Comparative Toxicity of *Bacillus thuringiensis* Berliner Strains to Larvae of Simuliidae (Insecta: Diptera)

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Abstract Blackflies (*Simulium* spp.) are widely distributed and can cause serious economic losses causing annoyance to humans and other animals and damage to health, agriculture and the tourist industry. In addition, they are vectors of diseases eg. onchocerciasis. One alternative to control this insect is the use of biopesticides based on *Bacillus thuringiensis* (*Bt*) serotype israelensis but other dipteran-active serotypes have not been tested against these insects. This study assessed the toxicity of standard *Bt* strains belonging to serotypes *israelensis*, *medellin* and *jegathesan*, known to kill dipteran insects, along a collection of *Bt* strains from Amazonia (a blackfly-endemic area) to find the most effective strains for *Simulium* control. Ninety-six strains were isolated from soil collected in the Amazon and two of these showed high toxicity against larvae of *Simulium* spp. The biochemical and molecular characterization of the two strains showed that both produce proteins with molecular weights of 130 kDa, 72 kDa and 30 kDa that are comparable with proteins Cry4A and Cry4B (130 kDa), Cry10 and Cry11 (72 kDa) and Cyt1 and Cyt2 (30 kDa) and corresponding genes *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1* and *cyt2*, similar to *Bt israelensis*, used as standard. These strains also showed similar toxicity to *Bt israelensis* and higher toxicity than *Bt medellin* and *jegathesan* against blackflies.

Keywords Biological control; Bioinsecticide; Blackflies; *Bacillus thuringiensis*

Background

Blackflies, *Simulium* spp. (Diptera: Simuliidae) have a broad geographical distribution and their immature stages inhabit almost all types of lentic and lotic aquatic environments (Adler, 1994). They have great importance in medical-veterinary health, as they are vectors of filariae such as *Mansonella ozzardi* (Manson) and *Onchocerca volvulus* Leuckart (Marcondes, 2001). When in high density, some species cause serious economic losses since blood feeding females annoy humans and other animals causing damage to health, agricultural productivity and tourism (Araújo-Coutinho et al., 1988; Adler and Mason, 1997) and producing serious economic impacts, eg in Brazil (Mardini et al., 2000; Regis et al., 2000). The control of blackfly larvae has been accomplished by the use of synthetic pesticides or biolarvicides (Mardini et al., 2000) but many populations have developed resistance to the chemical insecticides used (Mardini et al., 2000; Campos and Andrade, 2002). However, the use of biolarvicides based on *Bacillus thuringiensis israelensis*, is able to reduce the population of blackflies in these regions (Mardini et al., 2000).

B. thuringiensis (*Bt*) has a wide distribution, and is found in terrestrial and aquatic environments worldwide (Martin and Travers, 1989). Serotype H-14 (*B. thuringiensis israelensis*) was isolated from samples collected in breeding sites in Israel and produces a protein crystal that is toxic to some species of Diptera (de Barjac, 1978). *B. thuringiensis* serotype H-14 has been used in control programs in West Africa and South America with excellent results (Regis et al., 2000).

Despite the good prospects for the control of blackfly, there are few commercial products and the high cost of importation may limit their use in biological control programs. To facilitate local production of a bioinsecticide for production and use against *Simulium* in Brazil, this study investigated the activity of standard strains and new isolates of *Bt* from Brazil to identify the most effective strains for control. Accordingly, the present study aimed to isolate and characterize *B. thuringiensis* strains toxic to *Simulium* spp. from the blackfly-endemic Amazon region, which is known for its high biodiversity, and to compare their toxicity with strains from serotypes *israelensis*, *medellin*, *jegathesan*, known to be toxic to dipteran larvae.

1 Results and Discussion

From the 200 soil samples tested, 96 strains of *B. thuringiensis* were isolated. These strains were named and deposited in the Collection of Bacteria of Invertebrates belonging to EMBRAPA Genetic Resources and Biotechnology. Due to the limitations of culturing *Simulium* on a large scale, selective assays to identify dipteran-active strains from the collection were carried out using the larvae of the mosquitoes *Aedes aegypti* and *Culex quinque-fasciatus*. Among the 96 strains from Amazonia, only two, S2271 and S2272 caused 100% mortality in selective bioassays and were selected for use in further assays against blackflies. The other 94 caused less than 50% mortality.

