

## Synergy Between Zwittermicin A and *Bacillus thuringiensis* subsp. *kurstaki* Against Gypsy Moth (Lepidoptera: Lymantriidae)

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**ABSTRACT** *Bacillus cereus* French & French increased the mortality of 3rd-instar gypsy moths, *Lymantria dispar* (L.), caused by *Bacillus thuringiensis* subsp. *kurstaki* Berliner. *B. cereus* did not cause mortality of *L. dispar* when applied alone. The activity of various *B. cereus* strains was correlated positively with their accumulation of zwittermicin A, an aminopolyol antibiotic, in culture. When a constant dose of *B. thuringiensis* subsp. *kurstaki* and increasing concentrations of purified zwittermicin A were applied to artificial diet, mortality of larvae was directly proportional to the dose of zwittermicin A. Zwittermicin A by itself caused no mortality at the concentrations tested. Addition of zwittermicin A to a culture of a mutant strain of *B. cereus* that does not accumulate zwittermicin A restored synergistic activity. These results indicate synergy between *B. thuringiensis* subsp. *kurstaki* and zwittermicin A. The potential for enhancing efficacy of *B. thuringiensis* and delaying development of insect resistance are discussed.

**KEY WORDS** *Bacillus thuringiensis*, *Bacillus cereus*, zwittermicin A, *Lymantria dispar*, gypsy moth, synergism

BIOPESTICIDES ARE AN important component of pest management because they usually have fewer non-target effects and lower human toxicity than synthetic chemical pesticides. The most widely used biopesticide is *Bacillus thuringiensis* Berliner, a bacterium that kills insect larvae largely through the action of a protein known as crystal delta-endotoxin (Garczynski et al. 1991, Dubois and Dean 1995). Despite its ability to control insect populations and its general environmental safety, *B. thuringiensis* has several limitations. For example, efficacy varies considerably among insect species and strains of the same insect species (Heimpel and Angus 1959; Ramachandran et al. 1993a, b; van Frankenhuyzen et al. 1995), the residual period of efficacy is relatively brief, and performance varies considerably among similar insects feeding on various plant species (Appel and Schultz 1994, Farrar et al. 1996). As with all control measures, evolution of resistant insect biotypes can diminish efficacy (Tabashnik 1994, Moar et al. 1995, McGaughey et al. 1998).

The use of synergists has been proposed as one strategy to enhance the efficacy of *B. thuringiensis* by reducing the quantity needed to obtain control and lengthening residual activity (Dubois et al. 1989, Dubois and Dean 1995, Liu and Tabashnik 1997). The term synergist has acquired several usages: here we employ the convention of Walker et al. (1996) in

which the term refers to materials with no activity when applied alone that enhance the effects of active compounds. Synergists can also help delay the onset of resistant insect biotypes by compounding the evolutionary, physiological, and genetic barriers that an insect must overcome (Georghiou and Saito 1983, Raffa and Priester 1985, Brattsten et al. 1986).

Recent studies have identified a number of materials that increase the activity of *B. thuringiensis*. For example, crystal proteins together with spores of *B. thuringiensis* and spores and vegetative cells of several forest bacteria increase the mortality of gypsy moth, *Lymantria dispar* (L.), larvae (Dubois and Dean 1995). *B. thuringiensis* toxins cryIA(a) and cryIA(c) act synergistically on gypsy moth (Lee et al. 1995), and *B. thuringiensis* spores enhance activity of protoxin against diamondback moth (Tang et al. 1995). Moreover, combined use of *B. thuringiensis* spores and toxins increased mortality in diamondback moth populations that are resistant to the toxins alone (Liu et al. 1998).

