BioControl (2006) 51:779–794
DOI 10.1007/s10526-006-9018-4
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Characterization of *Bacillus thuringiensis* isolates from Argentina that are potentially useful in insect pest control

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Received 8 January 2005; accepted in revised form 12 April 2006

Abstract. In order to find novel strains of *Bacillus thuringiensis* that are toxic to some of the major pests that impact economically important crops in Argentina, we initiated a search for *B. thuringiensis* isolates native to Argentina. We succeeded in assembling a collection of 41 isolates, some of which show a high potential to be used in biological control programs against lepidopteran and coleopteran pests. About 90% of the strains showed toxicity against *Spodoptera frugiperda* and *Anticarsia gemmatalis*, two important lepidopteran pests in Argentina. It is noteworthy that only one of these strains contained a *cry1*-type gene, while another isolate showed a dual toxicity against the lepidopteran and coleopteran insects assayed. Genetic characterization of the strains suggests that the collection likely harbors novel Cry proteins that may be of potential use in biological insect pest control.

Key words: *Bacillus thuringiensis*, isolation, insecticidal activity, native strains, strain collection

Introduction

Although the use of chemical insecticides has proven to be very effective in agriculture and forestry, increased public concern regarding their potential adverse environmental effects has prompted the search of alternative strategies for insect pest control (Joung and Côté, 2000). Entomopathogens are efficient regulatory factors of insect populations, and as such can often be used as biological control agents of agricultural insect pests and of vectors of human and animal diseases. One of the most promising alternatives to the use of chemical pesticides is the application of *Bacillus thuringiensis*, a

Gram-positive bacterium characterized by the production of protein crystals during sporulation. The insecticidal activity of those microorganisms is associated with the parasporal proteinaceous inclusions (Lacey et al., 2001; de Maagd et al., 2001; Nester et al., 2002). Most *B. thuringiensis* strains produce a mixture of structurally different insecticidal crystal proteins (Cry proteins), which are encoded by *cry* genes. Each of these proteins may contribute to the insecticidal spectrum of a strain and can be selectively toxic to a wide variety of insects belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera and Mallophaga, as well as to other invertebrates (Beegle and Yamamoto, 1992; Feitelson et al., 1992; Schnepf et al., 1998; de Maagd et al., 2003).

It has long been believed that the occurrence of *B. thuringiensis* was closely related with insect-breeding environments. However, more recent studies have shown that it is an indigenous bacterium in many ecosystems and is distributed worldwide (Schnepf et al., 1998; Forsyth and Logan, 2000). Strains have been isolated from many habitats, including soil (DeLucca et al., 1981; Martin and Travers, 1989; Hastowo et al., 1992; Hossain et al., 1997), stored grains (Burges and Hurst, 1977; DeLucca et al., 1984; Meadows et al., 1992; Chaufaux et al., 1997), insect cadavers (Carozzi et al., 1991) and the phylloplane (Smith and Couche, 1991; Damgaard et al., 1998). The bacterium is probably best described as an opportunistic pathogen in insect habitats (Martin and Travers, 1989; Chilcott and Wigley, 1993; Bernhard et al., 1997; Chaufaux et al., 1997; Schnepf et al., 1998). Major collections of *B. thuringiensis* have been assembled during the past few decades as a result of intensive screening programs in many countries of the world, resulting also in the identification of thousands of different strains (Martin and Travers, 1989; Meadows et al., 1992; Chilcott and Wigley, 1993; Chak et al., 1994; Ben-Dov et al., 1997; Bernhard et al., 1997). However, very few screening programs have been carried out in Latin America (Bravo et al., 1998; Ibarra et al., 2003; Uribe et al., 2003). In Argentina, Dias et al. (1999) isolated 21 native strains that were toxic against Lepidoptera. Recently, five novel *B. thuringiensis* isolates were characterized primarily according to their crystal morphology, protein composition, presence of *cry*-type and *vip* genes and insecticidal activity; however, only three of these showed toxicity against Lepidoptera (Benintende et al., 1999, 2000; Franco-Rivera et al., 2004).

