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Occurrence of *Bacillus thuringiensis* on Cured Tobacco Leaves

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Abstract. A worldwide survey was conducted to evaluate the frequency and distribution of *Bacillus thuringiensis* populations on cured tobacco leaves during post-harvest storage. In total, 133 tobacco samples of different types and origins were analyzed. Nine percent of the samples showed the presence of *B. thuringiensis*, and 24 *B. thuringiensis* strains were isolated and characterized. The majority of the isolates produced bipyramidal crystals, and three fourths of them showed a second type of crystal protein (cuboidal or heterogeneous crystals). Only three isolates showed the rhomboidal crystal morphology characteristic of the anti-coleopteran *B. thuringiensis* subsp. *tenebrionis*. PCR analysis with primers specific for *cry1* and *cry3* genes revealed eight distinct *cry* gene profiles. The results of this study indicate that *B. thuringiensis* is naturally present at low frequency on the phylloplane of cured tobacco leaves and that its distribution is worldwide.

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium that produces insecticidal proteins that form crystalline inclusions during sporulation [25]. *B. thuringiensis* strains have been encountered in a large diversity of biotopes [2, 8] and have been isolated worldwide from many habitats, including soil [12, 21, 27], insects [19], sericulture [24], and stored-product environments [15, 18, 22]. Its isolation from the phylloplane of different temperate-climate trees [26], mulberry leaves [23], cabbage foliage [10], and grass foliage [11] indicates that *B. thuringiensis* is also part of the phylloplane microflora of many plants.

Tobacco (*Nicotiana tabacum* L.) leaves are submitted to a controlled post-harvest drying process called curing. Different types of curing are used, such as air curing for Burley and Maryland, sun curing for Oriental, and flue curing for Virginia or Flue-cured tobaccos. Cured tobaccos are then usually aged in redried form (relative humidity 11–12%) for 1–3 years to improve taste and aroma before being used. The heat and desiccation treatments of the leaves were shown to select spore-forming bacteria belonging predominantly to the genus *Bacillus* [14]. Our previous studies showed that *B. thuringiensis* is present at low level in the stored tobacco environment [15, 16] and can be of interest to control stored tobacco insect pests [17]. However, these *B. thuringiensis* strains were mostly isolated from dust, leaf debris, and insect cadavers. The presence of *B. thuringiensis* on the tobacco leaf surface was not investigated.

The objective of this study was to evaluate the frequency and distribution of *B. thuringiensis* populations on cured tobacco leaves. A worldwide survey was conducted in 1996–1997 on four different tobacco types from 18 different countries. *B. thuringiensis* crystal proteins were characterized by their electrophoresis profiles. Polymerase Chain Reaction (PCR) analysis was used to differentiate *B. thuringiensis* strains harboring *cry1* genes coding for anti-lepidopteran crystal proteins and *cry3* and *cry8* genes coding for anti-coleopteran proteins [9].

Materials and Methods

Reference strains and growth conditions. *B. thuringiensis* subsp. *kurstaki* (HD-1) and subsp. *israelensis* (HD-567) were obtained from the Institut Pasteur (Paris, France), and *B. thuringiensis* subsp. *tenebrionis* B1 256-83 was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). Dipel 2X™ was obtained from Abbott Laboratories (Chicago, USA). The *B. thuringiensis* strains were cultured as previously described [15].

Sample collection and isolation of *B. thuringiensis* strains. Four types of tobacco were evaluated: Flue cured, Burley, Oriental, and Maryland. Random leaf samples were collected in the following countries: Albania, Argentina, Brazil, Bulgaria, China, Former Yugosla-

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Table 1. *B. thuringiensis* isolation from cured tobacco leaves

Tobacco samples	Number of samples analyzed	Number of positive samples	Number of strains isolated
Flue cured	62	3	4
Burley	37	2	8
Oriental	25	4	6
Maryland	9	3	6
Total	133	12	24

vian Republic of Macedonia (FYROM), Greece, Italy, Malawi, Mexico, Philippines, Sri Lanka, Switzerland, Turkey, Uganda, USA, Zaire and Zimbabwe.

Bacillus thuringiensis strains were isolated from the cured tobacco leaves according to the acetate selection procedure developed by Travers et al. [27] and modified by Carozzi et al. [5], with the exception that large rod bacteria were first inoculated onto Mannitol Egg Yolk Agar. This medium allowed us to differentiate the *B. thuringiensis* group, forming pink colonies (mannitol-negative), from the other *Bacillus* spp., forming yellow colonies (mannitol-positive). The putative *B. thuringiensis* colonies were further subcultured on Tryptic Soy Agar plates (Difco, Detroit, MI) until sporulation and were selected for the presence of parasporal inclusions by phase-contrast microscopy. The isolation procedure was replicated five times for each tobacco sample.

