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EXPERIMENTAL ARTICLES

Characterization of a New *Bacillus thuringiensis* Strain That Is Toxic to Coleoptera

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Abstract—A new strain of *Bacillus thuringiensis* 2-7 was found to belong to the serotype H8. Cells of this strain contained irregular and flat crystalline inclusions and two large plasmids. The gene responsible for crystal formation is most likely located on the large plasmid greater than 105 MDa in size. Comparison of the *cry* gene of *B. thuringiensis* 2-7 and the *cryIIIA* gene of *B. thuringiensis* subsp. *tenebrionis* showed that their nucleotide sequences are identical.

Key words: *Bacillus thuringiensis*, crystal morphology plasmid composition, polymerase chain reaction

In recent years, a number of *Bacillus thuringiensis* strains with activity against the production of coleopteran larvae have been described. They all are natural isolates of serotype H8, whose cells contain flat or bipyramidal crystalline inclusions and a few large plasmids [1, 2]. The *cryIII* genes responsible for crystal formation are located on large plasmids with a molecular mass of approximately 88–90 MDa. The strains described so far differed in their insecticidal activity, molecular mass of the protoxins, and in the nucleotide sequences of the *cryIII* genes. It is the sequence of the genes that underlies their classification into the *cryIIIA*, *cryIIIB*, etc., categories. One of the most well-studied strains with crystallized inclusions *B. thuringiensis* subsp. *tenebrionis*, is now extensively used as the primary component in insecticides for Colorado potato beetles [1].

This work is devoted to the study of the *B. thuringiensis* strain isolated from the meal worm *Tenebrio molitor* larvae, which shows insecticidal activity against the Colorado beetle *Leptinotarsa decemleniata* larvae.

MATERIALS AND METHODS

The strains *B. thuringiensis* subsp. *galleriae* tet cry$^-$ (serotype 5) and *B. thuringiensis* subsp. *tenebrionis* (serotype 8) were obtained from the Collection of the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow. The *B. thuringiensis* isolate was obtained by plating the intestinal contents of the meal worm larvae on a sporulation medium of the following composition (%): trypcasin, 1; K_2HPO_4, 1; $MgSO_4 \times 7H_2O$, 0.03; $CaCl_2$, 0.008; $MnSO_4$, 0.005; Difco yeast extract, 0.2; and glucose, 0.4. Colonies grown for 72 h were suspended in 0.75% NaCl. The cell suspensions were incubated at 70°C for 20 min and again plated on the sporulation medium. The colonies were then analyzed for the presence of crystals in the cells by microscopic examination. Those containing crystals were tested for their activity against Colorado beetle larvae.

Crystals were isolated according to the procedure described by Chestukhina *et al.* [3]. The form of the crystals, stained with amido black and Ziehl carbolfuchsin, was studied under the microscope at ×1000 magnification of [4]. The size of the crystals was measured using an S-405 A scanning microscope.

The serotype of the *B. thuringiensis* strains was determined by an agglutination reaction using a standard kit of antisera against the flagellar H antigens of *B. thuringiensis* [5]. In addition, we also prepared antiserum to isolate the large crystals.

The insecticidal activity of crystal endotoxin was assessed in the following manner. Potato leaves were dipped into mixtures containing different concentrations of spores and crystals, or simply crystals, then dried, and then placed in petri dishes with 10 first-age larvae. After 48 and 72 h of incubation, the death rate of the larvae was estimated.

Plasmid transfer between *B. thuringiensis* strains was carried out using the method of Gonsales *et al.* [6]. For this experiment, a mixture of the donor and recipient strains of *B. thuringiensis* was incubated on a 0.45-μm-pore-size Millipore membrane for 72 h and then plated on the sporulation medium with tetracycline. The colonies grown were analyzed for their ability to produce crystals and for their serotype.

