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DETECTION OF *CRY*1 GENES IN *BACILLUS THURINGIENSIS* ISOLATES FROM SOUTH OF BRAZIL AND ACTIVITY AGAINST *ANTICARSIA GEMMATALIS* (LEPIDOPTERA:NOCTUIDAE)

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ABSTRACT

The bacterium *Bacillus thuringiensis* (Bt) is characterized by its ability to produce proteic crystalline inclusions during sporulation. Cry1 protein has insecticidal activity and is highly specific to certain insects and not toxic to unrelated insects, plants or vertebrates. In this work, the patogenicity of twelve Bt isolates was tested against *Anticarsia gemmatalis*, one of the most important insect pests of soybeans. Spore-crystal complex was applied to the surface of artificial diets and the mortality of *A. gemmatalis* larvae was assessed seven days after each treatment. When compared to a control Bt isolate known by its high toxicity to *A. gemmatalis* larvae, four novel Bt isolates exhibited even higher toxic activities against the insect, resulting in more than 90% mortality. PCR was used to amplify DNA fragments related to known *cry*1 genes. Bt strains with high toxicity produced expected PCR products of around 280 bp, whereas non-toxic or low toxic strains did not produce any PCR product or showed amplified fragments of different sizes. Toxic Bt isolates also exhibited an expected protein profile when total protein extracts were evaluated by SDS-PAGE.

Key words: *Bacillus thuringiensis*, velvetbean caterpillar, *Anticarsia gemmatalis*, *cry*1 gene, δ-endotoxin

INTRODUCTION

Bacillus thuringiensis (Bt) Berliner is a Gram-positive, aerobic or facultative anaerobic entomopathogenic bacterium found in soil. These bacteria are characterized by their ability to produce crystalline protein inclusions during sporulation that have toxic activity against different invertebrates, especially insects (19,21,25). Bt spores can survive for several years, whereas the stability of the protein crystal is highly variable, lasting from a few days to several months depending on environmental conditions (25). The Bt crystal is composed of proteins called δ-endotoxins, cry proteins or insecticidal crystal proteins (ICP) of molecular mass ranging from 25 kDa to 140 kDa (5,9). The morphology, size and number of parasporal inclusions vary among different Bt strains (22).

Many genes encoding toxin proteins have been cloned and sequenced, and are commonly named as *cry* or *cyt* genes. Many Bt strains contain multiple *cry* genes that are usually present on large plasmids (4,14). To date, over 100 *cry* gene sequences have been described and are classified in 22 families with different subclasses in relation to their amino acid composition (3,8). The toxic protein against lepidopteran, for instance, belong to the *cry*1, *cry*2 or *cry*9 groups (8). Within the cry1 protein group, there are around 10 different subclasses, each subclass has a specific range of activity against different lepidopteran insects (2,13,27).

Despite their classification, cry proteins have similar mechanisms of action. When ingested by insect larvae, the crystal protein is first dissolved in the alkaline midgut and proteolitically converted into smaller toxic polypeptides. These polypeptides bind to specific receptor sites on the apical microvilli of the insect

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midgut cells. An insertion of the toxin (or a part of it) occurs into the membrane after receptor interaction, forming a pore that causes osmotic swelling of the cell and subsequent lysis, leading to death of the insect (16,20,26).

The use of Bt as a commercial insecticide in agriculture for more than 40 years is based on its high specificity and efficiency to certain insect pests and on its non-toxicity to other unrelated insects, plants and vertebrates. These characteristics point to a clear advantage of Bt formulations over many chemical insecticides, especially when a particular pest is responsible for high losses in agriculture (4,9,15,19). The velvetbean caterpillar cause a lot of damage to soybeans world wide, determining great reductions of leaf areas and, consequently, lowering photosynthesis and productivity (24). Among the Bt strains known to be toxic to *A. gemmatalis* larvae is Bt *kurstaki* HD1, which is commercially available. This strain contains at least 5 *cry* genes including classes *cry*1 and *cry*2 (19). Together with strains Bt *kurstaki* HD73 and Bt *thuringiensis* 4412, that respectively contain *cry*1Ac and *cry*1B genes, HD1 is commonly used as a standard strain when evaluating new Bt isolates (19).

