African Journal of Biotechnology Vol. 6 (15), pp. 1821-1827, 6 August 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Characterisation of *Bacillus thuringiensis kurstaki* strains by toxicity, plasmid profiles and numerical analysis of their *cry*IA genes

S. Kashyap [1] and D. V. Amla [2]*

[1]National Bureau of Agriculturally Important Microorganisms (ICAR) Kushmaur Katholi, Maunath Bhangan, 275101 Uttar Pradesh, India.
[2]Molecular Biology and Genetic Engineering Division, National Botanical Research Institute, Rana Pratap Marg, P. B. # 436, Lucknow 226001, Uttar Pradesh, India.

Accepted 25 April, 2007

In the present comparative study, four *Bacillus thuringiensis kurstaki* strains (HD1, dipel, HD73, HD1dipel) were characterized by investigating their total plasmid profiling; *cry*IA genes profiling and toxicity towards local isolates of agricultural insects *Helicoverpa armigera* and *Spodoptera litura*. Result showed that LC_{50} for *S. litura* were 0.11 µg of HD73, 0.027 µg of HD1, 0.20 µg of dipel and 0.018 µg of HD1 dipel, while LC_{50} for *H. armigera* insect were 0.04 µg of HD73, 0.031 µg of HD1, 0.011 µg of dipel and 0.008 µg of HD1dipel. The native plasmid number and type varied from 4 - 8 among these strains and *Hind*III restriction showed 0.4 to 8.0 kb size fragments. The numerical classification of the *cry*IA gene profiles showed two distinct clusters at 48% similarity level. Cluster one of 80% similarity comprises of two strains HD73 and dipel, while cluster two of 65% consisted of HD1 and HD1dipel strains. Southern analysis of restricted plasmid combined with *cry*IA profile can provide an effective approach for investigation of taxonomic relationship within *Bacillus thuringiensis kurstaki* strains.

Key words: *Bacillus thuringiensis kurstaki, Helicoverpa armigera, Spodoptera litura,* δ-endotoxin, plasmids, Southern hybridization.

INTRODUCTION

The genus *Bacillus* is usually determined at species level by physiological reactions, but inconsistency in tests results can make identification difficult (Ash et al., 1991a). Description of the genus has improved by using information obtained from DNA base composition and DNA-DNA hybridization studies which was listed in Bergey's Manual of Systematic Bacteriology (Claus and Berkely, 1986). However in the literature there are newly identified strains which were shown to be genetically and phenol-typically distinct from other species and strains and have not been described in Bergey's Manual (Slepeck and Hemphill, 1992). Similar situation exists for the well known Gram positive bacterium *Bacillus thuringiensis*, a candidate with insecticidal properties against a number of agriculturally important insect pests and common invader of different ecological niches like soil, dead leaves, grains, rice dusts and snow (Hofte and Whiteley, 1989; Schnepf et al., 1998; Widener and Whiteley, 1990; Travers et al., 1987; Martins and Travers, 1989) and till now considered to be completely harmless for humans and mammals (Glare and O'Challaghan, 2000; Damgaard et al., 1997). The bacterium comprises of different sub-species and isolates each of which produces toxins that kills different insects belonging to various orders and groups (Adang et al., 1985). Although, it is one of the most widely used biopesticide in the world, in India it is

*Corresponding author. E-mail: sudhanshukshyp@yahoo.com.

Abbreviations: ICP, Insecticidal Crystal Protein; H.a., *Helicoverpa armigera*; S.p., *Spodoptera littura*; *Bt, Bacillus thuringiensis*; *Btk, Bacillus thuringiensis kurstaki*; RH, relative humidity; BSA, bovine serum albumin; kDa, kilodalton; SDS-PAGE, Sodium Dodeosyl Sulfate Poly-acrilamide Gel Electrophoresis; BME, ß-Mercaptoethanol; and DTT, Dithiothreitol.

still restricted to experimental field trials on few crops only.

A number of different methods have been used for typing these species by serotyping, bacteriophage typing, bacteriocin activity, antibiogram and biotyping, plasmid typing, analysis of fatty acid content, native PAGE, small-subunit – ribosomal RNA sequencing and genome analysis (Ash et al., 1991a; Berber and Cokmus, 2001). Although these methods have been used for identification at species level, characterization of these microorganisms is still not well defined (Ivanova et al., 1999). Second level information for a bacterial cell, other than sequencing of bacterial genome, can be obtained from cellular protein or gene profiling. Different types of electrophoresis were used to explore such profiles. The protein profiles produced by SDSPAGE of whole cell extract have been found to correlate closely with DNA- DNA hybridization results suggesting that it could be appropriate to use plasmid profiling and conserved phenotypic lineages for rapid identification. (Vauterin et al., 1990; Niemi et al., 1993; Berber et al., 2003). Combination of gene profiling with computerized analysis of profile provides an effective approach to investigate the taxonomic relationships among many bacterial species (Kestas, 1985; Costas, 1992).

