Molecular Characterization of Novel Serovars of *Bacillus thuringiensis* Isolates from India

Ketan D. Patel · Sanjay S. Ingle

Received: 23 March 2011 / Accepted: 16 November 2011 / Published online: 29 November 2011
© Association of Microbiologists of India 2011

Abstract Novel *Bacillus thuringiensis* isolates GS4, GN24 and UP1 were isolated and characterized by determination of serotyping, insecticidal protein by SDS-PAGE, plasmid composition, *cry* gene content and insect toxicity. Serologically two isolates GS4 and UP1 were allocated to the H3abce which is a new serovar while isolate GN24 was of H3ab type. Isolate GS4 produced flat crystal inclusions while UP1 produced cuboidal crystals. PCR analysis found that both isolates contained *cryI* and *cryI*Ac genes. The major protein bands found of isolate GS4 were of molecular weights 175, 135, 97, 88, 66, 54 and 27 kDa, isolate UP1 were of 85, 60 and 40 kDa and isolate GN24 were of 130, 90, 66 and 45 kDa. Though isolates GS4 and UP1 belonged to a new serovar H3abce, they showed different crystal inclusions and *cry* gene content. Isolate GS4 was toxic to lepidopteran insect larvae of *Helicoverpa armigera* but UP1 did not showed any toxicity.

Keywords Crystal inclusions · Cry proteins · Serovars · Plasmids

Introduction

Bacillus thuringiensis (*Bt*) is a potent insect pathogen with Cry, Cyt and Vip proteins as virulence factors [1]. *Bt* proved to be one of the most important biocontrol agent for use in agriculture, forestry and human disease vector

control [2]. With the ease of production it has been widely used as spore-crystal formulations. It has widespread use both as formulations and in transgenic plants. With this advent in use it has significantly reduced the use of chemical pesticides and contributed to control environmental pollution [3].

Worldwide *Bt* isolation and screening programs has led to find a large number of *Bt* strains [4–7]. However several reasons necessitate continuous isolation and characterization of novel *Bt* strains. Development of resistance in few insects to *Bt* toxins at field level [8] needs a special attention. Narrow spectrum of toxicity of *Bt* could become a drawback in case where more than one insects infest the same field. Recent and the most challenging is the emergence of secondary pests. Sap sucking pests such as whitefly (*Bemisia tabaci*), cotton aphids (*Aphis gossypii*), mealybugs (*Phenacoccus* sp., *Maconellicoccus* sp.) and mirids (*Creontiades* sp.) are major threats. These secondary pests bear no susceptibility to *Bt* toxins [9]. Outbreaks of Mirid insects are rising in India and China [10, 11]. Besides Cry, Cyt and Vip toxins, *Bt* also produces other toxic factors like thuringiensin (β -exotoxin or ‘heat-stable’ exotoxin) and phosphatidylinositol-specific phospho-lipase C (PIPLC, α -exotoxin or ‘heat-labile’ exotoxin) and chitinases [12, 13]. In addition to these virulence factors other valuable products like proteases, enzymes for dye degradation, PHA are also reported by *Bt* strains [14, 15]. Thus a continuous investigation on isolation and characterization of novel *Bt* strains is the need of time.

Present study describes characterization of new serovars from soil of India for crystal inclusion, Cry protein profile, plasmid pattern, *cry* gene content and insect toxicity. Characterization of these strains would contribute to knowledge of *cry* gene distribution in different serovars and their pathogenicity.

K. D. Patel · S. S. Ingle (✉)
Ground Floor Laboratory, Department of Microbiology
and Biotechnology Center, M.S. University of Baroda,
Vadodara, Gujarat, India
e-mail: ingle05@yahoo.co.in

Materials and Methods

Bt Isolation

Native *Bt* strains were isolated by *Bt* enrichment method as described in previous study [16]. Isolates GS4 and GN24 were isolated from soil of agricultural lands of Vyara and Bhavanagar districts, Gujarat respectively. Isolate UP1 was isolated from agricultural soil of Chandoli district, U.P., India.