In this work, we selected isolates from south of Brazil with high efficiency against velvetbean caterpillar and confirm *cry*1 genes presence using PCR techniques and protein spore-crystal analysis.

MATERIALS AND METHODS

Bacterial Strains

New Bt isolates were obtained from soil samples of different districts of the State Rio Grande do Sul (Brazil) for Polanczyk (1999). We used six isolates did not tested against *Anticarsia gemmatalis* named U68-5, U87-2, U90-1, U96-3, U98-1, U98-4. Strains Bt serovar *thuringiensis* (IP01), Bt serovar *alesti* (IP03), Bt serovar *fukuokaensis* (IP06), Bt serovar *kurstaki* (HD73), Bt serovar *thuringiensis* (4412), and Bt serovar *kurstaki* (HD1-Dipel) were obtained from WHO Collaborating Centre for Entomopathogenic *Bacillus*, Institut Pasteur (France).

Anticarsia gemmatalis larvae

Adult and larvae *A. gemmatalis* insects were obtained from the soybean crop plantations of south Brazil and colonies were maintained on artificial diets, prepared as described by Greene *et al.* (17), at 25 ± 1ºC with a photoperiod of 12:12 (L:D) and 70% relative humidity.

Culture conditions of Bt

Bt strains were grown in glycoside usual medium at 28º ± 1ºC under agitation of 180 rpm until complete autolysis was achieved according to Mahillon and Delcour (23). Spore-crystal complexes were harvested by centrifugation at 5,000 rpm for 20 min at 4ºC. To eliminate extracellular components, including β-exotoxins, known to accumulate in the cell culture supernatant (12), parasporal bodies and cell debris were washed twice in sterile-distilled water by centrifugation (5,000 rpm, 15 min). Pellets were suspended in 8 ml of sterile-distilled water. Cell number was monitored by counting in a Neubauer chamber under phase contrast microscopy (400X) and each suspension was diluted to achieve a title of 3×10^8 spore/ml.

Bioassays

The activity of different Bt isolates were screened among third-instar larvae of *A. gemmatalis* using a volume of 100 µl of Bt spore/crystal complex (3×10^8 cells/ml) according to Hernandez (18) were applied onto diet surface. In total, 24 larvae were tested for each isolate in this pre-selective assay. In a control group, Bt suspension was replaced by sterile-distilled water. Assays were maintained in a growth chamber at 25ºC ± 1ºC, 70% relative humidity and a photoperiod of 12:12 (L:D). Larval mortality was assessed after 7 days. Mortality of the control larvae reared on the toxin-free diet and under the same conditions was recorded and used to correct the test mortality with Abbot's formula (1).

Protein analysis

The protein composition of the spore-crystal complex was analyzed by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrilamide gel eletrophoresis). Gels were stained with Coomasie Blue. The molecular weight of proteins were estimated by comparasion with protein molecular weight standarts (High range, Gibco-BRL).

*Cry*1 gene related sequence detection by PCR

Bt strains were grown overnight at 30ºC in petri dishes containing solid culture media (3 g/l peptone, 5 g/l beef extract, 0.5 g/l yeast extract, 0.006 g/l $MnCl_2$, 0.08 g/l $CaCl_2$, 0.07 g/l $MgCl_2$, 1.5% agar, on bi-destilled water). The presence of crystals was confirmed under phase contrast microscopy. A loopful of cells from a single colony was transferred to 0.1 ml of sterile bidistilled water in a microfuge tube. The bacterial suspension was frozen at -20ºC for 20 min and directly boiled for 10 min to lyse the cells. The resulting cell lysate was centrifuged briefly at 10,000 rpm and 15 µl of the supernatant was used as DNA sample for PCR. PCR mixtures were prepared using 300 ng of the specific *cry*1 gene primers: *primer* 1: 5'-TGTAGAAGAGGAAGTCTATCCA-3' (CJI-1) and *primer* 2: 5'-TATCGGTTTCTGGGAAGTA-3' (CJI-2) (9), 0.5 U Taq DNA Polymerase (CENBIOT Enzimas®), 0.2 mM dNTP's, 10 mM Tris, 50 mM KCl and 3 mM $MgCl_2$. Amplification was performed in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400) using a single denaturation step (2 min at 95ºC), followed by a 30-cycle program, with each cycle consisting of a denaturation step of 95ºC for 1 min; an anneling step of 52ºC for 1 min, and an extension step of 72ºC for 1 min. A final extension step of 72ºC for 5 min was also included. Ten µl samples from the PCR mixtures were electrophoresed on polyacrylamide gel and stained with ethidium bromide (0.5 µl/ml).