Previous results indicate that certain strains of *B. thuringiensis* produce a compound that potentiates *B. thuringiensis* activity (Manker et al. 1994). Similarly, closely related *Bacillus cereus* (French & French) strains produce an antibiotic, zwittermicin A (Fig. 1) (He et al. 1994, Stabb et al. 1994), which is similar in structure to the molecule described as a potentiator of *B. thuringiensis* (Manker et al. 1994). Zwittermicin A is a linear aminopolyol (He et al. 1994, Silo-Suh et al. 1998) that inhibits the growth of a variety of gram-positive and gram-negative eubacteria as well as cer-

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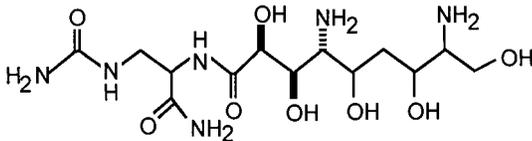


Fig. 1. Structure of zwittermicin A (He et al. 1994).

tain ascomycete and basidiomycete fungi (Silo-Suh et al. 1998). Zwittermicin A also suppresses alfalfa, *Medicago sativa* L., seedling disease caused by *Phytophthora medicaginis* (Drechs.), an oomycete pathogen, apparently by inhibiting the elongation of germ tubes from *P. medicaginis* (Silo-Suh et al. 1994).

The gypsy moth is the most damaging defoliator of deciduous trees in the eastern United States (Doane and McManus 1981, McFadden and McManus 1991). Outbreaks cause severe losses to forests and affect tree growth and survival, soil and water quality, wildlife habitat, and recreational value. Additional losses occur to residential property values and through quarantine restrictions on exporters of nursery stock, Christmas trees, and other forest products. *B. thuringiensis* subsp. *kurstaki* is the preferred treatment for control of outbreak gypsy moth populations in both forested habitats and residential areas (Montgomery and Wallner 1989, Reardon et al. 1994, Dubois and Dean 1995). However, gypsy moths continue to expand their range and cause severe, intermittent outbreaks. Gypsy moth has long been recognized as being one of the Lepidoptera species most tolerant to *B. thuringiensis* (Heimpel and Angus 1959, Dubois and Dean 1995), particularly on certain tree species (Farrar et al. 1996). Moreover, there is evidence of inter-population variation in gypsy moth susceptibility to *B. thuringiensis* subsp. *kurstaki* (Rossiter et al. 1990, Robison et al. 1994).

The objectives of this study were to determine whether *B. cereus* strains have a synergistic effect on *B. thuringiensis* subsp. *kurstaki* against gypsy moth and whether zwittermicin A is the principal synergistic component of *B. cereus* cultures.

## Materials and Methods

**Bacterial Strains and Culture Media.** *B. cereus* strains were isolated from soils from the United States, Australia, and Honduras (Table 1) and cultured as described previously (Stabb et al. 1994, Raffel et al.

1996, Silo-Suh et al. 1998). All cultures were grown in half-strength tryptic soy broth and shaken for 72 h. Culture density was determined by dilution plating and ranged from  $2 \times 10^8$  to  $2.5 \times 10^8$  CFU/ml (colony forming units/ml).

**Zwittermicin A Quantification and Purification.** Zwittermicin A was separated by high-voltage paper electrophoresis at pH 9.2 and detected by silver nitrate staining by comparison with known standards (Milner et al. 1995). Zwittermicin A concentration was determined by an endpoint dilution assay. Zwittermicin A was purified from *B. cereus* UW85 cultures by ion exchange chromatography and high performance liquid chromatography through a cyano-bonded column (Silo-Suh et al. 1998).

**Gypsy Moth Rearing.** Gypsy moth egg masses were obtained from culture NJSS (New Jersey Standard Strain) at the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) laboratory at the Otis Air National Guard (ANG) Base, Cape Cod, MA. Before hatching, 10–15 egg masses were surface sterilized in 40–50 ml of a solution of 10.2 ml Tween 80 (polyoxyethylene sorbitan monooleate) and 19.9 ml of bleach per liter of distilled water for 5 min, rinsed 3 times with distilled water, and dried under a vacuum hood for 30 min. Egg masses were then placed in petri dishes and both egg masses and insects were reared in an environmental chamber under a photoperiod of 16:8 (L:D) h at 25°C. Larvae were reared in a quarantine facility at the University of Wisconsin-Madison Department of Entomology. Upon emergence, the larvae (in groups of 100–150) were provided with  $\approx 2$  1-cm<sup>3</sup> cubes of artificial diet (USDA, Hamden formula), which were replaced every 48 h until the larvae were used for experiments. Detailed procedures are in Chenot and Raffa (1998).

