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Molecular Characterization of the *cry* Gene profile of *Bacillus thuringiensis* Isolated from a Caribbean Region of Colombia

PEDRO FRAGOSO[1], ALICIA ARMIJO[2], DORIS GÓMEZ[3], CLAUDIO GÓMEZ[4], MARCO BUGUEÑO[2], GITTITH SÁNCHEZ[5] and JUAN VENEGAS[2]*

[1] Research Group of Parasitology and Agroecology Mileno, Popular University of Cesar, Colombia
[2] Cellular and Molecular Biology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile
[3] UNIMOL Laboratory, Tropical Medicine, University of Cartagena, SUE-Caribe, Colombia
[4] Faculty of Science, Department of de Pharmacie. Nacional University of Colombia. UNIMOL Group, Bogotá DC, Colombia
[5] Human Genetic Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile

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Abstract

In order to characterize native strains of *Bacillus thuringiensis* of the Colombian Caribbean with toxic effect against insect vectors, 28 samples of bacteria identified as *B. thuringiensis* were isolated from different soils and muds around the city of Valledupar. Using a biological test, five isolates of *B. thuringiensis* showed toxic effect against larvae of *Aedes aegypti*. PCR methods were used to detect *cry1*, *cry2*, *cry4B*, *cry10* and *cyt1* genes. *Cry1* and *cry2* genes were detected in 35.7% and 32.1% of the 28 isolates analyzed, respectively. Surprisingly, reduced lengths of *cry4B* gene segments were detected in 28.6% of *B. thuringiensis* samples. The presence of *cry10* or *cyt1* was not detected in any of the 28 samples *of B. thuringiensis*, despite the high sensitivity of the assays used. The results show that *B. thuringiensis* samples from the Colombian Caribbean have atypical characteristics compared to those of Latin America and elsewhere in the world, which is consistent with the idea that the geographic origin of *B. thuringiensis* samples is associated with their biological and genetic characteristics.

Key words: *Bacillus thuringiensis* – Colombian strains, *cry* genes, *Aedes aegypti* larvae, PCR methods, biological test

Introduction

Current methods for controlling insect vectors of different infectious diseases are based on the use of chemical insecticides (Hemingway *et al.,* 2004; Macoris *et al.*, 2007). However, excessive use of these chemicals has caused damage to ecosystems, environmental pollution, toxicity in humans and animals and development of resistance in insect vectors (Soderlund and Knipple, 2003; Grisales *et al.*, 2013). These side effects have led to the search for new and more harmless control methods including biological methods, in which the bacterium *Bacillus thuringiensis* represents a very promising alternative (Boyce *et al.*, 2013; Shingote *et al.*, 2013; WHO 2012; 2016: Ingabire *et al.*, 2017).

In Central and South America, several infectious diseases which affect humans, animals and plants are transmitted by insect vectors from all orders (López-Pazos *et al.*, 2009; Hernández-Fernández 2011; Pérez *et al.*, 2016; WHO 2016; Camacho-Millán *et al.*, 2017). Among them are Diptera such as mosquitoes of *Anopheles* and *Aedes* genus involved in the transmission of malaria and dengue, respectively, which cause great impact in human heath (WHO 2016; Soares-da-Silva *et al.*, 2017). Among the insect vectors that cause great pests in agriculture in Latin America, are Lepidoptera such as *Tuta absoluta* which produces great loss in tomato production (Salazar and Araya, 2001; Hernández-Fernández, 2011) and Coleoptera such as *Premnotrypes vorax* and *Anthonomus grandis* that cause devastating pests for potato and cotton crops, respectively (López-Pazos *et al.*, 2009; Pérez *et al.*, 2016) . For these reasons it is very important in Latin America to seek more effective, sustainable and ecological methods to improve the campaigns to control and eradicate the various vectors of communicable diseases present

* Corresponding author: J. Venegas, Cellular and Molecular Biology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile; **e-mail: jvenega@med.uchile.cl**

in our countries (Prabakaran and Hoti, 2008; WHO 2016; Soares-da-Silva *et al.*, 2017). One of the possible alternatives for biological control of these vectors is *B. thuringiensis*.

