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Characterization of a Novel Strain of *Bacillus thuringiensis*

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***Bacillus thuringiensis* is a well-known species of entomopathogenic bacteria that is widely used as a biopesticide against many insect pests. Insecticidal proteins, coded for by genes located in plasmids, form typical parasporal, crystalline inclusions during sporulation. In this report, an unusual strain of *B. thuringiensis* subserovar oyamensis (LBIT-113), isolated from living larvae of *Anopheles pseudopunctipennis* in Mexico, was characterized by its ultrastructure, the protein composition of its parasporal crystal, plasmid pattern, and toxicological properties against several insect and noninsect targets. The parasporal crystal is enclosed within the spore's outermost envelope (exosporium), as determined by transmission electron microscopy, and exhibits a square, flat shape. Its main components are two proteins with sizes of 88 and 54 kDa. Despite some crystal morphology resemblance, both proteins are immunologically unrelated to the Cry IIIA protein, as shown by immunoblot analysis, when probed with antisera raised against the 88-kDa protein and the Cry IIIA protein. Partial N-terminal sequence of the 88-kDa protein revealed a unique amino acid arrangement among the Cry proteins. Solubilization of the crystal proteins was achieved at 3.3 M NaBr, and its digestion with trypsin showed only one ca. 60-kDa peptide, as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The patterns of three plasmids of strain LBIT-113 were considerably different from those of *B. thuringiensis* subspp. *kurstaki*, *tenebrionis*, and *israelensis*. Parasporal crystals showed no toxicity to larvae of four species of caterpillar, three species of mosquito, two species of beetle, one species of cricket, one species of ant, one species of aphid, one species of nematode, one species of ostracod, one species of ameba, and one species of rotifer.**

Bacillus thuringiensis is an aerobic, spore-forming, gram-positive bacterium that synthesizes crystalline proteins during sporulation (4). These proteins are specifically toxic to insect larvae and are widely used as bioinsecticides against lepidopteran, dipteran, and coleopteran pests. Crystal proteins from numerous strains have been classified according to the similarity of their amino acid sequences and their insecticidal specificity (14).

The morphology, size, and number of parasporal inclusions vary among different *B. thuringiensis* strains. However, five distinct morphologies are apparent: the typical bipyramidal crystal, related to Cry I proteins (2); cuboidal inclusions, related to Cry II proteins and usually associated with bipyramidal crystals (35); amorphous and composite crystals, related to Cry IV and Cyt proteins (10); flat, square crystals, related to Cry III proteins (13); and a bar-shaped inclusion, related to the Cry IVD protein (15). Only a few reports describe atypical morphologies (6, 16, 30), but in all cases these parasporal inclusions are formed outside of the endospore and are distinctly separated from it. Therefore, the crystal is liberated at the end of the autolysis process (4). In contrast, the *B. thuringiensis* subsp. *finitimus* typically exhibits a bipyramidal crystal surrounded by the endospore's exosporium and remains attached to the spore after lysis (9).

On the basis of their biological activity, *B. thuringiensis* strains are typically divided into three pathotypes: pathotype A, specific to lepidoptera (caterpillars); pathotype B, specific to diptera (mosquitoes and blackflies), and pathotype C, specific to coleoptera (beetles) (17). However, our knowledge of the host range has recently been expanded toward other insect (and noninsect) groups because of the discovery of new strains (11). However, little attention has been paid to nontoxic isolates, despite the fact that their distribution in natural environments is wider than that of the toxic ones (25).

In this report, we describe an unusual, nontoxic strain of *B. thuringiensis* (LBIT-113), subserotyped as oyamensis, whose flat, square parasporal crystal is enclosed within the endospore's exosporium and is composed of two proteins with sizes of 88 and 54 kDa.

