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ORIGINAL ARTICLE

# Diversity and distribution of lepidopteran-specific toxin genes in *Bacillus thuringiensis* strains from Argentina

**Diego H. Sauka***, **Graciela B. Benintende**

*Instituto de Microbiología y Zoología Agrícola INTA, CC No 25 (1712), Castelar, Buenos Aires, Argentina*

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*Epinotia aporema*;
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**Abstract** A total of 268 *Bacillus thuringiensis* strains obtained from different sources of Argentina were analyzed to determine the diversity and distribution of the *cry1*, *cry2*, *cry8*, *cry9* and *vip3A* genes encoding for lepidopteran-specific insecticidal proteins. Twin strains were excluded. Ten different profiles were detected among the 80 selected *B. thuringiensis* strains. Two of these profiles (*cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* (35/80), and *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* (25/80)) pooled 75% of the strains. The existence of this low diversity is rare, since in most of the studied collections a great diversity of insecticidal toxin gene profiles has been described. In addition, the most frequently detected profile was also most frequently derived from soil (70%), stored product dust (59%) and spider webs (50%). In contrast, the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* profiles were mainly detected in strains isolated from leaves (40%) and dead insect larvae (50%). Six of the identified insecticidal toxin gene profiles were discovered in strains isolated from stored product dust and leaves indicating higher diversity of profiles in these kinds of sources than in others. Some strains with high insecticidal activity against *Epinotia aporema* (Lepidoptera) larvae were identified, which is important to explore future microbial strategies for the control of this crop pest in the region.


* Corresponding author at: Insumos Bacterianos, Instituto de Microbiología y Zoología Agrícola, Instituto Nacional de Tecnología Agropecuaria, De los Reseros y Las Cabañas s/nro, CC 25, C.P. 1712, Castelar, Buenos Aires, Argentina.
*E-mail address:* sauka.diego@inta.gob.ar (D.H. Sauka).

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**PALABRAS CLAVE**
*Bacillus thuringiensis*;
*Cry*;
*Vip*;
Lepidoptera;
*Epinotia aporema*;
Argentina

**Diversidad y distribución de genes de toxinas insecticidas Lepidoptera-específicos en cepas de *Bacillus thuringiensis* de Argentina**

**Resumen** Se analizaron 268 cepas de *Bacillus thuringiensis* obtenidas de diferentes fuentes de Argentina con el objeto de determinar la diversidad y distribución de genes *cry1*, *cry2*, *cry8*, *cry9* y *vip3A*, *que codifican* proteínas insecticidas lepidóptero-específicas. Se excluyeron las cepas gemelas. Se detectaron solo diez perfiles diferentes entre los 80 *B. thuringiensis* seleccionados. Dos de estos perfiles, el *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* y *vip3Aa* (35/80) y el *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* y *vip3Aa* (25/80), comprendieron el 75% de las cepas seleccionadas. La existencia de esta baja diversidad es una rareza, ya que en la mayor parte de las colecciones estudiadas se ha descrito una gran diversidad de perfiles de genes de toxinas insecticidas. El perfil detectado con mayor frecuencia se obtuvo principalmente de cepas procedentes de suelo (el 70% de los de esa fuente lo tenían), también fue mayoritario entre los procedentes de polvo de producto almacenado (59%) y en los que procedían de telas de araña (50%). En cambio, el perfil *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* y *vip3Aa* se detectó principalmente en las cepas aisladas de hojas (40%) y de larvas de insectos muertos (50%). Seis de los perfiles identificados fueron encontrados en cepas aisladas de polvo de producto almacenado y de hojas, lo que indica una mayor diversidad de perfiles en estas fuentes que en otras. Se identificaron algunas cepas con alta actividad insecticida contra larvas de *Epinotia aporema* (Lepidoptera), hallazgo importante para explorar en el futuro estrategias microbianas para el control de esta plaga en la región.


