Curr Microbiol (2010) 61:13–18
DOI 10.1007/s00284-009-9569-6

Diversity of *Bacillus thuringiensis* Isolated from Western Ghats of Tamil Nadu State, India

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Received: 4 September 2009 / Accepted: 30 November 2009 / Published online: 24 December 2009

Abstract The Western Ghats of India is the one of the world's 10 "Hottest biodiversity hotspots" that runs along the western part of India through four states including Tamil Nadu. The only biodiversity reserve in the Western Ghats is the Nilgiri biosphere located in the Tamil Nadu state. In the present study, 525 soil samples were collected from all the 14 different divisions of the Western Ghats in Tamil Nadu state, India. A total of 316 new isolates of *Bacillus thuringiensis* (Bt) that produce parasporal crystalline inclusions were isolated from 525 soil samples. Seven different types of crystalline inclusions were observed in the 316 new isolates of Bt. Cuboidal inclusion was predominantly present in 26.9% of the Bt isolates when compared to other shapes. Further characterization of 70 of the 316 Bt isolates for crystal protein profile through SDS-PAGE revealed six different types of crystal protein profile viz., 135 and 65, 135, 95, 65, 43, and 30 kDa crystal proteins. Variation in the mass of crystal protein(s) purified from the isolates of Bt revealed molecular diversity of this bacterium prevalent in the Western Ghats of Tamil Nadu, India.

Introduction

Bacillus thuringiensis Berliner (Bt), a species of Gram positive sporulating soil bacteria that forms insecticidal crystal proteins during sporulation phase of its growth cycle is the major source for development of insect-resistant transgenic plants. These crystal (Cry) proteins are sequestered as protoxins in crystalline inclusions. After sporulation they mediate specific pathogenicity against insects [9]. Molecular potency of Bt toxins is high compared to the chemical pesticides, i.e., 300 times higher than synthetic pyrethroids and 80,000 times stronger than organophosphates [3]. These crystalline parasporal inclusions are toxic to a wide spectrum of insects including the orders of Lepidoptera, Coleoptera, and Diptera. Search for novel Bt strains may lead to the discovery of additional insecticidal proteins with higher toxicity and/or wider spectrum (7). Novel toxins are also important for providing alternatives to cope up with the emergence of resistant insect populations. Therefore, a large number of Bt strains have been isolated and many types of insecticidal crystal proteins genes have been cloned. The diversity of Bt strains facilitates isolation of new types of *cry* genes. New variants of the already known *cry* gene subgroups could encode crystal proteins with significant difference in the level and spectrum of toxicity due to variation in their sequences [12]. Diversity available in nature has to be collected systematically, classified, analyzed, and harnessed for whatever it is worth. It is a basic resource, and it has to be collected and made available in an easily usable form. The Western Ghats of India is one among the hotspots of biodiversity of the world and relatively undisturbed. The Western Ghats or Sahyadri mountains is a chain of highlands that runs along the western part of India through the states of Maharashtra, Goa, Karnataka, Kerala, and Tamil Nadu covering an area of 159,000 sq km. The only biodiversity reserve in the Western Ghats is the Nilgiri biosphere reserve of Tamil Nadu. It is estimated that about one-third of the flowering plant species in India is found in

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this area. Isolation and characterization of Bt from soils of the Western Ghats of Tamil Nadu may yield new isolates with novel *cry* gene sequences which could encode crystal proteins with significant difference in the level of toxicity due to variation in their sequences. Hence, the present study was carried out to explore diversity of Bt present in soil samples of Western Ghats in Tamil Nadu state, India.

Materials and Methods

Soil Samples of Western Ghats Forest Used for Isolation of Bt

A total of 525 soil samples from different spots of fourteen different divisions of the Western Ghats forest (Mudumalai, Gudalur, Nilgiris North, Nilgiris South, Coimbatore, Pollachi, Dindigul, Kodaikanal, Theni, Madurai, Srivilliputhur, Tirunelveli, Kalakkad, and Kanyakumari) in Tamil Nadu state, India were used for isolation of Bt (Fig. 1). All the soil samples were collected aseptically from top to a depth of 2–3 cm after scrapping off the surface material with a sterile spatula. The soil samples were stored in sterile containers.