MATERIALS AND METHODS

Bacterial strains and crystal purification. Strain LBIT-113 was isolated from living larvae of the mosquito *Anopheles pseudopunctipennis* collected in Irapuato, Guanajuato, within a nationwide isolation program of Mexican *B. thuringiensis* strains. Identification of the species *B. thuringiensis* was done according to the tests suggested by Slepecky and Hemphill (32) and Stahly et al. (33). Samples were sent for serotyping to the Institut Pasteur, Paris, France, and to Kyushu University, Fukuoka, Japan. Lyophilized samples of *B. thuringiensis* subsp. *kurstaki* (HD-1), *B. thuringiensis* subsp. *israelensis* (IPS-82), and *B. thuringiensis* subsp. *tenebrionis* (DSM 2803) were obtained from the Centro de Investigacion y de Estudios Avanzados' stock collection. All of the strains were grown in PMB medium, as previously described (20). Spores, parasporal bodies, and cell debris were washed three times in cold distilled water by centrifugation (10 min at 10,000 rpm in a Sorvall SS34 rotor). Crystals from strain LBIT-113 were separated from the spores by a freeze and thaw series. Crystals were purified on discontinuous sucrose gradients (56, 59, 63, 67, and 71% [wt/vol]) at 20,000 rpm for 1 h in a Sorvall HD-674 rotor. Bands were collected and washed three times in distilled water. Samples were freeze-dried and stored at −20°C until used.

SDS-PAGE. The protein composition of parasporal bodies was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted essentially as described by Laemmli and Favre (19) with a 3% stacking gel and 10% running gel in a Bio-Rad mini-Protean II cell slab vertical gel apparatus at 50 V for 15 min and 100 V for 1.5 h. Gels were stained with Coomassie blue. The molecular masses of the parasporal body proteins were estimated by comparison with a series of protein size standards (MW-SDS-200; Sigma).

Electron microscopy. (i) Scanning electron microscopy. Spore-crystal suspensions of isolate LBIT-113 were air-dried on aluminum mounts. Samples were coated with gold in an E. M. Fullan EMS-76M evaporator for 5 min and examined and photographed in a JEOL JMC-35C scanning electron microscope at a voltage of 15 kV.

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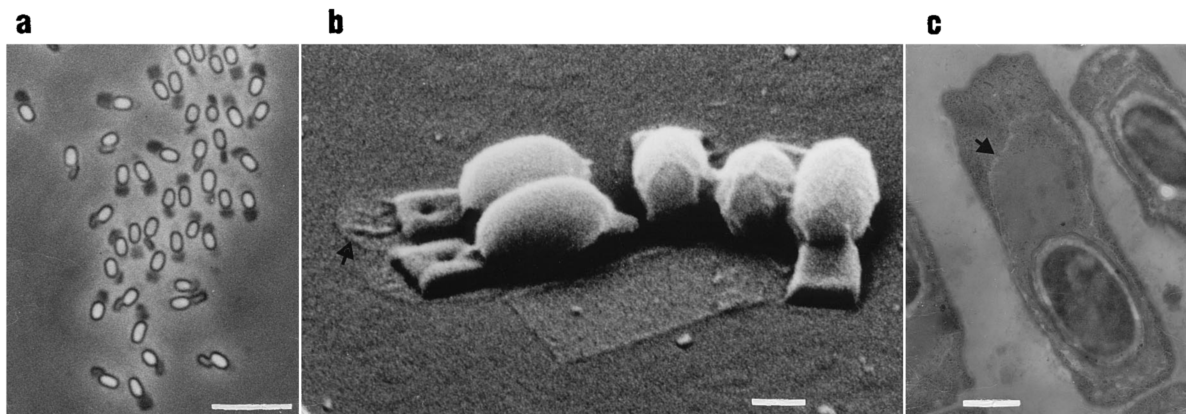

FIG. 1. Micrographs of strain LBIT-113. (a) Phase-contrast micrograph of the spore-crystal complex after autolysis. Bar, 5 μm. (b) Scanning electron micrograph of the spore-crystal complex after autolysis. Remains of the exosporium are indicated by an arrowhead. Bar, 500 nm. (c) Transmission electron micrograph of a sporulating cell containing a parasporal crystal enclosed within the exosporium, as indicated by an arrowhead. Bar, 500 nm.

