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Occurrence and Linkage Between Secreted Insecticidal Toxins in Natural Isolates of *Bacillus thuringiensis*

Sylvain Espinasse,[1] Josette Chaufaux,[1] Christophe Buisson,[1] Stéphane Perchat,[1] Michel Gohar,[1,2] Denis Bourguet,[1] Vincent Sanchis[1,3]

[1]Unité de Recherches de Lutte Biologique, INRA La Minière, 78285 Guyancourt Cedex, France

[2]Aventis Crop Science, Jozef Plateaustraat 22–B 9000 Ghent, Belgium

[3]Unité de Biochimie Microbienne, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

Abstract. Little is known about the occurrence and linkage between secreted insecticidal virulence factors in natural populations of *Bacillus thuringiensis* (*Bt*). We carried out a survey of 392 *Bt* strains isolated from various samples originating from 31 countries. The toxicity profile of the culture supernatants of these strains was determined individually against *Anthonomus grandis* (Coleoptera) and *Spodoptera littoralis* (Lepidoptera). We analyzed β-exotoxin I production and searched for the genes encoding Vip1–2, Vip3, and Cry1I toxins in 125 of these strains. Our results showed that these insecticidal toxins were widespread in *Bt* but that their distribution was nonrandom, with significant linkage observed between *vip3* and *cry1I* and between *vip1–2* and β-exotoxin I. Strains producing significant amounts of β-exotoxin I were more frequently isolated from invertebrate samples than from dust, water, soil, or plant samples.

Although microbial populations display apparent overall genotypic and phenotypic homogeneity, many microbial species exhibit considerable diversity, which has an impact on the biological properties of individual strains [16]. This is especially important for microorganisms with pathogenic properties, such as *Bacillus thuringiensis* (*Bt*). *Bt,* which has a worldwide distribution [19], is a sporogenic bacterium used in biological control that synthesizes specific insecticidal toxins [24]. To date, most studies on *Bt* insecticidal toxins have focused on δ-endotoxins, which are produced as parasporal inclusions, whereas little is known about the pathogenic potential of the compounds secreted into the culture medium [7, 15]. Many *Bt* strains secrete vegetative insecticidal proteins (Vips) during vegetative growth [27]. Two classes of Vip toxins have been described. The first consists of a binary system composed of two proteins, Vip1 and Vip2, which are 100 kDa and 52 kDa in size, respectively. These proteins are highly toxic to certain coleopteran species [27, 28]. The second class consists of an 82.5-kDa protein, Vip3, which is active against a wide spectrum of lepidopteran insects [8]. These two classes of protein do not display sequence homology. Cry1I (also referred as to CryV in the literature) is another insecticidal toxin secreted during early stationary phase. It is active against certain lepidopteran and coleopteran insect larvae [15]. Finally, several *Bt* strains also produce a thermostable exotoxin, known as β-exotoxin I or thuringiensin [7]. This compound is an adenine nucleotide analogue, highly toxic to a wide range of insect species [9], that is thought to inhibit RNA polymerase [25], thereby affecting insect molting and pupation, in some cases having teratogenic effects [5]. β-exotoxin I is also toxic to mammalian cells [3, 18] and is very persistent in the environment [4]. It has therefore been banned for public use in accordance with WHO recommendations [29]. If we are to improve the efficacy and ensure safety of *Bt* products, we need to increase our understanding of the genetic variability of natural *Bt* populations with respect to the expression of these secreted virulence factors. In this study, we analyzed the toxicity of culture supernatants of natural *Bt* isolates to larvae of *Spodoptera litto-*

Correspondence to: V. Sanchis; *email:* vsanchis@pasteur.fr

Table 1. Oligonucleotides used in this study

Primer	Sequence	Description, accession number	Product size (bp)
cry1I-fw	5′ aacactcagtatatgaat 3′	position 1059–1374, L49391	378
cry1I-rev	5′ cacatgtgatgctgaaat 3′	position 1437–1419, L49391	378
vip2-fw	5′ gggaaagaaaaagaaaaagagtgg 3′	position 231–255, A64136	914
vip2-rev	5′ catatatcctttgtcttcttt 3′	position 1145–1124, A64136	914
vip3-fw	5′ acatcctccctacactttctaatac 3′	position 1700–1725, L48811	678
vip3-rev	5′ tcttctatggacccgttctctac 3′	position 2378–2353, L48811	678

ralis (Lepidoptera: Noctuidae) and *Anthonomus grandis* (Coleoptera: Curculionidae). We then explored the genomes of 125 natural *Bt* isolates for the genes encoding the four known insecticidal toxins secreted by *Bt.* The prevalence of these toxins in this species was determined and their distribution in relation to sample origin analyzed. We found that the secreted insecticidal toxins were not randomly distributed among *Bt* populations.

