



Research Article

Available online at www.journal-advances-developmental-research.com

Journal of Advances in Developmental Research

ISSN: 0976-4704 (Print), e-ISSN: 0976-4844 (Online)

J.Adv.Dev.Res. Volume 2, No.1, June 2011

Diversity and Characterization of *Bacillus thuringiensis* Isolates from Alluvial Soil of Mahi River Basin, India

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Abstract

Bt strains were isolated from alluvial soils of two places and characterized by molecular and analytical methods. Diversity in protein profiles and *cry* gene profiles was observed in the isolates. Majority of them showed 95 kDa protein band on SDS-PAGE while rest showed 130, 44 and 29 kDa bands. PCR analysis revealed dominance of *cry1* and *cry2* genes in these isolates. Some of the isolates also demonstrated presence of *cry11* and *cyt* genes. RFLP analysis of full length *cry1* genes and partial *cry2* genes showed diverse *cry1* genes but similar *cry2* genes. Prevalence of similar *Bt* strains in the soils of two sites was observed.

Key words: alluvial soil, *Bacillus*, *cry* genes

Introduction

Bacillus thuringiensis (*Bt*) has become the most well known microbial biocontrol agent due to its *Cry*, *Vip* and *Cyt* toxins¹. The key feature for its wide acceptance as biocontrol agent lies in specific action of these insecticidal toxins especially *cry* proteins towards large group of insects. Ease of commercial production and use has added to its advantage over other microbial biopesticides. As a result of this, it occupies more than 90% of the world biopesticide market². Along with the use of *cry* genes in transgenic plants recently, it has contributed significantly to reduce environmental pollution by chemical pesticides.

Worldwide isolation of *Bt* have established it to be a natural inhabitant of soil^{3, 4, 5}. However isolation of *Bt* from diverse environments such as phylloplane, dead insects, stored grains, brackish sediments, aquatic environments etc. has created an enigma about the role of *Bt* in nature^{6, 7, 8}.

Isolation of *Bt* was possible from environments where no respective insect population was present³. Similarly a firm correlation between frequency of active strains and geography or type of sample was not observed⁹.

Isolation of *Bt* from alluvial samples would thus contribute to a better understanding of the role of *Bt* in nature. Also novel *Bt* isolates if found, could be used to test its potential against resistant insect pests or against sap sucking pests such as planthoppers and leafhoppers (Delphacidae & Cicadellidae). Considering these aspects, isolation of *Bt* from alluvial soil samples in present study was carried out.

Experimental

Soil sample collection

Two stratified exposed sections of alluvial soils unaffected by anthropogenic activities were selected at Rayka and Jaspur sites situated along

Mahi river basin, Gujarat, western India. Both sites were at fourteen kilometers of distance from each other. Soil samples were collected after removing five centimeter surface soil. Seven soil samples from Rayka and four from Jaspur were collected at heights of one meter interval from bottom of the exposed section. All samples were collected as triplicate composite mixture of soils.

Isolation of *Bt*

Bt isolation was carried out by three different methods. Sodium acetate selection and dry heat pretreatment was performed as described in reports^{10, 11}. Enrichment method was performed by aseptically adding one gram soil sample into 20 ml Glucose Yeast extract Salt medium prepared in 250 ml flask [GYS per liter: 1 g glucose, 2 g yeast extract powder, 2 g $\text{NH}_4(\text{SO}_4)_2$, 0.06 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g CaCl_2 , 5 g K_2HPO_4]. Mixture was incubated at 30°C with 200 rpm shaking conditions for 48 hours. One ml supernatant was withdrawn from this mixture and given brief centrifugation at 1000 rpm for 1 min., followed by heat treatment at 80°C for 3 min and serial dilution up to 10^{-8} . A 200 μl aliquot from 10^{-6} to 10^{-8} dilutions were spread on L.B. plates. Plates were incubated at 30°C for two days and *Bt* like colonies were observed. *Bt* like colonies were confirmed by microscopy, SDS-PAGE and PCR analysis.

SDS-PAGE analysis

Bt colonies were inoculated in 250 ml flask containing 20 ml GYS sporulation medium and incubated at 30°C for two days with 200 rpm shaking conditions. Two ml sample was centrifuged at 10,000 rpm for 10 minutes. One ml of Tris-EDTA-PMSF buffer (Tris 10 mM, EDTA 1 mM, phenyl methyl sulphonyl fluoride 1 mM) was added to the pellet, resuspended and incubated for 10 minutes at room temperature (RT) followed by centrifugation at 10,000 rpm for 10 minutes. Pellet was washed with 0.5 ml of 0.85% saline. Finally pellet was dissolved in 100 μl 0.1 N NaOH, incubated at RT for 5 min, 20 μl of 6X SDS-PAGE loading dye was added and boiled for ten minutes. 20 μl sample was loaded onto 10% SDS-PAGE gel and electrophoresis was carried out.

