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Insecticidal Activity of the Toxins from *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* Adsorbed and Bound on Pure and Soil Clays

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The release of transgenic plants and microorganisms expressing truncated genes from various subspecies of *Bacillus thuringiensis* that encode active insecticidal toxins rather than inactive protoxins could result in the accumulation of these active proteins in soil, especially when bound on clays and other soil particles. Toxins from *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *tenebrionis*, either free or adsorbed at equilibrium or bound on pure clay minerals (montmorillonite or kaolinite) or on the clay size fraction of soil, were toxic to larvae of the tobacco hornworm (*Manduca sexta*) and the Colorado potato beetle (*Leptinotarsa decemlineata*), respectively. The 50% lethal concentrations (LC_{50}) of free toxins from *B. thuringiensis* subsp. *kurstaki* were higher than those of both bound and adsorbed complexes of these toxins with clays, indicating that adsorption and binding of these toxins on clays increase their toxicity in diet bioassays. The LC_{50} of the toxin from *B. thuringiensis* subsp. *tenebrionis* that was either free or adsorbed on montmorillonite were comparable, whereas the toxin bound on this clay had higher LC_{50} and the toxin bound on kaolinite had lower LC_{50} than when adsorbed on this clay. Results obtained with the clay size fraction separated from unamended soil or soil amended with montmorillonite or kaolinite were similar to those obtained with the respective pure clay minerals. Therefore, insecticidal activity of these toxins is retained and sometimes enhanced by adsorption and binding on clays.

Bacillus thuringiensis produces a parasporal, proteinaceous, crystalline inclusion during sporulation. This parasporal inclusion, which may contain more than one type of insecticidal crystal protein, is released with the spore upon lysis of the sporangium. The inclusions (protoxins) are not toxic and require solubilization and enzymatic cleavage to yield the active toxins (7). Preparations of *B. thuringiensis*, usually as a mixture of cells, spores, and parasporal crystals, have been used as microbial insecticides for more than 30 years. Apparently, no unexpected toxicities have been observed, probably because *B. thuringiensis* does not survive or grow well in natural habitats, such as soil. Consequently, there is probably little or no production of the toxins in natural habitats, and the persistence of the introduced toxins is a function primarily of (i) the concentration added, (ii) the rate of consumption and inactivation by insect larvae, and (iii) the rate of degradation by the microbiota (16).

However, when the genes that code for the production of these toxins are genetically engineered into other organisms, e.g., plants and bacteria, that are indigenous or adapted to a specific habitat and, therefore, can persist and proliferate, the toxins may be synthesized in that habitat for extended times. Moreover, in the case of transgenic plants, only the usable portions of the plants will be harvested, and the remainder of the plant biomass containing the toxins will be incorporated into soil. Hence, the toxins will be present longer and at higher concentrations in soil than toxins introduced with commercial preparations of *B. thuringiensis*. These concentrations could exceed consumption, inactivation, and degradation, resulting in levels of toxins that could constitute a hazard to nontarget organisms and enhance the selection of toxin-resistant target insects, especially if the toxins are bound on soil constituents. Consequently, the effects of such interactions with soil particles on the persistence and activity of the toxins must be established to evaluate the potential risks associated with the release to the environment of transgenic plants and bacteria containing toxin genes. This is especially true for truncated genes that encode active toxins rather than the nontoxic protoxins. Currently, little information is available about the fate of the toxins from subspecies of *B. thuringiensis* in soil (17).

The toxins from *B. thuringiensis* subsp. *kurstaki* (active against lepidoptera) and subsp. *tenebrionis* (active against coleoptera) are rapidly adsorbed (at equilibrium) and tightly bound (after "ultimate" washing) on the clay minerals montmorillonite and kaolinite, either containing a mixed cation complement, homoionic to various cations ("clean" clays), or coated with two types of polymeric oxyhydroxides of Fe(III) ("dirty" clays), as well as on the clay size fraction separated from soil (16, 17, 19). Consequently, toxins released from transgenic plant or microbial biomass containing toxin genes from *B. thuringiensis* subsp. *kurstaki* or subsp. *tenebrionis* (calculated for transgenic plants to range from 5 to 100 ng of toxins per g of soil [17]) would be free and susceptible to microbial degradation in soil for only a short time. Studies with various proteins have shown that proteins bound on clay minerals become resistant to such degradation (14). Similar resistance was observed in preliminary studies with the toxins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* bound on clays (9). Moreover, the structure of these toxins did not appear to have been modified as the result of their binding on clays (16). When these toxins were added to soil, they were bound on the clay size fraction of soil and were still detectable by a dot blot enzyme-linked immunosorbent assay method (17) after 40 days in nonsterile soil.

