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Production of Kanosamine by *Bacillus cereus* UW85

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***Bacillus cereus* UW85 produces two antibiotics that contribute to its ability to suppress certain plant diseases (L. Silo-Suh, B. Lethbridge, S. J. Raffel, H. He, J. Clardy, and J. Handelsman, Appl. Environ. Microbiol. 60: 2023–2030, 1994). To enhance the understanding of disease suppression by UW85, we determined the chemical structure, regulation, and the target range of one of the antibiotics. The antibiotic was identified as 3-amino-3-deoxy-D-glucose, also known as kanosamine. Kanosamine was highly inhibitory to growth of plant-pathogenic oomycetes and moderately inhibitory to certain fungi and inhibited few bacterial species tested. Maximum accumulation of kanosamine in *B. cereus* UW85 culture supernatants coincided with sporulation. Kanosamine accumulation was enhanced by the addition of ferric iron and suppressed by addition of phosphate to rich medium. Kanosamine accumulation was also enhanced more than 300% by the addition of alfalfa seedling exudate to minimal medium.**

The use of microorganisms to control plant disease is an attractive alternative or supplement to synthetic pesticides and genetic resistance of crop plants for the management of plant disease. *Bacillus cereus* UW85 has a wide range of disease-suppressive and other biological activities on plants (10, 13–15, 25, 26, 30). The ability of *B. cereus* UW85 and other isolates of *B. cereus* to suppress damping-off disease of alfalfa caused by the oomycete pathogen, *Phytophthora medicaginis*, in a laboratory bioassay is associated with two antibiotics designated zwittermicin A and antibiotic B, which are found in the extracellular fraction of fully sporulated cultures (15, 28, 31). Mutants of UW85 lacking antibiotic production are deficient in disease suppressiveness, and purified zwittermicin A or antibiotic B each suppress plant disease (28). Knowledge of the chemical structure of these compounds is essential for understanding the mechanistic basis for disease suppression by *B. cereus* strains and the ecology of *B. cereus* on roots and in the soil. Zwittermicin A is a novel aminopolyol that may represent a previously undescribed class of antimicrobial compounds (16, 28). In this report, we identify antibiotic B as 3-amino-3-deoxy-D-glucose, also known as kanosamine (6, 8, 37, 38).

Elucidation of the chemical structure of antibiotic B. Antibiotic B was purified from 500-ml cultures of *B. cereus* UW85 (ATCC 53522) (15) grown in half-strength Trypticase soy broth (TSB; BBL, Cockeysville, Md.) by the Amberlite IRC50 high-voltage paper electrophoresis method described previously (28). The structure of antibiotic B was determined by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) of purified antibiotic B and its acetylated derivative. The chromatographic behavior of purified antibiotic B suggested that a single compound was present, and the NMR and MS spectra of this fraction indicated no detectable contaminants. Thus, the preparations were judged to be of sufficient purity for biological assays.

The electrospray ionization mass spectrum of antibiotic B exhibited a 100% abundance peak at *m/z* 180.0, and the fast atom bombardment MS showed a weak signal at the same position. The ^{1}H NMR spectrum (500 MHz, D_2O) displayed the majority of proton resonances in the 3.20- to 4.00-ppm region, which suggested that the molecule is a glycoside. Doublets at 5.28 ppm (J = 2.5 Hz) and 4.73 ppm (J = 8.0 Hz) that integrated to one proton indicated that both the α and β forms of the molecule were present in the purified material. The splitting patterns of the methine resonances indicated that the sugar has glucose-like configurations. In addition, the H-4 methine proton formed a triplet (J = 10 Hz), which was coupled *trans*-diaxially to an H-3 and an H-5, as is characteristic of glucose in the D form. In the ^{13}C NMR spectrum (100 MHz, D_2O), 12 distinct signals were observed, including two carbon resonances at 57.7 and 60.5 ppm, which suggested that the antibiotic is an amino sugar.