Materials and Methods

Bacterial cultures and supernatant preparation for toxicity assays. The INRA collection contains 1260 natural *Bt* strains, representing isolates from 101 countries over the world, isolated from various sources (soil, insects, dust, plants, animal waste, etc.). These strains were primarily characterized as *B. thuringiensis* on the basis of their ability to produce crystals during sporogenesis [6]; they are conserved as spores in 12% vol/vol glycerol suspension at −20°C. 392 *Bt* strains, representing isolates from France and from 31 other countries across the five continents, were randomly taken from our collection. The strains were grown on LB nutrient agar plates at 30°C. Liquid cultures, in 100-mL LB, were inoculated from a single colony and shaken for 24 h at 30°C. The cultures were harvested at mid-sporulation (before cell lysis) by centrifugation at 14,000 *g* for 10 min at 4°C. The supernatants were filtered twice through 0.2-μm Nalgene filter units (Nalgene). The resulting extracts, to be used for toxicity assays and β-exotoxin I determination, were stored at −20°C until bioassay.

Insect bioassays. The toxicity of the *Bt* supernatant preparations was determined using neonate larvae of *S. littoralis* and eggs of *A. grandis,* using a free ingestion technique. *S. littoralis* larvae were fed with an artificial diet [21], dispensed into 50-well plastic plates (1.65-cm^2 surface each). Supernatant (25 μL) was applied uniformly over the food surface and allowed to dry. One neonate larvae was placed on each of 35 wells and incubated for 10 days at 25°C, with a photoperiod of 16:8 (L:D) hours and 70% relative humidity (RH). Mortality was recorded at 3, 6, and 10 days. For *A. grandis,* the supernatant was incorporated into an artificial diet to a final concentration of 8% and dispensed into sterile 24-well plates. The diet consisted of a mixture of soya flour, cotton flour, wheat germ oil, cholesterol, sugar, vitamins, and agar. One egg of *A. grandis* was placed on the food in each well. Control larvae developed on untreated food for three weeks after hatching at 25°C. Percentages of mortality were calculated at three weeks after Abbott correction [1].

PCR amplifications for cry1I, vip3, and vip1-2 detection. 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 using Primer Select from the DNAStar software (DNAStar Inc). DNA was obtained from cells grown overnight on LB-agar plates and resuspended in 100 μL of distilled water. Cell membranes were disrupted by freezing at −70°C and immersion in a 98°C water bath (heat shock). The cells were centrifuged and the DNA suspension (2 μL) was mixed with 0.5 μL of each primer (0.1 μM), 0.5 U Taq polymerase, water (50 μL), $MgCl_2$, and buffer, according to the manufacturer's instructions (Gibco-BRL). 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, 1 min and 30 s at 72°C, and a final elongation for 10 min at 72°C. Amplifications were recorded as positive, only when specific fragments of the expected size were found after migration on a 1% agarose gel. 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 quantified by HPLC [11]. Briefly, for solvent extraction, acetone was added to the exotoxin in 0.2 mL of the culture supernatant to a final concentration of 90%, and the mixture was centrifuged. The pellet was resuspended in 0.2 mL of double distilled (dd) water. Acetonitrile was added to a final concentration of 40% and the mixture was then centrifuged. The pellet was discarded and the acetonitrile concentration of the supernatant brought up to 90%. The precipitate was collected by centrifugation and the pellet was resuspended in 100 μL of 50 mM potassium phosphate buffer, pH 2.5. For HPLC, we injected 25 μL of the sample into a Lichrospher (Merck) C18 endcapped 4- × 250-mm column. A gradient of 5% to 15% methanol in 50 mM potassium phosphate buffer, pH 2.5 was developed over 10 min. The flow rate was 1 mL/min, and UV absorption was monitored at 260 nm. β-exotoxin I was eluted at 5.5 min. The detection limit of this method for β-exotoxin I was 2 μg/mL. A standard sample (70% purity) of β-exotoxin I was kindly provided by I. Thiery from the Laboratoire des Bactéries Entomopathogènes (Institut Pasteur, Paris, France).

