

Characterization of genetic diversity of some serovars of *Bacillus thuringiensis* by RAPD

Debasis Pattanayak*, Swarup K Chakrabarti*, P Ananda Kumar*** & Prakash S Naik*

*Molecular Biology Laboratory, Division of Crop Improvement, Central Potato Research Institute, Shimla 171 001, India.

**National Research Center on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India.

Received 11 December 2000; revised 30 August 2001

RAPD based fingerprinting of 21 serovars of *Bacillus thuringiensis* (Bt) representing different serotypes was performed using 19 random decamer primers. A total of 172 polymorphic fragments, ranging in size from 161-2789 bp, were amplified from 13 of the 19 primers. Pairwise genetic similarity analysis revealed very low similarity values, ranging from 3-68%, among the serovars of Bt, indicating high genetic divergence. Nineteen serovars of Bt fell in two major clusters and remaining two formed solitary clusters in the dendrogram. Clustering of Bt strains established genetic relatedness between serovars and serotypes. It has been suggested that RAPD analysis can be used for genotypic characterization of Bt to complement flagellar serotyping.

Bacillus thuringiensis, the ubiquitous, gram-positive, aerobic, endospore forming bacterium, is used world over as biological pesticide in commercial agriculture, forest management and mosquito control. It also serves as a key source of genes for the generation of transgenic plants against insects and pests¹⁻³. Because of its economics and importance, thousands of strains have been isolated from soil samples, plant surfaces, dead insects and stored grains worldwide, and are deposited in a number of collections. Bt strains have been classified mainly based on flagellar antigens and phenotypic characters⁴. So far, more than 60 different flagellar serotypes and eight non-flagellated biotypes have been reported⁵. Identification and characterization of Bt have also been accomplished through molecular techniques such as DNA hybridization⁶, M13 fingerprinting⁷ and PCR analysis using conserved primers of rDNA sequences⁸. In our earlier work, we established high levels of genetic diversity among Bt serovars through AFLP fingerprinting⁹. Technical sophistication is the main constraint towards adoption of this technique for routine characterization of Bt strains. PCR-based randomly amplified polymorphic DNA (RAPD) technique is relatively simple, easy, has wide genome coverage and does not require prior sequence information for amplification. The objective of the present study is to ascertain the suitability of RAPD fingerprinting in characterization of serovars of Bt.

Materials and Methods

Twenty-one strains representing different serovars of Bt (obtained from *Bacillus* Genetic Stock Center, Columbus, Ohio, USA) comprised the experimental material (Table 1). Bacterial cultures were grown on Luria agar for at least 16 hr at 30°C. Single colonies were selected and used to inoculate 100 mL of Luria broth and incubated at 30°C for 12 hr or until turbidity was observed. Cells were harvested from the broth cultures by centrifugation at 5000 g for 10 min at 4°C. The pellet was resuspended in Tris-EDTA buffer (9.5 mL). Total genomic DNA was isolated through CTAB-NaCl method following the procedure of Ausubel *et al*¹⁰. DNA pellet was resuspended in 0.5 mL of TE buffer and treated with 5 µL of RNase A (10 mg mL⁻¹) at 37°C for 30 min. DNA was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The upper phase was collected and 0.1 volume of 3 M sodium acetate (pH 5.2) was added and mixed thoroughly. To this 2.5 volume of ice cold ethanol was added, mixed thoroughly and placed at -20°C for 30 min. DNA was pelleted by centrifugation at 12000 g for 10 min. Pellet was washed with 1 mL of 70% (v/v) ethanol and dissolved in 200 µL of TE.

RAPD analysis was performed using 19 random decamer primers. Polymerase chain reaction was carried out in a reaction volume of 12.5 µL containing 10 mM, Tris-HCl (pH 8.3); 50 mM, KCl; 0.1%, Triton X 100; 2.5 mM, MgCl₂; 200 µM each of dNTPs,

