MODIFICATIONS OF PLASMIDS AND *CRY* GENES OF *Bacillus thuringiensis* subsp. *kurstaki* HD-1 AFTER TREATMENT WITH ETHYLMETHANOSULFONATE (EMS) AND UV LIGHT

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ABSTRACT

Bacillus thuringiensis subsp. *kurstaki* HD-1 is a potential insecticidal bacterium producing five types of ä-endotoxin crystal proteins. This bacterium is widely commercialized due to its wide spectrum toxicity against both Lepidopteran and Dipteran larvae. The objective of this work was to create autolysin deficient mutant causing cell fails to lyse. This mutant yield intact cells within spore and ä-endotoxin crystal protein protected inside, from which an undamaged active bio-insecticide would be obtained. Two methods of mutagenesis, 2% of ethylmethanosulfonate and 10, 25, and 50 seconds of UV light exposure, resulted in *mot* (loss of motility) mutation. Observation showed that 14 of 20 survived mutants have lost some of its plasmids (varied from one to five), while the other six maintained their plasmids. By employing the polymerase chain reaction (PCR) the change on the *cry* genes was studied.

Keywords: UV and EMS mutagenesis, autolysin deficient mutant, plasmid, *cry* genes, *Bacillus thuringiensis* subsp. *kurstaki* HD-1.

ABSTRAK

Bacillus thuringiensis subsp. *Kurstaki* HD-1 merupakan galur bakteri insektisida potensial yang dapat memproduksi 5 macam protein kristal ä-endotoksin. Insektisida hayati dengan bahan galur bakteri ini sudah secara luas diperdagangkan karena memiliki spektrum toksisitas tinggi terhadap larva-larva serangga hama dari ordo Lepidoptera maupun Diptera. Kegiatan ini dilakukan untuk memperoleh mutan defisien autolisin yang menyebabkan sel bakteri ini tidak dapat lisis. Mutan ini akan memproduksi sel-sel utuh dimana spora dan protein kristal ä-endotoksin tetap berada di dalam dan terlindung sehingga diperoleh insektisida hayati aktif yang tidak mudah rusak. Dilakukan 2 cara mutagenesis: perlakuan dengan 2% etilmetanosulfonat dan penyinaran dengan sinar UV selama 10, 25 dan 50 detik, yang keduanya menghasilkan mutan-mutan *mot* (kehilangan kemampuan motilitas). Observasi yang dilakukan terhadap 20 mutan yang dapat bertahan hidup, 14 di antaranya kehilangan plasmid (bervariasi satu hingga 5), sedang selebihnya dapat mempertahankan keenam plasmidnya. Dengan menggunakan teknik *Polymerase Chain Reaction* (PCR), perubahan yang terjadi pada gen *cry* dipelajari.

Kata kunci: Mutagenesis dengan UV dan EMS, mutan defisien autolisin, plasmid, gen-gen *cry*, *Bacillus thuringiensis* subsp. *kurstaki* HD-1.

INTRODUCTION

Bacillus thuringiensis subsp. *kurstaki* HD-1 is commercially and widely used (approximately 80% of total sales). It has a broad spectrum of target insects and is commonly applied in agriculture and forestry to control pests (Morris-Cole, 1995). Strain HD-1 serotype 3-H antigen 3a, 3b is highly toxic to Lepidopteran larvae, which its esterase type was not determined (de Barjac & Bonnefoi, 1962). This strain was first isolated by Dulmage (1970) and allowed the major advance in the effectiveness of *B. thuringiensis* (BT) containing insecticide. It was reported that this strain has five protein types of ä-endotoxin (Aronson, 1993), they are: Cry IA(a) 133.2 kDa which targets *Bombyx mori*; Cry IA(b) 131.0 kDa for several Lepidopterans; Cry IA(c) 133.3 kDa for *Heliothis virescens* (Diptera); Cry IIA 70.9 kDa, low toxicity for Lepidopteran and Dipteran and Cry IIB 70.8 kDa (*cryptic*) for *Manduca sexta* (Lepidoptera). BT *israelensis* produces a parasporal crystal which is composed of 4 major polypeptides with mass weights of 28, 72, 128 and 135 kDa.

The *cry* genes sequences encoding ä-endotoxin are present in the plasmid in most strains, although some chromosomally located *cry* genes were also found. The number of plasmids in BT strains vary from one to 12, with the size vary from 2 to 150 MDa or 3.05 to 265.5 kbp in size (Carlton & Gonzalez, 1986). The number of plasmids contained in the strain *kurstaki* HD-1 according to Lereclus *et al.* (1982) are eight, their sizes are 2.3, 8.0, 8.5, 8.7, 14.5, 46.5, 72.84 and 83.7 kb. Piot (1987) denoted six, they are 8.0, 8.5, 8.7, 14.5, 31.0, and 46.5 kb. Hofte & Whiteley (1989) classified *cry* genes into 4 types: type I encodes 130 kDa protein specific for Lepidopteran, type II encodes 70 KDa protein that is specific for both Lepidopteran and Coleopteran, type III encodes also about 70 kDa protein that is specific to Coleopteran, and type IV is specific to Dipteran. In addition, Feiteillon *et al.* (1992) added 2 other types of *cry* genes named *cry* V and *cry* VI, both are specific for certain species of Nematodes.