(ii) Transmission electron microscopy. LBIT-113 was grown to the sporangium stage and then washed and fixed in 3% glutaraldehyde in phosphate buffer. The suspension was pelleted, dehydrated in an ethanol series, and embedded in PolyBed 812 resin mixture (Polysciences, Inc.), to be thin sectioned in a NOVA LKB ultramicrotome. Cuts were contrasted with uranyl acetate and lead citrate and examined and photographed in a JEOL JEM-2000 EX electron microscope operated at an accelerating voltage of 80 KV.

Polyclonal antibodies and Western (immunoblot) analysis. The 88-kDa crystal protein of isolate LBIT-113 was purified in 10% polyacrylamide gels. Bands were excised, equilibrated to pH 7.2 with phosphate buffer, and injected subcutaneously to New Zealand White rabbits. The first immunization was administered in incomplete Freund's adjuvant. Subsequent weekly immunizations were performed until completion of a total of 300 to 400 μg of protein per rabbit. Blood was collected from the ear vein 2 weeks after the last injection, and the serum was separated. The level of antiserum sensitivity was determined by dot immunoblotting. Western analyses of transferred SDS-PAGE samples were performed as described previously (29). Membranes were developed by chemiluminescence (31).

Crystal solubilization. Solubilization of LBIT-113 crystals was attempted in 50 mM $NaHCO_3$ (pH 8.3) and CAPS (3-[cyclohexylamino]-l-propanesulfonic acid) (pH 11), both solutions with and without 25 mM dithiothreitol (DTT). Solubilization was also tested in 50 mM NaOH (pH 12, 50 mM Na_2CO_3 (pH 10.5) with 25 mM DTT, 3.3 M NaBr. All assay mixtures were incubated at 37°C for 2 h. After incubation, the suspensions were centrifuged at 10,000 rpm, and supernatants were dialyzed against deionized water. Both proteins in supernatants and pellets were quantified by SDS-PAGE to examine the degree of solubilization. Crystals of strains HD-1 and DSM 2803 were dissolved at 37°C for 30 min in 50 mM Na_2CO_3 at pHs 10.5 and 9.5, respectively, as controls. DTT (25 mM) was added to the HD-1 crystal suspension.

Trypsinization of crystal proteins. Aliquots (100 μl) of solubilized crystal proteins of strains HD-1, DSM 2803, and LBIT-113 were subjected to trypsin treatment at 37°C in a 10:1 (vol/vol) soluble protein/trypsin ratio with 1 mg of trypsin (EC 3.4.21.4 [type IX from bovine pancreas]) per ml dissolved in deionized water. After a 2-h incubation period, samples were analyzed by SDS-PAGE.

N-terminal sequencing. LBIT-113 crystal proteins were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. These were stained with 0.1% amido black in methanol-acetic acid (45 and 7%, respectively) for 2 min and washed for 10 min in methanol-acetic acid only. Membranes were dried and stored at −20°C. The band corresponding to the 88-kDa protein was excised, and the N-terminal amino acid sequence was determined by automated Edman degradation (22) in an Applied Biosystems 470A protein sequencer. The N-terminal sequence of LBIT-113 was compared with other N-terminal sequences of Cry proteins from the GenBank database.

Plasmid purification. Plasmid extracts were obtained by a technique published previously (20). Plasmid patterns of strains LBIT-113, HD-1, IPS-82, and DSM 2803 were compared by 0.6% agarose gel electrophoresis.

Bioassays. Highly concentrated (100 to 1,000 μg/ml) spore-crystal mixtures, pure crystal suspensions, solubilized and trypsinized crystals, and vegetative cells of strain LBIT-113 were bioassayed on first instar larvae (caterpillars) of the lepidopterans *Spodoptera frugiperda*, *Helicoverpa zea*, *Manduca sexta*, and *Diatraea saccharalis*; fourth instar larvae of the mosquitoes *Aedes aegypti*, *A. pseudopunctipennis*, and *Culex quinquefasciatus*; first and third instar larvae of the coleopterans (beetles) *Callosobruchus maculatus* and *Leptinotarsa texana*, respectively; adults of the orthopteran *Acheta* sp. (cricket); adults of the hymenopteran *Atta* sp. (leafcutting ant); adults of an unidentified aphid (Aphididae); and juveniles of the nematode *Steinernema feltiae*, the ameba *Entamoeba histolytica*, an unidentified ostracod, and an unidentified rotifer. Mortality was recorded 1 to 7 days after treatment (depending on the organism). The effects on larval growth were also observed during this period.