Analysis of two-dimensional NMR experiments (correlation spectroscopy [COSY], ^{1}H-^{1}H correlation; heteronuclear multiple quantum coherence [HMQC], direct ^{13}C-^{1}H correlation; heteronuclear multiple band coherence [HMBC], long-range, two- to three-bond ^{13}C-^{1}H correlation; total correlation spectroscopy [TOCSY], long-range, four- to six-bond ^{1}H-^{1}H correlation) identified antibiotic B as 3-amino-3-deoxy-D-glucose, also known as kanosamine (Fig. 1). No evidence for a glycosidic linkage between the α and β monomers was detected. The assignment of the amino group in the 3 position was based on the lower chemical shift of C-3 and H-3 and their homonuclear and heteronuclear correlations. The NMR and MS analyses of the acetylated derivative of antibiotic B supported these conclusions. The fully acylated derivative of antibiotic B, obtained following reaction of antibiotic B with acetic anhydride in pyridine for 12 h and purification from a reverse-phase high-pressure liquid chromatography C_{18} column, afforded acetyl signals in the ^{1}H NMR corresponding to the α- and β-pentaacetate (high-resolution fast atom bombardment-MS, *m/z* $[M^+H^+]^+$ 390.140600, observed; 390.140021, calculated for $C_{16}H_{24}NO_{10}$). The ^{1}H NMR and ^{13}C NMR spectra of antibiotic B were also consistent with those previously reported for kanosamine (8). Kanosamine, first described as one of the monosaccharide units that compose kanamycin (35), accumu-

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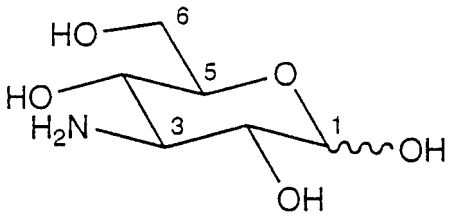

FIG. 1. Kanosamine (α- and β-anomers of 3-amino-3-deoxy-D-glucose).

lates as the free monosaccharide in culture filtrates of *Bacillus aminoglucosidicus* (37, 38), *Bacillus* sp. (8), and *Streptomyces lansus* (6).

Sensitivity of oomycetes to kanosamine. We previously reported that the oomycete *P. medicaginis* is sensitive to antibiotic B (25, 28). Thus, we determined the minimum amount of purified kanosamine that would inhibit *P. medicaginis* and other oomycetes that cause seedling damping-off or root-rot disease. Zoospores of *P. medicaginis* (28), *Pythium* spp. (27), and *Aphanomyces euteiches* (24) were spread on potato dextrose agar (PDA) (5), and kanosamine was introduced into wells cut in the agar as previously described (28).

Among the strains tested, *P. medicaginis* M2913 was the most sensitive to kanosamine, whereas *Pythium aphanidermatum* Pa138 and *Pythium torulosum* A25a were less sensitive, and *A. euteiches* WI-98 showed an intermediate level of sensitivity (Table 1). The oomycetes were more sensitive to kanosamine at pH 7.0 than at pH 5.6.

Sensitivity of bacteria to kanosamine. We tested representative soil and plant root-associated bacteria for sensitivity to kanosamine. For sensitivity testing, most bacterial strains were grown in Mueller-Hinton broth, pH 7.3 (MH broth; Difco Laboratories). Exceptions included *Rhodospirillum rubrum*, which was grown in MH broth amended with 1 mg of biotin ml^{-1}; *Clostridium pasteurianum*, which was grown in MH broth amended with 20 mg of sucrose ml^{-1}; and *Rhizobium meliloti*, *Rhizobium tropici*, and *Lactobacillus acidophilus*, which were grown in L broth (19). The lowest antibiotic concentration that inhibited visible growth was determined by inoculating bacterial strains into broth medium containing 50 to 400 μg of kanosamine ml^{-1}. Test cultures, inoculated with approximately 5×10^5 CFU ml^{-1} from a fresh broth culture, were incubated at 28°C with shaking for 24 or 48 h until control cultures without kanosamine incubated under the same conditions were saturated.

TABLE 1. Sensitivity of oomycetes to kanosamine

Oomycete[a]	Minimum inhibitory amt (μg of kanosamine)[b]	
	pH 5.6	pH 7.0
Aphanomyces euteiches WI-98	60	40
Pythium aphanidermatum Pa138	250	NT[c]
Pythium torulosum A25a	250	150
Phytophthora medicaginis M2913	25	NT

[a] Zoospores of *A. euteiches* (2×10^4) (24), *P. medicaginis* (5×10^4), *P. aphanidermatum* (10^3), and *P. torulosum* (10^3) (27), enumerated microscopically with a hemacytometer, were spread onto PDA, pH 5.6 (5) or 7.0. The pH of PDA was adjusted to 7.0 with MOPS. A known amount of purified kanosamine (50 to 100 μl) was introduced into 8-mm wells cut into the agar, the plates were incubated at room temperature, and zones of inhibition were measured from the well to visible mycelial growth after 48 h. Data reported are representative of two independent experiments.