*B. thuringiensis*is (*Bt*) is a Gram-positive, aerobic and facultatively anaerobic bacterium that during the sporulation phase synthesizes a set of proteins that produce parasporal crystals with toxic activity against insects. These crystals consist of several proteins, including the Cry and Cyt protein families (Santos *et al.*, 2012; Bravo *et al.*, 2013). Using a nomenclature based on the phylogenetic relationships of the amino acid sequences deduced from coding genes, four orders of identity were established (Crickmore *et al.*, 1998). Currently more than 700 *cry* gene sequences have been identified, classified into at least 70 groups, named cry1, cry2, cry3 ... cry70, whose corresponding insect toxicity is not as specific and simple as previously thought (Bravo *et al.*, 2013; Soares-da-Silva *et al.*, 2017).

Today it is relatively clear that both the intensity and specificity of the toxic effect against a particular insect order depends on the set of toxic proteins expressed by the bacterium. For example, larvicidal activity against Diptera larvae has been associated with proteins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1 and Cyt2 (Cantón *et al.*, 2011; Santo *et al.*, 2012). The classical example of a bacterium with this protein pattern is *B. thuringiensis* var. *israelensis*, used all over the world as commercial preparations against Diptera larvae (Cantón *et al.*, 2011; Santos *et al.*, 2012). However, in addition to this set of proteins, in some cases other complementary proteins such as Cry1 and Cry2, have been detected that enhance the toxic effect against Diptera (Ben-Dov *et al.*, 1997; Pinto *et al.*, 2012). Most of the genes encoding these δ-endotoxins are localized in plasmids, such as the megaplasmid pBtoxis of 127,923 bp that is present in the *B. thuringiensis* var. *israelensis* strain (Berry *et al.*, 2002). This plasmid encodes the six proteins toxic to Diptera mentioned above (Berry *et al.*, 2002; Cantón *et al.*, 2011; Santos *et al.*, 2012). However, the Cry1 and Cry2 proteins are encoded by other plasmids (Porcar and Caballero, 2000).

Studies of *B. thuringiensis* strains isolated from Latin America strongly suggest that there is a heterogeneous geographic distribution of *cry* genes associated with toxicity against different insect orders (Bravo *et al.*, 1998; Ibarra *et al.*, 2003; Uribe *et al.*, 2003; López-Pazos *et al.*, 2009; Santos *et al.*, 2012; Pérez *et al.*, 2016; Camacho-Millán *et al.*, 2017; Soares-da-Silva *et al.*, 2017). Colombian strains of *B. thuringiensis* have been isolated mainly from the central region, *e.g.* Medellin (Segura *et al.*, 2000; Ruiz *et al.*, 2004) and few studies on *B. thuringiensis* samples from Caribbean regions of Colombia have been reported (Uribe *et al.*, 2003; López-Pazos *et al.*, 2009). It is very interesting to note that *B. thuringiensis* samples isolated from tropical forests contained different *cry* gene sets and showed distinct toxicity to insect orders compared to those isolated from agricultural soils. The tropical forest samples showed *cry1, cry3* and *cry7* genes and were toxic to coleopteran species, while the samples from agricultural soils only had *cry1* genes and were toxic to Lepidoptera (Uribe *et al.*, 2003).

Based on the literature reports mentioned above that suggest association between geographic origin and *cry* gene variants of *B. thuringiensis*, the aim of the present study was to isolate and characterize new *B. thuringiensis* strains toxic against dipterans of importance in human health from a tropical region not previously studied.

Experimental

Materials and Methods

Strains. Twenty-eight isolates of *B. thuringiensis* were obtained from urban soil samples and mud from the water treatment plants of the city of Valledupar and stored in the laboratory of Parasitología Agroecología Milenio of the Universidad Popular del Cesar, Colombia (Table S1 in Supplementary Material). Two commercial strains of *B. thuringiensis* were used as positive controls in the toxicity tests; Turilav®, produced by Laverlam S.A., which is the strain *B. thuringiensis* var. *kurstaki*, and VectoBac® WDG, produced by Valent Biosciences Corporation, which is *B. thuringiensis* var. *israelensis*.

Production of spores and crystals. Isolates of *B. thuringiensis* containing spores and crystals conserved in filter paper were cultured as described (Santos *et al.*, 2012). Briefly, these samples were submerged in 2 ml of nutrient broth: 0.1% (w/v) meat extract, 0.2% (w/v) yeast extract, 0.5% (w/v) bacteriological peptone and 0.5% (w/v) NaCl, incubated at 28°C for 24 h. Then, 200 ml of culture medium N° 1 were added and the solution was incubated at 28°C for 96 h with gentle agitation. The suspensions of each isolate were washed 3 times with 1.5 M NaCl and 3 times with distilled water and centrifuged at 10000 g for 10 min; the spore-crystal complex was stored at –20°C.