RESULTS AND DISCUSSION

With the objective of isolating new genes coding *cry*1 proteins lethal to *A. gemmatalis* larvae, 6 new Bt isolates obtained from soil samples of Rio Grande do Sul (Brazil) were analyzed for their *"in vivo"* effect and compared to the three known active Bt strains Bt serovar *kurstaki* HD1 - Dipel, Bt serovar *kurstaki* HD73 and Bt serovar *thuringiensis* 4412 from WHO Collaborating Centre for Entomopathogenic *Bacillus* (Institut Pasteur, France). A series of bioassays were performed by feeding *A. gemmatalis* larvae with artificial diets containing the Bt spore-crystal complex. As presented in Fig. 1, four (U87-2; U98-1; U98-4 and IP01) out of the 9 Bt isolates tested against *A. gemmatalis* larvae presented equal or higher mortality than HD1, HD73 and 4412 strains. Although the mortality of *A. gemmatalis* determined by Bt isolates IP03 and IP06 (respectively 33 % and 51%) were not equivalent to HD1, larvae development was severely reduced when compared to control insects (result not shown). According to others authors, such effect over larval development can consistently reduce plant damage (7,24). Corroborating these observations, diets of larvae treated with IP03 and IP06 were much less consumed than diets given to control larvae (data not shown). Neglectable effects over *A. gemmatalis* larvae were observed when isolates U68-5, U91-1 and U96-3 were assayed.

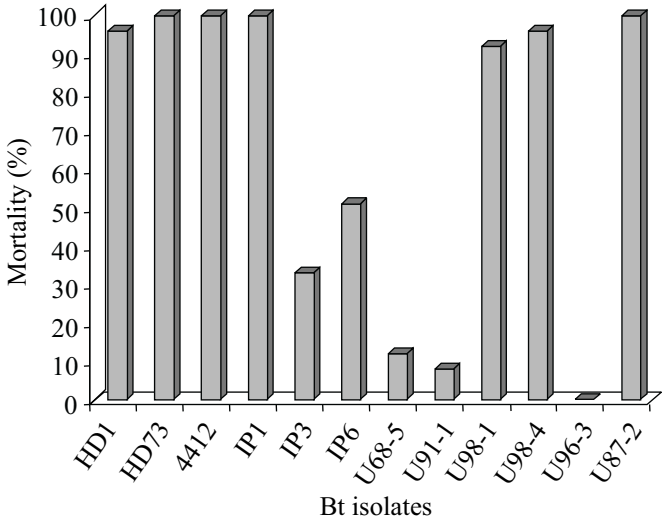

Figure 1. Mortality of *Anticarsia gemmatalis* larvae fed with artificial diets containing different *Bacillus thuringiensis* (Bt) strains: HD1, HD73 and 4412 represent standard Bt strains known to be toxic to *A. gemmatalis*, IP01, IP03 and IP06 are Bt strains from Institut Pasteur, France. New Brazilian isolates are designed by the initial letter U. The mortality was corrected by Abbot's fomula (1).

Cry1 proteins are known to be active against lepidopteran insects. In order to check for their presence in the new Bt isolates active against *A. gemmatalis*, total protein extracts were prepared from liquid bacterial cultures and assayed by SDS-PAGE. After Comassie blue staining, isolates IP01, IP06, U87-2 and U98-4 presented a clear band of around 135 kDa, similar to HD1 (Fig. 2). This is the expected weight for cry1 proteins as described by Schenpf *et al.* (27). Although we could not detect the protein band of 135 kDa with the highly toxic isolate U98-1, all isolates presenting this band demonstrated a mortality of over 90%, suggesting that it may be responsible for the observed activity.