*Correspondent author: Email- polumetla@hotmail.com

Table 1 — *Bacillus thuringiensis* serovars used in this study

Subspecies	Strain designation	Flagellar serotype	Description
<i>thuringiensis</i>	1715	1	Wild type
<i>alesti</i>	HD4	3a, 3c	Isolated in France from <i>Bombyx mori</i>
<i>kurstaki</i>	HD1	3a, 3b, 3c	Isolated in USA
<i>sotto</i>	sotto	4a, 4b	Wild type
<i>kenyae</i>	HD293	4a, 4c	Isolated in USA from <i>Cadra cautella</i>
<i>galleriae</i>	HD168	5a, 5b	Isolated in the USSR
<i>canadensis</i>	HD224	5a, 5c	Isolated in Canada
<i>entomocidus</i>	HD10	6	Isolated in Canada from <i>Plodia interpunctella</i>
<i>morrisoni</i>	HD12	8	Isolated in USA
<i>tolworthi</i>	HD537	9	Wild type
<i>darmstadiensis</i>	HD146(103)	10a, 10b	Isolated in UK
<i>toumanoffi</i>	HD201(B-30-2)	11a, 11b	Isolated in UK from <i>Galleria mellonella</i>
<i>thompsoni</i>	HD542	12	Wild type
<i>pakistani</i>	HD395	13	Isolated in Pakistan from <i>Cydia pomonella</i>
<i>israelensis</i>	4Q2-72	14	Plasmid cured mutant, bears only 72 kDa plasmid
<i>dakota</i>	Oats 43	15	Wild type
<i>indiana</i>	HD521	16	Wild type
<i>kyushuensis</i>	HD541 (74-F-6-18)	11a, 11c	Isolated in Japan from <i>B. mori</i>
<i>tohokuensis</i>	78-FS-29-17	17	Isolated in Japan from <i>B. mori</i>
<i>kumamotoensis</i>	HD867 (3-71)	18a, 18b	Isolated in Japan from <i>B. mori</i>
<i>tochiensis</i>	HD868 (117-72)	19	Isolated in Japan from soil

25 pmole primer (Operon Technology Inc., USA); 25 ng, genomic DNA and 0.5 unit of Taq DNA polymerase (Promega, USA). Amplification was performed for 45 cycles at 94°C for 1 min, 35.5°C for 1 min and 72°C for 2 min for denaturation, annealing and primer extension, respectively. All PCR samples were subjected to a 5 min preamplification at 94°C and 10 min postamplification at 72°C. Amplified products were separated by agarose gel electrophoresis (1.6 % in Tris-acetate buffer). Gel image was captured by Fluor-S™ MultiImager (Bio-Rad). DNA fragments were detected and aligned by diversity database software (Bio-Rad).

DNA fragment profiles representing a consensus of two replicates were scored in a binary fashion with '0' indicating absence and '1' indicating presence of a band. A similarity matrix was constructed from the binary data using Jaccard co-efficient. This was further subjected to UPGMA clustering analysis and a dendrogram was generated. A co-phenetic matrix was constructed using the similarity matrix to test the validity of clusters generated. Correlation (Mantel *t* test) between cophenetic matrix and similarity matrix was determined using MXCOMP module. All the

above analysis was done using the software package NTSYS-pc (version 2.0)¹¹.

Results and Discussion

Genomic DNA isolated from 21 strains of Bt belonging to different serovars and serotypes were characterized through RAPD using 19 random decamer primers. Six primers did not exhibit any amplification. Remaining 13 primers produced a total of 172 scorable fragments ranging from 161-2789 bp across the 21 Bt strains studied, with an average of 13 fragments per primer. Each primer was amplified twice and results were found to be reproducible. The fragment polymorphism was quite apparent among different serovars of Bt, as all the 172 fragments screened were found to be polymorphic (Table 2). The primer OPE 20 was most discriminatory which produced 28 polymorphic fragments (Fig. 1).

Pairwise similarity analysis revealed very low levels of genetic similarity among the serovars of Bt, ranging from 3 to 68%. The dendrogram generated based on RAPD data grouped serovars of Bt in two major clusters (Fig. 2). The highest similarity of 68% was recorded between serovar *dakota* (serotype 15)

and *indiana* (serotype 16). Serovar *alesti* (serotype 3a, 3c) and *sotto* (serotype 4a, 4b) also showed high similarity value of 60%. Serovar *tochigiensis* (serotype 19) and *kyushuensis* (serotype 11a, 11c) formed solitary clusters in the dendrogram with an average similarity value of 18% (SD 6.0) and 15% (SD 7.0), respectively, with other serovars. The lowest similarity value of 3% was observed between *kyushuensis* (serotype 11a, 11c) and *kurstaki* (serotype 3a, 3b, 3c), *kyushuensis* (serotype 11a, 11c) and *pakistani* (serotype 13), and *tochigiensis* (serotype 19) and *kyushuensis* (serotype 11a, 11c) (Fig. 2). The cophenetic correlation coefficient for the dendrogram was found to be high (0.834) thereby indicating the validity of the cluster generated from the present study. The cophenetic correlation coefficient measures the agreement between the similarity values implied by the dendrogram and those of the original similarity matrix¹².

