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Identification and Characterization of *cry* Genes Coding for Insecticidal Crystal Protein in *Bacillus thuringiensis* JC 590

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ABSTRACT

The detection of *cry*I, *cry*II and *cry*V genes in chromosomal and plasmid DNA of *Bacillus thuringiensis* JC 590 was done using polymerase chain reaction (PCR). The PCR products were cloned, sequenced and subject to BLAST search at NCBI. The results revealed that 5 genes of *cry*1 namely *cry*1Ab, *cry*1C, *cry*1D, *cry*1E and *cry*1I and 1 gene of *cry*2 namely *cry*2A were in both chromosomal and plasmid DNA. The full length DNA sequence of *cry*IC was cloned by PCR walking technique, sequenced, and analyzed. The result showed that the chromosomal *cry*1C gene consisted of 3,507 bp encoded for 1,169 of amino acid and the plasmid DNA *cry*1C gene had 3,450 bp encoded for 1,160 of amino acid in length. Sequence comparison with other *cry*1C genes in database at NCBI showed these sequences had highly homology with *cry*1C genes in other *B. thuringiensis* strains. The investigation of *cry* 1C in *B. thuringiensis* JC 590 could be the basic knowledge for future application in biocontrol because of its high toxicity.

Key words: *cry* genes, *Bacillus thuringiensis,* diamondback moth

INTRODUCTION

Bacillus thuringiensis was first isolated by the Japanese scientist, Professor S. Ishiwata, from silkworm larvae exhibiting the sotto disease in 1901 (Juarez-Perez and Ferrandis, 1997). *B. thuringiensis* is a spore-forming gram-positive bacterium. During sporulation, it produces a parasporal bipyramidal protein toxin called insecticidal crystal protein (ICP) or δ-endotoxin. These proteins are toxin to insect larvae in the Orders Lepidoptera, Diptera and Coleoptera (Kuo and Chak, 1996). There are two types of δ-endotoxins: the highly specific Cry (from crystal) toxins which act via specific receptors and non-specific Cyt (cytolytic) toxins, with no known receptors. Both families of toxins are classified exclusively on the basis of their amino acid sequence identity (Juarez-Perez and Ferrandis, 1997). Some *cry* genes have been cloned and sequenced. These genes have been organized into six different groups based on their sequence similarities and ranges of specificity (Luthy and Wolfersberger, 2000). The CryI, CryII, Cry III, Cry IV, CryV proteins are toxin to Lepidoptera, Lepidoptera and Diptera, Coleoptera, Diptera, and Coleoptera, nematode larvae and Lepidoptera, respectively (Crickmore *et al*., 1998). The CryVI

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proteins are also toxin to nematodes but their origins seem to be different from those of the other Cry proteins (Feitelson *et al*., 1992). The insecticidal properties of these toxins are of great importance to agriculture and public health worldwide (Ejiofor and Johnson, 2002).

The insecticidal activity of *B. thuringiensis* is mainly due to its ability to synthesize Cry proteins. During the sporulation phase, Cry proteins are synthesized as protoxins, which are further solubilized and activated through protease action in the insect mid gut. Toxins specifically bind to protein receptors in the epithelial insect mid gut and produce pores, leading to the loss of normal membrane function (Hofte and Whitely, 1989; Luthy and Wolfersberger, 2000; Schwart and Laprade, 2000). As the result of membrane permeability, the epithelial cells lyses and feeding activity is paralyzed. Finally, insects die of starvation, septicemia or a combination of both (Juarez-Perez and Ferrandis, 1997; Schwart and Laprade, 2000).

B. thuringiensis JC 590 was isolated from soil in Thailand (Chanpaisaeng *et al*., 2001). It was tested for its insecticidal activity against diamondback moth (*Plutella xylostella*) under laboratory condition, greenhouse and farmers' fields. The results showed that it had dramatically high toxicity and the preliminary study of *cry* genes distribution also showed that this bacterium carried *cry*I genes encoded insecticidal crystal protein against diamondback moth (Chanpaisaeng *et al*., 2001).

In this paper, the characterization and types of *cry* genes in *B. thuringiensis* JC 590 were investigated. The detection of *cry* gene effective to insects in Order Lepidoptera was carried out using polymerase chain reaction (PCR). The PCR products were cloned, sequenced and subjected to BLAST search at NCBI. The full length *cry*1C were also cloned by PCR walking technique, sequenced, and analyzed.