Fein & Rogers (1976) cited that the mutant of *pneumococci* might either have a deficient in autolytic enzymes formation or hyper-producer of lipotheicoic acid, an inhibitor of autolytic enzymes. Mutant of *B. substilis* with a temperature-sensitive autolytic amidase have been claimed to occur in the culture that has been treated with N-methyl-N'-nitro-N-nitrosoguanidine. Nonmotile, pleiotropic, partially autolytic enzyme deficient mutant of this organism that are hyper-producer of amylases and proteases have been isolated and studied genetically. The most powerful class of mutagen is alkylating agents and one of them is EMS (ethylmethanosulfonate) which can spontaneously transfer alkyl group to nitrogen ring in the base of DNA. Therefore, in general, mutagenic action may induce transition, transversion, or cell death, and the survivors are likely mutated.

PCR approaches using both forward and reverse primers are limited to detection of already known genes, and fail to detect and identify unknown or novel *cry* genes eventhough various strategies have been proposed to increase their efficiency. Juarez-Perez *et al.* (1997) used single primer by a technique called E-PCR (exclusive PCR) after aplicon exclusion process in the second step which allows both the identification of known *cry* genes in *B. thuringiensis* and detection of *cry-1* related sequence unrecognized by specific primers. This technique is used in this study on mutated genes due to complexity of nucleotide base changes.

The aim of the present work was to create randomly mutated BT subsp. *kurstaki* HD-1 harboring higher broad spectrum of insecticidal activity in its proteinous parasporal body by means of mutagenesis. These were then used to examine if the

mutation leads to deficiency in autolytic enzymes, lost of motility, lost or change of the number of plasmids and modification at the level of the *cry* genes.

MATERIALS AND METHODS

Microorganisms

The bacterial strains employed in this work were: BT *kurstaki* HD-1 wild type, a *str3*$^{-}$ mutant of BT *kurstaki* that loss of its resistantcy to streptomycin, and a strain of BT *kurstaki* Kto^{2} (harboring plasmid ^{2}). All these strains were provided by courtesy of Dr. J.C. Piot from Institut de Génétique et de Biologie Microbiennes – Université de Lausanne (Switzerland).

Growth media and culture conditions

The bacterial strains were cultured and maintained in SAT medium (Spizizens Amino Acid Tryptone). One liter of this medium contains: 10 g casein hydrolysate, 10 g glucose, 20 mg tryptone, 2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 g Na. citrate.$2H_2O$, 0.2 mg $MgSO_4.7H_2O$, and 0.85 mg $MnSO_4$. Solid medium was prepared by the addition of 1.5% Bacto agar to the SAT medium. For treatment during UV mutagenesis, the bacterial cells were suspended in minimum medium S containing 0.29% glucose, 50 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.22 mg/ml $MnSO_4$, 0.05 mM $ZnSO_4$, 0.05 mM $FeSO_4$, and 1 mM $CaCl_2$. The medium used for selection of the mutants was a semisolid enriched medium which in one liter contains of 10 g Bacto pepton, 80 g Bacto gelatin, 4 g Bacto agar, 5 g casein hydrolysate, 20 mg tryptophan, 20 mg glycine, 2 g $(NH_4)_2SO_4$, 14 g K_2HPO_4, 6 g KH_2PO_4, 1 g Na. citrate.$2H_2O$, 0.2 g $MgSO_4$, and 0.85 g $MnSO_4$. To grow the mutants obtained, the addition of 20 mg/l thymidine was applied to the LB medium (Sambrook *et al.*, 1989).

Production of mutants

Single colony was cultured into 5 ml SAT broth in a 25 ml tube, and shaked gently overnight to obtain seed culture. An equivalent of SL value (scattered light) of 5 starting culture was made in the 50 ml SAT broth in 100 ml aerated tube by adding the seed culture obtained, placed in water bath at 37°C and shaked moderately by aeration. The growth of cell was measured by nephelometer in every 30 mins until the exponential phase was reached equivalent to SL value = 100 or correspond to 2 x 10^8 cells/ml of medium. Approximately 15 ml of this culture were harvested by centrifugation at 7000 rpm for 10 mins. The pellets were then resuspended in minimum medium S and mutagenized by UV light according to the method previously described by Sherman *et al.* (1979), for 10 secs, 25 secs and 50 secs. The number of survivors were determined by plating this UV mutagenized culture on LB agar, and 0.5 ml of the culture was then transferred into 15 ml fresh medium of LB broth in 50 ml aerated tube, incubated at 37°C, shaked with moderate aeration overnight. The culture was then plated on LB agar in Petri dish in such manner to obtain about 50 colonies each. The auxothroph mutants were eliminated by putting these colonies appeared on solid minimum medium.

