Correspondence of High Levels of Beta-Exotoxin I and the Presence of *cry1B* in *Bacillus thuringiensis*

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Examination of 640 natural isolates of *Bacillus thuringiensis* showed that the 58 strains (9%) whose super-natants were toxic to *Anthonomus grandis* (Coleoptera: Curculionidae) produced between 10 and 175 μg of β-exotoxin I per ml. We also found that 55 (46%) of a sample of 118 strains whose culture supernatants were not toxic to *A. grandis* nevertheless produced between 2 and 5 μg/ml. However, these amounts of β-exotoxin I were below the threshold for detectable toxicity against this insect species. Secretion of large amounts of β-exotoxin I was strongly associated with the presence of *cry1B* and *vip2* genes in the 640 natural *B. thuringiensis* isolates studied. We concluded that strains carrying *cry1B* and *vip2* genes also possess, on the same plasmid, genetic determinants necessary to promote high levels of production of β-exotoxin I.

Bacillus thuringiensis is a sporogenic soil bacterium which forms characteristic crystalline inclusions composed of insecticidal crystal (Cry) proteins that are highly specific for the larvae of several insect pests (27). Because of this, *B. thuringiensis* has been widely used in biological pest control (19, 22a). Many isolates also produce an assortment of various other virulence factors that are secreted into the culture medium (9). These factors include the vegetative insecticidal proteins Vip1, Vip2, and Vip3, which do not display any sequence homology with each other or with any known protein (10, 29, 30), the Cry1I toxin (17), and β-exotoxin I (2, 5, 15, 23), a nonprotein-aceous toxin. Unlike the Vip and Cry toxins, β-exotoxin I is not specific and thus may have detrimental effects on nontarget organisms (16, 22); it is particularly active against dipteran species, but it is also active against coleopteran, lepidopteran, and some nematode species (12). The mechanism of action of β-exotoxin I is not fully understood. However, this toxin is an adenine nucleotide analogue (11) that has been found to interfere with RNA polymerase (1, 28). Thus, it has been proposed that this molecule inhibits the synthesis of RNA by competing with ATP for binding sites, thereby affecting insect molting and pupation and causing teratological effects at sublethal doses (4, 16). β-Exotoxin I displays some toxicity to mammalian cells (1, 22) and has been banned from public use based on World Health Organization advice (31). However, unless a bioassay or high-performance liquid chromatography (HPLC) analysis (6, 13, 14) is performed, it is impossible to predict whether a strain produces β-exotoxin I. Previous studies have shown that β-exotoxin I production is often linked to the presence of plasmids bearing *cry* genes (*cry* plasmids); several experiments have shown that the ability to secrete β-exotoxin I and the ability to produce crystals were transferred together to *Bacillus cereus* and *B. thuringiensis* recipient strains by conjugation (21, 24). Conversely, strains that had lost the capacity to synthesize crystal toxins following loss of *cry* plasmids also were unable to secrete β-exotoxin I, although the parental strains had the capacity to produce high levels of this compound. β-Exotoxin I production has been linked to the presence of plasmids of various sizes expressing Cry proteins with apparent molecular masses of 150, 140, 67, 23, and 21 kDa (18, 21). However, no direct relationship between the presence of specific *cry* plasmids and β-exotoxin I production has been established, and it is not known whether Cry toxins are associated with β-exotoxin I other than in a strain-specific fashion.

In this study we assessed the abilities of 640 natural *B. thuringiensis* strains, isolated from different places around the world, to produce β-exotoxin I by examining their crystal toxin profiles in order to determine whether the ability to synthesize β-exotoxin I is associated with the presence of a particular *cry* gene.