MATERIALS AND METHODS

Bacterial strains and media

B. thuringiensis JC590 was obtained from the culture collection of Department of Entomology, Kasetsart University. *E.coli* DH5α for PCR cloning was obtained from the bacterial stock of Department of Biotechnology, Kasetsart University. *B. thuringiensis* JC590 and *E.coli* DH5α were grown in single-strength Luria-Bertani broth (LB) at 37 °C and 30 °C, respectively.

Isolation of genomic DNA and plasmid DNA for PCR analysis

B. thuringiensis culture was incubated overnight at 30 °C in LB medium with vigorous shaking. *B. thuringiensis* genomic DNA was isolated as described by Harwood and Cutting (1990) while plasmid DNA was isolated using the method described by Birnboim and Doly (1979).

PCR

Primers for cry gene identification are listed in Table 1. Concentration of genetic DNA and plasmid DNA in PCRs was 100 ng/ml. DNA amplification was carried out in a Thermal cycle and carried out in 25 µl reaction mixture containing 1 µl of template DNA in reaction buffer, 50 mM of deoxynucleoide triphosphate (dNTP), 15 mM of each primer and 5U *Taq* DNA polymerase (Fermentus, Lithuania). Template DNA was preheated at 94 °C for 5 min. Then it was denatured at 94 °C for 1 min, annealed to primers at 45 °C for 45 s and extensions of PCR products were achieved at 72 °C for 2 min. The PCR was done for 30 cycles. Negative (without DNA template) and positive (with a standard template) controls were set for each pair of primers used. An aliquot of the reaction mixture (5 µl) was analyzed by agarose gel (1%) electrophoresis, and the products were stained with ethidium bromide and visualized under UV light. All PCR products were cloned into a pGEM-T easy vector (Promega) and

sequenced at Bio-Service Unit, National Center for Genetic Engineering and Biotechnology, Thailand. DNA sequences were compared with database at NCBI by BLAST.

PCR walking of *cry*1C on genomic DNA and plasmid DNA

Nucleotide sequences of *cry*1C available from GenBank were aligned using the ClustalW at NCBI. The highly conserved regions among all *cry*1C genes were selected and used in primer design. The designed primers for PCR walking are listed in Table 2.

RESULTS

Identification by universal primers

Two pairs of universal primers designed by Kuo and Chak (1996) were used to detect *cry* genes by the sizes of their PCR products (Table 1). The genomic DNA and plasmid DNA of *B. thuringiensis* JC 590 isolate served as the template

Table 1 Specific primer pairs used to the identification of *cry* and *cyt* genes.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
5′ end of *cry* gene	K5Un3 (CAATGCGTACCTTACAATTGTTTAAGTATT)	K3Un3(CCTCCTGTAAATCCTGGTCCT)
3′ end of *cry* gene	K5Un2 (AGG ACCAGGATTTACAGGAGG)	K3Un2 (GCTGTGACACGAAGGATATAGCCA)
*cry*1Aa	1Aa (TTCCCTTTATTTGGGAATGC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ab	1Ab(CGGATGCTCATAGAGGAGAA)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ac	1Ac (GGAAACTTTCTTTTTAATGG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ad	1Ad (ACCCGTACTGATCTCAACTA)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1Ae	1Ae (CTCTACTTTTTATAGAAACC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1B	1B (GGCTACCAATACTTCTATTA)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1C	1C (ATTTAATTTACGTGGTGTTG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1D	1D (CAGGCCTTGACAATTCAAAT)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1E	1E (TAGGGATAAATGTAGTACAG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1F	1F (GATTTCAGGAAGTGATTCAT)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1G	1G (GCTTCTCTCCAAACAACG)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1H	1H (ACTCTTTTCACACCAATAAC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1I	V(+)(ATGAAACTAAAGAATCCAGA)	V(-) (AGGATCCTTGTGTTGAGATA)
*cry*1J	1J (GCGCTTAATAATATTTCACC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*1K	1K (TGATATGATATTTCGTAACC)	I(-) (MDATYTCTAKRTCTTGACTA)
*cry*2A	II(+)(TAAAGAAAGTGGGGAGTCTT)	II(-) (AACTCCATCGTTATTTGTAG)
*cry*5	(TAAGCAAAGCGCGTAACCTC)	(GCTCCCCTCGATGTCAATG)
*cry*12	(CTCCCCCAACATTCCATCC)	(AATTACTTACACGTGCCATACCTG)
*cry*13A	cry13A(d)(CTTTGATTATTTAGGTTTAGTTCAA)	cry13A(r)(TTGTAGTACAGGCTTGTGATTC)
*cry*14	(ATAATGCGCGACCTACTGTTGT)	(TGCCGTTATCGCCGTTATT)

