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Isolation of *Bacillus thuringiensis* from Stored Tobacco and *Lasioderma serricorne* (F.)

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Bacillus thuringiensis was isolated from dried tobacco residues and dead tobacco beetles (*Lasioderma serricorne* (F.); Coleoptera: Anobiidae) collected in a large number of locations worldwide. Eighty-eight samples of stored tobacco were analyzed and yielded 78 *B. thuringiensis* strains which were characterized on the basis of parasporal crystal morphology, sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles, and the results of an immunoblot analysis of the insecticidal crystal proteins. Flagellar antigen identification was used to differentiate selected isolates. Strains that produced rhomboidal crystals associated with the Coleoptera-specific pathotype (Cry III group) were the most abundant strains (59% of the isolates). Preliminary toxicity assays were performed with *L. serricorne* larvae, and the results suggested that activity is not restricted to isolates related to the Coleoptera-specific group. The results of our survey indicate that *B. thuringiensis* is part of the natural microflora in the stored-tobacco environment and that this special habitat represents a source of *B. thuringiensis* isolates that may be used to control stored-product pests.

Tobacco (*Nicotiana tabacum* L.) is the economically most important nonfood crop worldwide (11), as well as the plant that is used most widely for genetic engineering studies (12, 29). The cured leaves, which are used for cigarette manufacturing, are stored in redried form (relative humidity, 11 to 12%) usually for 2 years, during which fungi, bacteria, and insects may affect the quality of the product. Two species of insects attack stored tobacco, the tobacco beetle (*Lasioderma serricorne* (F.)), the most widespread and destructive pest, and the tobacco moth (*Esphestia elutella* Hb).

To avoid considerable damage of raw or processed tobacco, chemical insecticide applications are often necessary to control the insect populations during tobacco ageing in industrial warehouses (21, 22). However, biopesticidal formulations based on the bacterium *Bacillus thuringiensis* may also be used to control stored-product lepidopteran pests (24, 28), including the tobacco moth, in curing barns (6, 22) or during farm storage.

B. thuringiensis produces crystalline protein inclusions during sporulation which may be toxic to several species of insect pests (2). The insecticidal crystal proteins (ICPs) have been grouped into the following four classes on the basis of their host ranges and sequence homologies (1, 13, 20): Cry I proteins, which are Lepidoptera specific; Cry II proteins, which are Lepidoptera and Diptera specific; Cry III proteins, which are Coleoptera specific; and Cry IV proteins, which are Diptera specific. Recently, new ICPs that are insecticidal for the larvae of both coleopteran and lepidopteran pests (32) or exhibit silent activity against members of the Coleoptera (19) have been discovered.

B. thuringiensis, a soil dwelling bacterium, has been isolated frequently from stored-product habitats (4, 8, 10, 16, 26) and ecosystems with no record of agricultural *B. thuringiensis*-based insecticide treatments (31), and this has led to a reconsideration of the role of this organism in the environment.

The objectives of this study were to survey the worldwide occurrence, distribution, and diversity of *B. thuringiensis* in stored tobacco and to explore new approaches to the control of the tobacco beetle.

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MATERIALS AND METHODS

Organisms and growth conditions. *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *israelensis* HD-567 were obtained from the Institut Pasteur, Paris, France, and *B. thuringiensis* subsp. *tenebrionis* B1 256-82 was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

B. thuringiensis strains were grown in sporulation liquid medium that contained (per liter) 10 g of glucose, 7.5 g of peptone from casein that was pancreatically digested (Merck), 6.8 g of KH_2PO_4, 123 mg of $MgSO_4 \cdot 7H_2O$, 2.23 mg of $MnSO_4 \cdot 4H_2O$, 14 mg of $ZnSO_4 \cdot 7H_2O$, and 20 mg of $Fe_2(SO_4)_3$ dissolved in 1 N NH_2SO_4 (pH 7.5). The cultures were incubated at 30°C with vigorous shaking (200 rpm).

The strains were stored at 4°C on nutrient agar containing 1% (wt/vol) glucose and 1 mM $MnCl_2$.

Sample collection. A total of 88 samples of stored tobacco and insects were collected in warehouses and manufacturing facilities; these samples included 55 samples consisting of dried leaf residues (dust, scraps), 6 samples consisting of raw dried leaf, 6 samples consisting of processed tobacco or finished product, 7 samples consisting of a mixture of leaf residues and dead tobacco beetles (*L. serricorne* (F.); Coleoptera: Anobiidae), and 14 samples consisting of dead beetles. The dead beetles were collected when they were found on leaf residues or, more often, on pheromone traps used for insect monitoring.