## Introduction

The extensive culture of crops requires the utilization of chemical insecticides to control the attack of lepidopteran pests, which may be toxic and may cause environmental hazards when used improperly. Therefore, the interest in the application of bioinsecticides based on the gram-positive entomopathogenic bacterium *Bacillus thuringiensis* as an option instead of chemical insecticides has contributed to establish *B. thuringiensis* collections all around the world, since they have a limited and particular spectrum of toxicity to a specific range of insect species[37]. Characterization of these collections has revealed the great variability, diversity and distribution of this bacterium in nature. *B. thuringiensis* is found in soil, in stored product dust, in the phylloplane of plants, and in insects and their habitats, even in spider webs[4,23,30,38,42,43]. Furthermore, some degree of relationship has been established between the distribution of *B. thuringiensis* and the type of sample, and between their distribution and the geographical or climate region of origin[23,43].

The insecticidal properties of *B. thuringiensis* are mainly attributed to the synthesis of insecticidal crystal proteins (Cry proteins) and/or vegetative insecticidal proteins (Vip proteins), which are synthesized during sporulation or vegetative growth, respectively. More than 700 *cry* and 130 *vip* genes have been identified and classified into classes and subclasses based on the percent of pairwise amino acid identity of their corresponding proteins[15]. It has been established that genes within the *cry1*, *cry2*, *cry9* and *vip3* groups encode proteins that are toxic against lepidopteran larvae[7]. Recently, a *cry8* gene has been included into this selected group of encoding lepidopteran active proteins[1]. Generally, Cry producer strains synthesize 130–140 kDa proteins contained in bipyramidal crystals and also synthesize 65 kDa proteins contained in smaller cuboidal crystals which have a somewhat extended toxicity spectrum, as some are also mildly toxic to mosquito larvae[9]. Still, some lepidopteran-active *B. thuringiensis* strains can produce 130 kDa proteins which occur as spherical inclusions[1,44]. Most *B. thuringiensis* strains harbor complex insecticidal gene combinations[5,8,27,43], such as the well-known HD-1 strain[46], whereas some others can harbor a single *cry* gene, such as strain HD-73 strain[29].

The aim of the present work was to characterize a *B. thuringiensis* collection from Argentina. We determined the diversity and distribution of lepidopteran-specific insecticidal toxin genes in these isolates and tried to correlate these gene profiles with the region and source of isolation. In addition, the toxicity of specific *B. thuringiensis* native isolates harboring at least one of these insecticidal toxin genes was analyzed through bioassays against *E. aporema*, a lepidopteran insect that may attack legume crops in Argentina and other regions of South America.

## Materials and methods

### *B. thuringiensis* isolates and strains

Two hundred and sixty eight *B. thuringiensis* strains collected from soils, stored product dust, leaves, spider webs, and dead insect larvae from different regions of Argentina were obtained from the Instituto de Microbiologia y Zoologia

Agricola – Instituto Nacional de Tecnologia Agropecuaria (IMYZA-INTA) bacterial collection. *B. thuringiensis* serovar *kurstaki* HD-1 and serovar *israelensis* HD-567 were kindly provided by the United States Department of Agriculture (USDA), Agricultural Research Service (Peoria, USA), and *B. thuringiensis* serovar *morrisoni* DSM2803 by the Centro de Investigacion y Estudios Avanzados (CINVESTAV, Irapuato, Mexico). Powders of spore-crystal complexes were obtained as previously described and kept at −20 °C until further use[38].

### Characterization of crystals and their protein composition

Parasporal inclusions of each isolate were primarily classified through phase-contrast microscopy. The protein composition of crystals was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 3% stacking gel and 10% running gel in a Bio-Rad Mini Protean 3 Cell system. Electrophoresis was carried out at 50 V for 15 min and 100 V for 2 h. Gels were stained with Coomassie Brilliant Blue. High molecular weight standard mixture (Sigma SDS-6H) was used to estimate molecular masses of crystal proteins.

