PCR-Based Identification of *Bacillus thuringiensis* Isolated from Soil Samples in Nigeria

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Six isolates of *Bacillus thuringiensis* isolated from soil samples confirmed to be toxic to mosquito larvae were differentiated using a PCR-Based technique. Three of these isolates initially identified using a serological technique were further differentiated with the PCR amplification of the δ-endotoxin target sequences. Using the total DNA of isolates as template, at least four isolates yielded amplicons one or all the crystal protein genes, cryI a, b, c, or II with sizes ranging from 238–1070 bp. None of these isolates yielded an amplicon for any of Cry IV A, B and D tested. Of the four isolates identified by PCR technique one isolate remained unidentified by serology.

Introduction

The gram positive bacterium *Bacillus thuringiensis* has been identified and used as a biopesticide in the control of both agricultural and medically important insects in several parts of the world. Strains of this bacterium have been identified to be pathogenic to specific orders of insect, namely lepidoptera, diptera, coleoptera and recently spodoptera (Hofte and Whiteley, 1989). This broad spectrum of activity has given it a widespread use in the control of these orders of insect, with new strains being identified having potential to control some members of these orders that are otherwise not known to be susceptible to the pathogenic substance, which are crystal proteins produced by previously identified *B. thuringiensis* strains. Identification of these bacterial strains had previously been done via the conventional microbiological culture techniques and serological assays, processes that are cumbersome and long. The use of the PCR technique offers a robust, efficient and faster alternative method to identify *B. thuringiensis* from other *Bacillus* isolated and even differentiates strains and subspecies of this bacterium (Bourque *et al.*, 1993; Brousseau *et al.*, 1993). This paper reports the PCR-based identification of dipteran and lepidopteran specific strain of *B. thuringiensis* isolated from soil samples collected from different locations in Nigeria and the types of crystal proteins produced in this strain.

Materials

Isolates used are listed in Table II. Reference strains used and primers were provided by Dr. Jin Byung-Rae, College of Agriculture and Natural Sciences, National University of Korea, Swon, Korea, Taq polymerase and deoxynucleotide triphosphates (dNTP) were purchased from Promega (Madison, WI 53711, USA). Amplification was done using the PCR apparatus from MJ Research, Watertown, MA, U. S. A; (Model PTC 150).

Isolation of Bacillus from soil samples

One gram of soil was subjected to acetate selection as described earlier (Travers *et al.*, 1987) combined with an anaerobic culture to inhibit the growth of obligate aerobes. An average of sixty colonies was obtained. After acetate selection, about 20% (10 colonies) of these colonies were picked and cultured onto a T_3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M NaH_2PO_4, pH 6.8 and 0.005 g $MnCl_2$). From these cultures *B. thuringiensis*-like isolate was picked

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and was identified further with the following biochemical identification tests: lecithinase activity, penicillin resistance, starch hydrolysis, salicin and esculin utilization, and microscopic examinations (Buchanan and Gibbons, 1974).

Isolates being positive to these tests were selected for growth at 42 °C and 50 °C respectively, followed by mortality test and growth in 7% sodium chloride broth.

Primer selection

Primers used in this study were selected according to known mosquito larvicidal *B. thuringiensis* δ-endotoxin gene sequences. Set of primers specific for target sequence of these genes were used for PCR amplification. These are summarized in Table I.

Preparation of DNA template for PCR

Plasmid DNA isolation was done by alkaline lysis method described earlier (Sambrook *et al.*, 1989), followed by phenol-chloroform extraction and DNA precipitated with ice cold absolute ethanol. Precipitate was centrifuged at 14,000 rpm for 15 min and pellet was washed with 70% ethanol at room temperature, pellet was allowed to dry and suspended in TE buffer (10 mM Tris buffer, (hydroxymethyl) aminomethane, 1 mM EDTA) pH 8.0.

PCR amplification and identification of crystal protein genes

DNA target amplicons were prepared using PCR technique. The DNA amplification was carried out using a primer set derived from Cry I, II, IVA, IVB and IVD genes. The amplification reaction mixture (100 μl) contained 10 mM Tris pH 9.0, 50 mM KCI, 1.8 mM $MgCl_2$, 0.001% Triton X-100, 200 μM each of the deoxynucleoside triphosphates (Promega), 1.5 units of Taq polymerase, 0.5 μM each of the primers and 2.0 μl of template plasmid DNA, and overlaid with 50 μl mineral oil in a 500 μl tube. The amplification was performed, with a 5 min initial denaturation at 95 °C followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min and extension at 72 °C for 2 min. The products (10 μl) from each reaction were resolved by electrophoresis for 40 min at a constant voltage of 100V in a 1.4% agarose gel in Tris-acetate-EDTA (TAE) buffer containing 0.5 μg/ ml ethidium bromide. Hae III digest of φ X 174 DNA was used as DNA size markers. The DNA bands in the gel were illuminated by UV light and photographed using Polaroid Film 667.