MATERIALS AND METHODS

Bacterial strains, electrophoretic Cry profiles, and supernatant preparations for toxicity assays. The Institut National de la Recherche Scientifique (INRA) collection contains 1,260 natural *B. thuringiensis* strains, including isolates from 101 countries, which were isolated from various sources (soil, insects, dust, plants, animal waste, etc.). These strains were characterized as *B. thuringiensis* primarily on the basis of the ability to produce crystals during sporogenesis; they are conserved as spores in 12% (vol/vol) glycerol suspensions at −20°C. Spore crystal preparations from 640 strains randomly selected from this collection were individually grown in liquid HCT medium (20) at 30°C for 48 h. They were then analyzed directly by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Spores and crystals were centrifuged, washed in distilled water, and subjected to Bradford protein quantification. They were then treated with 1% SDS and electrophoresed on 10.5% polyacrylamide gels. The sizes of the major protein bands obtained for each strain were recorded (8). The culture supernatants were also tested against the insect *Anthonomus grandis*. Supernatants were harvested from cultures at the midsporulation stage, grown in 100 ml of liquid Luria-Bertani nutrient broth at 30°C, and inoculated by using single colonies previously reisolated on agar plates. Supernatants were filtered through 0.2-μm-pore-size Nalgene filter units (Nalgene) and stored at −20°C until the bioassay was performed.

Insect bioassay. We used a free ingestion technique to assess the toxicity to *A. grandis* of bacterial culture supernatant preparations. Each supernatant was incorporated into an artificial diet at a final concentration of 8%. The diet

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TABLE 1. Oligonucleotides used in this study

Primer	Sequence	Position	Accession no.	Reference	Product size (bp)
CJ8 cry1B-fw	5′ CTTCATCACGATGGAGTAA 3′	1007–1025	X06711	7	367
CJ9 cry1B-rev	5′ CATAATTTGGTCGTTCTGTT 3′	1355–1077	X06711	7	367
crylI-fw	5′ AACACTCAGTATATGAAT 3′	1059–1374	L49391	17	378
crylI-rev	5′ CACATGTGATGCTGAAAT 3′	1437–1419	L49391	17	378
vip2-fw	5′ GGGAAAGAAAAAGAAAAAGAGTGG 3′	231–255	A64136	31	914
vip2-rev	5′ CATATATCCTTTGTCTTCTTT 3′	1145–1124	A64136	31	914
vip3-fw	5′ ACATCCTCCCTACACTTTCTAATAC 3′	1700–1725	L48811	10	678
vip3-rev	5′ TCTTCTATGGACCCGTTCTCTAC 3′	2378–2353	L48811	10	678

consisted of a mixture of soya flour, cotton flour, wheat germ oil, cholesterol, sugar, vitamins, and agar. It was poured into a sterile 24-well plate. One egg of *A. grandis* was placed on the food in each well. At 25°C, control larvae developed into adults when they were given untreated food for 3 weeks. *A. grandis* was highly sensitive to β-exotoxin I, as the concentration of toxin required to kill 50% of *A. grandis* larvae was 6 μg/ml, as calculated by PROBIT analysis (26) with 95% confidence intervals (2 to 10 μg/ml). In bioassays, culture supernatants were considered toxic when the level of mortality at 3 weeks was more than 80%, whereas the level of mortality in the controls was less than 10%.

PCR amplification of *cry1B*, *vip2*, *vip3*, and *cry1I*. The oligonucleotides used in this study are listed in Table 1. The specific primers used to detect the *cry1I*, *vip2*, and *vip3* genes were designed from the *cry1I*, *vip2*, and *vip3* gene sequences present in the databases by using Primer Select from the DNAStar software (DNAStar Inc.). The primers used for specific detection of *cry1B* were the previously described primers CJ8 and CJ9 (7). PCR amplifications were performed by using the general procedure described here to search for *vip2*, *vip3*, *cry1I*, and *cry1B* genes in wild-type *B. thuringiensis* cells. DNA templates were obtained from cells grown overnight on agar plates and resuspended in 100 μl of distilled H$_2$O. Cell membranes were disrupted by freezing at −70°C and immersion in a 98°C water bath (heat shock). The DNA solution (2 μl) was mixed with 0.5 μl of each primer (0.1 μM), 0.5 U of *Taq* polymerase, water (50 μl), MgCl$_2$, and buffer according to the manufacturer's instructions (Gibco-BRL). The PCR conditions were as follows: denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C and then a final elongation step consisting of 10 min at 72°C.

