APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1988, p. 2320–2321
0099-2240/88/092320-02$02.00/0

Vol. 54, No. 9

Toxicity of *Bacillus sphaericus* Crystal Toxin to Adult Mosquitoes

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Received 25 March 1988/Accepted 16 June 1988

Adult *Culex quinquefasciatus* mosquitoes were killed by alkaline-solubilized *Bacillus sphaericus* toxin when it was introduced by enema into the midgut of the insect but not when it was administered orally. Adult *Aedes aegypti* mosquitoes were not affected by the toxin.

Two species of bacteria, *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*, contain unique protein toxins that are lethal to the larvae of a variety of mosquitoes (9, 12, 15, 23, 24), but neither the molecular targets of these toxins nor their mechanisms of action are known. Because of their efficacy (12) and safety (7, 18, 20), these two bacilli provide an attractive alternative to the chemical control of mosquitoes (15, 22, 24). Although *B. thuringiensis* subsp. *israelensis* toxin is known to be lethal to adult mosquitoes (13, 14), the toxicity of *B. sphaericus* toxin to adult mosquitoes has not previously been demonstrated. Current advances in genetic engineering techniques make it likely that these toxins can be expressed in plants, and, because adult mosquitoes also feed on nectar, this phenomenon could supplement existing mosquito control systems in the same way that tomato plants containing the toxin gene of *B. thuringiensis* kill Lepidoptera larvae that feed on their leaves (10). In this paper we have demonstrated that adult *Culex quinquefasciatus* mosquitoes, but not *Aedes aegypti*, are killed by the soluble toxin of *B. sphaericus* when the toxin is administered via the anal route and that oral ingestion of this toxin is not lethal.

Bulk cultures of *B. sphaericus* 2362 were grown as described by Broadwell and Baumann (6) for 72 to 96 h to ensure >80% sporulation. The spore-crystal material was collected and washed twice in 0.1 M NaCl–0.01 M EDTA (pH 7.5) by centrifugation and stored at −20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described (16). The protein concentration was determined on samples solubilized in 0.3 M NaOH (0.25 ml) at 100°C for 10 min and mixed with 2.5 ml of a freshly prepared reagent consisting of 50 ml of 2% (wt/vol) Na_2CO_3 plus 1 ml of 0.5% (wt/vol) $CuSO_4 \cdot 5H_2O$ in 1% (wt/vol) sodium citrate; 0.25 ml of Folin reagent was then added (17). Insoluble material (e.g., spores) was removed by centrifugation before the absorbance was determined. Dry weight was determined after samples were heated at 105°C for 24 to 48 h.

Since spore-crystal mixtures plug the fine delivery tubes and syringe needles used in these procedures, soluble toxin had to be prepared. To obtain soluble toxin, the spore-crystal pellet was suspended by sonication in deionized water (1 g [wet weight] per 10 ml of water), washed twice in deionized water, and stirred in deionized water at room temperature for 3 h while being maintained at pH 12. After the insoluble material was removed by centrifugation, the pH was adjusted to 7.4 with 1 M Tris hydrochloride buffer, and the supernatant was sequentially filtered through cellulose acetate filters (0.45- and 0.22-μm pore sizes; Micro Filtration Systems, Dublin, Calif.). The sterile, soluble toxin was stored at −20°C. Spore counts taken before and after the alkaline extraction showed no detectable spore death during the extraction (data not presented). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of the solubilized material was similar to that obtained by Baumann et al. (3) (Fig. 1). The protein pattern, plus the lack of spore death, indicated that this material was a relatively pure form of the toxin complex similar to the forms obtained by others (3, 8). Spore-crystal mixtures for toxicity testing were prepared by lyophilization of unwashed cell pellets. The dried powder was stored at room temperature and diluted in deionized water for testing. *A. aegypti* (Linnaeus) and *C. quinquefasciatus* (Say) larval and adult mosquitoes were reared and bioassayed as previously described (13, 14) except that mortality counts were taken after 48 h, as recommended by the World Health Organization (21). The solubilized toxin was dialyzed for 10 to 14 h against insect saline containing 50 mM Tris hydrochloride (pH 7.4) prior to use (14). Statistical analyses were done by log probit analysis (SAS Institute, Cary, N.C.).

The concentration of spore-crystal powder that killed 50% of the *C. quinquefasciatus* larvae was 5 ng/ml, and for solubilized toxin the necessary concentration was 17.3 ng/ml. When administered to adult *C. quinquefasciatus* as an enema, the 50% lethal dose (expressed as the dose per milligram of mosquito body weight based on an average wet weight of 3.61 mg for *C. quinquefasciatus*) of the solubilized toxin was 11.1 ng/mg. Neither adult mosquitoes nor larvae of *A. aegypti* were susceptible to any form of the toxin, however, delivered, in agreement with previous studies showing *A. aegypti* insensitivity to *B. sphaericus* toxin (1, 8, 23). When fed orally as a single dose (200 ng) either in 10% sucrose or in saline, the *B. sphaericus* toxin did not cause any mortality to *C. quinquefasciatus*. The *B. sphaericus* toxin is reported to be activated by proteinases (6, 8), and since mosquito adults fed only sugar produce only a small amount of these enzymes (5), two doses of the soluble toxin were administered 24 h apart in an attempt to stimulate gut proteinase synthesis, but this procedure had no effect on adult-mosquito mortality. It is possible that the inability of orally administered *B. sphaericus* toxin to kill adult *C. quinquefasciatus* is due to its inactivation in the diverticulum before it passes into the midgut. Recently, Davidson et al. (8) and Broadwell and Baumann (6) have observed that gut extracts of *A. aegypti* larvae were as effective in activating the *B. sphaericus* toxin as extracts from *C. quinquefasciatus* larvae were, and they concluded that the difference in

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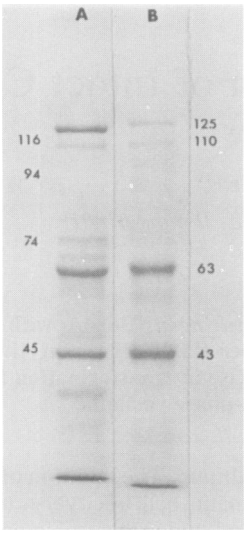

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of partially purified crystal toxin of *B. sphaericus*. Lane A, Crude spore-crystal mixture after harvesting and washing. Lane B, Soluble crystal toxin after alkaline solubilization and filtration. In both cases, 30 μg of protein was used. Numbers on the left indicate molecular weights (in thousands) of standards (the position of bovine albumin was defined as in reference 3), and those on the right indicate apparent molecular weights (in thousands) of the major proteins present in the samples.

susceptibility was associated with differences at the cellular level. If toxin susceptibility is related to the possession of specific midgut receptors, then it appears that these receptors are similar in both larval and adult mosquitoes, despite total replacement of the larval midgut epithelium during metamorphosis (19).

Because the orally ingested toxin of *B. sphaericus* in its present form is not lethal to adult mosquitoes, it does not appear to have much potential as a biological control agent produced by genetically engineered plants. However, knowledge of how the *B. sphaericus* toxin functions, combined with availability of the cloned toxin gene and knowledge of its gene sequence, could be used to produce a modified, orally toxic derivative. The recent cloning and sequencing of the *B. sphaericus* toxin gene in several laboratories makes production of such a derivative more likely (2, 4, 11, 12; R. E. Hurlbert et al., unpublished data).

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