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Characterization of INTA 51-3, a New Atypical Strain of *Bacillus thuringiensis* from Argentina

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Abstract. Several isolates of *Bacillus thuringiensis* native to Argentina obtained in a nationwide screening program showed atypical crystal morphology. One of these strains, INTA 51-3, was further characterized in order to determine other features like protein composition of its parasporal crystal, plasmid pattern, identification of *cry* genes and toxicological properties. *B. thuringiensis* INTA 51-3 (serovar *tohokuensis*) had an amorphous inclusion containing a major protein component of *ca*. 130 kDa. After trypsin digestion of solubilized crystals, SDS-PAGE resolved a unique protease-resistant peptide of *ca*. 90 kDa. The plasmid pattern from INTA 51-3 resembled that of the standard strain HD-1. However, Southern analysis showed no hybridization to fragments of *cry*1Aa, *cry*2Aa, *cry*3A, and *cry*11A genes. Degenerate primers were used for identification of the *cry*1 genes by PCR. Nevertheless, the presence of *cry*1 type gene(s) in *B. thuringiensis* INTA 51-3 was confirmed. Highly concentrated crystal suspensions showed to be weakly toxic only to lepidopteran species.

Bacillus thuringiensis is a ubiquitous soil bacterium that produces proteinaceous crystalline inclusions constituted of highly specific insecticidal toxins. Most of the toxins are active against lepidopteran species [14], but some of them show toxicity against dipteran and coleopteran species [10, 17].

B. thuringiensis isolates produce parasporal inclusions of a wide range of morphological types, which can be classified into five major types: bipyramidal, spherical-amorphous, flat squares, cubic, and bar-shaped [3]. Most of the isolates with lepidopteran activity contain bipyramidal and cubical crystals [6]; irregular spherical and bar-shaped inclusions can be mosquitocidal [7, 23]; while flat, square crystals are often active against certain coleopteran species [12]. Additionally, some atypical crystal morphologies have been found, but generally with no identified toxicological activity [1, 21, 25].

In spite of the variability of insecticidal proteins described up to date [4], numerous publications reporting the isolation and characterization of new *B. thuringiensis* strains prove the interest to find new insecticidal activities and new crystal proteins [2, 16, 20]. New strains may render both higher insecticidal activity and broader host range, which explains the progress on the isolation of several strains with new toxins active against other insects and non-insect groups [8, 9, 13].

A screening program of indigenous strains to Argentina has led to the finding of unusual *B. thuringiensis* strains [1]. In this report we describe the main features of a unique *B. thuringiensis* strain, registered as INTA 51-3, serotyped within the serovar *tohokuensis*, and whose amorphous crystal contains a trypsin-resistant peptide of *ca*. 90 kDa.

Materials and Methods

Bacteria and culture conditions. *Bacillus thuringiensis* INTA 51-3 was isolated from a soil sample obtained from a bean field at Salta province, Argentina, by using PEMBA medium [15]. The strain was primarily identified by the presence of parasporal inclusions by using phase-contrast microscopy. Serotyping of INTA 51-3 was kindly carried out by M. Lecadet at the Institut Pasteur, France. *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *israelensis* (IPS-82), and

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B. thuringiensis subsp. *tenebrionis* (DSM 2803) were used as reference strains. They were obtained from CINVESTAV's stock collection in Irapuato, Mexico. Strains were grown in 100 ml of PMB medium [20] at 340 rpm and 30°C, during 72 h or until complete autolysis was observed.

Crystal purification. In order to obtain spore-crystal complexes, autolyzed cultures were centrifuged at 4°C and 12,000 *g* for 15 min. The pellets were washed three times with distilled water by centrifugation under the same conditions and then resuspended in distilled water. Crystals were separated from spores in discontinuous sucrose gradients (62, 66, 70, 74, 78 and 82% wt/vol), which were centrifuged at 35,000 *g* for 1 h, at 15°C. Bands of crystals were collected and washed twice with distilled water and then stored at −70°C.

Electron microscopy. (a) Scanning electron microscopy: Spore-crystal suspensions of INTA 51-3 were air dried on aluminum mounts and coated with gold in an E.M. Fullan EMS-76M evaporator for 5 min. Mounts were observed and photographed in a JEOL JSM 6400 electron microscope operated at 20 Kv [21]. (b) Transmission electron microscopy: INTA 51-3 was grown until sporulation, washed twice, and fixed in 2% glutaraldehyde in phosphate buffer. The suspension was centrifuged, and the pellet was treated with osmium tetraoxide, included in 1% agarose, and dehydrated in an ethanol series. Ultrathin cuts were contrasted with uranyl acetate and examined and photographed in a JEOL JEM-1200 EX II electron microscope, operated at 80 Kv.