[b] The minimum inhibitory amount was defined as the lowest quantity of kanosamine that resulted in a zone of inhibition of at least 2 mm after 48 h of incubation.

[c] NT, not tested.

TABLE 2. Sensitivity of fungi to kanosamine

Fungal group, sp., and strain(s)	Growth inhibition (%)[a]
Ascomycetes	
Aspergillus flavus MP03	<30
Botrytis cinerea NRRL1684	<30
Candida utilus 1Y0-Y002	<30
Ophiostoma ulmi UWCC82	<30
Saccharomyces cerevisiae Y008	<30
Sclerotinia homoeocarpa KS20	<30
Sclerotinia sclerotiorum 91-26	<30
Venturia inaequalis UWCC365	>50
Basidiomycetes	
Rhizoctonia solani AG1, AG4	<30
Typhula incarnata SM93-34	<30
Ustilago maydis 521	>50
Deuteromycetes	
Alternaria alternata NRRL20593	30–50
Alternaria tagetica UWCC70	30–50
Colletotrichum phomoides UWCC37	<30
Colletotrichum trifolii SMM	<30
Cytospora cincta NRRL5185	<30
Drechslera poae KS58	30–50
Epicoccum nigrum NRRLA-10128	<30
Fusarium oxysporum UWCC62r1	<30
Fusarium solani 93.21	<30
Fusarium sporotrichioides CN-Z	30–50
Helminthosporium carbonum UWCC48	30–50
Helminthosporium sativum UWT84	30–50
Phomopsis obscurans UWCC95	30–50
Verticillium dahliae RNS87:1	<30
Verticillium albo-atrum Linden	<30

[a] For all tests, an 8-mm well was cut into the PDA (5 to 10 mm from the fungal plug for plates inoculated with plugs) into which 50 to 100 μl of purified kanosamine (200 μg) or sterile distilled water was placed (28). The plates were incubated at room temperature and scored for growth after 2 to 6 days. Growth of fungi on each test plate was compared with growth on a control plate that did not contain kanosamine (both PDA, pH 5.6). Results are representative of two independent experiments.

Kanosamine inhibited few bacterial species tested. Three *B. cereus* strains (UW85, 569, and BAR145) and representative strains from 15 additional bacterial species, including bacilli, pseudomonads, enterics, and rhizobia, were not sensitive to kanosamine at 400 μg ml^{-1}, which was the highest concentration tested. *L. acidophilus*, *Staphylococcus aureus*, and *Erwinia herbicola* LS005 were all inhibited by 400 μg of kanosamine ml^{-1}. *Cytophaga johnsonae*, which was inhibited by 300 μg of kanosamine ml^{-1}, was the most sensitive bacterium tested in this study. Kanosamine did not reduce the number of viable cells recovered from a culture of *C. johnsonae* (data not shown), which suggested that kanosamine is bacteriostatic at the concentrations tested. In previous studies, kanosamine also inhibited only a few bacterial species including *Diplococcus pneumoniae*, *Mycobacterium phlei*, and *S. aureus*, the latter by inhibition of cell wall synthesis (6, 17, 32, 37).

Sensitivity assays on certain strains conducted in MOPS (morpholinepropanesulfonic acid)-buffered MH broth, pH 8.0, indicated that kanosamine was more active against bacteria at the higher pH. *C. johnsonae* was inhibited by 50 μg of kanosamine ml^{-1} at pH 8.0. *Escherichia coli* K37 was inhibited by 300 μg of kanosamine ml^{-1} at pH 8.0, although it was not sensitive to 400 μg ml^{-1} at pH 7.3. This result was consistent with the observations for oomycetes (Table 1).