Phase Contrast Microscopy

Bt isolates were sporulated by inoculating in Glucose Yeast extract Salt (GYS) medium and incubating at 30°C with 200 rpm shaking conditions for two days. After complete sporulation, cultures were smeared on glass slides, air dried, heat fixed and stained with 0.5% crystal violet. Spore-crystal complex was observed under phase contrast microscopy at 1000× magnification.

Cry Protein Analysis

Sporulation of isolates was done in GYS medium as described above. Two ml of sporulated culture was centrifuged at 10,000 rpm for 10 min and resuspended in one ml of Tris–EDTA–PMSF buffer (Tris 10 mM, EDTA 1 mM, phenyl methyl sulphonyl fluoride 1 mM). After incubation at room temperature for 10 min, centrifugation was done at the same speed and time followed by wash with 0.5 ml of 0.85% saline. Pellets were resuspended in 100 µl of 100 mM NaOH, incubated at RT for 10 min. An aliquot of 20 µl loading dye (6X) was added and mixed well. Boiling treatment was given for 10 min and used for loading onto 10% SDS-PAGE gel. Molecular weight of each band was estimated by Alpha Ease FC software version 4.0.0, Alpha Innotech.

Bt Plasmid Extraction

It was done by Total DNA extraction method with some modifications as described in previous study [16]. Total DNA sample (25 µl) containing genomic and plasmid DNA was loaded onto 0.8% agarose gel stained with 0.5% ethidium bromide to observe bands of plasmids.

PCR Analysis

Total DNA sample (~25 ng) was used in PCR system with CJ1/CJ2 primers for universal *cryI* genes [17], II(+)/II(–) primers for *cry2* genes [18] and CJIII20/CJIII21 primers for *cry3*, 7, 8 genes [19]. Primers *gral-nem/gral-nem(r)*, *gral-cry11(d)/gral-cry11(r)* and *gral-cry13(d)/gral-cry13(r)*

were used for *cry5, 12, 14, 21, cry11, cry13* and *cyt1* genes respectively as described by Bravo et al. [5]. Primers Ac2kbF (TGGGGATTTGATGCCGCGACTAT) and Ac2kbR (CTTGCGTGCCCATCTTGCGTCT) were used as specific primers for the partial amplification of *cryIAc* gene at 56°C annealing temperature, yielding a 2 kb amplification product.

Serotyping

Flagellar H antigen serotyping of isolates was performed by tube agglutination tests using diluted H-specific antisera [20] at Graduate School of Bioresource & Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan.

Insect Bioassay

Laboratory reared 3rd instar larvae of *Helicoverpa armigera* were used for insect bioassay. Sporulated culture was prepared in GYS medium, centrifuged and washed with 0.85% saline. Pellet was resuspended with 0.1% Triton X100 so as to contain 1×10^7 spores/ml for *Bt* isolates and reference strain *Bacillus thuringiensis* var. *kurstaki* (*Btk*) HD1. Equal volume of the suspension (0.5 ml) was air dried on 5 cm² sections of cabbage leaf discs which was further cut into 1 cm² pieces and fed individually to larvae. Fifteen larvae were tested in triplicates for the isolates, reference strain and control. Mortality was recorded within 7 days.

Btk HD1 strain was used as positive control for microscopy, Cry protein analysis, PCR amplification for *cryI*, *cry2* and *cryIAc* genes and insect bioassay.

Results

Morphology of Crystal Inclusions

Isolate GS4 showed unusual type of flat crystal formation as shown in phase contrast micrograph. crystals whereas GN24 produced both bipyramidal and spherical crystals indicating presence of more than one type of Cry proteins (Fig. 1).

