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Diversity of *Bacillus thuringiensis* Strains Isolated from Citrus Orchards in Spain and Evaluation of Their Insecticidal Activity Against *Ceratitis capitata*

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A survey of *Bacillus thuringiensis* (Berliner) strains isolated from Spanish citrus orchards has been performed, and the strains were tested for insecticidal activity against the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), a key citrus pest in Spain. From a total of 150 environmental samples, 376 isolates were selected, recording a total *B. thuringiensis* index of 0.52. The collection was characterized by means of phase-contrast microscopy, SDS–PAGE, and PCR analysis with primer pairs detecting toxin genes *cry1*, *cry2*, *cry3*, *cry4*, *cry5*, *cry7*, *cry8*, *cry9*, *cry10*, *cry11*, *cry12*, *cry14*, *cry17*, *cry19*, *cry21*, *cry27*, *cry39*, *cry44*, *cyt1*, and *cyt2*. Diverse crystal inclusion morphologies were identified: bipyramidal (45%), round (40%), adhered to the spore (7%), small (5%), and irregular (3%). SDS–PAGE of spore-crystal preparations revealed 39 different electrophoresis patterns. All primer pairs used in PCR tests gave positive amplifications in strains of our collection, except for primers for detection of *cry3*, *cry19*, *cry39*, or *cry44* genes. Strains containing *cry1*, *cry2*, *cry4*, and *cry27* genes were the most abundant (48.7%, 46%, 11.2%, and 8.2% of the strains, respectively). Ten different genetic profiles were found, although a total of 109 strains did not amplify with the set of primers used. Screening for toxicity against *C. capitata* adults was performed using both spore-crystal and soluble fractions. Mortality levels were less than 30%. We have developed a large and diverse *B. thuringiensis* strain collection with huge potential to control several agricultural pests; however, further research is needed to find out Bt strains active against *C. capitata*.

Keywords: *Bacillus thuringiensis*, Mediterranean fruit fly, *cry*, *cyt*, biological control, PCR, SDS–PAGE, phase-contrast microscopy, bioassays

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B. thuringiensis is a ubiquitous Gram-positive, spore-forming bacterium that forms parasporal crystals at the onset of the sporulation phase of its growth cycle. *B. thuringiensis* insecticidal activity primarily lies in its parasporal crystals and these are predominantly composed of one or more types of proteins, known as δ-endotoxins: the Cry proteins and, in some strains, the Cyt (cytolitic) proteins [43]. Nevertheless, other factors may contribute to *B. thuringiensis* toxicity, such as the spore itself and extracellular soluble factors like β-exotoxins, VIP (vegetative insecticidal proteins) toxins, phospholipases, proteases, and chitinases [35].

Cry toxins are highly specific to their target insects, are harmless to humans, vertebrates, and plants, and are easily biodegradable [22]. To date, several invertebrates have been described to be susceptible to *B. thuringiensis* strains, mostly insects from Lepidoptera, Diptera, and Coleoptera, but also from other orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and also species from nematodes, mites, and protozoa. However, for many crystal-bearing *B. thuringiensis* strains, no toxic activity has been detected yet [43]. Thus, in recent decades, there has been great interest in the screening of *B. thuringiensis* collections to find isolates useful for pest control [4, 8, 10, 18, 21, 37].

As a coevolution process between *B. thuringiensis* and the insect host has been proposed [27], several *B. thuringiensis* screening programs have used samples taken from the target insect habitat or even from the insects themselves [2, 14, 29].

Many dipteran species have been found to be susceptible to *B. thuringiensis*; these include mosquitoes, blackflies, chironomids, tipulids, muscids, sciarids, drosophilids [1, 11], and tephritidis; such as the olive fly *Bactrocera oleae* [1, 29] and the Mexican fruit fly *Anastrepha ludens* [39, 45]. The Mediterranean fruit fly or Medfly, *Ceratitis capitata*, one of the most destructive pests of fruits worldwide, has also been reported to be susceptible to preparations obtained from

B. thuringiensis, but in that study the activity was attributed to β-exotoxin and not to δ-endotoxins [13]. In Spain, this pest is considered one of the most important for citrus orchards [9]. Its control in the Valencia region, the main area of citrus production in Spain, has been based on field monitoring of the population levels and aerial and ground treatments with Malathion bait sprays [36]. The continuous use of this insecticide has diminished its effectiveness by the appearance of resistance in field populations of Medfly [32, 33]. Moreover, Malathion will not longer be authorized in the European Union from 2008 (annex 1 of the Directive 91/414 CEE). In this scenario, there is need to develop environmentally friendly control methods. The utilization of the entomopathogenic bacteria *Bacillus thuringiensis* (Berliner) could be a promising alternative.

The identification of *cry* and *cyt* genes by PCR is a very useful method for isolates characterization and, to a certain extent, this technique allows the prediction of insecticidal activity of a specific *B. thuringiensis* strain [35]. In this sense, identification of *cry* and *cyt* genes detected on strains exhibiting toxic activity to dipterans could be of great interest for this study. To date, such a group includes gene subfamilies, *cry4*, *cry10*, *cry11*, *cyt1*, and *cyt2* [7]; *cry17*, and *cry25* [21]; *cry19* [20]; *cry21* [8]; *cry27* [42]; *cry39* [25]; and *cry44* [26].

Most common techniques used for *B. thuringiensis* characterization are determination of protein crystal morphology, protein electrophoresis of spore–crystal mixtures, PCR of *cry* and/or *cyt* genes, and flagellar serotyping by H-antigen agglutination [31]. To date, the majority of studies applying these methods have presented the results for each technique individually or only regarding some representative strains of the collection. Few reports have attempted to establish correlations of all data as a whole; for instance, Quesada-Moraga *et al.* [37] classified strains isolated from Spanish territory according to protein electrophoresis pattern, strain serotype, and geographical origin, but PCR tests were not performed.

