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Target Range of Zwittermicin A, an Aminopolyol Antibiotic from *Bacillus cereus*

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Abstract. Zwittermicin A is a novel antibiotic produced by *Bacillus cereus* UW85, which suppresses certain plant diseases in the laboratory and in the field. We developed a rapid method for large-scale purification of zwittermicin A and then studied the in vitro activity of zwittermicin A against bacteria, fungi, and protists. Zwittermicin A was highly active against the Oomycetes and their relatives, the algal protists, and had moderate activity against diverse Gram-negative bacteria and certain Gram-positive bacteria as well as against a wide range of plant pathogenic fungi. Zwittermicin A was more active against bacteria and fungi at pH 7–8 than at pH 5–6. When zwittermicin A was combined with kanosamine, another antibiotic produced by *B. cereus*, the two acted synergistically against *Escherichia coli* and additively against *Phytophthora medicaginis*, an Oomycete. The results indicate that there are diverse potential applications of this new class of antibiotic.

Discovery of new antibiotic-producing organisms will contribute to dealing with the challenges that confront medicine and agriculture. To maintain and improve the health of the human population, new drugs will be needed to manage the major human pathogens that have developed resistance to the antibiotics that have controlled them in the past [15]. Likewise, to maintain the quality of the food supply, we need to develop improved measures for control of crop diseases to replace fungicides that are currently in widespread use but are likely to be restricted in the future owing to safety concerns and the development of resistance in the pathogen populations [5, 7]. Thus, research is needed to find and characterize new antimicrobial agents for controlling infectious disease of plants and animals.

Bacillus cereus strain UW85 accumulates two antibiotics, zwittermicin A and kanosamine, in its culture supernatant. Zwittermicin A is a novel, linear aminopolyol (Fig. 1) and represents a new class of antibiotic [14]. Zwittermicin A contributes to the ability of UW85 to suppress alfalfa damping-off [28] and may be important for other biological activities of UW85, such as the control of fruit rot of cucumber [29] or the suppression of other plant diseases [12, 13, 22, 23] in the lab and in the field.

We are unable to make predictions about the target range or mode of action of zwittermicin A on the basis of its structure because it is structurally different from known antibiotics (Fig. 1). Knowledge of the target range of zwittermicin A may suggest productive avenues for research on, and application of, UW85 for biological control of plant diseases or as a producer of useful antibiotics, and may suggest an appropriate model system in which to study the mode of action of zwittermicin A in the target cell. Here, we report the in vitro activity of zwittermicin A against various bacteria, fungi, and protists.

Materials and Methods

Purification of zwittermicin A. Zwittermicin A was purified either by the method reported earlier [28] or by an HPLC-based method that provided more efficient purification. The zwittermicin A obtained by the two methods had the same ^{1}H-NMR profile and the same specific activity against *Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Erwinia herbicola*, *Salmonella typhimurium*, *Bacillus cereus*, and *Escherichia coli*. Yields of zwittermicin A were 2–4 mg/L of culture in

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the purification by paper electrophoresis [14] and 9–18 mg/L by the HPLC method. Yield was estimated by the endpoint dilution method [28], comparing the zwittermicin A present after purification to the amount present in the filtrate. Losses at each step were too small to quantify; however, by the final step the yield of activity of pure material was 50–75% of the initial culture filtrate activity.

In the HPLC-based method, zwittermicin A was purified from supernatants of sporulated cultures of *B. cereus* strain UW85 grown in ½-strength tryptic soy broth (TSB). In some experiments, ½-strength TSB was supplemented with 0.5 mM $FeCl_3$ to increase zwittermicin A yield [18]. Cultures were grown in 30-L fermenters at the University of Wisconsin Pilot Plant (Madison, WI), and supernatants were brought to neutral pH with the addition of HCl. Purification was a three-step process, and at each step the fraction(s) containing zwittermicin A were identified by high voltage paper electrophoresis at pH 9.2 and staining with silver nitrate [28].