Cry Protein Analysis

SDS-PAGE analysis was carried out in order to determine molecular weight of Cry proteins produced by the isolates (Table 1). Isolate GS4 showed multiple bands of proteins including 88 and 54 kDa as described by Lopez-Meza and Ibarra [21] (Fig. 2). It also showed presence of other bands of size 175, 135 and 97 kDa indicating presence of more

Table 1 Characteristics of novel *Bt* serovars

Isolates	Novel serovars	Crystal inclusion	Protein bands (kDa)	<i>cry</i> Gene content	Insect bioassay (% mortality)
GS4	H3abce	F	175, 135, 97, 88, 66, 54, 27	<i>cry1</i> , <i>cry1Ac</i>	33
GN24	H3ab	B & S	130, 90, 66, 45	<i>cry1</i>	0
UP1	H3abce	C	85, 60, 40	<i>cry2</i> , <i>cyt</i>	6

Fig. 1 Phase contrast microscopy photographs of crystal inclusions of *Bt* isolates **Fig. 2** SDS-PAGE analysis of sporulated cultures of *Bt* isolates. Lane M Broad range Protein molecular weight marker

than one Cry proteins. Isolate GN24 produced prominent bands of 130 and 66 kDa corresponding to Cry1 proteins [22]. Isolate UP1 showed a band of 60 kDa characteristic of Cry2 proteins [22] along with faint bands of 85 and 40 kDa.

Plasmid Pattern

All three isolates showed bands of plasmid of various sizes (Fig. 3). Isolates GS4 and GN24 produced megaplasmids of high molecular weight whereas UP1 produced small molecular weight sized plasmid bands (Fig. 3). Isolate GS4 showed three plasmid bands representing molecular mass ~28, 11 and 6.7 kb. Similarly isolate GN24 showed three bands representing ~28, 10.5 and 7.5 kb. In contrast, isolate UP1 produced plasmid bands representing 2.2 and 1.3 kb.

Fig. 3 Plasmid patterns of *Bt* isolates. Lane M λ DNA HindIII digest as molecular weight marker

Identification of *cry* Genes

PCR amplification with different primers was performed in order to confirm presence of various *cry* genes. Both isolates GS4 and GN24 showed expected amplifications of sizes 272 bp and 2000 bp for *cry1* and specific *cry1Ac* genes respectively, confirming presence of respective genes (Fig. 4). Isolate UP1 showed amplification of sizes 1.5 and 0.5 kb for *cry2* and *cyt* genes respectively. None of the isolates showed amplification with primers for *cry3*, 7, 8, *cry5*, 12, 14, 21, *cry11*, and *cry13* genes.

Serotyping

Isolates showed novel types of flagellar H3 antigen combinations (Table 1). Isolates GS4 and UP1 showed H3abce type of serovar whereas isolate GN24 showed H3ab type, which are not from the known serovars and thus are novel serovars from Indian soil.

Insect Bioassay

Insect toxicity assay of each isolates against *H. armigera* larvae was compared with that of reference strain *Bacillus thuringiensis* subs. *kurstaki* HD1 strain. Only isolate GS4 showed 33% mortality comparable to 44% mortality by reference strain (Table 1). Isolate UP1 showed low mortality whereas isolate GN24 showed no mortality.

Discussion

In this paper we report the characterization of novel serovars of *Bt* isolates from India. Characterization was based on crystal inclusion, Cry protein composition, plasmid pattern, *cry* gene content and insect toxicity.

Isolate GS4 was similar to *Bt* strain as described by Lopez-Meza and Ibarra [21] only for the flat type of crystal inclusion and protein bands of 88 and 54 kDa. In addition,

Bt GS4 also produced protein bands of size 175, 135, 97 and 27 kDa and had difference in megaplasmid pattern. Presence of *cry1* and *cry1Ac* genes, toxicity to *H. armigera* and serovar were also found to be different. Protein profile of the sporulated culture and serovar did not match to that of any other *Bt* strain in the literature.