The aim of this study was first to isolate and select *B. thuringiensis* strains from field-collected samples obtained from citrus plantations in eastern Spain, where *C. capitata* impact is very high. Second, characterization of these strains by morphological (phase-contrast microscopy) and molecular methods (protein electrophoresis and PCR analysis to identify a wide array of *cry* and *cyt* gene subfamilies); as well as the establishment of correlations among data provided by these techniques. Additionally, the collection obtained was tested against a laboratory colony of *C. capitata*.

Materials and Methods

Bacterial Strains and Culture Conditions

Known *B. thuringiensis* strains, other than those isolated in this study, were kindly supplied by Dr. D. R. Zeigler (Bacillus Genetic Stock Center, B.G.S.C., Ohio State University, Columbus, OH, U.S.A.) and Dr. J. Ferré (University of Valencia, Burjassot, Spain). Selected reference strains that have been reported to be active against dipterans were the following: serovar *israelensis*: strains HD567 (B.G.S.C. code 4Q1), HD500 (4Q2), and IPS70 (4Q3) [14]; serovar *morrisoni*: strains HD12 (4K1) and HD518 (4K3), serovar *leesis*: strain HL51 (4AK1); serovar *kyushuensis*: strain 74-F-6-18 (4U1) [15]; serovar *fukuokaensis*: strain T03C001 (4AP1); and serovar *darmstadiensis*: strain HD146 (4M1) [16]. Other reference strains used in this study were serovar *kurstaki*: strains HD1 and HD73 (4D4); serovar *thuringiensis*: strain HD2; serovar *finitimus*: strain HD19 (4B1); and serovar *morrisoni*: strains *san diego* (4AB1) and *tenebrionis* (4AA1). Additionally, *Bacillus sphaericus* strain WHO2297 (13A4) and Skeetal (Valent Biosciences Corporation, Libertyville, U.S.A.), a commercial product based on *B. thuringiensis* subsp. *israelensis*, were also bioassayed. Bacteria were grown in CCY medium [44] for 48 h at 28°C.

Sample Collection, Isolation of *B. thuringiensis*, and Strain Conservation

Samples were collected from three citrus orchards in Valencia, Spain. Sampling was performed from heterogeneous sources: soil underground surface (to avoid spore inactivation caused by UV radiation), phylloplane of citrus and ground cover, damaged fallen fruits, dead arthropods and gastropods, manure, and stagnant water from irrigation ditches. In all cases, samples were collected in 5-ml sterile plastic tubes and stored at −20°C until processed.

B. thuringiensis isolation was carried out as described previously [4]. Colonies with characteristic *B. thuringiensis* morphology and presence of parasporal inclusions in the sporangium were selected and plated again for single colony isolation. After a second microscopic observation to verify presence of protein inclusions, strains were cultured in CCY liquid medium, for 2 days until sporulation. Vegetative cells were eliminated by heating the sample at 70°C for 20 min. Aliquots were taken and stored at −80°C in 15% glycerol.

Crystal Characterization

Parasporal inclusions were examined before cell lysis by phase-contrast microscopy at 1,000× magnification. Two characteristics were recorded: crystal morphology and number of crystals per cell. Two categories were defined to record the number of crystals per cell: single crystal and multiple crystals (2 or more crystals per sporangium).

Protein Electrophoresis

Protein composition of spore–crystal mixtures was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), as previously described [30]. This analysis had two purposes; first, to assist the selection procedure for *B. thuringiensis* isolation (only strains isolated from the same sample that exhibited distinct protein profiles were selected, thus reducing redundancy) and, second, the characterization of each strain of the *B. thuringiensis* collection.

Polymerase Chain Reaction (PCR) Primers

Most primer pairs used for detection of *cry* and *cyt* gene subfamilies (*cry1*, *cry2*, *cry3*, *cry4*, *cry5*, *cry7*, *cry8*, *cry9A*, *cry10*, *cry11*, *cry12*, *cry14*, *cry17*, *cry19*, *cry21A*, *cry27*, *cry39*, *cyt1A*, *cyt2*) have already

been documented [5, 8, 16, 21]. In addition, a novel specific primer pair was designed to detect gene *cry44Aa* (GenBank Accession No. BAD08532), using the online application "Primer 3: WWW primer tool" (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were synthesized in a DNA synthesizer as specified by the manufacturer (Microsynth, Balgach, Switzerland). Table 1 shows sequences of all primers used in the study, the expected sizes of their PCR products, and the optimal annealing temperature for PCR.

PCR Analysis

B. thuringiensis strains were grown in 96-well vee-bottom plates (Starstedt, Newton, U.S.A.). Each well contained 150 µl of CCY medium without salts supplemented with 5% glycerol [44]. Plates were incubated at 28°C for 24 h without agitation. A 96-pin replicator (Boekel Scientific, Feasterville, U.S.A.) was used to directly transfer a small volume of cell suspensions to the 96-well ThermoQuick PCR plate Type 4 (Greiner bio-one, Stonehouse, U.K.), each well containing 5 µl of sterile milli-Q water (Millipore, Billerica, U.S.A.). The final volume of the PCR mixture was 20 µl, containing 100 µmol/l of each dNTP, 1 µmol/l of each primer, and 0.75 U of *Taq* DNA polymerase with its corresponding buffer (Biotools, Madrid, Spain). PCR was performed in an Eppendorf Mastercycler ep, and amplification conditions were as follows: an initial denaturation step for 5 min at 95°C, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 45 s at variable temperature depending on the primer pair (see Table 1), and extension for 30 s to 1 min at 72°C (depending on amplicon expected size), and a final extension step of 7 min at 72°C. PCR products were subsequently separated by electrophoresis in 1% agarose gels.