Only fragments of the expected size after migration on a 1% agarose gel were considered to result from specific amplification. PCR results were considered to be negative if no amplicon was obtained after two attempts with independent DNA preparations.

Detection and quantification of β-exotoxin I. β-Exotoxin I was extracted from the culture supernatant by solvent extraction and was quantified by HPLC, as previously described (13). Briefly, for solvent extraction, acetone was added to the exotoxin in 0.2 ml of culture supernatant to a final concentration of 90%, and the mixture was centrifuged. The pellet was solubilized in 0.2 ml of double-distilled H$_2$O. Acetonitrile was added to a final concentration of 40%, and the mixture was then centrifuged. The pellet was discarded, and the acetonitrile concentration in the supernatant was increased to 90%. The precipitate was collected by centrifugation, and the pellet was solubilized in 100 μl of 50 mM potassium phosphate buffer (pH 2.5). For HPLC, we injected 20 μl of the sample into a Lichrospher (Merck) C$_{18}$ end-capped column (4 by 250 mm). A 5 to 15% methanol gradient in 50 mM potassium phosphate buffer (pH 2.5) was applied for 10 min. The flow rate was 1 ml/min, and UV absorption was monitored at 260 nm. β-Exotoxin I eluted at 5.5 min. The detection limit of this method for β-exotoxin I was 2 μg/ml. A standard sample of β-exotoxin I (70% pure) was kindly provided by I. Thiery from Laboratoire des bactéries entomopathogènes (Institut Pasteur, Paris, France).

RESULTS

Distribution of Cry toxins in natural strains producing β-exotoxin I. We analyzed the supernatant toxicities and crystal contents of 640 strains randomly selected from our collection of 1,260 strains (8). We assessed the toxicity of culture supernatants of each of the strains individually against *A. grandis*. As β-exotoxin I was toxic at a concentration of 5 μg/ml or more, we used this value as a threshold to discriminate poten-

tial Exo$^+$ strains from Exo$^-$ strains. β-Exotoxin I was then characterized and precisely quantified by HPLC in all possible Exo$^+$ strains. We found 58 strains whose culture supernatants were toxic to *A. grandis*, and each of these strains secreted more than 10 μg of β-exotoxin I per ml (generally more than 50 μg/ml) into the culture supernatant, accounting for the activity against this species. We also determined precisely the amounts of β-exotoxin I present in the culture supernatants of 118 of the nontoxic isolates using the same quantification method. We found that 55 of these nontoxic strains produced between 2 and 5 μg of β-exotoxin I per ml and that 63 produced less than 2 μg/ml. These amounts of β-exotoxin I were below the threshold for detectable toxicity against *A. grandis* in our test conditions.

Analysis of the crystal protein profiles of the 640 strains revealed many different electrophoretic patterns (Fig. 1). Most of the patterns were consistent with those reported previously for the nontoxic Cry15 proteins (40 and 45 kDa), the Cry2 proteins (66 kDa) active against Lepidoptera and Diptera, the Cry3A protein (70 and 73 kDa) active against Coleoptera, and the Cry1 proteins (130- to 140-kDa polypeptides) active against Lepidoptera (27). However, many isolates contained crystals with unusual protein profiles. We created the following categories based on the major protein bands observed: 40 and 45 kDa, 66 kDa, 70 and 73 kDa, 128, 130, or 132 kDa, 140 kDa, and unusual profiles. These categories roughly reflected the Cry diversity of the *B. thuringiensis* strains (Table 2). We investigated whether there was a correlation between β-exotoxin I production and a particular protein profile by comparing the crystal protein profiles of the 58 Exo$^+$ strains with those obtained for the whole sample. Chi-square analysis showed that there was a highly significant difference between the Exo$^+$ strains and the whole strain collection and indicated that there was a strong link ($P < 0.0001$) between β-exotoxin I production and the presence of a 140-kDa polypeptide in the crystals of a given strain. Conversely, there was a strong negative correlation between the presence of the 40- and 45-kDa, 66-kDa, and 128-, 130-, or 132-kDa Cry proteins and the presence of β-exotoxin I ($P < 0.0001$, $P < 0.0021$, and $P < 0.0068$, respectively). Finally, 13% of the strains that produced unusual protein profiles produced β-exotoxin I, but the difference was not significant ($P = 0.1719$).