Isolate GN24 produced bipyramidal crystal inclusions, showed 130 and 66 kDa bands of protein and contained *cry1Ac* gene, but did not showed toxicity to *H. armigera*. Similarly isolate UP1, though produced cuboidal type crystal inclusions, protein band of 60 kDa and harbored *cry2* gene, showed very low toxicity to *H. armigera*. Thus low or no toxicity by these novel serovars could be attributed to lack of trypsin sites for activation or production of mutant toxins. However toxicity to other insects or towards other insect hosts cannot be ignored, since Cry1Ac and Cry2 toxins are shown to have toxicity to wide range of insects [23].

Isolate GS4 and GN24 showed many proteins bands of sporulated culture during SDS-PAGE analysis indicating presence of multiple *cry* genes. Isolate UP1 showed presence of very low molecular weight DNA bands (Fig. 4), which could not be considered megaplasmids as such. Thus novel serovars described in present study along with unusual characteristics should be considered as new strains of *Bt*.

Classification of *Bt* strains has also been done on basis of flagellar serotypes [24]. However isolates GS4 and UP1 representing same H3abce serovar showed presence of *cry1-cry1Ac* and *cry2-cyt* genes respectively. Results in the present study showed that the same serovars can have absolutely different *cry* gene content as well as different serovars can have the same *cry* gene content (Table 1). Change et al. [25] and Lee et al. [26] showed different *cry* gene content in *kurstaki* serovar compared to *Bt kurstaki* HD-1 strain. Quesada-Moraga et al. [27] also showed diversity in protein profiles of sporulated cultures of different serovars. Similarly Arango et al. [28] showed different levels of toxicity in *kurstaki* H3a, 3b, 3c serovar. Thus present study supports notion that firm correlation between serovar and *cry* gene content of *Bt* strains could not be established.

Acknowledgments Authors would like to acknowledge Dr. Kumiko Kagoshima and Prof. Michia Ohba, Kyushu University, Japan for serotyping of our *Bt* isolates.

References

1. Johnston RB, Nielsen-LeRoux PR, Lereclus D, Crickmore N (2010) *Bacillus thuringiensis*: An impotent pathogen? Trends Microbiol 18:189–194
2. Schnepf E, Crickmore N, van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Fig. 4 PCR analysis of *Bt* isolates for *cry1* (a), *cry1Ac* (b), *cry2* (c), and *cyt* (d) genes. Lanes M1 100 bp DNA ladder, M Mix DNA ladder