Preparation of *B. thuringiensis* Extracts for Bioassays

A loopful of cells from a single colony of each strain grown in CCY agar medium [44] were inoculated in 4.5 ml of liquid CCY medium (preculture) and grown for 48 h at 28°C and 200 rpm. An aliquot was taken to verify spore and crystal formation (over 90% sporulation is optimum), and the preculture was incubated for 20 min at 70°C to eliminate vegetative cells (synchronization). The main culture (40 ml) was inoculated with 1/1,000 volumes of synchronized preculture and grown as mentioned above. Optimal crystal formation was checked by phase-contrast microscopy. The whole culture was centrifuged at 9,000 ×*g* for 10 min. An aliquot of the supernatant (1 ml) was kept at −20°C for future bioassays. The

Table 1. PCR primers for *cry* or *cyt* genes identification.

Primer pair	Gene(s) detected	Amplicon size (bp)	Annealing temp. (°C)	Sequence[a]	Reference
Un1	*cry1*	277	50	5' CATGATTCATGCGGCAGATAAAC (dir) 5' TTGTGACACTTCTGCTTCCCATT (rev)	[5]
Un2	*cry2*	689-701	50	5' GTTATTCTTAATGCAGATGAATGGG (dir) 5' CGGATAAAATAATCTGGGAAATAGT (rev)	[5]
Un3	*cry3*	589-604	50	5' CGTTATCGCAGAGAGATGACATTAAC (dir) 5' CATCTGTTGTTTCTGGAGGCAAT (rev)	[5]
Un4	*cry4*	439	50	5' GCATATGATGTAGCGAAACAAGCC (dir) 5' GCGTGACATACCCATTTCCAGGTCC (rev)	[5]
Un7/8	*cry7* *cry8*	420 423	50	5' AAGCAGTGAATGCCTTGTTTAC (dir) 5' CTTCTAAACCTTGACTACTT (rev)	[5]
spe-cry9A	*cry9A*	571	51	5' GTTGATACCCGAGGCACA (dir) 5' CCGCTTCCAATAACATCTTTT (rev)	[8]
cry10spe	*cry10*	348	51	5' TCAATGCTCCATCCAATG (dir) 5' CTTGTATAGGCCTTCCTCCG (rev)	[21]
cry11spe	*cry11*	305	51	5' TTAGAAGATACGCCAGATCAAGC (dir) 5' CATTTGTACTTGAAGTTGTAATCCC (rev)	[8]
gral_nem	*cry5* *cry12* *cry14* *cry21A*	474 477 483 489	50	5' TTACGTAAATTGGTCAATCAAGCAAA (dir) 5' AAGACCAAATTCAATACCAGGGTT (rev)	[8]
cry17+27	*cry17Aa* *cry27Aa*	832 895	47	5' CATTGTTCTACTTGGTATAA (dir) 5' GATACAATTACATCTCCTCCTGTA (rev)	[21]
cry19+39	*cry19* *cry39Aa*	616–631 619	51	5' AAGCTGCGAATCTGCATTTACTTTT (dir) 5' CTCATAATTTTCCGTCCATAAAT (rev)	[21]
cry44Aa	*cry44Aa*	444	60	5' CATTACACGGGGTGCGTTAT (dir) 5' CCGCACTTACATGTGTCCAA (rev)	
gral-cyt	*cyt1A*	522–525	51	5' AACCCCTCAATCAACAGCAAGG (dir) 5' GGTACACAATACATAACGCCACC (rev)	[8]
cyt2	*cyt2*	469	42	5' AATACATTTCAAGGAGCTA (dir) 5' TTTCATTTTAACTTCATATC (rev)	[16]

[a]"dir" and "rev": direct and reverse primers, respectively

pellet was washed once with ice-cold 1 mol/l NaCl, 10 mmol/l EDTA solution. Finally, the pellet was suspended in 1 ml of 10 mmol/l KCl. OD_{600nm} was measured and suspensions were stored at -20°C until bioassayed. All steps after centrifugation were done on ice to limit proteolysis.

Insect Bioassays

C. capitata was reared as described elsewhere [33]. Mass screening of *B. thuringiensis* collections for toxicity against *C. capitata* was performed as follows: 10 adult flies (1–2 days old) per Petri dish (15 cm diameter) were fed with a 30% sucrose solution. The culture supernatants and the suspensions of spores and crystals (OD_{600nm} ranging from 50 to 100) were tested. These ingredients were applied by adding 5 drops (5 µl per drop) on a piece of Parafilm into the Petri dish; the drop composition was a 1:1 mixture of the material to test and 1% hydrolyzed protein bait (Biocebo, Agrodan SA, Madrid, Spain) or just 0.5% bait for controls. Approximately 5 to 50 µg of toxin was added per experimental unit. The climatic regime was 25°C at 16 h:8 h light/dark photoperiod. Mortality was recorded after 7 days. Three replicates per assay were carried out.