Sensitivity of fungi to kanosamine. We tested representa-

TABLE 3. Summary of medium designations and kanosamine accumulation

Medium designation[a]	Component(s)	Kanosamine accumulation[b]
TSB	Trypticase soy broth	±
TSB-Fe	TSB plus 0.5 mM $FeCl_3$	+
TSB-PO_4	TSB plus 100 mM K_2HPO_4	−
TSB-seedling exudate[c]	TSB plus alfalfa seedling exudate	±
MES-AA0	Unamended MES medium	NR
MES-AA1	MES-AA0 medium plus Thr, Val, Ser, Ala, Leu, and Asp	−
MES-AA3	MES-AA1 medium plus Glu, Arg, Ile, Met, Phe, and Trp	±
MES-CH	MES medium plus casein hydrolysate	±
MES-AA0-seedling exudate	MES-AA0 plus alfalfa seedling exudate	NR
MES-AA1-seedling exudate	MES-AA1 plus alfalfa seedling exudate	±
MES-AA3-seedling exudate	MES-AA3 plus alfalfa seedling exudate	+

[a] Media are described fully in the work of Milner et al. (23).

[b] Accumulation of kanosamine is indicated as follows: +, detectable accumulation; −, accumulation reduced by more than 80% in base medium with indicated addition; ±, accumulation was not consistently detected; NR, not relevant because *B. cereus* UW85 did not grow in the medium. For all media, assays were conducted in triplicate in at least three independent experiments. Quantitative data are reported in the text.

[c] Seedling exudate, designated sprout exudate in the work of Milner et al. (23), was diluted into *B. cereus* UW85 growth media to a final concentration equivalent to 50 mg of seed ml of culture medium^{-1}.

tives of three major groups of fungi, Ascomycetes, Basidiomycetes, and Deuteromycetes, for kanosamine sensitivity (Table 2). Most fungi were tested by inoculating PDA with a mycelial plug transferred from a stock plate. For *Candida utilus*, *Saccharomyces cerevisiae*, and *Ustilago maydis*, approximately 10^4 CFU were spread on PDA plates. For *Venturia inaequalis*, 2×10^5 conidia (34) were mixed with 25 ml of half-strength PDA and poured into a petri dish. Kanosamine was introduced into a well in the agar, and growth was scored after 2 to 6 days.

Kanosamine inhibited the growth of 1 of 8 ascomycetes, 1 of 3 basidiomycetes, and 7 of 15 deuteromycetes tested (Table 2). All of the fungi and oomycetes inhibited by kanosamine are pathogens of plants.

Influence of culture conditions on kanosamine accumulation. Triplicate cultures of *B. cereus* UW85 were grown in media defined in Table 3 and assayed for kanosamine accumulation after 4 days, unless otherwise indicated. Kanosamine was recovered from culture supernatant for quantitation with CM-Sep-Pak cartridges (Waters Chromatography Division, Millipore Corp.), a procedure in which kanosamine and zwittermicin A copurify (23). Kanosamine was quantified by high-voltage paper electrophoresis with the endpoint dilution assay described previously, a procedure that also separates kanosamine and zwittermicin A (23, 28). Unless otherwise indicated, the limit of kanosamine detection was 0.06 μg ml^{-1}. Within each experiment, there were no significant differences in the relative kanosamine accumulation among samples when the values were adjusted for dry cell weight determinations that were made for each culture (23). Therefore, we report kanosamine accumulation in units of micrograms per milliliter of original culture supernatant. Data were analyzed with the Statistical Analysis System software (SAS Institute, 1982). Table 3 provides a summary of descriptions of growth media and kanosamine accumulation.

To determine when kanosamine was produced during bacterial growth, we monitored accumulation of kanosamine, culture pH, cell density, and sporulation over 120 h in TSB or TSB-Fe inoculated with *B. cereus* UW85 (Table 3; Fig. 2). The pH of the supernatant increased from 7.4 at inoculation to 8.8 at 120 h after inoculation, and the maximum cell mass (3.7 ± 0.2 mg ml^{-1}) was reached at 48 h after inoculation. Unreleased spores were observed at 48 h after inoculation, and released spores predominated by 96 h after inoculation. In TSB, kanosamine accumulated to detectable levels by 24 h. From 72 to