Although the number of Cry proteins described to date is more than 300 (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html), it is still essential to search for novel *B. thuringiensis* strains

that may lead to the discovery of additional insecticidal proteins with a higher toxicity against uncontrolled pests. Novel toxins are also important for providing alternatives for coping with the emergence of resistant insect populations (Nester et al., 2002).

Since the Argentinean economy depends on agriculture, insect control is a priority as well as a necessity. In addition, due to the extensive use in Argentina of transgenic insect-resistant crops based on *cry*-type genes, there is a need for alternative *cry* gene sequences to meet the challenge of novel insect resistance. Consequently, we set out to establish and characterize a collection of *B. thuringiensis* isolates from samples collected in different Argentinean localities in order to find novel strains toxic against insect pests of economically important crops (like soybean and maize). We succeeded in assembling a *B. thuringiensis* collection (41 isolates) that includes 37 isolates with insecticidal activity against *Spodoptera frugiperda* and *Anticarsia gemmatalis*, two major lepidopteran pests in Argentina. In contrast to previous studies (Benintende et al., 1999, 2000; Franco-Rivera et al., 2004), the *cry1*-type gene was detected in only one of the strains found to be toxic against lepidopteran insects. A second noteworthy isolate of the collection showed dual activity, being toxic against the two Lepidoptera (*Spodoptera frugiperda* and *Anticarsia gemmatalis*) and the two Coleoptera (*Tenebrio molitor* and *Diabrotica speciosa*) species assayed.

Materials and methods

Sample collection

A total of 211 samples were collected aseptically from 18 sites located in eight of the 23 Argentinean provinces and stored at 4°C until processing. The samples included soil (5 cm below the surface from different crop fields and associated agricultural lands), stored grain dust, dead insects and spider webs. No commercial *B. thuringiensis* base product was used in any of the sampled areas.

Bacterial isolation and maintenance of strains

Pasteurized samples were plated on nutrient agar (Difco No. 001). Single colonies were microscopically identified by their inclusion bodies, isolated and then cultured for further studies. Strains were freeze-dried and kept at −20°C at the FIBA's stock collection.

Standard *B. thuringiensis* strains [*B. thuringiensis* subsp. *kurstaki* (HD1, *Bt-k*), *B. thuringiensis* subsp. *san diego* (*Bt-sd*) and *B. thuringiensis* subsp. *israelensis* (HD567, *Bt-i*)] were supplied by Dr. J. Ibarra from CINVESTAV, Irapuato, Mexico.

For each isolate, parasporal body morphology was examined in sporangia under phase contrast microscopy at a magnification of 1500×. For comparison, microscopy observations were also performed on autolyzed cells. The spore-forming bacteria with parasporal bodies were maintained as spores in sterile filter paper packed into sealed vials.

Culture conditions and spore-crystal formulation

Cultures of the isolates were grown in nutrient broth medium (Difco No. 001) at 28°C under orbital agitation until complete autolysis was achieved. The spore-crystal complexes were harvested by centrifugation at 7000 *g* for 10 min at 4°C. Pellets containing spores and parasporal bodies were exhaustively washed (at least for three times) with distilled water, freeze-dried and stored at −20°C until bioassayed.

Insect rearing and bioassay

The insecticidal activity of different *B. thuringiensis* strains was evaluated on five insect species: *Spodoptera frugiperda* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), *Diabrotica speciosa* (Coleoptera: Chrysomelidae), *Tenebrio molitor* (Coleoptera: Tenebrionidae) and *Aedes aegypti* (Diptera: Culicidae). Insect larvae were obtained from colonies grown at 28°C on artificial diets specific for each insect (Parra, 1998). The assays were performed at 28 ± 2°C under a 14/10-h (light/dark) photoperiod.

