APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2000, p. 1093–1097 Vol. 66, No. 3
0099-2240/00/$04.00+0

Cyt1A from *Bacillus thuringiensis* Synergizes Activity of *Bacillus sphaericus* against *Aedes aegypti* (Diptera: Culicidae)

MARGARET C. WIRTH,[1]* BRIAN A. FEDERICI,[1,2] AND WILLIAM E. WALTON[1]

Department of Entomology[1] and Interdepartmental Graduate Program in Genetics and Microbiology,[2] University of California, Riverside, California 92521

Received 23 August 1999/Accepted 15 December 1999

***Bacillus sphaericus* is a mosquitocidal bacterium recently developed as a commercial larvicide that is used worldwide to control pestiferous and vector mosquitoes. Whereas *B. sphaericus* is highly active against larvae of *Culex* and *Anopheles* mosquitoes, it is virtually nontoxic to *Aedes aegypti*, an important vector species. In the present study, we evaluated the capacity of the cytolytic protein Cyt1A from *Bacillus thuringiensis* subsp. *israelensis* to enhance the toxicity of *B. sphaericus* toward *A. aegypti*. Various combinations of these two materials were evaluated, and all were highly toxic. A ratio of 10:1 of *B. sphaericus* to Cyt1A was 3,600-fold more toxic to *A. aegypti* than *B. sphaericus* alone. Statistical analysis showed this high activity was due to synergism between the Cyt1A toxin and *B. sphaericus*. These results suggest that Cyt1A could be useful in expanding the host range of *B. sphaericus*.**

Bacillus thuringiensis strains pathogenic to insects produce two distinct types of toxin proteins, Cry and Cyt proteins (9, 28). Generally, the genes encoding these proteins are located on large plasmids, and the proteins are synthesized and form crystalline inclusions during sporulation. More than 100 different *cry* genes have been identified and sequenced, and significant homologies among the amino acid sequences of this group, in combination with experimental studies, suggest they have a common mode of action, colloid-osmotic lysis (10, 14). Cyt toxins, however, have only been found in *B. thuringiensis* strains that are mosquitocidal, have amino acid sequences that are unrelated to those of the Cry toxins, and can lyse a variety of cell types in vitro (14, 34). The mode of action of Cyt toxins has not been fully elucidated. However, research suggests that Cyt toxins are also involved in colloid-osmotic lysis (14), but may differ in the mechanism by which lesions are formed in the cell membrane (6, 7, 9).

One of the more interesting features of the Cry and Cyt toxin combination that is found in *B. thuringiensis* subsp. *israelensis*, the subspecies upon which many commercial mosquito larvicides are based, is the effect of this combination on toxicity. This subspecies produces a crystalline parasporal inclusion containing four major toxic proteins, Cry4A (134 kDa), Cry4B (128 kDa), Cry11A (66 kDa), and Cyt1A (27 kDa). These four proteins are assembled into separate inclusions that are enveloped together to form the parasporal body. The proportion of each protein in the parasporal body, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electron microscopy (15), is approximately 40% Cyt1A and 20% for each of the three Cry proteins. Studies of these proteins revealed that the toxicity of individual proteins in *B. thuringiensis* subsp. *israelensis* was much less than that of the intact parasporal crystal. Subsequently, it was shown that the Cry toxins in *B. thuringiensis* subsp. *israelensis* interact synergistically with the Cyt1A toxin, as well with each other, to produce this high level of activity (9, 15, 24, 41). This synergism has also been shown to be important in the relatively low rate of resistance development toward *B. thuringiensis* subsp. *israelensis* in *Culex* mosquitoes (12) and can suppress high levels of resistance to Cry4 and Cry11 toxins (37, 38). The mechanism of this synergism is not understood, but we have postulated that Cyt1A aids these toxins in binding to or inserting into the mosquito microvillar membrane (37). The synergistic capacity of Cyt1A was extended by the recent observation that sublethal concentrations of Cyt1A, combined with *B. sphaericus*, an unrelated mosquitocidal bacterium which does not have Cry-type toxins, were synergistic and toxic toward highly resistant *Culex quinquefasciatus* (40). Because this combination was synergistic against resistant mosquitoes that have lost the capacity to bind *B. sphaericus* toxins (21), we postulated that this same mixture might be synergistic toward a mosquito species that is naturally insensitive to *B. sphaericus*.

