)**

Zoospores of *Pythium torulosum* were added to each strain. Survival of seedlings in control treatments was as follows: UW85 without zoospores, 75·9%; water without zoospores, 75·2%; UW030 without zoospores, 74·6%; water with zoospores, 49·2%; TSB with zoospores, 27·6%.

Strain	*zmaR**	ZmA detected [μg (ml culture)$^{-1}$]†	Seedling survival (%)‡
UW85	+	14·6 ± 1·1	78·0 ± 3·9**
W35	+	9·3 ± 1·0	73·9 ± 5·1**
W6	+	12·8 ± 2·4	72·8 ± 4·4**
Z8	+	17·4 ± 3·7	72·8 ± 4·1**
Z39	+	6·8 ± 1·4	69·3 ± 3·0**
Z33	+	17·7 ± 2·4	68·1 ± 4·2**
W9	+	2·65 ± 0·5	66·3 ± 6·8
Z42	+	15·2 ± 1·1	64·3 ± 4·6**
Z54	+	13·9 ± 2·7	63·7 ± 5·4
W37	+	8·2 ± 0·2	60·2 ± 4·4
W28	+	7·6 ± 1·1	59·4 ± 5·5
W50	+	12·4 ± 1·5	58·9 ± 5·8
W21	+	3·5 ± 0·8	57·2 ± 4·8
W23	–	0	68·5 ± 5·6**
Z7	–	0	64·1 ± 4·2
Z49	–	0	62·6 ± 4·7
W48	–	0	61·1 ± 4·6
W38	–	0	60·2 ± 4·7
Z12	–	0	58·3 ± 4·9
W10	–	0	56·8 ± 4·8
W49	–	0	56·7 ± 4·7
Z55	–	0	51·1 ± 5·4
W8	–	0	50·2 ± 4·2
Z66	–	0	50·0 ± 5·2
Z53	–	0	47·0 ± 5·6
UW030	–	0	46·8 ± 5·2

* *zmaR* detected by PCR with primers A0677 and A0678.

† Zwittermicin A (ZmA) detected by endpoint dilution assay; detection limit is 0·25 μg.

‡ Seedling survival determined in alfalfa damping-off assay.

** Indicates that survival was significantly higher than the water plus pathogen control by Dunnett's one-tailed *t*-test ($P < 0·0001$).

Association between zwittermicin A production and biological control activity among *B. cereus* isolates

The 24 *B. cereus* isolates from Madison and Zamorano for which zwittermicin A production was quantified were tested for biological control activity in an assay for

damping-off of alfalfa caused by the pathogen *Pythium torulosum* (Table 3; controls are listed in the legend). Six of the 12 zwittermicin A producers identified with the *zma*R primers had significant biological control activity. One of the 12 non-producers, W23, also had significant biological control activity. As a group, the isolates that were positive in PCR with the *zma*R primers increased emergence of alfalfa seedlings significantly more than did isolates that were negative in the PCR test ($P = 0{\cdot}0001$).

DISCUSSION

We examined a world-wide collection of *B. cereus* and *B. thuringiensis* strains isolated from soil or obtained from culture collections for characteristics associated with the ability to produce zwittermicin A. We found a strong association between zwittermicin A production and cellular fatty acid composition. RAPD analysis and PCR amplification of a zwittermicin A resistance locus were also effective in distinguishing *B. cereus* strains that produce zwittermicin A from those that do not. Each method identified a greater proportion of zwittermicin A producers among the *B. cereus* strains screened than did previously described assays (Stabb *et al.*, 1994).

Since Abel *et al.* (1962) first proposed the link between fatty acid composition and taxonomic classification, fatty acid composition has proved to be a useful phylogenetic tool to determine genus or species affiliation (Lambert *et al.*, 1983; Chase *et al.*, 1992). Fatty acid composition has also been useful in identifying bacterial strains with particular traits, as illustrated by a cluster analysis of *B. sphaericus* FAME profiles, which indicated that mosquitocidal strains tended to group together (Frachon *et al.*, 1991). Our results indicate that zwittermicin A producers also tend to group together in cluster analysis of FAME profiles (Fig. 1), and that cluster or discriminant analysis of FAME profiles are useful methods for identifying zwittermicin A producers. The cluster analysis of FAME profiles, which placed the zwittermicin A producers in more than one grouping, also indicated diversity among zwittermicin A producers.