To test for potential toxicity of individual isolates, we used 20 second-instar larvae of *A. gemmatalis*, *S. frugiperda* or *T. molitor* in each bioassay. Plastic 24-well cell culture plates (well diameter: 2 cm^2) were filled with the diet that had been surface contaminated with 30 μg/cm^2 of spore-crystal complex (approximately 10^6 spores/ml). One larva was placed in each well and the plates were then sealed. The number of dead insects was recorded daily for 10 days. *D. speciosa* larvae were infected by exposing batches of 20 third-instar larvae to corn sprouts (1 cm length) that had been previously dipped into a spore-crystal complex suspension (concentration: 2 mg/ml) for 5 s. Larvae were placed individually into 24-well cell culture plates containing

autoclaved sand. After 48 h, fresh non-inoculated corn sprouts were supplied every 2 days (Consolo et al., 2003). The number of surviving lepidopteran and coleopteran insects were recorded after 7 days of treatment. To assay toxicity against *Aedes aegypti*, 0.2 mg of spore-crystal complex was added to disposable cups containing 100 ml of water and 20 second-instar larvae. The number of surviving larvae were counted 24 and 48 h after assay.

Each treatment was performed twice with three replicates each. In all cases, the mortality of control larvae, reared on a toxin-free diet (or water medium) and under the same environmental conditions as the experimental larvae, was recorded and used as a correction factor for the test result, as previously described (Bohorova et al., 1996).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis

The polypeptide composition of the spore-crystal complexes of the isolated strains was determined by SDS-PAGE on 10% polyacrylamide gels (Laemmli, 1970) and visualization with Coomassie blue. For immunoanalysis (Western blots), polypeptides were passively transferred onto a nitrocellulose membrane (HyBond C, Amersham, UK) as previously reported (Renart and Sandoval, 1984). The membranes were probed with polyclonal antibodies raised in rabbits following the procedure reported by Salerno et al. (1998). Anti-*Bt-k* polyclonal antibodies were prepared by inoculating rabbits with a preparation of the polyacrylamide gel band containing the approximately 130-kDa polypeptide; this polypeptide had been electrophoretically isolated (SDS-PAGE) from an aliquot of spore-crystal complex from *B. thuringiensis* subsp. *kurstaki* and localized by staining with Coomassie blue. A similar procedure was followed to raise anti-*Bt-sd* polyclonal antibodies from the *B. thuringiensis* subsp. *san diego* spore-crystal complex, only in this case the rabbits were inoculated with the approximately 70-kDa polypeptide gel band. The immunoanalysis of proteins present in the 41 isolates was performed in duplicate using three independent protein extracts.

PCR cry *gene analysis*

Native *B. thuringiensis* strains were characterized for the presence of the *cry* gene using different primer pairs: (1) Lep1A/Lep1B and Lep2A/Lep2B for detection of the *cry1Aa*, *cry1Ab* and *cry1Ac* genes;

(2) Col1A/Col1B and Col2A/Col2B for detection of the *cry3Aa* and *cry3Ba* genes; (3) Dip1A/Dip1B and Dip2A/Dip2B for detection of the *cry4Aa* and *cry4Ba* genes (Carozzi et al., 1991). The presence of *cry1*-type genes was also assayed for using general *cry*1 primers (Bravo et al., 1998). For detection of *cry2* genes, the *cry*2gral primer pair was used (Ibarra et al., 2003). Genomic DNA from *B. thuringiensis* strains for PCR amplification was isolated as previously described (Delécluse et al., 1991; Sambrook and Russell, 2001). PCR amplifications were carried on 0.2 μg of DNA template in a reaction mixture (total volume: 25 μl) containing 200 μ*M* of each deoxynucleoside triphosphate, 0.5–1 μ*M* of each primer and 0.5 U of *Taq* DNA polymerase dissolved in the corresponding reaction buffer (Promega, Madison, Wis.). Amplifications were performed with a PTC-100 thermal cycler (model-96V; MJ Research, Watertown, Mass.) under the following conditions: one cycle of 2 min at 94°C; 30 cycles of 45 s at 94°C (denaturation), 1 min at 45°C (annealing) and 1 min at 72°C (extension); an extra extension step of 5 min at 72°C.