In another 15 ml of culture growing in SAT medium, the culture was treated with 2% final concentration of EMS (ethylmethanosulfonate) for 90 mins without aeration in centrifuge tube coated with al-foil and shaked using mechanical agitation. To enrich for autolytic enzyme deficient mutant, 50 µg/ml of methicillin was added. The EMS mutagenized culture was then filtered and washed with physiological solution and the pellet obtained was resuspended in 3 ml LB broth. The number of survivors was determined by taking 0.1 ml of this suspension and the rest was transferred into

25 ml of LB broth and shaked by vigorous aeration. Microscopic observation of hazard deficient mutant was performed by taking this culture and plating it on LB agar.

Selection for non-motile and autolytic deficient mutants

The colonies appeared on LB agar both from UV light and EMS treatment were passed six times on growing cycles in enriched semi-solid medium. Non-motile and autolytic deficient mutants should show a non-dispersed compact colony in the center of dish. The autolytic activity of the suspected mutants was analyzed by pipeting the cell extract on the minimum agar containing the cell wall of BT and followed by coloring with procion brilliant red. Preparation of cell wall of BT and the extraction were done as described previously (Fein & Rogers, 1976).

Plasmid isolation

Plasmids were isolated according to the standard procedure (Cork & Khalil, 1996). Supernatan was filtered using *High Pure Spin Filter Tubes* (Boehringer Mannheim). The dried pellet resuspended in 200 µl TE pH 8.0. The DNA obtained was quantified using *RNA/DNA Calculator Gene Quant* (Pharmacia).

Identification *cry* gene types

Ten types of primers (20 mers) for different types of *cry* genes were synthesized based on the description of previous authors (Carozzi *et al.*, 1991; Juarez-Perez *et al.*, 1997). The primers were made synthetically by Cyber Gene AB (Sweden) (Table 1). Amplification of the *cry* genes was performed using a PCR kit, ready to go beads, obtained from Amersham Pharmacia Biotech. About 50 pg of DNA plasmid obtained after being diluted in water was used as the template and 25 pmol of a primer introduced to a PCR bead, sterilized dd-H_2O was added to a final volume of 25 µl. Amplification was accomplished with the DNA Thermal Cycler (Corbett Research FTS-960 Thermal Sequencer) by using the Step-Cycle program set to denature at 95°C for 30 secs, anneal at 37 – 65°C for 30 secs, and extend at 72°C for 1 mins, followed by a 4 secs per cycle extension for a total of 35 cycles.

Electrophoresis

The DNA profile of both plasmids and *cry* gene amplified bands were documented on 0.8% agarose gel. The electrophoresis were done at 75 V for 3 h for PCR product and 5 h for plasmid identification. Plasmid

Table 1. Type of primers and its nucleotide sequences used to determine the different *cry* genes

No.	Primer's type	Nucleotide sequence	References
1.	Cry IA	CAATAGTCGTTATAATGATT	Juarez-Prez *et al.* (1997)
2.	Cry IA(b)	CGGATGCTCATAGAGGAGAA	idem
3.	Cry IA(c)	GGAAACTTTCTTTTTAATGG	idem
4.	Cry IA(d)	ACCAGTACTGATCTCAACTA	idem
5.	Cry IB	GGCTACCAATACTTCTATTA	idem
6.	Cry IC	ATTTAATTTACGTGGTGTTG	idem
7.	Cry ID	CAGGCCTTGACAATTCAAAT	idem
8.	Cry IE	TAGGGATAAATGTAGTACAG	idem
9.	Cry IIIA	GTCCGCTGTATATTCAGGTG	Carozzi *et al.* (1991)
10.	Cry IVA	CAAGCCGCAAATCTTGTGGA	idem

isolated from wild type of BT *kurstaki* HD-1, 100 bp ladder (Amersham), and *DNA Molecular Weight Marker X (0.07 – 12.2 kb)* (Boehringer Mannheim) were used as markers.

RESULTS

The mutants obtained and the phenotypic character observed under light microscope after treatment with EMS and UV light exposure are presented in Table 2. The mutants mentioned in Table 2 were capable of growing in LB agar and conserved by lyophilization. Most of them were lost its

Table 2. List of the mutants obtained after mutagenesis with 2% EMS and 10, 25, and 50 secs UV light exposure with its phenotypic characters observed under light microscope.

