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NOVEL *CRY*-TYPE GENES DETECTED BY EXTENDED PCR SCREENING FROM FIELD-COLLECTED STRAINS OF *BACILLUS THURINGIENSIS*

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ABSTRACT

A rapid method for identification of *Bacillus thuringiensis* strains was established by using extended multiplex PCR (Ben Dov et al., 1997). Using this method we found seven distinct *cry*-type profiles and recorded their distribution in Israel, Uzbekistan and Kazakhstan. Three new genes with potential insecticidal activity were identified. One of them seems to be a naturally occurring recombinant, apparently composed of *cryI*- and *cryIV*-type genes. The other two (apparently homologous to *cryIIIF* and *cryIIIG*) seem to include various combinations with either *cryI* or *cryIV*.

KEY WORDS: PCR screening, *Bacillus thuringiensis, cry* genes, biological control of pests.

INTRODUCTION

The very properties that rendered the chemical pesticides useful — long residual action and toxicity to a wide spectrum of organisms, have brought about serious environmental problems. The emergence and spread of insecticide resistance in many species of vectors, the concern with environmental pollution and the high cost of the new chemical insecticides made it apparent that vector control can no longer depend upon the use of chemicals. Thus, increasing attention has been directed toward biological agents that would be highly toxic to the target organism while being safe to nontarget organisms, able to being mass-produced on an industrial scale, have a long shelf life, can be applied using conventional application technology and being transportable (Carlton et al., 1990; Bernhard and Utz, 1993; Van Frankenhuyzen, 1993; Margalit et al., 1995). The unique capacity of insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*Bt*) subspecies has spurred their use as natural control agents in agriculture, forestry and human health. The scientific benefits of the discovery of novel insecticidal bacteria will find their expression in environmentally safe bio-control practices and will lead to increased food production and post-harvest protection.

The use of *Bt* as a commercial insecticide is based on its remarkable ability to produce large quantities of insect-larvicidal proteins, that may reach as much as 20–30% of the dry cell mass. The insecticidal proteins, also known as δ-endotoxin, form crystalline inclusion bodies during

sporulation (Bulla et al., 1980; de Barjac and Sutherland, 1990). The ICP genes, normally associated with large plasmids, have been classified as *cryI-VI* (the old nomenclature) depending on the host specificity and the degree of amino acid homology. The *cryI* class is toxic to Lepidoptera, *cryII* — to Lepidoptera and Diptera, *cryIII* — to Coleoptera, *cryIV* — to Diptera, *cryV* — to Lepidoptera and Coleoptera and *cryVI* — to nematodes (Höfte and Whiteley, 1989; Feitelson et al., 1992; Margalit et al., 1995). The multitude of ICP genes directs the synthesis of related proteins to form either heterogeneous crystalline inclusions or separate crystals with distinct morphologies.

Isolation and identification of novel *Bt* isolates by bioassays is a long and complicated process, impeded by repeated isolation of the same strains. On the other hand serotypes of *Bt* strains do not directly reflect the specific *cry* gene class(es) in the corresponding bacteria. The Polymerase Chain Reaction (PCR), which is a highly sensitive method to detect rapidly and identify target DNA sequences (Saiki et al., 1988), requires minute amounts of DNA, and allows quick, simultaneous screening of many *Bt* samples, to classify them and predict their insecticidal activities. Several workers (Carozzi et al., 1991; Bourque et al., 1993; Kalman et al., 1993; Chak et al., 1994; Ceron et al., 1995; Kuo and Chak, 1996) have employed PCR in order to screen numerous *Bt* isolates: to predict insecticidal activity, to identify and determine distribution of *cry*-type genes and to detect new *cry*-type genes.

In this paper we used a PCR strategy for extended multiplex rapid screening of field-collected *Bt* strains that harbor genes from classes *cryI, cryII, cryIII* and *cryIV* (Ben-Dov et al., 1997). This strategy will enrich the existing arsenal of insecticidal strains, identify novel genes or new combinations of known genes and predict their insecticidal activities. Novel strains displaying unknown *cry* profiles (containing new genes or combinations) should be further characterized by bioassays.