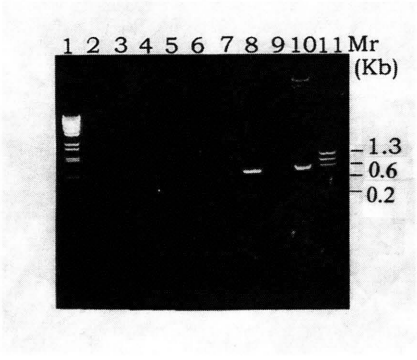
Results and Discussion

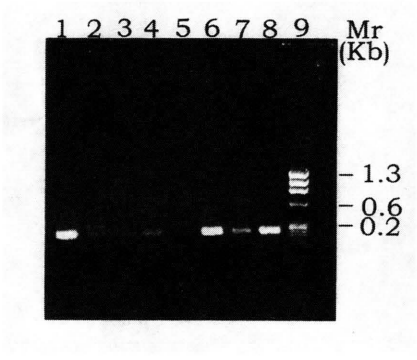
The isolates used in this study were recently isolated from soil samples in Nigeria. Identification of isolates becomes necessary when antibodies to the H-flagellar are not available. Besides unavailability, the generation of antibodies in mouse is cumbersome and could be unreliable sometimes because of cross-reactivity. Therefore, a technique such as the PCR is useful because of its specificity and ability to identify strains of bacteria within a very short time.

The PCR amplification using the primers listed in Table I for specific target sequences within the δ-endotoxin genes found in different *B. thuringiensis* strains with mosquito larvicidal activity showed that the amplicons of 4 isolates, OA-10, OA-48, OA-53 and OA-59 are not related to those found in *B. thuringiensis israelensis.* Primers selected for cryIAa, b, c, genes yielded amplicons that ranged between 238 and 782 bp. Each set of sense and anti-sense primer was tested separately for specificity by mixing with other primers in a multiplex amplification to confirm the specificity for their target regions. The result confirmed their specificity for the target regions with distinct bands visible in agarose gels and showed that each of the cryIA genes could be differentiated as observed in Figure 1a, b, and c. Also, primer sets designed on sequences of the mosquito larvicidal genes CryII, IVA, B, and D revealed that only the cryII gene was distinguishable on agarose gel with an amplicon size of 1070 bp (Fig. 2). The information obtained suggest that local isolates do not encode all the genes tested. PCR amplification using Cry IVD specific primers with DNA from isolates as template did not yield amplicons, neither did primers specific for Cry IVA, B and C yield amplicons. The amplification with a Cry II target region specific primers observed in *B. thuringiensis morrisoni* confirmed the presence of Cry II related toxin in this strain. PCR has proven efficient and

Table I. Primers used to amplify target regions.

Toxins	Primer Sequence	Amplicons	Target seq
Cry 1A(a)	5'- GAGCCAAGCGACTGGAGCAGTTTACACC(28)	782	1910–1943
Cry 1A(b)	5'- TCGAATTGAATTTGTTCC(18)	238	2453–2470
Cry 1A(c)	5'- TCACTTCCCATCGACATCTACC(22)	550	2159–2170
Cry 13 (Antisen)	5'- ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC(33)		2658–2690
Cry 115	5'- CAGATACCCTTGCTGGTGTAA(21)	1070	475–495
Cry 113	5'- ATAGGCCCGTGCCCACCAGG(21)		1524–1544
Cry IV A5	5- CGAGGTGAAATTTGCTCC(18)	1032	1926–1943
Cry IV A3	5'- ATGGCTTGTTTCGCTACATC(20)		2938–2957
Cry IV B5	5'- GGTGCTTCCTATTCTTTGGC(20)	2610	372–391
Cry IV B3	5'- ATGGCTTGTTTCGCTACATC(20)		2962–2981
Cry IV C5	5'- ATGAATCCATATCAAAATAAG(21)	2040	941–961
Cry IV C3	5'- AAGAACTTTGTTTTAATTAAC(21)		2960–2980
Cry IV D5	5'- ATGGAAGATAGTTCTTTAGAT(21)	1932	41–61
Cry IV D3	**5'- CTACTTTAGTAACGGATT(18)**		**1955–1972**