Results

B. thuringiensis isolation

A total of 150 samples from soil, citrus, and ground cover canopy, fallen citrus fruits, dead arthropods and gastropods, manure, and stagnant water were examined (Table 2). Based on the capacity of producing parasporal crystal inclusions, we detected the presence of *B. thuringiensis* in 78.7% of the samples. This feature differed considerably between sampling locations (72.1% to 91.2%) and sample sources (69.6% to 100%). *B. thuringiensis* strains could be isolated from all types of sample sources; recovery was higher in water and arthropod samples and lower in soil and fruit samples. The global *B. thuringiensis* index was 0.52 (Bt index: ratio between number of *B. thuringiensis* colonies and total number of sporulating colonies examined). Soil samples showed the lowest Bt index (0.34) whereas Bt indexes recorded from the rest of the samples were always over 0.60.

On the basis of microscopic observations, parasporal crystals were classified into five morphological classes (Fig. 1); in order of occurrence: bipyramidal (45% of the isolates), round shaped (40%), adhered to spore (7%), small (5%), and irregular (3%). Crystals were classified as "small" when they were not big enough to determine its shape at 1,000× magnification. About 32% of the strains of the collection produced more than 1 crystal per sporangium. Almost half of the strains with bipyramidal crystals produced multiple crystals per cell, and only 17% of the strains with round-shaped crystals did. Twenty-five strains were found to produce crystals that remained adhered to the spore after cell lysis, and four morphological types were observed among them (Fig. 1).

Molecular Characterization

Two molecular methods were used to characterize our collection: SDS–PAGE of spore and crystal suspensions to determine the number and size of Cry and/or Cyt protein bands, and PCR to identify *cry* and *cyt* genes content.

In a first step, all field-isolated crystal-producing bacteria were analyzed by SDS–PAGE. A great diversity of protein patterns, regarding both number and size of protein bands, was found among these isolates, even between strains isolated from the same sample. However, quite commonly, several strains from the sample showed identical protein profiles. In such cases, strains were considered as duplicates and only one strain per protein pattern and sample was selected. Based on this selection procedure, 376 out of 896 *B. thuringiensis* isolates were chosen to build up the collection (Table 2).

Table 2. Distribution of samples and *Bacillus thuringiensis* (Bt) recovery.

Sampling location	No. of samples examined	Samples with Bt recovery (%)	No. of colonies analyzed	No. of crystaliferous colonies	No. of strains selected[a]	Bt Index[b]
Olocau	61	72.1	584	211	126	0.36
Bétera	34	91.2	507	297	121	0.59
Alcudia	55	78.2	631	388	129	0.61
TOTAL	150	78.7	1,722	896	376	0.52
Sample source:						
Soil	46	69.6	896	303	114	0.34
Phylloplane	63	85.7	433	313	162	0.72
Fruits	27	70.4	232	140	56	0.60
Arthropods	7	100.0	90	73	28	0.81
Manure	5	80.0	27	23	8	0.85
Stagnant water	2	100.0	44	44	8	1.00

[a]Only *B. thuringiensis* strains that exhibited different protein profiles were selected from the same sample.
[b]*B. thuringiensis* colonies as a fraction of total analyzed colonies.

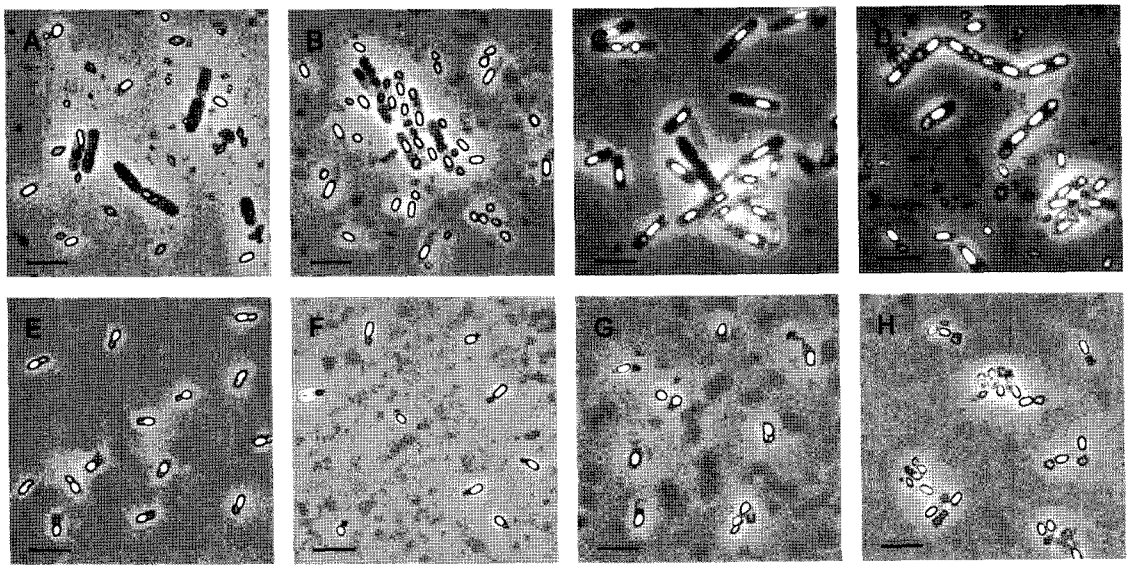


Fig. 1. Phase-contrast microscopy of sporulated cultures of eight *B. thuringiensis* isolates, showing typical parasporal inclusion structures.
A. Bipyramidal. **B**. Round. **C**. Small. **D**. Irregular. **E, F, G, H**. Four different types of crystals adhered to the spore. Bar=5 μm.