In view of these, present communication describes toxicity profile (phenotypic trait) and numerical analysis of *cry*IA gene profile (genetic trait) of four *Bacillus thuringiensis kurstaki* (*Btk)* strains belonging to same serotype for evaluating the usefulness of the technique as taxonomical and identification tool in the genus at subspecies level. The goal of present communication was, *Btk* isolates that differ in *cry*IA genes relatedness were examined for the presence of DNA sequence similar to *cry*IA linage alone correlates with genomic relatedness as establish by other comparisons (Jackson et al., 1999; Tricnor et al., 2001). Here we show that *Btk* strains distinctly separated within two clusters are further grouped by numerical analysis.

MATERIAL AND METHOD

Insect bioassay

The individual larvea of *Helicoverpa armigera* were collected from local okra (*Hibiscus esculentus* L.) and chickpeas (*Cicer arietinum* L.) fields and cultured on artificial diet at 26 ± 2°C; 85 - 90% R.H.; 8 – 10 h dark-light cycle. The adult moths were allowed to mate into large plastic boxes. During mating, 10% sucrose with honey in (1:1) was kept inside containers. Freshly laid eggs hatched and neonatal larvae were transferred to separate tubes containing artificial diet cubes. These larvae were maintained under control conditions and used for insect bioassay. Toxicity bioassay was carried out with artificial diet cubes of 2 mm^2 essentially consisting of Bengal gram power (110 gl^{-1}), yeast extract (20 gl^{-1}), ascorbic acid (2.6 gl^{-1}), hydroxybenzoate (2.0 gl^{-1}), multiplex (0.6 gl^{-1}), streptomycin (0.2 gl^{-1}), casein (10.0 gl^{-1}), vitroline (0.5 gl^{-1}), formaldehyde (10 gl^{-1}), agar agar (12 gl^{-1}), and desired dilution of protein was soaked into diet cubes and feed to neonatal larvae of *H. armigera* and *Spodoptera littura* 2nd instars larvae were fed with protein coated (18mm) leaf discs made from young castor leaf lamina. The protein dilution was made between 0.00 to 3.00 µg cm^2 of diet and 5 - 10 larvae were placed in three sets per dilution. The leaf disc and diet cubes were replaced at 24 h interval. Mortality of insects was monitored for 6 - 8 days.

Bacterial strains

Btk-HD73, *Btk*-HD1, *Btk* -dipel, obtained from Pasteur culture collection Paris, France and *Btk*-HD1 dipel obtained from personal culture collection of Prof. H. R. Whiteley, USA. They were grown in Luria- or Nutrient- Broth (Hi-Media, Mumbai, India) at 28°C on Rotatory shaker (200 rpm).

Protein estimation

Quantitative estimation of protein was done by procedure described by Ausubel et al. (2002) using BSA (fraction IV, Sigma) as standard.

Molecular technique

Plasmid DNA – isolation; purifications, quantification, electrophoresis, γ^{32} end labeling of oligo probe, Southern blotting and hybridization were carried out following standard protocols of Sambrook (Sambrook and Russell, 2001). DNA- restriction; -modifying enzymes (New England Biolabs USA) Nylon membrane (BioRad, USA) were used as per manufacturer's recommendations. Probe consisting of 20 mer oligo fragment derived from conserved *cry*IA gene region was used (Adang et al., 1985).

PCR analysis

Loopful of bacterial strains grown on LB agar were transferred to eppendorf tube containing lysis buffer (50 mM Tris , 50 mM EDTA, 3% SDS, 1% BME, pH 7.2) and heated at 65°C for 20 min with occasional gentle mixing. The lysate was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and phenol (water saturated) once. The aqueous phase was removed after centrifugation (15000 rpm. 15 min, 4°C). The total DNA was precipitated by *iso*propanol and pellet washed with 70% ethanol. Finally, pellet was air dried and resuspended in 200 µl TE buffer (0.1 mM Tris HCL, 1 mM EDTA pH 8.0) and allocates were directly utilized for PCR assay. The two oligo primers set used were 5' CCGAGAAGTCAAACATGGCG 3' and 3' TACATGCCCTTTCACGTTCC 5' which were specific for *cry*IA (Carozzi et al., 1991). The typical PCR reaction mixture consisted of 10 µ l DNA, 2 µ l each of primers 0.5 µl each of dNTP's 2 µ l PCR buffer (with $MgCl_2$), 0.5 µ l of *Taq* polymerase and 1.5 µ l of sterile water. Amplification was done by using step cycle program set to denature at 94°C for 1 min, anneal at 40°C for 1 min and extension at 72°C for 3 min. A final extension step of 15 min at 72°C was added at the end of 45 cycles. The amplificons were resolved on 0.8% agarose gel and after staining with ethidium bromide, gel was recorded on with gel doc system.

ICP purification

Late stationary phase culture of *Btk* was harvested by centrifugation (10000 g, 10 min and 4°C). The pellet was washed twice with 2 ml cold sterile of extraction buffer (20 mM sodium bicarbonate, 0.001% Triton X-100; pH 9.5). The suspension was sonicated (MSE Soniprep UK) at full energy level (4 cycles, 30 s, 4°C). The lysate was further subjected to biphasic separation to obtain pure crystal protein. Essentially, the sonicated lysate obtained from 2/ of sporulated culture was centrifuged (10000 g, 20 min, 4°C). The pellet consisting of spores and ICP was retained and washed three times

Table 1. Flagella serotype and growth pattern of four *Btk* strains.