To evaluate this possibility, we tested Cyt1A in combination with *B. sphaericus* against larvae of the mosquito *Aedes aegypti*. Although highly toxic to *Anopheles* and *Culex* species, *B. sphaericus* is not active against *A. aegypti* (18, 19). Thus, in *A. aegypti*, enhanced toxicity of *B. sphaericus* in the presence of Cyt1A would be due to the effect of the Cyt1A toxin. Here, we report that a mixture of Cyt1A with *B. sphaericus* strain 2362 was >3,600-fold more toxic than *B. sphaericus* toward a laboratory strain of *A. aegypti*.

MATERIALS AND METHODS

Mosquitoes. A laboratory colony of *A. aegypti* was obtained from M. Mulla, Department of Entomology, University of California, Riverside. This colony was established in culture in our laboratory and maintained by standard procedures described previously for this species (39). Early fourth-instar larvae were used in the bioassays.

Bacterial strains and toxins. Toxin preparations for this study were lyophilized powders of spore-crystal mixtures. Technical powder of *B. sphaericus* 2362 was obtained from Abbott Laboratories (North Chicago, Ill.). The Cyt1A powder was derived from a recombinant strain of *B. thuringiensis* subsp. *israelensis* that produces only the Cyt1Aa toxin (42). Two powders of Cyt1A were used in these experiments because the quantity of the first powder was insufficient to complete the bioassays. For the first powder, cells were grown on liquid media as previously described (22, 37). For the second powder, the cells were grown on wheat-germ media (Berdsk Co., Berdsk, Russia). After fermentation, the sporulated cells were washed in distilled water and sedimented, and the resultant pellets were lyophilized. Lyophilized powders of purified Cyt1A crystals were also used (42).

For the mosquito bioassays, stock suspensions of the powders were prepared in distilled water in 125-ml flasks containing approximately 25 glass beads. Sus-

* Corresponding author. Mailing address: Department of Entomology, University of California, Riverside, CA 92521. Phone: (909) 787-3918. Fax: (909) 787-3086. E-mail: mcwirth@mail.ucr.edu.

TABLE 1. Toxicity of *B. sphaericus* technical powder, Cyt1A spore-crystal powder, and combinations of *B. sphaericus* and Cyt1A against *A. aegypti*

Toxin(s)	No. tested	LC (μg/ml) of fourth-instar larvae (range)		Slope (standard error)	Synergism factor	
		50%	95%		LC_{50}	LC_{95}
B. sphaericus (strain 2362)						
24 h	500	266 (45.0–1,900)	660,117 (19,904–385,589)	0.5		
48 h	500	860 (636–1,320)	19,791 (8,612–74,715)	1.2 (0.14)		
Cyt1Aa (fermentation 1) 24 h	800	5.90 (2.86–12.0)	133.9 (41.4–448)	1.2 (0.21)		
Cyt1Aa (fermentation 2) 24 h	600	14.1 (6.63–30.0)	136.5 (28.8–667)	1.7 (0.31)		
B. sphaericus + Cyt1A (3:1)						
24 h	600	7.77 (6.96–8.67)	25.8 (21.5–32.4)	3.1 (0.23)	2.9	20.4
48 h	600	5.23 (1.58–17.3)	22.8 (2.29–228)	2.6 (1.0)		
B. sphaericus + Cyt1A (10:1)						
24 h	500	3.14 (2.84–3.47)	7.76 (6.67–9.42)	4.2 (0.32)	15.6	172
48 h	500	2.46 (0.399–15.6)	5.36 (0.160–252)	4.9 (1.2)		
B. sphaericus + Cyt1A (20:1)						
24 h	900	5.81 (4.98–6.86)	47.7 (34.4–72.8)	1.8 (0.13)	14.3	52.2
48 h	900	1.75 (1.45–2.12)	40.0 (27.2–64.2)	1.2 (0.07)		
B. sphaericus + CytA (50:1)						
24 h	700	8.29 (7.06–9.91)	64.3 (44.4–106.9)	1.8 (0.16)	17.0	103
48 h	700	3.17 (2.63–3.86)	59.6 (39.2–102)	1.3 (0.09)		