RESULTS

Isolation and identification. Strain LBIT-113 was isolated from living larvae of *A. pseudopunctipennis* collected in shallow waters of a small stream in Irapuato, Guanajuato, Mexico, as part of a strategy to isolate native *B. thuringiensis* strains from filter-feeding organisms. Preliminary identification of the species was based on its morphology and the presence of a parasporal body. Proper identification corroborated its fitness within this species (32, 33). Serotyping showed a reaction only against the antiserum H-6 (Institut Pasteur), which identifies the *B. thuringiensis* serovar entomocidus, and more specifically against the antiserum H-6a6c (Kyushu University), which indicates that the strain belongs to *B. thuringiensis* subserovar oyamensis (28).

Crystal morphology. Preliminary observations with phase-contrast microscopy indicated that after autolysis, spores and crystals of strain LBIT-113 remained attached to each other, and crystals appeared square and flat (Fig. 1a). This morphology was corroborated by scanning electron microscopy (Fig. 1b), by which the linkage between spore and crystals is clear. Crystals are approximately 800 nm per side, and many exhibit a peculiar central puncture. Also, a thin projection of the exosporium is observed around the crystal; however, the enclosure of the crystal within the exosporium is more evident under transmission electron microscopy (Fig. 1c), by which a thin layer is projected beyond the crystal.

Parasporal body composition and immunological relationship. When protein composition was analyzed by SDS-PAGE, crystals showed the presence of two major proteins with estimated molecular masses of 88 and 54 kDa (Fig. 2a). Other Cry proteins from strains HD-1, IPS-82, and DSM 2803 showed no comigration with these proteins. To detect any similarity between the 88-kDa protein of strain LBIT-113 and the Cry proteins present in strains HD-1, IPS-82, and DSM 2803, immunoblot analyses were carried out, which showed that antibodies raised against the 88-kDa protein cross-reacted selectively with its original antigen and faintly with the 54-kDa component of the same crystal (Fig. 2b). No cross-reaction was observed with the known Cry proteins of the standard strains. A possible immunological similarity with the Cry IIIA protein,

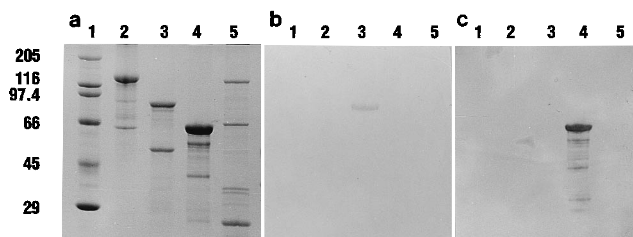

FIG. 2. Electrophoretic and immunoblot analyses of parasporal body proteins of *B. thuringiensis* standards and strain LBIT-113. (a) SDS-PAGE Coomassie blue-stained gels. (b) Immunoreaction of antibodies raised against the 88-kDa protein of LBIT-113. (c) Immunoreaction of antibodies raised against the Cry IIIA protein of DSM 2803. Lanes: 1, molecular mass (kilodaltons) markers; 2, *B. thuringiensis* subsp. *kurstaki* (HD-1); 3, strain LBIT-113; 4, *B. thuringiensis* subsp. *tenebrionis* (DSM 2803); 5, *B. thuringiensis* subsp. *israelensis* (IPS-82).

suggested by some morphological resemblance between parasporal bodies, was tested by using antibodies raised against this protein. Figure 2c shows a strong cross-reaction of these antibodies with their own antigen and no reaction with any of the LBIT-113 crystal components.