The insecticidal activity of purified toxins from *B. thuringien-*

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sis subsp. *kurstaki* and subsp. *tenebrionis*, free or adsorbed at equilibrium or bound on montmorillonite, kaolinite, or the clay size fraction from soil, has been evaluated. The results showed that the toxins adsorbed and bound on clay retain insecticidal activity.

MATERIALS AND METHODS

Preparation of toxins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis*. The toxins from *B. thuringiensis* subsp. *kurstaki* (M_r = 66,000) were purified from a commercial preparation of cells, spores, and parasporal crystals produced by Abbott Laboratories (Dipel 2X). Dipel was washed twice with 1 M NaCl and twice with deionized water (DI-H_2O). The washed sediment was extracted overnight (18 h) with MOPS buffer (0.1 M 3-*N*-morpholinopropanesulfonic acid [Sigma] [pH 7.8] containing 0.5 M dithiothreitol [Boehringer Mannheim Corp.] and 1 M KSCN [Sigma]), the extract was dialyzed against DI-H_2O for 6 to 8 h with hourly changes of DI-H_2O, and 17.5 g of $(NH_4)_2SO_4$ per 100 ml of dialysate was added to precipitate the proteins. After 2 to 3 h, the precipitate was centrifuged at 27,000 × *g*, resuspended in a minimum amount of DI-H_2O, dialyzed for 8 h against DI-H_2O with several changes of DI-H_2O, and lyophilized (16, 18).

The toxin from *B. thuringiensis* subsp. *tenebrionis* (M_r = 68,000) was purified from commercial M-One (Mycogen Corp.) by adding 1 M Na_2CO_3 to M-One (1:5), diluting twice with DI-H_2O (the pH was maintained at 10), stirring for 2 to 3 h, and centrifuging at 16,300 × *g* for 15 min. The supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 6.0) for 3 days, with a daily change of buffer. The precipitated protein was recovered by centrifugation at 26,300 × *g* for 30 min, redissolved in a minimum amount of 100 mM Na_2CO_3 (pH 10), dialyzed for 8 h against DI-H_2O with several changes of DI-H_2O, and lyophilized (11, 16).

Preparation of clay minerals. The <2-μm fraction of montmorillonite and kaolinite, prepared from bentonite and kaolin (Fisher Scientific Co.), respectively, contained a mixed-cation complement consisting primarily of Na and Ca (mont-mix and kaol-mix, respectively) (13). The clays were also made homoionic to various mono-, di-, and trivalent cations, as previously described (2, 6, 8, 16). Briefly, the clays were suspended in a solution of the chloride salt of the appropriate cation (0.5 M) and centrifuged at 40,000 × *g* for 10 min, the pellets were resuspended in the cationic chloride solution, and the process was repeated twice. The pellets were then washed repeatedly with DI-H_2O, with centrifugation at 40,000 × *g*, until the supernatants were free of chloride, as determined by the absence of a precipitate of AgCl after the addition of a 1% solution of $AgNO_3$.

Soils. Studies were conducted with Kitchawan soil (a sandy loam soil collected at the Kitchawan Research Laboratory of the Brooklyn Botanic Garden, Ossining, N.Y.) that was not amended (K-soil, which naturally contained only kaolinite) or amended to 6% (vol/vol) with montmorillonite (K6M-soil) or kaolinite (K6K-soil, used as an internal control). These soil-clay mixtures have been used extensively in this laboratory in numerous studies on the effects of various physicochemical factors on the ecology of microbes and viruses in soil; therefore, there is a large data base available for these mixtures (14). The physicochemical characteristics of these soil-clay mixtures have been described (1, 15).

Separation of the clay size fraction from soil. The clay size fraction was separated from the soil-clay mixtures by sedimentation according to Stokes' law (3, 17). The silt and sand size fractions were not collected, as previous studies have shown that these toxins do not bind on these particles from this soil (17).