In the first purification step [28], a column (5 cm diameter × 42 cm length) packed with amberlite IRC-50 cation exchange resin (Biorad, Hercules, CA) was equilibrated with 4 L of 5.0 mM $NH_4H_2PO_4$ (pH 7.0), the 30 L of culture supernatant was loaded on the column, the column was washed with 5 L of 5.0 mM $NH_4H_2PO_4$ (pH 7.0) and eluted with 1 M NH_4OH (pH 11.2); 200-ml fractions were collected and placed in a rotary evaporator until the pH was less than 8.0. Fractions containing zwittermicin A were combined and dried in a rotary evaporator at 45°C and resuspended in 60 ml distilled H_2O.

In the second step, a second column packed with amberlite IRC-50 cation exchange resin (2 cm diameter × 17 cm length) was equilibrated with 3 L of 10 mM ammonium acetate, loaded with the equivalent of 5 L of initial sample, and eluted with 375 ml ammonium acetate (pH 8.6), 375 ml ammonium acetate (pH 8.8), 1.5 L ammonium acetate (pH 9.0), 1.5 L ammonium acetate (pH 9.1), 1.5 L ammonium acetate (pH 9.3), 1.5 L ammonium acetate (pH 9.5), and 500 ml 1 M NH_4OH. Nine 750-ml fractions were collected, and the final 500 ml of eluate was collected as a tenth fraction. Each fraction was placed in a rotary evaporator until its pH was less than 8.0. If zwittermicin A was present in the last fraction, it was concentrated in a rotary evaporator at 45°C. Earlier fractions containing zwittermicin A were combined and concentrated by re-equilibrating the column with 500 ml 5 mM $NH_4H_2PO_4$, loading the zwittermicin A-containing fractions, eluting with 500 ml of 1 M NH_4OH, and concentrating as above. The combined zwittermicin A-containing samples were resuspended in 1 ml of water in a 1.5-ml microfuge tube and centrifuged to remove insoluble debris.

In the third step, the equivalent of 1 L of starting material was injected into a Beckman Model 332 Gradient Liquid Chromatograph System with a 10 mm × 25 cm Beckman Ultrasphere Cyano bonded-phase column (Beckman Instruments Inc., Fullerton CA). The mobile phase had a flow rate of 2 ml/min and consisted of water for the first 5 min, a gradient from 0 to 20 mM ammonium acetate established over the next 20 min, and 20 mM ammonium acetate for 25 min. Four-ml fractions were collected, and those containing zwittermicin A were concentrated in a Speed Vac Concentrator (Savant Instruments Inc., Farmington, NY), resuspended in water, centrifuged and filtered to remove any insoluble debris, dried, and weighed to determine yield.

Sensitivity testing of Oomycetes and protists. Susceptibility of Oomycetes to zwittermicin A was tested on potato dextrose agar (PDA) [8] at pH 5.6 and PDA buffered with 3-(n-morpholino) propanesulfonic acid (MOPS) to pH 7.0. *Phytophthora medicaginis* zoospores were prepared as described previously [28], *Pythium* spp. zoospores were prepared according to Rahimian and Banihashemi [25], and *Aphanomyces euteiches* zoospores were prepared by the method of Mitchell and Yang [21]. Conidia of *Venturia inaequalis* were prepared by the method of Tuite [34]. Zoospores were enumerated microscopically with a hemacytometer. Zoospores of *Aphanomyces euteiches* (2×10^4), *Phytophthora medicaginis* (5×10^4), *Pythium aphanidermatum* (1×10^3), and *Pythium torulosum* (1×10^3) were spread on PDA plates. A well was made in the center of the agar with a sterilized cork borer, purified antibiotic was placed in the well, and the plates were incubated at room temperature for 48 h. Zones of inhibition were measured from the well to visible mycelial growth. Minimal inhibitory concentrations (MICs) in this assay were defined as the lowest antibiotic concentration that resulted in a zone of inhibition.

