

***Bacillus Thuringiensis* Mutant Increase Activity Against *Spodoptera Frugiperda* Larvae**

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Abstract: *Bacillus thuringiensis* is a Gram-positive bacterium which main characteristic is the production of Cry proteins, that is toxic to some insects. These proteins, when ingested by susceptible insects, become active causing their death. In nature, it is possible to found *B. thuringiensis* strains which produce these proteins, but they differ in productivity (some of these isolates are more productive then others), and as to the toxicity levels of the produced proteins. Two *B. thuringiensis* strains that were highly effective against *Spodoptera frugiperda* larvae were chosen to verifying genetic mutation implication on Cry proteins productivity. One strain with a prolific spores production, while the other one only produced small amounts of spores. A genomic mutant library of these two isolates was, separately, constructed by genome Tn-5 transposon random insertion. Data analysis showed that mutation had a direct effect on the spores production, inducing an increase as well as a decrease in the production, according to the different strain observed. These results indicate, for the first time, that it is possible to use the described technique with *B. thuringiensis*, as well as the possibility to genetically breeding this bacteria. Another possibility introduced here is the possibility to do functional genetic studies mediated by mutagenesis in this bacterium.

Key words: Transposon, *cry* genes, mutant library, functional genomics.

INTRODUCTION

B. thuringiensis is a sporogenic Gram-positive bacterium that, while sporulating, produces crystalline inclusions (Hofte, 1989). These parasporal crystals have a toxic effect on some insects, such as those from orders Lepidoptera, Diptera and Coleoptera. These bacteria can be found in substrate from soil, water, plants surface, dead insects and stored grain (Miralles, 2004).

At the end of the logarithm growth phase, these bacteria go through a spore production process due to an available nutrients exhaustion, and produce parasporal crystals composed proteins. These crystals are called Cry proteins or δ -endotoxins, and are responsible for the entomopathogenic effect produced by the bacteria. The δ -endotoxin toxic effect depends on the crystal's dissolution by insects enzymes, in an alkaline environment. Therefore, the Cry protein is produced as a pre toxin, which, to be activated, has to be processed by protease enzymes found in the gut of susceptible insect larvae. This enzyme dissolution of the crystals liberates the toxic fragment Bravo, (2005) and produces the insecticide effect when these fragments bind to the receptors on the gut cell membrane and pores are formed. As a result, the cells are lysed and the intestines' integrity is ruptured, resulting in death of the larvae in a few days.

Cry proteins are highly specific and do not affect animals, non targeted insects, or the general environment. However, although highly specific, some *cry* genes exert a toxic effect on insects from more then one order (Zhong, 2000). Furthermore, there are bacteria strains that produce a large quantity of these proteins and others that produce these in small amounts. As *B. thuringiensis* is mostly used as a biocontrol agent by being placed directly on the targeted organism, the use of isolates that produce small amounts of spores aren't economically viable, even if the protein produced is highly efficient.

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Because of these aspects, researchers from around the world have been trying to obtain improved *B. thuringiensis* strains. These, being very productive, would reduce the cost of fermentation and would become a very competitive alternative to chemical products marketed today for this use.

Although an increase in spore productivity could be attained by improvement of the fermentative process and the use of growth media that would be appropriate and adequate to the metabolism of each strain of bacteria (Zouari, 1998; Zouari, 1999; Zouari, 2002) genetically improved strains can reach high levels of sporulation using low cost fermentative processes and growth media. This would permit the production of Cry proteins with low cost and investment (Stanbury, 1995).

Among the techniques used for genetic improvement of microorganisms, mutation, genetic selection and recombination are those most used. Initially, the mutation process was obtained by using physical and chemical compounds that, almost always, caused many random mutations. As these could affect a great number of metabolic pathways, obtaining viable mutant strains in satisfactory amounts was difficult. Even so, Ghribi, (2004) with more than 94% of cell mortality, were able to select highly productive *B. thuringiensis* mutant strains with the use of UV light and nitrous acid. After a protocol for attaining mutation mediated by transposon insertion was developed, the obtaining of many of mutant strains with high survival rates and with single mutations became much easier (Steggles, 2006; Wang, 2008; Duo, 2008). Furthermore, mutant bacteria obtained by the use of transposon complexes can have the mutant gene identified by DNA sequencing (Goryshin, 2000).

With these considerations in mind, the present work aimed to analyze the effect of random mutation obtained by insertion of labeled tags on the production of Cry protein by *B. thuringiensis*. The results obtained allowed the identification of mutant clones with a higher spore production than the wild-type *B. thuringiensis*. Until the present moment, this is the first report of a large-scale production of mutant forms of this species of bacteria by using transposon insertion.