Results

Bacillus thuringiensis *strain collection*

The *B. thuringiensis* strains isolated throughout this study originated from 211 samples collected at different localities in Argentina. From 1466 different colonies of spore-forming bacteria, 41 *B. thuringiensis* isolates were obtained after microscopic observation (Table 1). Soil samples were the most abundant and diverse source of *B. thuringiensis*. A majority of the isolates (85%) showed the presence of bipyramidal crystals, similar to those typical of isolates active against Lepidoptera. Different morphologies (rounded crystals) were only found in isolates obtained from soil samples.

Biochemical analysis of the isolates

The polypeptide composition of the parasporal bodies produced by the 41 isolates was analyzed by SDS-PAGE, revealing different protein patterns (Figure 1). Similar to the *Bt-k* standard strain (Figure 1A, lane 2), most of the isolates exhibited two major polypeptides with a relative molecular mass (M_r) in the range of 130 to 140 kDa and approximately 70 kDa, respectively (Figure 1, Table 2). In addition, a few strains showed only the 130- to 140-kDa polypeptide

Table 1. Distribution of *Bacillus thuringiensis* isolates in samples collected in different localities in Argentina

Province	Number of sites	Number of samples	Number of colonies	Number of *B. thuringiensis* isolates	Crystal morphology
Buenos Aires (S)[a]	3	80	544	22	Bipyramidal (91%) Rounded (9%)
Buenos Aires (SW)[a]	3	21	126	3	Bipyramidal
Buenos Aires (Misc.)[a]	2	5	16	5	Bipyramidal
Catamarca (S)	1	20	149	1	Rounded
Chaco (S)	1	30	237	0	–
Córdoba (S)	2	11	121	2	Rounded
Entre Ríos (S)	2	23	205	7	Bipyramidal
Neuquén (SW)	1	10	40	0	–
San Luis (S)	1	1	1	1	Bipyramidal
San Juan (S/SW)	2	10	27	0	–

[a]S, Soil; SW, spider web; Misc., miscellaneous that includes dead insects and grain dust.

(Figure 1C, lane 6; 1D, lane 7), and others lacked it entirely (Figure 1C, lane 12; 1E, lanes 8, 12 and 13). Immunoanalyses of the polypeptides present in the parasporal bodies using polyclonal antibodies anti-*Bt-k* revealed a faint protein band in only six native isolates (FCC 29, FCC 32–36) (Figure 2A, lanes 6, 9–13). When anti-*Bt-sd* was used, a very weak band was revealed in only two isolates (FCC 40 and FCC 42); this band had an M_r lower than that of the polypeptide present in the *Bt-sd* standard strain (Figure 2B and not shown).

Insecticidal activity of the B. thuringiensis *isolates*

To detect whether isolates had any toxic activity, spore-crystal complexes were bioassayed against five insect species belonging to the Lepidoptera, Coleoptera and Diptera. The assays were carried out with the velbetbean caterpillar *Anticarsia gemmatalis* (an economically relevant pest of soybean and other legume crops), the fall armyworm *Spodoptera frugiperda* (a poliphagous insect which is a pest of soybean, corn, wheat, sorghum, barley, rice, alfalfa, cotton, etc.), the mealworm beetle *Tenebrio molitor* (one of the largest pests found in