pensions were agitated for 5 min with a vortex mixer. Stocks were prepared monthly, and 10-fold serial dilutions were prepared weekly as needed. All stocks and dilutions were frozen at −20°C when not in use.

Bioassay procedures. Groups of 20 early fourth-instar larvae were exposed to a range of concentrations of the spore-crystal powders in 100 ml of deionized water in 237-ml plastic cups. This range, usually 5 to 10 different concentrations, resulted in mortality between 2 and 98% after 24 h for Cyt1A and 48 h for *B. sphaericus*. Bioassays were replicated on five different days. The different ratio combinations of *B. sphaericus* and Cyt1A were based on the dry weight of the respective powders. Cyt1A powder 1 was used for the tests of the ratios 3:1 and 10:1, whereas Cyt1A powder 2 was used for the tests of the ratios 20:1 and 50:1. Mortality in these bioassays was evaluated at both 24 and 48 h. Because of the limited quantity of Cyt1A crystal, bioassays utilizing the crystal in combination with *B. sphaericus* technical powder took place in 30-ml plastic cups, with 10 ml of deionized water and 10 early fourth-instar larvae. These bioassays were replicated on three different days. Mortality was evaluated after 48 h.

All data were analyzed by a probit program (11, 27). Lethal concentration values with overlapping fiducial limits were not considered significantly different. Synergism between *B. sphaericus* and Cyt1A was evaluated by the method described by Tabashnik (31). Theoretical lethal concentrations for the different combinations of *B. sphaericus* and Cyt1A were calculated from the weighted harmonic means of the individual values for these toxins. The synergism factor, defined as the ratio of the theoretical lethal concentration to the observed lethal concentration, was determined for each of the combinations. When the ratio was greater than 1, the toxin interaction was considered synergistic because toxicity exceeded the value expected from the individual additive toxicity. When the ratio was less than 1, the interaction was considered antagonistic, whereas a ratio of 1 indicated that the toxicity was additive.

RESULTS

B. sphaericus exhibited only a low level of toxicity against fourth-instar larvae of *A. aegypti*, yielding a 50% lethal concentration (LC_{50}) of 860 μg/ml (Table 1). In contrast, the powders of sporulated Cyt1A cells were much more toxic, with an LC_{50} of 5.90 μg/ml for Cyt1A powder 1 and an LC_{50} of 14.1 μg/ml for Cyt1A powder 2. These two values were not significantly different. Bioassays in the smaller cups yielded lower lethal concentrations for both *B. sphaericus* and Cyt1A, with LC_{50} values of 12.8 and 1.86 μg/ml, respectively (Table 2).