M = A or C, D = A G or T, Y = T or C, K = G or T, R = A or G

Table 2 Designed primers used for PCR walking of *cry*1C.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
cry 1C	1C(F,L)(ATGGAGGAAAATAATCAAAATCAATGC)	1C(R,L)(CCTCTGACTACAGAGGTACC)
	1C(F,R)(ACATGCGAAGCGACTCAGCG)	1C(R,R)(TTATTCCTCCATAAGGAGTAATTCC)

in reactions (Figure 1). The result showed that both chromosome DNA and plasmid DNA of *B. thuringiensis* JC 590 reacted positively to K5Un2 and K3Un2 by producing a fragment of 1600 bp, and to K5Un3 and K3Un3 by producing a fragment of 1400 bp.

Identification by specific primers

The specific primers designed by Juarez-Perez and Ferrandis (1997) were used to identify

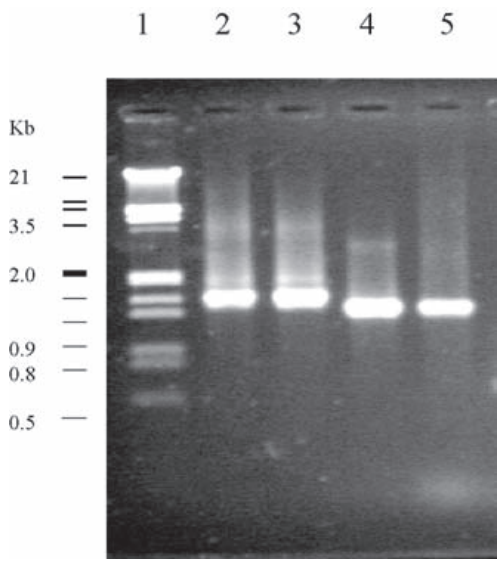

Figure 1 Agarose gel (1%) electrophoresis of PCR products amplified from *B. thuringiensis* JC 590 with universal primers. Lane 1, molecular weight markers (l DNA cleaved by *Hind* III and *Eco*RI) with sizes (in kilobases) as indicated on the left. Lane 2, plasmid DNA of *B. thuringiensis* JC 590 with K5UN2 and K3UN2 (1600 bp).Lane 3, chromosomal DNA of *B. thuringiensis* JC 590 with K5UN2 and K3UN2 (1600 bp).Lane 4, plasmid DNA of *B. thuringiensis* JC 590 with K5UN3 and K3UN3 (1400 bp).Lane 5, chromosomal DNA of *B. thuringiensis* JC 590 with K5UN3 and K3UN3 (1400 bp).

20 genes from the three *cry* groups, *cry*1, *cry*2 and *cry*5 genes (Table 1). The results showed eight different *cry* gene profiles in chromosomal DNA of *B. thuringiensis* JC 590; 5 genes of *cry*1 (namely *cry*1Ab, *cry*1C, *cry*1D, *cry*1E and *cry*1I), 1 gene of *cry*2 (namely *cry*2A), 2 genes of *cry*5 (namely *cry*12 *and cry*14) (Figure 2). Six different *cry* gene profiles were detected in plasmid DNA; 5 genes of *cry*1 (namely *cry*1Ab, *cry*1C, *cry*1D, *cry*1E and *cry*1I) and 1 gene of *cry*2 (namely *cry*2A) (Figure 3).

The PCR products were cloned, sequenced and subjected to BLAST search at NCBI. The result showed that both chromosomal DNA and plasmid DNA contained six different *cry* genes. In its chromosome, *cry*1Ab, *cry*1C, *cry*1D, *cry*1E, *cry*1I and *cry*2A consisted of 1,073, 1,139, 1,121, 1,136, 1,170, and 1,510 bp, respectively. The sequences of *cry*12 and *cry*14 PCR products cloned were not in *cry* gene groups in database. In plasmid DNA, *cry*1Ab, *cry*1C, *cry*1D, *cry*1E, *cry*1I, and *cry*2A consisted of 1,369, 1,138, 1,131, 1,126, 1,138, and 1,479 bp, respectively.