MATERIAL AND METHODS

2.1. Bacteria Strains:

The present work used a *Bacillus thuringiensis* strains from the collection from the Laboratory of Bacterial Genetics and Applied Biotechnology from the Department of Biology Applied from UNESP, Campus Jaboticabal-SP, Brazil.

2.2. Preparation of Electrocompetent *Bacillus Thuringiensis* Cells:

A pre-inoculum of cells from the chosen strain was produced in 3 ml of the TSB (Tryptone Soya Broth) growth media which was shaken at 200 rpm and 37 °C for 12 hours. Afterwards, 2 ml of the pre-inoculum was transferred to an Erlenmeyer containing 200 ml of the TSB growth medium (previously warmed to 37 °C), and then was shaken at 200 rpm and 37 °C until reaching an optic density (OD)₆₀₀ value between 0.2 and 0.4. After the desired OD was attained, the cell culture was divided into four 50 ml Falcon type test tubes and submitted to centrifugation at 5,204 g for 10 min at 4 °C. The supernatant produced was then discarded and 1.5 ml of the electroporation buffer (250 mM of sucrose; 1 mM of MgCl₂; 1 mM of Hepes buffer; 10% of glycerol), cold and sterilized by filtration, was added. Cell sediment from the culture was then gently re-suspended. After transferring the cell suspension to 1.5 ml tubes, the cells were once again centrifuged at 3,824 g for 5 min at 4 °C, and the supernatant produced was discarded. This procedure for washing the cells with the electroporation buffer was then repeated 4 more times under the same conditions as described above. After the fourth wash, the cells were re-suspended in 1,3 ml of the cold electroporation buffer and then 50 µl aliquots were placed each in a 1,5 ml tube. Afterwards, electroporation of these cells was performed using the intended DNA samples.

2.3. Transference of the Transposon to Competent Cells:

One microlitre of the transposon/transposase complex supplied with the compound of reactants EZ::TNTM <KAN-2> Insertion Kit (Epicentre), was added to 50 µl of electro-competent cells. This mixture was transferred to a 1 mm cuvette (BioRad) previously chilled, and the mixture was submitted to electroporation at 200 ohms, 25 µF and 2.0 kV. Immediately after applying the electrical pulse, 950 µl of the recuperation buffer (1 X TSB; 250 mM sucrose; 5mM MgCl₂ MgSO₄) was added and transferred to 1.5 ml tubes. After 2 h at 200 rpm and 37 °C, a 100 µl aliquot and another of 10 µl were individually inoculated in two Petri dishes containing solid BHI growth media supplemented with kanamycin (30 µg/ml).

These dishes were incubated at 37 °C until visualization of the bacterial colonies was possible, which occurred after approximately 16 h.

2.4. Construction of the Mutant Library:

The clones that multiplied in the kanamycin growth medium were harvested individually, using previously sterilized wood sticks, and were then transferred to 96 well plates, which contained 100 µl of the liquid BHI growth media supplemented with kanamycin (15 µg/ml). After 3 h of cell cultivation, 10 µl were then transferred to a “Deepwell” plate containing 1 ml of the liquid BHI growth media supplemented with kanamycin and then the cell multiplication was continued for approximately 16 h, at 200 rpm and 30 °C. The suspension then remaining on the plate received 100 µl of 40% glycerol, was homogenized and then stored in a freezer at – 80 °C.

2.5. Extraction of Mutant DNA:

The mutant *B. thuringiensis* clones were multiplied in 96 wells plates which contained 1 ml of the BHI growth medium supplemented with kanamycin (15 µg/ml) and were then incubated for 16 h at 30 °C while being shaken constantly at 200 rpm. After this period, the plates were submitted to centrifugation at 3,220 g at 25 °C. DNA extraction was done according to method described by Wilson, (1997) with some modifications such as the use of lysozyme (20 mg/ml) at the beginning and the use of RNase (10 µg/ml) at the end of the extraction.

The DNA samples obtained were analyzed by electrophoresis in 0.8% agarose gel containing 0.05 µg/ml of ethidium bromide, and the pattern of the samples extracted were compared to other samples of known concentrations.

2.6. Confirmation of Transposon Insertion into the *B. Thuringiensis* Genome:

Confirmation of the transposon insertion into the genome of *B. thuringiensis* was done using two different techniques: polymerase chain reaction (PCR) and Southern Blotting (2008).