Bioassays. The toxic effect of the isolates of *B. thuringiensis* on larvae of *A. aegypti* was determined following the methodology recommended by the World Health Organization (WHO 2005). Twenty five third or fourth instar larvae of the reference strain (Rockefeller) were transferred to transparent watch glasses with 150 ml capacity (Santos *et al.*, 2012). Determinations were performed in triplicate (Table S2 in supplementary material). The positive controls Turilav® and VectoBac® WDG were included in each assay. As negative control 100 larvae were placed in distilled water. Mortality was recorded after 48 h. Pupae were omitted in the analyses.

If the negative control had mortality > 20% the assay was eliminated. Bioassays were performed in laboratory conditions at a temperature of 28°C and relative humidity of 65%.

Detection and sequencing of *cry* genes. The *cry1* and *cry2* genes were analyzed based on the methods described by Ben-Dov *et al.* (1997), using the primers Un1(d) and Un1(r) for *cry1* and Un2(d) and Un(r) for *cry2*. The PCR conditions used for amplification of *cry1* were: DNA denaturation for 3 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension at 72°C for 10 min. Amplification of the *cry2* gene was similar to the *cry1* gene, only changing the number of cycles and annealing temperature to 40 cycles and 51°C, respectively. The primers and conditions used to detect *cry4B*, *cry10* and *cyt1* were based on Santos *et al.* (2012). The PCR protocol to amplify these genes was: a DNA denaturation step of 3 min at 94°C; 35 cycles for 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and a final extension of 10 min at 72°C.

DNA amplification was performed in a TECHNE TC 412 thermocycler. All reactions were performed in a volume of 20 µl. PCR products were visualized in 2.5% agarose gels with TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3), also including a 100 pb (base pair) DNA ladder (Invitrogen, Brazil).

Positive controls for *cry1*, *cry4B*, *cry10* and *cyt1* assays were performed using a DNA sample of *B. thuringiensis* var. *israelensis* generously donated by Mario Soberón, of the Instituto de Biotecnología, Universidad Nacional Autónoma de México.

PCR products of the *cry1*, *cry4B* and *cry11* segments were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and then sent for sequencing to the DNA Core Sequencing Facility, University of Illinois, EEUU, with the corresponding primers. The GenBank accession codes for the sequences Bt1-cry1 and Bt3-cry1 are MG271933 and MG271934, respectively.

Analysis of DNA sequences. Colombian *B. thuringiensis cry1* gene segments were aligned with *B. thuringiensis cry1A* gene for the insecticidal crystal protein segment located between 2780 and 3080 bp (GenBank accession code D17518.1). This information is available in the database http://www.ncbi.nlm.nih.gov/. Alignments were made with the CLUSTALX program and edition of the sequences was performed with the Bioedit software (Hall, 1999). The Colombian *cry4B* segment, corresponding to Bt-UPC-22 (Bt22), was aligned with the *B. thuringiensis* serovar *israelensis cry4B* gene coding sequence (*cry4B*, GenBank accession: D00247.1).

Bioassays. The highest mortality (27–30%) at the concentration 0.1 mg/l was caused by *B. thuringiensis* isolates Bt-UPC-15, Bt-UPC-20 and Bt-UPC-25 (Table S2, Supplementary Material). Two other isolates, Bt-UPC-5 and Bt-UPC-6, caused 17% and 23% mortality, respectively, at the highest concentration tested (10 mg/l). These five isolates with toxic activity came from urban soils (Table SI, Supplementary Material).

Results

Detection of *cry1* gene segments in three Colombian *B. thuringiensis* samples. The analysis of various isolates of *B. thuringiensis* to detect the *cry1* gene showed a single PCR amplification product in the expected range of 274-277 bp (Fig. 1, Bt-UPC samples 1, 3, 8, 16 and 17). The *cry1* was detected in 35.7% of the Bt-UPC samples analyzed (Table SII, Supplementary Material). However, the gene was not detected in any of the Bt-UPC samples that were toxic to mosquito larvae.

Two *cry1* gene segments from DNA samples Bt-UPC-1 and Bt-UPC-3 were sequenced (Fig. 2).