Solubilization and trypsin digestion of crystals. Experiments for optimal solubilizing conditions demonstrated that LBIT-113 crystals were poorly soluble when 50 mM $NaHCO_3$ (pH 8.3) without DTT and CAPS (pH 11) with or without 25 mM DTT were used. Partial solubilization was achieved with 50 mM NaOH (pH 12), 50 mM $NaHCO_3$ (pH 8.3) with 25 mM DTT, and 50 mM Na_2CO_3 with 25 mM DTT. However, more than 80% of the solubilization was achieved after a 2-h incubation when the crystals were suspended in 3.3 M NaBr.

The presence of any putative peptide fragment resistant to tryptic digestion in the LBIT-113 crystal was verified. After solubilization, proteins were treated with trypsin. Crystal proteins of control strains HD-1 and DSM 2803 underwent partial digestion, revealing trypsin-resistant peptides of the expected molecular masses on SDS-PAGE gels (65 and 55 kDa, respectively) (Fig. 3). Likewise, digested LBIT-113 crystals show one fragment with a size of ca. 60 kDa resistant to tryptic cleavage, similar to the trypsin-resistant fragment of Cry IIIA from DSM 2803 (Fig. 3).

N-terminal sequencing. The sequence Met-Lys-Ile-Tyr-Asp-Ile-Tyr was identified at the N-terminal end of the 88-kDa protein component of the LBIT-113 crystal. This sequence was compared with those of the known Cry proteins in the GenBank database. Six Cry proteins showed only one homologous position, other than the first methionine. Cry IVA and C showed only one coincident tyrosine in the fourth position; similarly, Cry V and the 53-kDa protein of *B. thuringiensis* subsp. *cameroun* exhibited a coincident lysine at the second position. Isoleucine at the third position is shared with three proteins with a putative effect against protozoa and nematodes. No further homology was found at the N-terminal end. Additional comparison of the 88-kDa protein N-terminal sequence with total sequences of Cry proteins also failed to find any similarity.

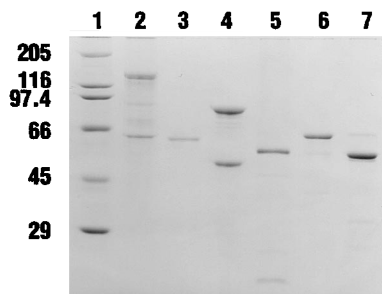

FIG. 3. Undigested (lanes 2, 4, and 6) and trypsin-digested (lanes 3, 5, and 7) solubilized crystal proteins of *B. thuringiensis* subsp. *kurstaki* (HD-1) (lanes 2 and 3), strain LBIT-113 (lanes 4 and 5), and *B. thuringiensis* subsp. *tenebrionis* (lanes 6 and 7). Molecular masses are given to the left in kilodaltons.

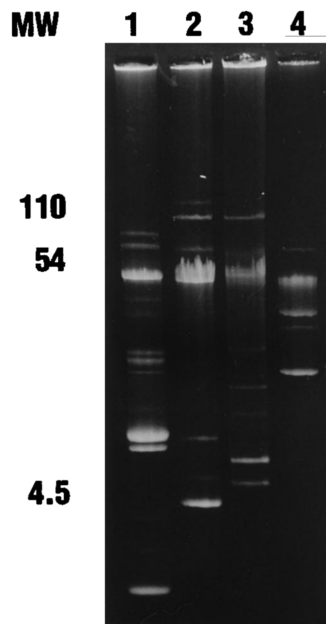

FIG. 4. Plasmid patterns of *B. thuringiensis* subsp. *kurstaki* (HD-1) (lane 1), strain LBIT-113 (lane 2), *B. thuringiensis* subsp. *israelensis* (IPS-82) (lane 3), and *B. thuringiensis* subsp. *tenebrionis* (DSM 2803) (lane 4). MW, estimated molecular size (in kilobases) of covalently closed circles of LBIT-113.