No.	Strains of BT *kurstaki*	Technique of mutant isolation	Phenotypic character identified	Mutant code number
1.	Ktoβ	10 secs UV exposure, found at LB agar for survivor calculation	Autolytic deficient enzymes	**Lyt-1**
2.	HD-1 *str*-3	*idem*	*idem*	**Lyt-2**
3.	HD-1 *str*-3	10 secs UV exposure, 6 x cycling in semi solid medium	no motility, only 2-3 flagel per cell, crystal present	**10-S-9**
4.	HD-1 *str*-3	25 secs UV exposure, filtration by glass filter, 6 x cycling in semi solid medium	no motility, no flagel, no crystal	**25-F-1**
5.	HD-1 *str*-3	*idem*	no motility	**25-F-2**
6.	HD-1 *str*-3	25 secs UV exposure, 6 x cycling in semi solid medium	no motility	**25-S-2**
7.	HD-1 *str*-3	*idem*	no motility	**25-S-3**
8.	HD-1 *str*-3	*idem*	no motility, crystal present	**25-S-6**
9.	HD-1 *str*-3	*idem*	no motility	**25-S-7**
10.	HD-1 *str*-3	50 secs UV exposure, 6 x cycling in semi solid medium	no motility	**50-S-1**
11.	HD-1 *str*-3	*idem*	no motility	**50-S-3**
12.	HD-1 *str*-3	*idem*	no motility	**50-S-4**
13.	HD-1 *str*-3	*idem*	no motility, only 5 flagel, crystal present	**50-S-10**
14.	HD-1 *str*-3	*idem*	no motility	**50-S-14**
15.	HD-1 *str*-3	*idem*	no motility	**50-S-15**
16.	HD-1	2% EMS, enriched by methicillin 50 µg/ml, direct selection on media cont. Procion Brilliant.	no motility	**2-EM-1**
17.	HD-1	*idem*	no motility, crystal present	**2-EM-2**
18.	HD-1	*idem*	no motility, crystal absent	**2-EM-3**
19.	HD-1	*idem*	no motility	**2-EM-4**
20.	HD-1	*idem*	no motility	**2-EM-5**
21	HD-1	*idem*	no motility, no flagel, crystal present	**2-EM-6**
22.	HD-1	*idem*	no motility, crystal present	**2-EM-7**

motility, while observation towards the flagellae and crystal showed that some mutants were difficult to identify in the presence of their proteineous crystal. It may be due to the low capacity of the light microscope used to distinguish between the spore and the crystal in the uncolored fresh medium of bacterial culture. Almost all the mutants showed lytic zone on the Procion agar except mutant 10S-9. From all the mutants listed in Table 2, after being stored for three years in -20°C, there were 18 survivors in Luria-Bertani agar medium.

Plasmid number after mutagenesis

Plasmid contents of each mutants showed as electrophoresis bands is presented in Figure 1. Bands appeared from wild type BT *kurstaki* HD-1 (lane 2) can be considered as an indicator for the molecular size of plasmid from mutants obtained. As described previously by Piot (1987) this wild type contains six plasmids of 8.0, 8.5, 8.7, 14.5, 31.0, and 46.4 kb. The DNA molecular weight marker available being used in this work can measure only the molecule size smaller than 12.216 kb. The mutants 25-S-2 (lane5), 25-S-3 (lane 6), 2-EM-3 (lane 17), and 2-EM-6 (lane 20) show the similar profile with the wild type. It could be interpreted that no change happened in the plasmid number for those mutants. Meanwhile, the mutants 10-S-9 (lane 3), 50-S-4 (lane 11), 50-S-14 (lane 13), 2-EM-2 (lane 16), and 2-EM-5 (lane 19) have lost four of their plasmids. Finally, the mutants named 25-F-2 (lane 4), 25-S-6 (lane 7), 25-S-7 (lane 8), 50-S-1 (lane 12), 50-S-15 (lane 14), and 2-EM-4 (lane 18) have lost almost all their plasmids and remained only one a 8.6 kb fragment. The mutant 2-EM-1 (lane 15) has remained four plasmids of sizes: 8.6, 14.5, 31.0, and 46.5 kb.

Modification of *cry* gene types

From ten types of primer, cry IA(a), cry IA(d), cry IB, cry IC, cry IE, and cry IVA did not give any products of amplification. It could be interpreted that no such type of gene to be found in the mutants obtained. The *cry* gene types identified in the mutants were: *cry* IA, *cry* IA(a), *cry* ID, and *cry* IIIA. The gene *cry* IA was found in five mutants: 50-S-1 (lane 8), 50-S-3 (lane 9), 2-EM-1 (lane 14), 2-EM-3 (lane 16), and 2-EM-4 (lane 17). The *cry* IA found in mutant 50-S-1 gave different sizes product from 200 until about 1500 bp in size consisting of 9 to 10 bands. The mutant