The sampling sites were in 15 different countries, including Albania, Australia, Egypt, France, Germany, India, Italy, Mexico, Panama, Poland, Senegal, Spain, Switzerland, The Netherlands, and Turkey. In most cases, the tobacco leaf stored in the warehouse or processed in the factory was imported from other locations.

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In addition, five bulk field samples consisting of green tobacco leaves and the corresponding soil were also collected on Swiss tobacco farms during the 1993 growing season. Several leaves from different stalk positions on a tobacco plant were combined and processed as a single sample. Approximately 50 g of soil from the tobacco plant rhizosphere was taken separately for each sample.

Isolation of _B. thuringiensis_. _B. thuringiensis_ strains were isolated by using the acetate selection procedure developed by Travers et al. (34) and modified by Carozzi et al. (5). The cultures were examined for colony morphology and the presence of parasporal crystals by phase-contrast microscopy.

All crystal-forming colonies were removed and subcultured on tryptic soy agar. Non-crystal-forming colonies were restreaked on tryptic soy agar and checked for crystal production after 48 h.

Serotyping. All serovar identities were determined at the Unité de Bactéries Entomopathogènes, Institut Pasteur, Paris, France.

Preparation and purification of the crystal proteins. _B. thuringiensis_ strains were cultured in liquid medium until sporulation was complete. After lysis of the cells, the cultures were harvested by centrifugation at $10,000 \times g$ for 15 min, washed three times with cold 1 M NaCl, and resuspended either in 1 M NaCl or in 100 mM Tris buffer (pH 8.0) containing 10 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride.

The crystal proteins of _B. thuringiensis_ subsp. _tenebrionis_ were purified by dissolving the protein crystals in 100 mM carbonate buffer (pH 10.5) for 2 h at the ambient temperature (3). The insoluble material was removed by centrifugation, and the supernatant was filtered by using a filter having a pore size of 0.2 μm. The crystal proteins of _B. thuringiensis_ subsp. _kurstaki_ and _B. thuringiensis_ subsp. _israelensis_ were purified similarly by first dissolving the crystals in 10 mM phosphate buffer (pH 7.0) containing 10% sodium dodecyl sulfate (SDS), 2% mercaptoethanol, and 6 M urea. The suspensions were incubated at 50°C for 15 min (27).

For the bioassays, the crystal proteins were purified by sucrose density gradient centrifugation; the spore-crystal suspensions were washed three times with cold sterile distilled H_2O before they were separated by centrifugation at $20,000 \times g$ for 2 h on a 60 to 100% sucrose gradient. The isolated crystal proteins were then washed twice with H_2O in order to remove the sucrose. The purity of all of the isolated proteins was controlled by gel electrophoresis, and the protein concentrations were determined by using the Bio-Rad protein assay with bovine plasma gamma globulins as the standard.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by using the discontinuous buffer system of Laemmli (18). Spore-crystal mixtures, as well as purified protein samples, were solubilized before loading by incubation at 100°C for 5 min in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.1% bromophenol blue.

Immunoblotting. Polyclonal antibodies were raised against purified proteins of _B. thuringiensis_ subsp. _tenebrionis_, _B. thuringiensis_ subsp. _kurstaki_, and _B. thuringiensis_ subsp. _israelensis_ at Eurogentec SA, Seraing, Belgium.

Crystal proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45 μm; Bio-Rad). Nonspecific sites were blocked with 3% gelatin in TBS (20 mM Tris, 500 mM NaCl; pH 7.5). All additional washing steps were done with TBS

TABLE 1. _B. thuringiensis_ isolation from tobacco and tobacco beetles

Sample material	No. of samples analyzed	No. of samples yielding _B. thuringiensis_	No. of _B. thuringiensis_ isolates
Tobacco residues	55	16	72
Dead _L. serricorne_	14	2	2
Tobacco and dead _L. serricorne_	7	1	3
Raw dried tobacco	6	1	1
Processed tobacco leaf	6	0	0
Green tobacco	5	0	0
Field soil	5	0	0

containing 0.05% Tween 20. Primary and secondary antibodies were diluted in TBS containing 0.05% Tween 20 and 1% gelatin.

The membranes were incubated with polyclonal antiserum for 2 h. Goat anti-rabbit antibodies conjugated with horseradish peroxidase (Bio-Rad) were used as the secondary antibody (1/3,000, vol/vol). The protein-antibody complexes were visualized by incubating the filters in TBS containing 0.015% H_2O_2 and 40 mM 4-chloro-1-naphthol previously dissolved in methanol.

