Screening of *Bacillus thuringiensis* strains effective against mosquitoes

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Abstract – The objective of this work was to evaluate 210 *Bacillus thuringiensis* strains against *Aedes aegypti* and *Culex quinquefasciatus* larvae to select the most effective. These strains were isolated from different regions of Brazil and are stored in a *Bacillus* spp. collection at Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil. The selected strains were characterized by morphological (microscopy), biochemical (SDS-PAGE 10%) and molecular (PCR) methods. Six *B. thuringiensis* strains were identified as mosquito-toxic after the selective bioassays. None of the strains produced the expected PCR products for detection of *cry4*, *cry11* and *cyt1A* genes. These results indicate that the activity of mosquitocidal Brazilian strains are not related with Cry4, Cry11 or Cyt proteins, so they could be used as an alternative bioinsecticide against mosquitoes.

Index terms: *Culex quinquefasciatus*, *Aedes aegypti*, bioinsecticide.

Prospecção de estirpes de *Bacillus thuringiensis* efetivas contra mosquitos

Resumo – Neste trabalho foram realizados testes de patogenicidade com 210 estirpes de *Bacillus thuringiensis* contra larvas de *Aedes aegypti* e *Culex quinquefasciatus*, a fim de se determinar as mais eficazes. Estas estirpes foram isoladas de diversas regiões do Brasil e estão armazenadas na coleção de *Bacillus* spp. da Embrapa Recursos Genéticos e Biotecnologia. As estirpes selecionadas foram caracterizadas por métodos morfológicos (microscopia), bioquímicos (SDS-PAGE 10%) e moleculares (Reação em Cadeia da Polimerase). Foram selecionadas seis estirpes entomopatogênicas de *Bacillus thuringiensis*. Nenhuma das estirpes de *Bacillus thuringiensis* apresentou produtos de PCR esperados para a detecção dos genes *cry4*, *cry11* e *cyt1A*. A patogenicidade das estirpes não está associada à presença das toxinas Cry4, Cry11 ou Cyt, assim, essas estirpes poderão ser utilizadas para a formatação de um bioinseticida alternativo contra mosquitos.

Termos para indexação: *Culex quinquefasciatus*, *Aedes aegypti*, bioinseticida.

Introduction

In Brazil, some mosquito species of the family Culicidae and Blackflies (Simulidae) are considerable vectors of human and animal diseases. For example, *Aedes aegypti* was responsible for more than 750,000 occurences of dengue fever in Brazil during the summer of 2001/2002 (Vilarinhos, 2002).

Among the strategies used to control the endemic vectors, the World Health Organization recommends the use of bioinsecticides based on *Bacillus thuringiensis* (WORLD HEALTH ORGANIZATION, 1985). The advantages in using this bacterium are its specificity, the absence of pollution and of toxicity to mammals, other vertebrates or against plants (Whiteley & Schnepf, 1986). This bacterium exhibits high genetic variability,

producing more than 40 different classes of toxins against insects of the orders Diptera, Lepidoptera and Coleoptera (Feitelson et al., 1992). Researchers in several parts of the world are looking for new strains of *B. thuringiensis* that are able to produce new toxins.

Products based on this bacterium have been commercialized around the world for more than 50 years. At present, in Brazil, there has been no commercial production of bioinsecticides based on this bacterium, so that the products currently used are expensive, in some cases preventing its utilization.

Embrapa Recursos Genéticos e Biotecnologia maintains a culture collection of entomopathogenic *Bacillus* spp. in which around 1,400 *B. thuringiensis* (Bt) strains are stored (Monnerat et al., 2001).

The aim of this work was to characterize the *B. thuringiensis* strains of the Embrapa culture collection and to identify strains that could be used to control *A. aegypti* and *Culex quinquefasciatus*.

Material and Methods

Two hundred and ten *B. thuringiensis* strains stored in the collection of Entomopathogenic *Bacillus* spp. of Embrapa Recursos Genéticos e Biotecnologia were used. These strains were originally isolated from water and soil samples collected from different Brazilian regions (Monnerat et al., 2001).

