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ORIGINAL RESEARCH PAPER

Characterization of native *Bacillus thuringiensis* strains and selection of an isolate active against *Spodoptera frugiperda* and *Peridroma saucia*

Analía Álvarez · Eduardo G. Virla · Licia M. Pera · Mario D. Baigorí

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Abstract Twelve *Bacillus thuringiensis* (*Bt*) strains, isolated from larvae and soil samples in Argentina, were molecularly and phenotypically characterized and their insecticidal activities against *Spodoptera frugiperda* and *Peridroma saucia* were determined. One isolate—*Bt* RT—produced more than 93% mortality on first instar larvae of both species, which was higher than that produced by the reference strain *Bt* 4D1. *Bt* RT carried a different *cry* gene profile than *Bt* 4D1. Scanning electron microscopy showed the presence of bipyramidal and cuboidal crystals. Phenotypic characterization revealed lytic enzymes that could contribute to *Bt* pathogenicity.

Keywords *Bacillus thuringiensis* · Biological activity · *Peridroma saucia* · Phenotypic properties · *Spodoptera frugiperda*

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A. Álvarez (✉)
Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, Miguel Lillo 205, T4001 MVB Tucumán, Argentina
e-mail: alvanalia@gmail.com

E. G. Virla · L. M. Pera · M. D. Baigorí
PROIMI-CONICET, Av. Belgrano y Pasaje Caseros, T4001 MVB Tucumán, Argentina

Introduction

Bacillus thuringiensis is a spore-forming bacterium well-known for its insecticidal properties due to its ability to produce crystal inclusions during sporulation. These inclusions are proteins encoded by *cry* genes and have shown to be toxic to a variety of insects and other organisms like nematodes and protozoa (Konecka et al. 2006). The activity spectrum of *Bt* toxins is continually increasing as the result of the ongoing isolation of new strains around the world. The diversity of toxins produced by *Bt* allows the formulation of a variety of bioinsecticides by using the bacteria themselves or by expressing their toxin genes in transgenic plants.

The fall armyworm, *Spodoptera frugiperda* (*Sf*) (Lepidoptera: Noctuidae), and the variegated cutworm, *Peridroma saucia* (*Ps*) (Lepidoptera: Noctuidae), are two agricultural pests that cause severe damage to a variety of crops. While the first one mainly attacks corn, rice, peanuts, cotton, soybeans, alfalfa and forage grasses, the second one targets peanuts, sunflowers, soybeans and grapevines, among others. Currently, control of both insects relies on chemical insecticides. The rapid increase in resistance to insecticides together with the potential adverse environmental effects produced by these chemicals has encouraged the development of alternative methods for Lepidoptera control. Among these methods the use of *Bt* as a biocontrol agent has been shown to be extremely valuable. However, due to the

extensive use of transgenic crops in developing countries (ca. 17.1 million ha; James 2005) it has become necessary to find new strains to meet the challenge of novel insect resistance. In addition, there is a need to develop knowledge about the biological properties and diversity of *Bt* strains. Phenotypical properties provide useful information for typing and comparative studies (Konecka et al. 2006) and these data allow a better understanding of the biological factors that determine insecticidal properties. Extracellular factors such as phospholipases, proteases and chitinases have shown to contribute to insecticidal activity by *Bt* (Soberon and Bravo 2001).

The current study shows a screening of 490 samples from 40 different agricultural environments in Argentina for *Bt* strains that exhibited toxicity against *Sf* and *Ps* larvae. Twelve *Bt* strains were isolated and phenotypically, genetically and biologically characterized. Analysis of larvicidal activity indicated that one of the strains (*Bt* RT) exhibited high toxicity against both *Sf* and *Ps* larvae; this toxicity was higher than that of the reference strain *Bt* 4D1.

Materials and methods

Microorganisms

Bacillus thuringiensis var. *kurstaki* 4D1 was used as reference strain and provided by the Bacillus Genetic Stock Center (BGSC), Columbus, Ohio, USA. Bacteria were isolated from larvae showing disease symptoms and soil samples collected from 40 sites in northwestern Argentina, including cultivated fields, wild grasslands and subtropical rain forests. Suspensions were made of all samples, heated at 80°C for 15 min, plated onto Luria–Bertani (LB) agar and incubated at 30 or 55°C for 24 h. Colonies that did not grow at 55°C were analyzed for parasporal crystals by microscopic examination (Sharif and Alaeddinoğlu 1988). Single colonies that presented parasporal bodies were selected for further studies and were characterized by conventional microbiological methods (Supplementary Table 1).

