Available online at www.sciencedirect.com
ScienceDirect
Biological Control 40 (2007) 65–68
Biological Control
www.elsevier.com/locate/ybcon

Characterization of *Bacillus thuringiensis* isolates toxic to cotton boll weevil (*Anthonomus grandis*)

Érica Soares Martins [a], Lílian Botelho Praça [a], Vinícius Fiúza Dumas [a], Joseilde O. Silva-Werneck [a], Eduardo Hideki Sone [a], Isabel C. Waga [a], Colin Berry [b], Rose Gomes Monnerat [a,*]

[a] *Embrapa Recursos Genéticos e Biotecnologia, SAIN-Parque Rural, Caixa postal 02372, 70770-900-Brasilia-DF, Brazil*
[b] *Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, Wales, UK*

Received 6 April 2006; accepted 15 September 2006
Available online 27 September 2006

Abstract

The cotton boll weevil (*Anthonomus grandis*) is the major cotton pest in the Americas. One of the alternatives for its control is the utilization of *Bacillus thuringiensis* (*Bt*), an entomopathogenic bacterium characterized by its production of insecticidal crystal proteins. Embrapa Genetic Resources and Biotechnology has a collection of Bacilli in which different isolates of *Bt* are stored. A method for rearing and maintenance of *Anthonomus grandis* on artificial diet in the laboratory was developed, and a robust larval bioassay protocol was established for the selection of *B. thuringiensis* isolates toxic to boll weevil. After preliminary bioassays performed with 215 isolates, 5 were selected that demonstrated a good level of toxicity and these were analyzed in more detail. The most toxic were S601 and S1806 presenting LC_{50} (lethal concentration to kill 50% if the larvae) of 0.14 mg/ml and 0.30 mg/ml, respectively. S601 showed an LC_{50} value that was half that of the standard *B. thuringiensis* subspecies *tenebrionis* and S1806 demonstrated similar values of LC_{50} to the standard. S601 contained the *cry1B* gene and S1806 had the *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1* and *cyt2* genes like *B. thuringiensis* subspecies *israelensis*.

Keywords: *Bacillus thuringiensis*; *Anthonomus grandis*; Biological control; Toxicity; *cry* genes

1. Introduction

Cotton is one of the most financially profitable crops worldwide; in the 1990s its price was higher than those of other important products such as soy, corn and wheat (Ponchio, 2001). In the textile industry, the cotton fiber is recognized as one of the most important and valuable in the market. The extensive plantation of monocultures like soy, corn, cotton, canola and other plants, requires a high investment in insecticides owing to their resident pests, especially from the lepidopteran and coleopteran orders. According to Santos et al. (2003), about US$ 3–5 billion are spent on pesticides per year, and of that US$ 645 million are spent in cotton plantations.

Cotton boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), is a significant pest of cotton production in the Americas (Busoli et al., 1994; Gallo et al., 2002). In order to decrease damage and loss of production, this pest has been controlled primarily by the use of chemical agents. However, while this has produced efficient cotton weevil control (Martin et al., 1987; Wolfenberger et al., 1997), these chemicals are expensive and may cause negative environmental impacts. Other control methods employed are the use of pheromones (Gallo et al., 2002), early budding varieties, gathering and destruction of fallen flower buds (Silvie et al., 2001), biological control (Pallini et al., 2006) and integrated pest management (Luttrell et al., 1994). In addition, there is a possibility of producing transgenic cotton containing a gene or genes to make the plant

* Corresponding author. Fax: +55 61 3448 4672.
E-mail address: rose@cenargen.embrapa.br (R.G. Monnerat).

1049-9644/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2006.09.009

resistant to this insect. Transgenic cotton plants encoding a *Bacillus thuringiensis* Berliner protein (*Bt* cotton) have been adopted by farmers in Mexico and the USA to control caterpillar infestations, and this approach has contributed to a decrease in insecticide applications by 50%. In Argentina, this reduction in insecticide use has reached 65% (Santos et al., 2003).