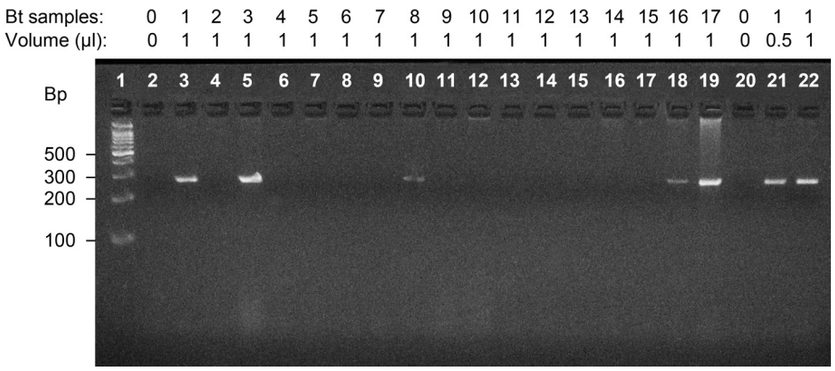

Fig. 1. Detection of the *cry1* gene in *Bacillus thuringiensis* DNA samples from a Caribbean region of Colombia.

Electrophoresis in 2.5% agarose gels, showing PCR amplification products for the *cry1* gene using the primers and protocols described by Ben-Dov *et al.* (1997). Molecular marker: 100 base pair (bp) DNA ladder (lane 1), without sample (lanes 2, 20). Lanes 3–19: *B. thuringiensis* samples (Bt-UPC-1 to Bt-UPC-17). Lanes 21, 22: different concentrations of Bt-UPC-1. The Bt DNA samples and the volume analyzed are indicated above the figure. Expected size 274–277 bp (See Material and Methods for more details).