Statistical analyses. ***Frequencies of virulence factors***. Total frequencies of occurrence for each virulence factor were calculated from the experimental data. Assuming that there was no linkage between the virulence factors, the expected frequencies for each combination of the virulence factors is the product of the frequencies of the presence or of the absence of each virulence factor. For example, let us consider the four virulence factors {a, b, c, d} present with frequencies of respectively $\{f_a, f_b, f_c, f_d\}$. The expected frequency of the combination (a, b, c) is $F_{abc} = f_a \cdot f_b \cdot f_c \cdot (1 - f_d)$. Expected frequencies were compared to the observed ones, using the Pearson chi-square test, the null hypothesis being no linkage between the virulence factors. The Pearson correlation coefficient was calculated for each pair of virulence factors. The significance of all the correlations in the matrix was tested with the Bartlett chi-square test, and the probabilities associated with each correlation were corrected for multiple tests using the Bonferroni method [13].

Table 2. Insecticidal activity of the culture supernatants of natural *Bacillus thuringiensis* isolates against larvae of *Spodoptera littoralis* and *Anthonomus grandis*

Number of supernatants	Mortality rate (%)	Number of strains with supernatants toxic to: Both *S. littoralis* and *A. grandis*	*S. littoralis* only	*A. grandis* only	Neither species
Toxic supernatants	71–100%	29	107	5	—
$n = 180$	31–70%	4	32	3	—
Nontoxic supernatants	6–30%	—	—	—	28
$n = 212$	0–5%	—	—	—	184
Total $n = 392$	—	33	139	8	212

n = number of *Bt* strains.

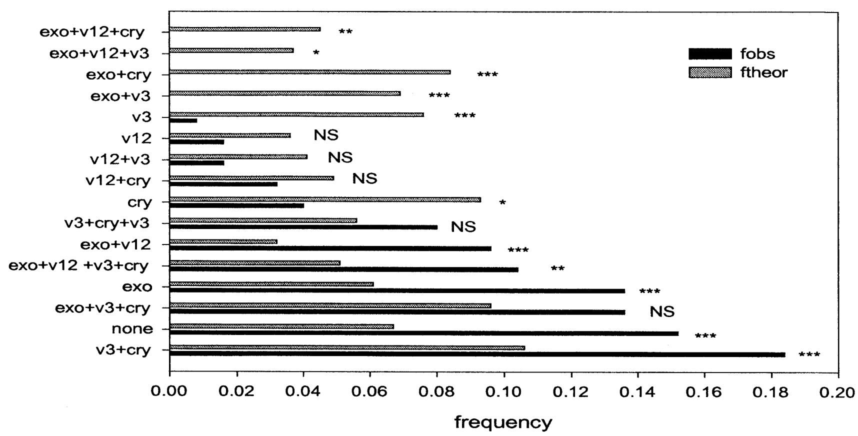

Fig. 1. Chi-square analysis of the linkage between various virulence factors in natural *Bt* isolates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; NS: not significantly different; exo, β-exotoxin I; v12, *vip1–2;* v3, *vip3;* cry, *cry1I;* fobs, frequency observed; ftheor, theoretical frequency).

Relationship between sample or geographic origin and genotypic or toxicity profiles. For each toxicity profile (i.e. nontoxic, toxic only to *S. littoralis,* or toxic only to *A. grandis*), the homogeneity of the frequencies observed for the different categories in sample or geographic origin were tested using a two-sided Fisher's exact test on contingency tables. Fisher's exact tests were performed using the STRUC program implemented in GENEPOP 3.2a [22].