A total of 39 different protein patterns were determined by SDS-PAGE. Table 3 shows the most frequent SDS-PAGE profiles found in this work. The most common group of protein profiles (20.5% of the collection) exhibited several polypeptides from a maximum molecular mass of 140 kDa to approximately 50 kDa (Fig. 2, No. 4). In addition, SDS–PAGE of an important group of strains (15.7%) showed a protein composition resembling that of the Lepidoptera-toxic *B. thuringiensis* subsp. *kurstaki* [6], consisting of two major polypeptides in the range of 130 to 140 kDa and 60 to 65 kDa, respectively (Fig. 2, No. 1). Almost 10% of the strains exhibited only the 130–140 kDa polypeptide (Fig. 2, No. 2), another usual profile in Lepidoptera-toxic strains. Another commonly represented pattern (8.2%) was composed of 4 polypeptides of 95, 45, 41, and 25 kDa, respectively (Fig. 2, No. 14). *B. thuringiensis* subsp. *israelensis* (Bti)-like profiles [10] were also found (6.8%), showing major polypeptides of 140, 65–70, and 24 kDa (see Fig. 2, No. 7).

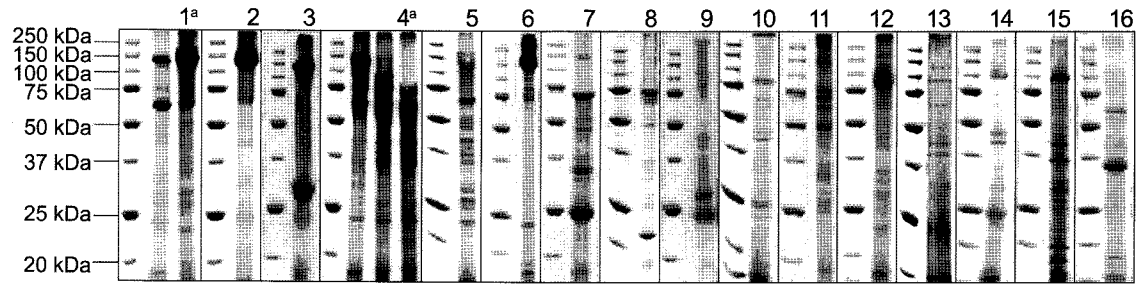
Table 3. Distribution of strains in relation to band size pattern of the spore-crystal mixtures determined by SDS-PAGE

Band size pattern (kDa)	Frequency (%)
190, 110, 90, 85	1.6
190, 80, 55	4.8
150	1.3
140, 75, 45, 22	2.9
140, 65–70, 24	6.6
140, 60	2.1
140–50[a]	20.5
130–140	9.8
130–140, 60–65	15.7
120, 44, 25	1.3
110, 40–50	4.5
100, 75, 45–50, 38, 25–30	4.0
100, 70, 25	1.6
95, 45, 41, 25	8.2
80, 60, 40–45, 20–25	1.1
80, 40–45, 20–25	1.1
30	1.6
Others[b]	11.2

[a]Multiple band sizes from 140 kDa to 50 kDa.
[b]Band profiles representing less than 1% of the *B. thuringiensis* collection (376 strains).

PCR was performed on selected strains using 14 primers pairs that can potentially detect 18 different *cry* gene groups and 2 *cyt* gene groups (Table 1). Strains containing *cry1* and *cry2* genes were the most abundant in our collection (48.7% and 46%, respectively) followed by *cry4* (11.2%), *cry27* (8.2%), *cry10* (6.6%), *cry11* (6.6%), *cyt1A* (6.6%), *cyt2* (6.6%), *cry7*, and/or *cry8* (2.1%), *cry9A* (1.1%), and nematicidal toxin coding gene(s) *cry5*, *cry12*, *cry14*, and/or *cry21A* (0.8%) detected by PCR with the "gral_nem" primer pair (Table 1). None of our field-collected strains was positive for *cry3*, *cry19*, *cry39*, or *cry44* genes.

Interestingly, PCR of 31 of our strains was positive using the "cry17+27" primer pair (Table 1). In all cases, these genes were identified as *cry27* and not *cry17*, based on different evidences: first, PCR amplicon size corresponded to that expected for the *cry27Aa* gene (895 bp for *cry27Aa* versus 832 bp for *cry17Aa*; Table 1); second, *cry17* has only been described in mosquitocidal bacteria *Clostridium bifermentans* [3]; and third, the molecular mass weight of the protoxin encoded by *cry27Aa* corresponds to one of the



cry 1+2	x	x	x	x	x											
cry 1		x		x		x										
cry 1+2+9A	x															
cry 1+9A		x														
cry 4+10+11 +*cyt* 1A+*cyt* 2							x									
cry 4								x	x	x	x					
cry 7/8												x				
cry 7/8+9A													x			
cry 27														x		
gral_nem															x	x

Fig. 2. SDS-PAGE patterns of spore-crystal suspensions of *B. thuringiensis* strains harbouring at least one of the *cry/cyt* genes analysed in the study (267 isolates).
Sixteen patterns are represented together with a molecular weight marker (Precision Plus Protein Standard, Bio-Rad): (1) I-BE17.1, I-OL33.6; (2) I-BE17.3; (3) I-OL20.5; (4) I-OL42.1, I-OL42.2, I-BE22.1; (5) I-BE26.40; (6) I-AL53.7; (7) I-BE26.15; (8) I-BE5.2; (9) I-OL40.16; (10) I-AL19.14; (11) I-AL5.9; (12) I-AL2.6; (13) I-OL57.1; (14) I-BE34.2; (15) I-BE25.13; (16) I-OL18.6. [a], SDS-PAGE classes represented by 2 or more specific patterns. Table bellow the figure shows the corresponding PCR profiles detected for each protein pattern.

polypeptides resolved by SDS–PAGE analysis of the strains harboring this gene (94 kDa instead of 72 kDa of Cry17Aa) (see Fig. 2, No.14).