**Bioassays.** Larvae were starved for 24 h after they molted to 3rd instar. We selected 10 larvae per treatment within a narrow weight range (10–20 mg) to minimize variability. Individual larvae were placed in the cells (4 by 2.5 by 1.5 cm) of rearing trays and covered with mylar. A constant dose of 0.65 IU ( $2 \times 10^6$  CFU/ml) of *B. thuringiensis* subsp. *kurstaki* Foray 76B (Novo Nordisk, currently owned by Abbott, North Chicago, IL, from strain HD-1) was used, which was previously estimated to cause  $\approx 25\%$  mortality to 3rd-instar gypsy moth (Chenot and Raffa 1998). Assays were first conducted using *B. cereus* strain UW85 (Handelsman et al. 1990). *B. cereus* culture was applied with the *B. thuringiensis* subsp. *kurstaki* culture at ratios (*B. cereus*:*B. thuringiensis* subsp. *kurstaki*) of 0:0, 0:1, 1:0, 1:10, 1:1, 10:1, 100:1, and 1,000:1. These ratios were determined based on CFU/ml of each culture. Each ratio was tested in triplicate, with each sample consisting of 10 newly molted 3rd instars using separate cohorts of gypsy moths provided by USDA-APHIS. Assays were repeated with UW85 and the other *B. cereus* strains using set culture volumes of 0.033, 0.067, 0.33, 0.67 (all culture dilutions in distilled water applied in 1- $\mu$ l doses), 3.3, 6.7, and 10  $\mu$ l. The culture mix was applied to a standard diet disc ( $\approx 4$  mm

Table 1. *B. cereus* strains used in study

<i>B. cereus</i> strains	Origin	Reference
UW85	Alfalfa root in Wisconsin soil	Handelsman et al. 1990
UW030	Mutant of UW85	Silo-Suh et al. 1994
UW226	Mutant of UW85	Silo-Suh et al. 1994
UW325	Mutant of UW85	Silo-Suh et al. 1998
DGA34	Australian soil	Stabb et al. 1994
MS1-9	Wisconsin soil	Stabb et al. 1994
Z33	Honduran soil	Raffel et al. 1996
W28	Wisconsin soil	Raffel et al. 1996

diameter, 1 mm high) by the method of Chenot and Raffa (1998), as modified from Dubois et al. (1989) and Appel and Schultz (1994), and fed to the larvae on 2 consecutive days. Mortality was recorded every 24 h for 5 d.

Zwittermicin A was purified using the method of Silo-Suh et al. (1998) and suspended in sterilized water at concentrations similar to those found in *B. cereus* UW85 culture. These concentrations were based on previous estimates of zwittermicin A concentration in UW85 culture ( $\approx 15 \mu\text{g}/\text{ml}$  of zwittermicin A) (Stabb et al. 1994, Milner et al. 1995, Silo-Suh et al. 1998). Zwittermicin A was applied in solution to diet disks in 1- $\mu\text{l}$  aliquots with *B. thuringiensis* subsp. *kurstaki* using the method of Chenot and Raffa (1998) and fed to larvae on 2 consecutive days. Mortality was recorded every 24 h for 5 d.

Purified zwittermicin A was added to a mutant strain of *B. cereus* that does not produce zwittermicin A (UW030) and does not synergize *B. thuringiensis*. The dosage of 15  $\mu\text{g}$  of purified zwittermicin A per milliliter of UW030 culture was used to simulate levels present in UW85 cultures (Silo-Suh et al. 1994).

**Statistical Analysis.** Data analysis was performed using Minitab (Minitab 1995). The analyses were performed fitting a quadratic regression of percent mortality versus a natural log transformation of culture volume. An imputed value for zero of  $10^{-2}$  was used for the *B. thuringiensis* control treatment with no addition of culture or purified antibiotic. All regressions were tested for lack of fit using Minitab (Minitab 1995).