Results

Toxicity of stationary-phase culture supernatants of *Bt* isolates to *S. littoralis* and *A. grandis*. We assessed the activity of the culture supernatants of 392 *Bt* isolates against larvae of *S. littoralis* and *A. grandis* (Table 2). *Bt* isolates were differentiated into four groups: nontoxic, toxic only to *S. littoralis,* toxic only to *A. grandis,* or toxic to both insects. We found that 212 culture supernatants (54.1%) were inactive or only slightly toxic to both insect species (0–30% mortality), 139 were toxic to *S. littoralis* only, 33 were toxic to both *S. littoralis* and *A. grandis,* and the supernatants of eight isolates were toxic only to *A. grandis.*

Screening of 125 *Bt* isolates for the presence of *cry1I, vip3,* and *vip1–2* genes and for the production of β-exotoxin I. We selected 125 isolates for further analysis and investigated whether these toxicity patterns were correlated with the presence of one or several of the four known secreted insecticidal toxins. These strains were representative of the various toxicity profiles observed among the 392 samples initially studied and originated from different locations and sampling sources. The number of samples with each of the four toxicity profiles was representative of the frequency of these profiles in the bioassay. For each strain, we determined the amount of β-exotoxin I present in the culture supernatant, using an improved quantification method based on reverse-phase HPLC [11]. β-exotoxin I production (>2 μg/mL) was detected in 47.2% of the strains. We also used PCR to investigate whether these 125 strains contained the *cry1I, vip3,* and *vip1–2* genes. The PCR products were of the expected sizes, 378 bp for *cry1I,* 676 bp for *vip3,* and 914 bp for *vip1–2* (Table 3). The *cry1I* gene was detected in 57.6%, *vip3* in 52.8%, and *vip1–2* in 34.4% of the 125 strains studied. Fifteen percent of the strains generated no PCR product for the three insecticidal toxin genes examined and produced less than 2 μg/mL β-exotoxin I. The 45 isolates with supernatants

Table 3. Detection of *cry1I, vip3,* and *vip1–2* genes by PCR analysis and quantification of β-exotoxin I in 125 isolates of *Bacillus thuringiensis*

Toxicity profile of the supernatants	Total number of isolates examined	Genotype screening by PCR			Determination of β-exotoxin I μg per mL of culture supernatant	Number of isolates
		cry1I	*vip3*	*vip1–2*		
Nontoxic	67 (53.6%)	−	−	−	0 < μg < 2	20
		−	−	−	2 < μg < 5	14
		−	−	−	7 μg	1
		+	+	−	0 < μg < 2	8
		+	+	−	2 < μg < 5	3
		+	+	+	0 < μg < 2	4
		+	+	+	2 < μg < 5	2
		+	−	+	0 < μg < 2	5
		+	−	−	0 < μg < 2	4
		−	+	−	0 < μg < 2	1
		−	−	+	0 < μg < 2	2
		−	−	+	2 < μg < 5	2
		−	+	+	0 < μg < 2	1
S. littoralis only	45 (36%)	+	+	−	0 < μg < 2	15
		+	+	−	2 < μg < 5	13
		+	+	−	10 μg	1
		+	+	+	0 < μg < 2	7
		+	+	+	2 < μg < 5	9
A. grandis only	3 (2.4%)	−	−	+	15 < μg < 50	3
S. littoralis and *A. grandis*	10 (8%)	−	−	−	> 50 μg	1
		+	+	+	> 50 μg	1
		−	+	−	0 < μg < 2	1
		−	−	+	> 50 μg	7
Total	125	72	66	43	57*	125

* > 2 μg of β-exotoxin I/mL.

toxic to *S. littoralis* harbored both the *vip3* and *cry1I* genes, the products of which are known to be active against certain lepidopteran insects but not against coleopterans [8], and produced small amounts of β-exotoxin I (<5 μg/mL). Twelve of the 13 isolates toxic to *A. grandis* produced significantly larger amounts of β-exotoxin I (>5 μg/mL) and harbored *vip1–2* genes. Three of these isolates were toxic to *A. grandis* (100% mortality in all cases) but not to *S. littoralis* (<15% mortality). We determined precisely the LC_{50} of purified β-exotoxin I for both insects and found that *A. grandis* was five times more susceptible than *S. littoralis* to this toxin (LC_{50}s of 6 μg/mL and of 30 μg/mL, respectively). The three strains toxic to *A. grandis* only produced intermediate amounts of β-exotoxin I (~16–50 μg/mL), consistent with the observed pattern of mortality. Nine of the 10 isolates toxic to both insects produced more than 50 μg/mL β-exotoxin I, accounting for their activity against both species. We found only one isolate toxic to *A. grandis* that did not produce β-exotoxin I or possess *vip1–2* genes. This particular isolate contained a *vip3*-like gene whose expression product was not toxic to *A. grandis.* This suggests that this strain secretes another, possibly novel, type of insecticidal compound highly active against *A. grandis* and/or *S. littoralis.*