Two kinds of bioassays were performed to determine the biological activity of the strains, a selective bioassay to determine the pathogenic activity of the strain and a quantified bioassay to determine the virulence of the strains through determination of 50% lethal concentration (LC_{50}).

All strains were grown in NYSM medium (Yousten, 1984) for 48 hours at 28°C and 200 rpm and tested against 3rd instar larvae of *C. quinquefasciatus* and *A. aegypti*. One mL of total culture of each strain was added to 200 mL cups in triplicate with 100 mL of distilled water and 25 larvae of *C. quinquefasciatus* or *A. aegypti*. One cup without bacteria was used as the control. Forty-eight hours later, the number of dead larvae was evaluated. The strains that killed more than 50% of the larvae were considered pathogenic (Silva-Werneck & Monnerat, 2001).

In order to determine the LC_{50}, several dilutions of the lyophilised final culture prepared as described above were used. One mL of these dilutions was added into 200 mL cups in triplicate, as for the procedure used in selective bioassays. Forty-eight hours later the numbers of dead larvae were recorded and the LC_{50} (lethal concentration necessary to kill 50% of larvae) was calculated by Probit analysis (Finney, 1971). *Bacillus thuringiensis israelensis* (IPS-82, from the Pasteur Institute) was used as standard.

For the analysis of protein profile, spore-crystal mixtures of the *B. thuringiensis* strains and the standard strain HD-1 were prepared by a rapid washing procedure (Lecadet et al., 1992). Samples (1.5 mL) of sporulated cultures were centrifuged at 11,750 *g* in a microcentrifuge for 20 minutes and washed once in 1.5 mL of 0.5 M NaCl and twice in cold sterilized water containing 1.0 mM of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF). The pellets were resuspended in 250 μL of

1.0 mM PMSF and kept frozen at -20°C. The protein composition of the spore-crystal mixtures was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with 10% acrylamide. Fifteen μL of the prepared sample was separated by electrophoresis at 125 V for two hours. *Bacillus thuringiensis israelensis* (IPS-82, from the Pasteur Institute) was used as standard.

The method used for analysis of the presence of *B. thuringiensis* toxin genes by polymerase chain reaction (PCR) was described by Bravo et al. (1998). *B. thuringiensis* strains were grown on NYSM agar for 16 hours, at 30°C. Cells were resuspended in MilliQ water and frozen at -80°C for one hour and then transferred to boiling water for 10 minutes to lyse the cells. Primers designed for detecting genes *cry4*, *cry11* and *cyt1A* were used. Fifteen μL of supernatant obtained from cell lysates of the *B. thuringiensis* strains were transferred to a 200-μL reaction tube containing 0.5 μM of each primer, 0.2 mM of each dNTP, 1x *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase in a final volume of 50 μL. PCR amplification was performed with a Programmable Thermal Controller. The conditions used for the PCRs were those described by Carozzi et al. (1991) (*cry4*) and Bravo et al. (1998) (*cry11* and *cyt*). After amplification, a 15-μL sample of the product from each PCR reaction was subjected to electrophoresis in a 2% (w/v) agarose gel in Tris-borate buffer at 100 V for one hour and stained with ethidium bromide.

Crystalline inclusions of each strain were purified from the sporulated culture by centrifugation in discontinuous sucrose gradient (Thomas & Ellar, 1983). Each strain was grown on five nutrient agar plates, at 30°C, for 48 hours. The culture was collected in 20 mL milliQ water and centrifuged at 12,800 *g* for 10 minutes at 15°C. The pellet was washed three times in 0.1 mM of the protease inhibitor PMSF, 0.01% Triton X100. The final pellet was resuspended in 2–5 mL of the following buffer: 0.1% Triton X100, Tris-HCl 0.05 M (pH 8.0), 0.01 M NaCl, and sonicated three times for one minute. The spore-crystal mixture was layered on top of a 28 mL discontinuous sucrose gradient, comprising 7 mL each of 67%, 72%, 79% and 84% (w/v) sucrose in 0.01% Triton X100, Tris-HCl 0.05 M (pH 8.0), 0.01 M NaCl. Centrifugation was carried out in an ultracentrifuge, in an AH627 rotor at 47,800 *g* for 30 min at 15°C. The bands were collected from the interfaces, washed once in the same volume of 0.01% Triton X100 by