PCR analysis

Total DNA extraction was carried out as described in report¹² with modifications in DNA spooling step. Instead of spooling, DNA precipitates

were centrifuged at 10,000 rpm for 10 min in 1.5 ml microfuge tube followed by dissolving pellet in 10mM Tris buffer pH 8.0. Total DNA (~25 ng) was used as template in a 30 μl PCR reaction. PCR analysis was carried out by using universal and specific primers for *cry* genes as shown in Table 1. Standard strain *Bacillus thuringiensis* var. *kurstaki* HD1 was used as positive control for *cry1* and *cry2* genes. Amplification products were cloned into *E. coli* DH5 α using InsTA clone kit, Fermentas, USA as per manufacturer instructions. Sequencing of the cloned amplification products was done commercially at Bangalore Genei Pvt. Ltd., Bangalore, India.

RFLP analysis

PCR products of size 3.4 kb were amplified for *cry1* genes to be used for RFLP analysis. Primers 1AF and GrC were used as forward primers for full length *cry1* gene amplification (Table 1). Primer CJ2¹³ was used as common reverse primer. Primers II(+) and II(-) (Table 1) was used for partial amplification of *cry2* genes (1.5 kb). Restriction enzyme digestion was carried out by using 5 units of *Hae* III enzyme and ~500 ng of PCR amplification products in 20 μl systems, incubated at 37°C overnight. Digests were resolved in 2% agarose and stained with Ethidium bromide (0.5 $\mu\text{g}/\text{ml}$).

Results

Isolation of *Bt*

Eight alluvial samples out of eleven were found to contain *Bt* isolates (Figure 1). *Bt* could not be isolated from two samples of Rayka site and one of Jaspur by any of the methods. Seventy isolates from Rayka site and eighty isolates from Jaspur showed *Bt* like colony morphology and crystal inclusions. *Bt* index in the samples varied broadly from the lowest 0.11 in Rayka 4 sample to the highest 0.72 in Jaspur 2 sample (Table 2). *Bt* index observed in present study was comparable to reports of *Bt* isolation from Indian soils^{14, 15}.

SDS-PAGE analysis

Isolates showed distinct protein profiles of sporulated mixture as shown by representative isolates (Figure 2). Many of the isolates from two sites showed similar protein profile. Surprisingly, a ~95 kDa protein band was found to be present in most of the distinct profiles of isolates. *Bt* strains

Table 1. Different pair of primers used to amplify respective *cry* genes

Primer pair	Sequence	Gene(s) recognized	Annealing Temp. (°C)	Reference
CJ1/	TTATACTTGGTTCAGGCC			
CJ2	TTGGAGCTCTCAAGGTGTAA	<i>cry1</i>	49.5	13
II(+)/ II(-)	TAAAGAAAGTGGGGAGTCTT AACTCCATCGTTATTTGTAG	<i>cry2</i>	50	32
CJIII20/ CJIII21	TTAACCGTTTTTCGCAGAGA TCCGCACTTCTATGTGTCCAAG	<i>cry3, 7 8</i>	50	13
gral-nem/ gral-nem(r)	TTACGTAAATTGGTCAATCAAGCAAA AAGACCAAATTC AATACCAGGGTT	<i>cry5, 12, 14, 21</i>	51	20
gral-cry11(d)/ gral-cry11(r)	TTAGAAGATACGCCAGATCAAGC CATTTGTA CT TGAAGTTGTAATCCC	<i>cry11</i>	51.5	20
gral-cry13(d)/ gral-cry13(r)	CTTTGATTATTTAGGTTTAGTTCAA TTGTAGTACAGGCTTGTGATTC	<i>cry13</i>	47	20
gral-cyt(d)/ gral-cyt(r)	AACCCCTCAATCAACAGCAAGG GGTACACAATACATAACGCCACC	<i>cyt1</i>	55	20
1AF/ CJ2	ATGGATAACAATCCGAACATC TTGGAGCTCTCAAGGTGTAA	<i>cry1A</i>	49.5	33
GrC CJ2	ATGGADATAARTMAYCARAA TTGGAGCTCTCAAGGTGTAA	<i>cry1C, 1D, 1G, 1J</i>	49.5	Present study

isolated from western Ghats, India also showed a band of ~95 kDa protein¹⁵. Some of the isolates revealed presence of 130-140 kDa bands of Cry proteins as reported by many authors^{16,17}. Interestingly, many of the isolates showing 130 kDa protein band did not showed 66 kDa band characteristic of activated Cry1A toxin. Few of the isolates showed an intense band of ~29 kDa size as reported¹⁸. Concurrent to report¹⁹, many of the isolates in present study also showed a band of 44 kDa protein along with other bands.