In taxonomic analysis of the larvae of *Simulium* spp. collected from the field for bioassay, over 90% of the specimens were identified as *S. perflavum* Roubaud.

Ten *B. thuringiensis* strains were tested against *Simulium*: the two dipteran active Amazonia strains above; a number of other Brazilian strains from the EMBRAPA collection representing different *Bt* serotypes; and three standard strains. Of these 10 strains, 6 caused 100% mortality of larvae of *S. perflavum* in selective bioassays (S2271 and S2272 from Amazon and S1785 (from EMBRAPA) and *Bt medellin* 163-0131 (from Colombia), and *Bt jegathesan* T28 A001 and *Bt israelensis* T14001 (from Institute Pasteur). Strains belonging to serotypes

aizawai (S1576), *kurstaki* (S1905 and S1450) and *tenebrionis* (S1122) showed no activity against the blackfly larvae. The 6 toxic strains were then subjected to further bioassay, to determine the lethal concentration that kills 50% of the target population (LC_{50}). Strains S1785, S2271 and *Bti* were the most toxic, presenting LC_{50} values between 9.5 mg/mL and 15.1 mg/mL. The strain S2272 showed an LC_{50} of 18.3 mg/mL, statistically different from the first group, and *Bt jegathesan* and *Bt medellin* were grouped in a third group of toxicity (lower) showing LC_{50} values of 89.1 mg/mL and 108.2 mg/mL, that were statistically indistinguishable, but low compared with the most toxic group (Table 1).

Table 1 Characterization of *Bt* strains toxic to *Simulium* spp. larvae: Serotype and LC_{50} (mg/mL)

Strains	Serotype	LC_{50} (mg/mL)
S2271	*israelensis*	9.5 (7.1~12.4)
S1785	*israelensis*	10.5 (7.2~12.6)
Bti T14001	*israelensis*	15.1 (10.3~21.8)
S2272	auto-agglutinant	18.3 (13.1~26.4)
Bt 163-0131	*medellin*	89.1 (72.2~106.3)
Bt T28 A001	*jegathesan*	108.2 (81.2~136.5)

The results of serological characterization showed that one of the Amazon strains belongs to serovar *israelensis* (S2271) and the other (S2272) is auto-agglutinating (Table 1). Strains S1785, S2271 and S2272 showed a protein profile similar to that of the standard *Bti* T14001 strain presenting proteins of molecular weight around 130 kDa, 72 kDa and 30 kDa, consistent with *Bti*-like proteins Cry4A and Cry4B (130 kDa), Cry10 and Cry11 (72 kDa) and Cyt1 and Cyt2 (30 kDa) (Figure 1). All these proteins are effective against dipteran larvae (Monnerat and Praça 2006). The protein profiles of the strains belonging to serotype *medellin* and *jegathesan* were distinct from those of serovar *israelensis*.

The two Amazon strains (S2271 and S2272) presented PCR products of the expected sizes when amplified with probes for *cry4A*, *cry4B*, *cry11*, *cyt1* and *cyt2* genes. These results complement the protein profile obtained for the strains showing similarity between these strains and serovar *israelensis*.
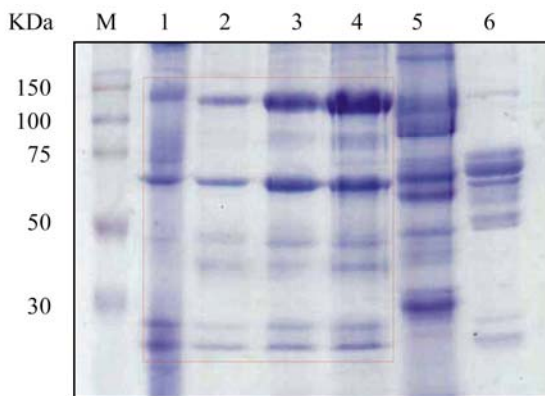
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Figure 1 SDS-PAGE 10% of *Bt* strains toxic to *Simulium* spp. Note: M=molecular marker, 1: *Bti* T 14001, 2: *Bti* S1785, 3: S2271, 4: S2272, 5: *Bt medellin* 163-0131, 6: *Bt jegathesan* T28 A001