### Detection and identification of insecticidal toxin genes

The DNA templates for PCR were obtained as previously described[35]. Five microliter of supernatant was used as DNA template in each reaction. Detection of *cry1* genes was carried out by following conditions, essentially as previously described by Juarez-Perez et al.[27], using the I(+) and I(−) group primers. Further identification of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1F* and *cry1G* was conducted as described by Ceron et al.[11,12] and Juarez-Perez et al.[27] PCR-restriction fragment length polymorphism (PCR-RFLP) methods previously described by Sauka et al.[34,35,39] were used to detect and identify *cry2*, *cry1I* and *vip3A* genes. Detection of *cry9* genes was carried out by following conditions as previously described[38], using the 9GP and 9GN group primers. For the detection of *cry8* genes, novel specific primers were designed based on the analysis of conserved regions by multiple alignments of DNA sequences in the ''Bt toxin nomenclature website''[15] using ClustalW (http://www.ebi.ac.uk/clustalw/) and Oligoanalyzer 3.1 (http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/). This PCR was carried out according to Sauka et al.[38], but using 2 mM $MgCl_2$ per reaction and each cycle consisting of an annealing step at 48 °C for 1 min. Primers used for the amplification of a DNA fragment of 927–945 bp in size of *cry8* genes and the others used during the course of this study are summarized in Table 1.

### Purification and solubilization of crystals

Crystals of selected *B. thuringiensis* strains were purified by continuous NaBr gradient differential centrifugation and solubilized as previously described[38].

### *Epinotia aporema* bioassays

Toxicity of *B. thuringiensis* spore-crystal suspensions was analyzed by bioassays against neonate larvae of *E. aporema* Wals. (Lepidoptera: Tortricidae). Spore-crystal suspensions (final concentration 2.5 μg/ml) were incorporated into polypropylene conical tubes containing a thermostatized (40 °C) artificial diet for *E. aporema* and poured into each well of a 24-well plate (Nunc 143982)[20]. Only sterile distilled water was added to the negative controls and also serovar *kurstaki* HD-1 strain was used as positive controls. Twenty four neonate *E. aporema* larvae were used per assay (three replicates at least). Mortality was registered after 5 days at 29 °C. *E. aporema* larvae were considered dead if they failed to respond to gentle probing. Bioassays with purified and solubilized crystals were conducted in the same way, except that a series of six concentrations (concentration range: 3.500–0.272 μg/ml; dilution factor: 0.600) were prepared in order to establish the concentration-response relationship by probit analysis. Twenty four larvae were tested for each concentration. Statistical restrictions were followed as mentioned earlier[24].

## Results and discussion

### Characterization of an Argentine *B. thuringiensis* collection

In this manuscript, we present the characterization of a *B. thuringiensis* collection built from strains isolated from different sources of Argentina. This characterization contributes to a better knowledge of *B. thuringiensis* diversity in Argentina and South America, where only a few large collections have been extensively characterized[3,6,17,41,42]. Moreover, even fewer studies have reported a detailed characterization of *B. thuringiensis* strain collections in terms of insecticidal toxin gene content[42]. In particular, this Argentine *B. thuringiensis* collection was characterized through different methods focused only on putative lepidopteran-active isolates. PCR and PCR-RFLP results showed that 195 strains out of 268 harbors, at least one anti-lepidopteran toxin gene. Those *B. thuringiensis* strains isolated from the same sample that share the same morphology of parasporal crystals, the same protein composition of crystals as revealed by SDS-PAGE and the same insecticidal toxin gene profile were considered twin strains. Exclusion of twin strains is important in order to get a real estimation of the diversity of the sampled areas[34]. That is, 80 of these isolates were selected for further studies in order to avoid an overestimation of distribution frequencies. A full list of these isolates and their main characteristics are presented in the Supplementary Table.

The specific characterization of these selected isolates produced ten different profiles of lepidopteran-specific insecticidal genes (Table 2). The *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa*, and *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* profiles were the most frequently detected. Both profiles pooled 75.0% of the selected strains. The low number of different profiles and this high frequency of strains harboring these two insecticidal toxin gene profiles that differ by a single gene would