Genotypic characterization of strains that produced 140-kDa protein bands. We further investigated the 39 Exo$^+$ and 2 Exo$^-$ strains that produced crystals containing 140-kDa Cry toxins in order to better characterize them genetically. The only known Cry toxin that produces a protoxin band at about
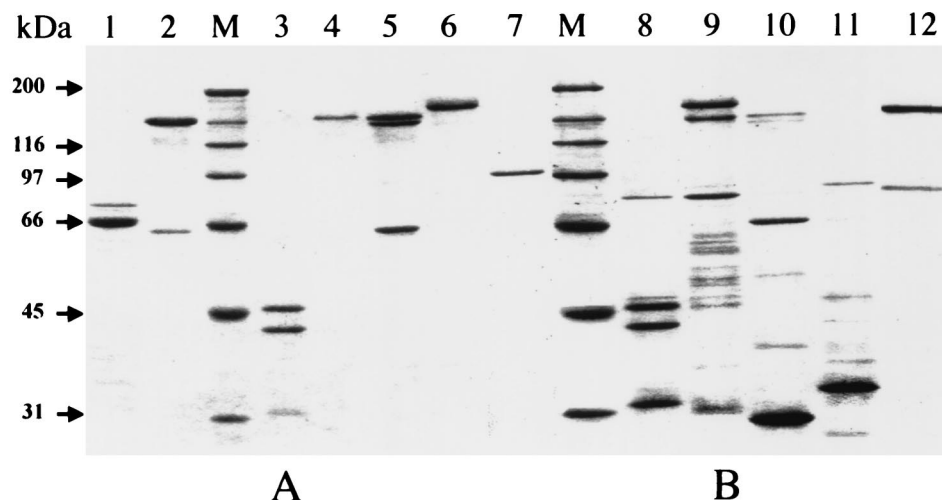
FIG. 1. SDS–10.5% PAGE of *B. thuringiensis* crystal proteins representative of the main profiles listed in Table 2. (A) Lane 1, strain LM63 (70 and 73 kDa); lane 2, strain LM651B (66 and 130 kDa); lane 3, strain LM381 (40 and 45 kDa); lane 4, strain LM380 (132 kDa); lane 5, LM 379 (66, 130, and 132 kDa); lane 6, strain LM378B (140 kDa). (B) Examples of unusual profiles. Lane 7, strain LM86 (98 kDa); lane 8, strain LM85 (34, 40, 45, and 80 kDa); lane 9, strain LM72 (32, 80, 130, and 140 kDa); lane 10, strain LM567 (28, 68, 125, and 135 kDa); lane 11, strain LM54 (36 and 90 kDa); lane 12, strain LM347 (85 and 135 kDa). Lanes M contained molecular mass markers, whose positions are indicated on the left.

140 kDa on SDS-PAGE gels is Cry1B (3, 27). On another hand, in a previous study S. Espinasse, J. Chaufaux, C. Buisson, S. Perchat, M. Gohar, D. Bourguet, and V. Sanchis, submitted for publication), we showed that there was a significant association between the presence of *vip2* genes and β-exotoxin I production in *B. thuringiensis* populations. We also found a negative correlation between β-exotoxin I production and the presence of *vip3* or *cry1I* genes encoding two other secreted insecticidal toxins. We therefore carried out PCR with primers specific for *cry1B*, *vip2*, *vip3*, and *cry1I* genes in these strains (Table 3). The *cry1B* gene was detected in all 39 strains that produced more than 10 μg of β-exotoxin I per ml. All of these strains also generated amplicons for *vip2* genes during PCR, indicating that there is a strong association between β-exotoxin I production and the presence of *vip2* and *cry1B* in a given strain. The two strains that produced only small amounts of β-exotoxin I but whose crystals contained a 140-kDa polypep-

tide did not contain these genes. The *vip3* and *cry1I* genes, although present in more than 50% of natural *B. thuringiensis* isolates, were underrepresented among the Exo⁺ strains harboring *cry1B*.