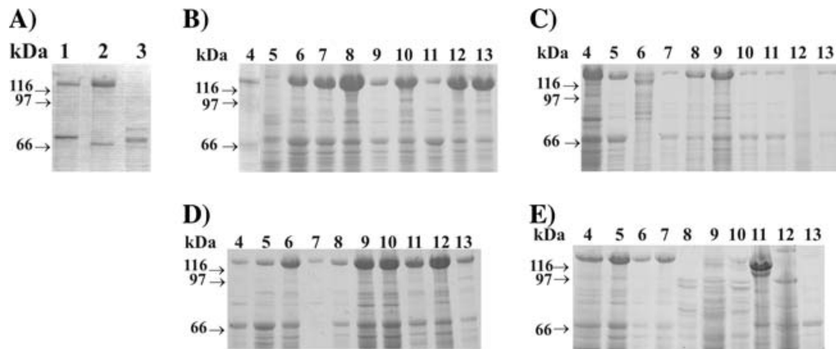

Figure 1. SDS-PAGE of spore-crystal suspensions of *B. thuringiensis* native isolates showing different polypeptide patterns. (**A**) Standard strains: *Bacillus thuringiensis* subsp. *israelensis* (*lane 1*), *B. thuringiensis* subsp. *kurstaki* (*lane 2*), *B. thuringiensis* subsp. *san diego* (*lane 3*). (**B**) FCC 4 (*lane 4*), FCC 7 (*lane 5*), FCC 8 (*lane 6*), FCC 9 (*lane 7*), FCC 10 (*lane 8*), FCC 11 (*lane 9*), FCC 12 (*lane 10*), FCC 13 (*lane 11*), FCC 14 (*lane 12*), FCC 15 (*lane 13*). (**C**) FCC 17 (*lane 4*), FCC 18 (*lane 5*), FCC 19 (*lane 6*), FCC 20 (*lane 7*), FCC 21 (*lane 8*), FCC 22 (*lane 9*), FCC 23 (*lane 10*), FCC 24 (*lane 11*), FCC 25 (*lane 12*), FCC 26 (*lane 13*). (**D**) FCC 27 (*lane 4*), FCC 28 (*lane 5*), FCC 29 (*lane 6*), FCC 30 (*lane 7*), FCC 31 (*lane 8*), FCC 32 (*lane 9*), FCC 33 (*lane 10*), FCC 34 (*lane 11*), FCC 35 (*lane 12*), FCC 36 (*lane 13*). (**E**) FCC 37 (*lane 4*), FCC 38 (*lane 5*), FCC 39 (*lane 6*), FCC 40 (*lane 7*), FCC 41 (*lane 8*), FCC 42 (*lane 9*), FCC 43 (*lane 10*), FCC 44 (*lane 11*), FCC 45 (*lane 12*), FCC 46 (*lane 13*). Polypeptides were visualized by Coomassie blue staining. *Arrows* indicate the positions of molecular mass markers (kDa).

stored grain products), the corn rootworms *Diabrotica speciosa* (capable of attacking most cultivated plants, particularly maize and cucurbits) and the mosquito *Aedes aegypti* (important vector of virus diseases).

The percentage of insect mortalities obtained with the 41 *B. thuringiensis* isolates are shown in Table 2. Approximately 90% of the strains showed toxicity towards the two lepidopteran insects. In particular, 44% of the isolates produced a mortality rate among *A. gemmatalis* of higher than 70%; however, only 5% of the strains presented similar level of toxicity against *Spodoptera frugiperda*. Notably, one of the isolates (FCC 7) exhibited a toxic activity against the two lepidopterans (*A. gemmatalis* and *S. frugiperda*) and the coleopterans (*T. molitor* and *D. speciosa*) assayed. FCC 7 was the only isolate that showed a significant toxicity against coleopterans. In addition, three of the isolates (FCC 11, FCC 27 and FCC 30) that showed bipyramidal crystals and harbored 130- to 140-kDa polypeptides did not show any insecticidal activity under the conditions

Table 2. Characteristics of *B. thuringiensis* isolates from Argentina (ND not determined)