Bacillus thuringiensis (*Bt*) is an aerobic, Gram-positive bacterium, characterized by the production of protein inclusions, called δ-endotoxin or Cry proteins (Feitelson et al., 1992). To date, more than 300 *cry* genes have been sequenced and the Cry proteins are classified into at least 49 groups organized into subgroups according to the percentage identity of their amino acid sequences (Crickmore et al., 1998). The toxins usually described as active against insects of the order Coleoptera are those of the Cry3 and Cry8 classes (Bravo et al., 1998; Abad et al., 2001), but lately some toxins from Cry1, Cry22, Cry34 and Cry35 classes have been described as toxic to this order as well (Tailor et al., 1992; Baum and Light Mettus, 2000; Baum et al., 2004). Embrapa, Genetic Resources and Biotechnology (Cenargen) has a collection of entomopathogenic *Bacillus* isolates (Monnerat et al., 2001), including isolates of *B. thuringiensis*. The aim of this work was to identify the isolates of *B. thuringiensis* showing toxic activity to cotton boll weevil, which may be used as a source of genes to create a transgenic plant.

2. Materials and methods

Two hundred and fifteen isolates of *B. thuringiensis* were used from a collection of entomopathogenic *Bacillus* isolates of Embrapa Genetic Resources and Biotechnology. These isolates were obtained from soil and water samples from different parts of Brazil and are stored as dry spores at room temperature (25 °C) (Monnerat et al., 2001).

The insects chosen for the bioassays came from the Cenargen laboratory of insect breeding where the mass culture of cotton boll weevil is performed. The insects were maintained in an environmentally controlled room under the following conditions: 25 °C, 60% relative humidity and a photoperiod of 12:12. Groups of up to 500 adults were maintained in a plastic cage with a mesh base (aperture size approximately 1 mm), positioned over a collecting tray into which eggs and feces can fall and accumulate. The adults were fed on an artificial diet comprised of 43.5 g agar (Biobrás, Brazil), 60 g Brewer's yeast, 60 g wheat germ, 40 pharmamedia (Traders Protein, Memphis, Tennessee, USA), 100 g soy protein, 60 g sucrose, 10 g mineral salts (21% $CaCO_4$, 0.039% Cu_2SO_4, 9% $MgSO_4$, 0.009% K_2SO_4, 12% KCl, 31% KH_2PO_4, 0.057% NaF, 14.9% Ca_3PO, 1.47% $FePO_4$, 0.02% $MnSO_4$, 0.009% $Al_2(SO_4)_3$, 0.005 KI, 10.5% NaCl), 20 g ascorbic acid, 2.4 g sorbic acid, 2 g nipagim (Vetec, Rio De Janeiro, Brazil) and 10 ml vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008 riboflavin, 0.004% thiamin HCl, 0.004% pyridoxine HCl, 0.008%) in a total volume of 1.5 l of water. This diet was cut into blocks of $1 \times 3 \times 0.5$ cm, which were placed in the cage on top of a plastic support, approximately 1 cm high. Every 2 days, the eggs were collected, together with the feces, and were placed in a solution of 18% $CuSO_4$. In this solution, the eggs float, whereas the feces sediment. The eggs were then collected and disinfected in 0.3% benzalconium chloride and separated into petri dishes containing the above diet, in groups of approximately 60. The eggs hatched and the larvae grew in these plates to the adult stage, when they were collected and transferred to the cage.

Two kinds of bioassays were carried out: (i) the selective or excluding one, the aim of which was to identify those isolates presenting toxic activity to the insect, that is, any which was capable of killing at least 50% of the larvae tested at a single dose; and (ii) the dose-dependent assay, the purpose of which was to quantify the toxicity of each isolate.