According to data available at the website maintained by Dr. Crickmore (Crickmore et al., 2010) strains of *B. thuringiensis* subsp. *israelensis* can express the proteins of approximately 135 kDa (Cry4Aa1, Cry4Aa2, Cry4Aa3); 128 kDa (Cry4Ba1, Cry4Ba2, Cry4Ba3, Cry4Ba4 and Cry4Ba5); 78 kDa (Cry10Aa1, Cry10Aa2 and Cry10Aa3); 72 kDa (Cry11Aa1, Cry11Aa2 and Cry11Aa3); 29 kDa (Cry60Aa2, Cry60Ba2, Cyt1Aa1, Cyt1Aa5, Cyt1Aa2; Cyt1Ca1; Cyt2Ba1, Cyt2Ba2 and Cyt2Ba9). The Cry4 and Cry11 are the most toxic Cry proteins to *A. aegypti* (Chen et al., 2009; Fernandez et al., 2009).

Bt medellin was first isolated in Colombia and presents high toxicity for some species of Culicidae (Orduz et al., 1991; Restrepo et al., 1997). According to Crickmore (2010), strains of serotype *medellin* can express proteins Cry11Bb1 (94 kDa), Cry29Aa1, Cry30Aa1, and Cyt1Ab1 (27 kDa) and Cyt2Bc1 (29.7 kDa).

Bt jegathesan was isolated in Malaysia and also shows toxicity to the larvae of Culicidae (Kawalek et al., 1995; Wirth et al., 2004). According to Crickmore (2010) strains of serotype *jegathesan* may express toxins Cry11Ba1 (81 kDa), Cry24Aa1 (75.9 kDa), Cry25Aa1 (76 kDa), and Cry30Ca2, Cry60Aa1 and Cyt2Bb1 (30 kDa). Work undertaken with *Bt jegathesan* strain 367 and *Bt medellin* strain 163~131 showed that although these strains are very toxic to mosquito larvae, they are 10 times less toxic

than *Bt israelensis* strains in laboratory conditions (Thiery et al., 1999).

In several studies, serovar israelensis is reported to be toxic to larvae of *Simulium* spp. (Myburgh and Nevill, 2003, Petry et al., 2004; Stoops and Adler, 2006), and a non-agglutinating strain of *Bt* has also recently been reported to have high toxicity against these insects (Cavados et al., 2005), however, to date there are no reports of the toxic activity of strains of serotypes *jegathesan* and *medellin* to blackflies.

The results of this study indicate that the Amazonian strains are promising for blackfly control with similar activities to those obtained with the strains of *Bt serovar israelensis*. This study, along with previous work (Cavados et al., 2005) also indicates that some auto-agglutinating strains can have good activity against these insects whereas strains of serotypes *medellin* and *jegathesan* significantly less effective for controlling blackfly larvae.

2 Materials and Methods

2.1 *Bacillus thuringiensis* strains

Strains used in this study were isolated from 200 soil samples from the city of Manaus and its surroundings, in the Amazon region. The isolation of strains was performed at the Laboratory of Entomopathogenic Bacteria of EMBRAPA Genetic Resources and Biotechnology, Brasília-DF/Brazil using the methodology described by the World Health Organization (WHO, 1987).

2.2 Selective bioassays against larvae of *Aedes aegypti* and *Culex quinquefasciatus*

To select the most toxic samples, we first carried out a pre-selection of strains of *B. thuringiensis* from soil of the Amazon by selective bioassay using larvae of *Aedes aegypti* (Linnaeus) and *Culex quinquefasciatus* Say (Diptera Culicidae), maintained at EMBRAPA Genetic Resources and Biotechnology.

Strains were grown in EMBRAPA medium (Monnerat et al., 2007) for 72 hours on shaker at 200 r/min and 28 ℃. After this time, optical microscopy was performed to observe the structures (spores and crystals). The bioassay was performed by placing 1 mL of sporulated culture in 100 mL of water in cups

containing 25 2nd instar larvae of *A. aegypti* or *C. quinquefasciatus*. *B. thuringiensis* subspecies *israelensis* (*Bti*) T14001, provided by the Institute Pasteur, Paris, France, was used as a standard and positive control. The test with larvae of *A. aegypti* was evaluated after 24 hours, and *C. quinquefasciatus* after 48 hours, when the numbers of deaths were determined. The strains that caused 50% mortality or more were considered pathogenic (Monnerat et al., 2005), and submitted to selective and dose bioassays with larvae of *Simulium* spp. Mosquito toxicity was used as predictive of blackly toxicity to restrict the number of strains to be taken forward to the more labour-intensive *Simulium* assay.