When lyophilized spore-crystal powders of the Cyt1A strain were combined with *B. sphaericus* technical powder and tested against *A. aegypti*, the resulting toxicity was much greater than that observed for *B. sphaericus* alone. As stated above, the LC_{50} of *B. sphaericus* was 860 μg/ml. In the presence of a 3:1 ratio of *B. sphaericus* to Cyt1A, this value was 5.23 μg/ml (Table 1; Fig. 1). When the proportion of Cyt1A in the combination was reduced, the mixtures continued to be highly toxic, with LC_{50} values of 2.46, 1.75, and 3.17 μg/ml for the ratios 10:1, 20:1, and 50:1, respectively. Similar high activity was observed at the LC_{95} (Table 1; Fig. 1). The concentration of Cyt1A in the latter three ratios was sublethal, whereas for the 3:1 ratio, Cyt1A could have contributed between 12 and 60% of the observed mortality. The 10:1 ratio of *B. sphaericus* and Cyt1A was the most active combination tested. Although toxicity was slightly lower at the 20:1 and 50:1 ratios, these combinations were still highly toxic.

The *B. sphaericus* and Cyt1A mixtures had an additional effect on toxicity. In contrast to *B. sphaericus*, which normally requires 48 h for maximum toxicity, high toxicity was observed 24 h after exposure to the combination of *B. sphaericus* and Cyt1A. In general, lethal concentrations did not increase significantly between 24 and 48 h of exposure to these combinations. However, when Cyt1A was present at a lower proportion in the mixture, 20:1 and 50:1, a significant increase in mortality after 48 h was noted at the LC_{50}. Slopes were higher for the ratios 3:1 and 10:1 than for the 20:1 and 50:1 ratios, which may reflect differences between the two powders of Cyt1A (Table 1; Fig. 1).

Calculation of the synergism factors from the 24-h data for

TABLE 2. The toxicity of a 10:1 ratio of *B. sphaericus* technical powder to Cyt1A crystals against *A. aegypti*

Toxin(s)	No.	LC (μg/ml) of fourth-instar larvae (range)		Slope (standard error)	Synergism factor	
		50%	95%		LC_{50}	LC_{95}
B. sphaericus (strain 2362), 48 h	240	12.8 (6.10–21.4)	933 (407–3,922)	0.88 (0.13)		
Cyt1A crystals, 24 h	190	1.86 (1.09–2.75)	36.5 (19.6–106)	1.3 (0.19)		
B. sphaericus + Cyt1A crystals (10:1), 48 h	140	3.80 (2.68–5.14)	31.5 (18.5–81.9)	1.8 (0.29)	2.1	8.6

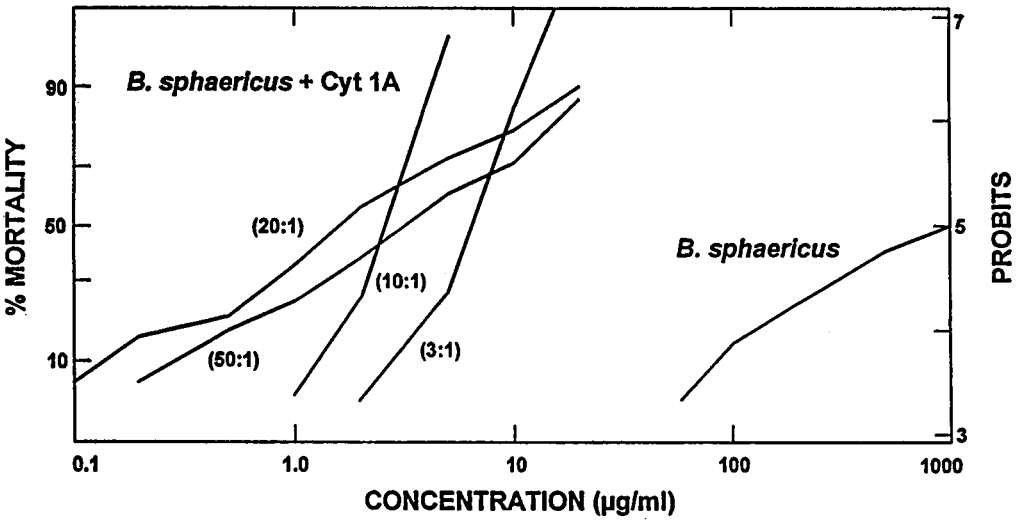

FIG. 1. Dose-response regression lines of *B. sphaericus* (strain 2362) technical powder and combinations of different ratios of *B. sphaericus* and Cyt1A spore-crystal powder from *B. thuringiensis* subsp. *israelensis* toward larvae of the mosquito *A. aegypti*.