Adsorption and binding studies. The lyophilized toxins were dissolved in 0.1 M phosphate buffer (pH 6), and any insoluble material was discarded after centrifugation at 26,300 × *g* for 20 min. The protein content of the toxin preparations was determined by the Lowry method (10), using bovine serum albumin as the standard, and adjusted to the desired concentrations with the buffer. The toxins were added to suspensions of montmorillonite, kaolinite, and the clay size soil fraction, and the clay-toxin mixtures were rotated at 40 rpm on a motorized wheel at 24 ± 2°C for 3 h; the mixtures were centrifuged at 26,300 × *g*, and the concentration of the toxins in the supernatants was determined by the Lowry method. The difference between the amounts of toxins added and the amounts of toxins detected in the supernatants was used to calculate the amounts of toxins adsorbed at equilibrium on the clays (16, 17, 19). Bound complexes were prepared by sequentially washing the adsorbed complexes with DI-H_2O (pH 5.8), with centrifugation at 26,300 × *g*, until no more toxins were desorbed. The supernatants were analyzed after each wash for the presence of protein. The amounts of the toxins bound on the clays were calculated by subtracting the total of the amounts of the toxins detected in the equilibrium supernatant and in all washes from the amounts of toxins added (16, 17, 19).

Insect bioassays. The insecticidal activity of the toxins from *B. thuringiensis* subsp. *kurstaki* was determined with the larvae of the tobacco hornworm (*Manduca sexta*). Eggs of *M. sexta* and food medium were obtained from Carolina Biological Supply Co. The eggs, dispensed on solidified medium in petri plates, were incubated at 29 ± 1°C under a 40-W lamp for 3 to 5 days, when the eggs hatched. The medium was dispensed, after microwaving, in 10-ml amounts into vials (3 cm in diameter and 6 cm tall) and allowed to solidify (12). Toxins (free, adsorbed, or bound) were serially diluted with DI-H_2O to yield 0.025 to 10 μg/ml. Aliquots (100 μl) of the dilutions were distributed over the surface of the medium (8.55 cm^2), and after air drying, four to five second-instar larvae were added to each of triplicate vials. Mortality was determined after 3 and 7 days. Controls consisted of particles or toxins alone at the same concentrations as in the particle-toxin complexes (i.e., the concentrations of toxins were the same for free, adsorbed, and bound toxins).

Bioassays of the insecticidal activity of the toxins from *B. thuringiensis* subsp. *tenebrionis*, done at the Department of Entomology, University of Maryland, used larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*). Toxins (free, adsorbed, or bound) were serially diluted in DI-H_2O containing Triton X-100 (0.5 ml/liter) as a surfactant to yield 1 to 500 μg/ml. Freshly prepared artificial diet (4) was dispensed into each of 96 wells (1 ml per well) in a plastic tray and allowed to solidify. Aliquots (50 μl per well) of each dilution of the clays, toxins, and clay-toxin complexes were distributed over the surface of the medium (1.69 cm^2) and dried at room temperature for 30 min. One second-instar larva was added to each well, a sheet of Mylar was glued over the top of the trays to seal them, and two holes per well were punched into the Mylar for ventilation. The trays were incubated under a 16-h:8-h light-dark photoperiod at 24 ± 2°C for 3 days, when the numbers of dead larvae were determined; a larva was considered dead if it did not move a leg when prodded. Each test consisted of clay-toxin complexes, toxins and clays alone, and surfactant-water controls, with 32 larvae per variable. Test data were discarded if the mortality of the controls exceeded 20%.

Statistics. Concentration-mortality responses were analyzed by the PC-POLO probit (5) procedure (Le Ora Software, 1987) to obtain regression statistics, which included slope, lethal concentration at which 50% of the larvae were killed (LC_{50}), and confidence intervals (CIs). Likelihood ratio tests of equality and parallelism were performed to determine whether the intercepts and slopes of the response lines for free toxins and for adsorbed and bound clay-toxin complexes were the same. If the slopes for each were the same, the potency ratio and 95% CI were estimated from a composite line. Experiments were done in triplicate and repeated at least twice.