Insect bioassays. In vivo bioassays were performed with _L. serricorne_ larvae. Single-organism bioassays were performed by using a modification of the method of Johnson et al. (14).

Small disks (area, approximately 2 cm^2) of dried tobacco leaves were placed into each well of a 24-well tissue culture plate and painted with spore-crystal suspensions or purified ICPs to give a final concentration of 10 μg of protein per mg of leaf. The leaves were allowed to dry before first instar larvae were placed individually into each well. The plates were incubated at 26°C and 65% relative humidity. The larvae were inspected each week, and mortality was recorded.

A total of 48 single-larva tests were done per sample.

RESULTS

Occurrence of _B. thuringiensis_ in tobacco storage warehouses and manufacturing facilities. A total of 88 samples consisting of tobacco residues, dead tobacco beetles, and raw and processed tobacco leaves were examined. The acetate selection method (34) was used to recover 78 _B. thuringiensis_ strains from 20 samples of stored tobacco and insects (Table 1).

In two cases, _B. thuringiensis_ was isolated from tobacco beetles (isolates 2603 and 8304). The bacterium was also found in one raw leaf sample; however, it was not recovered from processed leaves or finished products (Table 1).

The isolates were analyzed by phase-contrast microscopy and were classified as _B. thuringiensis_ when parasporal crystals were present. On the basis of microscopic observations the crystals were differentiated into the following three morphological classes (Fig. 1): rhomboidal, as in _B. thuringiensis_ subsp. _tenebrionis_ (59% of the isolates); bipyramidal, as in _B. thuringiensis_ subsp. _kurstaki_ (18% of the isolates); and heterogeneous, which included irregular cuboidal and pleomorphic crystals (23% of the isolates).

In addition, five bulk samples consisting of green tobacco leaves and five samples of soil were also analyzed to assess the presence of _B. thuringiensis_ on the tobacco phylloplane
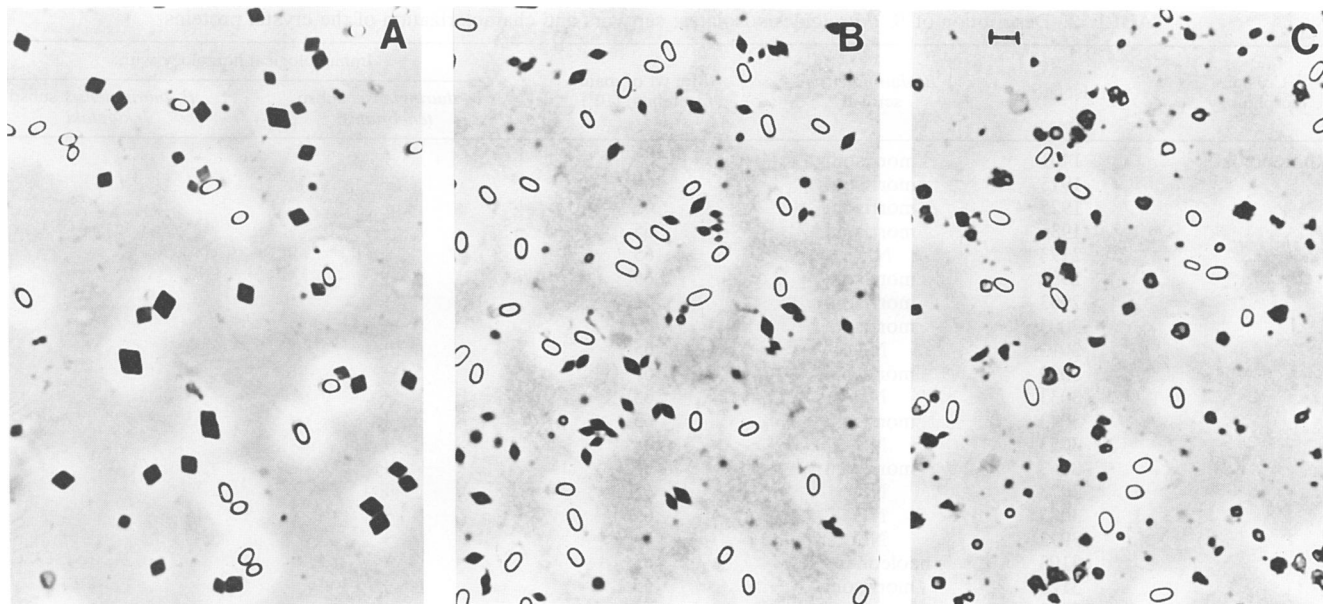
FIG. 1. Phase-contrast photomicrographs of sporulated cultures of three *B. thuringiensis* isolates, showing typical parasporal inclusion structures. (A) Rhomboidal. (B) Bipyramidal. (C) Heterogeneous. Bar = 2 μm.

and in the corresponding soil. None of the 10 field samples yielded *B. thuringiensis* (Table 1). However, larger samples and further investigations will be needed to test the hypothesis that *B. thuringiensis* is epiphytic on tobacco plants.