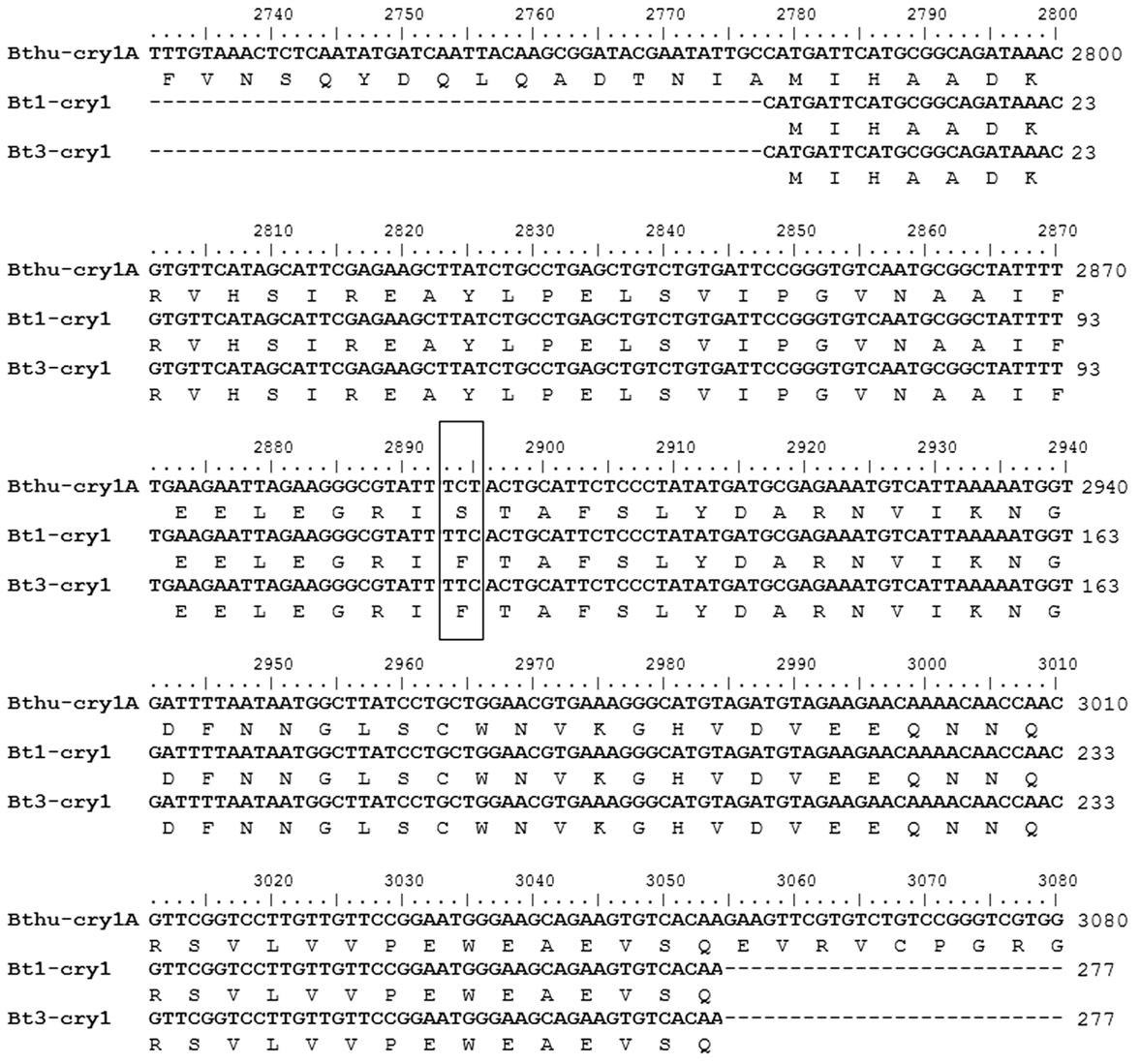

Fig. 2. Aligment of a *cry1* protein coding gene segment of two DNA samples from a Caribbean region of Colombia.
The corresponding deduced amino acid sequence is given below each nucleotide sequence. The first sequence corresponds to a segment of an open reading frame of the *B. thuringiensis* var. kurstaki HD-1 *cry1A* gene, from base pair 2731 to 3080 (Bthu-cry1A, Genbank code D17518.1). The second and third sequences are PCR products of *cry1* amplified from Colombian *B. thuringiensis* Bt-UPC-1 (Bt1-cry1, GenBank code MG271933) and Bt-UPC-3 (Bt3-cry1, GenBank code MG271934) DNA samples, respectively. The box indicates the two single nucleotide polymorphisms (SNP) between sequences, and the corresponding changes of Serine 965 to Phenylalanine in Bt sequences.

Importantly, the Bt1-cry1 and Bt3-cry1 sequences (GenBank accession numbers MG271933 and MG271934, respectively) are identical, and differ from the *cry1A* gene (GenBank accession number D17518.1) only in nucleotides 2894 and 2895 (indicated by a box into the figure). Interestingly, these nucleotide changes also produce a change in the aa (amino acid) encoded by the codon, from aa serine 965 to phenylalanine (S965F). The localization of the segment amplified by PCR with respect to the full length Cry1 amino acid sequence is shown in Fig. S1 in the Supplementary Material.

Detection of a *cry2* gene segment in Colombian *B. thuringiensis* samples. Using the method described by Ben-Dov *et al.* (1997) we detected the described segment of about of 700 bp in 32.1% of Colombian *B. thuringiensis* samples (Table SII, Supplementary Material). For instance, in Figure 3 there is clearly a single band of 700 bp in Bt samples 8, 17 and 18, and a very faint band is also observed in Bt sample 16. Interestingly, in Bt samples 8, 16, 17, 18, 26 and 28 both *cry1* and *cry2* were detected (Table SII, Supplementary Material). However, in Bt samples 22, 23 and 27 the *cry2* segments were accompanied by *cry4B* gene segments (Table SII, Supplementary Material).

Detection of *cry4B* gene segments in Colombian *B. thuringiensis* samples. The detection of *cry4B*

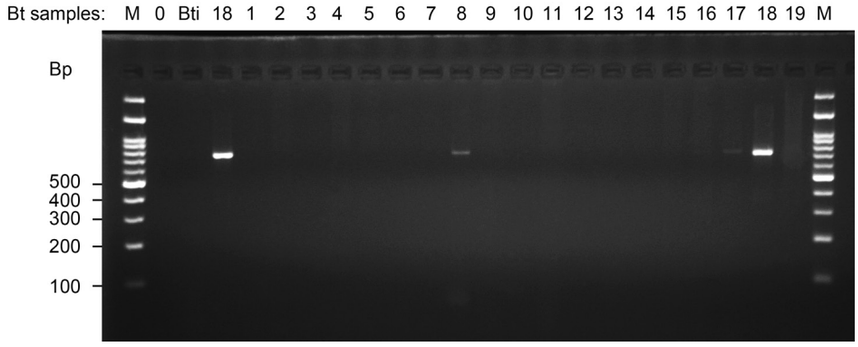

Fig. 3. Detection of the *cry2* gene in *Bacillus thuringiensis* DNA samples from a Caribbean region of Colombia.
Electrophoresis in 2.5% agarose gels, showing PCR amplification products for the *cry2* gene using the primers and protocols described by Ben-Dov *et al.* (1997). Molecular marker: 100 base pair (bp) DNA ladder (lane M), without sample (lane 0). DNA of *B. thuringiensis* var. *israelensi* (Bti), 0.34 µg. Colombian *B. thuringiensis* samples Bt-UPC-18, Bt-UPC-1 to 19 (lanes 18, 1–19, respectively). The volume analyzed for each sample was 1 µl. Expected size about 700 bp (See Material and Methods for more details).