Statistical analysis of toxicity and genotype data. The observed frequencies of the various genotypes among the 125 *Bt* strains were compared to the expected frequencies of the 16 possible genotypes in the absence of linkage among these four factors (the toxin genes and β-exotoxin I production). We observed an overall significant difference ($p < 0.0001$) between the observed and expected frequencies, indicating linkage disequilibrium between the virulence factors. The sample only contained representatives of twelve of the sixteen possible genotypes (Fig. 1, black bars) and seven of them accounted for most of the variability. For all but two of these categories (*vip3* + *cry1I* + β-exotoxin I and *vip3* + *cry1I* + *vip1–2*), the deviations of the observed frequencies from the expected frequencies was significant ($p < 0.01$). These linkages—i.e., *vip3* + *cry1I* (23 strains), none of the parameters examined (19 isolates), β-exotoxin I (>2 μg/mL) (17 isolates), all four toxins (13 isolates), and *vip1–2* and β-exotoxin I (12 isolates)—resulted in the frequencies of the most represented of the secreted virulence factors (i.e., Cry1I and Vip3), present singly in a strain, being much lower ($p < 0.05$ and $p < 0.005$ for *cry1I* and *vip3,* respectively) than expected. Similarly, the frequency of β-exotoxin I production in strains with *vip3* or *cry1I* genes was also lower than expected ($p < 0.005$). The other combinations were also underrepresented in the population. We used pairwise correlation analysis to investigate this differentiation further (Fig. 2). We found that there was a positive correlation between the *vip3* and *cry1I* genes ($r = 0.81$, $p < 0.001$) and between the presence of *vip1–2* and β-exotoxin I production ($r = 0.3$, $p = 0.002$). This is consistent with our finding that toxicity to *S. littoralis* was almost always associated with the presence of *vip3* and *cry1I* and that toxicity to *A. grandis* was associated with the presence of *vip1–2* and β-exotoxin I production. Conversely, we found that β-exotoxin I production was negatively correlated with the presence of *vip3* ($r = -0.24$, $p < 0.05$) and with the presence of *cry1I* in a given strain ($r = -0.28$, $p < 0.01$). We also analyzed the frequency of the various toxicity profiles as a function of the geographic origin or nature of the samples. The toxicity profile was not dependent ($p > 0.05$) on the geographic origin (Europe, Asia, Africa, or America) or the nature of the sample (invertebrates, dust, water, soil, or plants). However, strains producing more than 5 μg of β-exotoxin I were preferentially isolated from invertebrates (this difference being marginally significant: $p = 0.05$).

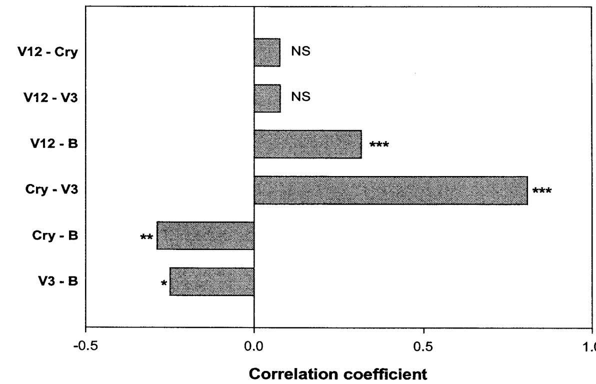

Fig. 2. Pairwise correlations between virulence factors in natural *Bt* isolates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; NS, not significantly different; B, β-exotoxin I; v12, *vip1–2;* v3, *vip3;* cry, *cry1I).*