Ten different gene combinations were found in our analysis (Fig. 3). Strains harboring both *cry1* and *cry2* were the most frequent (171 strains, representing 45.5%). Strains containing only *cry27* or gene combination *cry4+cry10+cry11+cyt1A+cyt2* were also quite abundant (8.3% and 6.7%, respectively). Additionally, 4.5% of our strains contained only *cry4*, and 2.4% were positive for *cry1* alone. One hundred and nine strains (29%) did not give any PCR product when assayed with our primers selection (Fig. 3). However, microscopic observation evidenced that these strains produced protein inclusions, suggesting that they may contain toxin genes not covered by the set of primers used in our PCR analysis or that they carry *cry* genes not yet described.

There is high correlation between molecular and morphological features analyzed in this study. Strains containing *cry1* and/or *cry2* (183) carried mainly bipyramidal crystals (93.4%) and, in a lower proportion, irregular or small crystals (4.4% and 2.2%, respectively). Moreover, no strain without *cry1* produced bipyramidal crystals, except for 2 strains that were positive for PCR with "gral_nem" primers and produced protein pattern No. 15 (Fig. 2). Protein electrophoresis patterns found in strains that contained at least one of the genes analyzed by PCR in

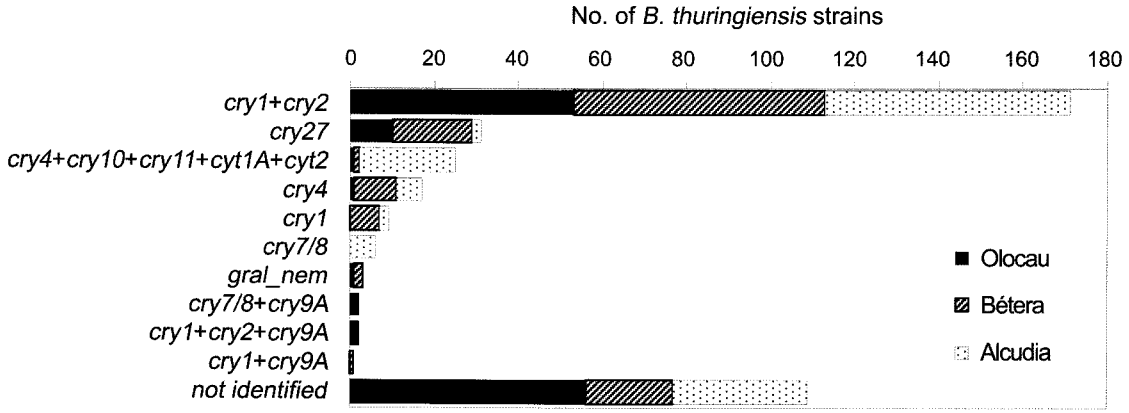


Fig. 3. Distribution of *cry* and *cyt*-type genes present in 376 field-collected strains of *B. thuringiensis* and identified by PCR analysis.

this study are shown in Fig. 2. Almost all the strains producing any of these SDS–PAGE patterns were found to harbor only one combination of genes; except for patterns No.1, 2, and 4 (Fig. 2). On the other hand, the same gene profile could be found in strains showing different SDS–PAGE patterns.

B. thuringiensis isolates that did not produce any PCR product with the primers tested were very diverse regarding their protein expression; 24 different SDS–PAGE patterns were found. Fig. 4 shows some of these patterns, which, in some cases, reflected very important protein expression level; for example, patterns No. 1, 4, or 12. The number and size of polypeptides resolved by electrophoresis were also very diverse. This group of strains (109) produced mainly round crystals (67.9%) and, to a lower extent, small and irregular crystals (7.3% and 1.8%, respectively). An important proportion (22.9%) of these isolates formed crystal inclusions that remained adhered to the spore even after cell lysis; 4 morphological types for these crystals were identified (Fig. 1E, 1F, 1G, 1H) that corresponded with 4 different SDS–PAGE profiles (Fig. 4, No. 9, 10, 11, and 12, respectively).

Insect Bioassays

Toxicity bioassays against *C. capitata* were conducted in parallel with the isolation, selection, and processing of the *B. thuringiensis* strains of our collection; therefore, the 376 strains were tested. Two kinds of materials were assayed; concentrated suspensions of spores and crystals, and culture supernatants. The maximum mortality level recorded was 30%; 5.9% of the strains caused 30% mortality when using spore–crystal suspensions, whereas 4.3% of the strains did it when testing supernatants. Additional bioassays were carried out using *B. thuringiensis* reference strains (see Materials and Methods), because they have been reported as toxic to some dipterans. Similar results were obtained (maximum mortality: 30%). A commercial product based on *B. thuringiensis israelensis* (Skeetal) and a *B. sphaericus* strain were also tested against *C. capitata*, showing no significantly higher mortality.

DISCUSSION

The data presented here show that the citrus agro-ecosystem is an important source of *B. thuringiensis*, regarding both richness and variability. Nearly 79% of the samples collected yielded colonies of *B. thuringiensis*, which is high with regard to *B. thuringiensis* recovery reported in previous works, ranging between 20% and 30% [23, 29, 37]. Nevertheless, our results are similar to that reported by Bel *et al.* [4] and Bravo *et al.* [8].