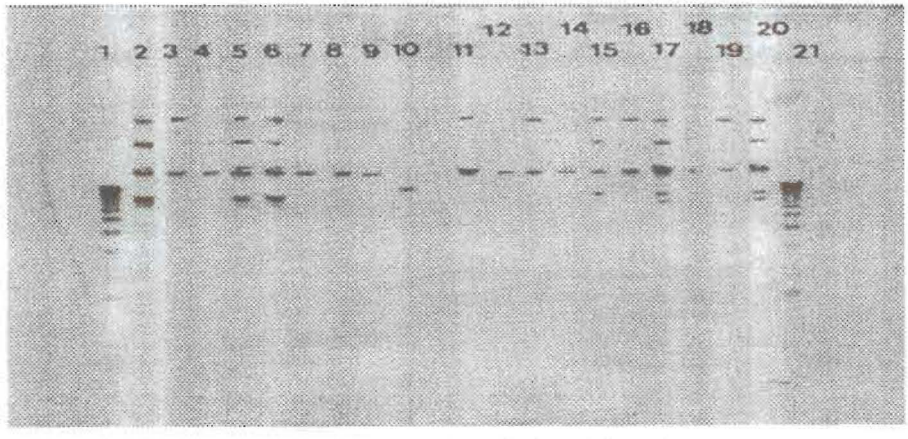

Figure 1. Electrophoresis gel showing the number of plasmid in the cell of wild type and mutagenized *B. thuringiensis* subsp. *kurstaki* HD-1. Lane **1** and **21** are DNA molecular weight marker X (00.7–12.2 kb), **2**. wild type HD-1 used as plasmids size indicator, **3**. 10-S-9, **4**. 25-F-2, **5**. 25-S-2, **6**.25-S-3, **7**.25-S-6, **8**. 25-S-7, **9**.50-S-1, **10**. 50-S-3 **11**. 50-S-4, **12**. 50-S-10, **13**. 50-S-14, **14**. 50-S-15, **15**. 2-EM-1, **16**. 2-EM-2, **17**. 2-EM-3, **18**. 2-EM-4, **19**. 2-EM-5, and **20**. 2-EM-6.

50-S-3 shows 5 bands of approximately 200, 350, 500, 650, and 1200 bp, meanwhile 2-EM-1 shows only 2 bands of 500 and 600 bp; 2-EM-4 estimated to produce 4 or 5 bands of size 400, 550, 600, 800, and an unclear band of about 1350 bp (Figure 2a).

The product of amplification of primer cry IA(b) are found almost in all mutants, the very thick bands appeared on the gel (Figure 2b) found in the mutants: 25-S-7 (lane 7), 50-S-1 (lane 8), 50-S-10 (lane 11), 50-S-14 (lane 12), 2-EM-5 (lane 18), and 2-EM-6

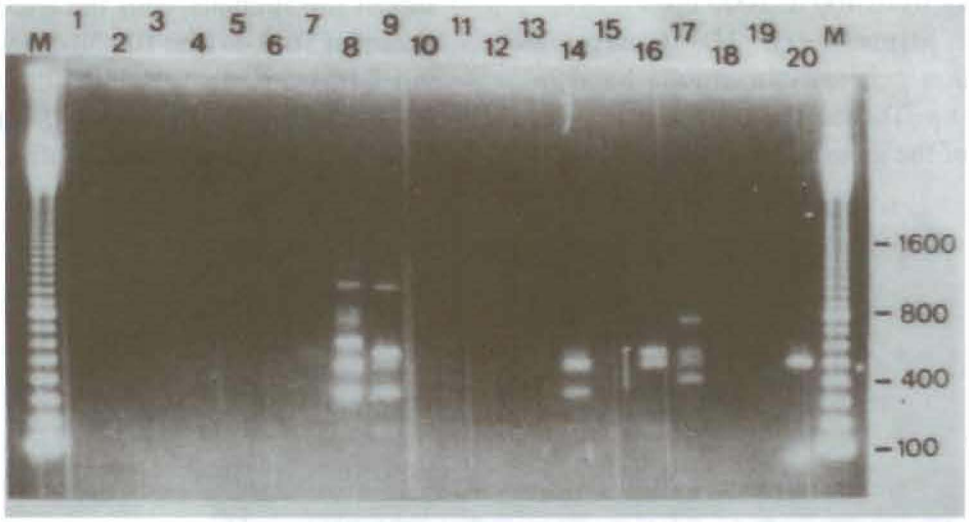

Figure 2a. The profile of bands produced from the amplification of plasmid DNA of wild type and mutants of *B. thuringiensis* subsp. *kurstaki* HD-1 using primer *Cry* IA (5'-CAATAGTCGTTATAATGATT-3'). **Lane M**. 100 bp of DNA ladder, **1**. wild type HD-1, **2**. 10-S-9, **3**. 25-F-2, **4**. 25-S-2, **5**. 25-S-3, **6**. 25-S-6, **7**. 25-S-7, **8**. 50-S-1, **9**. 50-S-3, **10**. 50-S-4, **11**. 50-S-10, **12**. 50-S-14, **13**. 50-S-15, **14**. 2-EM-1, **15**. 2-EM-2, **16**. 2-EM-3, **17**. 2-EM-4, **18**. 2-EM-5, **19**. 2-EM-6, and **20**. control of PCR primer.