Discussion

In this study, we investigated the prevalence and distribution of various insecticidal compounds, secreted by *Bt,* to obtain an insight into the biological diversity of natural isolates. Analysis of the toxicity spectra of the culture supernatants of the 392 *Bt* isolates revealed that over half the strains were not toxic to *S. littoralis* and *A. grandis.* This suggests that a large number of isolates do not secrete insecticidal compounds (at the concentrations tested) or that they produce toxic compounds active against other insect species. Among the toxic isolates those with activity against *S. littoralis* were the most frequent in this screening, consistent with previous observations that active *Bt* strains are generally more prevalent among lepidopteran species [6, 14], the larvae of which are commonly found on leaves of many plants [26].

Analysis of β-exotoxin I production and search for the genes encoding the secreted Vip1–2, Vip3, and Cry1I toxins in 125 of these isolates indicated that these four toxins are widely distributed among natural *Bt* populations. The *vip3* genes were detected in more than 50% of the strains tested; this figure was much higher than the 23% reported for the 125 colonies examined by Rice [23], and this difference was highly significant (two-tailed Fisher's exact test, $p < 10^{-5}$). Similarly, we found that 57% of the strains contained a copy of *cry1I,* whereas Gleave et al. [10] found that the *cry1I* gene was present in 7 of 21 (31%) *Bt* serotypes, a frequency also significantly lower than that obtained in this study (two-tailed Fisher's exact test, $p = 0.003$). The reasons for these discrepancies are unclear, but may reflect natural variation in bacterial population structures from different locations. On the other hand, the frequency of β-exotoxin I production reported here, in 47.2% of the strains, although lower than the 59% (17 of 29 isolates) reported by Perani et al. [20], is not significantly different (two-tailed Fisher's exact test, $p = 0.307$). Finally, we found that 34.5% of the isolates contained *vip1–2* genes indicating that Vip1–2 toxins are also common among *Bt* strains. Given the large number of natural isolates producing one or several of these toxins, it is likely that these compounds make a significant contribution towards determining the insecticidal host range of individual *Bt* strains. Our results also indicated that the toxicity pattern of the isolates was independent of the nature of the sample (soil, insect, or other sources) and of its geographic origin. This is not very surprising since the ecology and environmental distribution of spore forming bacteria is probably largely due to aerial dispersion. However, we found a fairly good correlation between the genotypic profiles of the isolates and their insecticidal properties. It is therefore possible that some combinations of toxin genes of which the host can take a particular advantage may have become fixed. None of the 67 nontoxic strains produced significant amounts of β-exotoxin I, and the majority of them neither contained *vip3, cry1I,* or *vip1–2.* Similarly, all of the isolates toxic to *A. grandis* produced more than 5 μg of β-exotoxin I, and the production of this compound in most of these strains was associated with the presence of *vip1–2* genes. This suggests that toxicity to *A. grandis* is associated mainly with the production of β-exotoxin I but that both toxins may play an important role for virulence against *A. grandis.* We also found that all of the isolates toxic only to *S. littoralis,* harbored both *vip3* and *cry1I.* It is not known whether the *vip* genes are present on the chromosome or on plasmids, but some of the genetic determinants involved in β-exotoxin I production have been shown to be present on the same plasmids as certain *cry* genes [12, 17]. We found a strong linkage between *vip1–2* and the genetic determinants of β-exotoxin I, on one hand, and between the *cry1I* and *vip3* genes, on the other hand. This provides indirect evidence that the genes encoding these four insecticidal toxins are located on plasmids. We therefore suggest that *vip1–2* and the genetic determinants of β-exotoxin I are generally present on the same plasmid and that *cry1I* and *vip3* are generally present on another plasmid. The evolution and dissemination of adaptative traits such as toxin production in bacterial populations is frequently mediated by DNA elements such as plasmids [2]. One possible driving force and mechanism responsible for the genetic shaping and divergence of natural *Bt* populations could be the transfer and fixation of advantageous plasmid/toxin genes combinations that allow a given strain to become established as a new clonal population and colonize a special niche. However, further research is needed to establish whether there is indeed a correlation between the genetic differentiation of *Bt* populations and the presence of certain plasmid types or specific plasmid-borne toxin genes combinations.

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