Bioassays

All experiments were conducted in a climate-controlled room at 25 ± 0.5°C, with a photoperiod of 14/10 (light/dark) and 70 ± 15% relative humidity. Data were recorded hourly by a data logger. Insecticidal activity was tested against *Spodoptera frugiperda* and *Peridroma saucia* grown on an artificial diet (Osores et al. 1982) previously soaked in a spore-crystal suspension. Bacterial inocula were prepared by suspending in sterilized water a 5 day culture ($OD_{610} = 2$, ca. 6×10^7 c.f.u. ml^{-1}) grown on LB agar at 30°C. Ten replicates of 10 neonate larvae were held in separate 2 ml Eppendorf vials. Each larva was supply with a piece of artificial diet (approximately 0.25 cm^2) immersed in a spore-crystal suspension of *Bt*. All *Bt* strains contained similar amount of crystals per field (×100). Control groups were fed with a similar piece of artificial diet but immersed in distilled water. Mortality was scored every 24 h during 7 days. The 50% lethal time (LT_{50}) was determined by Probit analysis.

PCR *cry* gene analysis

Strains were characterized in terms of presence of *cry1* and *cry2* genes by amplification with general primers (Bravo et al. 1998; Ibarra et al. 2003). The most pathogenic native *Bt* strain was characterized through additional PCR with specific primers to identify the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry2Aa*, *cry2Ab*, *cry2Ac*, *cry9A*, *cry9B* and *cry9C* genes (Ben-Dov et al. 1997; Bravo et al. 1998; Ceron et al. 1994). Total DNA was extracted from cells harvested in the mid-growth phase as described by Miller (1972). PCR amplification was performed in 25 µl containing 2.5 µl 10× STR reaction buffer (Promega), 20 ng total DNA, 0.5 µM of each primer and 1 U Taq DNA polymerase (Promega). PCR products were analyzed by electrophoresis in 2% (w/v) agarose gels.

Results

Phenotypic characterization

From 221 colonies of Gram-positive spore-forming bacilli here isolated from 490 samples, 12 were identified as *Bt* by the crystal-stain procedure. All contained bipyramidal crystals. They possessed typical cellular and colonial morphologies, as well as physiological, biochemical and nutritional features

that resembled *Bacillus* spp (Supplementary Table 1 and Fig. 1).

Insecticidal activity

One of the 12 strains analyzed presented high insecticidal activity. This strain, *Bt* RT, was isolated from a dead larva and produced 93 and 100% mortality of *Peridroma saucia* and *Spodoptera frugiperda* neonate larvae, respectively (Table 1; Fig. 1). 100% mortality of *Spodoptera frugiperda* was obtained after 5 days. According to the 50% lethal time (LT50) and the fiducial limit, the spore-crystal suspension of *Bt* RT killed the larvae of both species faster than reference strain *Bt* 4D1 (Table 1). *Bt* RT has been entered in the Bacillus Genetic Stock Center (accession number 4XX3).

Table 1 Comparison of 50% lethal time (LT_{50}) (h) and mortality (%) of first instar larvae of *Spodoptera frugiperda* (*Sf*) and *Peridroma saucia* (*Ps*) between native *Bt* RT and reference strain *Bt* 4D1

Bt strain	[a]Mortality (%) ± SD		[b]LT_{50} (95% fiducial limits)	
	Sf	*Ps*	*Sf*	*Ps*
Bt RT	100 ± 0	93 ± 10	9 (2–16)	7 (2–14)
Bt 4D1	91 ± 7	70 ± 19	38 (28–46)	80 (65–94)

[a] Ten individuals per treatment were observed and each treatment was repeated 10 times

[b] 50% lethal time (LT_{50}) was determined by Probit analysis. Mortality was scored every 24 h during 7 days

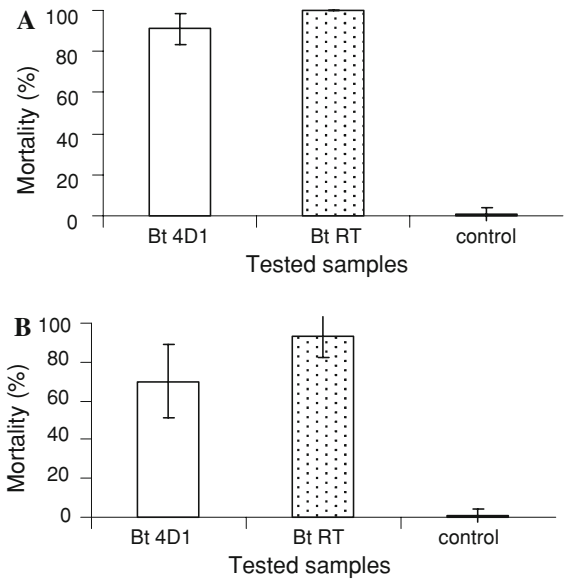

Fig. 1 Comparison of insecticidal activity of *Bt* RT and the reference strain *Bt* 4D1 against the first instar larvae of *Spodoptera frugiperda* (**a**) and *Peridroma saucia* (**b**). Mortality was measured at the 7th day of assay. Ten individuals per treatment were observed and each treatment was repeated 10 times. In both graphics (**a**, **b**) mortality differed significantly between all treatments ($P < 0.05$, Tukey test)