Strain	Flagellar serotype	Growth pattern	
		k (h^{-1})	g (h)
Btk HD73	3a3b3c	1.05	0.42
Btk HD1	3a3b3c	0.99	0.40
Btk dipel	3a3b3c	0.924	0.43
Btk HD1 dipel	3a3b3c	0.91	0.38

with sterile distilled water and one cycle with saline buffer (50 mM sodium phosphate, 150 mM sodium chloride pH7.5). The spore ICP pellet was finally suspended in sterile distilled water to get a suspension of 50 mg/ml (w/v). To 35 ml of this suspension 30 ml of 1% sodium sulfate (w/v) and 35 ml of carbon tetrachloride was added followed by extensive blending (800 rpm, 10 min, 25°C). The spore and crystal suspension was allowed to settle down for 12 to 18 h to separate into aqueous and organic phase. The upper aqueous phase consisting of 85 to 90% ICP was siphoned out using peristaltic pump. The crystal protein was separated out of aqueous phase by centrifugation (12000 g, 10 min and 4°C). The pellet was washed three times in cold sterile distilled water and dissolved in 2 ml bicarbonate buffer (sodium bicarbonate 0.02 M, pH 9.5). Undissolved material was removed by centrifugation. The clarified and partially purified ICP solution was stored at –20°C with 0.001 M DTT. The various components of ICP were separated on 10% SDSPAGE (Sambrook and Russell, 2001) and 135 - 140; 65 - 70 kDa ICP component were purified as per protocol of Harlow and Lane, (1988).

Calculations

Similarity Index of Toxicity activity ratio was performed by

LC_{50} of *H. a* ÷ LC_{50} of *S. l* = Index of Toxic Specificity

Specific growth rate k and generation time g was estimated according to equation Stainer et al., (1990)

$k = 2.3 (\log N_2 - \log N_1) T_2 - T_1$

and

$g = 0.693 \div k$

Data analysis and dendrogram construction

The data produced by Southern analysis of *Hin*dIII restricted total plasmid DNA of *Btk* strains were analyzed using the unweighted pair group method with arithmetic mean (UPGMA) of NTSYS-pc 2.02 (Rohlf, 1997). The dendrogram tree was constructed using TREE analysis of the Software.

RESULTS

Btk growth and sporulation

The growth profile was found similar in each strain and exhibited typical sigmoid pattern. Maximum specific growth rate was observed in case of *Btk* HD73 while lowest growth rate was exhibited by BtkHD1 dipel. The maximum generation time was observed for *Btk* dipel and shortest in case of BtkHD1 dipel. The values for growth rate and generation time for other strains were within the above mentioned values (Table 1). Sporulation and crystalline inclusion body formation occured consistently in mid to late stationery phase in each case.

Insect toxicity assay

Toxicity bioassay trials for *Btk* strains with *H. armigera* and *S. littura* showed differential pattern of susceptibility. The *S. littura* insect typically exhibited lack of appetite and stopped feeding after 2 - 3 days of toxic challenge due to gut paralysis and mortality occur-red within 2 to 7 days. The decreasing order of LC_{50} values revealed by *Btk* strains was HD1 dipel > HD1 > HD73 > dipel in *S. littura* and HD1dipel > dipel > HD1 > HD73 in *H. armigera.* The other regional isolates of *S. littura* and *H. armigera* also followed similar susceptibility pattern. Based on toxicity index, HD1 dipel showed highest score and other strain as HD1 > HD73 > dipel against local insects (Table 3).

Plasmid and *cry*IA gene profile

Summarized observations on number of native plasmid their *Hin*dIII restriction profile, Southern hybridization with *cry*IA gene probe of four strains is presented in Table 2 and Figures 1a and b. The native plasmid profile of all the four strains as observed after alkali lysis (Sambrook and Russell, 2001) on agarose gel electrophoresis showed comparable pattern as reported earlier and substantial deviations in polymorphism was not evident except in smaller cryptic plasmid's regions. The plasmid profile in *B. thuringiensis* has been shown to be influence by composition of medium-, growth- and stress- conditions, which also affect the stability and inheritance of plasmids by daughter cells. In the present case, cultures were routinely grown at 28 - 30°C in nutrient broth and higher temperatures resulted in loss of megaplasmids. Similarly, *Hin*dIII restriction of total plasmid DNA resulted into discrete 12 - 14 bands in each strain and dissimilarity/similarity calculation did not show sufficient variations among the strains. The Southern analysis was carried out using a 20- mer oiligo fragment derived from 5' conserved consensus sequence of *cry*IA genes (Adang et al., 1985). No hybridization was observed with small cryptic plasmid as previously reported (Andrup et al., 1995). Southern hybridization of *Hin*dIII restricted total plasmid DNA showed distinct polymorphism reflecting the presence of different classes of crystal protein genes in the strains and it was not assumed to be multiple copy of a gene *in lieu* of 4.5, 5.3 and 6.6 kb class gene concept of Kronstad (Kronstad and Whiteley, 1986). The PCR amplification with synthetic oligo primers specific for *cry*IAa and *cry*IAc genes produced 986 type amplificon while *cry*IAb gene gave 908 bp products. No additional products were observed in any case. Two main clusters were apparent in the dendrogram made by the SAHN analysis using similarity matrices based on hybridization

Table 2. Plasmids and *cry*IA gene profile of four *Btk* strains.