PCR walking of *cry*1C on genomic DNA and plasmid DNA

Reported nucleotide sequences of *cry*1C were chosen from GenBank. The highly conserved regions among all *cry*1C genes were selected and used to design primers. Two pairs of primers for PCR walking are listed in Table 2. PCR results of these primer are shown in Figure 4. The PCR products of each *cry*IC were cloned and sequenced. The result showed that the chromosomal *cry*1C gene consisted of 3,507 bp while that of the plasmid DNA consisted of 3,450 bp (Figures 5 and 6). Sequence comparison with other *cry*1C genes in database at NCBI with Blast-N program showed these sequences to have high homology with *cry*1C genes (M97880.1), 97% for chromosomal DNA and 95% for plasmid DNA. The sequences of the chromosomal and plasmid DNA *cry*1C gene were

translated at BCM (Boylor College of Medicine Search Launcher). The chromosomal *cry*1C gene encoded for 1,169 of amino acid and the plasmid DNA gave 1,160 of amino acid in length. Amino acid sequence compared with other Cry 1C in database at NCBI using Blast-P program, the amino acid sequences were highly homology with Cry 1C (P56953), 84% for chromosomal DNA and 54% for plasmid DNA (Figures 5 and 6).

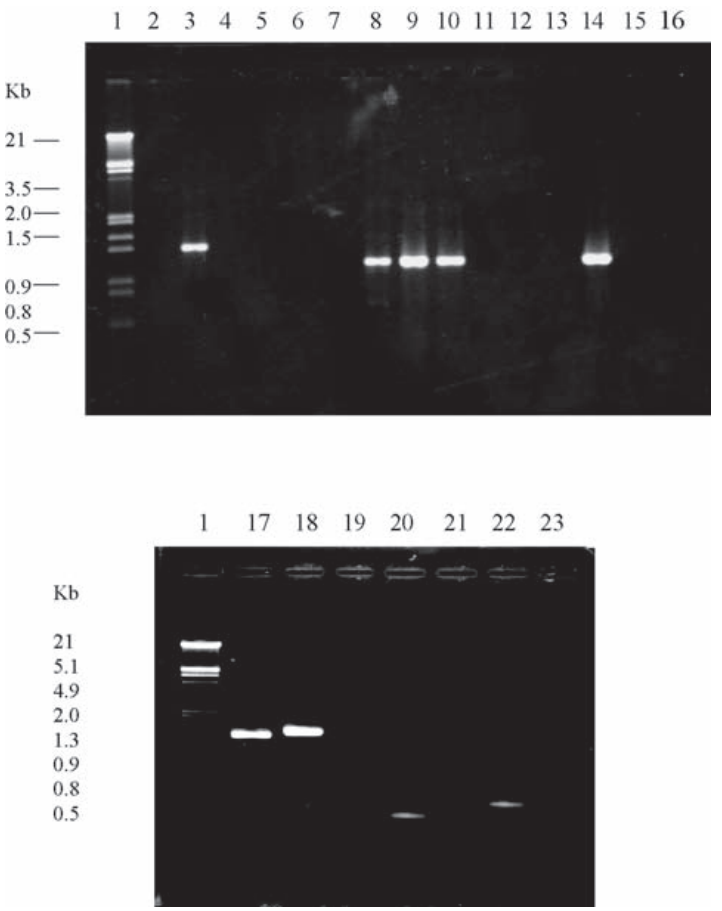

Figure 2 Agarose gel (1%) electrophoresis of PCR products amplified from *B. thuringiensis* JC 590 with specific primers. Lane 1, molecular weight markers (l DNA cleaved by *Hind* III and *Eco*RI) with sizes (in kilobases) as indicated on the left. Lane 2 to 23 , PCR products obtained from chromosomal DNA. Lane 2, primers for *cry*1Aa gene. Lane 3, primers for *cry*1Ab gene. Lane 4, primers for *cry*1Ac gene. Lane 5, primers for *cry*1Ad gene. Lane 6, primers for *cry*1Ae gene. Lane 7, primers for *cry*1B gene. Lane 8, primers for *cry*1C gene. Lane 9, primers for *cry*1D gene. Lane 10, primers for *cry*1E gene. Lane 11, primers