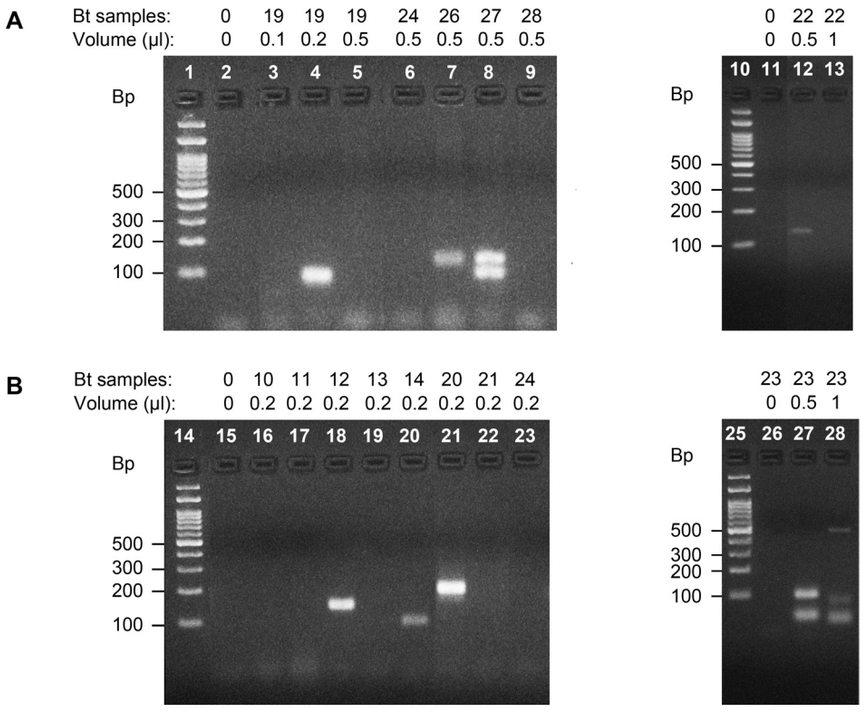

Fig. 4. Detection of the *cry4B* gene in different volumes of *Bacillus thuringiensis* DNA samples from a Caribbean region of Colombia.
The four electrophoreses were performed in 2.5% agarose gels, showing PCR amplification products for the *cry4B* gene using the primers and protocols described by Santos *et al.* (2012). Molecular marker: 100 base pair (bp) DNA ladder (lanes 1, 10, 14 and 25), without sample (lanes 2, 11, 15 and 26). Colombian *B. thuringiensis* samples (lanes 3–9, 12, 13, 16–23 and 27, 28, respectively). The corresponding Bt-UPC DNA samples and the volume analyzed are given above the figure.

was not easy, because it was necessary to try different volumes of each sample to observe any amplification product (Fig. 4). Thus in most Bt-UPC samples it was possible to obtain a PCR amplification product of 100 bp only with certain volumes (Lanes 4, 20 and 27), 150 bp (Lanes 7, 12 and 18) or 200 bp (Lane 21). At high DNA concentration in the PCR assay, a larger PCR product of about 500 bp was detected in some samples (Lane 28, Bt23 sample).To determine whether these small length products really encoded segments of the *cry4B* gene, several attempts were made to obtain sufficient quantity of DNA of different-sized fragments to be sequenced. It was possible to obtain enough DNA for sequencing only from the Bt-UPC-22 sample. This PCR product of 150 bp was partially sequenced in the inverse sense; as shown (Fig. 5), a 97 bp segment was sequenced which aligned with segment 1707–1817 of the *cry4B* gene from *B. thuringiensis* serovar *israelensis* (accession