ICP SDS-PAGE and Western blot analyses and flagellar serotyping. The ICPs isolated from sporulated cultures of the 78 tobacco isolates were characterized by SDS-PAGE profile and Western blot (immunoblot) analyses. Figure 2 shows the results obtained with selected isolates compared with known *B. thuringiensis* pathotypes belonging to the Cry I-Cry II, Cry III, and Cry IV groups. The ICPs associated with the rhomboidal crystal morphology (Fig. 2A, lanes 4, 7, 10, and 11) produced a protein pattern similar to that of Coleoptera-specific *B. thuringiensis* subsp. *tenebrionis* (lane 1). The isolates that exhibited a combination of different crystal morphologies (Fig. 1C) produced protein bands at 130 to 140 and 63 kDa (Fig. 2A, lanes 5, 6, and 9). A heterogeneous crystal morphology was also found in one *B. thuringiensis* strain isolated from *L. serricorne* (isolate 2603) (lane 5). A second strain isolated from tobacco beetles exhibited rhomboidal crystal morphology and produced 65-kDa ICPs (isolate 8304) (Table 2).

Bipyramidal crystals, which are typical of *B. thuringiensis* strains that are toxic to members of the Lepidoptera, were always associated with 130- to 140-kDa polypeptides (Fig. 2A, lanes 8 and 12). Isolates having the Lepidoptera-specific pathotype might represent residues of agricultural *B. thuringiensis* sprays (*B. thuringiensis* subsp. *kurstaki* HD-1) used for control of lepidopteran pests in the field or in curing barns. However, commercially available antilepidopteran *B.*
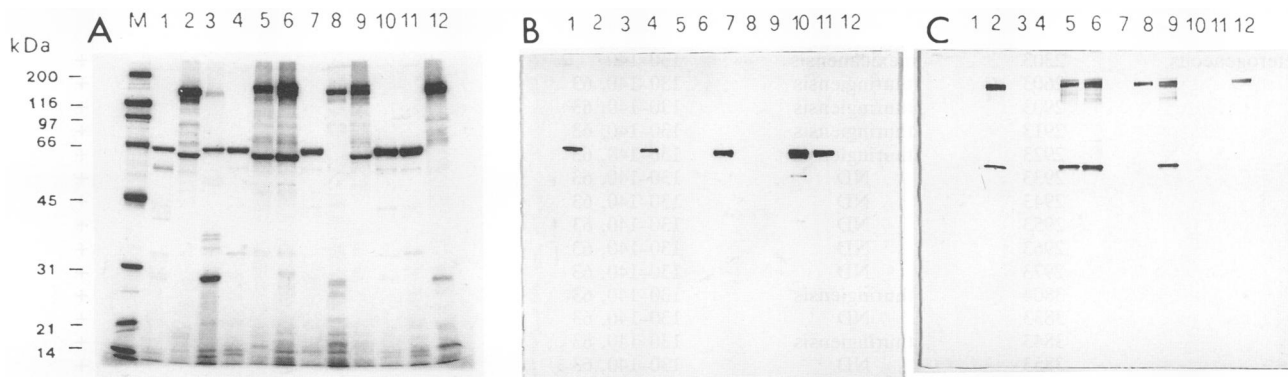

FIG. 2. SDS-PAGE and Western blot analyses of selected *B. thuringiensis* isolates. (A) Coomassie blue-stained 10% gel containing molecular mass standards (Bio-Rad) (lane M). (B and C) Immunoblots probed with antibodies prepared against the purified crystal proteins of *B. thuringiensis* subsp. *tenebrionis* (B) and *B. thuringiensis* subsp. *kurstaki* (C). Lanes 1 through 3, type strains of *B. thuringiensis* subsp. *tenebrionis*, *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *israelensis*, respectively; lanes 4 through 12, *B. thuringiensis* 1401, 2603, 2913, 3103, 3173, 3804, 3904, 5903, and 7600, respectively.