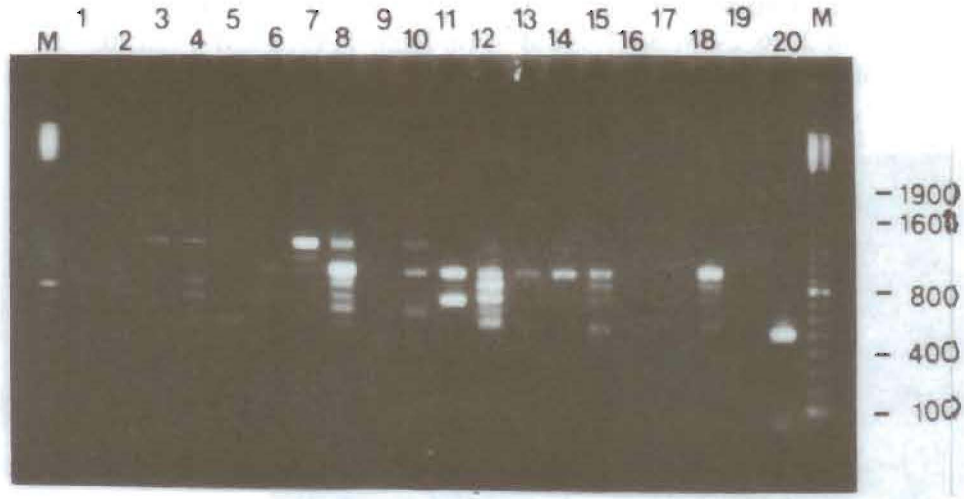

Figure 2b. The profile of bands produced from the amplification of plasmid DNA of wild type and mutants of *B. thuringiensis* subsp. *kurstaki* HD-1 using primer *Cry* IA(b) (5'-CGGATGCTCATAGAGGAGAA-3'). **Lane M**. 100 bp of DNA ladder, **1**. wild type HD-1, **2**. 10-S-9, **3**. 25-F-2, **4**. 25-S-2, **5**. 25-S-3, **6**. 25-S-6, **7**. 25-S-7, **8**. 50-S-1, **9**. 50-S-3, **10**. 50-S-4, **11**. 50-S-10, **12**. 50-S-14, **13**. 50-S-15, **14**. 2-EM-1, **15**. 2-EM-2, **16**. 2-EM-3, **17**. 2-EM-4, **18**. 2-EM-5, **19**. 2-EM-6 and **20**. control of PCR primer.

(lane 19). The wild type of HD-1 shows 3 bands of about 800, 1000, and 1700 bp (lane 1). The same profile with the wild type are found in the mutants: 50-S-14 (lane 12), 2-EM-1 (lane 14), 2-EM-2 (lane 15), and 2-EM-15 (lane 18), showing the thicker bands than others and all having 7 bands with the size ranging from 400 to 1000 bp.

The primer *cry* ID generates amplification generally in unique band in about 800 bp. The bacteria which show the existence of the gene of *cry* ID are: the wild type HD-1 (lane 1), mutants 10-S-9, (lane 2), 25-F-2 (lane 3), 25-S-3 (lane 5), 25-S-6 (lane 6), 25-S-7 (lane 7), 50-S-14 (lane 12), 2-EM-1 (lane 14), and 2-EM-5 (lane 18), as well as shown in Figure 2c.

Primer of *cry* IIIA has given extraordinary products of amplification in almost the mutants with the exception for mutant of 50-S-4 (lane 10), 50-S-15 (lane 13), and 2-EM-6 (lane 19) which did not show any amplification (Figure 2d). Some of them show the similarity of band profile as well as