2.3 *Simulium* spp. larval bioassay

Insects: The insects were collected daily from a river at an experimental farm belonging to EMBRAPA, located in Brasília (Distrito Federal). The identification of the *Simulium* species collected was based on morphological characteristics of larvae and pupae according to the taxonomic key of Coscarón (1987).

Selective bioassay: For the selective bioassay, we used two Amazon strains that killed more than 50% of the larvae of *C. quinquefasciatus* and *A. aegypti* and 5 strains from the Collection of EMBRAPA Genetic Resources and Biotechnology from different serotypes: *B. thuringiensis israelensis* (S1785), *B. thuringiensis aizawai* (S1576), 2 *B. thuringiensis kurstaki* (S1905 and S1450) and *B. thuringiensis tenebrionis* (S1122). As standards we used *B. thuringiensis jegathesan* T28 A001 and *B. thuringiensis israelensis* T14001 (provided by Institute Pasteur, Paris, France) and *B. thuringiensis medellin* 163-0131 (provided by CIB, Medellin, Colombia). Bacteria were cultivated in EMBRAPA medium (Monnerat et al., 2007) for 72 hours with shaking at 200 rpm and 28℃.

The tests were performed by adding 1 mL of final broth to a 500 mL beaker containing 100 mL of water from the river and 25 larvae of the second instar of *Simulium* spp. The beakers were then placed in a shaking incubator at 130 rpm at 28℃ and the experiments were performed in triplicate. As a negative control, we used only water without the addition of the bacteria. Data for larval mortality were

evaluated 24 hours after the beginning of the test.

Dosage bioassay: Strains that killed 50% or more of total larvae in the bioassays were subjected to selective dose bioassays against larvae of *Simulium* spp. to determine the lethal concentration required to kill 50% of the population tested (LC_{50}). To perform this assay, the strains were grown in EMBRAPA medium as before, then spores were harvested by centrifugation at 12 000 g for 30 min. Then they were frozen overnight, and lyophilized in a Labconco model Lyphlock lyophilizer for 18 h. Final doses ranged from 2.5 mg/mL to 500 mg/mL. The tests were performed in triplicate and a control was left without the addition of the bacteria. Counting the number of dead larvae was performed 24 hours after the start of the test. Mortality data were analyzed using Probit Analysis (Finney, 1971) to determine the LC_{50} (lethal concentration required to kill 50% of larvae tested).

2.4 Characterization of toxic strains

Serological characterization: The Amazon strains were serotyped according to the protocol described by de Barjac and Frachon (1991). Strains S1785, previously serotyped as *Bt israelensis*, and the *Bti* T14001, were used as positive controls.

Characterization of proteins by SDS-PAGE: The biochemical characterization of the strains was performed by protein electrophoresis in a denaturing polyacrylamide gel (SDS-PAGE 10%). Protein extraction was performed according to the protocol described by Lecadet et al. (1991). Samples were denatured at 100℃ for 5 minutes and loaded on a denaturing 10% polyacrylamide SDS-PAGE gel. The gel was stained in a solution of Coomassie Blue (40% methanol, 10% acetic acid and 25% blue Coomassie 250R) for 16 hours and placed in destain solution (40% methanol and 10% acetic acid) to complete visualization of protein bands.

Molecular characterization: The selected strains were characterized for the presence of genes encoding Cry proteins by PCR (polymerase chain reaction) using primers specific for detection of *cyt1*, *cyt2*, *cry4A*, *cry4B*, *cry10* and *cry11* genes (Bravo et al., 1998; Ibarra et al., 2003). DNA extraction was carried out as

described by Sambrook et al. (1989). PCR reactions were performed in a MJ Research, Inc. (PTC-100TM) thermal cycler and used 10 µL template DNA from each sample 8.0 µM of each specific primer, 5 mM dNTP mix, 10× Taq buffer and 2.5 U Taq DNA polymerase in a reaction of 50 µL. The amplification conditions were described by Bravo et al. (1998) and Ibarra et al. (2003). After amplification, 20 µL of each PCR product was loaded on a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light. The *Bti* strain T14001 was used as standard.

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