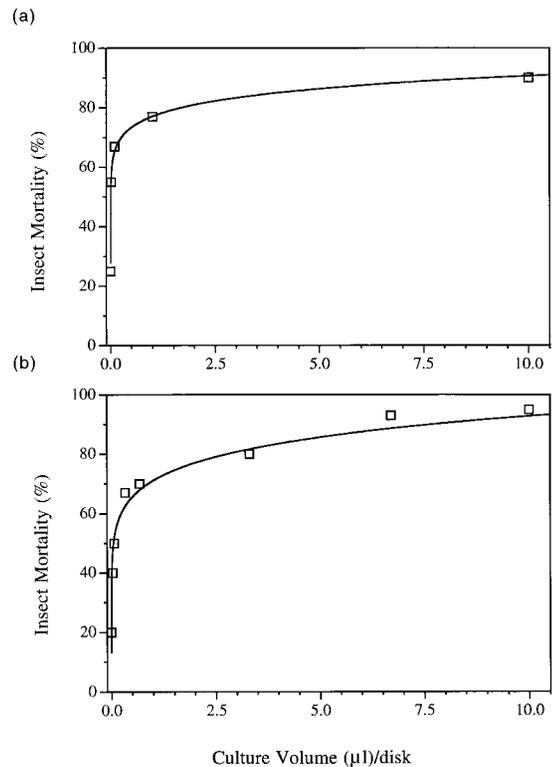
The  $\text{LD}_{50}$  and  $\text{LD}_{95}$  values denote the volume of *B. cereus* culture added to 0.65 IU of *B. thuringiensis* subsp. *kurstaki* needed to cause 50 and 95% mortality. The  $\text{LD}_{50}$  and  $\text{LD}_{95}$  values were determined only for cultures that resulted in mortality  $>50\%$ . Standard error was determined using PROC MEANS (SAS Institute 1990). Correlations between mortality and zwittermicin A concentration in *B. cereus* were performed using Minitab (Minitab 1995).

In assessing the effect of zwittermicin A on mutant UW030, we compared the slope of the reconstituted culture with the parent strain UW85 using Minitab (Draper and Smith 1998) (Minitab 1995).

The effect of zwittermicin A on the time required for *B. thuringiensis* to kill larvae was determined using PROC analysis of variance (SAS Institute 1990) of total mortality on day 3 of assay for each treatment.

## Results

**Effect of *B. cereus* UW85 Culture on *B. thuringiensis* Activity.** Addition of *B. cereus* UW85 culture significantly increased the mortality of gypsy moth larvae when added to a constant amount of *B. thuringiensis* subsp. *kurstaki* (Fig. 2; Table 2). UW85 had no effect on gypsy moth survival when applied alone (3.3%). The lowest concentration of UW85 culture (0.033  $\mu\text{l}$  per disk) nearly doubled the activity of *B. thuringiensis* subsp. *kurstaki* against the larvae, whereas *B. thuringiensis* subsp. *kurstaki* alone resulted in 20% mortality.



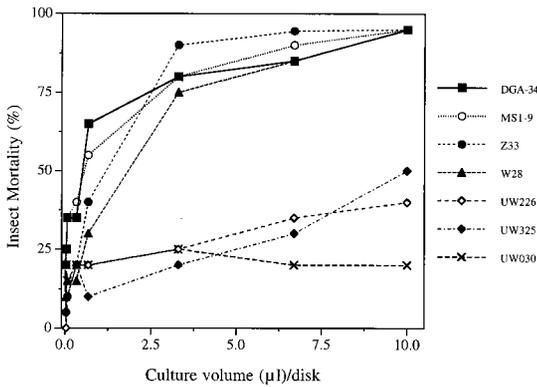
**Fig. 2.** Effect of *B. cereus* UW85 culture on *B. thuringiensis* subsp. *kurstaki* toxicity to 3rd-instar *L. dispar*. Each volume of UW85 was added to 0.65 IU *B. thuringiensis* subsp. *kurstaki*. Each point represents the mean mortality  $\pm$  SEM of a total of 30 larvae per dose (3 replications with 10 larvae each). Standard errors are included but are too small to be visible. (a) Experiment 1. (b) Experiment 2.

For strain UW85, the quadratic regression was not necessary, and therefore a linear regression of percent mortality and log transformed culture volume was used. Mortality increased with higher doses, reaching 95% with an addition of 10  $\mu\text{l}$  of culture. The  $\text{LD}_{50}$  and  $\text{LD}_{95}$  values estimated by the model are 0.10 and 8.39  $\mu\text{l}$ , respectively (Table 2). This result was highly reproducible because the 2 experiments yielded the same general relationships, with nearly identical parameters.

