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Mineralization of the *Bacillus thuringiensis* Cry1Ac Endotoxin in Soil

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Although a number of studies have been done describing the fate of *Bacillus thuringiensis* insecticidal endotoxins in soil, there is conflicting information on the persistence of this class of insecticidal toxins. This is partly due to methodological limitations in many of the previous studies. In the experiments reported here, ^{14}C-labeled *B. thuringiensis* Cry1Ac endotoxin was used to study its mineralization in soil incubated under controlled conditions. Fifty-nine percent of the radiolabeled Cry1Ac was recovered as $^{14}CO_2$ at the end of the 20 day incubation period. The addition of 4.5% corn residues stimulated mineralization of [^{14}C]Cry1Ac toxin, and mineralization of glucose was 3.6 times faster than that of the Cry1Ac toxin, indicating that the soil was microbiologically and metabolically active. Because only low mineralization (approximately 6%) of the radiolabeled toxin was observed in autoclaved soil, the current findings indicate that microbial processes play a major role in the dissipation of the Cry1Ac endotoxin in soil. The results of this study suggest that there may be limited risk of the bioaccumulation of Cry1Ac in soil due to the eventual release of this insecticidal toxin by Bt-protected crops.

KEYWORDS: ***Bacillus thuringiensis*** **crystal toxin; environmental fate; genetically modified crops; microbial degradation**

INTRODUCTION

The Gram-positive, spore-forming bacterium *Bacillus thuringiensis* (Bt) is widely distributed in the environment and has been isolated from several habitats, including soil, insect cadavers, stored grain, and the phyllosphere (*1, 2*). During sporulation, Bt cells produce parasporal, crystal, inclusion bodies composed of a mixture of crystal (Cry) endotoxins, which are lethal upon ingestion to a variety of insect larvae in the orders Lepidoptera, Diptera, and Coleoptera (*3*). Classification of Cry endotoxins is currently based on amino acid sequence identity and is roughly correlated with the taxonomic order of susceptible insect species. Bt-based formulations, consisting of formulated mixture of spores and crystals, have been used for more than four decades for the control of pests in agricultural crops and forestry (*4*). Due to their high selectivity and low toxicity to mammals and nontarget insects, Bt-based insecticides are compatible with integrated pest management and organic farming programs (*5*).

The use of genetically modified (GM) plants expressing Cry endotoxins has the potential to greatly reduce environmental and health costs associated with the use of synthetic chemical-based insecticides (*6, 7*). Since the mid-1990s, insect-resistant plants expressing the CryAc endotoxin have been used as an effective tool to control a wide range of insect pests (*8*).

Several studies have shown that Bt-protected crops may secrete Cry toxins from roots into the soil or release the toxins through decomposition of crop residues (*9–11*). In recent years, concerns about the impact of Cry toxins on the soil ecosystem have been expressed in public and scientific debate; however, they have not yet been adequately addressed (*12*). Some studies have demonstrated that Cry toxins are readily and strongly sorbed onto soil particles, thereby reducing their bioavailability to microbial degradation (*13*), and bound toxins have been shown to retain their insecticidal activity, persisting in soil for up to 6 months (*14, 15*). In contrast, other studies have reported rapid decay rates of Cry toxins in soil, thus excluding the risk of bioaccumulation in soil after continuous cultivation of Bt-protected crops (*16–18*). For example, Sims and Holden (*16*) estimated that Cry1Ac toxin added to a silt loam soil as corn residues or purified toxin had half-life values of 8.3 and 1.6 days, respectively. Similar results were reported by other authors (*11, 19, 20*). Contrasting results can partly be caused

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by differences in soil type, environmental conditions, and type of Cry toxin examined. However, one major source of differences seen in the soil persistence of Cry toxins is the lack of reliable and accurate analytical methods (*5*).

In the present study, we synthesized ^{14}C-labeled Cry1Ac toxin and used it in soil studies to determine if the toxin is actively degraded by the soil microbial community or if it persists within soil as a bound residue.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Escherichia coli* strain JM103(pOS4201) (*21*) encoding for the full-length CryAc *B. thuringiensis* toxins was obtained from the Bacillus Genetic Stock Center (Columbus, OH). The *E. coli* strain was grown for 72 h at 37 °C in modified E medium (*22*), containing 73 mM K_2HPO_4, 17 mM $NaNH_4PO_4 \cdot 4H_2O$, 10 mM citric acid, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.5% glucose, 50 μg mL^{-1} ampicillin, and 50 μCi mL^{-1} of [^{14}C]glucose (specific activity = 9.6 × 10^3 MBq mmol^{-1}, radiopurity ≥ 95%) (Sigma, St. Louis, MO). After a 24 h incubation period, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added to the culture to induce the overexpression of the insecticidal endotoxin (*23*).