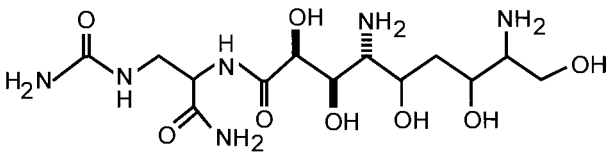

Fig. 1. Structure of zwittermicin A.

The protists *Ochromonas danica* and *Poterioochromonas malhamensis* were acquired from the Culture Collection of Algae at the University of Texas-Austin. Susceptibility of the protists to zwittermicin A was tested by the following method based on Thiemann and Beretta [33]. Cultures were grown in 100 ml of Ochromonas Medium (1.0 g glucose, 1.0 g tryptone, 1.0 g yeast extract, 40.0 ml liver extract infusion, 960 ml distilled H_2O) at room temperature (20–22°C) until they reached a density of 1×10^6 cells/ml. The 100-ml culture was then added to 100 ml of cooled Ochromonas Medium containing 0.4 g agar and poured into sterile petri dishes. A filter disk containing purified zwittermicin A was placed in the center of the agar plate, incubated at room temperature, and scored for zones of inhibition after 6 days.

Antibiotic was applied to the disks at 25 µg to 200 µg at twofold increasing concentrations. Organisms that were sensitive to 25 µg were retested at 1, 5, and 10 µg zwittermicin A.

Sensitivity testing of fungi. Unless otherwise indicated, fungi were tested for antibiotic susceptibility as follows: a plug of mycelia, produced with a cork borer, was placed in the center of a PDA plate. A well was cut into the agar 5–10 mm from the plug. Purified antibiotic (200 µg) or sterile distilled water was placed in the well, and the plates were incubated at room temperature. The plates were scored for growth after 2–6 days by measuring the distance of growth from the plug toward the well and by comparing the antibiotic-treated samples with the samples that contained sterile distilled water.

Candida utilus, *Saccharomyces cerevisiae*, and *Ustilago maydis* were tested on PDA plates as described for the Oomycetes above, and approximately 1×10^4 CFU were spread on the PDA plates. *Venturia inaequalis* was tested by mixing 2×10^5 conidia into 25 ml of ½-strength PDA. The agar was vortexed briefly and then poured into a petri plate. A well was made in the agar for the placement of antibiotic, and the plates were incubated at room temperature for 72 h.

Inhibition of growth was determined by visual examination. A "+" indicates that growth of the fungus from the plug toward the well with zwittermicin A in the well was less than 50% of the growth of the fungus on the plate with water in the well, or that a zone of inhibition developed around the well. A "±" indicates that growth was 50–70% of the water control, and a "−" indicates that growth was 70–100% of the control.

Sensitivity testing of bacteria. Sensitivity testing of *Rhizobium meliloti*, *R. tropici*, and *Lactobacillus acidophilus* was conducted in L-broth [17]. All other bacterial strains were tested in Mueller-Hinton (MH) broth (Sigma Chemicals, St. Louis, MO) at pH 7.3 and MH broth buffered with MOPs to pH 8.0. *Rhodospirillum rubrum* was grown in MH broth amended with 1 µg/ml biotin, and *Clostridium pasteurianum* was grown in MH broth amended with 20 µg/ml sucrose.