MATERIALS AND METHODS

Bacterial strains

Standard *Bt* strains were kindly supplied by Dr. D.R. Zeigler (Bacillus Genetic Stock Center, Columbus, Ohio). The other *Bt* strains that we used were isolated from soil and cadaver samples collected in Israel, Kazakhstan and Uzbekistan.

Isolation of *Bt* from soil samples

Sporeforming strains were isolated according to the acetate selection protocol of Travers et al. (1987). An aliquot (0.5 g) of soil or insect cadavers was added to 10 mL of LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter), buffered with 0.25 M sodium acetate, which selectively inhibits the germination of *Bt* spores. The mixture was shaken (250 rpm) in a 125 mL flask at 30°C for 4 h. At the end of this period a 1.5 mL sample was taken, heat-shocked at 80°C for 3 min, plated on L broth solid medium and incubated overnight at 30°C. Random colonies were inoculated into 5 mL T3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate [pH 6.8] and 0.005 g $MnCl_2$ per liter) and allowed to sporulate overnight at 30°C. Cultures were checked microscopically for the presence of crystals, which were used as the criterion for *Bt* isolates. For storage, stocks of the liquid T3 cultures were kept at –70°C after addition of glycerol to 15%. Lyophilized stocks were also prepared from 96-h cultures that were washed twice with sterile distilled water.

Oligonucleotide PCR primers

To identify *cryI, cryII, cryIII* and *cryIV*-type genes we used five pairs of universal and 13 specific primers as previously designed (Ben-Dov et al., 1997). The 10 specific primers for identification of eight *cryI* genes were used as previously designed by Kalman et al. (1993). PCR products were easily identified by electrophoresis in agarose gels.

Preparation of DNA templates and PCR

Templates were prepared from 16–18 h cultures in L or in tryptic soy (Difco) broth enriched with 0.3% (w/v) yeast extract. Aliquots of 3–4.5 mL were harvested by centrifugation, washed once in TES (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl), and the pellets resuspended in 100 µL of lysis buffer (25% sucrose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 4 mg/mL lysozyme). The cell suspension was incubated at 37°C for 1 h. DNA extraction was performed according to Birnboim and Doly (1979).

PCR amplifications (30 reaction cycles each) were routinely carried out in a 25 µL reaction volume in a DNA MiniCycler (MJ Research, Inc., Watertown, Massachusetts, USA). 1 µL of template DNA was mixed with PCR reaction buffer, 150 µM of each dNTP, 0.1–0.5 µM of each primer, and 0.5U Taq DNA Polymerase (Appligene). PCR cycle conditions were: denaturation of template DNA at 94°C for 1 min, annealing of templates and primers at 54–60°C for 40–50 sec, and extension of PCR products at 72°C for 1–1.30 min. Each experiment was done with a negative (without DNA template) and a positive (with a standard DNA template) control. An aliquot of the PCR reaction products (7–9 µL) was analyzed by agarose gel (0.8–2.5%) electrophoresis, after staining with ethidium bromide, and visualized under UV light.

The reliability of the *cryI, -II, -III* and *-IV* oligonucleotide primers for detection of *cry*-type genes from *Bt* strains has been verified by use of the well known *Bt* strains: *Bt kurstaki* HD-1 for *cryI* and *cryII* classes, *Bt tenebrionsis* and *dakota* for *cryIII* class and *Bt israelensis* for *crylV* class.