Based on the transposon sequence, an oligonucleotide was produced so that it was homologous to the area of transposon acknowledgement (“mosaic ends”). This area is composed of 19 bp present at the ends of the transposon (terminal peptides) and is required for the transposition. Once this sequence is present at the transposon extremities in an inverted position, only one oligonucleotide is sufficient to amplify the transposon sequence of nucleotides.

The primer Tn5_FR 5'-GTCTCTTATACACATCTCAACC-3' was used in the reactions which were composed by 30 ng of genomic DNA, 200 µM of a dNTP solution, 2.0 mM of MgCl₂, 0.5 µl of the initiator (5.0 pmoles), 1.0 U of the *Taq* DNA polymerase enzyme (Invitrogen), a buffer solution for the PCR reaction (equivalent to 10% of the reaction), and two times distilled water previously sterilized, in enough volume to complete a total volume of 20 µl. This reaction was submitted to a PCR amplification composed of an initial step for denaturation at 94 °C for 5 min, followed by 35 cycles composed of a denaturing phase (94 °C for 30 s), initiator pairing (50 °C for 30 s) and a phase for polymerization of the sequence (72 °C for 1 min and 30 s). At the end of the 35 cycle program, an extra polymerization phase was completed at 72 °C for 7 min, after which the tubes were maintained at 4 °C until they were taken out of the thermocycler. After electrophoresis of the samples, the presence of a 1,217 bp long fragment of amplified DNA confirmed the presence of the transposon in the bacteria's genome.

Besides the PCR amplification, hybridization of nucleic acids was also used to confirm the insertion of the transposon into the *B. thuringiensis* genome. Approximately 10 µg of the genomic DNA of each of the mutant strains were cleaved using the *Eco* RI restriction enzyme, which does not have a cleaving site on the transposon. The cleaving reaction was done in a final volume of 50 µl containing 10 µg of DNA, 5.0 µl of enzyme buffer (equivalent to 10% of the reaction), 1.0 µl of the enzyme (5000 U) and two times distilled water previously sterilized, in enough volume to complete a total volume of 50 µl. The cleaved samples were submitted to electrophoresis in a 1% agarose gel (1X TAE buffer) for 12 h at 25 V and, afterwards, stained using ethidium bromide and then photographically documented. The DNA was transferred to a nylon membrane (HYBOND N+, Amersham Biosciences) and hybridized with the labeled probe, using the group of reagents DIG High Prime DNA Labeling and Detection Starter kit II (Roche). All conditions of DNA transferal, hybridization, rinsing and exposing of the membranes after hybridization, were as recommended by the manufacturer.

2.7. Quantification of Spore Production by the Mutant Strains:

The mutant clones were taken from the freezers in which they were stored at -80°C and were harvested in BHI growth media supplemented with kanamycin ($15\text{ }\mu\text{g/ml}$) in a plate with 96 wells, which were then shaken at 200 rpm for 12 h to promote cell multiplication.

Immediately afterwards, $20\text{ }\mu\text{l}$ was transferred to Petri dishes containing NA (Nutrient Agar) growth media, and left to multiply for 5 days in an incubator at 30°C . Afterwards, all the contents of the Petri dishes were transferred to 10 ml of the spreading solution (500 ml of water + $25\text{ }\mu\text{l}$ of Tween) in Falcon 15 ml tubes. The contents of the tubes were then diluted 10 X and $150\text{ }\mu\text{l}$ of this now diluted solution was loaded in a hemocytometer to assess the number of spore produced. The same procedure was used for assessment of the wild-type *B. thuringiensis*, with the exception of the growth medium used for this strain, to which the kanamycin was not added. This whole protocol was repeated independently three times for confirmation of the obtained results.

Data was treated statistically using the R program (www.r-project.org) and statistic differences were tested using the Krustal-Wallis non parametric method. A Box-plot graph was constructed to permit visual analysis of the results and for identification of the differences between the mutant strains and between these and the wild-type bacteria.

2.8. Bioassays Using *Spodoptera Frugiperda* Larvae:

Bioassays of the wild-type and the mutant strains was done to determine the pathogenicity of the mutant clones. Ten mutant clones were screened for the bioassay: 5 that produced more and 5 that produced less spores than the wild type. The bioassays were done using *Spodoptera frugiperda* larvae, which were supplied by the Laboratory of Applied Ecology from the Department of Plant Health from UNESP/Campus de Jaboticabal. For the bioassays, each bacterial isolate was grown in NA at 30°C for 7 days.