TABLE 1. LC_{50} of bound clay-toxin complexes from *B. thuringiensis* subsp. *kurstaki* to larvae of *M. sexta* after 3 days of exposure

Clay[a]	Mortality with clay alone[b]	LC_{50} (ng/100 μl) of clay-toxin complex
Mont-K	0	0.5
Mont-H	0	5.0
Mont-Na	0	45.0
Mont-Mg	0	50.0
Mont-Ca	0	47.0
Mont-La	0	46.0
Kaol-Na	0	36.0
Kaol-Ca	0	49.0

[a] The clays were montmorillonite (mont) and kaolinite (kaol) homoionic to the indicated cations.

[b] Mortality at each clay concentration was not significantly different from the natural mortality of the controls.

RESULTS AND DISCUSSION

Toxins from *B. thuringiensis* subsp. *kurstaki*. All samples containing toxins from *B. thuringiensis* subsp. *kurstaki* (free or adsorbed or bound on clays) were toxic to the larvae of *M. sexta* (Tables 1 to 3). Complexes of the toxins with clays were prepared in this laboratory (16, 17) and bioassayed (Tables 1 to 3). Table 1 represents bioassay data from the Department of Molecular Biology, University of Wyoming, and Tables 2 and 3 are bioassay data from this laboratory. The results from both laboratories were comparable, although the data in Table 1 are considered preliminary, as the bioassays were not repeated, and no CIs are available. Moreover, the free toxins were not bioassayed. The protoxins (LC_{50} = 5 μg/ml) and toxins (LC_{50} = 36 μg/ml) from *B. thuringiensis* subsp. *kurstaki* purified in this laboratory were also toxic to the third-instar larvae of *Trichoplusia ni* (18).

The data in Tables 1 and 2 were based on the assessment of mortality after 3 days, whereas the data in Table 3 were based on assessment after 7 days, which resulted in lower LC_{50} (i.e., more mortality at lower concentrations). The LC_{50} of the free toxins were consistent with the data of Schesser et al. (12), who

TABLE 2. LC_{50} of free and bound clay-toxin complexes from *B. thuringiensis* subsp. *kurstaki* for larvae of *M. sexta* after 3 days of exposure

Clay[a]	Mortality with clay alone[b]	Clay-toxin complex	
		LC_{50} (ng/100 μl)[c]	95% CI
Mont-H	0	18.6	10.73–29.75
Mont-K	0	31.9	21.82–45.29
Mont-Mg	0	30.3	16.55–52.64
Mont-La	0	50.2	28.12–114.52
Mont-mix	0	29.5	13.00–55.44
Kaol-mix	0	55.4	27.61–99.18

[a] The clays were montmorillonite (mont) and kaolinite (kaol) either with a mixed-cation complement (mont-mix and kaol-mix) or homoionic to the indicated cation.
[b] Mortality at each clay concentration was not significantly different from the natural mortality of the controls.
[c] LC_{50} of free toxins = 172.7 ng/100 μl with a 95% CI of 122.56 to 256.48 ng/100 μl.

reported an LC_{50} of 59.6 ng/100 μl with a 95% CI of 47.22 to 72.97 ng/100 μl. Higher LC_{50} were obtained with free toxins than with complexes of the toxins with clays, both adsorbed and bound, indicating that adsorption and binding of the toxins from *B. thuringiensis* subsp. *kurstaki* increased their toxicity. This increase in apparent toxicity may have been a result of the toxins being localized by binding on the clays; i.e., more toxins were ingested by the larvae as clay-toxin complexes than when the free toxins were uniformly spread over the surface of the food medium. No mortality was observed with clays alone, even when the amounts of the clays were greater than those present in the clay-toxin complexes.

Montmorillonite (a swelling 2:1 Si-Al clay mineral with a high cation-exchange capacity and specific surface area [14]) bound greater amounts of the toxins from *B. thuringiensis* subsp. *kurstaki* than kaolinite (a nonswelling 1:1 Si-Al clay), and the toxins partially entered (intercalated) the interlayer space of montmorillonite but not that of kaolinite (16). However, the LC_{50} of toxins complexed with montmorillonite or kaolinite were not significantly different (Tables 1 to 3). Adsorption of the toxins from *B. thuringiensis* subsp. *kurstaki* on montmorillonite was affected by the type of cation to which the clay was made homoionic. Adsorption and binding of the toxins generally decreased as the valency of the charge-compensating cation increased (16). This was reflected in the LC_{50}, which were generally lower for complexes with clays made homoionic to monovalent cations (Tables 1 and 2). Similar LC_{50} were obtained with toxins adsorbed and bound on the same clay (Table 3). Only about 10% of the toxins from *B. thuringiensis* subsp. *kurstaki* adsorbed at equilibrium on the clays were desorbed by washing with DI-H_2O (16), indicating that the toxins in the bound complexes were as insecticidal as those loosely adsorbed and more insecticidal than free toxins. The LC_{50} of toxins bound on the clay size fraction separated from soil, either unamended (K-soil) or amended with montmorillonite (K6M-soil), were similar to those obtained with toxins bound on montmorillonite and kaolinite (Table 3), indicating that pure clays can be used to model the adsorption-binding and bioactivity of the toxins from *B. thuringiensis* subsp. *kurstaki* on soil clays.