Crystal protein composition. To analyze the crystal protein composition of INTA 51-3, we subjected pure crystals to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a discontinuous buffer system [18] with a 3% stacking gel and 10% running gel in a Bio-Rad mini Protean II cell slab vertical gel apparatus. Electrophoresis was carried out at 50 V for 15 min and 100 V for 2 h. Gels were stained with Coomasie Brilliant Blue. High molecular weight standard mixture (Sigma SDS-6H) was used to estimate molecular masses of crystal proteins.

Solubilization and proteolytic digestion of crystal proteins. Pure parasporal inclusions of INTA 51-3 were solubilized through incubation in 50 mM Na_2CO_3, pH 10.5, 25 mM dithiothreitol (DTT) at 37°C for 2 h. Crystals of standard *B. thuringiensis* strains HD-1 and DSM 2803 were solubilized as described previously [21]. Insoluble material was pelleted by centrifugation at 12,000 *g* for 10 min. Proteolytic digestion of crystal proteins was accomplished by trypsin treatment (EC 3.4.21.4, Sigma, Type IX from bovine pancreas). Samples were incubated at 37°C for 2 h in a 1:10 (vol/vol) trypsin solution (1 mg/ml)/protein ratio, at a pH of 8.3. Digested samples were immediately analyzed by SDS-PAGE.

DNA purification, plasmid pattern, and Southern blot analysis. Plasmid DNA of *B. thuringiensis* strains was obtained essentially by following the procedure described previously [20]. Plasmid profiles were compared by electrophoresis in 0.6% agarose gels, carried out for 2 h at 60 V. In order to detect sequence homology to some of the known *cry* genes in the plasmid profiles of INTA 51-3, *cry*1Aa (2 kb, *Sph*I-*Xho*I fragment), *cry*2Aa (complete gene), *cry*3A (0.7-kb *Eco*RI fragment), and *cry*11A (complete gene) genes, were used as probes in Southern hybridization experiments [20].

Identification of *cry* genes by PCR. Identification of known *cry*1 genes was conducted according to Juárez Pérez et al. [16], by using degenerate primers designed according to highly conserved regions among all *cry*1 genes. PCR amplification was conducted on 100 ng of plasmid DNA, mixed with 2.5 U of *Taq* DNA polymerase, 200 nM each deoxynucleoside triphosphate, 0.5 μM each primer [I(−) 5′ MDATYTCTAKRTCTTGACTA 3′ and I(+) 5′ TRACRHTDDBDGTATTAGAT 3′], and 3 mM $MgCl_2$ in a final volume of 50 μl. Amplifications were performed on a Ericomp DeltaCycler I thermal cycler under the following conditions: 5 min of initial denaturation at 94°C, followed by 25 cycles of amplification at 94°C for 1 min (denaturation), 45°C for 45 s (annealing), and 72°C for 2 min (extension). An extra extension step of 10 min at 72°C was added after completion of the 25 cycles. PCR products were visualized and analyzed by 1% agarose gel electrophoresis in Tris-acetate-EDTA buffer [26].

Toxicity tests. Toxicity of *B. thuringiensis* INTA 51-3 pure crystal was qualitatively analyzed by bioassays with neonate larvae of *Manduca sexta*, *Anticarsia gemmatalis*, fourth-instar larvae of *Culex quinquefasciatus*, *Aedes aegypty*, and third-instar larvae of *Leptinotarsa texana*. Highly concentrated pure crystals suspensions (10–75 μg/ml or cm^2 diet) were applied on the top of the artificial diet of the lepidopteran pests, or incorporated into dechlorinated water for mosquitoes, or added to droplets, according the procedure described by De Leon and Ibarra [5] for the coleopteran larvae. Mortality was determined after 4 days in mosquito bioassays and 10 days in lepidopteran and coleopteran species.

Results

Serotyping and crystal morphology. *B. thuringiensis* INTA 51-3 isolate was obtained through a screening program of native strains to Argentina. Preliminary identification was based on its morphology and the presence of parasporal crystal. The serological characterization with H antigens showed that INTA 51-3 cross-reacted with antiserum H-17 that identifies *B. thuringiensis* serovar *tohokuensis*.