TABLE 2. Description of *B. thuringiensis* isolates: serovars and characterization of the crystal proteins

Crystal morphology	Isolate	*B. thuringiensis* serovar	Mol wt of main protein(s) (10^3)	Immunological homology with:	
				B. thuringiensis subsp. *tenebrionis*	*B. thuringiensis* subsp. *kurstaki*
Rhomboidal	1401	morrisoni	65	+	
	1915	morrisoni	65	+	
	1925	morrisoni	65	+	
	1935	morrisoni	65	+	
	2173	ND[a]	65	+	
	2983	morrisoni	65	+	
	2993	morrisoni	65	+	
	3003	morrisoni	65	+	
	3013	ND	65	+	
	3023	morrisoni	65	+	
	3033	ND	65	+	
	3043	morrisoni	65	+	
	3053	ND	65	+	
	3063	morrisoni	65	+	
	3073	ND	65	+	
	3083	ND	65	+	
	3093	ND	65	+	
	3103	neoleonensis	65	+	
	3123	morrisoni	65	+	
	3203	morrisoni	65	+	
	3213	morrisoni	65	+	
	3273	morrisoni	65	+	
	3283	morrisoni	65	+	
	3904	morrisoni	65	+	
	3924	ND	65	+	
	3953	ND	65	+	
	3973	morrisoni	65	+	
	3993	ND	65	+	
	5903	neoleonensis	65	+	
	5913	ND	65	+	
	5923	ND	65	+	
	5933	ND	65	+	
	5943	ND	65	+	
	5953	ND	65	+	
	5963	ND	65	+	
	5973	ND	65	+	
	5983	ND	65	+	
	5993	neoleonensis	65	+	
	6003	neoleonensis	65	+	
	7703	morrisoni	65	+	
	7713	ND	65	+	
	7723	ND	65	+	
	8304	ND	65	+	
	8534	ND	65	+	
	8814	ND	65	+	
	8924	ND	65	+	
Heterogeneous	2303	mexicanensis	130–140		+
	2603	thuringiensis	130–140, 63		+
	2903	thuringiensis	130–140, 63		+
	2913	thuringiensis	130–140, 63		+
	2923	thuringiensis	130–140, 63		+
	2933	ND	130–140, 63		+
	2943	ND	130–140, 63		+
	2953	ND	130–140, 63		+
	2963	ND	130–140, 63		+
	2973	ND	130–140, 63		+
	3804	thuringiensis	130–140, 63		+
	3833	ND	130–140, 63		+
	3843	thuringiensis	130–140, 63		+
	3853	ND	130–140, 63		+
	3863	thuringiensis	130–140, 63		+
	3873	ND	130–140, 63		+
	3883	ND	130–140, 63		+
	8845	ND	130–140, 63		+

TABLE 2—*Continued*

Crystal morphology	Isolate	*B. thuringiensis* serovar	Mol wt of main protein(s) (10^3)	Immunological homology with:	
				B. thuringiensis subsp. *tenebrionis*	*B. thuringiensis* subsp. *kurstaki*
Bipyramidal	1242	ND	130–140		+
	3113	kurstaki	130–140		+
	3133	kurstaki	130–140		+
	3143	ND	130–140		+
	3153	kurstaki	130–140		+
	3163	ND	130–140		+
	3173	kurstaki	130–140		+
	3183	ND	130–140		+
	3193	ND	130–140		+
	3243	kurstaki	130–140		+
	3263	kurstaki	130–140		+
	3293	kurstaki	130–140		+
	3963	kurstaki	130–140		+
	7600	thuringiensis	130–140		+

[a] ND, not determined.

thuringiensis preparations registered for use on tobacco crops were analyzed by SDS-PAGE and Western blotting (data not shown), which revealed the presence of both Cry I and Cry II ICPs (130 to 140 and 65 to 70 kDa, respectively), whereas the *B. thuringiensis* subsp. *kurstaki* tobacco isolates lacked the smaller Cry II protein (Table 2). Western blot analyses performed with Cry IIIA-, Cry IA-, or Cry IV-specific polyclonal antisera were used to investigate the immunological relatedness of the *B. thuringiensis* pathotypes to the tobacco isolates and to predict their insecticidal activities (30) (Fig. 2B and C). A consistent correlation between the morphology and the electrophoretic and immunological characteristics of the proteins was observed (Table 2).

All of the isolates that produced 130- to 140-kDa proteins, as well as the strains that produced 130- to 140- and 63-kDa major proteins, exhibited immunological homology with *B. thuringiensis* subsp. *kurstaki*, while the isolates that produced 65-kDa crystal proteins were related to *B. thuringiensis* subsp. *tenebrionis*.

None of the isolates exhibited ICP antigenic relationships with *B. thuringiensis* subsp. *israelensis*.