for *cry*1F gene. Lane 12, primers for cry1G gene. Lane 13, primers for *cry*1H gene. Lane 14, primers for *cry*1I gene. Lane 15, primers for *cry*1J gene. Lane 16, primers for *cry*1K gene. Lane 17, positive control. Lane 18, primers for *cry*2A gene. Lane 19, primers for *cry*5 gene. Lane 20, primers for *cry*12 gene. Lane 21, primers for *cry*13A gene. Lane 22, primers for *cry*14 gene Lane 23, water control.

DISCUSSION

B. thuringiensis can produce insecticidal crystal protein toxin and it is widely used as biopesticide for pest control as the model organism. In this research, we selected *B. thuringiensis* JC 590 because of its high efficiency when compared with commercial strains. Identification of *cry*I genes in chromosomal DNA and plasmid DNA of *B. thuringiensis* JC 590 by

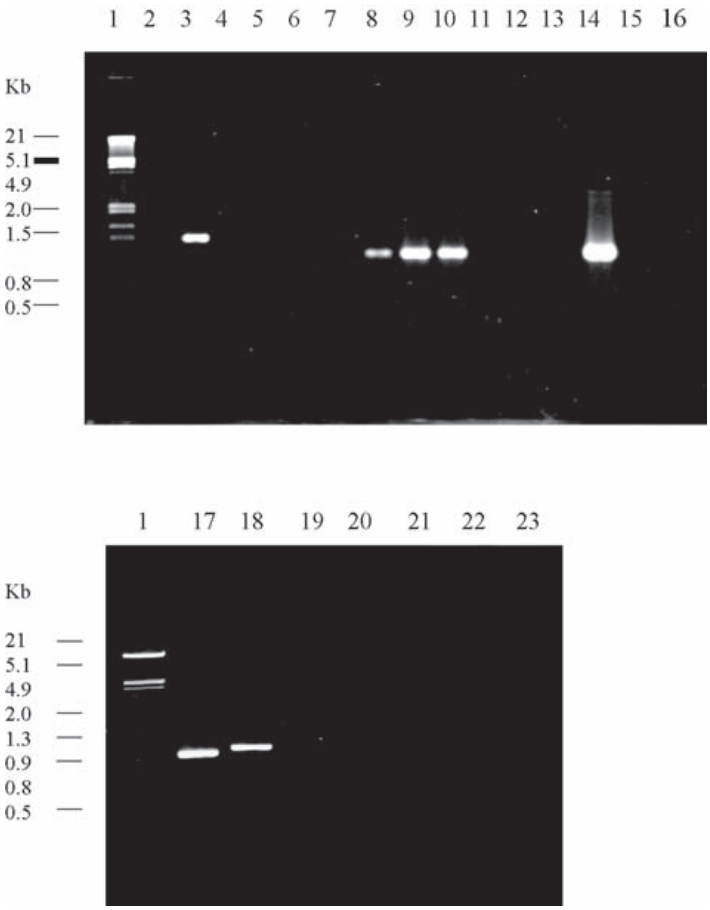

Figure 3 Agarose gel (1%) electrophoresis of PCR products amplified from *B. thuringiensis* JC 590 with specific primers. Lane 1, molecular weight markers (l DNA cleaved by *Hind* III and *Eco*RI) with sizes (in kilobases) as indicated on the left. Lane 2 to 23, PCR products obtained from plasmid DNA. Lane 2, primers for *cry*1Aa gene. Lane 3, primers for *cry*1Ab gene. Lane 4, primers for *cry*1Ac gene. Lane 5, primers for *cry*1Ad gene. Lane 6, primers for *cry*1Ae gene. Lane 7, primers for *cry*1B gene. Lane 8, primers for *cry*1C gene. Lane 9, primers for *cry*1D gene. Lane 10, primers for *cry*1E gene. Lane 11, primers for *cry*1F gene. Lane 12, primers for cry1G gene. Lane 13, primers for *cry*1H gene. Lane 14, primers for *cry*1I gene. Lane 15, primers for *cry*1J gene. Lane 16, primers for *cry*1K gene. Lane 17, positive control. Lane 18, primers for *cry*2A gene. Lane 19, primers for *cry*5 gene. Lane 20, primers for *cry*12 gene. Lane 21, primers for *cry*13A gene. Lane 22, primers for *cry*14 gene Lane 23, water control.