TABLE 3. LC_{50} of free and clay-toxin complexes from *B. thuringiensis* subsp. *kurstaki* for larvae of *M. sexta* after 7 days of exposure[a]

Clay[b]	Mortality with clay alone[c]	Clay-toxin complex			
		Adsorbed		Bound	
		LC_{50} (ng/100 μl)[d]	95% CI	LC_{50} (ng/100 μl)[d]	95% CI
Mont-mix	0	22.0	16.42–27.80	30.7	20.10–50.43
Kaol-mix	0	18.0	11.33–27.55	23.0	14.01–40.32
K-soil	0	21.9	18.67–25.77	21.4	15.82–29.12
K6M-soil	0	23.3	18.21–29.12	22.5	18.71–27.00

[a] The toxins were evaluated after both equilibrium adsorption and binding on the clays.
[b] The clays were montmorillonite (mont) and kaolinite (kaol) with a mixed-cation complement (mont-mix and kaol-mix) and the clay size fraction separated from Kitchawan soil, unamended (K-soil) or amended with 6% montmorillonite (K6M-soil).
[c] Mortality at each clay concentration was not significantly different from the natural mortality of the controls.
[d] LC_{50} of free toxins = 90.4 ng/100 μl with a 95% CI of 58.58 to 144.06 ng/100 μl.

Toxin from *B. thuringiensis* subsp. *tenebrionis*. All samples containing toxin from *B. thuringiensis* subsp. *tenebrionis* (free or adsorbed or bound on clays) were toxic to the larvae of *L. decemlineata* (Table 4). Similar results were obtained in preliminary studies with a surrogate (not identified for proprietary reasons) for the Colorado potato beetle in bioassays conducted by Mycogen Corp. with bound clay-toxin complexes prepared in this laboratory (data not shown). No statistically significant mortality of the larvae of *L. decemlineata* was observed with the clays alone or in the surfactant-water controls. Two batches of toxin from *B. thuringiensis* subsp. *tenebrionis*, purified from different containers of M-One several months apart, were used in the preparation of the clay-toxin complexes, which may explain the differences in LC_{50} between the two batches. For each batch, LC_{50} obtained with free toxin and those obtained with toxin adsorbed at equilibrium were generally similar. In contrast to the results obtained with the toxins from *B. thuringiensis* subsp. *kurstaki*, the toxin from *B. thuringiensis* subsp. *tenebrionis* bound on montmorillonite had significantly higher LC_{50} than free and adsorbed toxin. Approximately 30% of the toxin from *B. thuringiensis* subsp. *tenebrionis* adsorbed at equilibrium on montmorillonite and kaolinite was desorbed by washing with DI-H_2O (16), resulting in the bound complexes containing significantly less toxin than the equilibrium adsorption complexes. Apparently, the larvae had to ingest considerably more clay to get an equivalent dose of toxin. This effect was not apparent with adsorbed and bound complexes with toxins from *B. thuringiensis* subsp. *kurstaki*, probably because the clay/toxin ratio of the complexes differed by only about 10%. However, this difference in the content of toxin from *B. thuringiensis* subsp. *tenebrionis* does not account for the large differences in LC_{50} between adsorbed and bound complexes with montmorillonite, suggesting that the toxin from *B. thuringiensis* subsp. *tenebrionis* bound on montmorillonite was less insecticidal than that loosely adsorbed or free.