Strains	Number of plasmid	*Hin*dIII fragments bearing *cry*IA gene type	PCR product (bp)
Btk HD73	4-5	6.6 kb type	986
Btk HD1	5-7	4.5, 6.6 kb type	986
Btk dipel	6-7	5.3, 6.6 kb type	986, 908
Btk HD1 dipel	7-8	4.5, 5.3, 6.6 kb type	986, 908

Table 3. Toxicity profile of four *Btk* strains.

Strain	Toxicity to insects♣		
	S. littur (LC_{50})	*H. armigera* (LC_{50})	I. S.♦
Btk HD73	0.110	0.04	2.75
Btk HD1	0.021	0.031	6.75
Btk dipel	0.20	0.011	1.80
Btk HD1 dipel	0.018	0.008	22.5

♣Toxicity values are protein ng/cm^2 of diet.
♦Index of specificity (I. S.).

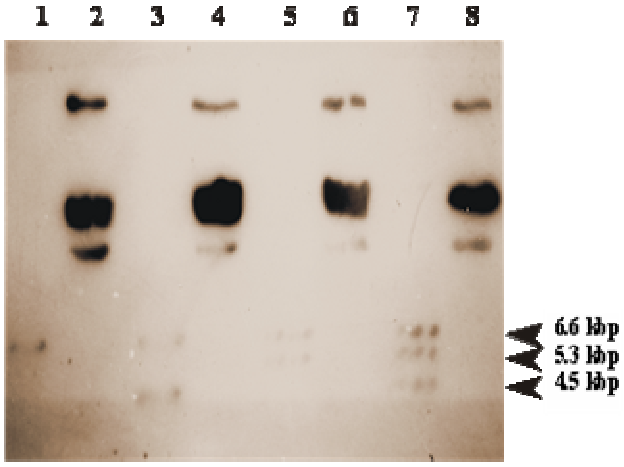

Figure Ib. Southern hybridization of four *Btk* strains with heterologous *cry*1A probe. Lane 1, *Hin*dIII restricted *Btk* HD73 plasmids; Lane 2, Native *Btk* HD73 plasmids; Lane 3, *Hin*dIII restricted *Btk* HD1plasmids; Lane 4, Native *Btk* HD1 plas-mids; Lane 5, *Hin*dIII restricted *Btk* dipel plasmids; Lane 6, Native *Btk*dipel plasmids; Lane 7, *Hin*dIII restricted *Btk* HD1dipel plasmids; and Lane 8, Native *Btk* HD1dipel plasmids.

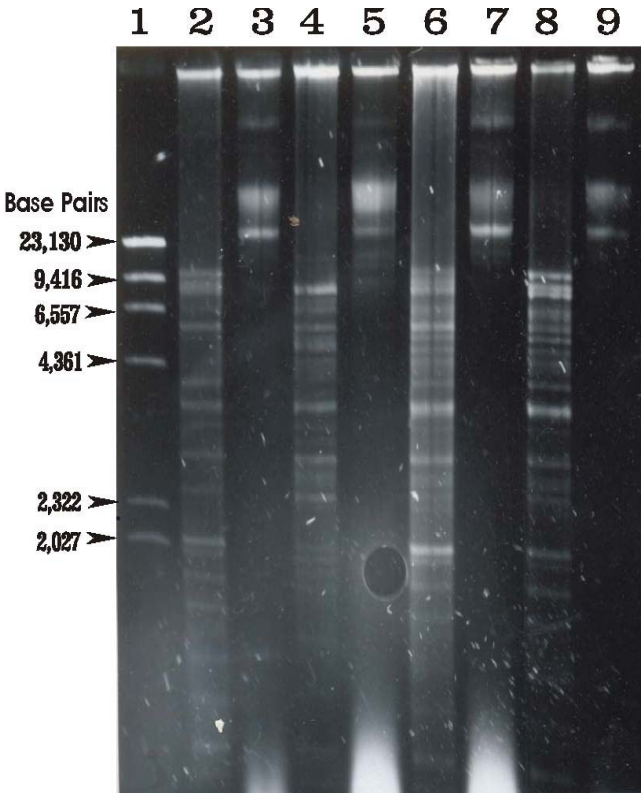

Figure Ia. Restriction and Native plasmid profile of four *Btk* strains. Lane 1, λ *Hin*dIII marker; Lane 2, *Hin*dIII restricted *Btk* HD73 plasmids; Lane 3, Native *Btk* HD73 plasmids; Lane 4, *Hin*dIII restricted *Btk* HD1plasmids; Lane 5, Native *Btk* HD1 plasmids; Lane 6, *Hin*dIII restricted *Btk* dipel plasmids; Lane 7, Native *Btk*dipel plasmids; Lane 8, *Hin*dIII restricted *Btk* HD1dipel plasmids; and Lane 9, Native *Btk* HD1dipel plasmids.

information (Figure 2). The *Btk* HD73 and *Btk* dipel was found to be closer than rest of strains.