Protein Purification. The CryIAc endotoxin was purified from *E. coli* JM103(pOS4201) cells using a slight modification of the method reported by Almond and Dean (*24*). Briefly, cells were centrifuged and resuspended in 50 mL of lysis buffer containing 15% sucrose, 50 mM EDTA, 50 mM Tris (pH 8.0), 10 μg mL^{-1} lysozyme, and two complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). The suspension was incubated for 1 h at 37 °C and then overnight at 4 °C. Cells were lysed by sonification using a model W-225R cell disruptor (Misonix, formerly Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The solution was centrifuged, and the resulting pellet was washed three times with 0.5 M NaCl containing 2% Triton X-100, followed by six washes with 0.5 M NaCl and one final wash with ultrapure deionized water. The CryIAc toxin crystals were solubilized by incubation for 2 h at 37 °C in 50 mM Tris (pH 9.5) and 10 mM dithiothreitol. Solubilized crystal crude extracts were subjected to 0–20% ammonium sulfate fractionation, and the partially purified crystal toxin was dialyzed for 2 days in 50 mM Tris (pH 9.5) containing 1 mM dithiothreitol. After dialysis, samples were concentrated using a Centricon concentrator (Millipore Co., Milford, MA), and total protein concentration was determined using the Bradford assay (*25*). Protein in the sample was separated by electrophoresis on 12.5% SDS-PAGE gels for a minimum of 4 h at 120 V. The 130 kDa Cry toxin band was excised from gels, and the protein was electroeluted from gel bands using a Bio-Rad (Hercules, CA) model 422 electroelutor.

The presence of the Cry endotoxin in the eluate was verified by an enzyme-linked immunosorbent assay using the QuantiPlate kit for Cry1Ab/Cry1Ac toxins (Envirologix, Portland, ME) and the procedure described by Accinelli et al. (*26*). The overall incorporation of the ^{14}C into Cry1Ac was determined by using a liquid scintillation counting on the eluted protein. The radiolabeled Cry1Ac endotoxin dissolved in the elution buffer (3 mM Tris base, 192 mM glycine, and 0.1% SDS) was concentrated by rotary evaporation and directly used in the mineralization study outlined below.

Mineralization Study. An agricultural soil from Becker, MN, was selected for this study. The Becker soil used is classified as a Hubbard loamy sand, with a pH of 5.8, 86.0% sand, 6.0% clay, and 1.5% organic carbon. Soil texture and organic carbon content were determined by the hydrometer method and by dichromate oxidation, respectively. Soil pH was measured in a 1:2 (w/w) soil/deionized water mixture. Soil samples were collected from the upper 15 cm soil layer, passed through a 2 mm diameter sieve, and stored at 4 °C until used. For sterile soil studies, soil was autoclaved at 120 °C for 60 min on three successive days. Two gram aliquots of natural, microbiologically active or autoclaved soil were weighed into 20 mL scintillation vials, and ^{14}C-labeled Cry1Ac endotoxin (approximately 2.2 × 10^{-4} MBq mL^{-1}) was added to attain a final concentration of 10 μg g^{-1} of soil. Sterile and nonsterile soils receiving only elution buffer were used as negative controls.

Samples of microbiologically active soil amended with corn residues were also included in this study. Corn residues were obtained from a non-Bt corn hybrid harvested at physiological maturity from a corn field located at Becker, MN. Dried residues consisting of leaves and stalks (0.62% total N, 42.0% total C) were ground at 20 mesh and incorporated into soil samples to a final concentration of 4.5%. Soil moisture was adjusted to the gravimetric content at −33 kPa using sterile water, and the soil was gently mixed with a sterile spatula. Scintillation vials were fitted with 1.5 mL microcentrifuge tubes containing 1 mL of a 1 M NaOH solution and sealed with rubber stoppers. Samples were incubated for 20 days in the dark at 25 °C. Endotoxin mineralization was monitored by trapping the evolved $^{14}CO_2$ in the NaOH solution. Solution was replaced at each sampling time, which also facilitated aeration. Trapped $^{14}CO_2$ in 1 mL aliquots of NaOH solution was determined by liquid scintillation counting using a Packard (Meriden, CT) model 1500 Tri-Carb analyzer as previously described (*26*). Samples were kept in the dark for approximately 24 h prior to scintillation counting to eliminate potential chemiluminescence.