Preparation and electrophoresis of crystal proteins. *B. thuringiensis* strains were cultured in liquid medium until sporulation was complete. After cell lysis, the spores and crystals were harvested by centrifugation (17,000 *g* for 15 min), washed twice with cold NaCl (1 M), and resuspended in water.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the standard discontinuous buffer system of Laemmli [20]. Spore-crystal mixtures were solubilized at 100°C for 5 min in sample buffer: Tris-HCl (62.5 mM, pH 6.8), SDS (2%), glycerol (10%), β-mercaptoethanol (5%), and bromophenol blue (0.1%), before being loaded onto 10% acrylamide gels.

Immunoblotting. Western blot analysis was carried out to determine the immunological relatedness of the crystal proteins to the proteins of *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *tenebrionis*, and *B. thuringiensis* subsp. *israelensis*, with Cry1-, Cry3-, or Cry4-specific polyclonal antisera, respectively [15]. Crystal proteins separated by SDS-PAGE were electrophoretically transferred onto 0.45-µm nitrocellulose membranes, and immunoblotting reactions were performed as already described [15].

PCR analysis. *B. thuringiensis* cultures were grown overnight in LB medium for the isolation of bacterial DNA. One milliliter culture was centrifuged (13,000 *g*, 5 min), and the pellet was washed once with sterile H_2O, 200 µl of InstaGene matrix (Bio-Rad, Hercules, CA) was mixed with the cells, and the mixture was incubated at 56°C for 30 min. The suspension was then vortexed at high speed for 10 s and incubated at 100°C for 8 min. The suspension was vortexed again and centrifuged at 12,000 rpm for 3 min. The resulting supernatant was used directly for PCR analysis or stored at −20°C.

The primers used in this study were described by Ceron et al. [6, 7] (genes were renamed according to [9]). In a first phase, general primers selected from highly conserved regions were used to detect *cry1* or *cry3* genes. In a second phase, specific primers designed from highly variable regions in the genes were used to identify specific *cry1* and *cry3* gene types. *B. thuringiensis* subsp. *kurstaki* HD-1 (Btk), *B. thuringiensis* subsp. *tenebrionis* (Btt), and the commercial product Dipel 2X™ were used as control strains. PCR amplifications were carried out in a 50-µl reaction volume, containing 1–2 µl template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, and 1.25 U of Taq polymerase (Gibco BRL, Life Sciences, Bethesda, MD). Amplification steps were performed as described by Ceron et al. [6, 7], and the PCR products were analyzed by electrophoresis on 2% agarose gels.

Results and Discussion

One hundred and thirty-three samples of cured tobacco leaves of different types and origins were screened for the presence of *B. thuringiensis* strains, which were recovered from 9% of the samples (Table 1). The highest proportion of positive samples was obtained with Maryland tobacco, where a third of the samples showed the presence of *B. thuringiensis* strains. Twenty-four *B. thuringiensis* strains were isolated.

While the vast majority of the strains (21 out of 24) produced bipyramidal crystals, 16 isolates showed a second type of crystal protein (cuboidal or heterogeneous crystals; Table 2). Only three isolates showed the rhomboidal crystal morphology characteristic of the coleopteran-specific *B. thuringiensis* subsp. *tenebrionis*. The proteins associated with the rhomboidal crystal morphology showed a protein pattern similar to that of *B. thuringiensis* subsp. *tenebrionis* with a major band at about 60 kDa. Bipyramidal crystals were mostly associated with polypeptides of 130–135 kDa. The isolates exhibiting a combination of different crystal morphologies produced protein bands at 120–135 and 60–63 kDa. Three isolates showed a band of 150–155 kDa (Table 2). Immunological analyses confirmed the relatedness of bipyramidal and heterogeneous crystals to *B. thuringiensis* subsp. *kurstaki* proteins and of rhomboidal crystals to *B. thuringiensis* subsp. *tenebrionis* proteins. None of the isolates showed antigenic relationships with proteins of *B. thuringiensis* subsp. *israelensis* (results not shown).