For selective bioassays, bacterial cultures were grown to sporulation in NYSM medium (Yousten, 1984) for 48 h at 28 °C and 200 rpm in a rotating shaker. Ten milliliter of these cultures were added to 35 ml of the above artificial diet at about 50 °C, just before it was poured into petri dishes. As the artificial diet solidified, 15 holes of approximately 2 mm diameter were punched into the surface of each petri dish. One neonate larva was placed in each hole. Each assay comprised four replicates (4 petri dishes) and was repeated four times and one dish was left without bacteria, as a control. Bioassays were assessed after 7 days (Praça et al., 2004).

The isolates that killed more than 50% of the larvae in the selective bioassays were lyophilized after being cultivated for 72 h in NYSM at 28 °C and 200 rpm to carry out the dose dependence bioassays. Cultures (600 ml) were centrifuged at 12,800*g* for 30 min, at 4 °C (BR4i centrifuge Jouan), the cell pellets were frozen for 16 h and lyophilized for 18 h in Labconco model Lyphlock 18 freeze-dryer. Afterwards, the material was weighed for use in the bioassay. The required weight of powder for each dilution was taken up in 5 ml 0.01% Tween 20 to achieve a more homogeneous suspension and this was added to 35 ml of the artificial diet before it was poured out into petri dishes and was punched with 48 holes. Each hole received a neonate larva. During the experiment, five concentrations (from 0.10 to 1.5 mg/ml) were tested and one bacteria free control was included. The bioassay was kept in an incubator with photoperiod of 14:10 at 27 °C. A week later, the bioassay was read and the LC_{50} was determined through probit analysis (Finney, 1971). *B. thuringiensis* subsp. *tenebrionis* T08 017 was obtained from the Collection of *Bacillus thuringiensis* and *Bacillus sphaericus* at the Institut Pasteur, Paris and was used as the reference of coleopteran-active isolate (Hofte et al., 1987). The bioassay was repeated three times and the LC_{50} were compared by ANOVA through Sigmastat program (Kuo et al., 1992).

The protein profiles of the isolates that showed the best toxicity were analyzed by SDS–PAGE in 10% acrylamide gels. The proteins were extracted from the culture according

to Lecadet et al. (1991) and 15 µl of each sample was loaded on the gel. After electrophoresis, the gel was stained and fixed in 40% methanol, 10% acetic acid and Coomassie blue (0.1%) for about 16 h, under slight shaking and destained in 40% methanol and 10% acetic acid for 2 h with agitation. *B. thuringiensis* subsp. *tenebrionis* T08017 was again used as the reference isolate.

The molecular characterization was performed through PCR to identify the toxin-coding genes, by using a variety of oligonucleotide pairs able to amplify the following genes/gene families: *cry1*, *cry2*, *cry3*, *cry4*, *cry8*, *cry9*, *cry10*, *cry11*, *cyt1* and *cyt2* (Ceron et al., 1994, 1995; Bravo et al., 1998; Ibarra et al., 2003). The extraction methodology for DNA was described by Sambrook et al. (2001).

Isolate S1806 was serotyped according to de Barjac (1981) using H-specific antisera kindly provided by Institut Pasteur.

3. Results and discussion

Among the 215 isolates examined, 5 produced greater than 50% larval mortality in the selective assay and were selected for the dose-dependence bioassay. From the LC_{50} results, four statistically different groups could be seen among the isolates evaluated (ANOVA $H = 16.62$; 6 d.f.; $p = 0.011$) (Table 1). The S601 isolate was classified as belonging to group A and showed the best LC_{50} result, which was 0.15 mg/ml. The reference isolate (*B. thuringiensis* subsp. *tenebrionis*) and S1806 were classified as belonging to group B with LC_{50} of 0.32 and 0.30 mg/ml, respectively, showing no statistical difference between the two isolates. Group C contained only one isolate, *B. thuringiensis* subsp. *israelensis* IPS82 obtained from the Collection of *Bacillus thuringiensis* and *Bacillus sphaericus* Meyer and Neide at the Institut Pasteur, Paris, which is a standard isolate active against the dipteran order, presenting a LC_{50} of 0.74 mg/ml against *A. grandis*. The other isolates, S811, S785 and S 325 were classified as belonging to group D, showing LC_{50} of 1.5, 3.5 and 8.1 mg/ml (Table 1) respectively.