Characterization of selected strains by flagellar serotyping revealed the presence of five different serovars (Table 2); *B. thuringiensis* serovar morrisoni and *B. thuringiensis* serovar neoleonensis isolates produced rhomboidal crystals, *B. thuringiensis* serovar kurstaki isolates produced bipyramidal crystals, and *B. thuringiensis* serovar thuringiensis and *B. thuringiensis* serovar mexicanensis isolates appeared to be related to the strains containing heterogeneous parasporal inclusions.

Insect bioassays. On the basis of the ICP analysis results (Table 2), representative *B. thuringiensis* isolates were selected for preliminary toxicity bioassays. The tests were performed by using crude suspensions containing spores and crystals. The results (Table 3) showed that toxic effects were observed with a strain that produced proteins homologous to Coleoptera-specific proteins and a strain that produced proteins homologous to Lepidoptera-specific proteins (strains 3003 and 7600, respectively).

However, in most cases, about 50% of the mortality occurred within a few days after exposure of neonate larvae, while the remaining activity increased with time until the larvae reached the second or third instar stage (2 to 3 weeks).

Bioassays carried out with purified crystal proteins isolated from the most active strains revealed decreased efficacy of the proteins compared with the crude preparation activity (data not shown). These results suggest that synergism between spores and crystals or septicemia may be responsible for the larval mortality observed.

Low levels of activity were observed with *B. thuringiensis* subsp. *tenebrionis*. The two *B. thuringiensis* strains isolated from dead tobacco beetles were tested and appeared to be nontoxic for *L. serricorne*.

DISCUSSION

The results of this survey showed that *B. thuringiensis* can be recovered from residues of dried tobacco leaves and beetles collected in warehouses and manufacturing plants and that isolates that have characteristics of the Coleoptera-specific group are predominant.

Industrial tobacco warehouses and processing plants are normally characterized by low insect activity and are not treated with *B. thuringiensis*-based pesticides. Nonetheless, samples collected in this environment yielded a considerable number of *B. thuringiensis* strains, whereas *B. thuringiensis* was not found in either green leaf or tobacco field soil samples, suggesting that diverse populations of this organism may be part of the stored-tobacco microflora. *B. thuringiensis* may become associated with the tobacco microflora as a dust contaminant during curing and farm storage. The dry and protected stored-product environment may provide an ecological niche for this bacterium, as hypothe-

TABLE 3. Insecticidal activity of *B. thuringiensis* isolates against *L. serricorne*

Immunological group[a]	Activity[b]	No. of strains tested
−	C	13
+	C	1
−	L	6
+	L	1

[a] C, Coleoptera specific; L, Lepidoptera specific.
[b] +, >50% mortality; −, <50% mortality (determined after 4 weeks). The average level of mortality for the controls was 7%.

sized by Meadows (25). In addition, previous studies have shown that the exposure of tobacco to heat treatment and desiccation select resistant spore-forming bacteria belonging to the genus *Bacillus* (15). The predominant species occurring on dried tobacco leaves are *Bacillus pumilus*, *Bacillus subtilis*, and *Bacillus licheniformis*, which are normally found at concentrations of 10^5 to 10^6 CFU/g of cured leaf. Moreover, stored-tobacco trade practices and exposure to postharvest insects during storage may contribute to further diversity of *Bacillus* spp. populations in this stored-product habitat.

Although experimental evidence suggests that the primary habitat of *B. thuringiensis* is soil (9, 23, 34), different strains of this bacterium have been isolated from leaf surfaces, indicating that it may be considered part of the phylloplane microflora of many plants and that its ecological role in the environment should be reconsidered (7, 25, 31).

In contrast to the high diversity of *B. thuringiensis* strains isolated from stored-product residues by Meadows et al. (26), our tobacco isolates could be differentiated into only three subgroups on the basis of crystal morphology and protein characteristics (Table 2). One subgroup, the most abundant included strains that produce rhomboidal crystals and belong to *B. thuringiensis* serovar morrisoni and *B. thuringiensis* serovar neoleonensis (serotypes 8a8b and 24, respectively). The other subgroups, which were found in tobacco samples at approximately the same frequency, comprised isolates that exhibited heterogeneous ICP morphology and belonged to *B. thuringiensis* serovar thuringiensis and *B. thuringiensis* serovar mexicanensis (serotypes H1 and H27, respectively) and strains that belonged to *B. thuringiensis* serovar kurstaki (serotype 3a3b3c) and produced bipyramidal crystals. In some cases, the three subgroups were present in a single sample. However, considering the wide variety of sample sources (e.g., diverse collection spots and tobacco types and origins), the limited diversity of the *B. thuringiensis* isolates which we recovered is interesting. In two cases, *B. thuringiensis* strains were isolated from dead adults of *L. serricorne*. The first isolate (strain 2603) appeared not to be associated with the Cry III group, while the second isolate (strain 8304) exhibited characteristics of this pathotype (Table 2). Previous workers (33) reported the isolation of a spore-forming, noncrystalliferous bacterium from dead tobacco beetles and identified this organism as *Bacillus cereus*. This bacterium was shown to have pathogenic effects against *L. serricorne*.