centrifugation at 12,800 *g* for 10 minutes, at 15°C, and twice in 1 mL of this solution. The final pellet was resuspended in 500 μL of 0.1 μM PMSF, the crystals observed by phase-contrast microscopy and stored at -20°C.

The crystalline inclusions of strains were lyophilized and deposited under a metallic support. The samples were covered with gold for 180 seconds, using sputter EMITECH model K550 and observed in a scanning microscope.

Results and Discussion

Six *B. thuringiensis* strains were identified as mosquito toxic in the selective bioassays. Four of them showed toxicity against *A. aegypti* and two against both *A. aegypti* and *C. quinquefasciatus* (Table 1).

Even though strains were pathogenic, none of them showed lower LC_{50} than *B. thuringiensis israelensis* against *A. aegypti*. These Brazilian pathogenic strains to *A. aegypti* presented similar LC_{50}, with S479 being the most effective among them (Table 1). Between the pathogenic strains to *C. quinquefasciatus*, S550 was as toxic as *B. thuringiensis israelensis*, while S447 showed a lower efficiency (Table 1).

All strains presented different protein profiles (Figure 1). However, in all cases there is a major protein around 130 kDa, a size similar to that of the Cry4 proteins, encoded by *B. thuringiensis israelensis*. On the other hand, none of the Brazilian strains produced the expected PCR products for *cry4* genes whereas the control strain Bti IPS82 produced the 797 bp amplicon. Strains S447, S479, S685 and S1255 also produced a protein around 70 kDa, a similar size to the Cry11 protein (Figure 1), but again none of the Brazilian strains yielded the 305 bp PCR amplicon that was produced by Bti using the *cry11* specific primers. The same happened when Cyt proteins were analyzed, the strains S447 and S479 exhibited a 30 kDa protein (Figure 1) but the expected PCR product for *cyt* gene (525 bp) was not amplified. These results indicated that the activity of mosquitocidal Brazilian strains is not related with Cry4, Cry11 or Cyt proteins.

Morphological analysis by scanning microscopy showed that all strains produce round crystals. Strains S447, S479, S550 and 1255 exhibited round crystals with a size similar to Bti crystals (Figure 2). All strains except S1255 showed also a round crystal smaller and different from Bti crystals. It is likely that mosquitocidal activity of these strains is linked to the presence of round crystals.

Further studies are needed to characterize the proteins detected in this work, however, as the toxins produced by all strains appear to be distinct from the toxins of Bti, these strains could be used as an alternative in programs of mosquito control.

Table 1. LC_{50} (μg/mL) of the most toxic *Bacillus thuringiensis* strains identified after selective bioassays against *Aedes aegypti* and *Culex quinquefasciatus*.

