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Evidence of two mechanisms involved in *Bacillus thuringiensis israelensis* decreased toxicity against mosquito larvae: Genome dynamic and toxins stability

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ABSTRACT

Biopesticides based on *Bacillus thuringiensis israelensis* are the most used and most successful around the world. This bacterium is characterized by a dynamic genome able to win or lose genetic materials which leads to a decrease in its effectiveness. The detection of such phenomena is of great importance to monitor the stability of *B. thuringiensis* strains in industrial production processes of biopesticides. New local *B. thuringiensis israelensis* isolates were investigated. They present variable levels of delta-endotoxins production and insecticidal activities against *Aedes aegypti* larvae. Searching on the origin of this variability, molecular and biochemical analyses were performed. The obtained results describe two main reasons of the decrease of *B. thuringiensis israelensis* insecticidal activity. The first reason was the deletion of *cry4Aa* and *cry10Aa* genes from the 128-kb pBtoxis plasmid as evidenced in three strains (BLB124, BLB199 and BLB506) among five. The second was the early degradation of Cry toxins by proteases in larvae midgut mainly due to some amino acids substitutions evidenced in Cry4Ba and Cry11Aa δ-endotoxins detected in BLB356. Before biological treatment based on *B. thuringiensis israelensis*, the studies of microflore in each ecosystem have a great importance to succeed pest management programs.

1. Introduction

Bacillus thuringiensis israelensis was first isolated by Goldberg and Margalit (1977), from dead *Culex pipiens* larvae. It is a spore forming bacterium able to produce, during the sporulation phase, crystalline inclusions highly toxic toward mosquitoes but safe for the environment (Wirth et al., 2010). Currently, it is considered as the most powerful environmental-friendly biological alternative component used in integrated programs to control disease vectors (Ben-Dov, 2014). *B. thuringiensis israelensis* crystals are composed by a combination of six main proteins having synergistic interactions: four delta-endotoxins (encoded by *cry4Aa*, *cry4Ba*, *cry10Aa*, and *cry11Aa* genes) and two haemolytic factors (encoded by *cyt1Aa* and *cyt2Ba* genes) (Ben-Dov, 2014). All there corresponding genes are often present in combination in the same strains. Nevertheless, some of them could be absent (Schnepf et al., 1998). These toxin protein genes are mainly located on large conjugative plasmid: pBtoxis (Berry et al., 2002). This mega plasmid, of 128 kb, contains several insertion sequences and co-integrative transposons that are commonly associated with insecticidal crystal protein genes (Berry et al., 2002). In fact, over 23% of the genes on this plasmid resemble transposon-related genes, which facilitate recombination events and plasmid exchange between *B. thuringiensis* strains (Berry et al., 2002). For instance, *cry4A* gene is flanked by two insertion sequences in opposite orientation designated IS*240* (Bourgouin et al., 1988). The later insertion sequence (IS*240*) is widely distributed in *B. thuringiensis* and is invariably present in known dipteran-active strains (Rosso and Delcluse, 1997). An IS*240* variant has been found in *B. thuringiensis jegathesan* upstream *cry11B* gene (Délecluse et al., 1995) and in a plasmid of the dipteran-active strain belonging to *fukuokaensis* subsp. (Dunn and Ellar, 1997). The structural dynamics of pBtoxis could delete or inactivate *cry* genes and therefore causes partial or complete insecticidal activity loss (Zghal and Jaoua, 2006). The insecticidal process of *B.*

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thuringiensis delta-endotoxins starts with crystal inclusion dissolution under alkaline condition in digestive juices at midgut lumen (Schnepf et al., 1998; Wirth et al., 2010). Solubilization generates inactive protoxin which must be activated with gut proteases to be harmful to insect larvae (Bravo et al., 2007). The proteolytic cleavage takes place at both the C- and N-terminal protein end (Choma et al., 1991). Activated Cry toxin binds to specific receptors on the larval gut (Bravo et al., 2007, 2004) which leads to membrane penetration. Then cation-selective channels were formed that cause osmotic lysis and death of larval midgut epithelial cells (Ben-Dov, 2014; Bravo et al., 2007). Both, protoxins activation and activated toxins binding to specific midgut receptors are key steps that account in insecticidal crystal proteins activity (Federici et al., 2010). The present study evidenced two main mechanisms leading to a decrease in *B. thuringiensis israelensis* larvicidal activity against *A. aegypti*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *B. thuringiensis israelensis* studied strains were isolated in the laboratory, from different Tunisian bioinsecticide free soil samples, using the method reported by Jaoua et al. (1996).