The group A isolate S601 produced a major 130 kDa protein and four other proteins of lower molecular mass (Fig. 1). This major protein is consistent with the presence of the *cry1B* gene that was detected by PCR analysis (Table 2) and the product of which was previously described to be toxic to coleopteran insects (Brizzard and Whiteley, 1988). It is possible that the toxicity of S601 is due to the presence of this protein.

Table 1
Toxicity of *Bacillus thuringiensis* (*Bt*) isolates against *Anthonomus grandis*

Strain	LC_{50} (mg/ml)[a]
S601	0.14 (0.11–0.17) A
S1806	0.30 (0.25–0.36) B
Bt subsp. *tenebrionis*[b]	0.32 (0.23–0.44) B
Bt subsp. *israelensis*	0.74 (0.61–0.91) C
S811	1.5 (0.97–2.9) D
S785	3.5 (1.8–16) D
S325	8.1 (2.9–16.) D

[a] LC_{50} values are shown (with confidence limits at a level of 95%). Different uppercase letters indicate significant differences between LC_{50} values.
[b] Reference for coleopteran toxicity.

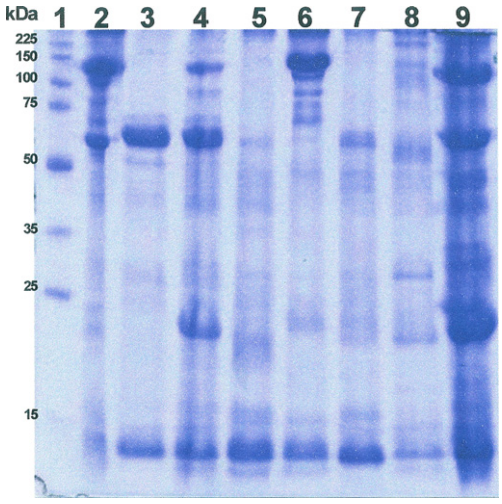

Fig. 1. SDS–PAGE of *Bacillus thuringiensis* crystal proteins from the selected isolates. Spore/crystal preparations from sporulated cultures were subjected to electrophoresis in 10 acrylamide gels. M, rainbow molecular weight markers (Amersham), 1, *B. thuringiensis* subsp. *tenebrionis*, 2, *B. thuringiensis* subsp. *israelensis*, 3, S325, 4, S601, 5, S785, 6, S811 and 7, S1806.

Table 2
Profile of *cry* genes/families in the selected *Bacillus thuringiensis* (*Bt*) isolates determined by PCR

Isolate	Genes/gene families
S601	*cry1B*
S1806	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt 1*
Bt subsp. *tenebrionis*	*cry3A*, *cry8*
Bt subsp. *israelensis*	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt 1*
S811	*cry1Ab*,*cry 1I*,*cry 8*
S785	*cry2*
S325	*cry2*

The group B isolate S1806 was serotyped as a subsp. *israelensis* isolate and exhibited a protein profile (Fig. 1) and *cry* genes similar to *B. thuringiensis* subsp. *israelensis* IPS82 (Table 2). S1806 presented the same level of toxicity as *B. thuringiensis* subsp. *tenebrionis* showing that the mosquitocidal isolate S1806 (unpublished data) could also be toxic to coleopteran larvae. This result reflects a previous study demonstrating *B. thuringiensis* subsp. *israelensis* isolate activities against insects of the coleopteran order (Méndez-López et al., 2003). The standard *B. thuringiensis* subsp. *israelensis* isolate also exhibited high level toxicity, albeit of a lower level than S1806.

S811 produced major proteins of 130 and 65 kDa (Fig. 1) consistent with the *cry1Ab*, *cry1I* and *cry8* genes detected by PCR (Table 2). Cry1I and Cry8 have been described as toxic to coleopteran larvae (Tailor et al., 1992; Abad et al., 2001). S785 and S325 presented a 65 kDa protein (Fig. 1) consistent with the presence of a *cry2* gene (Table 2).