To prepare the total and plasmid DNA, cultures were grown for 8 h in a Spizizen medium supplemented with 0.1% yeast extract and 0.2% casein hydrolysate. The cells were then harvested by centrifugation. Further steps were performed according to the method of

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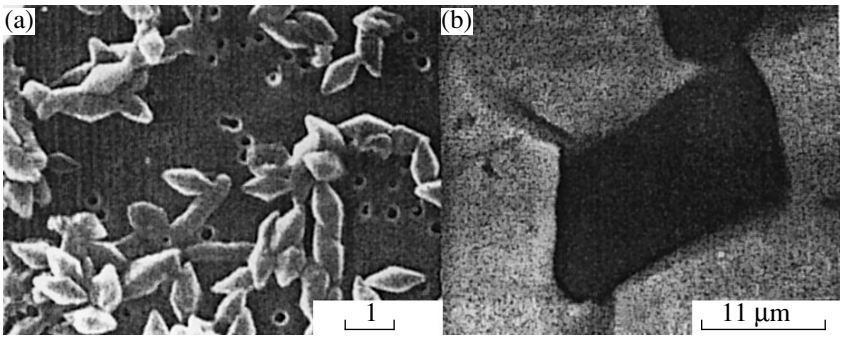

Fig. 1. Different types of endotoxin crystals: (a) small bipyramidal crystals and (b) large, flat crystals formed in *B. thuringiensis* 2-7 cells.

Kronstad *et al.* [7]. The nucleotide composition and the size of plasmids were determined with the use of 3-h electrophoresis in a 0.6% agarose gel prepared with Tris-borate buffer.

Crystal proteins were studied using the method of Laemli [8]. Dissolved crystal proteins were analyzed by electrophoresis in 12.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS). The molecular weights of the proteins were determined using a molecular weight marker kit.

Polymerase chain reaction (PCR) was performed using the method of Sambrook *et al.* [9] with oligonucleotide primers generously provided by Yu.L. Dorokhov (Belozerskii Institute of Molecular Biology and Bioorganic Chemistry, Moscow State University). The primers were synthesized in accordance with the nucleotide sequence of the *cryIIIA* gene of *B. thuringiensis* subsp. *tenebrionis*: TATGGATCCGGGAGAGAAGAAAAATGAATCCGAA (primer 1); CCAAGCTTTAATTCACTGGAATAAATTCAA (primer 2); CTGCATAGAATTCAATTTCAC (primer 3); TTGGAACCGCGTGAAATTGA (primer 4); AACAGATGAACCTCTAGAAAA (primer 5); and GCTATCCCTTTTCTAGAGGT (primer 6).

RESULTS AND DISCUSSION

Microscopic examination of one of the isolates showed that it produces small (1×1 μm) bipyramidal and large (1.8×2.4 μm) flat crystals (Fig. 1). Plating this isolate onto the sporulation medium allowed us to separate it into two strains. The strain with small crystals was found to be only slightly toxic to the Colorado potato beetle larvae, while the strain forming large crystals (designated *B. thuringiensis* 2-7) possessed an insecticidal activity comparable with that of the known insecticidal strain *B. thuringiensis* subsp. *tenebrionis*.

The serotype of the *B. thuringiensis* 2-7 strain, which was determined by an agglutination reaction using a standard set of antisera against the flagellar H antigen, turned out to be H8.

Generally, *B. thuringiensis* strains of serotype H8 contain at least five plasmids with a high molecular weight. The chromatographic analysis of the plasmid DNA of *B. thuringiensis* 2-7 revealed only two large plasmids with molecular masses of approximately 58 and 56 MDa. The possibility that larger plasmids (greater than 105 MDa in size) are not seen on the electrophoretogram (Fig. 2) cannot be excluded.

Since it is known that the genes encoding insecticidal crystal proteins are commonly located on the *B. thuringiensis* plasmids, we performed relevant studies on the *B. thuringiensis* 2-7 isolate.

There were two types of experiments. In the plasmid curing experiments, we attempted to eliminate plasmids by exposing the isolate to 43°C, to discover if the loss of plasmids correlated with the loss of insecticidal activity. In the experiments on plasmid transfer, the crystalliferous *B. thuringiensis* 2-7 strain was incubated with the non-crystalliferous *B. thuringiensis* subsp. *galleriae* strain, and the latter was then tested for the presence of plasmids and insecticidal activity.

Incubation of the *B. thuringiensis* 2-7 cells at 43°C and then plating them on the sporulation medium resulted in variants that did not produce insecticidal crystals. The plasmid composition of these variants was similar to that of the parent strain, as evidenced by the same two plasmid bands on the electrophoretograms of the native and cured strains (Fig. 2). This suggests that the ability of *B. thuringiensis* 2-7 to form insecticidal crystals is not related to plasmids with molecular masses of 58 and 56 MDa.