**Table 1** Characteristics of primers used for insecticidal toxin gene detection and identification

| Primer pair | Sequence | Expected size (bp) | Gene/s recognized | References |
|---|---|---|---|---|
| I(+) | TRACRHTDDBDGTATTAGAT | 1500–1600 | *cry1* | [27] |
| I(−) | MDATYTCTAKRTCTTGACTA | | | |
| IAb | CGGATGCTCATAGAGGAGAA | ~1400 | *cry1Ab* | [27] |
| I(−) | MDATYTCTAKRTCTTGACTA | | | |
| CJ1 | TTATACTTGGTTCAGGCCC | 246 | *cry1Aa*/*cry1Ad* | [11] |
| CJ2 | TTGGAGCTCTCAAGGTGTAA | | | |
| CJ3 | CAGCCGATTTACCTTCTA | 171 | *cry1Ad* | [11] |
| CJ2 | TTGGAGCTCTCAAGGTGTAA | | | |
| CJ4 | AACAACTATCTGTTCTTGAC | 216 | *cry1Ab*/*cry1Ac* | [11] |
| CJ5 | CTCTTATTATACTTACACTAC | | | |
| CJ6 | GTTAGATTAAATAGTAGTGG | 180 | *cry1Ac* | [11] |
| CJ7 | TGTAGCTGGTACTGTATTG | | | |
| CJ8 | CTTCATCACGATGGAGTAA | 367 | *cry1B* | [11] |
| CJ9 | CATAATTTGGTCGTTCTGTT | | | |
| CJ10 | AAAGATCTGGAACACCTTT | 130 | *cry1 C* | [11] |
| CJ11 | CAAACTCTAAATCCTTTCAC | | | |
| CJ12 | CTGCAGCAAGCTATCCAA | 290 | *cry1D* | [11] |
| CJ13 | ATTTGAATTGTCAAGGCCTG | | | |
| CJ14 | GGAACCAAGACGAACTATTGC | 147 | *cry1E* | [12] |
| CJ15 | GGTTGAATGAACCCTACTCCC | | | |
| CJ16 | TGAGGATTCTCCAGTTTCTGC | 177 | *cry1F* | [12] |
| CJ17 | CGGTTACCAGCCGTATTTCG | | | |
| CJ18 | ATATGGAGTGAATAGGGCG | 235 | *cry1G* | [12] |
| CJ19 | TGAACGGCGATTACATGC | | | |
| 1IF | CTCAACACAAGGRTCTAC | 665 | *cry1I* | [35] |
| 1IR | CTAAGTCCTCTCCTCTATTC | | | |
| II(+) | TAAAGAAAGTGGGGAGTCTT | ~1556 | *cry2* | [32] |
| II(−) | AACTCCATCGTTATTTGTAG | | | |
| S8F | GAATGCCTATCSGATGA | 927–945 | *cry8* | This study |
| S8R | CCABGCTTGTTGGAGTC | | | |
| 9GP | CGGCAAATTTAGTGTCRTGCTTATC | 640–643 | *cry9* | [38] |
| 9GN | AATTCAAGATTTCCTARCGTCGC | | | |
| DS3AF | GTGAAAACAAGTGGCAGTG | 608 | *vip3A* | [39] |
| DS3AR | TCCGCTTCACTTGATTCTACT | | | |

**Table 2** Distribution of insecticidal toxin genes in the Argentine *Bacillus thuringiensis* collection ($n = 80$)

| Profiles of insecticidal toxin genes | Crystal morphologies | Protein size (kDa) | No. of strains/frequency |
|---|---|---|---|
| *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* | B and C | 130 and 65 | 1 (1.2%) |
| *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | B and C | 130 and 65 | 25 (31.3%) |
| *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, *cry1Ia*, *cry2Ab*, *cry9* and *vip3Aa* | B | 130 | 1 (1.2%) |
| *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | B and C | 130 and 65 | 35 (43.8%) |
| *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | B and C | 130 and 65 | 4 (5.0%) |
| *cry1Ac*, *cry1E*, *cry1Ib*, *cry2Aa*, *cry2Ab* and *vip3Aa* | B and C | 130 and 65 | 5 (6.3%) |
| *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | B and C | 130 and 65 | 1 (1.2%) |
| *cry1Ac*, *cry2Ab* and *vip3Aa* | B | 130 | 2 (2.5%) |
| *cry8* | O | 130 | 4 (5.0%) |
| *cry9* | O | 130 | 2 (2.5%) |

B: bipyramidal; C: cuboidal; O: ovoid.

only indicate low diversity in native strains of Argentina. The existence of this low diversity is a rarity, since in most of the studied collections a great diversity of insecticidal toxin gene profiles has been described[4,5,8,18,19,23,42,43,45]. Some others were detected at very low frequencies. That is the case of the *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, *cry1Ia*, *cry2Ab*, *cry9* and *vip3Aa* profiles. The simultaneous presence of *cry1C* and *cry1D* genes is a common feature in most of the

screening studies of insecticidal toxin genes but it is not in our collection[5,19,23,43].