the various combinations indicated that all of the mixtures were synergistic. Synergism factors ranged from 2.9 to 17.0 at the LC_{50} and from 20.4 to 172 at the LC_{95}. The synergism factors, in agreement with the toxicity levels, were greatest with the 10:1 ratio of *B. sphaericus* to Cyt1A.

When *B. sphaericus* technical powder was combined with purified Cyt1A crystals at a 10:1 ratio and tested in the smaller bioassay system, we observed increased toxicity toward *A. aegypti*, with LC_{50} and LC_{95} values of 3.8 and 31.5 μg/ml, respectively, and synergism factors of 2.1 and 8.6, respectively (Table 2).

DISCUSSION

Low concentrations of the Cyt1A protein from *B. thuringiensis* subsp. *israelensis* dramatically altered the toxicity of *B. sphaericus* against the normally insensitive mosquito species, *A. aegypti*. A ratio of 10:1 of *B. sphaericus* technical powder and Cyt1A spore-crystal powder was 3,600-fold more toxic at the LC_{95} against *A. aegypti* than was *B. sphaericus*, and this high level of activity resulted from synergism between the Cyt1A toxin and *B. sphaericus*.

The mechanism of synergism between Cyt1A and *B. sphaericus* is not known. Previous work has demonstrated that Cyt1A has little or no effect on the toxicity of *B. sphaericus* toward susceptible *C. quinquefasciatus* (40). However, in a laboratory-selected, resistant population of that species, low concentrations of Cyt1A combined with *B. sphaericus* suppressed >30,000-fold resistance to *B. sphaericus*. Resistance in this strain of mosquitoes is due to failure of the binary *B. sphaericus* toxin to bind to midgut microvilli (21). *B. sphaericus* is poorly toxic toward *A. aegypti* because this species either lacks a specific receptor for the binary toxin of *B. sphaericus* or has an extremely low concentration of such receptors (20). Other factors that could affect the activity of *B. sphaericus*, such as the rate of ingestion of the toxins or differences in proteolytic activation, have not been found to be significantly different between *C. quinquefasciatus* and *A. aegypti* (1, 5). Our results, therefore, suggest that Cyt1A may enhance toxicity by facilitating the binding or insertion of the binary toxins to the microvillar membrane.

Our tests revealed that purified Cyt1A crystals combined with *B. sphaericus* technical powder were more toxic to *A. aegypti* than *B. sphaericus* alone, and the combination was synergistic. However, the levels of synergism were lower than those observed with Cyt1A spore-crystal powder in the large-cup bioassay system. The reduction in the synergism factor primarily resulted from the increased toxicity of *B. sphaericus* in the smaller cups relative to its toxicity in the larger cups (67-fold more toxic at the LC_{50}). Because larval density can have a significant effect on the toxicity of *B. sphaericus* (21) and *B. thuringiensis* subsp. *israelensis* (2), the higher density in the small cups was probably a contributing factor. Moreover, Cyt1A crystals in the small cups were only three- to fourfold more toxic than Cyt1A spore-crystal powder in the large cups. This is not unexpected because of the proportionally higher concentration of Cyt1A in the crystal powder (on a weight basis) relative to spore-crystal powder. Because the highest proportion of Cyt1A-to-spore-crystal was associated with the lowest synergism level, the higher proportion of Cyt1A in tests using crystal may also have contributed to the decreased synergism. However, the role of spores in the observed synergism cannot be ruled out because *B. sphaericus* 2362 spores have been shown to be toxic to *Aedes* (4).