The plasmid transfer experiments were carried out with the donor strains *B. thuringiensis* subsp. *tenebrionis* (control hybridization), *B. thuringiensis* 2-7 (experimental hybridization), and the recipient strain *B. thuringiensis* subsp. *galleriae* tet cry$^-$ of serotype H5. The criteria for selection of the transcipients was their resistance to tetracycline and their affiliation to serotype H5. The transfer frequency of the cry$^+$ character (the ability to form crystalline inclusions) was high (0.5 and 0.1%) during both the control and experimental hybridizations.

The cry$^+$ transcipients of both control and experimental types of hybridization possessed insecticidal

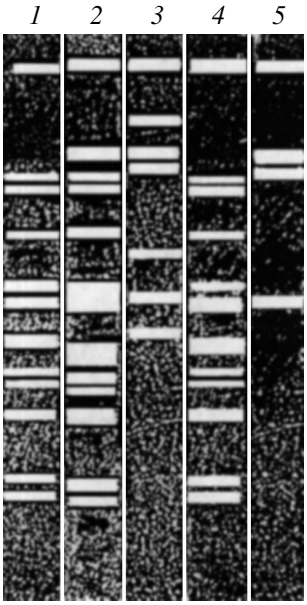

Fig. 2. Plasmids in donor and recipient *B. thuringiensis* strains and their transcipients. Lanes: (*1*) *B. thuringiensis* subsp. *galleriae* tet cry$^-$; (*2*) transcipient *B. thuringiensis* subsp. *galleriae* tet cry$^-$ × *B. thuringiensis* subsp. *tenebrionis*; (*3*) *B. thuringiensis* subsp. *tenebrionis*; (*4*) transcipient *B. thuringiensis* subsp. *galleriae* tet cry$^-$ × *B. thuringiensis* 2-7; and (*5*) *B. thuringiensis* 2-7.

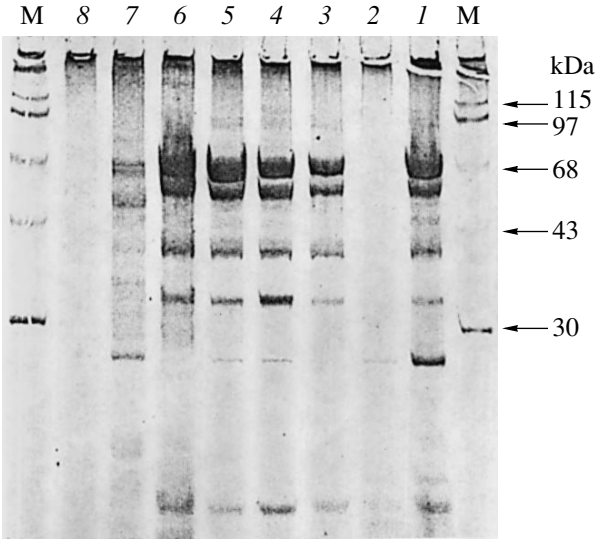

Fig. 3. Electrophoresis of soluble crystal proteins. Lanes: (*1*) *B. thuringiensis* 2-7; (*2*) *B. thuringiensis* 2-7 cry$^-$; (*3*) *B. thuringiensis* subsp. *tenebrionis*; (*4–6*) transcipients *B. thuringiensis* 2-7 × *B. thuringiensis* subsp. *galleriae* tet cry$^-$; (*7*) transcipient *B. thuringiensis* subsp. *tenebrionis* × *B. thuringiensis* subsp. *galleriae* tet cry$^-$; and (*8*) *B. thuringiensis* subsp. *galleriae* tet cry$^-$. M denotes molecular weight markers.

activity, but the activity of the experimentally hybridized clones was more pronounced. Furthermore, unlike the control hybridization clones, the clones from the experimental hybridization contained large crystals of their donor strain.

Our studies showed that the 90-MDa plasmid of the donor strain *B. thuringiensis* subsp. *tenebrionis* was transferred to the control cry$^+$ transcipients (Fig. 2). The experimental transcipients that formed insecticidal crystals had no plasmids typical of their donor strain, *B. thuringiensis* 2-7.