The reference strain H14 and the acrystalliferous strain 4Q7, belonging to *B. thuringiensis* subsp. *israelensis*, were obtained from the Bacillus Stock Centre at Ohio State University (Columbus, OH, U.S.A.). Luria-Bertani (LB) medium broth was used for *B. thuringiensis israelensis* strains growth (Sambrook et al., 1989). Solid T3 medium was used for sporulation and crystal inclusion bodies production by *B. thuringiensis israelensis* studied strains (Zouari and Jaoua, 1997; Travers et al., 1987).

For delta-endotoxin production and bioassays, inocula were prepared by transferring a single pure colony from nutritive agar slants into 3 ml of LB medium with shaking, incubated for 16 h at 30 °C then transferred into 250 ml erlenmeyer flasks containing 50 ml of LB medium. After 6 h of incubation at 30 °C the culture was used to inoculate the glucose medium described by Zouari et al. (1998) to start with an initial O.D.600 of 0.15, in 1 l shake flasks at on a rotary shaker set at 200 rpm.

2.2. Plasmid DNA extraction, DNA probe preparation and PCR amplification

Plasmid DNA was extracted from *B. thuringiensis israelensis* isolates using the standard alkaline lysis method of Sambrook et al. (1989) including the step involving lysozyme treatment. Plasmid DNAs profiles were separated by running on a 0.8% agarose gel at 17 V for a night. Specific primer pairs (Table 1), synthesized by the "Centre de Génétique Moléculaire, CNRS, GENSET, Orsay, France" were used to detect *B. thuringiensis israelensis* specific genes (*cry4A*, *cry4B*, *cry10A*, *cry11A* and *cyt1A*). *cry4Ba* specific probe was prepared from H14 reference strain by PCR using the primers pair Prom33/Dip1B. The amplification was accomplished as described by Jaoua et al. (1996) using DNA extracted from *B. thuringiensis israelensis* isolates as template. PCR amplification was performed in a "Gene Amp PCR System 2700" (Applied Biosystems) thermal cycler. DNA sequences were obtained using a taq Dye Deoxy terminator cycle Sequencing kit and a 3700 ABI Prism DNA sequencer (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's instructions. The resulting sequences were deposited in the GenBank database and were subjected to a blastx homology search against the protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Nucleic acid hybridization

Restricted plasmid DNAs were size separated by electrophoresis in horizontal 0.8% agarose gels and transferred to nylon membrane (Hybond NC; Amersham) by the procedure of Southern (1975). Prehybridization and hybridization of filters were carried out as described by Shin et al. (1995). *cry4Ba* specific probe was labeled and purified as described by Zghal and Jaoua (2006).

2.4. Preparation of A. aegypti gut proteases

Third instar larvae of *A. aegypti* were chilled on ice for 30 min. Then, the whole larvae guts were collected in cold MET buffer (Mannitol, 300 mM; EDTA, 5 mM; Tris, 20 mM; pH 7.2) (Rouis et al., 2007). After that, they were disrupted into a blender and centrifuged for 10 min at 13,000 × *g*. The supernatants corresponding to larvae protease extract were recovered and the protein contents were determined by the method of Bradford (Bradford, 1976) using Bio-Rad reagent (Bio-Rad Protein assay, Cat. 500-0006).

2.5. Parasporal protein preparation, proteolysis assay and SDS-PAGE analysis

B. thuringiensis israelensis strains were grown in solid T3 medium until complete sporulation. Spores and crystals were harvested by centrifugation, washed three times with NaCl (1 M) and three times with cold bi-distilled water, respectively. Then spore-crystal mixtures were solubilized in 50 mM sodium carbonate buffer (pH 9.5) at 37 °C for 2 h with constant agitation. After centrifugation for 10 min at 13,000 × *g*, solubilized delta-endotoxins were recovered in the soluble fractions and their concentrations were determined by using the Bio-Rad Protein Assay (Germany) according to the method of Bradford (Bradford, 1976).

Solubilized Cry proteins were mixed with *A. aegypti* larval gut protease extracts at a ratio of 50:1 (w/w). The mixtures were incubated under controlled temperature of 37 °C with constant agitation for diverse incubation periods. All protein samples were suspended in Laemmli sample buffer, boiled for 5 min and size separated by SDS-PAGE (10%) (Laemmli, 1970). Gels were stained with Coomassie blue dye.