two pairs of universal primers designed by Kuo and Chak (1996) showed that both chromosomal DNA and plasmid DNA carried *cry*I genes. PCR analyses with specific primers effective to insect in Order Lepidoptera (Table 1) showed that both chromosomal DNA and plasmid DNA carried 5 genes of *cry*1 namely *cry*1Ab, *cry*1C, *cry*1D, *cry*1E and *cry*1I and 1 gene of *cry*2 namely *cry*2A. Tabashnik *et. al*. (1994) also reported that these genes in *B. thuringiensis* JC 590 (*cry*1Ab, *cry*1C and *cry*2A) had high toxicity to diamondback moth. The presence of these genes in chromosomal DNA can result in higher stability of these genes because there are many reports of plasmid loss in *B. thuringiensis* (Gonzalez *et al*., 1982).

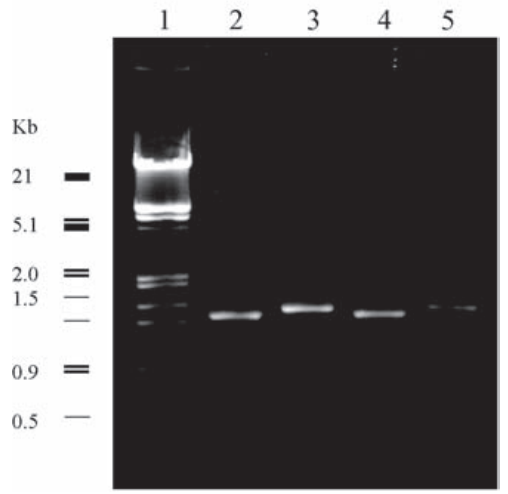

Figure 4 Agarose gel (1%) electrophoresis of PCR products amplified from *B. thuringiensis* JC 590 with specific genes. Lane 1, molecular weight markers (l DNA cleaved by *Hind* III and *Eco*RI) with sizes (in kilobases) as indicated on the left. Lane 2 and 3, PCR products obtained from chromosomal DNA; Lane 2 primers 1C(F,L) and 1C(R,L), Lane 3, primers 1C(F,R) and 1C(R,R). Lane 4 and 5, PCR products obtained from plasmid DNA; Lane 4 primers 1C(F,L) and 1C(R,L), Lane 5, primers 1C(F,R) and 1C(R,R).

Appearance of the same *cry* genes both chromosomal DNA and plasmid DNA could be the best explanation for high efficiency of *B. thuringiensis* JC 590 against diamondback moth and other pest.

DNA sequence of a full sequence *cry*IC in chromosome DNA showed that the chromosomal *cry*1C gene consisted of 3,507 bp encoded for 1,169 of amino acid. But on plasmid DNA, there were 3,450 bp of *cry*1C encoded for 1,160 of amino acid in length. Sequence comparison with other *cry*1C genes in database at NCBI showed that these sequences had high homology with *cry*1C genes in other *B. thuringiensis* strains. Amino acid sequence comparison with other Cry 1C in database at NCBI also showed these amino acid sequences to have high homology with Cry 1C in other *B. thuringiensis* strains. The DNA and amino sequences of *cry* 1C and Cry 1C were not significantly different from the previous reports. However, the presence of cry 1C in both chromosomal and plasmid DNA could draw interest because the toxicity of Cry 1C to the important insect pest (Tabashnik *et. al*., 1994). The knowledge of these genes might lead to the novel application by mean of genetic engineering. The result presented here is a preliminary report of *cry* genes study in *B. thuringiensis* JC 590. This result can be the first step in understanding gene organization, expression and control in this bacterium, leading to the better application of *B. thuringiensis* as bio-pesticide in the future and provided basic knowledge for future study on *cry* gene expression and insect resistance to *B. thuringiensis*.