The acquisition of the ability to form crystals with insecticidal activity in the control variant of hybridization correlated with the transfer of 90-MDa plasmid. No such correlation was observed during the experimental-type hybridization. In the latter case, however, the transcipients might acquire the ability to form insecticidal crystals through the transfer of a large plasmid, which is invisible on the electrophoretograms of the donor strain *B. thuringiensis* 2-7 and transcipient strains because of its degradation in the plasmid isolation process.

The spore formation capacity of *B. thuringiensis* subsp. *tenebrionis* was found to be 20% higher than that of *B. thuringiensis* 2-7. The efficiency of crystal formation in these strains, determined by evaluating the amount of insecticidal crystal protein in cell-free homogenates, was higher in *B. thuringiensis* 2-7 (0.9 mg/ml) than in *B. thuringiensis* subsp. *tenebrionis* (0.3 mg/ml). This difference was probably due to a higher spore content in the homogenate of *B. thuringiensis* subsp. *tenebrionis* cells, since the amounts of crystal protein in the cell-free homogenates of the two strains after the removal of spores were almost identical.

Electrophoresis in 12.5% polyacrylamide gel with SDS showed that both of these strains contained two protoxins with molecular masses of 68 and 65 kDa (Fig. 3). The protoxins of the two strains probably possess the same insecticidal activity, since preparations containing equal amounts of these crystals in their strains had similar activities.

Based on the nucleotide sequences of *cry* genes [9, 10], the Cry proteins of *B. thuringiensis* are divided into 14 types, 13 of which are grouped into four classes. The crystal proteins toxic to Coleoptera are encoded by the *cryIII* genes. This class of genes has recently been divided into six subclasses of *cryIIIA* through *cryIIIF.*

To discern whether the protoxin of *B. thuringiensis* 2-7 is encoded by the *cryIII*-type gene, we compared the nucleotide sequence of the gene controlling the production of crystal protein in this strain with the known nucleotide sequence of the *cry* gene of *B. thuringiensis* subsp. *tenebrionis.* To do this, we amplified the total and plasmid DNA of *B. thuringiensis* 2-7 with templates composed of the oligonucleotides corresponding to the three overlapping fragments of the *cryIII* gene (Fig. 4) [11]. The analysis revealed 100% homology of the PCR products of the total DNA to the known nucleotide sequence of the *cryIII* gene. When the plasmid DNA was used for amplification, PCR products were not formed. Therefore, the gene controlling crystal formation in *B. thuringiensis* 2-7 belongs to the *cryIIIA*-

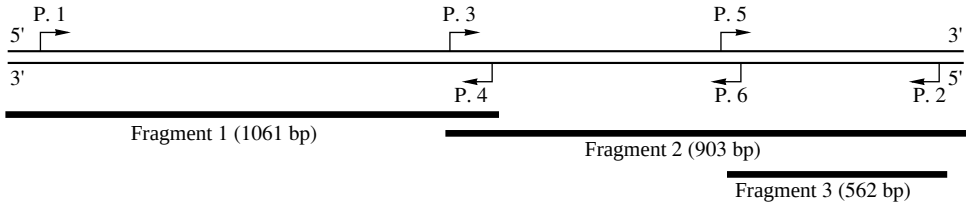

Fig. 4. Diagram illustrating the PCR analysis of the *cryIII* gene (2400 kb) of *B. thuringiensis* 2-7 with the use of various primers (P): fragment 1 (P1–P4); fragment 2 (P3–P2); and fragment 3 (P5–P2).

subclass of genes (this was confirmed by sequencing the data). The absence of amplification products in the plasmid DNA can be explained by the location of the *cry* gene on a very large plasmid, which, due to its extreme size, occurs in the chromosomal fraction of bacterial DNA.

Thus, due to its specific relevant characteristics, such as double the usual size of the endotoxin crystals, the presence of two or three plasmids (such a number of plasmids is not typical of other strains of *B. thuringiensis* with insecticidal activity), and the location of the *cryIIIA* gene on a very large plasmid, *Bacillus thuringiensis* 2-7 can be considered to be a new insecticidal strain active against Coleoptera.

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