2.6. Spore and delta-endotoxin production

The *B. thuringiensis israelensis* strains were grown in liquid glucose medium at the optimal conditions for delta-endotoxin production as described by Zouari et al. (1998). After 72 h, the cultures were examined under a light microscope. They were only composed of a mixture of spores, crystals and minor cell debris. The spores' number was estimated by the counting of colony forming units (CFU). 1 ml from each culture was heated at 80 °C for 10 min and appropriate dilutions were plated on a solid LB medium (Sambrook et al., 1989). Delta-endotoxin concentration was determined as previously described by Ghribi et al. (2004). The acrystalliferous strain 4Q7, was used as negative control, in order to take into account, in delta-endotoxin determinations, the possibly contaminating dissolved proteins from spore coat and cell debris (Zouari et al., 1998). The delta-endotoxin production yield was calculated as the ratio of delta-endotoxin concentration (mg l^{-1}) and CFU (spores l^{-1}). The obtained yields of delta-endotoxin production were the average of three replicates of three separate experiments for each strain.

2.7. Insecticidal activity against A. aegypti

A. aegypti eggs were provided by "*Laboratoire de Lutte contre les Insectes Nuisibles* (*LIN*), *Montpellier*, *France*" in filter paper

Table 1
List of primers designed and used in this study.

Primer pairs	Sequences	PCR products size (bp)	Reference
4AF 4AR	5′ATTAAATCCCCTCCTGTATGAC3 5′TATCCAATAGAAAATAGTCCAAAA 3′	1616	This study
Dip2A Dip2B	5′GGTGCTTCCTATTCTTTGGC 3′ 5′TGACCAGGTCCCTTGATTAC 3′	1290	Carozzi et al. (1991)
Cry10ASI Cry10R40180	5′ GTGTCTAATTTGAGCCCGGGAGGAATAG 3′ 5′ AGGCCATCTGGTTACCTGTA 3′	1119	This study
Cry11F Cry11R	5′ CCAGCATTAATAGCAGTAGCTCC 3′ 5′ TGCCRTCTGTTGCTTGATC 3′	1460	Sauka et al. (2010)
D23 D24	5′GTTGTAAGCTTATGGAAAAT3′ 5′TTAGAAGCTTCCATTAATA3′	750	Zghal et al. (2008)
Prom33 Dip1B	5′ AACCACTCGAGATATGTATG 3′ 5′ ATGGCTTGTTTCGCTACATC 3′	3183	This study Carozzi et al. (1991)
Cry4Bst **Dip2B**	**5′ ATGAATTCAGGCTATCCGTT 3′** **5′TGACCAGGTCCCTTGATTAC 3′**	**1395**	**This study** **Carozzi et al. (1991)**
$Cry11A_1$ **$Cry11A_2$**	**5′ CGAACCTACTATTGCGCC 3′** **5′ TATAGGATGGACGCCACG 3′**	**800**	**This study**

Table 2
Insecticidal activity of *B. thuringiensis israelensis* isolates against 3rd instar larvae of *A. aegypti*.

+Strains	CL_{50} ($\mu g\,l^{-1}$)
H14	$0.15^{a} \pm 0.01$
BLB124	$4.5^{b} \pm 0.11$
BLB199	$3.3^{c} \pm 0.12$
BLB354	$0.19^{a} \pm 0.02$
BLB356	$0.47^{d} \pm 0.08$
BLB506	$3.8^{e} \pm 0.14$

Different letters indicate significant difference among means at $p = 0.05$ upon Duncan multiple range test.

strips. When larvae are needed, the paper strip is immersed in distilled water then larvae were reared at 28 °C, 87% relative humidity, and a 12:12 dark/light photoperiod following WHO protocols (WHO, 2005). The screening of *B. thuringiensis israelensis* isolates for toxicity against *A. aegypti* 3rd instar larvae was performed using standard WHO bioassays (WHO, 2005). In a plastic cup containing 100 ml of distilled water, twenty-five *A. aegypti* larvae were placed. Seven concentrations of the spore-crystal mixtures of each strain were added. Three replicates were performed for the all tested concentrations and for a non-treated control group in each experiment. Plastic cups were incubated at 28 °C, 87% relative humidity and a 12:12 dark/light photoperiod. After 24 h, the numbers of dead larvae were recorded. The mean 50% lethal concentration and confidence limits were estimated by WIN DL32 software (version 2.0).

2.8. Statistical analysis of results

All the data related to the determination of delta-endotoxin production and lethal concentrations were statistically analyzed by SPSS software (version 17.0) using the Duncan test, performed after ANOVA.