Isolation of Bt from Soil Samples and Maintenance of Bt Strains

One gram of soil sample was suspended in 10 ml of sterile distilled water (10^{-1}) in a boiling tube. The boiling tube was subjected for heat treatment at 65°C for 30 min and allowed to settle. One ml of heat treated suspension was taken and added to four ml of saline (0.85% NaCl), which gives 5^{-1} dilution. Similarly, dilutions were prepared up to 5^{-5}. One ml aliquots of dilutions 10^{-1}, 5^{-1}–5^{-5} were taken in six different petri plates over which melted T_3 agar medium (7) was poured and mixed clockwise and anti clockwise directions. The plates were incubated at 30°C for 2–3 days. From each soil samples, around 12 colonies resembling Bt were selected and subcultured as ribbon streak (four colonies per plate) on T_3 agar medium. After 48 h of incubation, smear is prepared from ribbon streak cultures on glass slide and heat fixed. After heat fixing, drops of the Coomassie Brilliant Blue stain (0.133% Coomassie Brilliant Blue G250 in 50% acetic acid) were added and kept as such for 1 min. Then, the smear is washed gently in running tap water. After blot drying with blotting paper, the stained cultures were observed through bright field microscopy for presence of crystalline inclusions. The isolates showing the presence of crystalline inclusions were selected as Bt and streaked on T_3

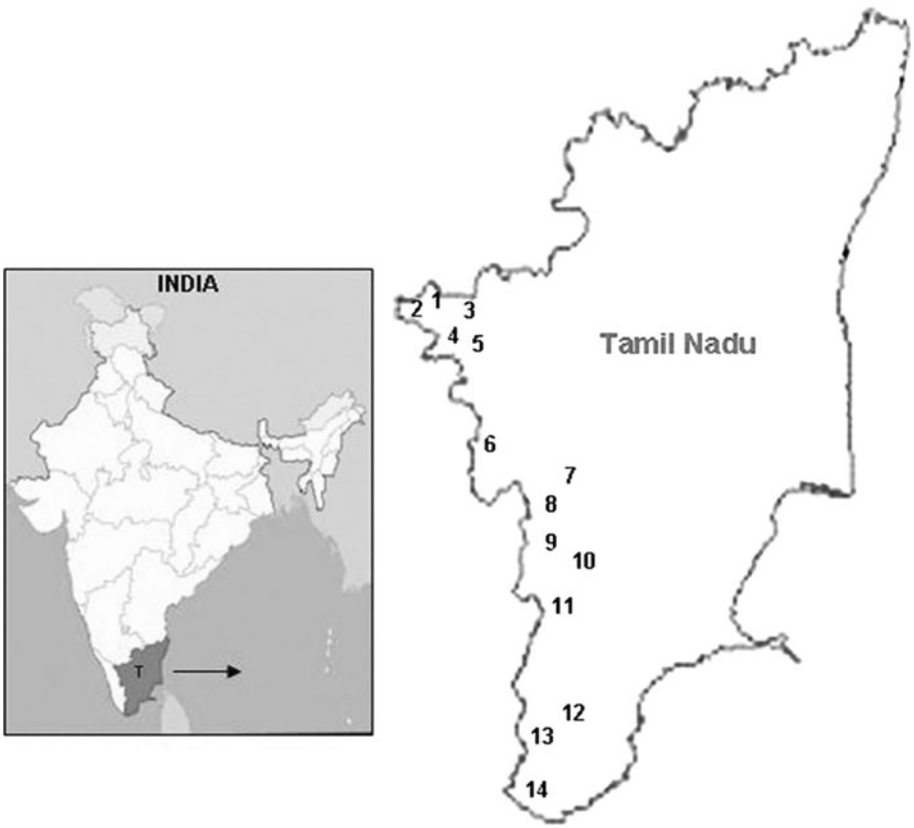

Fig. 1 Fourteen divisions of the Western Ghats in Tamil Nadu (T) State, India. 1: Mudumalai; 2: Gudalur; 3: Nilgiris South; 4: Nilgris North; 5: Coimbatore; 6: Pollachi; 7: Dindigul; 8: Kodaikanal; 9: Theni; 10: Madurai; 11: Srivilliputhur; 12: Tirunelveli; 13: Kalakkad; 14: Kanyakumari

agar medium for single colony purification. Broth culture was made from the isolated single colonies of crystal positive Bt isolates. Glycerol stocks were prepared using 24 h old broth culture and stored at −70°C for further studies.