The predominance of isolates showing characteristics of anti-lepidopteran proteins might suggest that they were residues of *B. thuringiensis*-based insecticides, although *B. thuringiensis* finds only limited use as foliar insecticide in the USA to control the tobacco budworm, *Heliothis virescens* (F.) and the tobacco hornworm, *Manduca sexta* (F.), with Dipel 2X™ (*B. thuringiensis* subsp. *kurstaki* HD-1) being the most widely used product [4, 13]. Tobacco and storage areas can also be treated with Dipel 2X™ to help prevent tobacco moth [*Ephestia elutella* (Hb)] infestation. No published information was found on *B. thuringiensis* insecticide usage in other tobacco-growing countries.

PCR, which is a very sensitive method to rapidly detect and identify target DNA sequences, has been employed by several researchers to identify *B. thuringien-*

Table 2. Crystal protein and gene features of the *B. thuringiensis* isolates

Strains[a]	Origin of strains[b]	Crystal morphologies[c]	Protein size (kDa)[d]	Genes detected by PCR
Btk HD-1	Reference strain	B	130, 62	*cry1Aa, cry1Ab, cry1Ac*
Dipel 2X	Reference strain	B	142, 65	*cry1Aa, cry1Ab, cry1Ac*
Btt	Reference strain	R	62	*cry3Aa*
RT-001	Or, Turkey	B, H	133, 61	*cry1Aa, cry1Ab, cry1Ac*
RT-002	Bu, Malawi	B	133	*cry1Aa, cry1Ab, cry1Ca, cry1Da*
RT-003	Bu, Malawi	B	132	*cry1Aa, cry1Ab, cry1Ca, cry1Da*
RT-004	Ma, USA	B, C	130	*cry1Aa, cry1Ab, cry1Ca, cry1Da, cry1Ea*
RT-005	FC, USA	B, C	130, 61	*cry1Aa, cry1Ab, cry1Ac*
RT-006	Ma, USA	B, C	125, 61	*cry1Aa, cry1Ab, cry1Ac*
RT-007	Ma, USA	R	61	*cry3Aa*
RT-008	Ma, USA	B, C	150, 140	*cry1Ab, cry1Ba, cry1Ea*
RT-009	Ma, USA	B, C	153, 144	*cry1Ab, cry1Ba, cry1Ea*
RT-010	Ma, USA	B, C	151, 142	*cry1Ab, cry1Ba, cry1Ea*
RT-011	Or, Turkey	B	133	*cry1Ab, cry1Da, cry1Ea*
RT-012	Bu, Mexico	B, C	133, 63	*cry1Aa, cry1Ab, cry1Ac*
RT-013	Bu, Mexico	B, C	130, 62	*cry1Aa, cry1Ab, cry1Ac*
RT-014	Bu, Mexico	B, C	127, 61	*cry1Aa, cry1Ab, cry1Ac*
RT-015	Bu, Mexico	B, C	122, 60	*cry1Aa, cry1Ab, cry1Ac*
RT-016	Bu, Mexico	B, C	122, 60	*cry1Aa, cry1Ab, cry1Ac*
RT-017	Bu, Mexico	B, C	129, 61	*cry1Aa, cry1Ab, cry1Ac*
RT-018	FC, USA	B, C	132, 61	*cry1Aa, cry1Ab*
RT-019	FC, Switzerland	R	60	*cry3Aa*
RT-020	FC, Switzerland	R	60	*cry3Aa*
RT-021	Or, Turkey	B, H	131	*cry1Ab, cry1Da*
RT-022	Or, Turkey	B	134	*cry1Ab, cry1Da, cry1Ea*
RT-023	Or, Turkey	B	137	*cry1Ab, cry1Da, cry1Ea*
RT-024	Or, FYROM	B, H	129, 61	*cry1Aa, cry1Ab, cry1Ac*

[a] Btk, *B. thuringiensis* subsp. *kurstaki;* Btt, *B. thuringiensis* subsp. *tenebrionis.*
[b] Tobacco type and country of collection; Bu, Burley tobacco; FC, Flue cured tobacco; Ma, Maryland tobacco; Or, Oriental tobacco.
[c] B, bipyramidal; C, cuboidal; H, heterogeneous; R, rhomboidal.
[d] Major protein bands on SDS-PAGE gels.