**Table 2.** Results of regression analysis of *B. cereus* strains

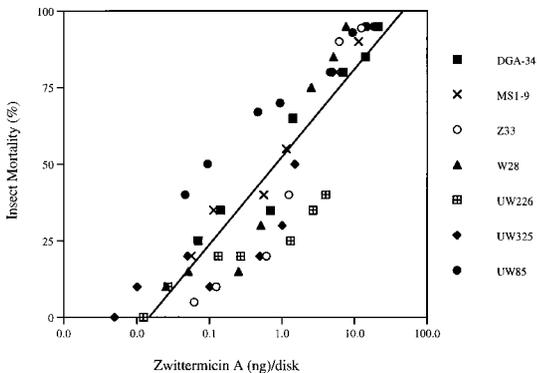
Strain	<i>n</i>	<i>F</i>	df	<i>P</i>	$\text{LC}_{50}$ ( $\mu\text{l}$ )	$\text{LC}_{95}$ ( $\mu\text{l}$ )
UW85	30	182.68	23	0.000	0.10	8.39
DGA34	30	49.41	23	0.000	0.37	9.39
MS1-9	30	49.87	23	0.000	0.47	8.71
Z33	30	135.01	23	0.000	1.26	6.95
W28	30	167.73	23	0.000	1.65	8.45
UW325	30	73.01	23	0.000		
UW226	30	25.18	23	0.000		
UW030	30	0.01	23	0.906		

$\text{LC}_{50}$  and  $\text{LC}_{95}$  values show quantity needed to raise *B. thuringiensis* subsp. *kurstaki* toxicity to 50 and 95%.

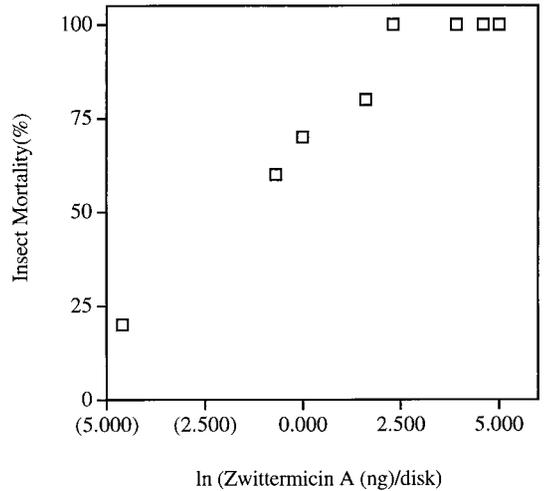


**Fig. 3.** Effect of wild-type and mutant *B. cereus* strains on toxicity of *B. thuringiensis* subsp. *kurstaki* to 3rd-instar *L. dispar*. Each point represents the mean mortality  $\pm$  SEM of a total of 30 larvae per dose (3 replications with 10 larvae each). Standard errors are included but are too small to be visible.

**Effect of Diverse *B. cereus* Strains with Varying Zwittermicin A Content on *B. thuringiensis* Activity.** The results from genetically diverse strains of *B. cereus* that differ in accumulation of zwittermicin A are shown in Fig. 3. Six of the 7 strains increased the efficacy of *B. thuringiensis* subsp. *kurstaki*. None caused mortality by themselves (range, 0–7%). The 6 cultures that affected *B. thuringiensis* subsp. *kurstaki* activity showed diverging responses. Wild strains DGA34, MS1-9, Z33, and W28 increased the larval mortality to 95% at the highest dosage (10  $\mu$ l per disk). Each of these generated highly significant exponential relationships between culture volume and mortality (Fig. 3; Table 2). UW85 mutants, UW226, and UW325 increased mortality to a lesser extent than the wild strains. UW030 culture did not affect mortality in the presence or absence of *B. thuringiensis* subsp. *kurstaki*.



**Fig. 4.** Relationship between zwittermicin A concentration in treatments (Fig. 3 legend) of *B. cereus* wild and mutant strains and synergy of *B. thuringiensis* subsp. *kurstaki* on 3rd-instar *L. dispar* larvae. Each point represents the mean mortality  $\pm$  SEM of a total of 30 larvae per dose (3 replications with 10 larvae each). Standard errors are included but are too small to be visible.



**Fig. 5.** Effect of zwittermicin A on *B. thuringiensis* subsp. *kurstaki* toxicity to *L. dispar*. Each point represents the average mortality of a total of 30 larvae per dose (3 replications with 10 larvae each). Standard errors are included but are too small to be visible.