B. thuringiensis strain	Crystal morphology	Polypeptide pattern (approx. kDa)	Mortality (%)[a]					Immuno-reactivity with anti-*Bt-k*
			Ag[b]	Sf[c]	Tm[d]	Ds[e]	Aa[f]	
FCC 4	Bipyramidal	130/60	70	35	10	0	5	–
FCC 7	Rounded	130/70	90	100	90	50	20	–
FCC 8	Bipyramidal	130/70	100	25	0	0	0	–
FCC 9	Bipyramidal	130/70	100	17	0	10	0	–
FCC 10	Bipyramidal	130/70	60	20	0	0	20	–
FCC 11	Bipyramidal	130/70	0	0	0	0	0	–
FCC 12	Bipyramidal	130/70	60	12	20	0	10	–
FCC 13	Bipyramidal	130/70	80	30	0	10	0	–
FCC 14	Bipyramidal	130/70	100	8	0	0	0	–
FCC 15	Bipyramidal	130/70	100	0	10	0	3	–
FCC 16	Bipyramidal	ND	20	0	0	0	0	–
FCC 17	Bipyramidal	130/70	65	20	0	0	0	–
FCC 18	Bipyramidal	130/70	55	55	30	0	15	–
FCC 19	Bipyramidal	130	15	30	0	10	0	–
FCC 20	Bipyramidal	130/70	95	17	0	0	5	–
FCC 21	Bipyramidal	130/70	100	8	0	0	3	–
FCC 22	Bipyramidal	130/70	65	30	0	0	0	–
FCC 23	Bipyramidal	130/70	50	35	0	0	15	–
FCC 24	Bipyramidal	130/70	40	5	20	0	5	–
FCC 25	Bipyramidal	70	95	67	20	0	0	–
FCC 26	Bipyramidal	130/70	65	58	0	0	0	–
FCC 27	Bipyramidal	130/70	0	0	0	10	0	–
FCC 28	Bipyramidal	130/70	75	15	20	0	0	–
FCC 29	Bipyramidal	130/70	0	15	20	0	5	+
FCC 30	Bipyramidal	130	0	0	0	0	0	–
FCC 31	Bipyramidal	130/70	70	8	0	0	5	–
FCC 32	Bipyramidal	130/70	55	8	0	0	0	+
FCC 33	Bipyramidal	130/70	30	17	0	0	0	+
FCC 34	Bipyramidal	130/70	65	55	0	0	0	+
FCC 35	Bipyramidal	130/70	50	50	0	0	0	+
FCC 36	Bipyramidal	130/70	70	45	0	0	0	+
FCC 37	Bipyramidal	130/70	95	35	0	0	0	–
FCC 38	Bipyramidal	130/70	95	55	0	0	0	–

Table 2. Continued

B. thuringiensis strain	Crystal morphology	Polypeptide pattern (approx. kDa)	Mortality (%)[a]					Immuno-reactivity with anti-*Bt-k*
			Ag[b]	Sf[c]	Tm[d]	Ds[e]	Aa[f]	
FCC 39	Bipyramidal	130/70	100	60	0	0	3	–
FCC 40	Bipyramidal	130/70	85	70	0	0	0	–
FCC 41	Rounded	80–75	65	40	0	0	98	–
FCC 42	Rounded	130/80–75	30	40	0	0	3	–
FCC 43	Rounded	130/80–75	74	45	0	0	0	–
FCC 44	Rounded	130/70	35	40	0	0	0	–
FCC 45	Rounded	100–70	40	45	0	0	0	–
FCC 46	Bipyramidal	70	65	0	0	0	3	–

[a]Each treatment was carried out in two independent experiments, with three replicates.
[b]Ag, *Anticarsia gemmatalis*.
[c]Sf, *Spodoptera frugiperda*.
[d]Tm, *Tenebrio molitor*.
[e]Ds, *Diabrotica speciosa*.
[f]Aa, *Aedes aegypti*.

tested. One of the isolates of the collection (FCC 41) induced a 98% mortality rate among *A. aegypti*.

Molecular characterization of the isolates

In order to perform a preliminary screening for the presence of known *cry* genes in the native isolates, we investigated the PCR-amplification product profiles using primers reported to be able to detect the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry3Aa*, *cry3Ba*, *cry4Aa* and *cry4Ba* genes. Only with the Lep1A/Lep1B oligonucleotides, which can amplify *cry1Aa*, *cry1Ab*, *cry1Ac* gene sequences, was it possible to obtain an amplification product of the expected size (490 bp) in isolate FCC 25 (Figure 3A, lane 5), which was confirmed with the *cry1* general primers (Figure 3B, lane 4). Also, no amplification product was detected in the 41 strains using Col1A/Col1B, Col2A/Col2B, Dip1A/Dip1B, Dip2A/Dip2B and the *cry2* general primer pairs (Figure 3C and not shown).