The metabolic potential of microorganisms in soil samples was estimated by measuring mineralization of [^{14}C]glucose as described by Becker et al. (*27*). An aqueous solution of ^{14}C-labeled (3.7 × 10^{-3} MBq mL^{-1}) and unlabeled glucose was applied to soil mixture to obtain a final concentration of 1 μg g^{-1} glucose. Unlabeled glucose (chemical purity > 99%) and uniformly labeled [^{14}C]glucose (specific activity = 9.6 MBq mmol^{-1}, radiopurity ≥ 98%) were purchased from Sigma (St. Louis, MO). After the experiment had ended (after 5 and 20 days of incubation for the glucose and Cry1Ac experiments, respectively), the remaining ^{14}C residues in soil were quantified using a model 307 sample oxidizer (Packard Instrument Co.). Triplicate 0.3 g (dry weight) soil subsamples from each of the three replicates from the ^{14}C-labeled Cry1Ac and [^{14}C]glucose experiments were combusted. The radioactivity of trapped $^{14}CO_2$ was counted using a liquid scintillation counter as described above. Radiolabeled standards were combusted prior, during, and after the experimental samples to determine oxidation and trapping efficiency.

RESULTS AND DISCUSSION

Mineralization of the ^{14}C-labeled Cry1Ac endotoxin in both unamended and amended nonsterile Becker soil is shown in **Figure 1**. Mineralization did not proceed rapidly until after a 24 h lag period. Once evolution of $^{14}CO_2$ occurred, samples incubated with additional organic material in the form of corn residue mineralized the toxin at a faster rate than those samples without added organic material (**Table 1**). Cry1Ac toxin-containing soil samples incubated with 4.5% corn residues mineralized the toxin nearly twice as fast as those receiving Cry1Ac alone, with mineralization rates of 0.76 versus 0.41 μg g^{-1} day^{-1} for Cry1Ac toxin with and without added organic material, respectively. Overall, mineralization of the [^{14}C] Cry1Ac toxin was effective, with 63 and 59% converted to $^{14}CO_2$ within samples incubated with and without additional organic material, respectively, over the 20 day period. In contrast, there was little $^{14}CO_2$ evolution in both sterile (7.5% evolved) and nonsterile soil (6% evolved) receiving only elution buffer (**Figure 1**). It has been previously shown that autoclaving does not stop all microbial functions (*28*), which is consistent with the slight increase in $^{14}CO_2$ evolution within the sterilized samples.

After the experiment had run its course, triplicate soil samples were oxidized to determine the amount of radioactivity remaining in the soil as either biomass or sorbed to the soil particles. As shown in **Table 2**, the majority of the radioactivity remained within the soil when applied to sterilized soils. In contrast, less than half this amount remained as bound residue in nonsterile, natural soil, further supporting the contention that an active microbial population is needed to degrade the labeled toxin.

^{14}C-Labeled glucose was used in the present study to illustrate the metabolic potential of the soil microbial community within

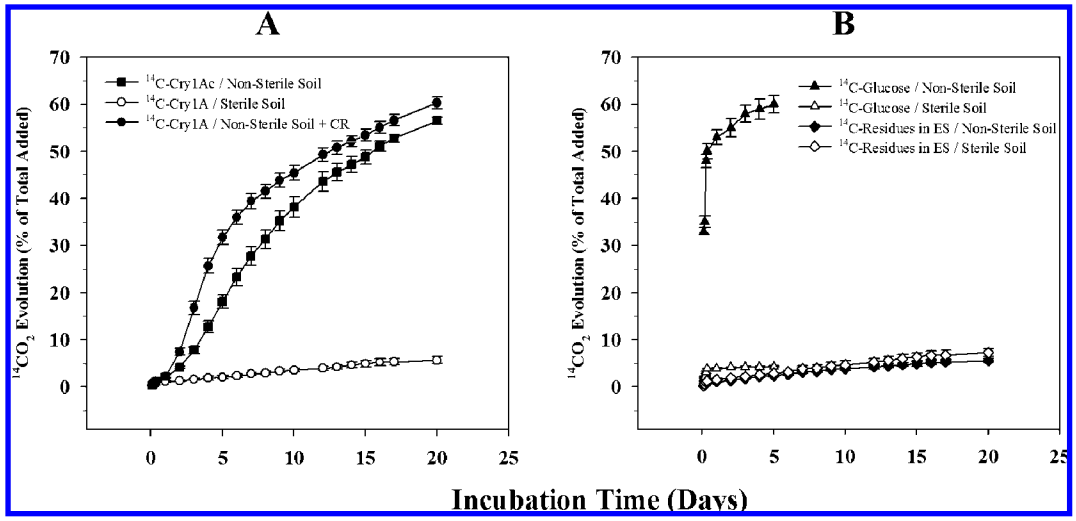

Figure 1. Mineralization of [^{14}C]Cry1Ac toxin (**A**) and [^{14}C]glucose (**B**) in nonsterile and sterile soil with or without addition of corn residues (CR). The efficiency of ^{14}C incorporation in the endotoxin was estimated by measuring mineralization of radiolabeled residues remaining in the elution solution (ES) from nonsterile and sterile soil.