The Cry2 protein has not previously been described as showing toxicity to coleopteran larvae. However, none of the isolates showed PCR amplicons for the *cry3* gene, the product of which has been described previously as active against Coleoptera (Bravo et al., 1998).

These analyses prove the utility of the boll weevil bioassay described and indicate its potential to select native *B. thuringiensis* isolates that may be used either directly in the biological control of boll weevil or as the source of genes to produce transgenic cotton plants resistant to this insect.

References

Abad, A.R., Duck, N.B., Feng, X., Flannagan, R.D., Kahn, T.W., Sims, L.E., 2001. Genes encoding novel proteins with pesticidal activity against coleopterans. Patent: WO 0234774-A 02-MAY-2001; E.I. Du Pont De Nemours AND COMPANY (US).

Baum, J.A., Light Mettus, A.-M., 2000. Polypeptide compositions toxic to *Diabrotica* insects, obtained from *Bacillus thuringiensis*; CryET70, and methods of use. Patent: PCT WO/00/26378-B 11-MAY-2000. Monsanto Company; 700 Chesterfield Parkway North, GG4C; Plant Protection; St. Louis, MO, USA.

Baum, J.A., Chu, C., Rupar, M., Brown, G., Donovan, W., Huessing, J., Ilagan, O., Malvar, T., Pleau, M., Walters, M., Vaughn, T., 2004. Binary toxins from *Bacillus thuringiensis* active against the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Appl. Environ. Microbiol. 70, 4889–4898.

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, F.J., Peña, G., Nuñez-Valdez, M.E., Soberón, M., Quintero, R., 1998. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. 64, 4965–4972.

Brizzard, B.L., Whiteley, H.R., 1988. Nucleotide sequence of an additional crystal protein gene cloned from *Bacillus thuringiensis* subsp. *thuringiensis*. Nucl. Acids Res. 16, 2723–2724.

Busoli, A.C., Soares, J.J., Lara, F.M., 1994. O bicudo do algodoeiro e seu manejo. FUNEP, Jaboticabal: . 32 p. (Boletim, 5).

Ceron, J., Covarrubias, L., Quintero, R., Ortiz, A., Ortiz, M., Aranda, E., Lina, L., Bravo, A., 1994. PCR analysis of the *cryI* insecticidal crystal family genes from *Bacillus thuringiensis*. Appl. Environ. Microbiol. 60, 353–356.

Ceron, J., Ortiz, A., Quintero, R., Guereca, L., Bravo, A., 1995. Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. 61, 3826–3831.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D.H., 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Molec. Biol. Rev. 62, 807–813.

de Barjac, H., 1981. Identification of H-serotypes of *Bacillus thuringiensis*. In: Burges, H. (Ed.), Microbial Control of Pests and Plants Diseases 1970–1980. Academic Press, London, pp. 35–43.

Feitelson, J.S., Payne, J., Kim, L., 1992. *Bacillus thuringiensis* insects and beyond. Biotechnology 10, 271–275.

Finney, D., 1971. Probit Analysis. Cambridge University Press, Cambridge, UK.

Gallo, D., Nakano, O., Silveira Neto, S., Carvalho, R.P.L., De Baptista, G.C., Berti Filho, E., Parra, J.R.P., Zucchi, R.A., Alves, S.B., Vendarmin, J.D., Marchini, L.C., Lopes, J.R.S., Omoto, C., 2002. Entomol. agrícola. FEALQ, Piracicaba, p. 920.

Hofte, H., Seurinck, J., Van Houtven, A., Vaeck, M., 1987. Nucleotide sequence of a gene encoding an insecticidal protein of *Bacillus thuringiensis* var. *tenebrionis* toxic against Coleoptera. Nucleic Acids Res. 15, 7183.