DISCUSSION

Although the genetic determinants of β-exotoxin I have not yet been characterized, β-exotoxin I production has been associated with the presence of plasmids harboring *cry* genes (18, 21). Ozawa and Iwahana (24), by transferring a single 62-MDa plasmid from a *B. thuringiensis* subsp. *darmstadiensis* strain by conjugation, even managed to demonstrate production of both β-exotoxin I and crystals in a strain of *B. cereus*, indicating that at least some of the β-exotoxin I genetic elements and *cry* genes were present on a single plasmid.

The toxic compound produced by the 58 strains whose su-

TABLE 2. Crystal protein profiles of *B. thuringiensis* strains based on β-exotoxin I production[a]

Category	Protein profiles of parasporal inclusions	Toxic supernatants (β-exotoxin I concn, >10 μg/ml)		Nontoxic supernatants (β-exotoxin I concn, <5 μg/ml)		P value
		No. of strains	% of total	No. of strains	% of total	
40 and 45 kDa	40- and 45-kDa protein bands	1	1.7	231	39.7	<0.0001
66 kDa	66-kDa protein band in association with one or several protein bands at about 130 kDa	4	7	169	29	0.0021
70 and 73 kDa	70- and 73-kDa protein bands alone or in association with one or two protein bands at about 130 kDa	0	0	10	1.7	0.320
128, 130, or 132 kDa	One or several protein bands at about 130 kDa	0	0	74	12.7	0.0068
140 kDa	140-kDa protein band alone or in association with protein bands at about 66 or 130 kDa	39	67	2	0.4	<0.0001
Unusual profile	One or several protein bands of various sizes unique among the known Cry proteins	14	24.3	96	16.5	0.1719

[a] Each strain was characterized individually on the basis of its crystal protein profile determined by SDS-PAGE and the amount of β-exotoxin I produced (<5 or >10 μg/ml). P values were calculated by chi-square analysis for a total of 640 strains.

TABLE 3. Genotypic characterization of the strains
producing 140-kDa crystal proteins[a]

Strain	β-Exotoxin I concn (μg/ml)[b]	Cry toxin(s) (kDa)	PCR analysis results			
			cry1B	vip2	vip3	cry1I
Strains active against *A. grandis*						
177	11	128, 130, 140	+	+	+	+
180	89	128, 130, 140	+	+	+	−
181	66	140	+	+	−	−
240	54	66, 140	+	+	+	+
286	50, 93	128, 140	+	+	−	−
376A	79	140	+	+	−	−
421B	65	130, 140	+	+	+	+
461	225.3	140	+	+	−	+
550	79.5	140	+	+	−	−
563	>30	140	+	+	−	+
572	16	140	+	+	−	−
594	>30	140	+	+	−	+
601A	94	140	+	+	−	−
603	176	130, 140, UCPB	+	+	−	−
604	157	130, 140, UCBP	+	+	−	−
614B	103, 147	140	+	+	−	−
619	107	140	+	+	−	−
621	107	140	+	+	−	−
637	21	140	+	+	−	−
639	145	140	+	+	−	−
640	40	140	+	+	+	+
813A	80, 87	66, 130, 132, 140	+	+	+	+
830	76	140	+	+	−	−
999	92	140	+	+	−	−
1022	65	140	+	+	−	−
1077A	122	140	+	+	−	−
1260	125	140	+	+	−	−
1830	104	66, 130, 140	+	+	+	+
1836	131	66, 130, 140	+	+	+	+
1866	120	140	+	+	+	+
1925	30	130, 140	+	+	−	−
1934	95	130, 140	+	+	−	−
2689	39	66, 130, 132, 140	+	+	+	+
2690A	61	66, 130, 132, 140	+	+	+	+
2691B	88, 90	66, 130, 132, 140	+	+	+	+
3449	43	130, 140	+	+	−	−
3522	68	66, 130, 132, 140	+	+	+	+
3647	>50	130, 140	+	+	−	−
4355A	64.5	140	+	+	+	−
Nonactive strains						
3689	0.6, 3.7	140	−	−	−	+
4393	3, 3.7	140, UCBP	−	−	+	+