Table 1. Mineralization Rates of [^{14}C]Cry1Ac (10 μg g^{-1}) and [^{14}C]Glucose (1 μg g^{-1}) in Unamended, Nonsterile, Soils and in Nonsterile Soils Amended with 4.5% Corn Residue (CR)[a]

treatment	mineralization rate (μg g^{-1} day^{-1})
[^{14}C]Cry1Ac	0.41 ± 0.04
[^{14}C]Cry1Ac + CR	0.76 ± 0.05
[^{14}C]glucose	2.8 ± 0.7

[a] Rates were determined from the linear portions ($r^2 = 0.94$) of the degradation profiles, at days 1−5 for the Cry1Ac and for the first 4 h for the glucose amendments.

Table 2. Proportion of the Total Radioactivity of Applied [^{14}C]Cry1Ac and [^{14}C]Glucose Mineralized as $^{14}CO_2$ and Remaining ^{14}C Residues in Nonsterile and Sterile Soils[a]

soil status	treatment	% mineralized	% remaining in soil
nonsterile	[^{14}C]Cry1Ac	58.8 ± 1.4	45.8 ± 2.1
	[^{14}C]Cry1Ac + CR	63.0 ± 1.4	41.8 ± 2.0
	[^{14}C]glucose	58.1 ± 1.2	44.6 ± 2.5
sterilized	[^{14}C]Cry1Ac	6.0 ± 0.8	81.4 ± 5.7
	[^{14}C]glucose	4.4 ± 0.2	75.1 ± 5.0

[a] Experiments conducted with the [^{14}C]Cry1Ac toxin included samples amended with 4.5% corn residue (CR). Numbers represent the mean of triplicate samples ± standard errors.

the Becker soil. It has been shown that mineralization of glucose reflects the metabolic potential of a particular soil because it is so easily incorporated into the TCA cycle (*27*). If a soil contains an active microbial community, glucose should be degraded relatively quickly. In the Becker soil used for this study, uniformly labeled [^{14}C]glucose was degraded rapidly (**Figure 1B**). Within the first 2 h after the addition of 1 μg g^{-1} glucose, nearly 34% was mineralized to $^{14}CO_2$ with evolution of $^{14}CO_2$ leveling off within the first 8 h of incubation. This is indicative of a highly active microbial community within the soil. The higher percentage of ^{14}C residue remaining in the sterilized soil further supports this contention (**Table 2**). One possible explanation for the difference in glucose mineralization compared to that of the insecticidal endotoxin is the fact that glucose is easily incorporated into the TCA cycle and efficiently converted to energy, whereas the endotoxin is a rather large 130 kDa protein. This size molecule would most likely need to be initially broken down by exoenzymes from the microbes and then imported into the cells, where it could then be used to create energy and biomass.

Results from the present study clearly demonstrate that the tested Bt toxin was degraded by the soil microbial community and did not persist within the soil as has been previously reported (*29, 30*). Rather, the toxin was actively metabolized by the soil microbial community at a fairly fast rate within the first 5 days of incubation. Approximately 50% of the toxin was mineralized after 12 and 15 days in soil amended or nonamended with organic matter, respectively.

Conflicting data exist as to the effect of Bt toxin on soil microbial communities. In contrast to our results, several studies have reported that the toxin may persist within the soil for several months, which could result in changes to the soil microbial community. For example, Vettori et al. (*30*) reported that *B. thuringiensis* subsp. *kurstaki* toxin persisted in soil for up to 28 months. Other studies, however, reported that Bt toxin did not affect the functional components of the soil microbial communities. For example, Flores et al. (*31*) showed no significant difference in the enzymatic activity of soils amended with biomass from Bt or non-Bt corn with respect to the enzymes important in plant degradation. Likewise, Griffiths et al. (*32*) observed that only soil type resulted in significant differences in the soil microbial community response to Bt toxin addition to soil. Similarly, Shen et al. (*33*) observed no difference in the functional diversity, microbial species richness, or microbial enzyme activities in microbial communities in the rhizospheres of Bt- and non-Bt cotton. In contrast, Mulder et al. (*34*) reported short-term effects of Bt on soil microbial communities. These effects included a spike in microbial respiration as well as an overall increase in bacterial numbers and an increase in microbial diversity, illustrating the dynamic nature of the soil microbial community.

In summary, to our knowledge, this is the first study that used ^{14}C-labeled Cry toxins to determine the direct effects of the toxin on mineralization by the soil microbial community. The results presented here support earlier studies outlined above, showing that Bt toxin appears to have no significant negative effect on the soil microbial community. Moreover, our results support the contention that Bt toxin eventually released into the soil by the incorporation of plant residues and/or from root exudation by GM crops is actively degraded by the soil

microbial community and does not persist in soil for significant lengths of time.

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