The absolute polymorphism in RAPD fragment pattern and low level of similarity between different serovars of Bt implies high levels of genetic diversity which is reflected by the diversity in flagellar H-antigen agglutination reactions and presence of different toxins with different insect specificities¹³. The remarkable genetic divergence of Bt is because of the presence of many different plasmids in each strain and their conjugal transfer mechanism, and transposon like inverted repeats flanking the endotoxin genes, which helps in high frequency of DNA rearrangements¹⁴.

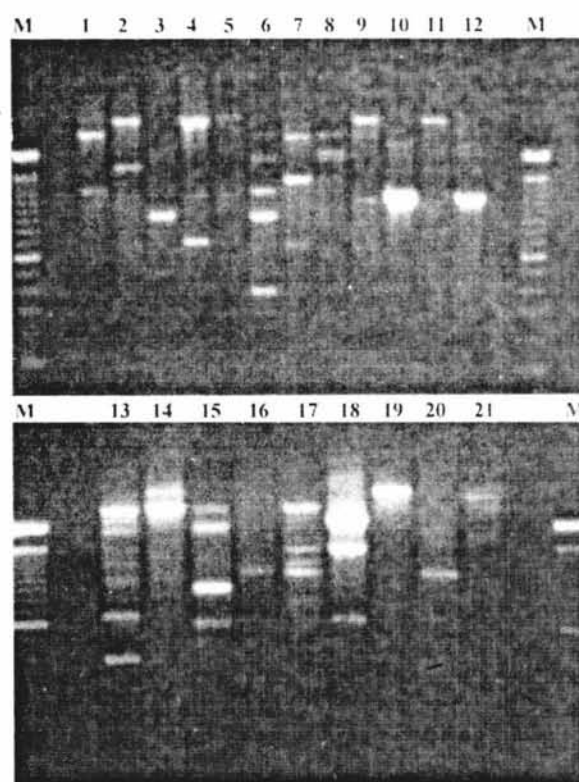


Fig. 1 — RAPD pattern of 21 serovars of *Bacillus thuringiensis* generated by primer OPE 20. M, 100 bp DNA ladder; 1, *thuringiensis*; 2, *alesti*; 3, *kurstaki*; 4, *sotto*; 5, *kenyae*; 6, *galleriae*; 7, *canadensis*; 8, *entomocidus*; 9, *morrisoni*; 10, *tolworthi*; 11, *darmstadiensis*; 12, *toumanoffi*; 13, *thompsoni*; 14, *pakistani*; 15, *israelensis*; 16, *dakota*; 17, *indiana*; 18, *kyushuensis*; 19, *toho-kuensis*; 20, *kumamotoensis*; 21, *tochigiensis*.

Table 2 — PCR amplification summary of serovars of *Bacillus thuringiensis* using random decamer primers.

Primer	Sequence (5'-3')	Number of fragments generated	Polymorphic fragments	Size range (bp)
OPA 03	AGTCAGCCAC	21	21	198-2697
OPA 04	AATCGGGCTG	14	14	256-2719
OPA 05	AGGGGTCTTG	3	3	309-1126
OPA 07	AATCGGGCTG	Nil	—	—
OPA 08	GTGACGTAGG	10	10	161-1148
OPA 10	GTGATCGCAG	11	11	298-1116
OPA 14	TCTGTGCTGG	Nil	—	—
OPA 17	GACCGCTTGT	5	5	388-2347
OPA 20	GTTGCGATCC	Nil	—	—
OPB 01	GTTTCGCTCC	10	10	1034-2553
OPB 03	CATCCCCCTG	11	11	331-2129
OPC 01	TTCGAGCCAG	13	13	593-2308
OPC 03	GGGGGTCTTT	Nil	—	—
OPC 04	CCGCATCTAC	20	20	336-2761
OPC 13	AAGCCTCGTC	Nil	—	—
OPC 19	GTTGCCAGCC	13	13	758-2619
OPC 20	ACTTCGCCAC	13	13	579-2789
OPE 09	CTTCACCCGA	Nil	—	—
OPE 20	AACGGTGACC	28	28	392-2565