All Bt strains tested in this work were also analyzed for the presence of gene sequences similar to *cry*1. To do so, synthetic oligonucleotides designed by Cerón *et al.* (9) were used in PCR. The designed primers are specific to *cry*1 genes, resulting in the amplification of a DNA fragment of around 280 bp after PCR. Results are illustrated in Fig. 3. Fragments with sizes ranging from 270 bp to 290 bp were observed when DNA from standard strains HD1, HD73 and 4412 (9) and from Bt isolates U87-2, U98-4, IP01 and IP06 were assayed. Isolates IP03 and U98-1 presented similar patterns of DNA amplification with two common DNA bands of approximately 900bp and 200 bp. An extra amplification product of around 1000 bp could also be visualized with the IP03 isolate. No amplification products could be detected after PCR using DNA from isolates U68-5, U91-1 and U96-3. The primers CJI-1 and CJI-2 used in the PCRs help to certify the presence of *cry*1 genes despite their classification (8). Bt *kurstaki* HD1 is known to present genes *cry*1Aa, *cry*1Ab and *cry*1Ac; Bt *kurstaki* HD73 harbours the *cry*1Ac gene; and the

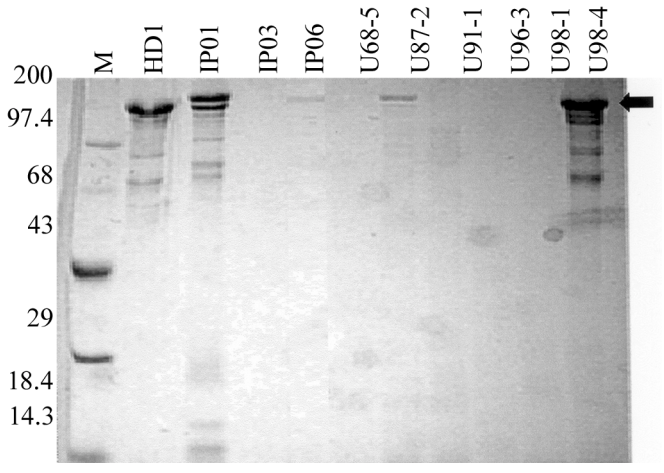

Figure 2. SDS-PAGE (10%) showing proteins from suspension spore-crystal by different *Bacillus thuringiensis* (Bt) isolates. M, molecular weight marker (kDa); HD1 represent standard Bt strains known to be toxic to *Anticarsia gemmatalis*; IP01, IP03 and IP06 are Bt isolates from Institut Pasteur, France; new Brazilian isolates are designed by the initial letter U. Protein band of expected size, around 130 kDa at 140 kDa, is indicated by an arrow.

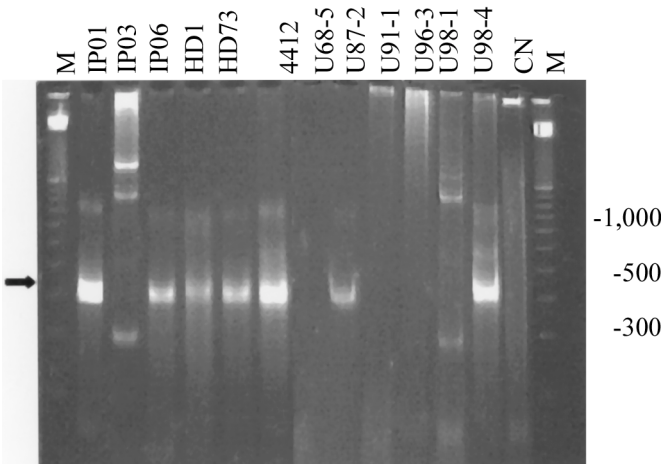

Figure 3. Detection of *cry*1 gene sequences in DNA from different Bt isolates. A volume consisting of 10% of each PCR reaction was applied in a polyacrilamide gel, electrophoresed and stained with ethidium bromide. HD1, HD73 and 4412 represent standard Bt strains known to be toxic to *Anticarsia gemmatalis*. IP01, IP03 and IP06 are Bt isolates from Institut Pasteur, France. New Brazilian isolates are designed by the initial letter U. Fragment of expected size, around 280 bp is indicated by an arrow. M indicates the 100 bp ladder (Boehringer-Mannheim).