The hypothesis that tobacco isolates belonging to *B. thuringiensis* serovar kurstaki might represent residues of agricultural *B. thuringiensis* sprays (e.g., Dipel 2X [Abbott Laboratories, Chicago, Ill.] or Biobit [Novo Nordisk A/S, Bagsvaerd, Denmark]) based on *B. thuringiensis* subsp. *kurstaki* HD-1 is not supported by the fact that both Cry I and Cry II ICPs are present in these commercial formulations, whereas only Cry I proteins were observed in the isolates examined (Fig. 1, lane 8, and Table 2).

The preliminary results of the experiment to determine the activity of selected isolates against *L. serricorne* larvae suggested that strains associated with both Coleoptera-specific and Lepidoptera-specific pathotypes exhibited toxic effects on the survival and development of the larvae. The only other record of *B. thuringiensis* strains that are toxic to tobacco beetles is found in the results of bioassays performed with *L. serricorne* at the USDA-ARS Crops Research Laboratory at Oxford, N.C., with commercial formulations based on *B. thuringiensis* subsp. *san diego* (M-One and M-One Plus; Mycogen Corp., San Diego, Calif.) and

other Coleoptera-specific strains, which caused a significant decrease in the rate of larval development in the treated insects (17).

The results of this study suggest that *B. thuringiensis* is part of the *Bacillus* microflora of stored tobacco and may be associated with the tobacco beetle. Moreover, we found that a special habitat like stored tobacco leaves may be an additional source of *B. thuringiensis* isolates for use in the control of stored-product pests. The isolates which we obtained will be further differentiated by performing PCR product profile analyses (5).

Unexpectedly, pathogenicity against *L. serricorne* larvae did not seem to be restricted to the strains previously associated with the subgroups active against members of the Coleoptera, although the bioactivity of all the *B. thuringiensis* isolates should be tested further.

REFERENCES

1. **Adang, M. J.** 1991. *Bacillus thuringiensis* insecticidal proteins: gene structure, action, and utilization, p. 3–24. *In* K. Maramorosch (ed.), Biotechnology for biological control of pests and vectors. CRC Press, Inc., Boca Raton, Fla.
2. **Andrews, R. E., Jr., R. M. Faust, H. Wabiko, K. C. Raymond, and L. A. Bulla, Jr.** 1987. The biotechnology of *Bacillus thuringiensis*. Crit. Rev. Biotechnol. **6:**163–232.
3. **Bernhard, K.** 1986. Studies on the delta-endotoxin of *Bacillus thuringiensis* var. *tenebrionis*. FEMS Microbiol. Lett. **33:**261–265.
4. **Burges, H. D., and J. A. Hurst.** 1977. Ecology of *Bacillus thuringiensis* in storage moths. J. Invertebr. Pathol. **30:**131–139.
5. **Carozzi, N. B., V. C. Kramer, G. W. Warren, S. Evola, and M. G. Koziel.** 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol. **57:**3057–3061.
6. **Collins, W. K., and S. N. Hawks, Jr.** 1993. Principles of flue-cured tobacco production, 1st ed. North Carolina State University, Raleigh.
7. **Damgaard, P. H., P. H. Smits, J. Heilenberg, and J. C. Pedersen.** 1992. Study on the natural occurrence and dynamics of *Bacillus thuringiensis*, abstr. 214, p. 240. Program Abstr. 25th Annu. Meet. Soc. Invertebr. Pathol. 1992.
8. **De Lucca, A. J., M. S. Palmgren, and A. Ciegler.** 1982. *Bacillus thuringiensis* in grain elevator dusts. Can. J. Microbiol. **28:**452–456.
9. **De Lucca, A. J., J. G. Simonson, and A. D. Larson.** 1981. *Bacillus thuringiensis* distribution in soils of the United States. Can. J. Microbiol. **27:**865–870.
10. **Donovan, W. P., J. M. Gonzalez, M. P. Gilbert, and K. Dankoscik.** 1988. Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the gene. Mol. Gen. Genet. **214:**365–372.
11. **Food and Agriculture Organization.** 1989. The economic significance of tobacco. F.A.O. Economic and Social Development Paper no. 85. U.N. Food and Agriculture Organization, Rome.
12. **Gadani, F., D. Ayers, and W. Hempfling.** Unpublished data.
13. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
14. **Johnson, D. E., W. H. McGaughey, and B. D. Barnett.** 1991. Small scale bioassay for the determination of *Bacillus thuringiensis* toxicity toward *Plodia interpunctella*. J. Invertebr. Pathol. **57:**159–165.