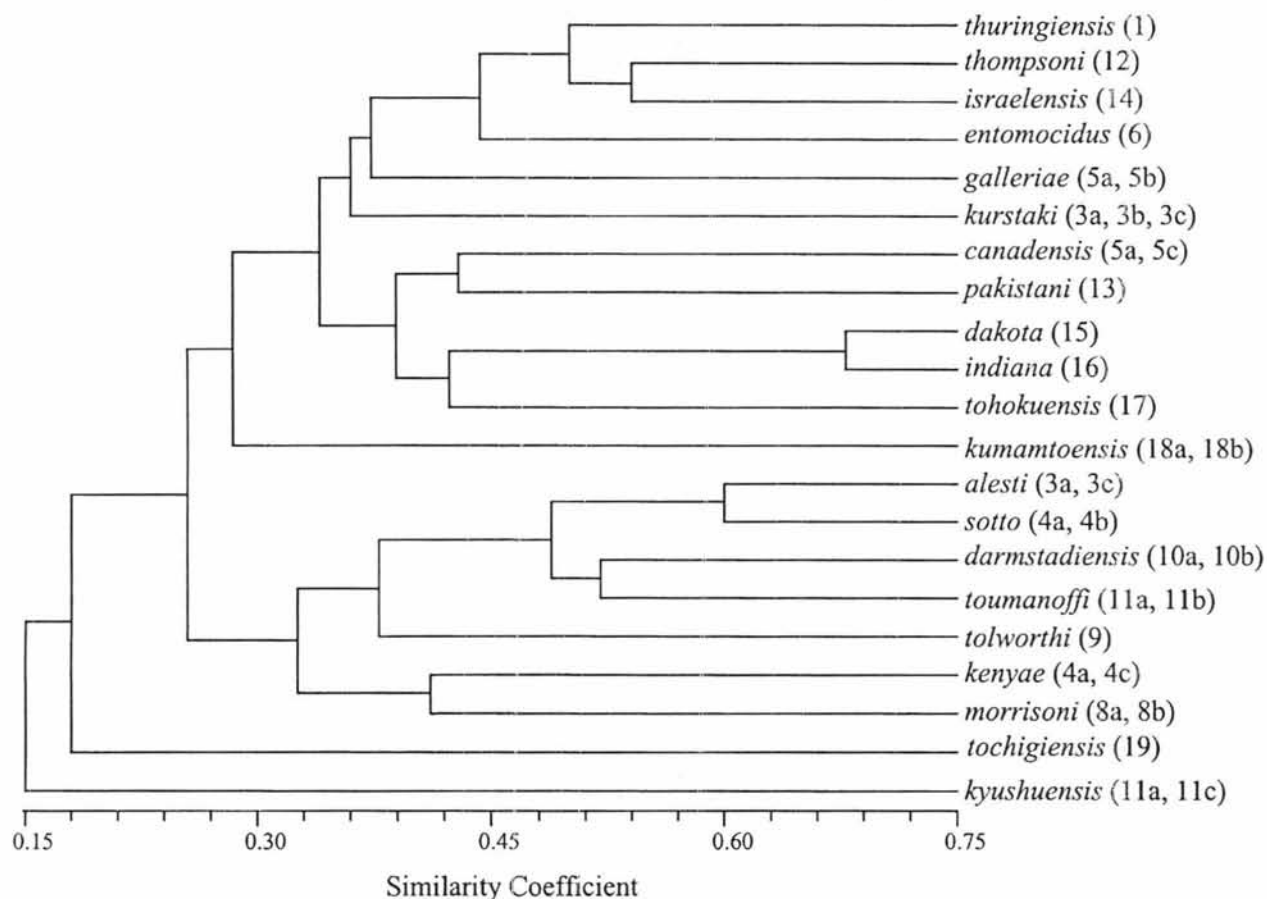


Fig. 2 — Dendrograms generated based on RAPD analysis with the program NTSYS-pc (version 2.0) using UPGMA cluster analysis, showing the degree of genetic relatedness between *Bacillus thuringiensis* serovars. Parentheses indicate serotype.