The artificial diets were prepared according to Barreto *et al* (1999) and, afterwards, a bacterial suspension was prepared at a 3×10^8 spores/ml concentration, which was overlaid on the diets. After the diets absorbed the bacterial suspensions, they were placed in trays which had 32 cells each, together with newborn *S. frugiperda* larvae. The control was prepared using the same procedure, although the diet was soaked in distilled water instead of receiving the bacterial suspension.

This procedure was repeated four times, using thirty-two insects each, for each *B. thuringiensis* strain. Mortality was scored for the first time after 24 h and this assessment continued until the seventh day after the inoculation of bacteria. Mortality scoring was evaluated by counting all larvae found dead for each strain assessed.

2.9 Extraction of the Cry Proteins:

The mutant clones as well as the wild type were all multiplied for 16 hours in solid BHI growth medium containing kanamycin. Afterwards, they were transferred to fresh NA media containing kanamycin and were shaken for 7 days so total spore production could take place.

Afterwards, the clones were centrifuged at $5,930 \times g$ for 20 min at 4°C . The sediment that was then formed was washed twice with 20 ml of 0.5 M NaCl and centrifuged at $5,930 \times g$ and 4°C for 20 min and then washed twice again at 4°C , this time using cold distilled water, under the same conditions of centrifugation. The sediment was re-suspended in 1 ml of the buffer solution (10 ml of phosphate buffer pH 8.0: 0.2 M NaH_2PO_4 + 0.2 M of NaHPO_4 + 10 ml of 5 M NaCl and the reaction volume was completed up to 100 ml), $30\text{ }\mu\text{l}$ of lysozyme and $10\text{ }\mu\text{l}$ of DNase were added, and then the suspension was incubated at 37°C for 30 min. Afterwards, the suspension was sonicated 3 times (for 60 s each with a 10 s pause between them), to promote lysis of any cells that could possibly still be whole, and the liberation of proteinaceous crystals. The obtained proteins were stored at -20°C in freezer until the time of use.

2.10. Electrophoresis in Polyacrylamide SDS-PAGE Gel:

For the preparation of $100\text{ }\mu\text{l}$ protein samples, $40\text{ }\mu\text{l}$ of the protein suspension was diluted in $10\text{ }\mu\text{l}$ of sterile water and to this was added $50\text{ }\mu\text{l}$ of the sample buffer (62 mM Tris-HCl pH 6.8; 4% SDS; 20% Glycerol; 5% β -mercaptanol and 0.02% Bromophenol Blue) and was then warmed for 5 min to 100°C . The samples were then stored on ice until their use. The protein separation gel was prepared as according to Laemmli (1970) and the samples were submitted to 30 mA for approximately 4 h. After the electrophoresis, the gel was fixed using 40% methanol, 10% glacial acetic acid and Coomassie Blue (0.1%) for approximately 14 h and then discolored using 40% methanol and 10% acetic acid for another 4 h.

3. Results:

Based on protocol developed for *B. cereus* (Turgeon, 2006), and on growth curve (Data not show), it was decided to produce electrocompetent *B. thuringiensis* cells from cell culture with an OD₆₀₀ value between 0.2 and 0.4, achieved between 2 and 3 h after growth.

Total DNA extraction was carried out based on protocol developed by Wilson (1997) with some modifications: lysozyme and RNase was added and no CsCl was used (see Methods). Since the present work proposed to analyze many mutants, the DNA extraction was, therefore, optimized. A new protocol for extracting genomic DNA from *B. thuringiensis* on 96 well plates was made from which developed by Laia (2009) for *Xanthomonas axopodis* pv. *citri*.

3.1. Mutant Library Construction:

All mutants were submitted to PCR and to Southern blot analysis to confirm the genome transposon insertion. The PCR results showed positive amplification (a fragment of 1217 pb) for 85 mutant clones (Figure 1). Therefore, of all the mutants obtained, only 11.5% (11 clones) were not confirmed.

In the same way, Southern blot analysis was done using two different procedures. In one, the mutant strain's genomic DNA PCR product, obtained using the Tn-FR primers, was separated in agarose gel and transferred to the nylon membrane (Figure 2). In the second procedure (Data not show), the mutants' genomic DNA was cleaved using the Eco RI enzyme and then was also transferred to the nylon membrane. This specific enzyme was chosen because it does not cleave the transposon sequence. DNA hybridizations confirmed that the amplifications were a part of the transposon (Figure 2).