DISCUSSION

Gram-positive, rod-shaped, aerobic or facultative anaerobic spore forming bacteria have been assigned to the genus *Bacillus* (Cohn, 1872). The genus *Bacillus* has

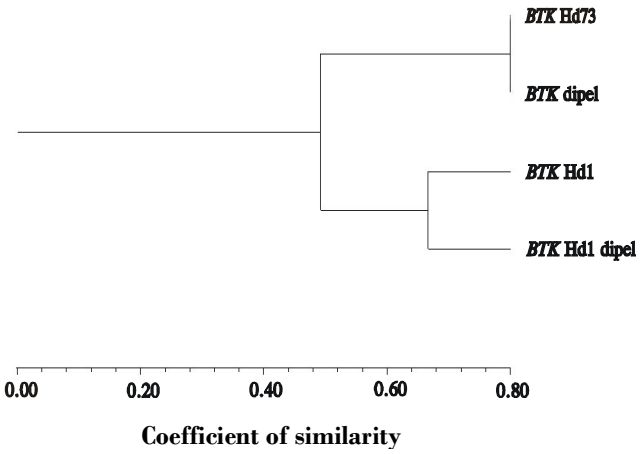

Figure 2. Dendrogram showing the genetic relationship among four *Bacillus thuringiensis kurstaki* strains**.**

undergone considerable taxonomic changes and is phenotypically heterogeneous with its members exhibiting an extremely wide range of nutritional requirements, growth conditions, metabolic diversity and DNA base composition (Claus and Berkely, 1986) *B. thuringiensis* is a member of *Bacillus cereus, Bacillus anthracis* phenotypic group (Ash et al., 1991b; Carlson and Kolse, 1993; Keim et al., 1997; Helgason et al., 2000). They are in principal mesophilic and neutrophilic and are placed in 16S rRNA/DNA group I. Classical features to distinguish this group from all other aerobic endospore forming bacteria are their ability to produce acids from mannitol and their production of lecithinase. Within the group, phenotypic differentiation is difficult. In general, the developmental process in *Bacillus* is temporally regulated at the transcription level by the successive action of different sigma factors to induce various sporulation dependent genes and cellular differentiation including crystal formation in *Bt* (Agaisse and Lereclus, 1995). Efficient sporulation may be linked, for enhanced production of insecticidal crystal protein in these bacterial strains (Agaisse and Lereclus, 1995).

The four different strains of *Btk* are evaluated for their comparative toxicity against two locally available common pest insects *H. armigera* and *S. littura* the mean LC_{50} values is different among each strain for both the insects. The quantitative and qualitative difference in production and composition of protoxin of *B. thuringiensis* isolates is postulated by various investigators (Grouchski et al., 1995; Du and Nickerson, 1996), the larval stage (Chenot and Raffa, 1998) and combined dosage effect of different toxins was also found to play determinative role (Chilcott and Tabashnik, 1997). The LC_{50} values strongly favour the view that more than one protoxin is present and they are efficiently processed within the gut to provide sufficient active toxin molecules in HD1 dipel compared to remaining three isolates. Other studies have also shown similar variability in toxicity within Btk serovars (Saitoh et al., 1996). To achieve unbiased values reflecting degree of lethality, index of specific toxicity was estimated (Table 1). It was found to be highest in case of *Btk* HD1 Dipel. These values showed prominent relationship with *cry*IA gene(s) presence on megaplasmids. Similar relationship was not evident either with generation time, specific growth rate and sporulation timing. The parental line of these insects may have already been exposed to synthetic insecticides in field but are never exposed to biopesticides preparation based on *B. thuringiensis*, therefore a difference in midgut physiological condition (Elghar et al., 1995) and nature of membrane receptors was expected in terms of susceptibility (Rao and Krishnayya, 1996; Reger et al., 1996; Chaufaux et al., 1997). Carrozzi et al. (1991) used 20 different primer sets to differentiate *B. thuringiensis* strains effective against lepidopteran, coleopteran and dipteran orders. Ben-Don et al. (1997) used five primer sets to probe highly conserved sequence of *cry* genes to distinguish Colombian *B. thuringiensis* isolates. The PCR amplificons in present work, were similar as in Carrozzi's work, no substantial difference in band size or intensity was observed to relate with copy number and proportion of *cry*IA genes to evaluate gene dosage effect in individual strains, further RTPCR work in this regard is in progress. The member of *Btk* group are virtually indistinguishable by 16S rDNA sequence analysis (Barns et al., 1999; Ash et al., 1991b) reconfirming the insufficiency in defining this genera on basis of phenotypic criteria alone (Woese, 1987; Ivanova et al., 1999). Multilocus enzyme electrophoresis and comparative DNA sequences analysis suggest that *B. cereus* group may represent single species (Helgasa et al., 2000). The conventional tests based on the phenotypic characteristics can clearly lead to misclassification in some bacterial taxa. Recently, it has been reported that the electrophoretic technique, as a practical method, is necessary for integrated use of phenotypic character in identification of bacterial genera at all level (Murray et al., 1990). Protein electrophoresis has been of grate value for delineation of numerous bacterial taxa (Vauterine et al., 1992). Each of the different electrophoretic technique has its own discrimination level and field of application. It is also widely acknowledged that the electrophoretic separation of plasmids is a sensitive technique that mainly provides information on the similarity of the strain at below species level. In addition, generally it is accepted that objective comparison of electrophoretic pattern provides a reliable measure of genomic inter-relationship. Many of the functional differences between *B. cereus, B. thuringiensis* and *B. anthracis* are due to presence of plasmid that varies in number and size (Carlson and Kolse, 1993; Ombui et al., 1996). Loss of crystal protein formation has been attributed to the loss or suppression of megaplasmid replication in several species of *B. thuringiensis* (Levinson et al., 1990). These mega-plasmids are known to be highly interrelated and replicate involving a single