3. Result

3.1. Insecticidal activity against A. aegypti

Mosquitocidal bioassays of the *B. thuringiensis israelensis* isolates were performed against *A. aegypti* third instars larvae (Table 2). The toxicity of the crystal-spore mixtures obtained from the studied isolates indicated that the 50% lethal concentration (LC_{50}) varied from 0.19 to 4.5 $\mu g\,l^{-1}$. Among the tested isolates, the most pathogenic one was BLB354 with LC_{50} of 0.19 $\mu g\,l^{-1}$, statistically equal to that exhibited by H14 reference strain. However, BLB356, BLB124, BLB199 and BLB506 showed a mosquitocidal activity lower than that of the standard strain of about 3–25 times. The delta-endotoxin coding genes of these strains were explored to know the main reasons of the decrease of the insecticidal activities.

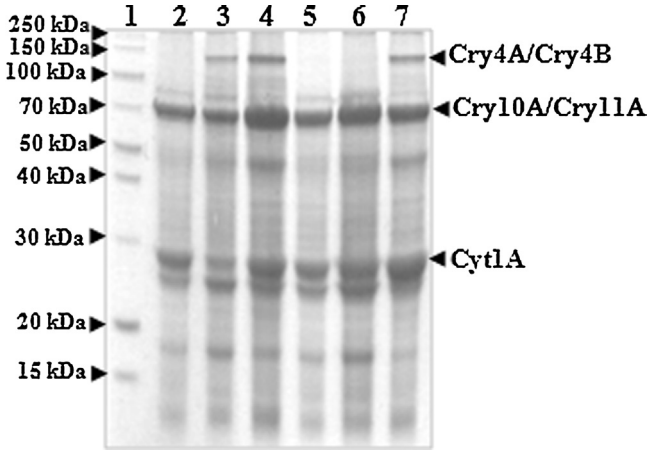

Fig. 1. Crystal protein profiles of *B. thuringiensis israelensis* studied strains. Lanes: 1, molecular weight markers (Fermentas); 2, BLB506; 3, BLB356; 4, BLB354; 5, BLB199; 6, BLB124; 7, H14.

3.2. Study of delta-endotoxin production and content

The delta-endotoxin production of new isolates of *B. thuringiensis israelensis* was compared with that of the reference strain (H14). Results are summarized in Table 2. Growing in the glucose medium (Zouari et al., 1998), the studied strains showed different delta-endotoxin yields (Table 3). Compared to the H14 reference strain, a great reduction of delta-endotoxin production level (about 50%) was observed for BLB124, BLB199 and BLB506 strains. However, both strains BLB354 and BLB356 produce statistically the same delta-endotoxin yields as the wild type H14. To compare the polypeptide composition of parasporal proteins from these *B. thuringiensis israelensis* isolates, seven μg of the solubilized crystal proteins mixture from each strain were analyzed by SDS-PAGE (Fig. 1). All studied profiles contained the specific bands of *B. thuringiensis israelensis* subspecies: 130 kDa, 70 kDa and

Table 3
Study of the delta-endotoxin production of *B. thuringiensis israelensis* isolates.

Strains	Toxin (mg l^{-1})	CFU (10^9 spores l^{-1})	Toxin yield [mg toxins (10^9 spores)$^{-1}$]
H14	$1103.77^a \pm 8.22$	$4.1^a \pm 0.3$	$270.07^a \pm 17.80$
BLB124	$512.74^b \pm 8.44$	$3.6^a \pm 0.6$	$144.87^b \pm 22.11$
BLB199	$533.90^b \pm 8.28$	$3.8^a \pm 0.3$	$140.96^b \pm 8.99$
BLB354	$1097.94^a \pm 6.46$	$3.7^a \pm 0.4$	$298.95^a \pm 30.75$
BLB356	$1084.95^a \pm 6.77$	$4.3^a \pm 0.8$	$258.14^a \pm 47.27$
BLB506	$500.39^b \pm 7.88$	$3.3^a \pm 0.6$	$154.79^b \pm 26.19$

Means followed by the same lower-case letter in a column are not significantly different (at $p = 0.05$) with Duncan's multiple range test.