The clustering of the FAME profiles of zwittermicin A producers could represent phylogenetic relatedness among zwittermicin A producers, or it could be due to a direct biochemical link between some fatty acid(s) and zwittermicin A production. The fact that ten fatty acids were significantly ($P < 0{\cdot}01$) correlated with the zwittermicin A phenotype (Table 1) may suggest a general relatedness rather than a specific physiological link. This is intriguing given the puzzling taxonomy of the *B. cereus* group, which includes *B. mycoides*, *B. thuringiensis* and *B. anthracis*. Others have shown that fatty acid profiles distinguished *B. anthracis* from *B. cereus* (Kaneda, 1968), while *B. thuringiensis*, *B. mycoides* and *B. cereus* were indistinguishable (Kaneda, 1968; Väisänen *et al.*, 1991). These findings are consistent with other taxonomic methods that were unable to distinguish *B. thuringiensis* and *B. cereus* (Gordon, 1975; Lysenko, 1982; Logan & Berkeley, 1984; Zahner *et al.*, 1989; Ash *et al.*, 1991a, b; Priest, 1993; Carlson *et al.*, 1994). Thus, while *B. cereus* and *B. thuringiensis* may not represent distinct species (Lysenko, 1983), zwittermicin A production, a trait found in members of both *B. cereus* and *B. thuringiensis*, may indicate a phylogenetic branch within the *B. cereus* group.

Primers designed to amplify genes of known function have been used to identify organisms at the genus, species or strain level (Böddinghaus *et al.*, 1990; Giesendorf *et al.*, 1992; Gustafson *et al.*, 1992; Bej *et al.*, 1994; Darrase *et al.*, 1994; Leite *et al.*, 1994; Li *et al.*, 1994; Reif *et al.*, 1994; Dreier *et al.*, 1995; Kreader, 1995; Schraft & Griffiths, 1995). Identification of zwittermicin A producers was more accurate with the *zma*R primers than with the random primers. This is not surprising since strains that produce zwittermicin A are likely to carry a resistance gene to avoid self-inhibition by the antibiotic. All of the strains resistant to zwittermicin A carried the resistance gene, *zma*R, as indicated by PCR, and all that were sensitive, except strains ALF83 and 33018, did not carry *zma*R (Table 2). ALF83 and 33018 may have acquired mutations in the resistance gene and the biosynthetic genes or they may contain genes that have sequence similarity to *zma*R but do not serve the same function. *B. cereus* strain 569 also appears to carry the resistance gene based on the PCR assay and its zwittermicin A-resistance phenotype, but it does not produce detectable quantities of zwittermicin A in culture (Table 2). This strain may have evolved from a strain that produces the antibiotic, or it may have acquired the resistance gene without the biosynthetic genes by gene transfer. The correlation between *zma*R, zwittermicin A resistance and zwittermicin A production validates the use of the *zma*R primers for identifying zwittermicin A producers, and it suggests the possibility that zwittermicin A producers may be selected directly from a mixed *B. cereus* population by culturing on zwittermicin A-containing media.

We predicted that a fragment of approximately 1·0 kb should be generated by primers A0677 and A0678. We attribute the 0·3 kb band to a putative primer binding site with 55% similarity to primer A0677, which occurs 466 nucleotides from the start of the *zma*R coding sequence (E. A. Stohl, personal communication). It is possible that the 0·3 kb fragment acts as a primer and PCR then generates the 1·1 kb band, but we do not have data to explain the 1·1 kb fragment at this time.