15. **Kaelin, P., J.-M. Renaud, and M. Hofer.** 1990. Biocontrol of tobacco microflora by D-alanine, abstr. APTS 01, p. 96. Abstr. Coresta Symp. 1990.

16. **Karamanlidou, G., A. F. Lambropoulos, S. I. Koliais, T. Manousis, D. Ellar, and C. Kastritsis.** 1991. Toxicity of *Bacillus thuringiensis* to laboratory populations of the olive fruit fly (*Dacus oleae*). Appl. Environ. Microbiol. **57:**2277–2282.

17. **Keever, D. W.** (U.S. Department of Agriculture, Agricultural Research Service). 1992. Personal communication.

18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

19. **Lambert, B., H. Hofte, K. Annys, S. Jansens, P. Soetaert, and M. Peferoen.** 1992. Novel *Bacillus thuringiensis* insecticidal crystal protein with a silent activity against coleopteran larvae. Appl. Environ. Microbiol. **58:**2536–2542.

20. **Lereclus, D., A. Delécluse, and M.-M. Lecadet.** 1993. Diversity of *Bacillus thuringiensis* toxins and genes, p. 37–69. *In* P. F. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley & Sons, Ltd., Chichester, England.

21. **Manzelli, M. A.** 1982. Management of stored tobacco pests, the cigarette beetle (Coleoptera: Anobiidae) and tobacco moth (Lepidoptera: Pyralidae), with methoprene. J. Econ. Entomol. **75:**721–723.

22. **Manzelli, M. A.** 1987. Control of the cigarette beetle in stored and processed tobacco. Tob. J. Int. **6:**373–378.

23. **Martin, P. A. W., and R. S. Travers.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. **55:**2437–2442.

24. **McGaughey, W. H., E. B. Dicke, K. F. Finney, L. C. Bolte, and M. D. Shogren.** 1980. Spores in dockage and mill fractions of wheat treated with *Bacillus thuringiensis*. J. Econ. Entomol. **73:**775–778.

25. **Meadows, M. P.** 1993. *Bacillus thuringiensis* in the environment: ecology and risk assessment, p. 193–220. *In* P. F. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley & Sons, Ltd., Chichester, England.

26. **Meadows, M. P., D. J. Ellis, J. Butt, P. Jarrett, and H. D. Burges.** 1992. Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. Appl. Environ. Microbiol. **58:**1344–1350.

27. **Nickerson, K. W., and J. D. Swanson.** 1981. Removal of contaminating protease from *Bacillus thuringiensis* parasporal crystal by density gradient centrifugation in NaBr. Eur. J. Appl. Microbiol. Biotechnol. **13:**213–215.

28. **Rassman, W.** 1986. Untersuchungen zur Wirksamkeit eines *Bacillus thuringiensis*-Präparates gegen vorratsschädliche Motten in der Getreidelagerung. Nachrichtenbl. Dtsch. Pflanzenschutzdienst (Berl) **38:**61–63.

29. **Schell, J. S.** 1987. Molecular biology and breeding of plants with tobacco as a model, p. 61–72. *In* C. Greenslit Lunsford (ed.), Natural products research: the impact of scientific advances. Proceedings of the 5th Philip Morris Science Symposium. Philip Morris, Inc., New York.

30. **Smith, R. A.** 1987. Use of crystal serology to differentiate among varieties of *Bacillus thuringiensis*. J. Invertebr. Pathol. **50:**1–8.

31. **Smith, R. A., and G. A. Couche.** 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. Appl. Environ. Microbiol. **57:**311–315.

32. **Tailor, R., J. Tippett, G. Gibb, S. Pells, D. Pike, L. Jordan, and S. Ely.** 1992. Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol. Microbiol. **6:**1211–1217.

33. **Thompson, J. V., and L. W. Fletcher.** 1972. A pathogenic strain of *Bacillus cereus* isolated from the cigarette beetle, *Lasioderma serricorne*. J. Invertebr. Pathol. **20:**341–350.

34. **Travers, R. S., P. A. W. Martin, and C. F. Reichelderfer.** 1987. Selective process for efficient isolation of soil *Bacillus* sp. Appl. Environ. Microbiol. **53:**1263–1266.