The combination of insecticidal toxin genes seems to be not random. For example, strong associations were observed between the three subclasses of *cry1A* genes. As shown in Table 2, *cry1Aa*/*cry1Ac*, and *cry1Aa*/*cry1Ab*/*cry1Ac* have a strong tendency to occur together. The existence of strains harboring these *cry* gene combinations may be explained by previous studies on *B. thuringiensis* serovar *kurstaki* HD-1. Carlton and González[10] suggested that the *cry1Aa* and *cry1Ac* genes would be located together in a smaller and unstable ~44-MDa plasmid that can be cured easily. While *cry1*, *cry2* and *vip3Aa* genes tend to be found together, these results also suggest that this association does not exist between them and *cry8* or *cry9* genes. Some specific classes of the *cry1* and *cry2* genes were found together very frequently. The presence of one *cry1* gene at least with one *cry2* was detected in 92.5% of the Argentine strains. This kind of association is known and has been reported previously[2,5,23,31,33,42]. Among *cry2* genes, a strong association was observed between *cry2Aa* and *cry2Ab*; 87.5% of the strains showed to harbor these genes. This combination was also the most frequent (90.4%) in *B. thuringiensis* strains isolated from China[28]. Ben-Dov et al.[5] reported that this kind of association was found in strains from Israel, Kazakhstan and Uzbekistan but less frequently (34.4%).

Wang et al.[43] reported a strong association between *cry1A* and *cry1I* genes in strains isolated from China. In our study, we observed this association in 71.0% of the strains. This strong association is attributed to the relationship between the three subclasses of *cry1A* detected in our study and the *cry1Ia* gene and, between *cry1Ac* and *cry1Ib*. He et al.[21] reported that *B. thuringiensis* subsp. *chinensis* CT-43 harbors the *cry1Aa3*, *cry1Ia14*, *cry2Aa9*, *cry2Ab* and *vip3Aa10* genes close to each other in the largest plasmid of the strain. This observation can explain the strong association between these genes reported in 75.0% of the strains in our study. Lately, Zhu et al.[46] have confirmed that strain HD-1 harbors six crystal protein genes. Four of these proteins (*cry1Aa*, *cry1Ia*, *cry2Aa*, and *cry2Ab*) are located on the large plasmid pBMB299 with the vegetative insecticidal protein gene *vip3Aa* where they form a pathogenicity island; two additional proteins are located on plasmids pBMB95 (*cry1Ac*) and pBMB65 (*cry1Ab*). Although it is clear that some profiles of insecticidal toxin genes are more common in nature than others, probably providing a major biologic advantage to their host, further studies are required to understand this relationships.

It is worth noting that 88.8% of the Argentine strains that harbor *cry1* and *cry2* genes also show bipyramidal and cuboidal crystals as observed by phase-contrast microscopy, and protein patterns of approximately 130 and 65 kDa as revealed by SDS-PAGE (Table 2). These characteristics are typical of strains expressing Cry1 and Cry2 proteins[40]. Strains harboring *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, *cry1Ia*, *cry2Ab*, *cry9*, *vip3Aa* (1.2%) or *cry1Ac*, *cry2Ab*, *vip3Aa* (1.2%) profiles produced only bipyramidal crystals and a unique band of *approximately* 130 kDa (Table 2). However, cuboidal inclusions and a *approximately* 65 kDa band at the SDS-PAGE gel typical of strains expressing Cry2 proteins[22] were not detected in these Argentine strains. It is noteworthy that other *B. thuringiensis* strains that harbor a *cry2Ab* gene contain little or no Cry2Ab protein in their crystalline inclusions[16]. Lack of expression of *cry2Ab* genes has been related to mutations that lead to a loss in the coding frame[14], to the lack of a functional promoter[25], and to the lack of expression due to a post-transcriptional factor[38]. Since the *cry1I* and *vip3Aa* genes encode insecticidal proteins secreted during the vegetative phase of growth of *B. thuringiensis*[37], they do not form parasporal crystal, thus their protein pattern is not expected to be in our SDS-PAGE gels. Those strains that harbor simply *cry9* (2.5%) or *cry8* (5.0%) genes exhibited an ovoid shape, containing a major protein of approximately 130 kDa (Table 2). To our knowledge, the association among *cry9* genes, this type of crystal shape and this major protein component kDa have not been reported previously. By contrast, Cry8-associated ovoid crystals resembled the parasporal bodies of some atypical Lepidopteran toxic strains previously reported by our group and other researchers[1,44].