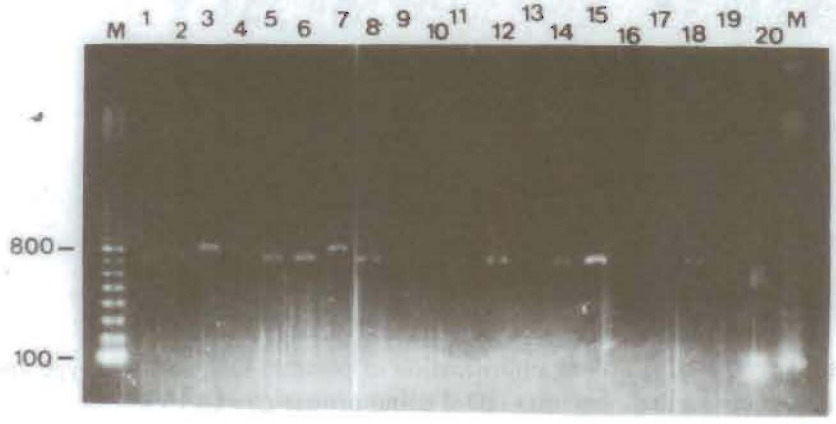

Figure 2c. The profile of bands produced from the amplification of plasmid DNA of wild type and mutants of *B. thuringiensis* subsp. *kurstaki* HD-1 using primer *Cry* ID (5'-CAGGCCTTGACAATTCAAAT-3'). **Lane M.** 100 bp of DNA ladder, **1.** wild type HD-1, **2.** 10-S-9, **3.** 25-F-2, **4.** 25-S-2, **5.** 25-S-3, **6.** 25-S-6, **7.** 25-S-7, **8.** 50-S-1, **9.** 50-S-3, **10.** 50-S-4, **11.** 50-S-10, **12.** 50-S-14, **13.** 50-S-15, **14.** 2-EM-1, **15.** 2-EM-2, **16.** 2-EM-3, **17.** 2-EM-4, **18.** 2-EM-5, **19.** 2-EM-6 and **20.** control of PCR primer.

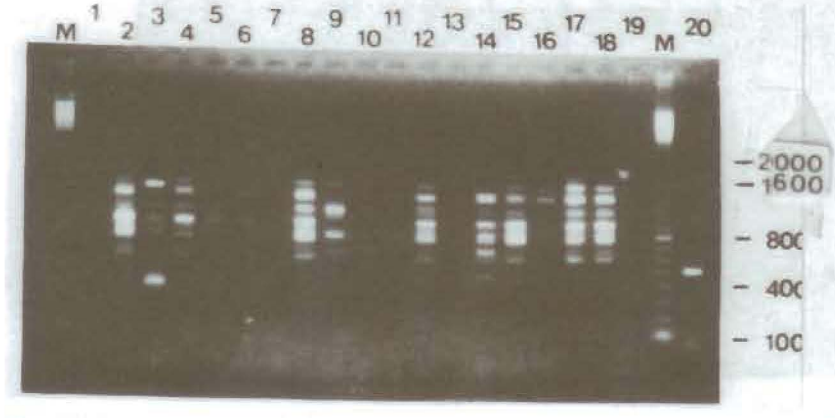

Figure 2d. The profile of bands produced from the amplification of plasmid DNA of wild type and mutants of *B. thuringiensis* subsp. *kurstaki* HD-1using primer *Cry* IIIA (5'-GTCCGCTGTATATTCAGGTG-3'). **Lane M.** 100 bp of DNA ladder, **1.** wild type HD-1, **2.** 10-S-9, **3.** 25-F-2, **4.** 25-S-2, **5.** 25-S-3, **6.** 25-S-6, **7.** 25-S-7, **8.** 50-S-1, **9.** 50-S-3, **10.** 50-S-4, **11.** 50-S-10, **12.** 50-S-14, **13.** 50-S-15, **14.** 2-EM-1, **15.** 2-EM-2, **16.** 2-EM-3, **17.** 2-EM-4, **18.** 2-EM-5, **19.** 2-EM-6 and **20.** control of PCR primer.

regarded among the mutants of: 10-S-2 (lane 2), 50-S-14 (lane 12), and 25-S-2 (lane 4) with 7 bands of size 600 to 1700 bp; also among the mutants of 2-EM-3 (lane 15), 2-EM-4 (lane 17), and 2-EM-5 (lane 18) which show the similarity in the number of band but not in their thickness. The total results yielded of PCR product using ten available primers towards the mutants obtained from the mutagenesis of BT *kurstaki* HD-1 is presented in Table 3.

Table 3. The result yielded of PCR product of 4 primers presenting the amplification towards the *cry* gene of the mutants obtained from the mutagenesis of *B. thuringiensis* subsp. *kurstaki* HD-1.

Bacterial strains		PCR product (*in bp*) yielded from following primers			
No.	Mutant code number	Cry IA	Cry IA(b)	Cry ID	Cry IIIA
1.	Lyt-1 (no growth)	-	-	-	-
2.	Lyt-2 (no growth)	-	-	-	-
3.	HD-1 (wild type)	no product	850, 1700	750	900
4.	10-S-9	no product	850, 950	750	400, 800, 1000 1200, 1500
5.	25-F-1 (no growth)	-	-	-	-
6.	25-F-2	no product	1300	800	800, 1000. 1200, 1400, 1500
7.	25-S-2	no product	700,800, 1200	no product	800, 1000, 1200, 1400, 1600
8.	25-S-3	no product	400, 1400	750	1200, 1400,
9.	25-S-6	no product	1000	750	1500
10.	25-S-7	no product	1200	800	1200, 1400, 1500
11.	50-S-1	200, 300, 500, 600, 700, 800, 1100	400, 500, 700, 800, 900, 1000, 1200	750	2000 700, 800, 1000, 1200, 1400,
12.	50-S-3	200, 400, 550, 1800	no product	no product	1600, 1900, 2200 800, 1200, 1600
13.	50-S-4	no product	500, 800, 1200, 1700	no product	no product
14.	50-S-10	no product	600, 800	no product	1500
15.	50-S-14	no product	400, 550, 600, 800	750	600, 800, 1200, 1400, 1600
16.	50-S-15	no product	800, 1700	no product	no product
17.	2-EM-1	200, 500	800	750	700, 800, 1000, 1400
18.	2-EM-2	no product	400, 600, 700, 800	750	600, 800, 1000, 1200, 1600, 1800, 2000
19.	2-EM-3	500, 550, 1100	no product	no product	1500, 1600
20.	2-EM-4	450, 500, 550, 1000	no product	no product	600, 800, 1000, 1200, 1600, 1800, 2000
21.	2-EM-5	no product	700, 800	750	600, 800, 1000, 1200, 1600, 1800
22.	2-EM-6	no product	no product	no product	800
23.	2-EM-7 (no growth)	-	-	-	-