PCR analysis

Abundance of *cry1* and *cry2* genes was observed in *Bt* isolates from both sites (Figure 2). Simultaneous presence of both genes was found in most of the isolates. Few isolates revealed presence of *cry11* and *cyt* genes along with *cry1* and *cry2* genes. However, none of the isolates from both sites used in present study showed amplification with

Table 2. Characterization of *Bt* isolates from alluvial soil samples

Soil sample	<i>Bt</i> index	Major protein profile bands (kDa)	<i>cry</i> genes
Rayka 1	5.6	130, 95, 55, 44	<i>cry1, cry2</i>
Rayka 2	6.0	95, 80, 29	<i>cry1, cry2, cyt</i>
Rayka 3	0.0	—	—
Rayka 4	1.1	130, 95, 29	<i>cry1, cry2, cry11</i>
Rayka 5	1.6	130, 95, 80,	<i>cry1, cry2, cry11</i>
Rayka 6	0.0	—	—
Rayka 7	6.6	130, 95, 80, 55,	<i>cry1, cry2</i>
Jaspur 1	3.3	140, 130, 95, 80, 55, 29	<i>cry1, cry2, cyt</i>
Jaspur 2	7.2	140, 130, 95, 80, 29	<i>cry1, cry2, cry11</i>
Jaspur 3	6.6	130, 95, 80	<i>cry1, cry2, cyt</i>
Jaspur 4	0.0	—	—

primers for *cry3*, 7, 8, *cry4*, *cry5*, 12, 14, 21, *cry9* and *cry13* genes. Diversity in *cry* gene profiles was observed with isolates. Isolates GE R18 and GE R38 from Rayka site and GE J44 from Jaspur site harbored only *cry2* gene, whereas isolates GE R30 from Rayka site and GE J82 from Jaspur site had only *cry1* gene. Few of the isolates from both sites were found to contain *cry1-cry2-cry11* and *cry1-cry2-cyt* gene combinations.

RFLP analysis

RFLP analysis was performed for *cry1* and *cry2* genes in order to explore diversity of these genes in the isolates. Restriction enzyme *Hae* III producing distinct digestion pattern for each of the known *cry1* and *cry2* genes was selected on basis of *in silico* restriction digestion analysis using NEB cutter V2 online software. A similar digestion pattern was observed in *cry2* gene partial amplification products of all isolates tested in present study (data not shown). Thus it indicated the probable presence of the same *cry2* gene in all isolates. Full length amplified *cry1* gene instead showed four distinct digestion patterns (Figure 3). Three distinct digestion patterns were observed in isolates from Jaspur 2, Rayka 2 and Rayka 7 samples indicating presence of different *cry1* genes in these samples. One digestion pattern matching to that of *in silico cry1Ac Hae* III digestion pattern was observed to be the most frequent pattern in isolates from all three sites (Figure 3, lanes 1, 2, 4, 6, 10, 12, 13 and 15). Sequencing of the partial *cry1* gene amplification product of the isolate GE J25 confirmed it as *cry1Ac* gene. Isolate GE R15 showed a unique digestion pattern. Few other patterns did not match to any of the known *cry1* genes and thus could be novel *cry1* gene or variant of a known gene (Figure 3, lanes 5, 8, 9, 11, 16).

Discussion

Isolation of *Bt* from nine out of eleven alluvial soil samples which are unaffected by any anthropogenic activities indicated *Bt* to be a natural inhabitant of soils. Alluvial soil samples being the sedimentary samples deposited by river since ages and devoid of insect, suggested *Bt* survived in these soils since long time without obligate interaction with insects. Thus results of the *Bt* isolation in present study supports survival of *Bt* irrespective of insects being present as reported³.

Distinct protein profiles of isolates demonstrated high diversity in isolates from both

sites. However, similar protein profiles observed in isolates from two sites indicated prevalence of similar strains of *Bt*. Isolates showing protein bands of 130 kDa without 66 kDa band could possess novel *cry* proteins other than *cry1* as reported¹⁶. Similarly isolates producing 95 kDa protein could harbor novel *cry* genes as reported¹⁵ for native Indian *Bt* isolates. Thus, presence of diverse and novel *cry* proteins could be expected in alluvial samples.