3.3. Spores Quantification:

Bacillus thuringiensis mutants spores production was very different. While some clones produced a large amount of spores, others produced very small amounts, and others produced spores in numbers that were very similar to those produced by the wild type (Figure3). Besides the variation in numbers of spores produced, some of the strains were found to maintain the spores produced inside the cells.

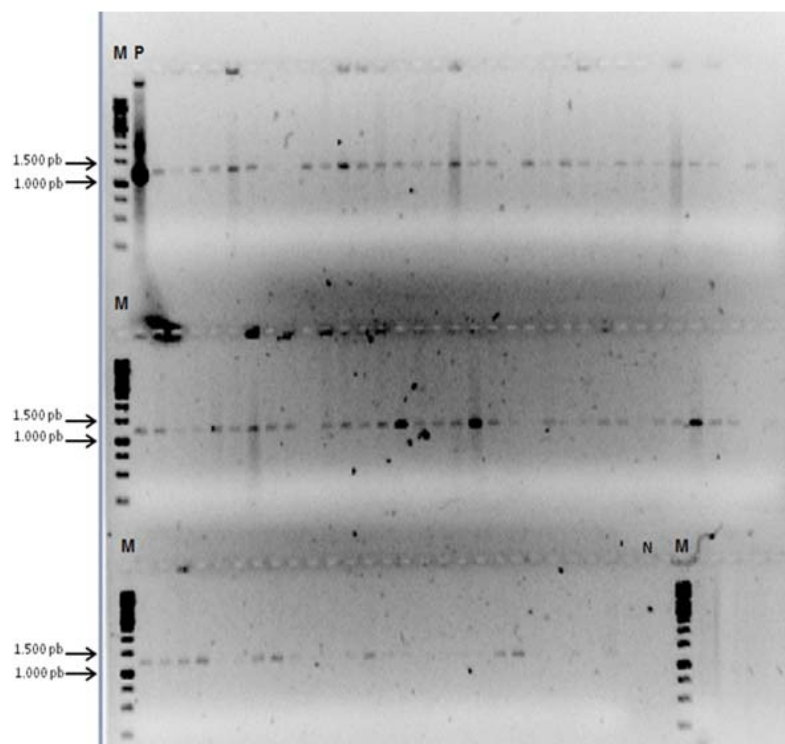


Fig. 1: Results concerning the amplification of 85 mutant clones of the Br55 *B. thuringiensis* strain using the initiating oligonucleotide Tn_FR, which is specific for amplifying a 1.217 pb fragment inside the Tn5 transposon (Epicentre). M= 1 kb DNA ladder Gene Ruler™, P= positive control (Tn5 cloned in pBluescript SKII plasmid); N= negative control (water instead of mold DNA in the reaction).

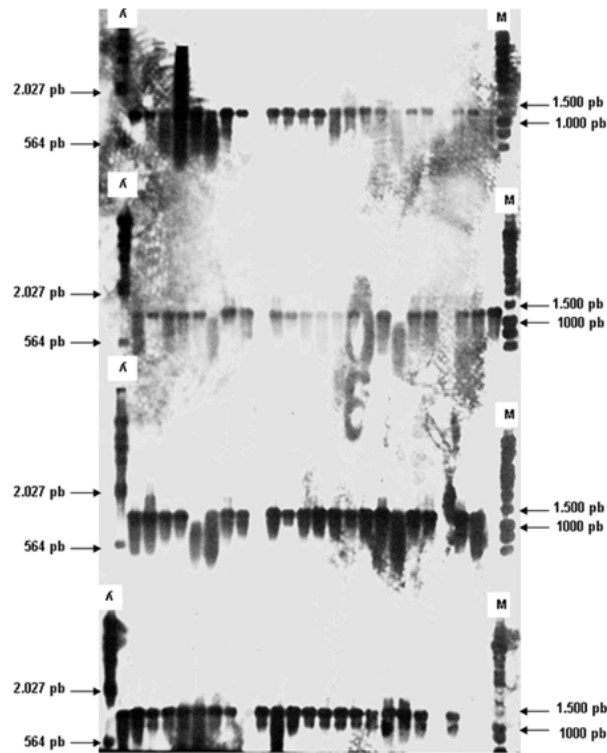


Fig. 2: *B. thuringiensis* mutant library screening. Each line was a different mutant. M = 1Kb Ladder DNA size maker; λ = lambda phage DNA *Hind* III cleaved.