It is interesting that certain strains are exceptions to more than one method for identifying zwittermicin A producers. For instance, SNY73 was previously identified as the only strain in a large collection that was sensitive to phage P7 (usually an indication of zwittermicin A production) but did not produce zwittermicin A (Stabb *et al.*, 1994). In the present study, SNY73 also behaved like most zwittermicin A producers in the RAPD analysis. Strain LUTZ58, which does not produce zwittermicin A, fitted the model of a zwittermicin A producer in our discriminant analysis of FAME profiles and in our RAPD analysis. Similarly ALF83, which does not produce zwittermicin A, was classified as a zwittermicin A producer both in the discriminant analysis of FAME profiles and by amplification of the *zma*R gene. Finally,

cluster analysis of FAME profiles revealed several clusters, one of which contained a mixture of zwittermicin A producers and non-producers. Strains SNY73 and ALF83 mentioned above, as well as LN100 and VGA118, which were exceptions to the trend in RAPD analysis, are contained in this heterogeneous cluster.

We identified zwittermicin A-producing isolates from soils from Wisconsin and Honduras using PCR with primers designed to amplify the zwittermicin A-resistance gene. PCR with these primers was useful in identifying isolates (such as W35, Z8 and W6) that produced zwittermicin A and were effective in suppression of alfalfa damping-off. However, the PCR method would not identify isolates such as W23, which did not produce detectable zwittermicin A, as effective biological control agents. It will be interesting to study the mechanism of disease suppression by W23, since it may suppress disease by a mechanism not previously described.

Four of the strains described in Table 2, 6464, 33018, F4810/72 and F0837/76, have atypically small chromosomes, making them useful for genetic mapping studies (Carlson & Kolstø, 1994). One of these strains, 6464, contained the *zmaR* gene, was zwittermicin A resistant, and produced zwittermicin A (Table 2). Since the chromosome of strain 6464 is roughly half the size of a typical *B. cereus* chromosome (Carlson & Kolstø, 1994), this strain may be useful for the genetic mapping of *zmaR* as well as the cloning and mapping of zwittermicin A biosynthetic genes.

The methods described here have two powerful applications. First, they will facilitate the construction of a diverse collection of strains that produce zwittermicin A. Such a collection may be useful in phylogenetic studies of the *B. cereus* group and may provide insight into the evolution of the bacteria and the origin of the ability to produce zwittermicin A. The collection will also provide an array of strains that suppress damping-off in the laboratory. These strains may provide the basis for new strategies to improve biological control of plant disease through the use of mixtures of strains (Pierson & Weller, 1994) or strains that are adapted to a particular host or geographical location (Stabb *et al.*, 1994; Keel *et al.*, 1996). Developing biological control strategies with many strains that share a common mechanism of disease suppression would mean that knowledge of one strain could be transferred to other strains, maximizing the use of results of fundamental research. Many members of *B. cereus* suppress plant disease (Stabb *et al.*, 1994); therefore, study of genetic diversity within the *B. cereus* group is attractive both for an understanding of the biology of the members of the group and for application of this knowledge to disease suppression on plants. Furthermore, as evidenced by the small-chromosome-containing, zwittermicin A-producing strain 6464, this collection may contain zwittermicin A producers amenable to the genetic study of zwittermicin A production and resistance. For example, a diverse collection of zwittermicin A producers may contain strains that are easily transformable, or strains that can be infected by transducing phage.

Second, the molecular tools may provide sufficiently sensitive and specific probes to study populations of zwittermicin A-producing *B. cereus* strains directly on plant roots or in soil, and such a study will contribute to our understanding of the ecology of these disease-suppressive organisms (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Hahn *et al.*, 1992; Szewzyk *et al.*, 1994; Assmus *et al.*, 1995). Furthermore, the ability to monitor populations of zwittermicin A-producing strains in the rhizosphere may facilitate breeding or engineering of plants that support populations of zwittermicin A-producing *B. cereus* on their roots to enhance biological control by inoculated bacteria or by bacteria recruited by the plants directly from soil (Stabb *et al.*, 1994; Milner *et al.*, 1996a; O'Connell *et al.*, 1996). Future work will test the usefulness of the methods developed here for studying the ecology of *B. cereus* and enhancing plant health in the agroecosystem.

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