Crystals of *B. thuringiensis* INTA 51-3 were primarily observed under phase contrast microscopy showing big spherical inclusions. However, under scanning electron microscopy, INTA 51-3 crystals showed a very atypical shape, similar to deflated balls, as seen in Fig 1A. When ultra-thin sections of INTA 51-3 sporulating cells were analyzed under transmission electron microscopy, crystals appeared uniform, amorphous inclusions enveloped by a dense membrane, totally separated from the spores, and ranging in size from 800 to 1000 nm (Fig. 1B). No difference in electron density was observed across the crystal.

Crystal protein composition, solubilization, and enzymatic digestion. SDS-PAGE of purified crystals of *B. thuringiensis* INTA 51-3 resolved a major band of *ca.* 130 kDa (Fig. 2), which co-migrated with the Cry1A proteins of the reference strain HD-1.

Solubilization of INTA 51-3 crystals was achieved by incubation in 50 mM Na_2CO_3, pH 10.5, with 25 mM dithiothreitol (DTT) at 37°C after 2 h.

The trypsinization of solubilized proteins of INTA 51-3 showed very interesting results, as shown in Fig. 2. After enzymatic digestion of the solubilized crystal proteins of INTA 51-3, SDS-PAGE resolved a unique pro-

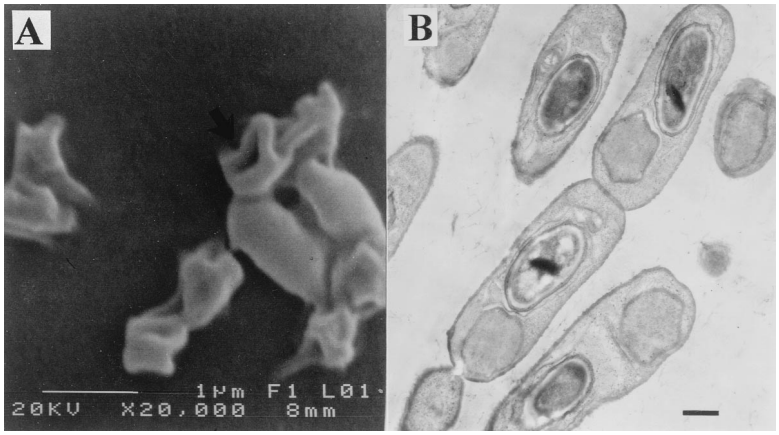

Fig. 1. Electron micrographs of *Bacillus thuringiensis* INTA 51-3. (A) Scanning electron micrograph of spores and crystals. Bar = 1 μm. × 20,000. (B) Transmission electron micrograph of sporulating cells, showing spores and crystals surrounded by a thick envelop. Bar = 500 nm. ×12,000.

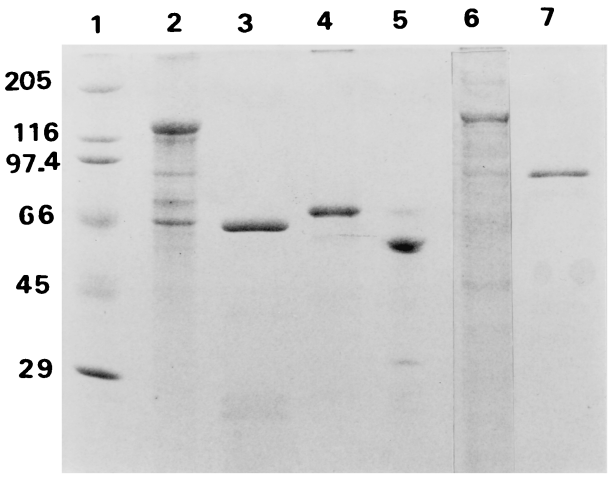

Fig. 2. Electrophoretic analysis of complete crystal proteins (lanes 2, 4, and 6) and trypsin digests of solubilized proteins (lanes 3, 5, and 7) of *B. thuringiensis* strains HD-1 (lanes 2 and 3), DSM 2803 (lanes 4 and 5), and INTA 51-3 (lanes 6 and 7). Lane 1: molecular weight markers (kDa).