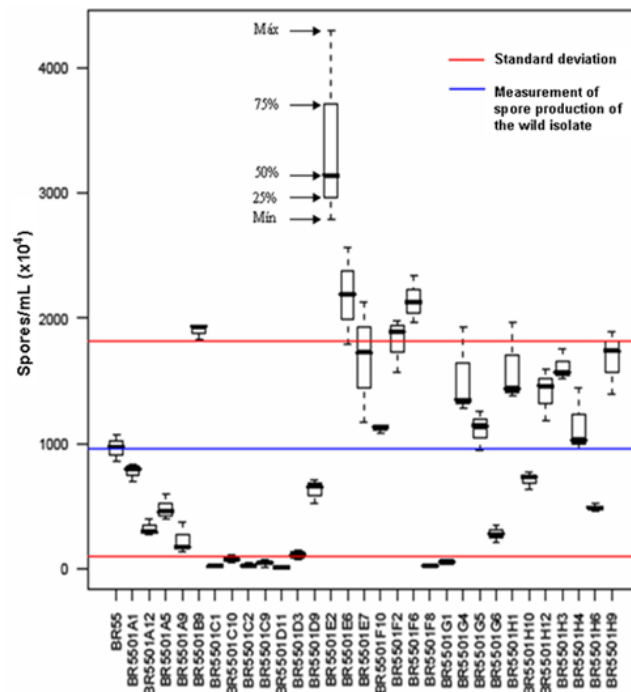


Fig. 3: Spore production regarding the 30 mutant and one *B. thuringiensis* wild type (BR₅₅). The blue line represents the wild type mean and the red ones represent the standard deviation.

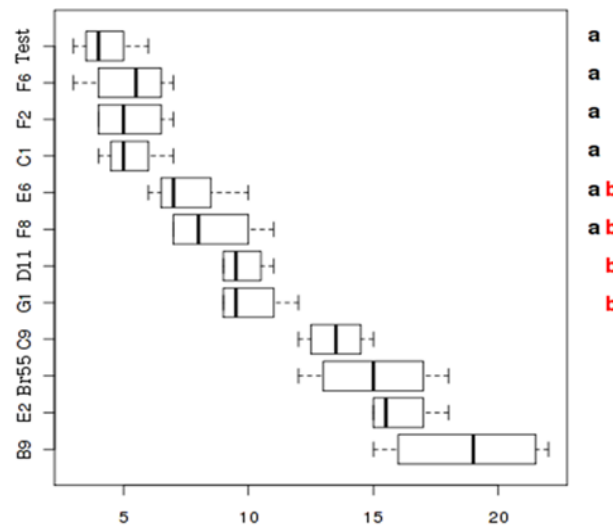


Fig. 4: Box plots showing the variance in efficiency in controlling newly eclosed *Spodoptera frugiperda* larvae and the results of statistical analysis. Mutant clones followed by the same letter did not differ at 5% significance according to Tukey's HSD test.