Strains	LC_{50}	Fiducial limits
Aedes aegypti		
S479	6.8	2.73–29.6
S1255	9.8	4.24–11.5
S550	15	4.31–96.1
S447	20	4.08–84.2
S285	24	6.61–181
S685	42	9.72–503
IPS82-(Bti)	1.1	0.82–1.43
Culex quinquefasciatus		
S550	0.073	0.048–0.11
S447	0.089	0.069–0.12
IPS82-(Bti)	0.034	0.020–0.065
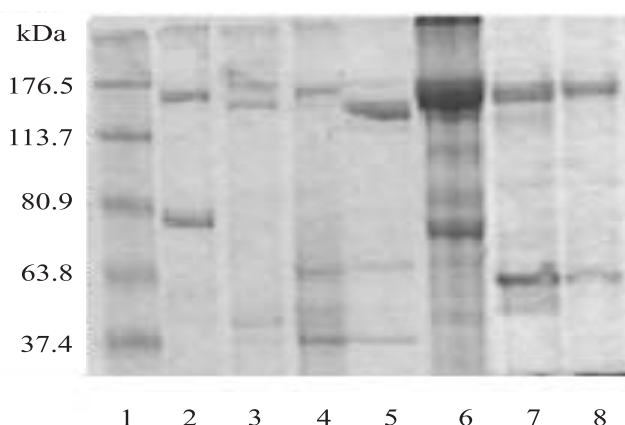
Figure 1. SDS-PAGE of spore-crystal from *Bacillus thuringiensis* strains. 1: Molecular marker Gibco BRL; 2: Bti; 3: S285; 4: S447; 5: S479; 6: S550; 7: S685; 8: S1255.
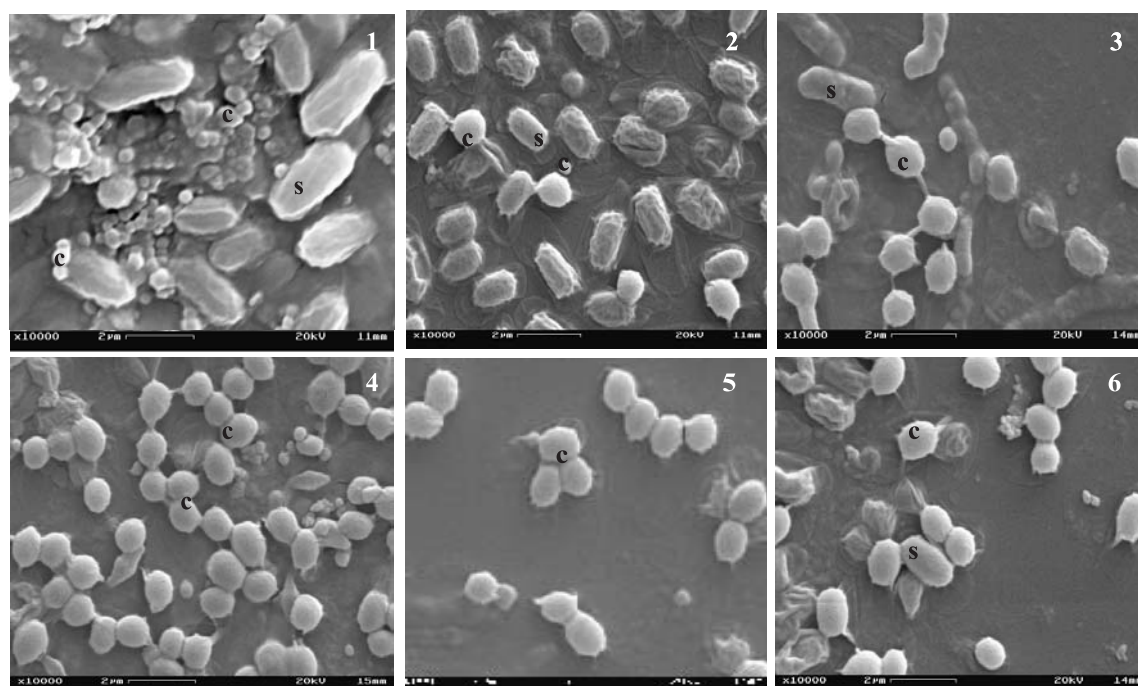
Figure 2. Scanning electron microscopy of spore (s) and crystals (c) of strains S285 (1); S447 (2); S479 (3); S550 (4); S1255 (5); and Bti (6).

Conclusion

Among 210 *Bacillus thuringiensis* strains of the Collection of Entomopathogenic *Bacillus* spp. of Embrapa Recursos Genéticos e Biotecnologia, six are toxic to *Aedes aegypti* and *Culex quinquefasciatus* larvae.

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