Figure 2. Immunoblot analysis of parasporal body proteins of native *B. thuringiensis* isolates. Polypeptides were immunorevealed with anti-*Bt-k* (**A**) and anti-*Bt-sd* (**B**). Standard strains: *B. thuringiensis* subsp. *kurstaki* (*lane 1*), *B. thuringiensis* subsp. *san diego* (*lane 2*), *B. thuringiensis* subsp. *israelensis* (*lane 3*). Native isolates: (**A**) FCC 27 (*lane 4*), FCC 28 (*lane 5*), FCC 29 (*lane 6*), FCC 30 (*lane 7*), FCC 31 (*lane 8*), FCC 32 (*lane 9*), FCC 33 (*lane 10*), FCC 34 (*lane 11*), FCC 35 (*lane 12*), FCC 36 (*lane 13*); (**B**) FCC 27 (*lane 4*), FCC 28 (*lane 5*), FCC 29 (*lane 6*), FCC 30 (*lane 7*), FCC 31 (*lane 8*), FCC 32 (*lane 9*), FCC 33 (*lane 10*), FCC 34 (*lane 11*), FCC 35 (*lane 12*), and FCC 36 (*lane 13*).

Discussion

We have characterized a *B. thuringiensis* strain collection assembled from different agricultural regions in Argentina. Although the type of sample and its origin had no relationship with the characteristics of the isolates, we concluded that the abundance of *B. thuringiensis* strains was higher in the soil samples than in the other source materials. This screening contributes to our knowledge of *B. thuringiensis* diversity in Latin America where to date very few collections have been described (Bravo et al., 1998; Uribe et al., 2003). In particular, there are very few reports on Argentinean *B. thuringiensis* strains. Of the few that have been published, Dias et al. (1999) described the isolation, pathogenic evaluation and polypeptide patterns of 21 Argentinian *B. thuringiensis* isolates. In other studies, five additional strains were characterized by serotype determination, toxicity, plasmid composition and insecticidal gene content (Benintende et al., 1999, 2000; Franco-Rivera et al., 2004). However, all of the toxic strains reported in these studies were active only against lepidopteran species, and only *cry1* genes were detected by *cry* genotyping analysis (Benintende et al., 2000; Franco-Rivera et al., 2004). Our analysis of the 41 *B. thuringiensis* isolates in the present investigation showed that most of the isolates formed bipyramidal crystals, similar to those typical of