3. Qaim M (2003) *Bt* cotton in India: field trial results and economic projections. *World Dev* 31:2115–2127
4. Martin PA, Travers RS (1989) Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl Environ Microbiol* 55:2437–2442
5. Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos FJ, Pena G, Nunez-Valdez M-E, Soberon M, Rodolfo Quintero (1998) Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* 64:4965–4972
6. Chen F-C, Tsai M-C, Peng C-H, Chak K-F (2004) Dissection of *cry* gene profiles of *Bacillus thuringiensis* isolates in Taiwan. *Curr Microbiol* 48:270–275
7. Das J, Dangar TK (2007) Diversity of *Bacillus thuringiensis* in the rice field soils of different ecologies in India. *Indian J Microbiol* 47:364–368
8. Heckel DG, Gahan LJ, Baxter SW, Zhao J-Z, Shelton AM, Gould F, Tabashnik BE (2007) The diversity of *Bt* resistance genes in species of Lepidoptera. *J Invertebr Pathol* 95:192–197
9. Vennila S, Biradar VK, Gadpayle JG, Panchbhai PR, Ramteke MS, Deole SA (2004) Field evaluation of *Bt* transgenic cotton hybrids against sucking pests and bollworms. *Indian J Plant Prot* 32:1–10
10. Udikeri SS, Patil SB, Shaila HM, Guruprasad GS, Patil SS, Kranthi KR (2008) Mirid menace—a potential emerging sucking pest problem in cotton. Paper presented at the Fourth meeting of Asian Cotton Research and Development Network, Anyang, China. http://www.icac.org/tis/regional_networks/documents/asian/papers/udikeri.pdf
11. Lu Y, Wu K, Jiang Y, Xia B, Li P, Feng H (2010) Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of *Bt* cotton in China. *Science* 328:1151–1154
12. Whiteley HR, Schnepf HE (1986) The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Ann Rev Microbiol* 40:549–576
13. Barboza-Corona JE, Reyes-Rios DM, Salcedo-Hernandez R, Bideshi DK (2008) Molecular and biochemical characterization of an endochitinase (ChiA-HD73) from *Bacillus thuringiensis* subsp. *kurstaki* HD-73. *Mol Biotechnol* 39:29–37
14. Desetty RD, Mahajan VS, Khan BM, Rawal SK (2008) Isolation and heterologous expression of PHA synthesizing genes from *Bacillus thuringiensis* R1. *World J Microbiol Biotechnol* 24:1769–1774
15. Dave SR, Dave RH (2009) Isolation and characterization of *Bacillus thuringiensis* for Acid red 119 dye decolourisation. *Biores Technol* 100:249–253
16. Patel KD, Bhansali F, Ingle SS (2011) Diversity and characterization of *Bacillus thuringiensis* isolates from alluvial soils of Mahi river basin, India. *J Adv Dev Res* 2:14–20
17. Ceron J, Covarrubias L, Quintero R, Ortiz A, Ortiz M, Aranda E, Lina L, Bravo (1994) PCR analysis of the *cryI* insecticidal crystal family genes from *Bacillus thuringiensis*. *Appl Environ Microbiol* 60:353–356
18. Masson L, Erlandson M, Puzstai-Carey M, Brousseau R, Juarez-Perez V, Frutos R (1998) A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. *Appl Environ Microbiol* 64:4782–4788
19. Ceron J, Ortiz A, Quintero R, Guereca L, Bravo A (1995) Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* 61:3826–3831
20. Thiery I, Frachon et al (1997) Identification, isolation, culture and preservation of entomopathogenic bacteria. In: Lacey L (ed) *Manual of techniques in insect pathology* edn. Academic Press, London, pp 56–77
21. Lopez-Meza JE, Ibarra JE (1996) Characterization of a novel strain of *Bacillus thuringiensis*. *Appl Environ Microbiol* 62:1306–1310
22. Crickmore N, Zeigler DR, Fietelson J, Schnepf E, van Rie J, Lerecluse D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62:807–813
23. van Frankenhuyzen K (2009) Insecticidal activity of *Bacillus thuringiensis* crystal proteins. *J Invertebr Pathol* 101:1–16
24. Lecadet M-M, Frachon E, Dumanoir VC, Ripouteau H, Hamon S, Laurent P, Thiery I (1999) Updating the H-antigen classification of *Bacillus thuringiensis*. *J Appl Microbiol* 86:660–672
25. Chang JH, Roh JY, Je YH, Park HW, Jin BR, Woo SD, Kang SK (1998) Isolation of a strain of *Bacillus thuringiensis* ssp. *kurstaki* HD-1 encoding δ -endotoxin CryIE. *Lett Appl Microbiol* 26:387–390
26. Lee IH, Je YH, Chang JH, Roh JY, Oh HW, Lee SG, Shin SC, Boo KS (2001) Isolation and characterization of a *Bacillus thuringiensis* ssp. *kurstaki* strain toxic to *Spodoptera exigua* and *Culex pipiens*. *Curr Microbiol* 43:284–287
27. Quesada-Moraga E, Garcia-Tovar E, Valverde-Garcia P, Santiago-Alvarez C (2004) Isolation, geographical diversity and insecticidal activity of *Bacillus thuringiensis* from soils in Spain. *Microbiol Res* 159:59–71
28. Arango JA, Romero M, Orduz S (2002) Diversity of *Bacillus thuringiensis* strains from Colombia with insecticidal activity against *Spodoptera frugiperda* (Lepidoptera:Noctuidae). *J Appl Microbiol* 92:466–474