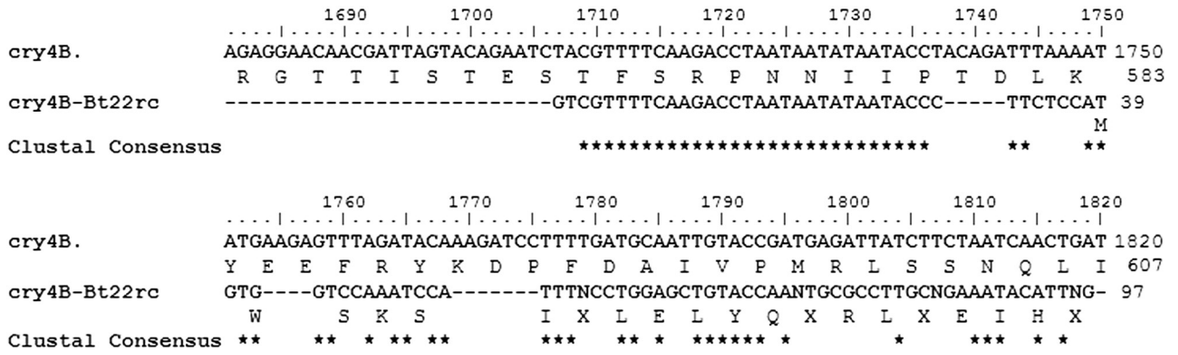

Fig. 5. Alignment of the *cry4B* segments from the Colombian *B. thuringiensis* Bt – UPC – 22 sample and from the *B. thuringiensis* serovar *israelensis* bacterium.

These nucleotide sequences correspond to a segment of 140 bp from *B. thuringiensis* serovar *israelensis cry4B* gene coding sequence (cry4B, GenBank code: D00247.1) and the 97 bp nucleotide segment sequenced from Colombian *B. thuringiensis* DNA sample Bt22 (cry4B-Bt22rc). Below each nucleotide sequence is the corresponding deduced amino acid segment.

code D00247.1). Three gaps and multiple nucleotide differences are shown between the sequences, corresponding to an identity of 50%. The localization of this *cry4B* segment amplified by PCR with respect to the full length Cry4B amino acid sequence is shown in Figure S1 (Supplementary Material).

***Cry10* and *cyt1* genes were not detected in any samples of the Colombian Caribbean.** Several attempts were made using diverse dilutions and PCR conditions to detect segments of genes *cry10* and *cyt1*, as shown in Figure S2, in which both assays clearly showed the expected fragments of 348 and 480 bp described for these gene segments, respectively, that were detected in the positive control with samples of *B. thuringiensis* var. *israelensis* (panels A and B, lanes 3–5). It must be mentioned that a very small amount of these *Bt* genes can be detected in the assay, even at dilutions higher than 1/1000, corresponding to a concentration lower than 17 ng/µl.

Discussion

B. thuringiensis has been isolated from different ecosystems; the soil is one of the habitats in which it is most widely distributed (Arango *et al.,* 2002; Jara *et al.*, 2006; Pérez *et al.*, 2016; Camacho-Millán *et al.*, 2017; Soares-da-Silva *et al.*, 2017). This fact, coupled with the wide variety of climatic regions and high diversity of insects in Colombia, provides opportunities to isolate new strains with toxic activity against Diptera or other insect orders (Ibarra *et al.*, 2003). However, in spite of the importance of dipterans as etiological agents of infectious diseases, few studies have reported the molecular characterization of collections of *B. thuringiensis* in relation to the content of *cry* and *cyt* genes lethal to species of this order in Colombia (Segura *et al.*, 2000; Ibarra *et al.*, 2003; Ruiz *et al.*, 2004).