RESULTS AND DISCUSSION

Several hundred field-collected samples were isolated in Israel, Uzbekistan and Kazakhstan; of these, about two hundred spore-forming *Bt* isolates were identified by the *cry* universal (Un) primers and the positive 126 isolates were further specified by the appropriate specific primers (Ben-Dov et al., 1997). Based on their *cry*-type gene content (by Un primers), they can be divided into several groups with different potential larvicidal activities (Fig. 1). In Israel and Uzbekistan we found three distinct *cry* profiles from which only one (*cryIV*) was identical. In Kazakhstan we found six distinct *cry* profiles; two of these profiles (*cryI* and *cryII*) were unique to Kazakhstan. The different *cry*-type profiles of *Bt* strains isolated in the three countries may be explained by biological and environmental variations between the different sampling locations. Climatic variations, for example, were shown to affect the distribution of *cry*-type genes of *Bt* isolates (Chak et al., 1994.).

Profiles *cryI+cryII+cryIII* and *cryIV+cryIII* apparently contain new combinations of *cry* genes, with promising potential properties to be utilized as biological control agents against pests that are not susceptible to the known *Bt* strains. For example, the new combination of the endotoxins CryIA(b), CryIB and CryIIA in a *Bt* isolate were toxic to the common house fly, *Musca domestica* (Hodgman et al., 1993).