Previous attempts have been made to improve the activity and host range of *B. sphaericus* by incorporating toxin genes from *B. thuringiensis* into the former species and expressing the combined products. Cyt1Ab1 from *B. thuringiensis* subsp. *jegathesan* was cloned and expressed in *B. sphaericus* 2297; however, no improvement in activity toward *A. aegypti* was detected (33). Cry toxins, or combinations of Cry toxins and Cyt1A, also have been incorporated into *B. sphaericus*. The activity of these engineered strains was extended to *A. aegypti* and toxicity was enhanced between 10- and 100-fold (3, 23, 25, 29, 35). However, these improvements in toxicity were considerably lower than the levels we have reported here, and the contribution of *B. sphaericus* to the observed activity was not established. Synergism, although implied, was not demonstrated directly.

From a practical perspective, Cyt1A may prove useful in expanding the host range of *B. sphaericus* against mosquito species that have heretofore been refractory to control with this bacterium. For example, the LC_{95} of a 10:1 ratio of *B. sphaericus* to Cyt1A spore-crystal powder was 5.36 μg/ml. This toxicity level is 11.5-fold higher than that of *B. sphaericus*

toward *C. quinquefasciatus* (40), a primary target of *B. sphaericus*-based insecticides. Our data indicate that even extremely low concentrations of Cyt1A combined with *B. sphaericus* were beneficial in enhancing toxicity toward *A. aegypti*. As many *Aedes* species are sensitive to *B. thuringiensis* subsp. *israelensis* and therefore are likely to be sensitive to Cyt1A, this combination may provide a practical mechanism for extending the host range of *B. sphaericus*. The *B. sphaericus* and Cyt1A combination may also have the potential to reduce the risk for developing *B. sphaericus* resistance. Studies of the development of resistance to *B. thuringiensis* subsp. *israelensis* have provided strong evidence that Cyt1A can delay resistance to the Cry toxins in this material (12). This is particularly important in view of the cases of *B. sphaericus* field resistance that have been reported in several parts of the world (26, 30; G. Sinègre, M. Babinot, J. M. Quernal, and B. Gaven, VII Eur. Meeting Soc. Vector Ecol., abstract, 1994).

The Cyt toxin group now contains several different toxins, including Cyt1Aa and Cyt2Ba from *B. thuringiensis* subsp. *israelensis* (13, 36), Cyt2Aa from *B. thuringiensis* subsp. *kyushuensis* (17), Cyt1Ab1 from *B. thuringiensis* subsp. *medellin* (33), and Cyt2Bb from *B. thuringiensis* subsp. *jegathesan* (8). These Cyt proteins vary in their toxicity to mosquitoes and their ability to synergize toxins. The differences among the Cyt proteins may provide insight into their mode of action, as well as a practical means to manage resistance and extend the host range of mosquitocidal bacterial insecticides.

ACKNOWLEDGMENTS

The assistance of Jeff Johnson and Ludmilla V. Kuzina is gratefully acknowledged.

This research was partially funded by grants to W.E.W. and B.A.F. from the University of California Mosquito Control Research Program.