Plasmid patterns. Figure 4 shows the typical plasmid pattern of strain LBIT-113 compared with those of control strains HD-1, IPS-82, and DSM 2803. This pattern shows only three plasmids, corresponding to sizes of ca. 4.5, 54, and 110 kb. Plasmid comigration was observed with plasmids of IPS-82; however, restriction patterns generated by plasmid preparations digested with the enzyme *Hin*dIII were totally different between these two strains (data not shown), suggesting that the resemblance is limited to mass.

Bioassays. In order to detect any toxic activity of the isolate LBIT-113, spore-crystal mixtures, pure crystal suspensions, solubilized and trypsinized crystals, and vegetative cells were bioassayed against 12 insect species within the orders Lepidoptera, Diptera, Coleoptera, Orthoptera, Homoptera, and Hymenoptera and four noninsect invertebrates (see Materials and Methods). Despite the high concentrations tested, no mortality was observed in any of the insect and noninsect species examined, nor was any effect on larval growth noticed.

DISCUSSION

The results presented in this report indicate that the isolate LBIT-113 of *B. thuringiensis* exhibits very peculiar features compared with those of other strains of this species. Morphological and biochemical attributes of its parasporal body as well as its plasmid pattern are unique among other *B. thuringiensis* strains. Other characteristics, such as its lack of toxicity toward

known susceptible insects and its serotyping, contribute to its peculiarity.

Conventional biochemical identification of LBIT-113 corroborated preliminary microscopical observations regarding the species identity. Furthermore, serotyping of this strain confirmed its relationship to other *B. thuringiensis* strains. Two separate laboratories (Institut Pasteur and Kyushu University) had similar results. Because until now *B. thuringiensis* subserovar oyamensis (antigen H-6a6c) has had no official recognition in the Institut Pasteur, LBIT-113 was identified as *B. thuringiensis* serovar entomocidus in this institute, while a more specific serotyping was able to discriminate between H-6 and H-6a6c in Kyushu University. A morphological comparison of the original *B. thuringiensis* serovar oyamensis isolate and the LBIT-113 strain exhibited no common features, because the former displays a parasporal crystal very similar to that of *B. thuringiensis* subsp. *israelensis* (from spherical to irregular). However, similarly to LBIT-113, the original *B. thuringiensis* serovar oyamensis isolate shows no toxicity against lepidopteran or mosquito larvae (28).

Strain LBIT-113 exhibits a distinct crystal morphology, which is associated with other peculiarities such as its crystal protein composition, solubilization and digestibility, plasmid pattern, and lack of toxicity to known susceptible insects. However, this strain's crystal morphology is the most remarkable characteristic. Despite resembling, to some extent, the crystal morphology displayed by strains of pathotype C (represented by strain DSM 2803), the resemblance is superficial. The most conspicuous difference is the attachment of the LBIT-113 crystal to the spore. Lateral projections of the exosporium in *B. thuringiensis* are frequently observed (24); however, the distinct identity of the LBIT-113 crystal is apparent and should not be confused with these projections, because the crystal is rather surrounded by the exosporium. This enclosure is visible in both transmission and scanning electron microscopy (Fig. 1a and b). A similar phenomenon is observed in *B. thuringiensis* subsp. *finitimus*, in which the exosporium encloses the crystal (9); however, its bipiramidal shape differs significantly from the LBIT-113 crystal, in addition to its 135-kDa protein component. These observations indicate that, although uncommon, the enclosure of the crystal by the exosporium is a feature that can be found in unrelated strains of *B. thuringiensis*, as well as in other related *Bacillus* species such as *B. sphaericus*, *B. cereus* subspp. *fowler* and *lewin*, and *B. popilliae* type A1. These species display parasporal crystals attached to the spore, in which the exosporium surrounds the spore and the crystal in one enclosing unit (*B. popilliae* and *B. cereus* subspp. *fowler* and *lewin*) (3) or separately (*B. sphaericus*) (36).

The protein composition of the LBIT-113 crystal shows two major protein components. Their estimated molecular masses (88 and 54 kDa) correspond to those of none of the typical Cry proteins. Only Cry V (34), reported with activity against nematodes (GenBank accession numbers L07022 and L07023), and the crystal protein components of *B. thuringiensis* subsp. *cameroun* (16) show some similarity, because their molecular masses have been estimated at 81.2, 88.1, 54, and 53 kDa, respectively. Other characteristics of the corresponding strains are significantly different.