Minimal inhibitory concentrations (MICs) of the antibiotic were

Table 1. In vitro activity of zwittermicin A against protists

	Zwittermicin A MIC (μg/well or filter disk)[a]
Oomycetes tested[b]	
Aphanomyces euteiches WI-98	4
Phytophthora medicaginis M2913	1
Pythium aphanidermatum PAL38	40
Pythium torulosum A25a	80
Chrysophytes tested[c]	
Ochromonas danica	25
Poterioochromonas malhamensis	25

[a] MIC indicates the minimum inhibitory concentration of antibiotic required to produce a zone of inhibition on agar plates.
[b] Oomycetes were tested for sensitivity to zwittermicin A on potato dextrose agar plates at pH 5.6. The data are representative of two independent experiments.
[c] Chrysophytes were tested for sensitivity to zwittermicin A on Ochromonas medium soft-agar plates. The data are representative of two independent experiments.

determined by inoculating bacterial strains into broth medium containing various concentrations of the antibiotic. Bacterial inocula were prepared from fresh broth cultures and diluted to provide inoculum concentrations of approximately 5×10^5 CFU/ml. Bacteria were enumerated in the culture used for inocula by dilution plating on MH agar plates, which were incubated at 28°C for 1–4 days. Antibiotic was added in twofold increasing concentrations, ranging from 50 μg/ml to 400 μg/ml, and each test tube contained 1 ml of MH broth. All cultures were incubated at 28°C with shaking for 24 h, except for *Lactobacillus acidophilus*, *Streptomyces griseus*, *Rhizobium meliloti*, *R. tropici*, *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum*, which were incubated for 48 h. *Clostridium pasteurianum* was tested under anaerobic conditions by overlaying the culture with 3 ml of sterile mineral oil and then growing the culture for 4 days at room temperature. The MIC was defined as the lowest antibiotic concentration that prevented visible growth. All MICs were determined at least three times and did not vary with the various preparations of the antibiotics made during the course of this work, although slight differences in MICs were observed between these experiments and previous work [19]. Bacteria that were inhibited by 50 μg/ml for zwittermicin A were retested at concentrations between 10 and 50 μg/ml antibiotic, in increasing 10-μg increments. Minimal bactericidal concentrations (MBCs) were determined for each bacterial strain by spreading 0.1 ml from each test culture without visible growth on MH agar plates containing no antibiotics. The plates were scored for bacterial growth after incubation at 28°C for 24–48 h. MBCs were defined as the lowest concentration of antibiotic that resulted in no growth when the treated culture was spread on antibiotic-free agar plates.

Results

Rapid method for purification of zwittermicin A. To facilitate further study of zwittermicin A, we needed a rapid method for producing the antibiotic, and thus developed a large-scale purification method based on ion-exchange chromatography and HPLC. The method yielded 9–18 mg of zwittermicin A from each liter of culture, in contrast with the previously published method, which led to recovery of 2–4 mg/L.

Sensitivity of Oomycetes and protists to zwittermicin A. The Oomycetes are zoosporic water molds long thought to be fungi [1], but now known to be more closely related to the algal protists [2, 9]. The Oomycetes *Phytophthora* and *Aphanomyces* were most sensitive to zwittermicin A, and the Chrysophytes (golden-brown algae), *Ochromonas danica* and *Poterioochromonas malhamensis*, were sensitive to zwittermicin A at similar concentrations (Table 1).

Sensitivity of fungi to zwittermicin A. We tested zwittermicin A against fungi representative of the Ascomycetes, Basidiomycetes, and Deuteromycetes, the three major groups of true fungi. Zwittermicin A strongly inhibited many, but not all, of the fungi in all groups at 200 μg/ml (Table 2).

Sensitivity of bacteria to zwittermicin A. Zwittermicin A inhibited four members of the enterobacteriaceae, two phototrophic bacteria, and two members of the rhizobiaceae at a concentration of 100 μg/ml or less (Table 3). Zwittermicin A was generally less inhibitory to Gram-positive than to Gram-negative bacteria, although strains of *B. cereus* that do not produce zwittermicin A are generally sensitive to it (Table 3 and [24]).

To determine whether zwittermicin A was bacteristatic or bactericidal, we tested the cultures for growth after removal of the antibiotic. Increasing concentrations of zwittermicin A tended to decrease the number of viable cells that could be recovered from a culture (data not shown), but only seven strains had minimal bactericidal concentrations (MBCs) within the concentrations of antibiotic tested (Table 3). The MBCs were generally two- to fivefold greater than the MIC for each strain except in the cases of *Bradyrhizobium japonicum* and *Rhizobium meliloti*, for which the MIC and MBC for zwittermicin A were the same.