Abundance of *cry1* gene in *Bt* isolates from alluvial soils supported dominance of this gene worldwide as reported by many authors^{6, 20, 21, 22}. Abundance of *cry1* gene in *Bt* strains isolated from mountain soil, phylloplane and aquatic environments have been reported^{22, 23, 24, 25}. Thus dominance of *cry1* gene could be concluded to be established in diverse environments including alluvial soil. *Cry2* gene was observed as second dominant gene in present study. In support to reports^{4, 6, 26}, *cry2* genes were found in combination with *cry1* gene in most of the isolates. Striking feature for *cry2* gene dominance was that the band of 66 kDa size protein representing *cry2* proteins was not observed in any of the isolates in SDS-PAGE analysis. It could be due to low or no expression of the gene as reported by^{22, 23}. Many authors reported simultaneous presence of *cry11* and *cyt* genes^{6, 20, 27, 28, 29}. Interestingly, in present study presence of *cry11* and *cyt* genes was not observed simultaneously but in combination with *cry1* and *cry2* genes. Low frequency of *cry3* and *cry5* genes in tropical regions has been reported^{6, 20, 23}. Sites selected for *Bt* isolation in present study being present under tropical region, absence of *cry3* and *cry5* genes could be attributed to it. However absence of *cry4*, *cry9* and *cry13* genes cannot be explained.

RFLP analysis indicated prevalence of probably one *cry2* gene in contrast to five different *cry1* genes in isolates from two sites. Similarly just one *cry2* gene compared to 12 *cry1* genes from 178 *Bt* strains was found²¹. Other reports^{26, 30} showed low diversity with just three *cry2* genes. It could be attributed to the reason that only five different *cry2* genes are reported worldwide compared to more than 42 *cry1* genes³¹. It indicates low diversity in *cry2* genes globally. Thus, probably there are fewer chances for *cry2* gene diversity in an environment such as alluvial soil in present study. Nevertheless, digestion with another tetra cutter restriction enzyme or sequencing needs to be performed in order to conclude presence of a single *cry2* gene in the isolates. Results of similar protein profile in *Bt* isolates from both sites were supported

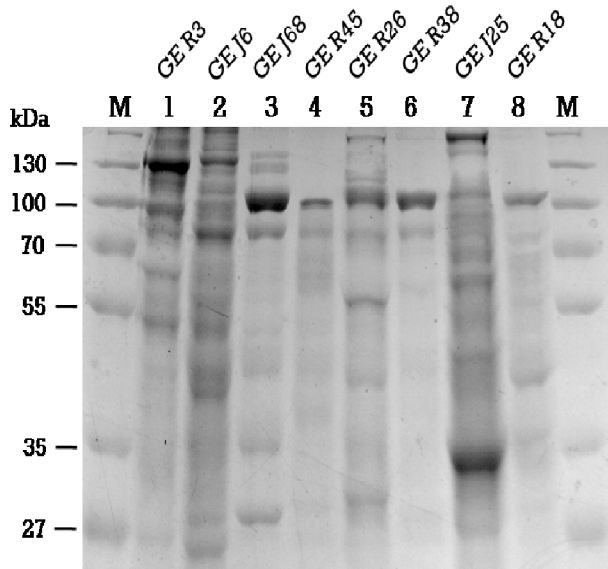


Fig. 1. SDS-PAGE Analysis of Representative *Bt* Isolates. Lane M: Protein Molecular Weight Marker, Lanes 1-8: *Bt* Isolates From Alluvial Samples