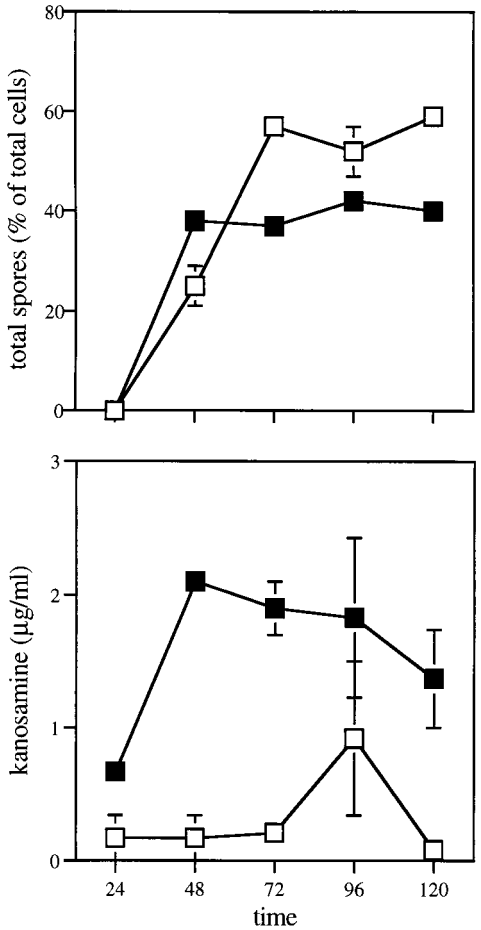

FIG. 2. Time course of kanosamine accumulation in TSB and TSB-Fe. *B. cereus* UW85 was grown in TSB (open squares) or TSB-Fe (closed squares). Error bars represent standard errors of the mean for three determinations. Where error bars are not visible, they are within the symbol. Kanosamine accumulation and the proportion of cells present as spores were measured at 24-h intervals as described in the text. The cell dry weight (micrograms per milliliter of culture) was measured at each time point (23) and was not significantly different between TSB- and TSB-Fe-grown cultures at a given time point.

120 h, the concentration did not fluctuate significantly from the concentration detected at 24 h. Thus, kanosamine was produced in culture prior to the detection of spores and before the maximum cell mass was reached. This observation is consistent with previous studies with *B. aminoglucosidicus* in which the maximum concentration of kanosamine accumulated by 48 h after inoculation in a soybean meal-based rich medium (36, 37).

Iron supplementation of fermentation media stimulates the production of many antibiotics (20, 22, 23, 29, 39). Likewise, kanosamine accumulation was strongly influenced by iron in the culture medium (Fig. 2). In TSB supplemented with 0.5 mM ferric iron (TSB-Fe), kanosamine accumulated to 2 μg ml^{-1} at 48 h after inoculation, which was 12-fold higher than the accumulation in TSB at the same point during growth (Fig. 2). Enhanced kanosamine accumulation was observed when 0.25 to 1.0 mM $FeCl_3$ ferric iron was added to TSB (data not shown). The enhanced kanosamine accumulation was specific to the ferric ion because the addition of 1.0 mM of another chloride salt, NaCl, had no effect on kanosamine accumulation in TSB (data not shown).

We observed that phosphate, a potent inhibitor of secondary metabolite production (20–23, 29, 39), inhibited kanosamine production. Kanosamine accumulated to concentrations 4.4-fold higher in TSB (2.2 ± 0.5 μg of kanosamine ml^{-1}) than in TSB supplemented with potassium phosphate at concentrations of 100 mM (0.4 ± 0.02 μg of kanosamine ml^{-1}). A similar effect was observed when TSB was supplemented with 50 mM K_2HPO_4 (TSB-PO_4). To determine whether the addition of other salts inhibited accumulation, we substituted 100 mM KCl or NaCl for K_2HPO_4. Accumulation of kanosamine was not affected by either chloride salt, which demonstrates that the suppression of accumulation was specific to phosphate and not to potassium or to increased salt concentrations.

Kanosamine accumulation was significantly higher in TSB supplemented with manganese (0.9 μM $MnSO_4$) than in TSB (1.3 ± 0.3 μg of kanosamine ml^{-1} compared with 0.7 ± 0.1 μg of kanosamine ml^{-1}, respectively). Supplementation of TSB with four other inorganic micronutrients at micromolar concentrations (boron, copper, molybdenum, or zinc) did not alter kanosamine accumulation by *B. cereus* UW85 (data not shown).