stranded DNA intermediate. The additional features are plus and minus origin of replication and presence of crystal protein gene (Gonzales et al., 1982; Andrup et al., 1995; Vilas-Boas et al., 1998). In HD73, these megaplasmid were studied in detail by Gonzalez et al. (1981) and are known as pAMB-1 family of theta replicative plasmids usually present in broad range of Gram positive species (Wilcks et al., 1998; Gonzalez et al., 1981). Our result indicate that *cry*IA gene profile of *Btk* strains can provide valuable information which may be utilized in direct identification of these strains providing a reliable measure of genomic interrelationship. These results are in good agreement with previous investigations (Lewis et al., 1987; Cockmus and Yousten, 1984; Zheng and Slavik, 1999; Berber and Cockmus, 2001). This approach allows rapid characterization of isolates toxic to insect pests of order Lepidoptera. In Asia, *Btk* is the most common biochemical type (Martins and Travers, 1989). Thus, by using toxicity as well as hybridization studies, it is possible to characterize a large number of *Bacillus* native isolates up to subspecies level. Our study exhibited presence of more than one *cry*IA gene in *Btk* HD1 dipel and its performance better in *in vitro* toxicity assay against local *H. armigera* and *S. litura* insects (Regev et al., 1995) and *Btk* HD1, *Btk* dipel, *Btk* HD73 and *Btk* HD1 dipel strains can be distinguished at the level of toxicity and *cry*IA gene polymorphism by use of hybridization analysis as the potential diagnostic tool to distinguish strains when no specific variation could be detected by restriction analysis using same restriction endonucleases. Methods for bacterial identification are of great importance for taxonomical purpose, especially when they are accurate, rapid and easily applicable to large number of strains. Our numerical analysis of *cry*IA gene profiles provides a useful approach towards clarifying relationships at the sub-species, level and such sub-species clusters had characteristically distinct banding pattern of the genes. We suggest that simple comparison of the native plasmid profile provides a rapid means of identifying isolates from various sources in combination of restricted plasmid DNA profiling. We conclude, that numerical analysis of plasmid borne functional conserved genes is extremely useful in identification and taxonomic assessment of different isolates and it strengthens the Jackson's view for redefinition of species and subspecies in a group (Jackson et al., 1999), and to a large extent, will influence the consideration of taxonomists about where and how to discriminate sub-species from species.

ACKNOWLEDGEMENTS

The authors are thankful to the Council of Scientific and Industrial Research, New Delhi for fellowship to S. K. and Prof. H. R. Whiteley for bacterial strain. We are grateful to DBT, GOI New Delhi for grant of project to D.V.A. Infrastructure facilities and encouragements by Director, NBRI and Director, NBAIM is duly acknowledged.

REFERENCES

Adang MJ, Staver MJ, Rocheleau TA, Leigfhton J, Barker RF, Thomson DV (1985). Characterization of full – length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HDF-73 and their toxicity to *Manduca sexta*. Gene. 36: 289-300.

Agaisse H, Lereclus D (1995.). How does *Bacillus thuringiensis* produces so much insecticidal crystal protein? J. Bacteriol. 177: 6027-6032.

Andrup L, Bendixen HH, Jensen GB (1995). Mobilization of Bacillus thuringiensis plasmid ptx14-3. Plasmid. 33: 159-167.

Ash E, Farrow JAE, Wallbanks S, Collins MD (1991a). Phylogenetic hetrogenity of the genus Bacillus revealed by comparative analysis of small –subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. 13: 2002-2006.

Ash C, Farroow JAE, Dorsch M, Stackebrandt E, Collins MD (1991b). Comparative analysis of Bacillus antracis, Bacillus cereus and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Sys. Bacteriol. 175: 1053-1060.

Ausubal FM, Brent R, Kiogston RE, Moorre DD, Seidman JG, Smith JA, Struhl K (2002). Short protocols in Molecular Biology, 5ed.A Compendium of methods fro current protocols in molecular biology. John Wiley and Sons. Inc. USA, 2002, 1: 10-9 10.10, ISBN-0-47125092-9

Barns SM, Hill KK, Jackson PS, Kuske CR (1999). 16s ribosomal sequence- based phylogeny of Bacillus species and the *B. cereus* group. Los Almos unclassified report. LAVR 99-5628. LOS Almos National Laboratory, Los Almos NM , USA.

Ben-Don E, Zarisky A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraev A, Troiskaya E, Dubisky A, Berezina N, Margalith Y (1997). Extended screening by PCR for seven *cry* group genes from field collected strains of *B. thuringiensis*. Appl. Environ. Microbiol. 63: 4883- 4890.