strain 4412 contains the *cry*1B gene (11,19). The PCR strategy used in this work confirmed the results obtained by Cerón *et al.* (9) and is a proof for the proposed usefulness of the designed primers. All three standard strains showed single amplification products ranging from 272 bp to 292 bp after PCR with the proposed primers. Although the absence of the 270-290 bp fragment does not prove the inexistence of *cry*1 genes, our positive PCR results with U87-2, U98-4, IP01 and IP06 strongly indicate that these isolates harbor sequences similar to *cry*1 genes. Similar results were obtain to strains very effective against *Spodoptera frugiperda* using these primer, all strain present positive results for *cry*1 genes (28). Sequencing of the amplified products and cross-hybridizations are underway to confirm this conclusion.

Once again, the amplification of the 270 bp to 290 bp DNA products after PCR may be correlated with the toxicity of the Bt isolates found in our bioassays. With the exception of U98-1, all isolates demonstrating activities superior to 90% against *A. gemmatalis* larvae clearly presented this PCR product. Absence of this band for isolates U68-5, U91-1 and U96-1 correlates with their absent or low toxic effect against larvae of *A. gemmatalis*. Bt isolates containing genes type *cry*1Aa, *cry*1Ab and *cry*1Ac independently or in combination were more efficient in bioassays against *Spodoptera* sp. and *Manduca sexta* than isolates containing either *cry*1C or *cry*1D (10). Since the primers used in the PCRs cannot distinguish among *cry*1 genes, the presence of different *cry*1 types in the Bt isolates tested may explain their toxicity differences.

Bt serovar *kurstaki* HD1 is being used as a standard strain in most of the pathogenicity tests developed over lepidopteran species (6,24,28). Considering the HD1 toxic activity described against lepidopteran insects like *Plutella xylostella* (28), *Spodoptera* sp., *Diatrea* sp., *Helicoverpa* sp. (6) and *Anticarsia* sp. (24; this work), the highly active Bt isolates here presented are very promising for new insecticide formulations against *A. gemmatalis*. More exciting, the results of gene amplification and protein profile here described for the new isolates strongly point them as good sources of genes for the generation of transgenic plants resistant to insect pests.

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RESUMO

Detecção de genes *cry*1 em isolados de *Bacillus thuringiensis* do sul do Brasil e sua atividade contra *Anticarsia gemmatalis* (Lepidoptera:Noctuidae)

A bactéria *Bacillus thuringiensis* (Bt) é caracterizada pela sua habilidade de produção de corpos paraesporais durante a esporulação. As proteínas cry1 têm atividade inseticida e são altamente específicas para certos insetos e não são tóxicas para outros insetos, plantas e vertebrados. Neste trabalho, a patogenicidade de doze isolados foram testados contra *Anticarsia gemmatalis*, um dos mais importantes insetos pragas da cultura da soja. Para tanto, foi aplicada sobre a superfície da dieta uma suspensão de esporo-cristal e a mortalidade de lagartas de *Anticarsia gemmatalis* foi avaliada sete dias após a aplicação. Quando comparado com uma linhagem de Bt controle, conhecido pela sua alta toxicidade para lagartas da soja, quatro novos isolados exibiram atividade tóxica superior, acima de 90% de mortalidade. Foi utilizada a técnica de PCR para amplificar fragmentos de DNA de regiões codificantes de genes *cry*1. Os isolados de Bt com alta mortalidade produziram produtos de PCR de tamanho esperado, em torno de 280 pb, isolados não tóxicos ou pouco tóxico não produziram qualquer produto de PCR ou mostraram fragmentos amplificados com padrões diferentes do esperado. Os isolados de Bt com alta atividade contra a lagarta da soja mostraram a presença de proteínas com tamanho de aproximadamente 150 kDa, quando o extrato protéico total foi analisado em SDS-PAGE.

Palavras-chave: *Bacillus thuringiensis*, lagarta da soja, *Anticarsia gemmatalis*, gene *cry*1, δ-endotoxina

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