The accumulation of zwittermicin A for each strain was as follows: DGA34 = 20  $\mu$ g/ml, Z33 = 18  $\mu$ g/ml, (MS1-9) = 17  $\mu$ g/ml, UW85 = 15  $\mu$ g/ml, W28 = 7.6  $\mu$ g/ml, UW226 = 4  $\mu$ g/ml, UW325 = 1.5  $\mu$ g/ml, UW030 = 0  $\mu$ g/ml and *B. thuringiensis* subsp. *kurstaki* formulation 0  $\mu$ g/ml (limit of detection is 0.2  $\mu$ g/ml). Based on these and previous measurements of zwittermicin A accumulation in these strains (Silo-Suh et al. 1994, Raffel et al. 1996), we suggest an association between synergistic activity and zwittermicin A accumulation in culture.

To determine whether zwittermicin A accumulation was related with the synergistic effect of the *B. cereus* cultures, we correlated zwittermicin A concentration on insect mortality (Fig. 4). This procedure yielded a significant positive correlation. The correlation between mortality and zwittermicin A concentration in all cultures was 0.817. Individual correlations between zwittermicin A concentration in *B. cereus* culture and mortality were as follows: UW226 = 0.855, UW325 = 0.894, DGA34 = 0.867, (MS1-9) = 0.892, UW030 = 0.0, UW85 = 0.803, W28 = 0.936, and Z33 = 0.885.

**Effect of Purified Zwittermicin A on *B. thuringiensis* Activity.** Purified zwittermicin A significantly increased the mortality of gypsy moth larvae ( $F = 186.65$ ,  $df = 23$ ,  $P = 0.000$ ,  $LD_{95} = 9.40$  ng,  $LD_{50} = 207$  pg) (Fig. 5). Supplementation with the smallest concentration (500 pg per disk) caused 60% mortality, compared with 25% mortality obtained with *B. thuringiensis* subsp. *kurstaki* alone. The  $LD_{95}$  value for pure zwittermicin A, in combination with 0.65 IU of *B. thuringiensis* subsp. *kurstaki*, was only 9.40 ng. Mortality increased with zwittermicin A concentration and reached 100% with 10 ng per disk of zwittermicin A (Fig. 5). Zwittermicin A had no effect on mortality

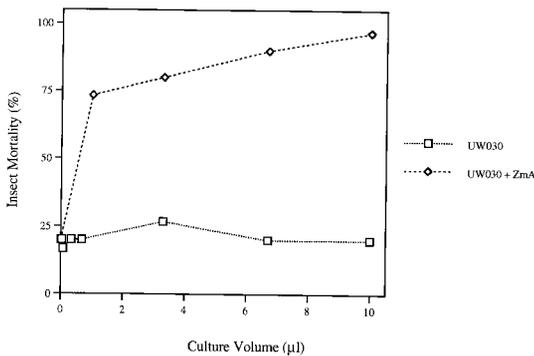


Fig. 6. Effect of the addition of 15 ng/ $\mu$ l zwittermicin A (ZmA) to the nonproducing mutant UW030. Each point represents the average mortality of a total of 30 larvae per dose (3 replications with 10 larvae each). Standard errors are included but are too small to be visible.

in the absence of *B. thuringiensis* subsp. *kurstaki* (3.3%). Zwittermicin A did have a significant effect on the time required for *B. thuringiensis* to kill larvae ( $F = 11.98$ ,  $df = 7$ ,  $P > 0.0001$ ). By day 3 of the assay, only 17% total mortality was after *B. thuringiensis* treatment. However, for those treated with zwittermicin A, day 3 mortality ranged from 61% for the lowest treatment (500 pg) to 93% for the highest treatment (150 ng).

The addition of a constant dose of zwittermicin A (15  $\mu$ g/ml) to the mutant, UW030, derived from *B. cereus* UW85, which does not produce zwittermicin A, induced mortality levels similar to those found with UW85 ( $F = 226.97$ ,  $df = 14$ ,  $P = 0.000$ ) (Fig. 6). Using Minitab (Minitab 1995), we determined the slopes of UW85 and the reconstituted UW030 culture were not significantly different ( $F = 0.283$ ,  $df = 38$ ,  $P = 0.06$ ), indicating that zwittermicin A is responsible for much of the synergistic activity of UW85 culture.