The toxicity of *B. thuringiensis* isolates against insect larvae is a complex phenomenon which depends upon several factors (Martínez and Caballero, 2002; Santos *et al.*, 2012; Soares-da-Silva *et al.*, 2017). These can be classified into bacterial, larval and environmental factors. Bacterial factors include the concentration of spores and crystals, presence and expression of toxic genes against insects (Martínez and Caballero, 2002; Santos *et al.*, 2012). Larval factors include the genus, species, strain and geographic origin of larvae, as well as the presence in their midgut of receptors which interact with the different toxic proteins expressed by the bacterium (Martínez and Caballero, 2002; Santos *et al.,* 2012; Soares-da-Silva *et al.,* 2017). Environmental factors include the temperature and pH of the water in which the larvae are infected with bacteria (Arunachalam *et al.*, 2010; Santos *et al.*, 2012). Thus it has been demonstrated that more acidic water yields higher toxicity of *B. thuringiensis* isolates against larvae (Martínez and Caballero, 2002; Santos *et al.*, 2012; Soares-da-Silva *et al.*, 2017). These authors suggest that one possible mechanism could be the stimulation of the ingestion of the bacteria by larvae. It appears that there is a range of optimal temperatures for high metabolism and ingestion of bacteria by larvae which is from 20–30°C (Arunachalam *et al.*, 2010), but this range could change depending on the strain or origin of the larvae (Santos *et al.*, 2012). In our study anti-larval activity was detected in 5 of the 28 *B. thuringiensis* isolates (17.9%), with mortality that ranged from 17% to 30% (Table SII, Supplementary Material). Considering the complex phenomenon of toxicity against larvae produced by toxins expressed by *B. thuringiensis*, we cannot rule out environmental factors in the low toxic effect observed in the present study.

Cry1 and *cry2* genes have been associated mainly with toxic effects against Lepidoptera (Morse *et al.*, 2001; Zhao *et al.*, 2005; Mandal *et al.*, 2007; Camacho-

Millán *et al.*, 2017), but in association with other *cry* genes toxic effects have also been reported against Dipteran larvae (Ben-Dov *et al.*, 1997). In order to know whether *cry1* and *cry2* genes might be involved in toxic effects against *Aedes aegypti* larvae detected in Colombian Bt samples, the presence of these genes was investigated (Fig. 1–3). As mentioned above, the presence of *cry1* and *cry2* genes was not detected in any of the Bt-UPC samples that were toxic to mosquito larvae (Table SII). This result is concordant with results previously reported in Bt samples from Latin America, showing that *cry1* and *cry2* genes both together and separately are associated with toxicity against Lepidoptera and Coleoptera but not against larvae of Diptera (Uribe *et al.*, 2003; Lopez-Pazos *et al.*, 2009; Santos *et al.*, 2012; Camacho-Millán *et al.*, 2017; Pérez *et al.*, 2016).

It is noteworthy that the sequenced *cry1* gene segments of the two Colombian *B. thuringiensis* samples were almost identical to the respective *cry1A* homolog of the subspecies named *Bt kurstaki* HD-1 isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago) (Kondo *et al.*, 1987), differing from the database sequence only in two nucleotides which caused a non-silent mutation from serine to phenylalanine (Fig. 2).

Interestingly, detection of the *cry4B* gene was very difficult; in almost all samples we were only able to detect segments much shorter than expected. Segments of 100, 150 and 200 bp were found instead of 321 bp segments (Fig. 4). In some samples two fragments were detected. In order to confirm that at least some of these fragments indeed correspond to segments of the *cry4B* gene, we tried to sequence them. It was only possible to sequence a PCR product of 150 bp from the Bt22 sample, obtaining a sequence of 97 nucleotides (Fig. 5). This segment corresponds to the delta-endotoxin C region of the respective Cry4B protein. Our results show that indeed, at least the 150 bp PCR fragment corresponds to a segment of the *cry4B* gene (Fig. 5). Further studies will be necessary to determine the total length of these genes in these Colombian samples and confirm whether the other fragments also are *cry4B* gene segments.

Based on the reduced length of *cry4B* gene segments detected in our samples, one explanation for the null or low larvicidal effect against *A. aegypti* larvae could be that these *cry4B* segments are pseudogenes. Confirming or refuting this hypothesis will require further study.

Conclusions

B. thuringiensis samples from the Colombian Caribbean area show atypical characteristics, which is consistent with the idea that the geographic origin of *B. thuringiensis* samples is associated with their biological and genetic characteristics.

Five of the twenty-eight isolates of *B. thuringiensis* showed a lethal effect against larvae of *A. aegypti*, the mosquito that transmits serious diseases such as dengue. *Cry1* and *cry2* genes were detected in 35.7% and 32.1% of the samples, respectively, but in none of the five Bt samples that were toxic against mosquito larvae.

Surprisingly, a reduced length of *cry4B* gene segments was detected in 28.6% of Bt samples, 100–200 bp instead of the expected 321 bp; neither of these segments was detected in the five *Bt* Colombian samples toxic to *A. aegypti* larvae. *Cry10* and *cyt1* were not detected in these Colombian *B. thuringiensis* samples.

Further studies will be required in order to identify other genes involved in the larvicidal effect observed in these Bt samples.

Acknowledgments

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