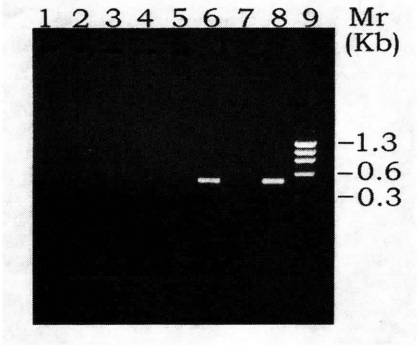


Fig. 1. Amplification of specific region of *B. thuringiensis* isolates crystal protein genes using different cryIA primers. (a) Photograph of TAE-Agarose gel (1.4%) electrophoresis of nucleic acid amplification products of Cry IAa primers. Primers corresponding to specific region of cryIAa gene were used to amplify a 782 bp target from total DNA of *B. thuringiensis,* lane 1-BstEII λ-DNA digest, lane 2 -*B. thuringiensis morrisoni*, lane 3 -*B. thuringiensis israeliensis*, lane 4 -OA 37, lane 5 -OA 48, lane 6 -OA13, lane 7. -OA 53, lane 8 -OA 59, lane 9 -OA 10 and lane 10 -φ Hae III digest. Fig. 1b. Amplification was performed using primers specific for cryIA 238 bp region. lanes 1–9 are described in figure1a (lanes 2–10). Fig. 1 c. Amplification was performed using primers specific for cryIAb 550 bp region. Lanes 1–9 are as described in figure 1a, lanes 2–10.

specific for the identification of unknown isolates and the technique is faster and less cumbersome when compared with conventional serological agglutination using flagellar antibodies (Bourgue *et al.*, 1993; Brousseau *et al.*, 1993 and Bravo *et al.*, 1998). The results obtained from the PCR amplification is consistent with the confirmatory agglutination test with each of H1-H27 antibody used in serological analysis of isolates. This strain was reported to be toxic to lepidopteran and dipteran (Widner and Whiteley, 1989). While isolates OA-37, OA-48 and OA-13 could not be identified by serological technique, however, isolate OA-48 was identified with a cry II related gene as evidenced from the PCR amplicon. Isolates OA-37 and OA-13 could not be identified with any of the selected

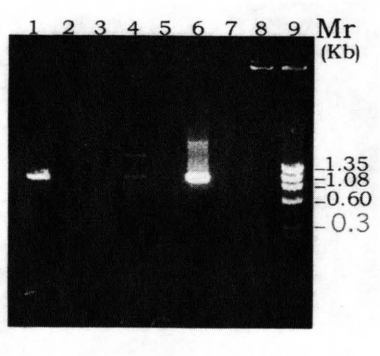


Fig. 2. Amplification was performed using primers specific for the cryII 1070 bp region. Lanes 1–9 are described in figure1a as lanes 2–10.

Table II. Distribution of different cry-genes found in isolates using PCR amplification technique.

PCR amplification Profiles of cry-genes found in isolates					
Isolates	CryIAa	CryIAb	CryIAc	CryII	CryIV
B. thuringiesis OA-10	+	+	+	–	–
B. thuringiensis OA-59	–	+	+	–	–
B. thuringiensis OA-53	+	+	+	+	–
B. thuringiensis OA-37	–	+	–	–	–
B. thuringiensis OA-48	–	+	+	+	–
B. thuringiensis OA-13	–	+	–	–	–
Bti HD-14	–	+	–	–	+
Btm HD-2	–	+	–	–	+

Bti represents *B. thuringiensis israelensis* HD-14, Btm represents *B. thuringiensis morrisoni* (positive controls) and PCR is polymerase chain reaction.

primers. In short, PCR-based identification of bacteria strains is efficient and may become a popular identification technique that can comfortably circumvent usual biochemical and serological identification procedures.

In this report, isolates were identified easily with PCR technique and differentiated according to the types of crystal proteins encoded by each of these isolates, therefore, the primers for target regions within the δ-endotoxin genes of cry Ia, b, c and II are suitable for the identification purpose in this study. The information obtained from the PCR amplification revealed the identity of the different δ-endotoxin genes harboured by each of OA-10, OA-59 and OA-53 isolates confirmed that these isolates might be identical. While the other three may be related or unidentified isolates by the available H-flagellar antibodies even though one of these was identified to harbour a crystal protein II gene but its actual serotype has still not been confirmed.

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