Berber I, Cokmus C (2001). Characterization of Bacillus spheaericus strains by Native –PAGE. Bull. Pure Appl. Sci. 20(1): 17-21.

Berber I, Cokmus C, Atalan E (2003). Characterization of Staphylococcus species by SDS-PAGE of Whole –Cell and Extracellular Proteins. Microbiol. 72(1): 42-47.

Carlson C, Kolse AB (1993). A complete physical map of a *Bacillus thuringiensis* chromosomes. J. Bacteriol. 41: 343-346.

Carozzi NB, Kramaer VC, Warreen GW, Evola S, Kozid MG. (1991). Prediction of Insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profile. Appl. Environ. Microbiol. 57: 3057-3061.

Chaufaux J, Muller CJ, Buisson C, Sanchis V, Lereclus D, Pasteur N (1997). Inheritance of resistance to the *Bacillus thuringiensis* CryIC toxin in *Spodoptera littoralis* (Boisd) (Lep. Noctuide) J. Econ. Entomol. 90: 873-878.

Chenot AB, Raffa KF (1995). Effect of parasitoid strains and host instar on the interaction of *Bacillus thuriengensis* subsp. *kurstaki* with the gypsy mouth (Lepidoptera: Lymantriidae) larvae parasitoid Cotesia melanoscela (Hymenoptera: braconidae) Environ Entimol. 27: 137-147.

Chilcott CF, Tabashnik BK (1997). Independent and combined effects of Bacillus thuringiensis and the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) on susceptible and resistant Diamondback mouth (Lepidoptera; plutellidae) J. Econ. Entomol. 90: 397-403.

Claus D, Berkeley CW (1986). The genus Bacillus In: Bergey's Manual of Systematic Bacteriology. vol 2. Sneath PHA (ed) Williams, Wilkins, Baltimore. 34: 1105-1139.

Cockmus C, Yousten AA (1987). Characterization of *Bacillus spharecius* strains by SDS-PAGE. J. Invertb. Pathol. 64: 267-3678.

Cohn F (1872). Untersuchungen uber Bakterien. In Beitrage zur Biologie der Pflanzen1.J.U. Kern's verlag (Max Muler) Braesiau 1875. (1): 127-224.

Costas M (1992). Classification, Identification and typing of bacteria by the analysis of their one dimensional polyacrylamide gel electrophoretic protein patterns In: Advances in Electrophorasis Vol 5. Chrambach A, Dunn NJ, Radole BJ (ed.). pp. 351-408.

Damgaard PH, Hansen BN, Pedersen JC, Eilenberg J (1997). Natural occurrence of *Bacillus thuringiensis* on cabbage foliage and in insects associated with cabbage crops. J. Appl. Microbiol. 82: 253-225.

Du C, Nickerson KW (1996). The *Bacillus thuringiensis* insecticidal toxic binds biotin- containing proteins. Appl. Environ. Microbiol. 62: 2932-2939.

Elghar G, Radwan HSA, Elbermawy ZA, Zidan LTM (1995). Inhibitory effect of thuringiensin and abamectin on digestive enzymes and non-specific esterase of *Spodoptera litoralis* (Boisd.) (Lep., Noctuidae) larvae. J. Appl. Entomol. 62: 355-359.

Glare TR, O'Callaghan M (2000). *Bacillus thuringiensis*: Biology, Ecology and Safety. New York; John Wiley and Sons, 350. ISBN 0-471-49630-8.

Gonzales JM, Brown BJ, Carlton BC (1982). Transfer of *Bacillus thuringiensis* plasmid coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Nat. Acad. Sci. USA 79: 6951-6955.

Gonzalez JM, Dulmage HT, Carlton BC (1981). Correlation between specific plasmid and delta- endotoxin production in *Bacillus thuringiensis*. Plasmid 5: 351-365.

Grouchski P, Manon L, Borisora S, Pusztai-cary M, Schwartz JL, Brouusseaus R, Lygler M (1995). *Bacillus thuringiensis cry*IA(a) insecticidal toxin: Crystal structure and channel formation . J. Mol. Biol. 254: 447-464.

Harlow ED, Lane D (1988). Antibodies, A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolste AB (2000). *Bacillus anthracis, Bacillus cereus* and *Bacillus thuringiensis*: one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66: 2627-2630.

Hofte H, Whitely HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbial. Res. 53: 242-255

Ivanova PE, Vysotskii MV, Svetonhev VV, Yamoto N, Shigeri Y, Taguchi T, Yoshikawa S (1999). Characterization of Bacillus strains of marine origin. Int. Microbiol. 2: 267-271.

Jackson PJ, Hill KK, Laker MT, Tricknor LO, Keim P (1999). Genetic comparison of *Bacillus anthracis* and its close relatives using AFLP and PCR analysis. J. Appl. Microbial. 87: 263- 269.

Keim P, Kalif A, Schupp J, Hill K, Travis SE, Richmound K, Adair DM, Hugh –Jones M, Kaske C R, Jackson PJ (1997). Molecular evolution and diversity in Bacillus anthracis as detected by amplified fragment polymorphism markers J. Bacteriol. 179: 818-824.