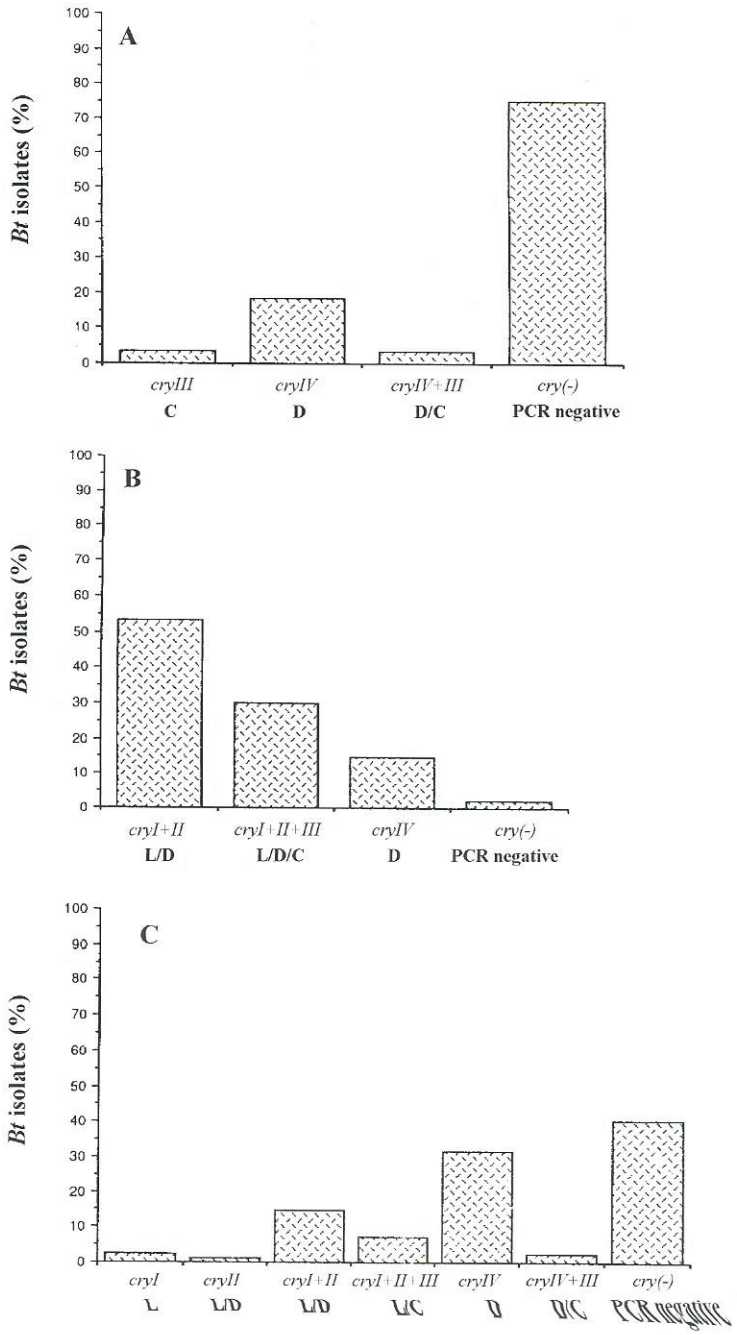

Fig. 1. Distribution of *cry*-type gene profiles identified by universal primers among the *Bt* isolates. A: in Israel; B: in Uzbekistan; C: in Kazakhstan. Predicted target organisms: L, Lepidoptera; D, Diptera; C, Coleoptera.

Many of the new *Bt* isolates did not give any PCR products with all of our universal primers. Some of these negative isolates may not harbor any toxin gene(s); however some may contain new Cry proteins with novel insecticidal activity and thus represent potentially new biological control agents.

We found no strain that harbors *cryIIIA, -B, -D* gene. We did, however, find several isolates that gave the appropriate DNA fragment with the universal primers Un7,8 for *cryIIIC, -E, -F, -G* genes. It is noteworthy that none of the latter *cryIII* isolates reacted with the specific primers of this *cry* group. Some of our *cryIII* positive isolates amplified either of two unexpected products (~700 or ~300 bp), using two direct specific primers EE-8B(d) and EE-8C(d) (Ben-Dov et al., 1997). These field-collected isolates apparently contain a new variant(s) of the *cryIII*-type gene(s).

One of our isolates (DL-1) produced a 1.8 kb fragment (Fig. 2) with a mixture of two pairs of the universal primers (Un1 and Un4; Ben-Dov et al., 1997). Further analysis revealed that it was obtained with the direct primers Un1(d) and Un4(d). This isolate also produced another, minor PCR product, of ~400 bp (Fig. 2), with the reverse primer Un4(r) alone. Further efforts to identify this crystal forming strain are now in progress.

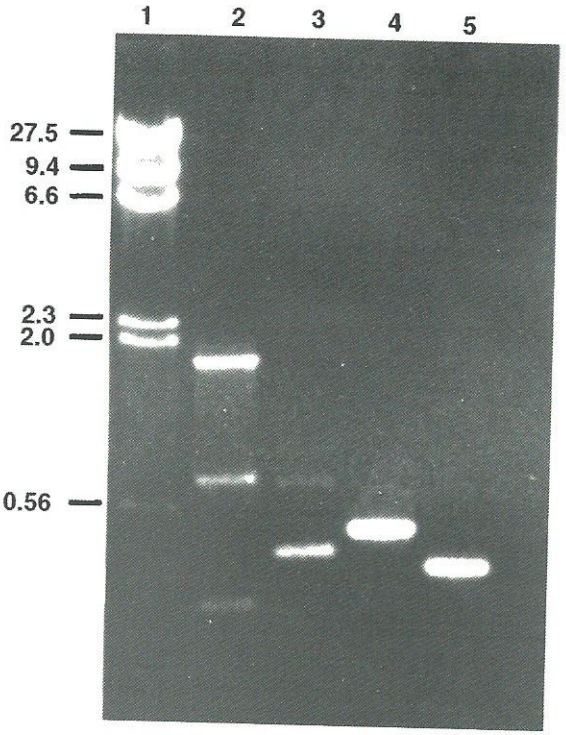

Fig. 2. Agarose gel electrophoresis analysis of PCR products from strain DL-1 obtained by using *cryI* and *cryIV* universal primers (Un). Line 1, molecular weight markers (λDNA cleaved by *Hin*dIII), with sizes (in kb) indicated on left; line 2, strain DL-1 with Un1(d) and Un4(d) primers; line 3, strain DL-1 with Un4(r) primer alone; line 4, *Bt israelensis* 4Q5 with Un4(d) and Un4(r) primers for *cryIV* genes; line 5, *Bt kurstaki* HD-1 with Un1(d) and Un1(r) primers for *cryI* genes.

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