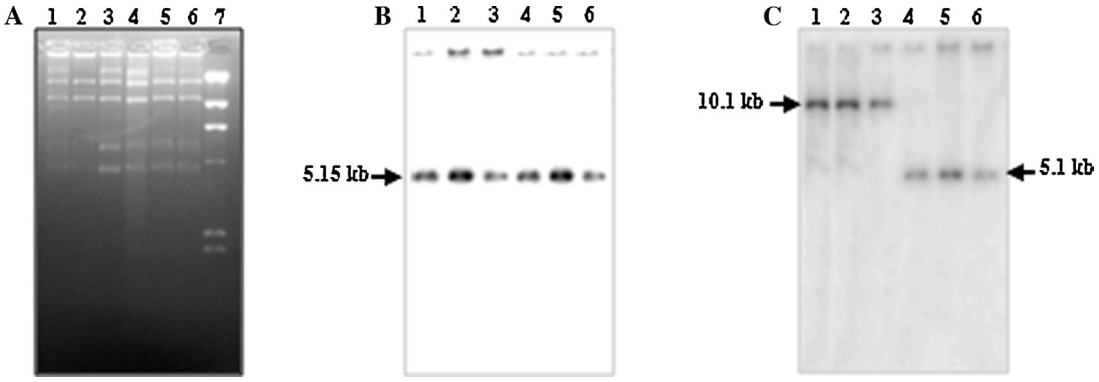

Fig. 2. Plasmid patterns of *B. thuringiensis* studied strains (A). Autoradiogramms corresponding to the Southern blot hybridization of *B. thuringiensis israelensis* plasmid DNA digested with *Hind*III (B) and *Eco*RI (C) and hybridized with a probe specific to *cry4Ba* genes. Lanes: 1, H14; 2, BLB356; 3, BLB354; 4, BLB199; 5, BLB124; 6, BLB506; 7, λ*Hind*III (23.5 kb; 9.5 kb; 6.64 kb; 4.35 kb; 2.29 kb; 1.94 kb; 0.58 kb).

27 kDa corresponding to Cry4A/Cry4B, Cry10A/Cry11A and Cyt1A, respectively. BLB354 and BLB356 showed protein patterns identical to that of H14, used as a reference, whereas BLB124, BLB199 and BLB506 produced thin proteins band of about 130 kDa.

3.3. *Plasmid patterns and* cry *gene content*

Plasmid patterns of mosquitocidal studied strains were analyzed (Fig. 2A). Here, all obtained plasmid profiles were almost identical, including that of the standard strain H14 of serovar *israelensis*.

The PCR analysis of the strain *cry* and *cyt* gene content, using primer pair's specific to *B. thuringiensis israelensis cry*, demonstrated that BLB354 and BLB356 contained *cry4A*, *cry4B*, *cry10A*, *cry11A* and *cyt1A* similar to those of H14 used as positive control. For BLB124, BLB199 and BLB506, PCR analysis showed the presence of *cry4B*, *cry11A* and *cyt1A* genes. While, they did not show any amplification using primers for *cry4A* and *cry10A* genes (Fig. 3). To check the DNA rearrangements in the pBtoxis plasmid described by Zghal and Jaoua (2006), a restriction-fragment-length-polymorphism (RFLP) hybridization experiment was carried out. Therefore, plasmid DNAs isolated from the studied strains were digested with *Hind*III and *Eco*RI restriction enzymes, respectively. Then, the obtained DNA fragments were separated on agarose gels and their corresponding blots were hybridized to radiolabeled probe specific to *cry4Ba* gene amplified by PCR from the reference strain H14. Autoradiogram corresponding to DNA plasmid restricted by *Hind*III shows that *cry4B* gene was present in all studied strains and located on a *Hind*III fragment of 5.15 kb similarly to the reference strain H14 (Fig. 2B). For plasmid DNA restricted by *Eco*RI, bands of 10.1 kb were observed, in autoradiogram, for BLB354 and BLB356 as well as for the H14 reference strain (Fig. 2C). The obtained results indicated that *cry4B* gene was located in *Eco*RI fragment of about 10.1 kb for BLB354 and BLB356. However, the autoradiogram showed the hybridization of *cry4B* probe with a fragment of about 5.1 kb for BLB124, BLB199 and BLB506 (Fig. 2C). The generated bands of 5.1 kb fragment demonstrated that a new *Eco*RI site was created downstream *cry4Ba* gene for BLB124, BLB199 and BLB506 strains.