The 88-kDa protein is the primary constituent of the LBIT-113 crystal, according to the relative intensity of bands in SDS-PAGE analysis. Antibodies raised against this protein showed that both components are slightly related, because a weak reaction with the 54-kDa crystal component was observed in some Western blots. This result may indicate either that the 54-kDa protein is independent but similar to the 88-kDa component or that the former is a degradation product of the latter. Degradation due to proteases related to parasporal crystals is well documented for *B. thuringiensis* (7).

The polyclonal antibodies raised against the 88-kDa protein of the LBIT-113 crystal proved the uniqueness of this protein when null reactivity was observed against other typical Cry proteins. On the basis of only presumable morphological similarities between pathotype C and the LBIT-113 parasporal crystals (described above), the cross-reactivity of antibodies raised against the Cry IIIA protein was tested. Western analysis confirmed the lack of immunological relationship between these two proteins.

Crystal solubilization tests indicated that, unlike other *B. thuringiensis* crystals, the LBIT-113 crystal is not soluble under alkaline conditions. This observation suggests a possible reason for its lack of toxicity toward the bioassayed insects, because most of the toxic crystals must be dissolved in the alkaline content of the insect midgut, prior to the crystals' activation into delta-endotoxins. However, bioassays were conducted both with intact spore-crystal complexes and solubilized crystals. Interestingly, crystals of pathotype C (toxic to coleopterans) are soluble in NaBr solutions (5), similar to LBIT-113 crystals.

Furthermore, similar to other Cry proteins, the 88-kDa protein component of the LBIT-113 crystal exhibits a fragment resistant to trypsin digestion. This 60-kDa fragment is similar in molecular mass to other delta-endotoxins (1). This peculiarity of the 88-kDa protein may support the idea of a toxic activity against a particular group of insect species, because protease resistance is a requirement of all of the active delta-endotoxins. Results relating to the insecticidal activity of strain LBIT-113 presented in this report show its presumable lack of toxicity, but this conclusion should be limited only to the insect species tested. The possibility of finding insect (or noninsect) species susceptible to this strain is still open, because it is impossible to bioassay all possible organisms. Bioassays against other insect (and noninsect) species are under way. It is important to notice that there is evidence indicating that nontoxic *B. thuringiensis* strains are isolated with a much higher frequency than those which show insecticidal activity (21, 23, 26, 27).

Another peculiarity of the LBIT-113 strain is its plasmid pattern. All of the known *B. thuringiensis* strains harbor a set of plasmids, ranging from 2 to 17 in number (12). Their variations in pattern (numbers and molecular masses) are representative of divergent *B. thuringiensis* strains. Likewise, LBIT-113 contains an unusual set of plasmids, including small and large plasmids. Two of them can be considered megaplasmids, which seemingly comigrate with two plasmids of strain IPS-82. Actual comigration occurs only with the 110-kb plasmid, because the 54-kb plasmid comigrates as an open circle with the covalently closed circle of the IPS-82 plasmid. The covalently closed circle form of the 54-kb plasmid of LBIT-113 is mostly covered by chromosomal DNA, and only a few gels show both forms.

In conclusion, the characterization of the isolate LBIT-113 has demonstrated its uniqueness among other *B. thuringiensis* strains. Because flagellar serotyping is no longer the basis for subspecific classification (8), and no criteria have been proposed for that purpose, so far, a proposed subspecies name could be unrecognizable. However, there are previous references in which H serotyping was disregarded, because of the notorious differences with the known strain (i.e., of *B. thuringiensis* subsp. *tenebrionis*) (18). This indicates that a thorough review of the subspecies classification of *B. thuringiensis* is required in which several attributes could be taken as the basis for subspecies discrimination. For this reason, we propose the

name *B. thuringiensis* subsp. *irapuatensis* for the isolate LBIT-113.

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