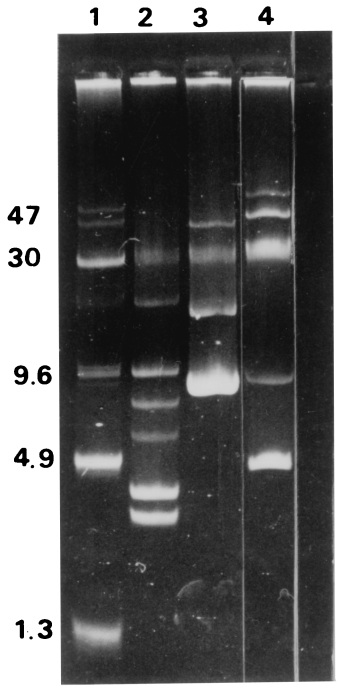

Fig. 3. Plasmid patterns of *B. thuringiensis* strains HD-1 (lane 1), IPS-82 (lane 2), DSM 2803 (lane 3), and INTA 51-3 (lane 4). Numbers on the left indicate molecular masses in mDa.

tease-resistant peptide of *ca.* 90 kDa. Solubilized proteins of standard *B. thuringiensis* strains (HD-1 and DSM 2803) produced fragments resistant to tryptic cleavage with the expected molecular masses (65 and 55 kDa, respectively).

Plasmid pattern and Southern hybridization. Electrophoresis of plasmid preparations in agarose gels revealed that *B. thuringiensis* INTA 51-3 contained a pattern of extrachromosomal DNA elements similar to that of the HD-1 strain. Their plasmidic profiles are shown in Fig. 3. INTA 51-3 showed a plasmid array of four elements, (corresponding to sizes of *ca.* 4.9, 9.6, 30, and 47 mDa). Most of them showed similar electrophoretic mobility to those of HD-1 strain. The only noticeable difference was the lack of a 1.3-mDa plasmid, always present in HD-1 plasmid profiles. The number of plasmids as well as their estimated molecular weights resulted from observations made of three agarose gels.

When Southern blot analysis was used to determine the homology with some known *cry* genes, no hybridization was observed when complete *cry*1Aa, *cry*2Aa, *cry*3A, and *cry*11A genes or fragments of them were used as probes (data not shown). These results evidence that INTA 51-3 plasmids contain no gene(s) similar to those mentioned above.

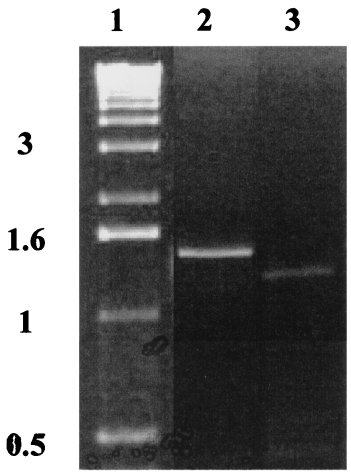

Fig. 4. PCR amplification products from strains HD-1 (lane 2) and INTA 51-3 (lane 3) of *B. thuringiensis*, with *cry*1-specific primers. Lane 1: molecular weight marker (kb).

Identification of *cry* genes by PCR. Degenerate primers I(+) and I(−) [16] were used to search for *cry*1 genes in *B. thuringiensis* INTA 51-3 strain. After amplification by PCR, one band of *ca*. 1.3 kb was observed when the amplification mixture was subjected to electrophoresis (Fig. 4), indicating the possible presence of a *cry*1 type gene. The size of this band was slightly smaller than that obtained from the HD-1 strain, probably representing a new *cry*1 gene. Cloning and sequencing of this fragment is in progress.

Toxicity. Qualitative tests of insect toxicity were carried out by using lepidopteran, dipteran, and coleopteran targets. In spite of the high concentrations tested, pure crystals of *B. thuringiensis* INTA 51-3 showed only a weak larvicidal activity against lepidopteran pests. *M. sexta* showed 60% mortality after 8 days of assay, and *A. gemmatalis* only 50% after 10 days. It is important to notice that larvae of both species died after one or two molts, unlike the effect of other *B. thuringiensis* strains of the same pathotype [6]. In spite of the high concentrations tested, no mortality was observed in any of the other insect examined, nor any effect on larval growth was noticed.