Isolation of Spore–Crystal Mixture from Bt Isolates

The spore–crystal mixture was isolated from the 70 new isolates of Bt and a reference strain, HD1. The reference strain, HD1 was originally obtained from Bacillus Genetic Stock Centre, Ohio State University, Columbus, Ohio, USA. For each Bt strain, a single colony was inoculated into 5 ml T3 broth and incubated in a rotatory shaker, maintained at 30°C at 200 rpm for nearly 48–60 h, and the bacterial sporulation was monitored through a phase contrast microscope. When more than 90% of cells had lysed, the sporulated broth culture was transferred to 4°C, at least half-an-hour before harvesting. The T3 broth containing spore-crystal mixture was centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was washed once with 5 ml of ice-cold Tris–EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)], once with 5 ml of ice-cold 0.5 M NaCl followed by two more washes with 5 ml of Tris–EDTA buffer with 0.5 mM PMSF by centrifuging at the same speed and time. Finally, the spore-crystal pellet was suspended in 100 µl of sterile distilled water containing 1 mM PMSF and stored in −20°C. Aliquots of spore–crystal mixture (5ul) of Bt isolates were analysed by SDS-PAGE [6].

Results

Isolation of Bt Strains from Soil Samples of the Western Ghats

Out of 525 soil samples of the Western Ghats used, Bt isolates were obtained from 292 soil samples (56% frequency). The highest frequency for isolation of Bt (64%) was recorded in the soil samples of Pollachi and Kodaikanal divisions. The lowest frequency (40%) for isolation of Bt was observed in the soil samples of Srivillipudhur division. For this purpose, a total of 6,629 bacterial colonies were subcultured from 525 soil samples and subjected to microscopic observation for the presence of proteinaceous crystals. From 6,629 bacterial colonies observed through bright field microscopy, 316 isolates were identified as Bt based on the presence of crystalline inclusions (Table 1). The isolates with crystalline inclusions were further purified as single colonies and stored as glycerol stocks at −70°C for future studies.

Morphology of Crystalline Inclusions in Bt Isolates of Western Ghats

The shape of the crystalline inclusions varied among the Bt isolates. Based on crystal morphology, the 316 isolates of Bt fall into the following seven groups: bipyramidal, cuboidal, bipyramidal with cuboidal, rectangular, spherical, small spherical, and crystal attached to spore (Fig. 2).

Table 1 Isolation of *B. thuringiensis* from soil samples of the Western Ghats

S. No.	Western Ghats forest division	Number of soil samples			Number of colonies		
		Examined	Bt* positive		Examined	Crystalliferous	
			Number	%		Number	%
1	Mudumalai	20	12	60	260	12	4.6
2	Gudalur	25	12	48	315	13	4.1
3	Nilgiris South	50	30	60	638	32	5.0
4	Nilgiris North	50	27	54	620	34	5.5
5	Coimbatore	25	10	40	300	11	3.7
6	Pollachi	45	29	64	546	31	5.7
7	Dindigul	20	12	60	250	12	4.8
8	Kodaikonal	45	29	64	640	34	5.3
9	Theni	50	26	52	610	26	4.2
10	Madurai	15	9	60	200	9	4.5
11	Srivilliputhur	50	20	40	640	22	3.4
12	Tirunelveli	35	20	57	420	21	5.0
13	Kalakkad	70	41	59	880	43	4.9
14	Kanyakumari	25	15	60	310	16	5.1
	Total	525	292	56	6629	316	4.8