Ibarra, J., Rincon, C., Ordúz, S., Benintende, G., Monnerat, R., Regis, L., Sánchez, J., Bravo, A., 2003. Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquitoes species. Appl. Environ. Microbiol. 69, 5269–5274.

Kuo, J., Fox, E., MacDonald, S., 1992. Sigmastat: statistical software for working scientists. Users manual. Jandel Scientific, San Francisco, CA.

Lecadet, M.M., Chaufaux, J., Ribier, J., Lereclus, D., 1991. Construction of novel *Bacillus thuringiensis* strain with different insecticidal activities by transduction and transformation. Appl. Environ. Microbiol. 58, 840–849.

Luttrell, R.G., Fitt, G.P., Ramalho, F.S., Sugonyaev, E.S., 1994. Cotton pest management: Part 1. A worldwide perspective. Annu. Rev. Entomol. 39, 517–526.

Martin, D.F., Barbosa, S., Campanhola, C., 1987. Observações preliminares e comentário sobre o bicudo do algodoeiro, no Estado de São Paulo. Jaguariúna, EMBRAPA-CNPDA. 21 p. (EMBRAPA-CNPDA, Circular Técnica, 1).

Méndez-López, I., Basurto-Ríos, R., Ibarra, J., 2003. *Bacillus thuringiensis* serovar *israelensis* is highly toxic to the coffee berry borer, *Hypothenemus hampei* Ferr. (Coleoptera: Scolytidae). FEMS Microbiol. Lett. 226, 73–77.

Monnerat, R.G., Silva, S.F., Silva-Werneck, J.O., 2001. Catalogo do Banco de germoplasma de bactérias entomopatogênicas do gênero *Bacillus*. Brasília: Embrapa Recursos Genéticos e Biotecnologia, 65 p.

Pallini, A., Silvie, P., Monnerat, R.G., Ramalho, F.F.S., Songa, J.M., Birch, A.N.E., 2006. Non-target and biodiversity impacts on parasitoids. In: Hilbeck, A., Andow, D.A., Fontes, E.M.G. (Eds.), Environmental Risk Assessment of Genetically Modified Organisms volume 2: Methodologies for Assessing *Bt* Cotton in Brazil. first ed., CABI Publishing, Wallingford, UK, pp. 200–224.

Ponchio, L.A., 2001. Paridade de preços nos mercados nacional e internacional do algodão e a competitividade da cotonicultura brasileira. Tese de Graduação. ESALQ, Piracicaba, 48 p.

Praça, L.B., Martins, E.S., Gomes, A.C.M.M., Falcão, R., Monnerat, R.G., 2004. Prospecção de estirpes de *Bacillus thuringiensis* efetivas contra insetos das ordens Lepdoptera, Coleoptera e Díptera. Pesquisa Agropecuária Brasileira 39, 11–16.

Sambrook, J., Fritsch, E.F., Maniatis, T., 2001. Molecular Cloning: A Laboratory Manual, Third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santos, R.C., Marcellino, L.H., Monnerat, R.G., Gander, E.G., 2003. Mechanical damage in cotton buds caused by the boll weevil. Pesquisa Agropecuária Brasileira 38, 1351–1355.

Silvie, P., Leroy,T., Belot, J,L., Michel, B., 2001. Manual de identificação das pragas, e seus danos no algodoeiro. 1st ed. Cascavel: COODETEC/CIRADCA, 2001. 74 p. (B.T.N.° 35).

Tailor, R., Tippet, J., Gibb, G., Pells, S., Pike, D., Jordan, L., Ely, S., 1992. Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol. Microbiol. 6, 1211–1217.

Wolfenberger, D.A., Hamed, A.A., Luttrell, R.G., 1997. Toxicity of *Bacillus thuringiensis* against the boll weevil *Anthonomus grandis* Boh. (Coleoptera, Curculionidae). Beltsville Cotton Conf., 1296–1300.

Yousten, A.A., 1984. *Bacillus sphaericus*: Microbiological factors related to its potential as a mosquito larvicide. Adv. Biotechnol. Proc. 3, 315–343.