3.4. *BLB356 parasporal proteins activation*

Despite the comparable *cry* gene contents and protein crystal composition, BLB356 shows an insecticidal activity 3 times lower than that exhibited by the H14 reference strain. In order to determine if the crystal protein activation step among the delta-endotoxins mode of action was involved in the lower activity

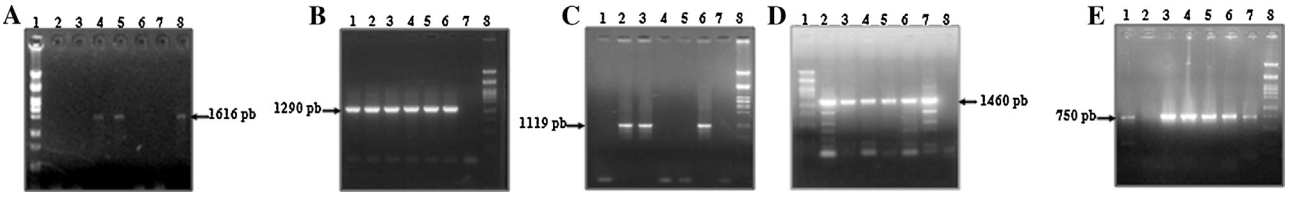

Fig. 3. PCR products amplified from *B. thuringiensis israelensis* isolates using primers specific to *cry4A* (A), *cry4B* (B), *cry10A* (C), *cry11A* (D) and *cyt1A* (E). (A) Lanes: 1 – λ*Pst*I; 2 – negative control; 3 – BLB506; 4 – BLB356; 5 – BLB354; 6 – BLB199; 7 – BLB124; 8 – H14. (B) Lanes: 1 – H14; 2 – BLB124; 3 – BLB199; 4 – BLB354; 5 – BLB356; 6 – BLB506; 7 – negative control; 8 – λ *Pst*I. (C) Lanes: 1 – BLB506; 2 – BLB356; 3 – BLB354; 4 – BLB199; 5 – BLB124; 6 – H14; 7 – negative control; 8 – λ *Pst*I. (D) Lanes: 1 – λ *Pst*I; 2 – H14; 3 – BLB124; 4 – BLB199; 5 – BLB354; 6 – BLB356; 7 – BLB506; 8 – negative control. (E) Lanes: 1 – H14; 2 – negative control; 3 – BLB124; 4 – BLB199; 5 – BLB354; 6 – BLB356; 7 – BLB506; 8 – λ *Pst*I. (λ *Pst*I: 11.5 kb; 5.07 kb; 4.74 kb; 4.5 kb; 2.83 kb; 2.55 kb; 2.45 kb; 2.44 kb; 2.14 kb; 1.98 kb; 1.7 kb; 1.09 kb; 0.8 kb; 0.51 kb; 0.46 kb; 0.44 kb; 0.33 kb; 0.26 kb; 0.24 kb.)

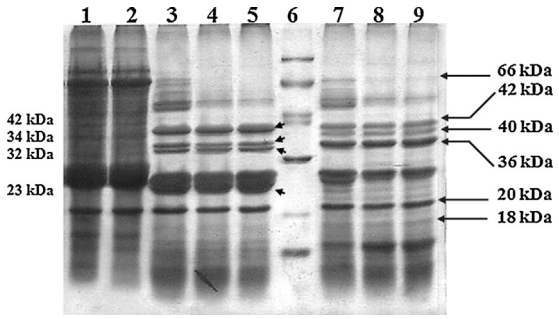

Fig. 4. *In vitro* processing of BLB356 and H14 protoxins with *A. aegypti* larvae protease extract after different incubation periods. Lanes: 1, BLB356 solubilized delta-endotoxins incubated without proteases; 2, H14 solubilized delta-endotoxins incubated without proteases; 3, BLB356 solubilized delta-endotoxins incubated with proteases for 10 min; 4, BLB356 solubilized delta-endotoxins incubated with proteases for 30 min; 5, BLB356 solubilized delta-endotoxins incubated with proteases for 60 min; 6, molecular weight markers (LMW: 97, 66, 45, 30, 20.1, 14.4 kDa; Amersham); 7, H14 solubilized delta-endotoxins incubated with proteases for 10 min; 8, H14 solubilized delta-endotoxins incubated with proteases for 30 min; 9, H14 solubilized delta-endotoxins incubated with proteases for 60 min.

of BLB356 against *A. aegypti*, the protoxin activation process was studied *in vitro* (Karumbaiah et al., 2007).