* Crystalliferous bacterium

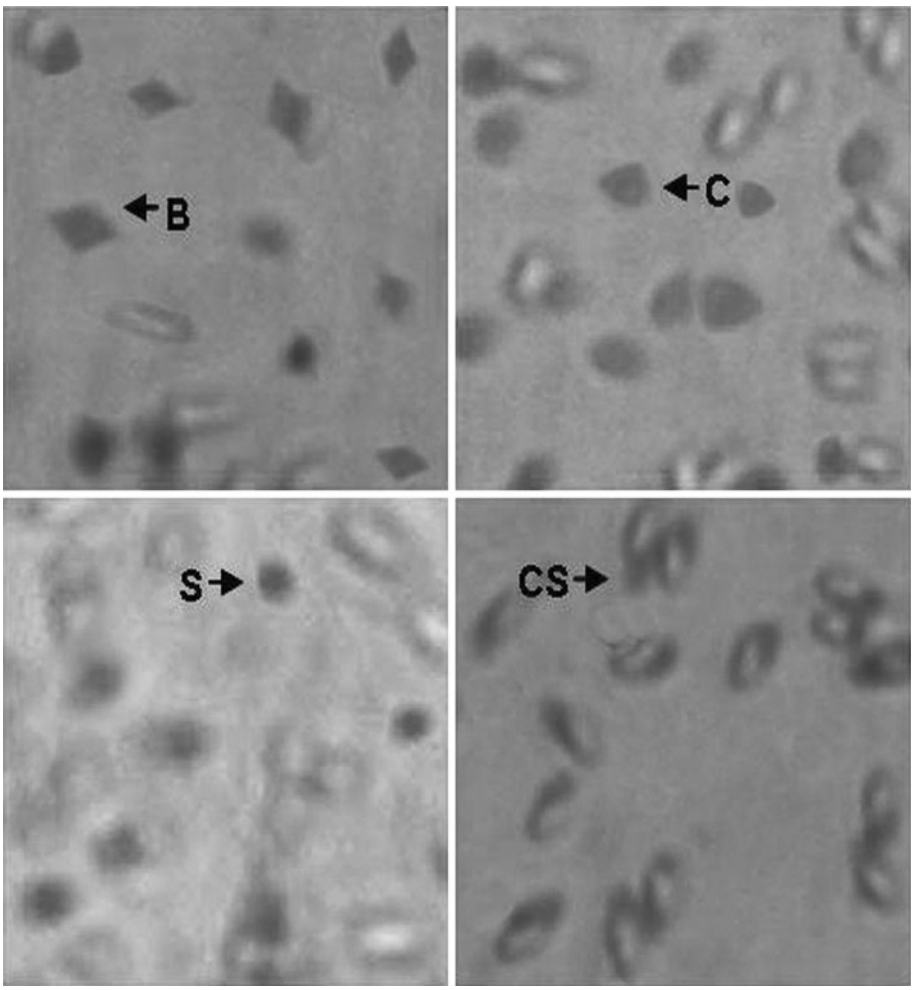

Fig. 2 Different crystal morphology of the Bt isolates from the Western Ghats. B: Bipyramidal; C: Cuboidal; S: Spherical; CS: Crystal attached to spore

Table 2 Types of crystalline inclusions in *B. thuringiensis* isolates of Western Ghats

S. No.	Crystal morphology	Bt isolates	
		Number	%
1.	Bipyramidal	67	21.2
2.	Cuboidal	85	26.9
3.	Bipyramidal and cuboidal	48	15.2
4.	Rectangular	15	4.7
5.	Spherical	51	16.1
6.	Small spherical	12	3.8
7.	Crystal attached to spore	38	12.0
Total		316	

The cuboidal inclusion was observed at the highest frequency (26.9%) followed by bipyramidal inclusion (21.2%). The small spherical inclusion was observed at the lowest (3.8%) frequency when compared to other shapes of crystal (Table 2).