Effect of pH on zwittermicin A activity. Zwittermicin A was more active at higher pH than at the lower pH against bacteria and fungi. At pH 7.3, the MIC for zwittermicin A against *E. coli* was 100 μg/ml and at pH 8.0 it was 40 μg/ml, and similarly, a twofold lower concentration of antibiotic was sufficient to inhibit the Oomycetes at the higher pH than at the lower pH (Table 4).

Activity of zwittermicin A with kanosamine. *Bacillus cereus* UW85 produces kanosamine, an aminoglycoside antibiotic, as well as zwittermicin A. To determine the effect of zwittermicin A in the presence of kanosamine, we tested the antibiotics together. Activities are defined as

Table 2. Activity of zwittermicin A against fungi

	Causal agent of	Inhibition by ZmA[a]
Alternaria alternata NRRL20593	Leaf blight on beet	+
Alternaria panax 1268	Leaf spot of ginseng	+
Alternaria tagetica UWCC70	Leaf and petal blight	+
Aspergillus flavus MP03	Nonpathogenic	−
Botrytis cinerea NRRL1684	Molds and rots of stored fruits and vegetables	+
Candida utilus 1 Y0-Y002	Nonpathogenic	+
Colletotrichum phomoides UWCC37	Anthracnose of tomato	+/−
Colletotrichum trifolii SMM	Anthracnose of alfalfa	+
Cytospora cincta NRRL5185	Branch canker of fruit trees	+
Drechslera poae KS58	Leaf spot/foot rot of grasses	+
Epicoccum nigrum NRRLA-10128	Leaf spot of magnolia	+
Fusarium graminaerum	Corn root rot, stalk rot, ear rot	+
Fusarium oxysporum UWCC62r1	Vascular wilt of tomato	−
Fusarium solani 93.21	Root rot of bean	+
Fusarium solani Cora 7		+
Fusarium solani Mont.1		+
Fusarium solani T8		+
Fusarium sporotrichioides CN-Z	Blight of barley/sunflower	+
Helminthosporium carbonum UWCC48	Leaf spot of corn	+
Helminthosporium sativum UWT84	Foot rot of grasses	+
Monilinia oxycocci	Cottonball of cranberry	+
Ophiostoma ulmi UWCC82	Dutch elm disease	+/−
Phomopsis obscurans UWCC95	Leaf blight of strawberry	+
Rhizoctonia solani (AG1, AG4)	Root rot of fruits/vegetables	+
Saccharomyces cerevisiae Y008	Nonpathogenic	−
Sclerotinia homoeocarpa KS20	Dollar spot of turf	−
Sclerotinia sclerotiorum 91-26	Rots of most crops	+
Septoria musiva	Leaf spot of poplar	+
Typhula incarnata SM93-34	Snowmold of turf/grasses	−
Ustilago maydis 521	Common smut of corn	+
Ustilago maydis UM002	Common smut of corn	+
Venturia inaequalis UWCC365	Scab of apple	+
Verticillium albo-atrum Linden	Wilt of alfalfa	+/−
Verticillium dahliae RNS87:1	Wilt of potato	+/−

[a] Fungi were tested for sensitivity to 200 µg/well of zwittermicin A (ZmA) on potato dextrose agar plates at pH 5.6. The data are representative of two independent experiments. Growth of fungi on each test plate was compared with growth on a control plate that did not contain antibiotic. % inhibition was determined by visual assessment. "+" indicates that growth was less than 50% of the water control or that a zone of inhibition developed around the well. "+/−" indicates that growth was 50–70% of the water control, and "−" indicates that growth was 70–100% of the control.