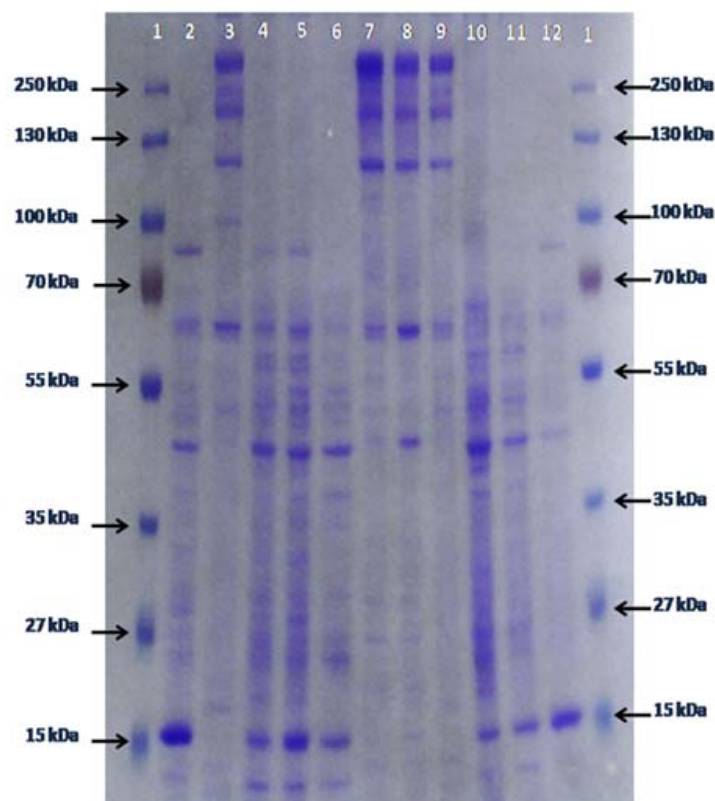


Fig. 5: Analysis by SDS-Page of the spore-crystal mixture of mutant clones compared to the Br₅₅ wild type. Rut 1: Marker of molecular size Fermentas Page Ruler™ Prestained protein Ladder Plus, 2: isolate Br₅₅, 3: clone B9, 4: clone C1, 5: clone C9, 6: clone D11, 7: clone E2, 8: clone E6, 9: clone F6, 10: clone F2, 11: clone F8 and 12: clone G1.

From the 30 isolates, 9 mutants produced smaller amounts of spores (Br_{5501A1}, Br_{5501A12}, Br_{5501A5}, Br_{5501A9}, Br_{5501D3}, Br_{5501D9}, Br_{5501G6}, Br_{5501H10}, Br_{5501H6}) and 9 produced larger amounts of spores (Br_{5501E7}, Br_{5501F10}, Br_{5501G4}, Br_{5501G5}, Br_{5501H1}, Br_{5501H12}, Br_{5501H3}, Br_{5501H4}, Br_{5501H9}) when compared to the wild type. However, all of these 18 mutants can be placed inside the area delimited by the values that determine the standard deviation for the assessed groups (Figure 3). On the other hand, 7 mutants (Br_{5501C1}, Br_{5501C10}, Br_{5501C2}, Br_{5501C9}, Br_{5501D11}, Br_{5501F8} and Br_{5501G1}) produced less spores than the inferior value delimiting the standard deviation and 5 mutants (Br_{5501B9}, Br_{5501E2}, Br_{5501E6}, Br_{5501F2} and Br_{5501F6}) produced larger amounts of spores than the superior standard deviation value (Figure 3). Morphological assessment showed the mutant colonies to be circular and opaque, indicating no phenotype alterations that could have occurred.

3.4. Bioassays:

From the selected mutants for the bioassays, two clones with high mortality rates were most prominent, corresponding to the clones Br_{5501B9} and Br_{5501E2}. These did not differ statistically from the Br55 wild type. The clones that did not differ from the controls were those that produced large amounts of spores (Br_{5501E6}, Br_{5501F2} and Br_{5501F6}) and the clones that produced very small amounts of spores (Br_{5501C1} and Br_{5501F8}) (Figure 4).

The proteinaceous crystal produced by the clones was characterized by SDS-PAGE of the spore-crystal suspension (Figure 5). The protein pattern bands produced by the Br55 wild type were compared to the pattern presented by the mutant clones, and the presence or the absence of protein bands was analyzed.

All the clones showed similarity to the wild type regarding the proteins of molecular weight ranging from 55 and 70 kDa, but were different when considering the proteins of 70 to 100 kDa. Considering these, only the C1, C9 and G1 clones shared similarity with the wild type. When observing the band of proteins ranging from 100 kDa to 130 kDa, a new protein, not shared by the wild type, was found from the isolates B9, E2, E6 and F6. However, when considering the absence of proteins between 35 and 55 kDa, the B9, E2 and F6 clones differ from all the other strains and also from the wild type. The difference between these three clones is that only the B9 and the E2 clones attained good mortality rates when comparing these to the wild type.

4. Discussion:

Many methods for obtaining electrocompetent *B. thuringiensis* cells were developed, however, all result in the production of very small amounts of clones. Using the method developed by Turgeon, (2006) the production of mutant clones by transposon insertion into the bacteria's genome was more successful.

The methodology used in the present study for preparing the electro-competent cells obtained the desired results, which could be due to the alterations made in the phase in which the cells were harvested (OD₆₀₀ between 0.2 and 0.4). According to Bhattacharya, (2000) the log phase starts after 3 to 4 h of incubation and between 10 to 12 h the stationary phase is reached. Sporulation starts 4-5 h after the beginning of the log phase. These periods are similar to those presented by the mutant strains as well as those presented by the wild type.

Due to the existence of a very large amount of clones, the method for DNA extraction was adapted for 96 well plates for a high-throughput mutagenesis analysis.