[a] The 41 strains that produced a 140-kDa Cry toxin are shown. The strain designations are those of the INRA collection held at INRA La Minière. The amount(s) of β-exotoxin I produced, the protein electrophoretic band(s), and the PCR results for detection of *cry1B, vip2, vip3, and cry1I* are indicated for each strain. UCPB, unusual crystal protein band.
[b] Two values on the same line are results from two independent experiments.

pernatants were toxic to *A. grandis* was clearly identified as β-exotoxin I by using HPLC. In bioassays with *A. grandis*, in which the larvae ingested crude supernatants, the typical inhibition of growth and molting was observed, followed by death, as described in several studies on β-exotoxin I (2). Quantification by HPLC indicated that in all cases the concentration of β-exotoxin I in the supernatants was more than 10 μg/ml. These results indicated that the toxicity of these strains was due to β-exotoxin I, eliminating possible confusion with any other insecticidal metabolite, such as β-exotoxin II (21). In addition, we found that 46% of a sample of 118 *B. thuringiensis* strains whose supernatants were not toxic to *A. grandis* also

produced small but detectable amounts of β-exotoxin I (between 2 and 5 μg/ml). We did not check for lower levels of β-exotoxin I production, but a larger number of β-exotoxin I-producing *B. thuringiensis* strains would probably be identified if the detection threshold for this compound was lowered. Given the large number of natural isolates that produce a constant low level of β-exotoxin I and given that in our culture conditions 9% of *B. thuringiensis* strains have the capacity to produce high levels of this compound, understanding the environmental factors or mutations that enhance β-exotoxin I production seems to be important.

We found a highly significant association between the presence of the *cry1B* gene and β-exotoxin I production. Such an association was previously reported by Perani et al. (25), but these authors did not find a statistical relationship between the ability to produce this metabolite and the presence of genes of the *cry1B* subfamily. In our study, only two strains that produced 140-kDa crystal protein bands did not give an amplicon with *cry1B*-specific primers (7), which suggests that these strains produce a unique 140-kDa polypeptide that is different from Cry1B. Remarkably, the 39 strains that produced large amounts of β-exotoxin I also gave amplicons with *vip2* primers, suggesting that *cry1B*, *vip2* (29), and the genetic determinants of β-exotoxin I are located on the same plasmid. In contrast, the vast majority of *B. thuringiensis* strains that produce 40- and 45-kDa, 66-kDa, and 128-, 130-, or 132-kDa crystal proteins, potentially corresponding to various known Cry toxins active against Lepidoptera, did not produce β-exotoxin I or produced only small amounts. Thus, certain Cry toxins are clearly only rarely associated with the determinants necessary to promote production of high levels of β-exotoxin I, suggesting that the genes encoding these proteins are present on different plasmids. We identified no strains that produced β-exotoxin I along with 70- and 73-kDa crystal proteins. However, we studied only a limited number of strains producing this profile, too few to establish a significant correlation for this category of strains.

In summary, this study shows that, in *B. thuringiensis*, production of high levels of β-exotoxin I is linked to the presence of *cry* plasmids preferentially harboring the *cry1B* crystal protein gene. We also found a strong association between some determinants that enhance production of β-exotoxin I and the *cry1B* and *vip2* genes, which suggests that these genes are generally located on the same plasmid. Further work should aim to characterize the various plasmid-borne and chromosomal genes involved in the regulation and/or biosynthesis of β-exotoxin I.

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