REFERENCES

1. **Aly, C., M. S. Mulla, and B. A. Federici.** 1989. Ingestion, dissolution, and proteolysis of the *Bacillus sphaericus* toxin by mosquito larvae. J. Invertebr. Pathol. **53:**12–20.
2. **Aly, C., M. S. Mulla, B.-Z. Xu, and W. Schnetter.** 1988. Rate of ingestion by mosquito larvae (Diptera: Culicidae) as a factor in the effectiveness of a bacterial insecticide. J. Med. Entomol. **25:**191–196.
3. **Bar, E., N. Sandler, M. Makayoto, and A. Keynan.** 1998. Expression of chromosomally inserted *Bacillus thuringiensis israelensis* toxin genes in *Bacillus sphaericus*. J. Invertebr. Pathol. **72:**206–213.
4. **Berry, C., J. Hindley, A. F. Ehrhardt, T. Grounds, I. De Souza, and E. W. Davidson.** 1993. Genetic determinants of host range of *Bacillus sphaericus* mosquito larvicidal toxins. J. Bacteriol. **175:**510–518.
5. **Broadwell, A., and P. Baumann.** 1986. Sporulation-associated activation of *Bacillus sphaericus* larvicide. Appl. Environ. Microbiol. **52:**758–764.
6. **Butko, P., F. Huang, M. Pusztai-Carey, and W. K. K. Surewicz.** 1996. Membrane permeabilization induced by cytolytic delta-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*. Biochemistry **35:**11355–11360.
7. **Butko, P., F. Huang, M. Pusztai-Carey, and W. K. K. Surewicz.** 1997. Interaction of the δ-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis* with lipid membranes. Biochemistry **36:**12862–12868.
8. **Cheong, H., and S. S. Gill.** 1997. Cloning and characterization of a cytolytic and mosquitocidal δ-endotoxin from *Bacillus thuringiensis* subsp. *jegathesan*. Appl. Environ. Microbiol. **63:**3254–3260.
9. **Crickmore, N., E. J. Bone, J. A. Williams, and D. J. Ellar.** 1995. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. **131:**249–254.
10. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean.** 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:**807–813.
11. **Finney, D. J.** 1971. Probit analysis. Cambridge University Press, Cambridge, United Kingdom.
12. **Georghiou, G. P., and M. C. Wirth.** 1997. Influence of single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. **63:**1095–1101.
13. **Guerchicoff, A., R. A. Ugalde, and C. P. Rubenstein.** 1997. Identification and characterization of a previously undescribed *cyt* gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **63:**2716–2721.
14. **Höfte, H., and H. Whitely.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
15. **Ibarra, J., and B. A. Federici.** 1986. Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **165:**527–533.
16. **Knowles, B. H., and D. J. Ellar.** 1987. Colloid-osmotic lysis is a general mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim. Biophys. Acta **924:**509–518.
17. **Koni, P., and D. J. Ellar.** 1994. Biochemical characterization of *Bacillus thuringiensis* cytolytic δ-endotoxins. Microbiology **140:**1869–1880.
18. **Lacey, L. A., and S. Singer.** 1982. Larvicidal activity of new isolates of *Bacillus sphaericus* and *Bacillus thuringiensis* (H-14) against anopheline and culicine mosquitoes. Mosq. News **42:**537–543.
19. **Mulla, M. S., H. A. Darwazeh, E. W. Davidson, H. T. Dulmage, and S. Singer.** 1984. Larvicidal activity and field efficacy of *Bacillus sphaericus* strains against mosquito larvae and their safety to nontarget organisms. Mosq. News **44:**336–342.
20. **Nielsen-LeRoux, C., and J.-F. Charles.** 1992. Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. Eur. J. Biochem. **210:**585–590.
21. **Nielsen-LeRoux, C., F. Pasquier, J.-F. Charles, G. Sinègre, B. Gaven, and N. Pasteur.** 1997. Resistance to *Bacillus sphaericus* involves different mechanisms in *Culex pipiens* (Diptera: Culicidae). J. Med. Entomol. **34:**321–327.
22. **Park, H.-W., B. Ge, L. S. Bauer, and B. A. Federici.** 1998. Optimization of Cry3A yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl. Environ. Microbiol. **64:**3932–3928.