Table 3. Activity of zwittermicin A against bacteria

	MIC (µg/ml)[a]	MBC (µg/ml)[b]
Gram-negative bacteria:		
Agrobacterium tumefaciens A759	40	>400
Bradyrhizobium japonicum USDA 110	100	100
Cytophaga johnsonae 9408	>400	>400
Erwinia carotovora 8064	40	100
Erwinia herbicola IRQ	>400	>400
Erwinia herbicola LS005	50	200
Escherichia coli K37	100	400
Klebsiella pneumoniae 8030	200	>400
Pseudomonas aeruginosa 9020	>400	>400
Pseudomonas fluorescens 9023	>400	>400
Rhizobium meliloti 1021	50	50
Rhizobium tropici CIAT 899	100	200
Rhodobacter sphaeroides 9502	50	100
Rhodospirillum rubrum 9405	50	>400
Salmonella typhimurium LT2	100	>400
Vibrio cholerae F115A	400	>400
Yersinia pseudotuberculosis	100	NT
Gram-positive bacteria:		
Bacillus megaterium	100	NT
Bacillus cereus 569	>400	>400
Bacillus cereus UW85	>400	>400
Bacillus cereus BAR145	>400	>400
Bacillus cereus SN14	>400	>400
Bacillus subtilis 168	>400	>400
Bacillus thuringiensis 4A9	>400	>400
Bacillus thuringiensis 4D6	>400	>400
Clostridium pasteurianum 5002	>400	>400
Lactobacillus acidophilus 4003	100	>400
Staphylococcus aureus 3001	200	>400
Streptomyces griseus 6501	400	>400

[a] Bacteria were tested for sensitivity to zwittermicin A in Mueller-Hinton medium at pH 7.3 or in L-broth. The data are representative of two independent experiments. MIC indicates the minimum inhibitory concentration of antibiotic that prevented visible growth.
[b] MBC indicates the minimum bactericidal concentration that results in no growth when the treated culture was spread on agar plates.

synergistic if the activity of the antibiotics in combination is greater than the sum of activities of the antibiotics alone [4]. To describe the interaction, we present the data as an isobol, plotting the concentrations of the two antibiotics on either axis and connecting the points that represent the MIC for each combination. A concave isobol represents a synergistic interaction, a convex isobol represents an antagonistic interaction, and a straight line indicates an additive effect. The isobol obtained for the combined activity of zwittermicin A and kanosamine against *E. coli* is somewhat concave (Fig. 2a), suggesting that the antibiotics are weakly synergistic. In contrast, the combined activity of zwittermicin A and kanosamine against *P. medicaginis* on PDA plates produced a straight line isobol, indicating that the antibiotics against *P. medicaginis* have an additive effect (Fig. 2b).

Table 4. Effect of pH on the activity of zwittermicin A against bacteria and Oomycetes

	MIC (μg/ml) of zwittermicin A[a]			
	pH 7.3	pH 8.0	pH 5.6	pH 7.0
E. coli	100	40	nt	nt
Vibrio cholerae	400	150	nt	nt
Pythium torulosum	nt	nt	80	40
Aphanomyces euteiches	nt	nt	4	2

Bacteria were tested for sensitivity to zwittermicin A in Mueller-Hinton media at pH 7.3 or pH 8.0. Zoospores were tested for sensitivity to zwittermicin A on potato dextrose agar plates at pH 5.6 and pH 7.0. Data are representative of two independent experiments.

[a] MIC indicates the minimum inhibitory concentration of antibiotic required to prevent visible growth of the bacteria in Mueller-Hinton broth or to produce a zone of inhibition on agar plates containing the Oomycetes.