### Discussion

These results demonstrate synergy by *B. cereus* strains and zwittermicin A of the insecticidal activity of *B. thuringiensis* subsp. *kurstaki* on gypsy moth. Three lines of evidence indicate that zwittermicin A is largely responsible for the synergistic effect of *B. cereus* on *B. thuringiensis* subsp. *kurstaki*. First, the synergistic activity of *B. cereus* strains correlated with the amount of zwittermicin A they accumulate in culture (Fig. 4). Second, purified zwittermicin A increased the insecticidal activity of *B. thuringiensis* subsp. *kurstaki*, while having no insecticidal activity alone (Fig. 5). Zwittermicin A was more active when added in the pure form than when an equivalent amount was present in a *B. cereus* culture. Third, the addition of zwittermicin A to the mutant, UW030, restored its synergistic activity with *B. thuringiensis* subsp. *kurstaki* to the level of the wild-type (zwittermicin A-producing) parent strain.

Our results also suggest the possibility of interactions, both positive and negative, between additional

components of *B. cereus* cultures and the insecticidal activity of *B. thuringiensis* subsp. *kurstaki*. For example, the mortality of gypsy moth caused by the addition of UW85 was consistently higher than would be expected based solely on its content of zwittermicin A (Fig. 4). Likewise, the lower than expected mortality with UW226 (Fig. 4) suggests that other factors could influence activity. Also, the higher  $LD_{95}$  and  $LD_{50}$  values for the combination of UW030 and zwittermicin A ( $LD_{95} = 198.25$  ng and  $LD_{50} = 705$  pg) (versus pure zwittermicin A alone [ $LD_{95} = 9.40$  ng, and  $LD_{50} = 207$  pg]) suggests that some component of the culture may inhibit activity.

The data presented here, indicating that zwittermicin A is a synergist of *B. thuringiensis* subsp. *kurstaki*, may explain some previous reports of synergy between components of *B. thuringiensis* cultures (Lee et al. 1995, Tang et al. 1995). Our previous work demonstrated that some *B. thuringiensis* strains, such as HD-1, produce zwittermicin A (Stabb et al. 1994). It is also possible that the previously reported synergy between spores and toxins present in *B. thuringiensis* (Liu et al. 1998) might be the result of spore-associated zwittermicin A.

Zwittermicin A could be used to reduce the amount of *B. thuringiensis* needed in the field and could also delay the rate of resistance development. Addition of as little as 207 pg of zwittermicin A per disk would increase the mortality caused by 0.65 IU of *B. thuringiensis* subsp. *kurstaki* to 50%; by comparison an additional 1.15 IU of *B. thuringiensis* subsp. *kurstaki* would be needed to have the same effect (Chenot and Raffa 1998).

There are several possible mechanisms by which zwittermicin A might enhance *B. thuringiensis* activity. Zwittermicin A may act directly on insect cells once they become accessible through disruption of the midgut epithelium by *B. thuringiensis*. Zwittermicin A might also, have a direct effect on aspects of midgut function, such as disruption of the peritrophic membrane to remove a physical barrier, stimulation of proteases necessary to *B. thuringiensis* solubilization and activation, and alteration of midgut epithelium properties to facilitate *B. thuringiensis* binding and pore formation.

However, our preferred hypothesis is that the antimicrobial properties of zwittermicin A may alter the composition of gut microflora in the gypsy moth. Although the gut microbial communities of only a few lepidopteran species have been studied, McKillip et al. (1997) recently characterized the midgut microbial community of the leafroller, *Pandemis pyrusana* (Tortricidae) Kearfott. Our preliminary results from microscopy and culturing found some culturable bacteria and that these populations drop below the limit of detection after the larvae are fed a diet containing zwittermicin A. Gut microflora are essential for many insect activities, such as normal growth and development, reproduction, digestion, and nutrition (Buchner 1965, Nolte 1977, Dasch et al. 1984, Cruden and Markovetz 1987, Mittler 1988, Campbell 1989). Disruption of such relationships could potentially alter

the potency of *B. thuringiensis*. If so, insight into the microfloral composition of insect midguts, and the effects of antibiotics and antibiotic-producing organisms, may yield a diverse collection of enhancers of *B. thuringiensis*.

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