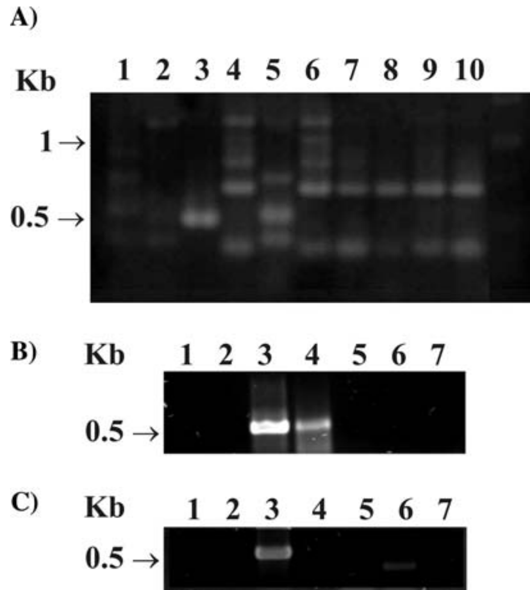

Figure 3. PCR amplification products from different *B. thuringiensis* native strains separated by agarose electrophoresis. The following primer pairs were used in the PCR amplification mixtures: Lep1A and Lep1B (**A**), gral-*cry1* (**B**) and *cry2*gral (**C**). Standard strains: *B. thuringiensis* subsp. *israelensis* (**A–C**, *lane 1*), *B. thuringiensis* subsp. *san diego* (**A–C**, *lane 2*), *B. thuringiensis* subsp. *kurstaki* (**A–C**, *lane 3*). Native isolates: (**A**) FCC 24 (*lane 4*), FCC 25 (*lane 5*), FCC 26 (*lane 6*), FCC 27 (*lane 7*), FCC 28 (*lane 8*), FCC 29 (*lane 9*), and FCC 30 (*lane 10*). (**B**,**C**) FCC 25 (*lane 4*), FCC 26 (*lane 5*), FCC 27 (*lane 6*), and FCC 28 (*lane 7*). DNA fragments were visualized with ethidium bromide staining. *Arrows* indicate the positions of molecular-weight markers (corresponding to 1-kb DNA ladder, GIBCO-BRL, Gaithersburg, Md.).

strains active against Lepidoptera. However, a small number of other isolates that exhibited this same toxicity were found to have rounded crystal inclusions. It can not be disregarded that the abundance of isolates producing bipyramidal crystals may have been artificially enhanced by the methodology of identifying *B. thuringiensis* strains, which was based principally on phase-contrast microscopy, and in phase-contrast microscopy bipyramidal crystals are more easily distinguished rounded ones (Bravo et al., 1998). When the toxicity of the native isolates was determined towards selected target insects belonging to Lepidoptera and Coleoptera, some strains attracted our interest more than others because of their special characteristics. Thus, 37 crystal-producing strains of two different morphologies were toxic against lepidopterans. In particular, the FCC 43 isolate, which produced rounded crystals, showed toxicity against lepidopterans (74 and

45% mortality of *A. gemmatalis* and *S. frugiperda* larvae, respectively), and the FCC 7 isolate was toxic against the lepidotperans and coleopterans assayed (Table 2). Mosquitocidal activity was also included to provide a more complete spectrum of the toxicological properties of the collection. Isolate FCC 41 was found to have insecticidal activity against mosquito larvae (Table 2). Based on the varied parasporal polypeptide patterns of the isolates, we conclude that this *B. thuringiensis* collection has quite an attractive diversity of Cry proteins. The *cry* genotype analysis of the 41 isolates using general primers for the *cry1*, *cry2*, *cry3* and *cry4* genes showed that only one isolate (FCC 25) harbored a *cry1*-type gene.

The toxicity evaluation, the varied polypeptide patterns and the fact that the more common *cry* genes were not detected led us to conclude that the isolates may be harboring non-frequent or novel genes. Consequently, we carried out a complementary study to develop a new strategy for the amplification and further identification of the unknown *cry* sequences. Using this methodology, we have recently reported the presence of a *cry8*-like, a *cry24*-like and a potentially novel *cry* gene in the FCC 7, FCC 41, and FCC 4 isolates, respectively (Berón et al., 2005).

In summary, an important feature of the *B. thuringiensis* collection described in this study is the presence of isolates that are toxic against lepidopterans. These isolates differ in terms of their crystal morphology and the lack of *cry1*-type genes. The isolation of a toxic strain against coleopterans (FCC 7) may also provide an alternative tool to be used in the control of beetle pests. Efforts to fully characterize these genes are in progress in our laboratory as are efforts to find additional evidence on the potential of these strains as biological control agents against new targets.

Acknowledgments

The authors thank Dr. Ed Etxeberria (Citrus Research Center, University of Florida) and Dr. Robert Igarashi (Department of Plant and Microbial Biology, University of California, Berkeley) for their critical reading of the manuscript, and Dr. Guillermo Cabrera Walsh (South American Biological Control Laboratory – Hurlingham, Argentina-USDA), Dr. Juan José García (CEPAVE, Argentina) and Dr. Rose Monnerat (CENARGEN – EMBRAPA, Brasilia, Brazil) for providing insect larvae. This work was supported by grants from the Universidad Nacional de Mar del Plata and FIBA, Argentina.

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