Although adsorption of the toxins from *B. thuringiensis* subsp. *tenebrionis* generally decreased as the valency of the charge-compensating cations increased (16), this was not reflected in the LC_{50}, except with montmorillonite homoionic to La, which had higher LC_{50} for both adsorbed and bound complexes, similar to what was observed with the toxins from *B. thuringiensis* subsp. *kurstaki* complexed with this homoionic clay (Table 2). The LC_{50} of the toxins from *B. thuringiensis* subsp. *tenebrionis* adsorbed at equilibrium on the clay size fraction separated from soil, both unamended (K-soil) and amended with montmorillonite (K6M-soil) or kaolinite (K6K-soil), were also lower than those of the respective bound complexes. Different results were obtained with the toxin from *B.*

TABLE 4. LC_{50} and relative potencies of free and clay-toxin complexes from *B. thuringiensis* subsp. *tenebrionis* for larvae of *L. decemlineata* after 3 days of exposure[a]

Clay[b]	Mortality with clay alone[c]	Clay-toxin complex					
		Adsorbed			Bound		
		LC_{50} (μg/50 μl)	95% CI	Relative potency[d]	LC_{50} (μg/50 μl)	95% CI	Relative potency
Batch 1[e]							
Mont-Na	0	1.5	1.07–2.05	0.86	17.8	12.93–24.57	0.07
Mont-K	0	1.7	1.13–2.52	0.77	13.4	8.95–20.18	0.10
Mont-Mg	0	1.2	0.81–1.71	1.05	9.5	6.51–13.43	0.13
Mont-La	0	2.6	1.80–3.56	0.52	96.1	40.69–264.32	0.01
Mont-mix	0	1.3	0.75–2.13	1.05	14.1	9.45–21.05	0.10
Kaol-mix	0	4.5	3.05–6.61	0.28	1.4	0.95–1.91	0.91
K6M-soil	0	0.5	0.33–0.79	2.38	1.8	1.16–2.64	0.70
Batch 2[f]							
Kaol-K	0	0.4	0.15–0.75	0.47	0.2	0.08–0.34	0.95
K-soil	0	0.3	0.16–0.38	0.88	2.3	1.30–4.40	0.10
K6K-soil	0	0.2	0.10–0.27	1.16	0.4	0.25–0.63	0.51

[a] The toxins were evaluated after both equilibrium adsorption and binding on the clays. Two different batches of *B. thuringiensis* subsp. *tenebrionis* toxins (M-One) were used.
[b] The clays were montmorillonite (mont) and kaolinite (kaol) with a mixed-cation complement (mont-mix or kaol-mix) or homoionic to the indicated cations and the clay size fraction separated from Kitchawan soil, unamended (K-soil) or amended with 6% montmorillonite (K6M-soil) or 6% kaolinite (K6K-soil).
[c] Mortality at each clay concentration was not significantly different from the natural mortality of the controls.
[d] Potency of adsorbed or bound toxins relative to the potency of free toxins at all concentrations.
[e] LC_{50} of free toxins from batch 1 = 1.3 μg/50 μl with a 95% CI of 1.02 to 1.62 μg/50 μl.
[f] LC_{50} of free toxins from batch 2 = 0.2 μg/50 μl with a 95% CI of 0.10 to 0.28 μg/50 μl.

thuringiensis subsp. *tenebrionis* adsorbed or bound on kaolinite: toxin from batch 1 bound on kaolinite with a mixed-cation complement (kaol-mix) and from batch 2 bound on kaolinite homoionic to K had lower LC_{50} than the respective adsorbed clay-toxin complexes.

Direct comparison of the LC_{50} between batches 1 and 2 was not possible because of the differences in the LC_{50} of the free toxin. However, relative potency values, calculated on the basis of the potency of the free toxin prepared from each batch being equal to 1, enable some comparison of the data. All bound complexes, with the exception of those with kaol-mix and kaolinite-K, which approached a potency of 1, had lower potencies than both the respective adsorbed complexes and the free toxin. In contrast, the potencies of the adsorbed complexes were either higher than or close to those of the free toxin, with the exception of complexes with montmorillonite-La, kaolinite-K, and kaol-mix.