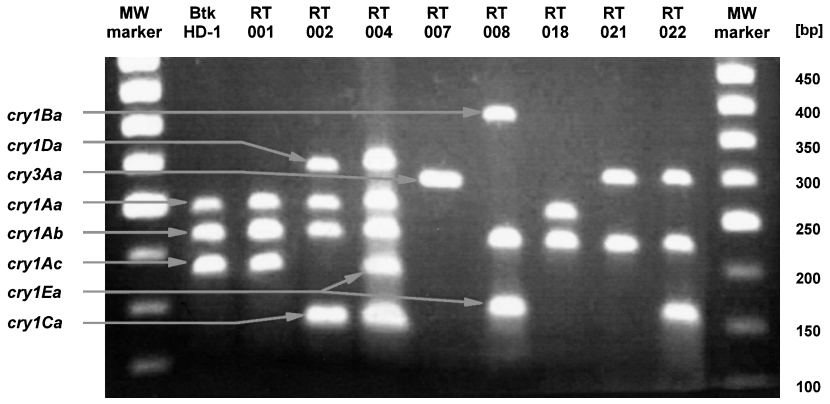

Fig. 1. PCR profiles of *B. thuringiensis* isolated from cured tobacco leaves. The PCR reactions were performed separately for each *cry* gene, and the PCR products corresponding to each isolate were mixed before being loaded on the gel.

sis cry genes [1, 3, 7] and to predict their insecticidal activities [5]. In our study, PCR was used to investigate the *B. thuringiensis* tobacco isolates for the presence of *cry1* and *cry3* genes, with *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *tenebrionis*, and Dipel 2X™ used as reference strains. PCR products detected with the general primers ranged from 272 to 290 bp and from 652 to 733 bp for *cry1* and *cry3* genes, respectively (Fig. 1). With the specific primers, seven different *cry1* genes and one *cry3* gene were detected (Table 2). It is suggested that *B. thuringiensis* isolates RT-012, 013, 014, 015, 016, and 017 were most likely redundant isolations of the same strain. The following groups of strains: RT-002 and RT-003; RT-008, RT-009, and RT-010; RT-019 and RT-020; and RT-022 and RT-023 probably also represented redundant isolations, as the strains within

these groups showed identical genetic patterns and originated from the same sample (Table 2). Strain RT-004 produced a larger PCR product than the expected band of 147 bp when it was assayed with the *cry1Ea*-specific primers. The *cry3Aa* gene was identified in three isolates, while no strains harboring the *cry1Fa* gene, *cry3Ba*, *cry3Bb*, *cry3Ca*, or *cry8Aa* genes were isolated, even though appropriate primers were used [7].

The genetic diversity observed among the strains harboring *cry1* genes (Fig. 1) confirm our previous report on the presence of diverse populations of *B. thuringiensis* on the phyllosphere of tobacco [15, 16]. This observation is consistent with that of several studies reporting that *B. thuringiensis* are naturally occurring organisms on the phylloplane of many plants [11, 23, 26]. The presence of isolates showing the *cry1Aa*, *cry1Ab*, and *cry1Ac* profile characteristic of *B. thuringiensis* subsp. *kurstaki* HD-1 (Dipel) may possibly point at residues of insecticidal sprays. The occurrence of *B. thuringiensis* on cured leaves of the four main industrial tobacco types is not surprising, considering the selection of spore-forming bacteria, predominantly *Bacillus* spp., in this dry environment [14, 16]. In contrast to our previous study, which investigated the presence of *B. thuringiensis* in the stored tobacco habitat [15, 16], our present results showed a much lower incidence of *B. thuringiensis* strains on the cured tobacco phylloplane and a higher proportion of isolates harboring Cry1-type proteins. The predominance of isolates producing anti-lepidopteran type proteins was also reported for *B. thuringiensis* strains recovered from other plants [10, 26], although with higher recovery frequency [23, 26]. This might reflect the decrease of bacterial populations of tobacco leaves associated with the curing process. Although the data collected do not allow appropriate statistical analysis, the results obtained indicate a higher *B. thuringiensis* frequency in air- or sun-cured tobaccos than in flue-cured tobaccos (Table 1). Air and sun curing processes take place at ambient temperature over weeks, whereas flue curing is carried out over one week, under a temperature cycle that peaks at about 72°C for a few hours. This may influence the bacterial density and diversity on the leaf.

In conclusion, the results of this study indicate that *B. thuringiensis* is naturally present at low frequency on the phylloplane of the main four tobacco types used in cigarette manufacturing and that it displays a worldwide distribution. The genetic diversity observed among the *cry1*-harboring isolates suggests that cured tobacco leaves could represent a specific source of new strains for the control of the tobacco moth, *E. elutella* (Hb), a major pest of stored tobacco.

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