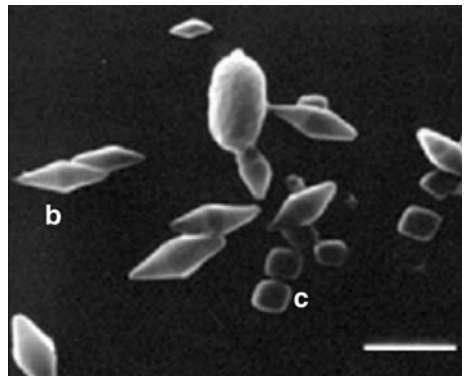

Fig. 2 Scanning electron microscopy (SEM) of spore-crystal proteins from *Bt* RT washed twice with ethanol/water (1:1, v/v). Concentrated spore-crystal suspensions were placed on a microscope lid and air-dried overnight. Samples were then coated with gold and examined using a scanning electron microscope. Both bipyramidal (**b**) and cuboidal (**c**) pesticidal crystal proteins are observed. *Scale bar* = 1 μm

Molecular and microscopic characterization

While PCR analysis showed presence of *cry1* and *cry2* genes in most of the strains, *cry9*C was not amplified in any of them. *Bt* RT was characterized for the presence of additional *cry* genes by PCR analysis (Supplementary Table 1).

Both bipyramidal and cuboidal crystal proteins were present in *Bt* RT (Fig. 2).

Discussion

Phenotypic characterization of the strains permitted identification of properties that are relevant for selecting effective strains for particular agricultural environments. Lytic activity and presence of Cry proteins are two of these properties that are highly important to the effectiveness of *Bt* as a bioinsecticide (Stefanova et al. 1999). Our results indicate that the isolates responded diversely regarding proteolytic, cellulolytic

and/or chitinolytic activity (Supplementary Table 1). Chitinolytic activity is a contributing factor in *Bt* pathogenicity (Soberon and Bravo 2001) which our most pathogenic strain possessed (*Bt* RT).

Although most of the isolates presented similar biochemical and phenotypical characteristics compared with reference strain *Bt* 4D1 (Group A) (Supplementary Fig. 1), they clearly differed in their toxicity to *Spodoptera frugiperda* (*Sf*) and *Peridroma saucia* (*Ps*). *Bt* RT was found to be highly pathogenic, even more than *Bt* 4D1 (Table 1; Fig. 1). This strong biological effect was represented by both a shorter LT_{50} and a higher mortality, which reached 100% in the case of *Sf* after 5 days. This result is extremely relevant considering that *Sf* is believed a pest with low sensitivity to *Bt* toxins (Del Rincón-Castro et al. 2006). In addition, the concentration of the spore-crystal suspension employed in our assays was lower than some commercial *Bt* formulations; while our crystal spore suspensions presented a dose of 10^7 c.f.u. ml^{-1}, *Bt* kurstaki preparations generally present at 10^9 c.f.u. ml^{-1}.

Cry genes are a family of genes associated with the virulence of *Bt* against insects. While *cry1* codes for bipyramidal proteins and is related to toxicity to Lepidoptera (Bravo et al. 1998) *cry2* codes for cuboidal proteins, toxic to Lepidoptera and Diptera (Al-Momani et al. 2002). Our molecular and electron microscopy analyses of *Bt* RT are in agreement with all this evidence, indicating that this highly pathogenic strain has both genes (Supplementary Table 1) and both kinds of proteins (Fig. 2). In contrast, TMAN2 and THM30, strains that also harbor the *cry1* and *cry2* genes (Supplementary Table 1) showed low toxicity against *Sf* and *Ps*. This indicates that the presence of these two genes is not the sole determining factor for the pathogenic activity against both *Sf* and *Ps*. The difference in insecticidal activity can be cause by variations in the amino acid sequences of Cry1 and Cry2 proteins or alternatively, by other factors such as differences in their chitin hydrolysis capacity (Supplementary Table 1). Besides, *cry*-specific PCR showed that *Bt* RT and *Bt* 4D1 had different *cry*-specific contents (Supplementary Table 1), although both strains showed similar pathogenic effects. The high toxicity of *Bt* RT compared to that of *Bt* 4D1 suggests that the *cry1Aa* and *cry2Aa* genes, present in *Bt* 4D1 but not in *Bt* RT, only contribute little to the toxicity against *Sf* and *Ps*.

The discovery of a highly toxic isolate against *Sf* and *Ps* reveals the usefulness of screening studies for novel *Bt* strains. It would be important to optimize the production conditions of *Bt* RT with low-cost substrates.

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