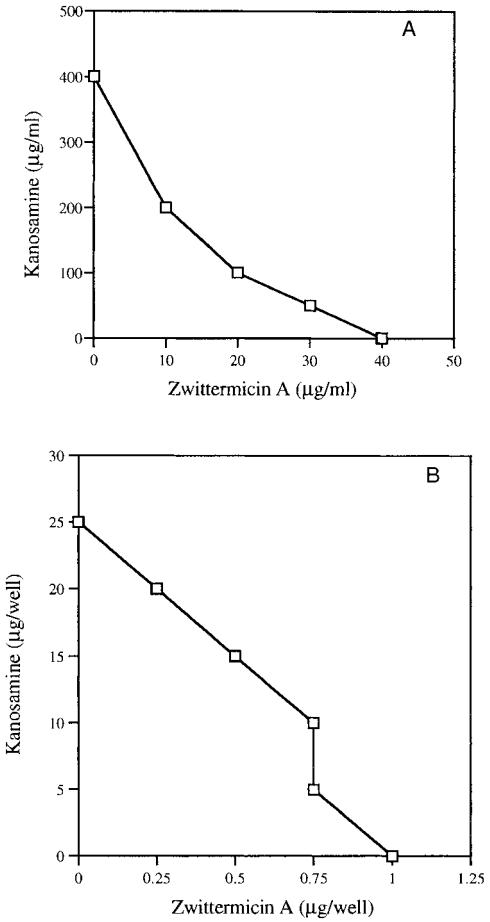

Fig. 2. (A) Combined activity of zwittermicin A and kanosamine against *E. coli*. *E. coli* was tested for sensitivity to zwittermicin A and kanosamine, individually or in combination, in Mueller-Hinton medium at pH 8.0. Under these conditions, *E. coli* was inhibited by kanosamine at 400 μg/ml [28]. Data points represent the concentrations of the antibiotics in combinations that inhibited growth of *E. coli*. Growth of *E. coli* strain K37 was scored as visible (+) or no visible growth (−). (B) Combined activity of zwittermicin A and kanosamine against *P. medicaginis*. *P. medicaginis* was tested for sensitivity to zwittermicin A and kanosamine, individually or in combination, on potato dextrose agar plates at pH 5.6. *P. medicaginas* was inhibited by kanosamine at 25 μg/well. Data points represent the concentrations of the antibiotics in combinations that inhibited growth of *P. medicaginis*. The data are representative of two independent experiments.

Discussion

Zwittermicin A inhibits diverse protists, Oomycetes, fungi, and bacteria, is more active at higher pH than lower, and acts synergistically with kanosamine against *E. coli* and additively with kanosamine against *Phytophthora*, an Oomycete. The broad target range of zwittermicin A suggests that bacteria that produce zwittermicin A, such as *B. cereus* UW85, might be useful for control of a wide range of foliar and soilborne plant diseases.

We identified several organisms that may be useful in future investigations into the activities of the antibiotics. The high sensitivity of *E. coli* to zwittermicin A on Mueller-Hinton medium at pH 8.0 and the powerful genetic techniques available for *E. coli* will be useful in the identification of genes for resistance to zwittermicin A and study of its mode of action [19, 20, 30, 31].

The target range and certain structural features of zwittermicin A are similar to chitosan, which is the deacetylated form of chitin and the polymeric form of glucosamine. Both chitosan and zwittermicin A are polycations. Chitosan and zwittermicin A are most active against the Oomycetes [3], both have antibacterial activity [32], and both can be phytotoxic at high concentrations [27, 35 and data not shown]. The biological activities of chitosan, such as the disruption of cell walls [16, 32], inhibition of RNA synthesis in fungi [11], binding of DNA [10], and induction of a host resistance response in plants [10] are due to the polycationic nature of chitosan.

The inhibitory activity of zwittermicin A against the lower eukaryotes, such as Oomyctes and Chrysophytes, suggests that zwittermicin A and other aminopolyol antibiotics may have application against the protist pathogens of humans. These organisms, including *Trichomonas* and *Giardia* [6, 26], are of growing significance in both basic research and human health. Further investigation should address the relationship between the structure of zwittermicin A and its broad-spectrum activity, the identity of other aminopolyol antibiotics, and whether this group of antibiotics has application in managing human infectious disease.

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