## Distribution of insecticidal toxin gene profiles in *B. thuringiensis* strains isolated from different sources

The analysis of the insecticidal toxin gene distribution according to the sample source of the strains is presented in Table 3. We found that the strains containing the most common profiles *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* were more frequently derived from soil (70.0%), stored product dust (59.0%) and spider webs (50.0%). On the other hand, the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* were mainly detected in strains isolated from leaves (40.0%) and dead insect larvae (50.0%). Six of ten of the identified insecticidal toxin gene profiles were observed in strains isolated from stored product dust and leaves. These data may indicate that the diversity of insecticidal gene profiles is somewhat higher in these kinds of sample sources than in soil, dead insect larvae and spider web samples. In previous studies, some authors have suggested that stored product dust[23,43] and phylloplane of plants[26] are a rich source of diversity of *B. thuringiensis*. These results imply that stored product dust and leaf samples are a rich source of diversity of *B. thuringiensis* and confirms previous findings.

## Disaggregate analysis of the insecticidal toxin gene content

The insecticidal toxin gene composition of the *B. thuringiensis* strains was also analyzed (Fig. 1). Strains containing *cry1A* or *cry2A* genes were the most abundant and represent 74 of the 80 (92.5%) studied *B. thuringiensis* (Fig. 1); 91.3% of the strains harbor a *vip3A* gene and 88.8% a *cry1I* gene. Just 5.0% and 2.5% of the strains contained a *cry8* or *cry9* gene respectively. The *cry1Ac* (91.3%), *cry2Ab* (91.3%), *vip3Aa* (91.3%), *cry2Aa* (88.8%), *cry1Ia* (82.5%), *cry1Aa* (77.5%), and *cry1Ab* (38.8%) genes were the most frequently detected, while *cry1Ib* (6.3%), and *cry1E* (6.3%) genes were less abundant. Very few *cry1C* (1.3%) and *cry1D* (1.3%) genes were found. Not all of the searched classes and subclasses of *cry1*, *cry2* and *vip3* genes were identified in the Argentine strains.

**Table 3** Insecticidal toxin gene distribution according to sample origin

| Profiles of insecticidal toxin genes | Soils (n = 10) | Dead insect larvae (n = 10) | Stored product dust (n = 22) | Leaves (n = 30) | Spider webs (n = 8) |
|---|---|---|---|---|---|
| *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* | 0 | 0 | 0 | 1 (3.3%) | 0 |
| *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 1 (10.0%) | 5 (50.0%) | 5 (22.6%) | 12 (40.0%) | 2 (25.0%) |
| *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, *cry1Ia*, *cry2Ab*, *cry9* and *vip3Aa* | 0 | 0 | 1 (4.6%) | 0 | 0 |
| *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 7 (70.0%) | 1 (10.0%) | 13 (59.0%) | 10 (33.3%) | 4 (50.0%) |
| *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 0 | 0 | 1 (4.6%) | 3 (10.0%) | 0 |
| *cry1Ac*, *cry1E*, *cry1Ib*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 0 | 4 (40.0%) | 1 (4.6%) | 0 | 0 |
| *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 1 (10.0%) | 0 | 0 | 0 | 0 |
| *cry1Ac*, *cry2Ab* and *vip3Aa* | 0 | 0 | 1 (4.6%) | 0 | 1 (12.5%) |
| *cry8* | 1 (10.0%) | 0 | 0 | 2 (6.7%) | 1 (12.5%) |
| *cry9* | 0 | 0 | 0 | 2 (6.7%) | 0 |