23. **Poncet, S., A. Delécluse, G. Anello, A. Klier, and G. Rapoport.** 1994. Transfer and expression of the *crylVB* and *crylVD* genes of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus* 2297. FEMS Microbiol. Lett. **117:**91–96.
24. **Poncet, S., A. Delécluse, A. Klier, and G. Rapoport.** 1994. Evaluation of synergistic interactions among CrylVA, CrylVB, and CrylVD toxic components of *Bacillus thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
25. **Poncet, S., C. Barnard, E. Dervyn, J. Cayley, A. Klier, and G. Rapoport.** 1997. Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the Cry11A toxin gene from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **63:**4413–4420.
26. **Rao, D. R., T. R. Mani, R. Rajendran, A. S. Joseph, A. Gajanana, and R. Reuben.** 1995. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. J. Am. Mosq. Control Assoc. **11:**1–5.
27. **Raymond, M., G. Prato, and D. Ratsira.** 1995. Probability analysis of mortality assays displaying quantal response, version 3.3. Praxeme, Saint Georges d'Orques, France.
28. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:**775–806.
29. **Servant, P., M.-L. Rosso, S. Hamon, S. Poncet, A. Delécluse, and G. Rapoport.** 1999. Production of Cry11A and Cry11Ba toxins in *Bacillus sphaericus* confers toxicity towards *Aedes aegypti* and resistant *Culex* populations. Appl. Environ. Microbiol. **65:**3021–3026.
30. **Silva-Filha, M.-H., L. Regis, C. Nielsen-LeRoux, and J.-F. Charles.** 1995. Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). J. Econ. Entomol. **88:**525–530.
31. **Tabashnik, B. E.** 1992. Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. **58:**3343–3346.
32. **Thiéry, I., A. Delécluse, M. C. Tamayo, and S. Orduz.** 1997. Identification of a gene for a cyt1-like hemolysin from *Bacillus thuringiensis* subsp. *medellin* and expression in a crystal-negative *B. thuringiensis* strain. Appl. Environ. Microbiol. **63:**468–473.
33. **Thiéry, I., S. Hamon, A. Delécluse, and S. Orduz.** 1998. The introduction into *Bacillus sphaericus* of the *Bacillus thuringiensis* subsp. *medellin cyt1Ab1* gene results in higher susceptibility of resistant mosquito larva populations to *B. sphaericus*. Appl. Environ. Microbiol. **64:**3910–3916.
34. **Thomas, W. E., and D. J. Ellar.** 1983. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal δ-endotoxin. FEBS Lett. **154:**362–368.
35. **Trisrisook, M., S. Pantuwatana, A. Bhumiratana, and W. Panbangred.** 1990. Molecular cloning of the 130-kilodalton mosquitocidal δ-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. Appl. Environ. Microbiol. **56:**1710–1716.
36. **Waalwijk, C., A. M. Dullemans, M. E. S. van Workman, and B. Visser.** 1985. Molecular cloning and nucleotide sequence of the Mr 28,000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. Nucleic Acids Res. **13:**8207–8217.
37. **Wirth, M. C., G. P. Georghiou, and B. A. Federici.** 1997. CytA enables CrylV endotoxins of *Bacillus thuringiensis* to overcome high levels of CrylV resistance in the mosquito *Culex quinquefasciatus*. Proc. Natl. Acad. Sci. USA **94:**10536–10540.
38. **Wirth, M. C., A. Delécluse, B. A. Federici, and W. E. Walton.** 1998. Variable

cross-resistance to Cry11B from *Bacillus thuringiensis* subsp. *jegathesan* in *Culex quinquefasciatus* (Diptera: Culicidae) resistant to single or multiple toxins of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **64:**4174–4179.

39. **Wirth, M. C., and G. P. Georghiou.** 1999. Selection and characterization of temephos resistance in a population of *Aedes aegypti* from Tortola, British Virgin Islands. J. Am. Mosq. Control Assoc. **15:**315–320.
40. **Wirth, M. C., W. E. Walton, and B. A. Federici.** Cyt1A from *Bacillus thuringiensis* restores toxicity of *Bacillus sphaericus* against resistant *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Entomol., in press.
41. **Wu, D., and F. N. Chang.** 1985. Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. FEBS Lett. **190:**232–236.
42. **Wu, D., and B. A. Federici.** 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. **175:**5276–5280.