BLB356 and H14 crystals were solubilized, and the obtained protoxins were digested by *A. aegypti* gut proteases at different incubation periods. SDS-PAGE analysis shows the bands of 130 kDa of both BLB356 and H14, corresponding to Cry4A/Cry4B protoxins, disappeared rapidly *via* the larvae proteases extract and were replaced by the activated toxins of about 66 kDa. As shown in Fig. 4, we did not find detectable band of 130 kDa after 10 min. By increasing incubation time, Cry4 active toxins of H14 reference strain remained relatively stable. In fact, activated toxins were already evidenced after 60 min of incubation with the *A. aegypti* gut proteases. While, this active form in BLB356 digestion profile vanished almost completely beyond 30 min. It was progressively proteolysed. The disappearance of Cry4 active form, for BLB356, was accompanied by the emergence of more abundant proteolytic products compared to H14 reference strain (Fig. 4). In fact, the intensity of bands of about 42, 23 and 20 kDa in BLB356 digestion profile were greater than that in H14 digestion profile (Fig. 4). Furthermore, the SDS-PAGE profiles of H14 digested crystal proteins showed the presence of bands of 40 and 36 kDa corresponding to Cry11A active toxins (Masashi et al., 2002). Whereas, polypeptides of 34 and 32 kDa were present in digestion profile of BLB356 and did not appear in that of H14 (Fig. 4). Those polypeptides may correspond to processed forms of Cry11A active toxins (Masashi et al., 2002). Such results should give evidence on the higher instability of BLB356 active toxins to *A. aegypti* larval protease extract compared to H14 suggesting the involvement of protoxin activation process in reducing BLB356 toxicity.

3.5. Analysis of partial δ-endotoxins sequences from BLB356 strain

Regions of *cry4Ba* and *cry11Aa* genes, coding part of Cry4Ba and Cry11Aa active toxins, were amplified from BLB356 strain using Cry4Bst/Dip2B and $Cry11A_1$/$Cry11A_2$ primer pairs, respectively. The obtained amplicons were sequenced and the resulting sequences were deposited in the GenBank database. The corresponding accession numbers are KR010368 and KR010369 belonging to *cry4Ba* and *cry11Aa* genes, respectively. Compared to the published sequences, both PCR product nucleotide sequences showed several substitutions.

For *cry4Ba* gene of BLB356 strain, the deduced amino acid sequence corresponding to the PCR fragment (KR010368) showed five mutations compared to that of the reference strain H14 (CAD30095) (Fig. 5A): $M^{15}I$, $M^{253}I$, $Y^{267}F$, $S^{300}F$ and $T^{312}S$. According to the published structure of Cry4Ba toxin (Boonserm et al., 2005) $M^{253}I$ and $Y^{267}F$ lying within helix α7, whereas, $S^{300}F$ and $T^{312}S$ are located upstream of β2 sheet.

The alignment of amino acids sequence corresponding to the amplified fragment from *cry11Aa* gene (800 pb) of BLB356 isolate with that of the reference strain H14 (CAD30081) revealed several differences (Fig. 5B). In fact, there were substitutions of two amino acids at positions 307 and 308 ($G^{307}A$ and $V^{308}P$) lying within loop 1. Interestingly, a substitution of 8 successive amino acids were found at position 310 to 317 ($P^{310}R$, $I^{311}N$, $Y^{312}I$, $D^{313}K$, $P^{314}F$, $S^{315}R$, $S^{316}Q$ and $G^{317}H$) located in β3 sheet.

4. Discussion

Toxicity level is species-dependent which is related to the insecticidal crystal proteins. Any insecticidal crystal proteins changes affect the toxicity of *B. thuringiensis israelensis* strains to mosquito larvae (Sarrafzadeh et al., 2005). The verification of *cry* genes combinations in the novel isolates was carried by PCR analysis and RFLP hybridization experiments. The absence of *cry4A* and *cry10A* genes in three new isolates, BLB124, BLB199 and BLB506, was confirmed. The lack of *cry4A* and *cry10A* genes was accompanied by the presence of a DNA rearrangement in pBtoxis plasmid as that described by Zghal and Jaoua (2006) for BLB124, BLB199 and BLB506 strains. This result could explain the observed decrease of delta-endotoxins production levels and also the detected difference in the protein patterns compared to the wild type strain H14. Although the activity of Cry4Aa and Cry10Aa, against *A. aegypti* larvae, were lower than that of Cry4Ba (Beltrão and Silva-Filha, 2007; Hernández-Soto et al., 2009), the loss of Cry4Aa and Cry10Aa affects greatly the toxicity of BLB124, BLB199 and BLB506 strains. These findings are in agreement with the results obtained in previous studies, pointing out that no combination is as active as the native *B. thuringiensis israelensis* crystal due to the synergistic interactions between *B. thuringiensis israelensis* toxins (Schnepf et al., 1998). Plasmid transfer between *B. thuringiensis israelensis* and other closely related *Bacillus* isolates could lead to the loss or the inactivation of some *cry* genes (Gonzalez et al., 1982).