Kronstad JW, Whiteley HR (1986). Three class of homologous *Bacillus thuringiensis* crystal protein gene. Gene. 43: 29-40.

KyongLee MI, Curtiss A, Alcantara E, Dean DH (1996). Synergistic Effect of the *Bacillus thuringiensis* toxin CryIA and CryIAc on the Gypsy Moth, *Lamantria dispar*. Appl. Environ. Microbial. 62: 583-586.

Levinson BL, Kasyan KJ, Chiu SS, Currier TC, Gonzalez JM (1990). Identification of beta- exotoxin production , plasmids encoding beta-exotoxin and a new exotoxin in *Bacillus thuringiensis* by using high performance liquid chromatography. J. Bacteriol. 172: 3172-3179.

Lewis LO, Yousten AA, Murray RGE (1987). Characterization of the surface protein layers of the mosquito pathogenic strains of *Bacillus sphaericus*. J. Bacteriol 169(1): 72-79.

Martins PAW, Travers RS (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. 55: 2437-2442.

Murray RGE, Brenner DJ, Cowell QR, Devos P, Goodfellow M, Grimont PAD, Pfenning N, Stackbrant E, Zavarzin GA (1990). Report of the ad hoc committee on approaches to taxonomy within the proteobacteria. Int. J. Syst. Bacterial. 40: 213-215.

Niemi RM, Niemela SI, Baoorford DH, Hantala J, Hyvaarienen T, Forsten T, Raateland A (1993). Presumptive fecal Streptococci in environmental samples characterized by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophorasis . Appl. Envi. Microbiol. 59(7): 2190-2196.

Ombui JN, Mathenge JM, Kimotho AM, Macharia JK, Nudhiu G (1996). Frequency of antimicrobial resistance and plasmid profiles of *Bacillus cereus* strains isolated from milk. East. Afr. Med. J. 73: 380-384.

Rao BM, Krishnayya PV (1996). Effect of diflubenzuron and *Bacillus thuringiensis*var.*kurstaki* baits on the growth and development of Spodotera litura (Fab.) larvae. Pestic. Res. J. 8: 80-83.

Reger A, Keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, Konz C, Schell J, Zlberstein A (1996). Synergistic activity of a *Bacillus thuringiensis* delta- endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl. Environ. Microbiol. 62: 3584-3586.

Rohlf F J (1997). Ntsys pc: Numerical taxonomy and Multivariate analysis system Version 2.02h. Exeter Software, New York.

Saitoh H, Higuchi K, Mizuki E, Ohba M (1996). Larvicidal activity of Bacillus thuringiensis natural isolates, indigenous to Japan, against two nematoceran insect pests occurring in urban sewage environments. Microbiol. Res. 151: 263-271.

Sambrook Joseph, Russell David W (2001). Molecular cloning: A laboratory manual. New York; Cold Spring Harbor Laboratory Press, Cold Spring Harbor,1610. ISBN 0-87969-577-3.

Schnepf E, Crickmore N, Van Rie J, Lerecus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998). *Bacillus thuringiensis* and its pesticidal crystal protein. Microbial. Mol. Biol. Rev. 62: 775-806.

Slepeck RA, Hemphill HE (1992). The Genus Bacillus Nonmedical in "The Prokaryotes" vol II. Balows A, Truper HG, Drowkin M, Tno WH, Schleifer KH, (Ed.) Springer-Verlag, New-York , Berlin. Heiderlberg, London, Paris, Tokyo, Hong-Kong, Barcelona, Budapest, 48: 1697-1745.

Stanier RY, Ingraham Jl, Wheelis ML, Painter PR (1990). In Microbial World. Prentice-hall Inc. USA. p. 183.

Travers RS, Martins PAW, Reichelderfer IF (1987). Selective process for efficient isolation of soil *Bacillus* spp. Appl. Environ,.Microbiol. 53: 1263-1266.

Tricnor lO, Kolsto AB, Hill KK, Keim P, Laker MT, Tonks M, Jackson PJ (2001). Fluorescent AFLP analysis of Norwegian *Bacillus cereus*/ *Bacillus thuringiensis* soil isolates. Appl. Environ Microbial. 67: 4863-4873.

Vauterin L, Vantomme R, Pot B, Host B, Swings J, Kersters K (1990). Taxonomic analysis of Xantrhomonas campestris pv. Begonidae and X. campestris pv. Pelangonii by means of phytopathological, phenotypic protein/electrophoretic and DNA hybridization methods. Syst. Appl. Bacteriol 13: 166-167.

Vilas-Boas GFLT, Vilas-Boas LA, Lereclus D, Arantes OMN (1998). *Bacillus thuringiensis* conjugation under environmental conditions FEMS Microbiol. Lett. 25: 369-374.

Widener WR, Whiteley HR (1990). Two highkey related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* posses different host range specificities. J. Bacteriol. 171: 965-974.

Wilcks A, Jayaswal N, Lerecus D, Andrup I (1998). Characterization of plasmid pAW63, a second self –transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. Microbiol. 144: 1263-1270.

Woese CR (1987). Bacterial Evolution. Microbiol. Rev. 51: 221-271.

Zheng G, Slavik MF (1999). Isolation, Partial purification and Characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strains Lett. Appl. Microbiol. 28: 363-367.