To identify minimal nutritional requirements for kanosamine accumulation, we grew *B. cereus* UW85 in a previously described MES (morpholineethanesulfonic acid)-based defined minimal medium (23) (Table 3). Kanosamine accumulated to similar concentrations when malate (1.71 ± 0.44 μg of kanosamine ml^{-1}) or glucose (1.67 ± 0.05 μg of kanosamine ml^{-1}) was supplied as the carbon source in MES-AA3 medium. Several other carbon sources (sucrose, maltose, acetate, and propionate) supported significantly less kanosamine accumulation. Thus, malate was routinely supplied as the carbon source in MES media. We tested all combinations of amino acids to define the minimum combination required for growth and antibiotic production. Six amino acids were sufficient to support growth of *B. cereus* UW85 (threonine, valine, serine, alanine, leucine, and aspartate), but they did not support kanosamine accumulation (<0.2 μg of kanosamine ml^{-1}, MES-AA1). However, kanosamine accumulated to 0.9 ± 0.1 μg ml^{-1} when *B. cereus* UW85 was grown in MES medium supplemented with six additional amino acids (MES-AA3 medium): glutamine, arginine, isoleucine, methionine, phenylalanine, and tryptophan. This level of accumulation was comparable to accumulation in TSB (Fig. 2). Kanosamine did not accumulate to detectable levels (<0.06 μg of kanosamine ml^{-1}) in MES-casein hydrolysate medium, perhaps because the tryptophan component of casein hydrolysate, which is heat labile, was destroyed during sterilization of the medium by autoclaving. MES-AA3 was filter sterilized to avoid this problem (23). We also observed that kanosamine was not consistently produced in MES-AA3: it was detected in five of eight independent experiments.

Kanosamine production is correlated with the ability of *B. cereus* UW85, mutants of UW85, and other *B. cereus* isolates to suppress a seedling disease of alfalfa (28, 31). Therefore, we examined the possibility that plant-derived factors influence production of compounds associated with disease suppression by *B. cereus*. Alfalfa seedling exudate (23) added to TSB, a rich medium, had no effect on kanosamine accumulation. However, accumulation was enhanced 3.8-fold in MES-AA3 supplemented with seedling exudate (0.45 ± 0.03 μg of kanosamine ml^{-1}) compared with MES-AA3 (0.12 ± 0.02 μg of kanosamine ml^{-1}). MES-AA3–seedling exudate was the only MES-based medium that we tested in which kanosamine was reproducibly accumulated in three separate experiments. This result suggests that an alfalfa seedling-derived factor(s) enhances kanosamine production by a mechanism that is not understood.

Conclusions. Our previous work showed that diverse *B. cereus* strains isolated from soil samples from five continents produce antibiotic B and that antibiotic B contributes to suppression of plant disease by *B. cereus* UW85 as demonstrated by biochemical and genetic analyses (15, 28, 31). Here we show that antibiotic B is kanosamine. Therefore, kanosamine-producing bacteria are much more widespread than indicated by previous reports in which three kanosamine-producing bacterial strains were described (6, 8, 38), and kanosamine is a key factor in biological control of plant disease.

In culture, antibiotic production by many organisms is influenced by carbon and nitrogen sources, inorganic compounds, and growth phase (1, 3, 7, 11, 18, 20–23, 29, 39). Soil and plant-derived factors may have profound effects on the production of antimicrobial compounds during growth by root-associated bacteria (4, 9, 33). Kanosamine production by *B. cereus* UW85 was suppressed by phosphate, enhanced by ferric iron, and enhanced by alfalfa seedling exudate. However, *B. cereus* UW85 produced, at most, a thousandfold less kanosamine than *B. aminoglucosidicus* (36, 37) under our test conditions. Identification of organic and inorganic factors that influence kanosamine accumulation suggest approaches to enhancing disease suppression through regulating antibiotic production by soil amendments or breeding host plants to supply an environment that is conducive to antibiotic production.

Kanosamine was highly inhibitory to plant-pathogenic oomycetes. Oomycetes have two forms of asexual reproduction: mycelial growth, which morphologically resembles fungi, and motile zoospores, which morphologically resemble protists. Although oomycetes have traditionally been classified as fungi, modern phylogenetic analyses suggest that they are more appropriately classified with the protists (2, 12). Thus, kanosamine may be useful in the study of the biology of this poorly understood group of organisms and in the control of detrimental plant-associated and animal-associated protists.

We found that kanosamine production is regulated by plant factors and a broad range of soilborne oomycetes and fungi are sensitive to kanosamine. These findings will contribute to our understanding of the mechanism of suppression of plant disease by strains of *B. cereus* and the ecology of *B. cereus* and the microbial community on roots and in the soil.

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