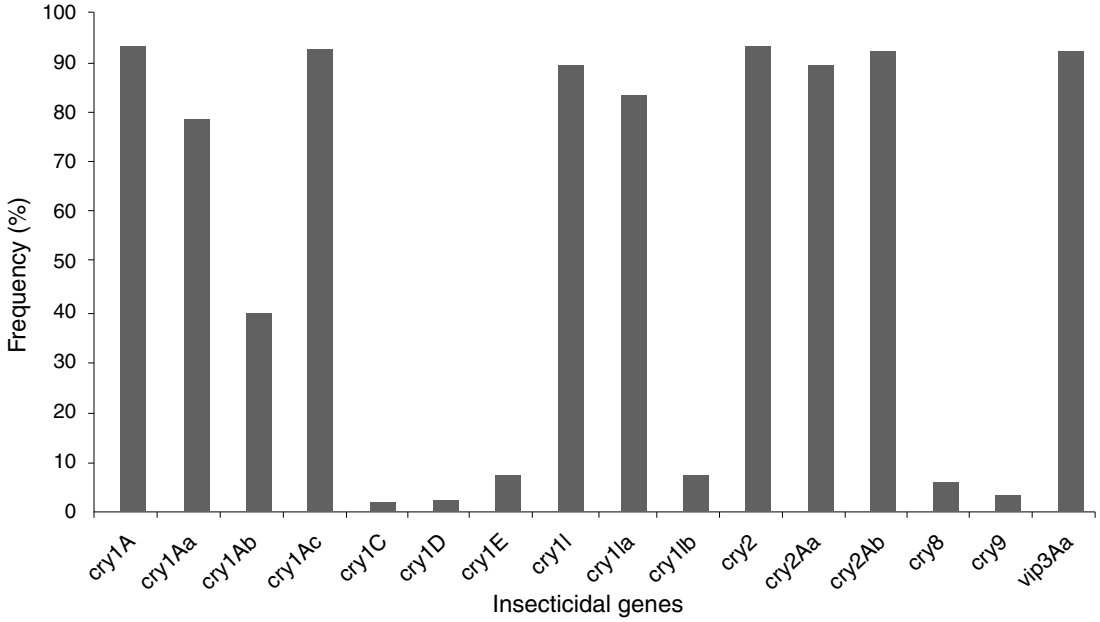


**Figure 1** Insecticidal toxin gene composition of Argentine *B. thuringiensis*.

## Correlation between the toxicity of Argentine *B. thuringiensis* strains against *E. aporema* and their insecticidal toxin gene profiles

Preliminary bioassays with spore-crystal suspensions (final concentration 2.5 μg/ml) of the 80 Argentine *B. thuringiensis* strains were performed with neonate larvae of *E. aporema*. These strains showed a mortality range of 0.0–52.5% (see Supplementary Table). A classification of the strains according to their toxicity and insecticidal toxin gene profiles is shown in Table 4. Most strains are classified in the category of 20–40% mortality. However, there were

**Table 4** Classification of the selected *B. thuringiensis* strains into groups according to their mortality rate against *E. aporema*

| Profiles of insecticidal toxin genes | Mortality | | |
|---|---|---|---|
| | <20% | 20–40% | >40% |
| *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* | 0 | 1 (100.0%) | 0 |
| *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 5 (20.0%) | 18 (72.0%) | 2 (8.0%) |
| *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, *cry1Ia*, *cry2Ab*, *cry9* and *vip3Aa* | 1 (100.0%) | 0 | 0 |
| *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 7 (20.0%) | 25 (71.4%) | 3 (8.6%) |
| *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 1 (25.0%) | 2 (50.0%) | 1 (25.0%) |
| *cry1Ac*, *cry1E*, *cry1Ib*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 4 (80.0%) | 1 (20.0%) | 0 |
| *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* | 1 (100.0%) | 0 | 0 |
| *cry1Ac*, *cry2Ab* and *vip3Aa* | 0 | 2 (100.0%) | 0 |
| *cry8* | 4 (100.0%) | 0 | 0 |
| *cry9* | 2 (100.0%) | 0 | 0 |

Samples tested n=80.