By means of a biological assay, *A. aegypti* larvae showed to be less sensitive to BLB356 delta-endotoxins compared to BLB354 and H14. Lightwood et al. (2000) reported that the proteolysis of delta-endotoxin was a major factor of *B. thuringiensis* toxicity. The study of the activation step of BLB356 delta-endotoxins was performed, *in vitro*. In the presence of proteases gut of *A. aegypti*, toxins of BLB356 (Cry4 and Cry11A) were less stable than that of H14. This finding may be explained by the detected variances in primary sequences of Cry4Ba and Cry11Aa delta-endotoxins from BLB356 that could induce changes in the number and/or the accessibility of the protease cleavage sites on account of tertiary structure modifications. In fact, for Cry4Ba toxin the occurrence of two F residues, due to $Y^{267}F$ and $S^{300}F$ substitutions, increases the number of theoretical cleavage sites of chymotrypsin which is one of the main digestive proteases in larval midgut (Park et al., 2012; Yang and Davies, 1971). In addition, both substitutions $M^{253}I$ and $Y^{267}F$ were located in the helix α7 which is implicated in pore formation by acting as a binding sensor (Boonserm et al., 2005; Tiewsiri et al., 2009; Tiewsiri and Angsuthanasombat, 2006). Regarding Cry11Aa toxin, $G^{307}A$ and $V^{308}P$ substitutions, lying within loop 1, could influence BLB356 toxicity since loop 1 is important determinant for receptor interaction and toxicity against mosquitoes in the case of dipteran-specific toxins (Fernandez et al., 2005; Smith and Ellar, 1994). Moreover, the substitution of 8 successive amino acids, founded in β3 sheet of Cry11Aa toxin, could lead to variances of its

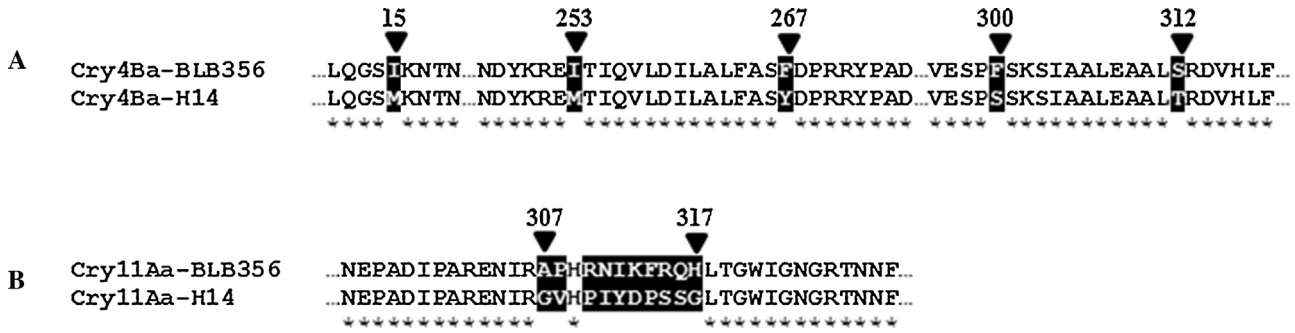

Fig. 5. Comparison of the deduced amino acid sequences of Cry4Ba (A) and Cry11Aa (B) from BLB356 with those of H14 reference strain. Only the regions containing differences are presented. The black boxes represent the residues showing variations. The vertical downward arrows indicate amino acid positions.

spatial configuration. Thus, detected mutations evidenced for Cry4Ba and Cry11Aa toxins from BLB356 could be the major cause of their early degradation by larvae midgut proteases. Thus, this finding was hypothesized to be a leading cause of the partial loss of larvicidal activity for BLB356. Therefore, the prediction of *B. thuringiensis israelensis* isolates toxicity could be achieved by the assessment of the efficiency based on active toxins stability in the midgut of mosquito larvae (Miranda et al., 2001). The detected diversity in *B. thuringiensis israelensis* local population strains should be considered as an important feature in *B. thuringiensis israelensis* use in biological control program. Further molecular investigations of these *B. thuringiensis israelensis* local isolates will be our major focus.

5. Conclusion

The present study reported two factors affecting *B. thuringiensis israelensis* mosquitocidal activity: (i) the deletion of *cry4Aa* and *cry10Aa* genes after a DNA dynamic of pBtoxis plasmid (ii) amino acids substitutions in Cry toxins causing their early degradation by larval proteases. The existence of such particular strains in the environment is a threat because the phenomena of conjugation and recombination could cause a decrease in the larvicidal activity of the strain used as biopesticide. Thus, the molecular investigation of the isolates isolated from the environment should be done for each ecosystem especially before biological treatment based on *B. thuringiensis israelensis*.

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