The Bt index value, which depends on the isolation procedures as well as the sampled materials, is an estimation of the success of a *B. thuringiensis* isolation. The average Bt index recorded in this study was 0.52, which means that more than half of the sporulating colonies examined contained crystal inclusions. This level is high when compared with some previous isolation programs (less than 0.1 to 0.3) [4, 6, 8, 10, 18, 23, 24, 37], but very similar to that found in the screening performed in 2004 with soil samples from Canary Islands (0.55) [40]. The Bt index is also dependent on the type of substrate used for isolation. In our work, soil samples showed the lowest Bt index (0.34), whereas Bt indexes recorded from the rest of the samples were always over 0.60 (Table 2). This evidences a higher rate of sporulating bacteria other than Bt in soils sampled from citrus fields.

Most of our field-isolated strains produced bipyramidal (45%) or round (40%) crystals; these levels are similar to those obtained by other authors [17, 28]. Interestingly, 25 isolates produced protein crystals that were not released after the lytic phase of their growth. As a result, crystals

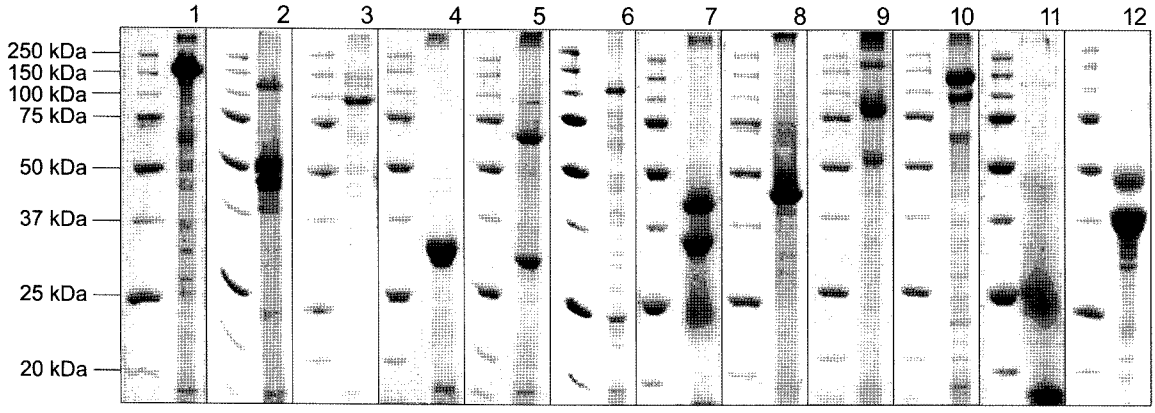


Fig. 4. SDS–PAGE patterns of spore–crystal suspensions of some *B. thuringiensis* strains that did not harbor any of the *cry*/*cyt* genes analyzed in the study.

Twelve patterns are represented together with a molecular mass marker (Precision Plus Protein Standard, Bio-Rad): (1) I-OL48.2; (2) I-OL10.1; (3) I-OL27.1; (4) I-BE5.9; (5) I-OL51.4; (6) I-BE14.1; (7) I-OL1.1; (8) I-AL41.1; (9) I-BE23.2; (10) I-AL37.5; (11) I-OL22.3; (12) I-BE26.2.

remained adhered to the spore, like in *Bacillus sphaericus* [2] but exhibiting clearly different cell morphology.

This study is one of the most extensive *B. thuringiensis* screenings performed to date in terms of *cry* and *cyt* genes identification. PCR analysis was performed on 376 isolates using a set of 14 general and specific primer pairs that could detect 18 *cry* genes and 2 *cyt* gene groups (Table 1). As published elsewhere, the most common *cry* genes found in nature are those of the *cry1* subfamily, with about half of the strains or more. *cry2* genes are also very frequent, especially among *cry1*-containing strains [35], which is in agreement with our findings. Almost half of our strains (48.7%) contained *cry1* gene(s) and 95% of those strains carried *cry2* gene(s). In previous works, it has been reported that the *cry2* occurrence in a strain is almost always associated to *cry1* presence [5]; other studies, however, have identified *cry2* in a much higher frequency than *cry1* (85% and 55% of the strains, respectively) [2]. In our work, all the strains that were positive for *cry2* were also positive for *cry1*.

Cry27 endotoxin was first described in a *B. thuringiensis* serovar *higo* mosquitocidal strain (H44) [42], but later attempts with other mosquitocidal strains failed in the identification of this gene [21]. In the present work, no serological analysis was carried out; hence the serotype of the 31 strains bearing *cry27* has not been identified. Further research is needed to clarify this; however, our strains do not share the same phenotype as strain H44 (serovar *higo*), as the SDS–PAGE pattern reported for this strain (major bands of 98, 91, 71, 63, 59, 50, 44, 27 kDa) [41] does not match with the pattern observed in our study (major bands of 94, 45, 41, 25 kDa; Fig. 2). Accordingly, the possibility of having found a new *cry27* gene other than that reported by Saitoh *et al.* [41] cannot be discarded.

The use of "general primers" in our PCR analysis allowed us to potentially detect more than 70% of the known variability existing on *cry* and *cyt* genes (297 of 409 known sequences) (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/; data from October 10th, 2007), even though almost 30% of our strain collection remains unidentified by PCR. This fact, together with the observation that protein profiles produced by these strains are different from those produced by strains containing genes analyzed in our study, reinforces the possibility of existence of novel *cry* genes in our collection.