Analysis of Crystal Protein Profile in Bt Isolates of Western Ghats

Among the 316 isolates of Bt obtained from the Western Ghats, 70 Bt isolates (five isolates from each division of the Western Ghats) were selected and studied for crystal protein profile(s) by SDS-PAGE along with the reference strain of Bt, HD1. The reference strain, HD1 showed that 135 and 65 kDa crystal proteins. The new isolates of Bt showed that six different types of crystal protein profile viz., 135 and 65, 135, 95, 65, 43, and 30 kDa (Fig. 3a–c). Out of the 70 Bt isolates analyzed by SDS-PAGE, 17 isolates (24.2%) exhibited two major polypeptide bands with molecular weights in the range of 135 and 65 kDa as in the case of HD1. Whereas, crystal protein(s) of 135, 95, 65, 43, 30 kDa were observed in 15 (21.4%), 12 (17.1%), 7 (10%), 4 (5.7%), and 7 (10%) Bt isolates, respectively (Table 3). Eight Bt isolates did not show any distinct band of crystal protein(s).

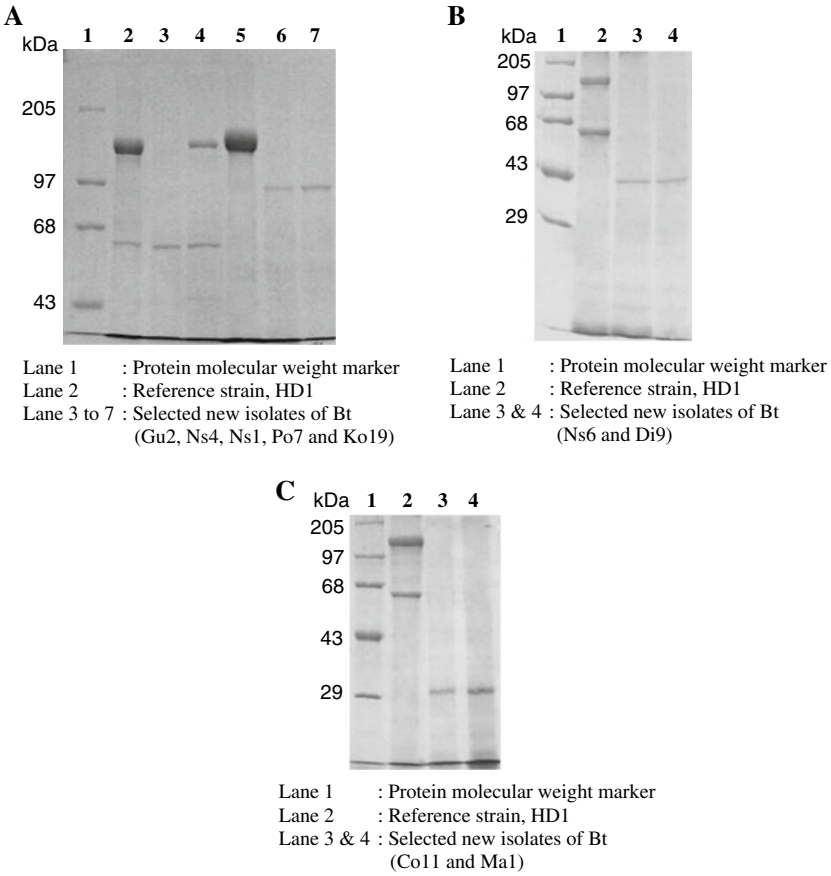

Fig. 3 SDS-PAGE analysis of spore–crystal mixture isolated from selected new isolates of Bt. **a** showing 135 and 65, 135, 95, and 65 kDa protein(s) (in lanes 4, 5, 6 and 3, respectively); **b** showing 43 kDa protein (in lane 3); **c** showing 30 kDa protein (in lane 3)

Table 3 Grouping of *B. thuringiensis* isolates of Western Ghats based on crystal protein profile

S. No.	Group number	Molecular weight of crystal protein(s) (kDa)	Bt isolates	
			Total	Per cent
1	I	135 and 65	17	24.2
2	II	135	15	21.4
3	III	95	12	17.1
4	IV	65	7	10.0
5	V	43	4	5.7
6	VI	30	7	10.0
7		No discrete band	8	11.4
Total			70	