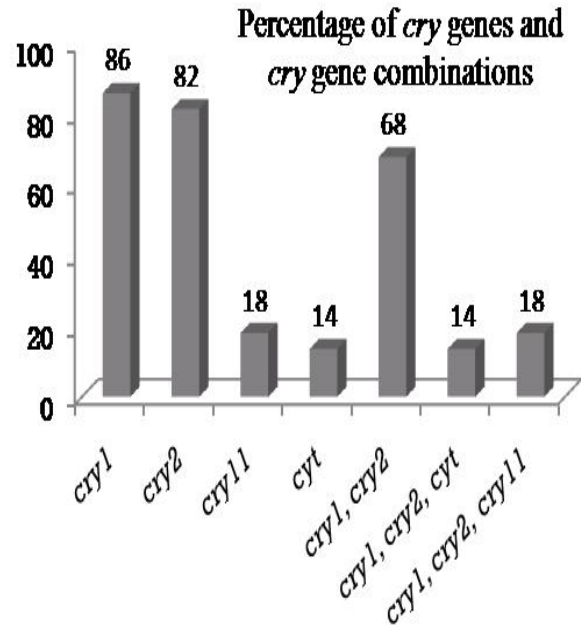


Fig. 2. Percentage of *cry* Genes and *cry* Gene Profiles

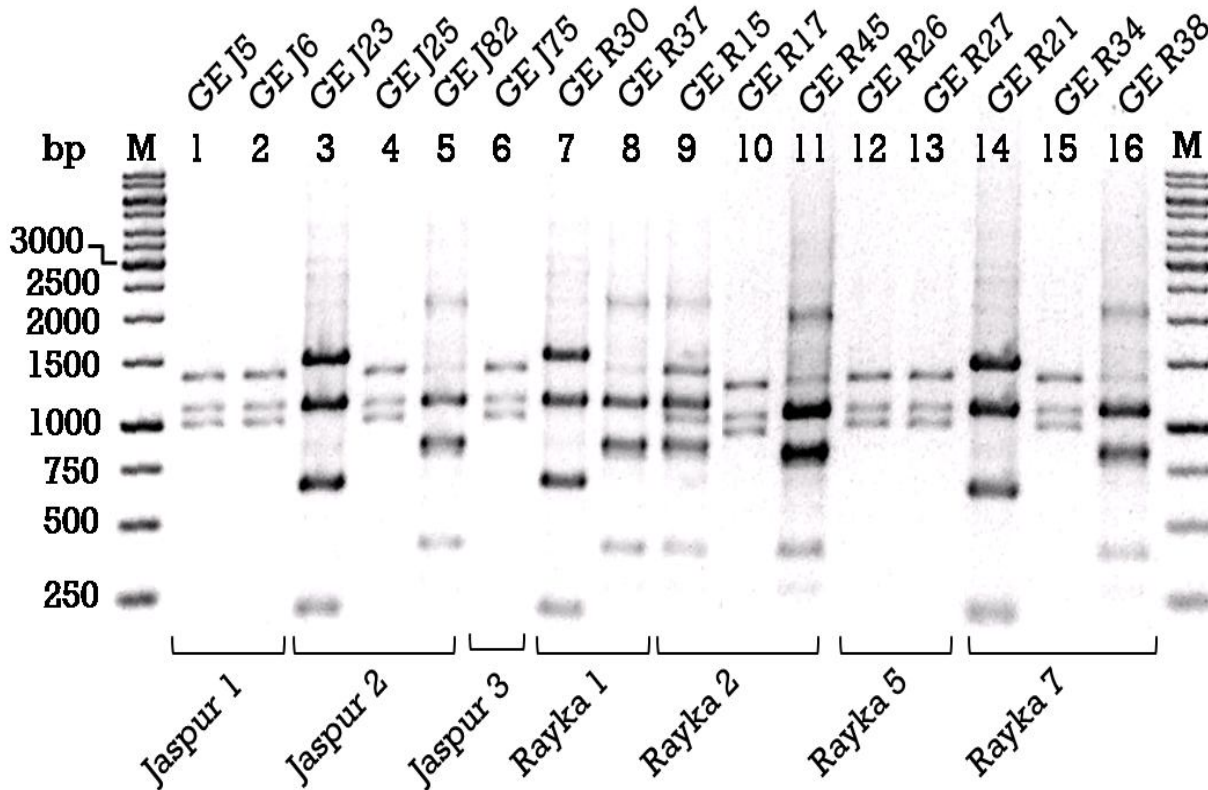


Fig. 3. RFLP Analysis of *cry1* Genes. Lane M: DNA Molecular Weight Marker, Lanes 1-16: *Bt* Isolates From Alluvial Samples

by occurrence of common *cry1* gene digestion patterns. Thus prevalence of diverse *Bt* strains but common between the two locations can be concluded.

Bt isolates showing 130 kDa protein band and presence of *cry1* genes could be toxic to Lepidopteran insects. Similarly isolates showing 44 kDa and 29 kDa bands along with *cry11* or *cyt* genes could be toxic to dipteran insects. Isolates with unmatched digestion pattern could harbor novel *cry1* genes. Thus future investigation of this study will include bioassays against resistant insects and various other insects to explore their toxic potential and cloning of novel *cry* genes.

Acknowledgements

All authors are greatly thankful to the grant provided by University Grant Commission (UGC), Government of India, New Delhi, for the project and research fellowship. We would like to acknowledge Mr. Gangavarpu Subrahmaniam Chaudhary and Dr. G. Archana for kindly providing alluvial soil samples.

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