DISCUSSION

Mutation can be defined as any permanent alteration in the DNA sequence, even if this alteration does not have detectable phenotypic effects. All the changes probably contribute to the mutagenic action of the alkylating agents, which induce both transition and transversion. DNA absorbs UV light strongly; the absorption on maximum of DNA lies at a wavelength of 260 nm. Cells are rapidly killed by UV absorption, and high rate mutation occurs among the survivors. According to Stanier *et al.* (1984), if some bacteria are immediately irradiated with visible light in the range of 300 to 400 nm, both mutation frequency and lethality are greatly reduced, a phenomenon known as photoreactivation. All cells also posses an elaborate set of enzymes that affect the dark repair of UV light damaged DNA. Most of the base pair substitution induced by UV are formed by copying errors during the repair synthesis that constitute one step in this recombination process. Mutations induced through the above mechanism are generally caused by base pairs substitution. Frameshift mutation resulted from the addition or removal of base pairs in one of multiple sites causes the translation process to lose its proper frame of reference. (Drake & Boltz, 1976).

Almost all the mutants obtained in this study were *mot*- mutants or which lost all or half of their peritrich flagellae. The incapability of four mutants obtained (Lyt-1, Lyt-2, 25-F-1, and 2_EM-7) to grow on Luria-Bertani agar medium, may reflect alteration to become full auxothroph where more nutritious media are required to become full auxothroph. The survivors lost its motility due to the loss of half or all of the flagellae. Flagella and autolysin gene are generally presence in the chromosome so that most mutations occur at the chromosomal level. While the plasmids generally harbor non-essential genes such as resistant to antibiotics and toxin production. Wild type of HD-1 showed six plasmids containing the cry IA(b), cry II, and cry IIIA. The mutants obtained after treatment with EMS and UV showed variation in plasmid and *cry* gene; and it could not be classified based on the kinds of mutagenic agent nor its doses. Mutant 25-S-3 which shows crystal, conserves its six plasmids and its *cry* gene as well as wild type, but mutant 2-EM-3 also produces crystal and conserves its *cry* gene, but shows appearance of *cry* IA which did not exist in the wild type. Mutant 50-S-10 for example, remains only one plasmid, but in microscopic observation has shown the existence of crystal, and the PCR result showed the existence of *cry* IA(b) and *cry* IIIA in which *cry* ID has lost. It can be speculated that both of these genes are harbored in one plasmid. Mutant 2-EM-6 which shows the presence of crystal has lost 4 plasmids where PCR analysis shows one band of 800 bp of *cry* IIIA. None of the mutants survived have lost all their plasmids, at least one remained, they were: 25-F-2, 25-S-6, 25-S-7, 50-S-1, 50-S-3, 50-S-10, 50-S-15, and 2-EM-4. Among them only mutant 25-S-6 and mutant 50-S-10 showing the crystal observed under microscope. The difference is found in the mutant 2-EM-3 conserving its six plasmids but no one crystal observed, although PCR analysis showed the existence of *cry* IA and *cry* IIIA genes. It may be that frameshift mutation by removal or addition nucleotide in its sequence affected the failure of translation. Although most of the plasmids in these mutants were lost, but some of them are still capable of showing the existence of the *cry* genes. The result of gene identification by PCR must actually yield only one band. The oligonucleotide sequences of the primers used for identification of the *cry* genes in this PCR work were cited from previous authors (Carrozi *et al.*, 1991; Juarez-Perez *et al.*, 1997). Those all were possibly forward primers giving some electrophoresis bands range from 200 to 1700 bp of nucleotide

sequences for each primer. The reverse primers were not used in this case so the bands appeared did not represent the *cry* genes or complete sequence of such gene. Lereclus *et al.* (1982) showed a remarkable characteristics of the region of the gene coding proteinous crystal of the existence of repetitive sequence of nucleotide being identical with some sequence in that gene. This repetitive sequence has been detected primarily in BT *kurstaki* presenting multiple copies on the plasmid carrying *cry* gene in the big plasmids; and also found in the chromosome of some subspecies of BT (Kronstad & Whiteley, 1984). It is greatly possible that they are responsible for great diversity of *cry* gene localization.

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