Discussion

Several isolates of *B. thuringiensis* showing atypical crystal morphology were obtained in a screening program of native strains to Argentina. One of these strains, INTA 51-3, was further characterized. Results presented in this report provide evidences that a new strain of *B. thuringiensis* has been discovered, mostly based on a unique combination of morphological, biochemical, molecular, and toxicological properties.

The INTA 51-3 strain was serotyped as *B. thuringiensis* serovar *tohokuensis*. Ohba et al. [22] described bacteriological and serological attributes of the Japanese type strain of serovar *tohokuensis*. The parasporal inclusions produced by this isolate were typically rhomboidal and usually showed two or three inclusions in a single sporangium. In spite of sharing the same H serotype, the INTA 51-3 strain showed a big spherical crystal when observed under phase contrast microscopy, resembling more those of *B. thuringiensis* serovar *shandongiensis* [24]. Further observations revealed that crystals were rather amorphous, like deflated balls, and surrounded by a dense envelope, when observed under electron microscopy. This morphology may also suggest some external similarity to the parasporal crystal of *B. thuringiensis* serovar *israelensis* [7]; however, INTA 51-3 crystals showed a very uniform composition, with no differential electron-dense areas conforming the crystal, beside some other differences. Primarily, crystal morphology of INTA 51-3 appears to be the most distinctive feature of this strain.

Apparently, specific crystal morphology of *B. thuringiensis* strains is generally associated with other properties such as crystal protein composition, solubilization conditions of parasporal inclusions, presence and size of a trypsin-resistant peptide, plasmid profile, *cry* genes, toxicity, etc. [14]. In the case of INTA 51-3, the main crystal component is a protein of *ca.* 130 kDa, similar to several insecticidal pro-toxins [14]. Also, comparable to most of the *B. thuringiensis* crystals, INTA 51-3 parasporal inclusions readily dissolved under alkaline conditions; however, after enzymatic digestion of solubilized proteins, SDS-PAGE resolved a unique protease-resistant peptide of *ca*. 90 kDa. This is another unique and very important peculiarity of *B. thuringiensis* INTA 51-3 strain, as all known Cry proteins produce δ-endotoxins of smaller molecular masses, ranging from 55 to 70 kDa [19].

This peculiar trypsin-resistant fragment (putative δ-endotoxin) may be related to the results observed during the toxicity tests; in spite of the high concentrations tested, pure crystals of INTA 51-3 showed only a weak larvicidal activity when assayed against lepidopteran pests. Because the bioassays were limited to the insect species tested, further bioassays are under way, with both usual (other lepidopteran species) and unusual targets.

Further characterization of INTA 51-3 was based on its plasmid components, as variations in the number and molecular masses of plasmid elements have been considered representative features of each *B. thuringiensis* strain [11]. The plasmid pattern of the INTA 51-3 strain

resembled to some extent that of the HD-1 standard strain. However, this resemblance may be artificial, as precise co-migration of some bands is ambiguous. Only in-depth genetic studies of each plasmid would clarify these doubts. On the other hand, Southern analysis showed a lack of nucleotide sequence homology to four well-known *cry* genes, including *cry*1Aa. These results evidence that *B. thuringiensis* INTA 51-3 plasmids contain no gene(s) similar to those mentioned above, especially to *cry*1Aa. However, when degenerate primers designed to recognize all currently known *cry*1 genes [16] were used in PCR analysis, the amplification of a band, slightly smaller than that expected (1.5–1.6 kb), was detected in *B. thuringiensis* INTA 51-3. This result may indicate the presence of a *cry*1-like gene, with at least some homology to the primers. Cloning and sequencing of the 1.3-kb amplified fragment is in progress and will clarify this uncertainty. Also, the use of primers designed to amplify other *cry* genes may be useful, especially those sharing homology with *cry*1 genes, like *cry*7 [4].

The *B. thuringiensis* INTA 51-3 strain presents a distinctive combination of features, which makes it notoriously different not only to the type strain of the serovar *tohokuensis*, but to the rest of the *B. thuringiensis* strains. Crystal morphology and the molecular mass of the putative δ-endotoxin are the most unique features. It is clear that serotyping, by itself, has many shortcomings as a means of sub-specific classification, suggesting that many other attributes should be used for this purpose. Also, molecular tools have been only partially used for the characterization of *B. thuringiensis* strains, even when their potential as sub-specific classification tools has been proven in other bacteria. It is evident that a review on the sub-specific classification of *B. thuringiensis* is necessary [21].

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