CONCLUSION

The results of detection of *cry* genes in chromosomal and plasmid DNA using polymerase chain reaction (PCR) revealed that 5 genes of *cry*1 namely *cry*1Ab, *cry*1C, *cry*1D, *cry*1E and *cry*1I and 1 gene of *cry*2 namely *cry*2A were found in

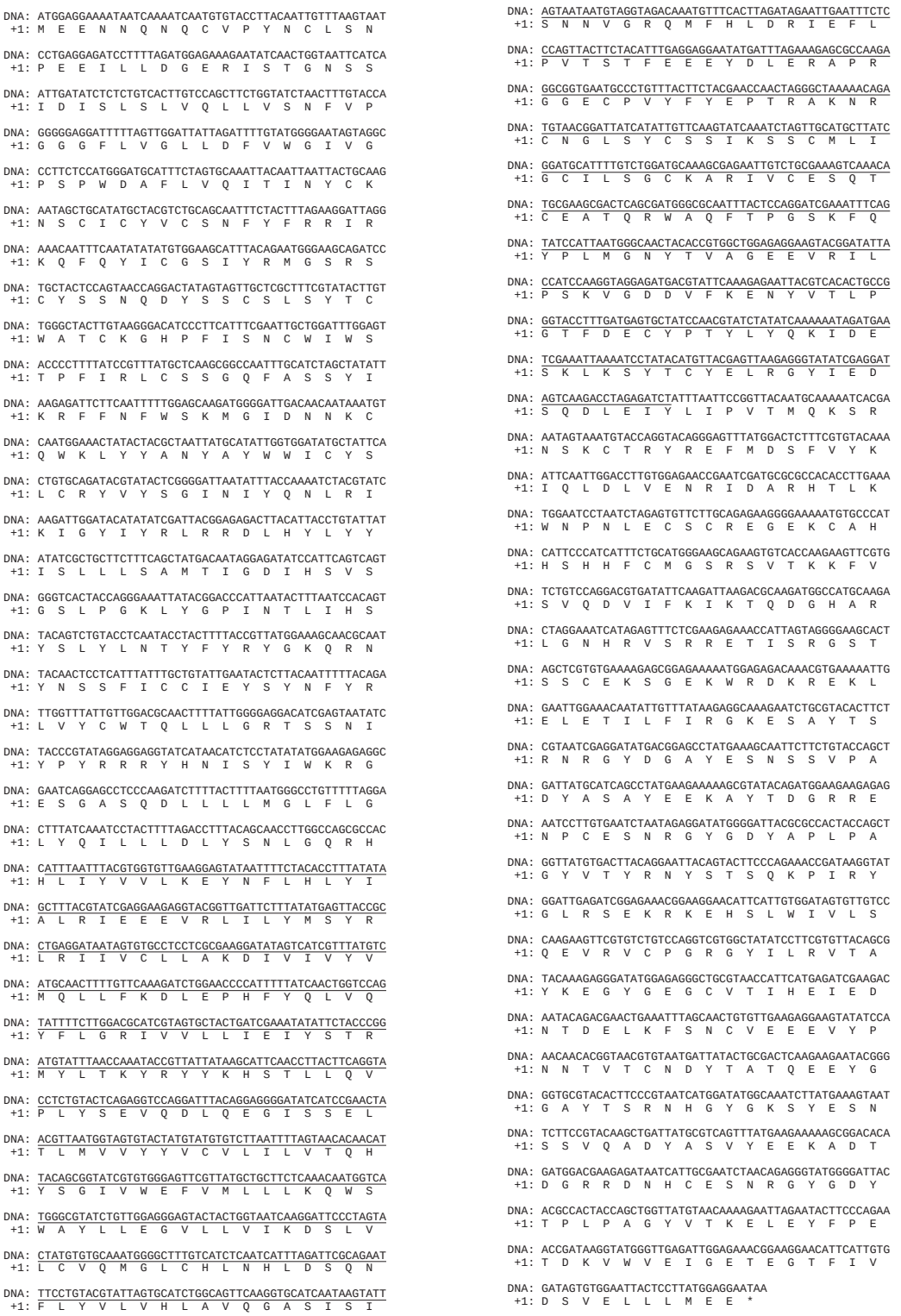

Figure 5 Nucleotide sequence and predicted amino acid sequence of putative *cry*1C gene in chromosome of *Bacillus thuringiensis* JC 590.