SDS–PAGE analysis revealed an important diversity within our collection (39 different patterns in 376 strains), similar to the levels reported in previous works [23, 24, 37] and much greater diversity than that found in others [10, 28]. Comparison of observed SDS–PAGE profiles and corresponding gene profiles in our collection has provided, in many cases, straightforward results. However, correspondence between polypeptides observed *via* SDS–PAGE and *cry/cyt* genes detected by PCR has not been so clear for all strains. Observation of SDS–PAGE protein profiles that do not correspond to the expected sizes regarding the gene or genes detected in a particular strain may be due to different reasons. First, the strain may produce endotoxins whose genes have not been analyzed here. An example could be the 27 kDa polypeptide observed in strains producing SDS–PAGE pattern No. 3 (Fig. 2); the protein size fits with that expected for Cyt toxins, but PCR analysis did not detect *cyt* genes in these strains. In addition, as no protease inhibitor was added to the SDS–PAGE samples, the protein bands resolved by electrophoresis could be either protoxins, activated toxins, or proteolysis intermediates. It is well known that *B. thuringiensis* produces endogenous proteases and that their production may vary considerably among strains [38]. It has been reported that Cry1Aa and Cry1Ab protoxins (≈133 kDa) subjected to *in vitro* proteolysis may produce 7 intermediates prior to formation of the active toxin (≈57 kDa) [34]. In Bti-like patterns (Fig. 2, No. 7), protein bands were detected apart from those that directly correspond to Bti protoxins: polypeptides in the range of 45–50 kDa match with digestion products reported for Cry4Aa and Cry4Ba [12] and polypeptides from 30 to 37 kDa may correspond to the two processed fragments that form the biologically active heterodimer derived from Cry11A [46].

PCR analysis can be considered, with some limitations, a tool to predict the biological activity of a *B. thuringiensis* isolate. From the genes identified in this study, *cry1*, *cry2*, and *cry9A* encode for toxins potentially active against lepidopterans; *cry4*, *cry10*, *cry11*, *cry21Aa*, *cry27Aa*, *cyt1A*, and *cyt2* produce toxins potentially active against dipterans; *cry7* and *cry8* encode for coleopteran-toxic proteins; and *cry5*, *cry12*, and *cry14* encode for toxins with nematicidal activity. As a result, 185 strains of our collection are potentially active against Lepidoptera; 76 strains are potentially active against Diptera; 8 strains may be toxic to Coleoptera; and 3 strains may be nematicidal.

Our screening of toxicity against Medfly was not restricted to promising strains inferred from PCR analysis, but to the complete collection (376 isolates). Some studies have shown that some individual Cry1 and Cry2 toxins can be moderately toxic to dipterans [19, 24]; taking this into account, the number of strains in our collection with potential activity against dipterans would increase. Furthermore, a study performed with the tephritid *Bactrocera oleae* found 23 *B. thuringiensis* isolates that were toxic to adults or larvae of this species [1]. These isolates were characterized by PCR including primers for most of the known diptera-active genes. They reported that 11 isolates harbored only *cry1* and/or *cry2* genes, whereas the remaining 12 did not contain any of the genes that were screened.

No *cry* or *cyt* gene could be identified by PCR in almost 30% of our collection, 109 strains; therefore, their potential biological activity remains unknown, and thus, these isolates should be regarded as an interesting reservoir.

None of our field-collected isolates of *B. thuringiensis* killed more than 30% of *C. capitata* adults, which is much

lower than the level recorded previously on other adult tephritids (80% mortality in 6 to 10 days) [1, 39]. This difference can be explained by the intrinsic variations of the tephritid species tested, the *B. thuringiensis* strains, and the bioassay conditions, or a combination of all. However, it should be pointed out that the efficacy of the best *B. thuringiensis* isolates against tephritids described to date is substantially lower than that reported for other related insects like mosquitoes. The amount of toxic material fed to flies in our bioassays was by far lower than that reported in previous studies about *B. thuringiensis* toxicity on tephritids. Thus, Robacker *et al.* [39] performed their tests exposing flies to pellets equivalent to 7.5 to 22.5 ml of *B. thuringiensis* sporulated culture, whereas in our work, we added 5 drops of spore–crystal suspensions equivalent to 0.5 ml of culture. Another important variation is that whereas some studies forced ingestion of *B. thuringiensis* toxic ingredients by mixing them with a complete diet (source of carbohydrates, proteins, and water) [1], we only mixed them with hydrolyzed protein as attractant; water and sugar were added independently. Our experimental design intended to identify *B. thuringiensis* strains that could be used to control *C. capitata* in the field, where alternative nutrient sources exist and the concentration of *B. thuringiensis* toxins that adult flies would encounter by spraying should be low. In any case, the amount and concentration of endotoxins applied in our experiments were much higher than that necessary to kill other close insects like mosquitoes (visit the Toxin Specificity Database at http://www.glfc.cfs.nrcan.gc.ca/bacillus/BtSearch.cfm).

On the other hand, the target of our tests was *C. capitata* adults exclusively, and not larvae. Larvae of fruit flies develop entirely inside the fruit and, therefore, sprays applied on the surface would not be effective for controlling larval stages. It has been reported that susceptibility to *B. thuringiensis* of the larval stages of Tephritidae seems to be higher than that of the adults [29]. However, even if we found active strains against *C. capitata* larvae, this would not necessarily mean that these isolates were also active against adult flies, as it has been proven in previous reports [1, 29, 39].

Altogether, we can conclude that we have developed a large and diverse *B. thuringiensis* strain collection with huge potential to control different agricultural pests, although further research is needed to find the *B. thuringiensis* strains active against *C. capitata*. In addition, an innovative approach has been presented to integrate the complex information provided by some of the most common techniques used in *B. thuringiensis* characterization. Further research regarding the biochemical mechanisms (protoxin solubilization, proteolytic activation, and binding to midgut receptors) would be of interest for the case where protein engineering is planned with some potentially useful *B. thuringiensis* crystal proteins.

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