Southern blot analysis confirm the transposon presence in the bacteria's genome. Besides this confirmation, the results from this analysis also identifies if transposon was inserted in a single copy, a fact that has been reported regarding other organisms submitted to the same procedure such as *Xylella fastidiosa*. To confirm the desired results of the procedure applied to this species of bacteria, ten mutant clones obtained by transposon insertion were isolated and submitted to Southern Blot analysis with the results of this assessment indicating that the insertions occurred in single copy and as independent events (Guilhabert, 2001). In the study of the efficiency of mutagenesis by transposon insertion done using *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, the mutagenesis was also analyzed by Southern Blot, showing that almost the totality of clones tested presented random single-copy insertions of the transposon (Sun, 2003).

PCR hybridization was important since it showed the insertion of the transposon in most of the collected clones, a result that differed from that obtained from the hybridization with the DNA cleaved by enzymes. This could have occurred due to the fact that the DNA was not totally cleaved or due to an error during the process of hybridization and membrane washings.

As to the quantification of spores, the mutant clones produced larger amounts of spores than the wild-type. Ghribi, (2004) identified a frequent appearance of highly productive mutants, some of which produced 59% more proteinaceous crystals than the wild-type.

However, the gene sites which were affected were not identified. Mahler and Halvorson (1980) showed that the resistance to erythromycin could affect the process of sporulation in *B. thuringiensis* and in *B. subtilis*, respectively.

Since the synthesis of δ -endotoxins in *B. thuringiensis* strains depends on the balance between vegetative growth and sporulation, on the regulation of catabolic repression and on nutritional necessities (Zouari, 1998) an impact on the random mutations occurred on many DNA loci was expected. This could have a positive or a negative effect, and these mutations could affect the production of the Cry proteins. The expression of *cry* genes is regulated by two different mechanisms: one which depends on specific sigma factors from the sporulation phase (where most of the *cry* genes are classified), and the other, independent of sporulation, such as with *cry3*, which factors typically appear during the phase of vegetative growth (Valadares-Inglis, 1998). Both these mechanisms could have been affected by insertion of the transposon, causing inefficient production of Cry proteins.

The mutants in which were found small amounts of spores usually presented many vegetative cells, and inside some of these cells, spores were seen. The opposite situation was verified regarding the mutant clones which produced the largest amounts of spores in which few vegetative cells and many spores were found. Regarding the changes in spore production, the results obtained in the present work differ from those found by Fedhila *et al.*, (2004) who found that mutation did not significantly affect the capacity of *B. thuringiensis* cells to produce spores (data not shown). As to the low production of spores found regarding some of the strains, Bhattacharya (2000) obtained mutant strains that also presented a spore production considered deficient compared to the wild-type, even those that were considered to be highly toxic. This find would be contrary to some reports that the production of toxins is dependent of the sporulating phase, besides other factors (Zouari, 1998; Zhong, 2000; Bing-Lan, 1999).

The results of the bioassay indicate that the two mutant strains that produced large amounts of spores were the same that were prominent in causing mortality of *S. frugiperda* larvae. The other clones, which did not produce enough spores, were not efficient in controlling the insect. These facts could indicate the occurrence of a mutation in a gene which does not interfere with sporulation, but in another site, important to the synthesis of Cry proteins. These genes could be inactive and under the control of an inefficient promoter that could have suffered mutation.

All the clones presented a protein profile ranging from ~45 kDa to ~120 kDa, which is characteristic of strains that are active against lepidopterans. Some proteins were not detected in some clones, which could be due to changes to the *cry* gene promoter which corresponds to that particular protein and, therefore, its expression would have been suppressed (as in the case of the ~45 kDa and ~80 kDa proteins).

All the clones presented a ~65 kDa band, which could refer to the *cry2* protein. However, the presence of this band does not indicate that the gene is in its native form. In the other clones that did not obtain significant mortality rates, the *cry* gene that corresponds to this protein could have suffered changes in its sequence due to the insertion of the transposon. The insertion of mutations into the sequence in the active part of the toxin, or even in the part that would be responsible for linking it to its receptors, would have caused them to not have any effect on this pest, interrupting the gene's activity. The clones that caused good larvae mortality rates (B9 and E2) presented only the ~65 kDa protein similar to the wild type, where it would be in its native form and responsible for the insecticide effect, since the ~45 kDa and the ~80 kDa proteins were lost. The insecticide effect could have been enhanced by the presence of a ~120 kDa band, which did not exist in the wild type. This could represent a *cry1* gene that would now have been expressed.

Finally, the results obtained in the present work demonstrate the possibility of genetically improving *B. thuringiensis* with the intention of augmenting the production of Cry proteins. This would make the commercial use of low production strains viable by way of mutagenesis mediated by transposon, and would pave the way for studies of metabolic pathways involved in this production.

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