**Table 5** Probit analysis of the most toxic strains against *E. aporema*

| Isolate/strain | Genes[a] | $LC_{50}$[b] (μg/ml) | CV[c] (%) | $LC_{90}$[d] (μg/ml) | Slope | $\chi^2$ (4 gl)[e] |
|---|---|---|---|---|---|---|
| HD-1 | 1 | 0.69 (0.54–0.85) | 12.14 | 2.12 (1.58–3.43) | 2.68 | 1.23 |
| INTA H48-34 | 1 | 0.91 (0.74–1.10) | 9.27 | 2.27 (1.77–3.34) | 3.25 | 1.87 |
| INTA H46-18 | 1 | 0.79 (0.61–0.98) | 19.39 | 2.56 (1.88–4.32) | 2.50 | 0.97 |
| INTA Fo3-2 | 2 | 1.11 (0.91–1.36) | 16.82 | 2.88 (2.20–4.39) | 3.15 | 3.32 |
| INTA Mo9-1 | 2 | 1.35 (1.10–1.69) | 7.83 | 2.80 (2.80–6.43) | 2.84 | 2.33 |
| INTA TA20-6 | 2 | 1.50 (1.22–1.87) | 18.06 | 4.25 (3.08–7.45) | 2.84 | 0.55 |

[a] 1, *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa*; 2, *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa*.
[b] Mean lethal concentration and 95% confidence interval.
[c] Coefficient of variation.
[d] 90% lethal concentration and 95% confidence interval.
[e] Chi-square.

six strains that caused more than 40% mortality. The most toxic strains were isolated from phylloplane, soil, stored product dust and spider webs and harbored *cry1A*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* genes. On the contrary, the six less toxic/nontoxic strains showed mortality rates under 4% and were mainly isolated from phylloplane and one from soil. These strains harbored *cry8* or *cry9* genes. These results suggest that multiple insecticidal toxin gene profiles may be used as markers for the spectrum of insecticidal activity of *B. thuringiensis* strains as previously suggested[13]. In addition, the mortality rate was not always associated with the insecticidal toxin gene content of particular strains. Strains harboring the same insecticidal gene profile differed in their level of toxicity against *E. aporema* (Table 4). For example, strains INTA H3-5 and INTA H6-3 that shared the same insecticidal toxin gene profile (*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa*) showed 38.3% and 12.7% mortality. This kind of results may be observed in previous reports[23,31]; however, the reasons of those variations still remain to be studied. Furthermore, it is clear that the toxic levels of *B. thuringiensis* in the target insects are influenced by many factors including the insecticidal toxin gene profiles, the level of expression of these genes, possible synergism between insecticidal proteins, and other unknown or undetected virulence factors. These reasons may hamper the PCR ability as a precise predictive tool for insecticidal activity in *B. thuringiensis* strains. The genome sequencing of all strains may overcome some of these factors. However, this partial solution is difficult for now because of the high cost of sequencing a large number of strains. Despite of all this, our results showed that different *B. thuringiensis* strains obtained from the same sample showed certain relationship between the level of toxicity and specific insecticidal toxin gene profiles. In general, strains harboring genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa* were more toxic than those harboring genes *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa*, and these more than those harboring genes *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, *cry2Ab* and *vip3Aa*. In a previous study, Cry1Ab and Cry1Ac had shown to be the most active proteins against neonate larvae of *E. aporema*, followed by Cry1Aa with $LC_{50}$ values of 0.55, 1.39 and 4.14 μg/ml, respectively[36].

## Bioassays with solubilized pure crystals of Argentine *B. thuringiensis* strains.

The five most toxic isolates were further bioassayed with serial dilutions of solubilized crystals for $LC_{50}$ estimation (Table 5). According to the $LC_{50}$ values and their fiducial limits, *B. thuringiensis* serovar *kurstaki* HD-1, *B. thuringiensis* INTA H46-18 and INTA H48-34 were the most toxic against *E. aporema*, although the difference among them was not statistically significant. The two Argentine strains were

isolated from samples of phylloplane and both shared the same insecticidal toxin gene profile. In contrast, the other less toxic strains harbored a similar insecticidal toxin gene profile lacking the *cry1Ab* gene. *B. thuringiensis* H46-18 and INTA H48-34 could be used as active ingredients of pesticides formulated for controlling *E. aporema*.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Conflict of interest

The authors declare that they have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2017.02.003.

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