Discussion

Bacillus thuringiensis (Bt) strains have been found worldwide from diverse habitats, including soil, insects, stored products, dust, deciduous and coniferous leaves, phyllospheres, and other miscellaneous habitats [4, 5, 10]. The soil samples of Western Ghats (one of the biodiversity hotspots) may yield new isolates of Bt with novel Cry proteins which could be used for control of various pests. In the present study, 525 soil samples from 14 different divisions of the Western Ghats were used as source material for isolation of indigenous Bt strains. Results showed that about 56% of the 525 samples were positive for Bt and yielded 316 isolates. Earlier studies reported varied frequency for isolation of Bt from soil samples ranging from 3 to 85% [7, 11]. Moderate frequency for isolation of Bt from the Western Ghats forest soil samples of the present study may be due to large amount of nutrients in the soil itself, allowing optimum survival and enrichment in the soils.

Initial identification of Bt is mainly based on the presence of crystalline inclusions. The bright field microscopy is more useful than phase contrast microscopy for high throughput evaluation of bacterial colonies for the presence

of crystals and also for identification of small crystals [8]. In the present study, 316 of the 6,629-stained bacterial colonies observed through bright field microscopy showed that the presence of crystalline inclusions, and were identified as Bt. Based on the number and shape of the crystalline inclusions, the 316 new isolates of Bt were characterized into seven groups viz., bipyramidal, cuboidal, bipyramidal with cuboidal, rectangular, spherical, small spherical, and crystals attached to spore. About 27% of the 316 Bt isolates showed that cuboidal crystal, followed by bipyramidal crystal in 21% of the isolates. These findings differed from the earlier reports [1, 7], wherein strains with bipyramidal crystals were predominant (46%). In addition to other shapes, notable shape of crystals viz., crystals attached to spore (12%) and small spherical (3.8%) were also observed in Bt isolates of Western Ghats. Rampersad and Ammons [8] reported that 30 and 3.8% of the 79 isolates characterized by them contained dark staining body which appeared as a cap on the spore and small parasporal bodies, respectively. Differences observed in the morphology of crystalline inclusions of Bt suggested presence of diversity in the Bt isolates of Western Ghats.

Grouping of Bt isolates according to crystal protein(s) profile studied by SDS-PAGE will give a prelude for the presence of diversity in *cry* genes. The *cry1* genes encoding the 130–138 kDa lepidopteran-active Cry proteins form bipyramidal crystalline inclusions. The Cry2 and Cry3 proteins are 65 and 70 kDa, respectively. The dipteran active, Cry4 and Cry10 or Cry11 proteins are 135 and 80 kDa, respectively, and form spherical inclusions [2]. Therefore, analysis of crystal proteins(s) profile could be useful to predict the presence of *cry* genes. In the present study, 44.2% of the 70 isolates are having 135 and/or 65 kDa proteins suggesting the presence of genes related to *cry1* and *cry2* families. Other isolates showed that the presence of 95 or 43 or 30 kDa proteins indicating the presence of other novel *cry* genes also. These results lead us to suggest the presence of diversity in Bt isolates of Western Ghats. Results of preliminary studies on PCR–RFLP and partial sequencing of nucleotides of *cry* genes also indicated variations in the Bt isolates of Western Ghats (data not shown). Further studies on cloning and characterization of cry genes from these new isolates of Bt will be useful to open new vistas in the area of integrated pest management for sustainable agriculture.

Acknowledgments Authors express sincere thanks to Dr. V. Balasubramani, Assoc. Prof., Dr. P. S. Shanmugam, Senior Research Fellow and Dr. V. Maheswaran, Senior Research Fellow, Tamil Nadu Agricultural University, for their help in the collection of soil samples. Authors are thankful to Dr. P. U. Krishnaraj, Project Coordinator, University of Agricultural Sciences, Dharwad, India. This research was supported by a grant from the Department of Biotechnology, Government of India, New Delhi (BT/PR5349/AGR/02/274/2004).

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