These considerations explain the high RAPD polymorphism and low level of similarity between different serovars of Bt observed in the present study. However, RAPD analysis was able to distinguish serotypes of Bt and established genetic relatedness between serovars and serotypes.

Earlier, attempts have been made to identify, discriminate and characterize Bt strains by M13 fingerprinting⁷, DNA hybridization using variable region of 16S rDNA (Ref. 6), PCR using conserved primers for 16S to 23S ribosomal intergenic spacer sequences⁸ and AFLP fingerprinting⁹. Ribotyping either by PCR or DNA hybridization failed to detect the diversity among Bt strains, probably because of the use of single gene or operon and evolutionarily conserved nature of rDNA. However, whole genome analysis through M13 fingerprinting and AFLP fingerprinting were able to detect high levels of diversity among Bt serovars. The genetic relatedness and groupings of serovars of Bt based on RAPD analysis have been found to be similar to that of

AFLP analysis. AFLP fingerprinting, although more sensitive and powerful, requires sophisticated detection technique. In contrast, RAPD is simple, easy and reproducible, and does not require any sophistication in detection. Therefore, RAPD based fingerprinting of Bt can be used for characterization and classification of serovars of Bt in broad perspective to complement flagellar serotyping of Bt.

Acknowledgement

Authors are grateful to Professor Donald Dean and Dr Daniel Zeigler of Ohio State University for providing Bt strains to National Research Center on Plant Biotechnology, IARI. Authors also thank Director, CPRI, for encouragement and providing facilities.

References

- 1 Estruch J J, Carozzi N B, Desai N, Duck N B, Warren G W & Koziel M G, Transgenic plants: an emerging approach to pest control, *Nature Biotech*, 15(1997) 137.

- 2 Schuler T H, Poppy G M, Kerry B R & Denholm I, Insect-resistant transgenic plants, *Trends Biotech*, 16(1998) 168.
- 3 De Maagd R A, Bosch D & Stiekema W, *Bacillus thuringiensis* toxin-mediated insect resistance in plants, *Trends Plant Sci*, 4(1999) 9.
- 4 De Barjac H & Franchon E, Classification of *Bacillus thuringiensis* strains, *Entomophaga*, 35(1990) 233.
- 5 Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler D R & Dean D H, *Bacillus thuringiensis* and its pesticidal crystal proteins, *Microbiol Mol Biol Rev*, 62(1998) 775.
- 6 Te Giffel M C, Beumer R R, Klijn N, Wagendrop A & Rombouts F M, Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA, *FEMS Microbiol Lett*, 146(1997) 47.
- 7 Miteva V, Abadjieva A & Grigorova R, Differentiation among strains and serotypes of *Bacillus thuringiensis* by M13 DNA fingerprinting, *J Gen Microbiol*, 137(1991) 593.
- 8 Bourque S N, Valero J R, Lavoie M C & Levesque R C, Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species, *Appl Environ Microbiol*, 61(1995) 1623.
- 9 Pattanayak D, Srinivasan K, Mandaokar A D, Shukla A, Bhalla R & Kumar P A, AFLP fingerprinting and genotypic characterization of some serovars of *Bacillus thuringiensis*, *World J Microbiol Biotechnol*, 16(2000) 667.
- 10 Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A & Struhl K, in *Short protocols in molecular biology* (John Wiley & Sons, Inc.) 1995.
- 11 Rohlf F J, *NTSYS-pc. Numerical taxonomy and multivariate analysis system*, Version 2.0, (Exeter Software, New York) 1998, 1-31.
- 12 Sneath P H A & Sokal R R, in *Numerical taxonomy: The principles and practice of numerical classification* (W. H. Freeman, San Francisco) 1973.
- 13 Kumar P A, Sharma R P & Malik V S, The insecticidal proteins of *Bacillus thuringiensis*, *Adv Appl Microbiol*, 42(1996)1.
- 14 Aronson A I, Beckman W & Dunn P, *Bacillus thuringiensis* and related insect pathogens, *Microbiol Rev*, 30(1986) 1.