In conclusion, insecticidal activity was retained and sometimes even enhanced when the toxins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* were adsorbed and bound on pure clay minerals or on the clay size fraction of soil. Adsorption on clays was rapid and essentially complete within 30 min (16, 19), and toxins bound on clay were more resistant to degradation by microorganisms than free toxins (9), indicating that these toxins in transgenic biomass would not be present long in soil in the free state susceptible to biodegradation. Consequently, the toxins could accumulate and retain insecticidal activity when bound on clays in soil. These results are important in evaluating the potential risks associated with the release to the environment of transgenic plants and bacteria containing toxin genes from subspecies of *B. thuringiensis*. This is especially relevant to transgenic organisms containing truncated toxin genes that code for active toxins rather than for inactive protoxins, as larvae that ingest the toxins do not require a high gut pH and appropriate proteases for solubilization and cleavage of the protoxins, respectively (7), which reduces the specificity of the toxins. Therefore, nontarget larvae could be susceptible to the toxins, and the accumulation of the toxins could also result in the selection of toxin-resistant target species.

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REFERENCES

1. **Babich, H., and G. Stotzky.** 1977. Effect of cadmium on fungi and on interactions between fungi and bacteria in soil: influence of clay minerals and pH. Appl. Environ. Microbiol. **33:**1059–1066.
2. **Dashman, T., and G. Stotzky.** 1982. Adsorption and binding of amino acids on homoionic montmorillonite and kaolinite. Soil Biol. Biochem. **14:**447–456.
3. **Day, P.** 1965. Particle fractionation and particle size analysis, p. 545–567. *In* C. A. Black (ed.), Methods of soil analysis. Part 1. Physical and mineral properties, including statistics of measurement and sampling. American Society of Agronomy, Madison, Wis.
4. **Everich, R. C., O. T. Forrester, and G. P. Dively.** Unpublished data.
5. **Finney, D. J.** 1971. Probit analysis. Cambridge University Press, Cambridge.
6. **Harter, R. D., and G. Stotzky.** 1971. Formation of clay-protein complexes. Soil Sci. Soc. Am. Proc. **35:**383–389.
7. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
8. **Jackson, M. L.** 1969. Soil chemical analysis, advanced course, 2nd ed. Department of Soil Science, University of Wisconsin, Madison.
9. **Koskella, J., and G. Stotzky.** 1994. Resistance of the toxins from *Bacillus thuringiensis* to biodegradation when bound on clay minerals, abstr. Q-71, p. 400. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
11. **MacIntosh, S. C., S. L. McPherson, F. J. Perlak, P. G. Harrone, and R. L. Fuchs.** 1990. Purification and characterization of *Bacillus thuringiensis* var.

tenebrionis insecticidal proteins produced in *E. coli*. Biochem. Biophys. Res. Commun. **170:**665–672.
12. **Schesser, J. H., K. J. Kramer, and L. A. Bulla, Jr.** 1977. Bioassay for homogeneous parasporal crystal of *Bacillus thuringiensis* using the tobacco hornworm, *Manduca sexta*. Appl. Environ. Microbiol. **33:**878–880.
13. **Stotzky, G.** 1966. Influence of clay minerals on microorganisms. II. Effect of various clay species, homoionic clays, and other particles on bacteria. Can. J. Microbiol. **12:**831–848.
14. **Stotzky, G.** 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses, p. 305–428. *In* P. M. Huang and M. Schnitzer (ed.), Interactions of soil minerals with natural organics and microbes. Soil Science Society of America, Madison, Wis.
15. **Stotzky, G., M. W. Broder, J. D. Doyle, and R. A. Jones.** 1993. Selected methods for the detection and assessment of ecological effects resulting from the release of genetically engineered microorganisms to the terrestrial environment. Adv. Appl. Microbiol. **38:**1–98.
16. **Tapp, H., L. Calamai, and G. Stotzky.** 1994. Adsorption and binding of the insecticidal proteins of *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* on clay minerals. Soil Biol. Biochem. **26:**663–679.
17. **Tapp, H., and G. Stotzky.** 1995. Dot blot enzyme-linked immunosorbent assay for monitoring the fate of insecticidal toxins from *Bacillus thuringiensis* in soil. Appl. Environ. Microbiol. **61:**602–609.
18. **Venkateswerlu, G., and G. Stotzky.** 1990. A simple method for the isolation of the antilepidopteran toxin from *Bacillus thuringiensis* subsp. *kurstaki*. Biotechnol. Appl. Biochem. **12:**245–251.
19. **Venkateswerlu, G., and G. Stotzky.** 1992. Binding of the protoxin and toxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* on clay minerals. Curr. Microbiol. **25:**1–9.