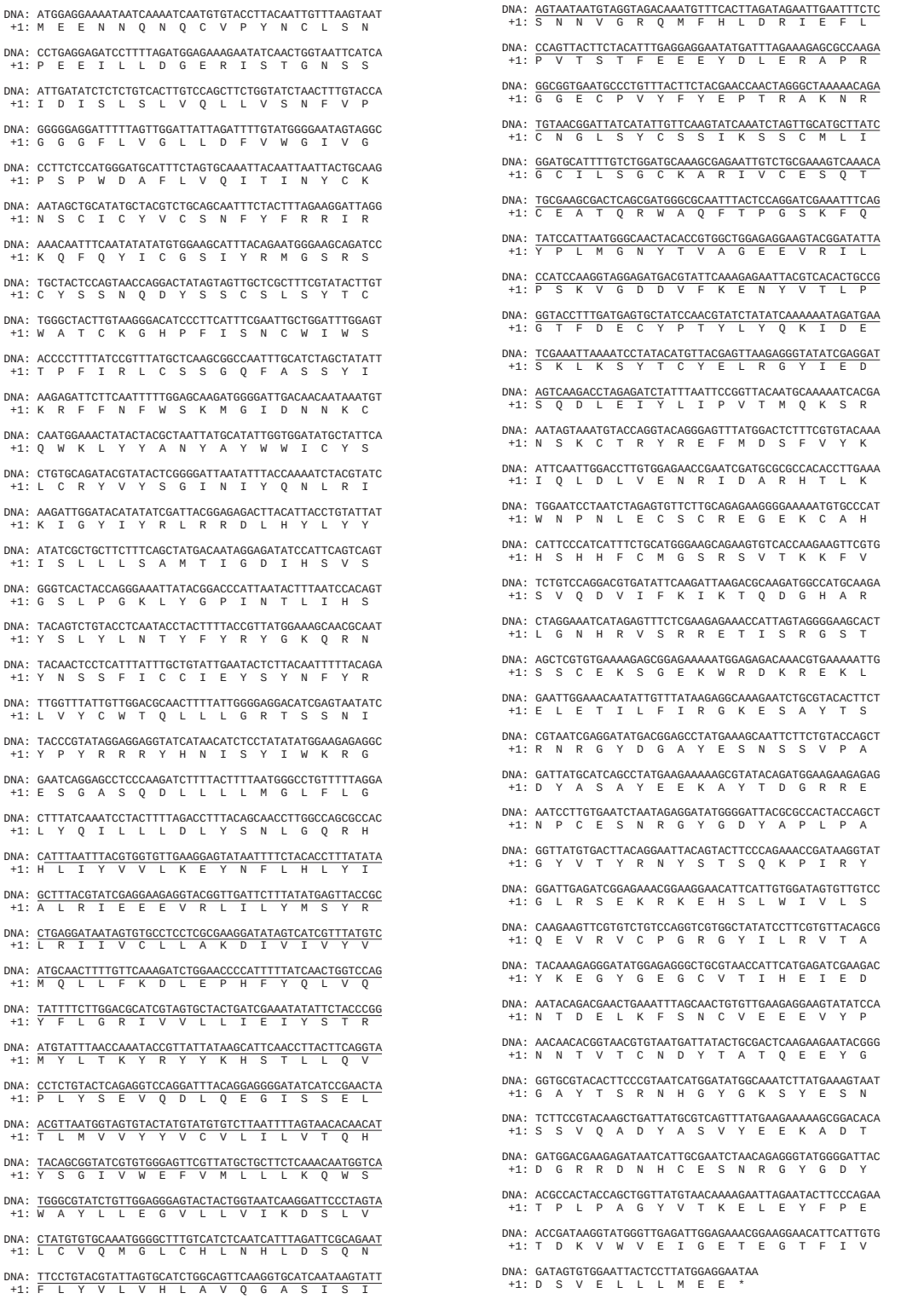

Figure 6 Nucleotide sequence and predicted amino acid sequence of putative *cry*1C gene in plasmid of *Bacillus thuringiensis* JC 590.

both chromosomal and plasmid DNA. The full length *cry*IC genes cloned by PCR walking technique, sequenced, and analyzed showed that the chromosomal *cry*1C gene consisted of 3,507 bp encoded for 1,169 of amino acid and the plasmid DNA *cry*1C gene had 3,450 bp encoded for 1,160 of amino acid in length. Sequence comparison with other *cry*1C genes in database at NCBI showed them to be high homology with *cry*1C genes of other *B. thuringiensis* strains in the database.

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