Diversity of *Bacillus thuringiensis* in the rice field soils of different ecologies in India

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Abstract Diversity of the *Bacillus thuringiensis* (Bt) in the rice field soils of different ecologies viz. the island (Port Blair), the Himalayan (Srinagar), brackish water (Mahe) and coastal mesophilic (Mangalore) habitats was analyzed by phenotypic characterization of 5, 66, 14 and 54 Bt isolates, respectively. The Bt isolates produced either monotypic (bipyramidal or spherical) or heterotypic (polymorphic-bipyramidal or bipyramidal-rhomboidal) crystals. The organisms were generally resistant to the penicillin group of antibiotics, tolerated 5–12% NaCl and 0.5M Na-acetate. The Bt isolates contained 1–5 plasmids of 0.89–58.61 kbp sizes. The plasmid profiles had no correlation with crystal morphology or salt tolerance of different bacteria. Each soil was inhabited by different types of Bt. Two Bt strains of Mangalore and one strain each of the other places were phenotypically similar. One Bt strain each of Port Blair and Srinagar was different from all other strains.

Keywords *Bacillus thuringiensis* · Diversity · Plasmid · Rice · Salt tolerance

Introduction

Bacillus thuringiensis (Bt) is the most widely exploited entomopathogen in agriculture, forestry and public health¹. It is ubiquitous in soil, phyllosphere, stored food, dead insects, air, water etc. but soil is the primary habitat^{1,2}. Out of 11000 soils of 30 countries, Bt (27000) habited in 50–70% total, 94.3% Asian and 87.1% agricultural soils^{1–4}. The Bt infects all insect orders and in the rice fields more than 50% insects recorded infection^{1–3}. However, information on the diversity and distribution of the Bt in South-East Asian countries, especially in the rice field soils of India, are negligible^{3–5}. Rice is the staple food of 80–90% Asian/Indian population. So, yield reduction due to salinity in about 23 mha (25% irrigated land) area in India and second largest pesticide consumption for pest control demand effective salt tolerant Bt to reduce the pesticide load in both the mesophilic and saline ecologies. So, the rice field soils of highly diverse ecologies viz. the coastal, brackish water, island and the Himalayan regions of India were studied to understand the distribution, diversity, potency and identity of the indigenous Bt resources to select and exploit them as biopesticides.

The soil samples (1 cm below the top layer) were collected (sealed in polybags) from the rice fields of the island (Port Blair, UT of Andaman and Nicobar), brackish water (Mahe, UT of Pudhucherry situated in coastal Kerala), the Arabian sea coastal mesophilic (Mangalore, Karnataka) and the Himalayan (Srinagar, Jammu and Kashmir) ecologies. Each soil (1g) sample was suspended in 9 ml sterilized (121°C, 15 min) distilled water, diluted up to 10^{–3} level and heated at 60°C for 30 min. An aliquot (100 µl, 10^{–3} dilution) of the suspension was mixed with 100 ml nutrient agar (NA) (g/l: peptone 5, beef extract 3, NaCl 3, agar 18,

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Table 1 Phenotypic characteristics of the bacteria isolated from different soils.

Location	Bacteria (BT)	Total isolate ¹	Colony character ²	Bacterium ³	Spore ⁴	Crystal	Tolerance		Antibiotic	
							NaCl (%)	Na-acetate (M)	Sensitive	Resistant
Port Blair	1	19	C, W, M, R, E, G, 3–5.1	4.8–5.2 x 1.1–1.2	1.8–1.9 x 0.9–1.2	Polymorphic/bipyramidal	8	0.5	a	b, c, d
	2	21	C, W, M, R, U, G, 5.1–7.2	4.8–5.1 x 0.9–1.1	1.7–1.8 x 0.9–1.1	Bipyramidal	8	0.5	a	b, c, d
	3	11	I, BW, M, R, E, G, 3.1–4.9	4.5–4.7 x 0.9–1.1	0.8–0.9 x 0.6–0.7	Spherical	5	0.5	a, b, c, d	None
Srinagar	4	9	I, W, M, R, E, G, 3–4	4.8–5.1 x 1.1–1.4	1.8–2 x 0.8–1.3	Bipyramidal	9	0.5	a, d	b, c
	5	17	I, W, M, R, U, G, 6.1–8.1	4.9–5.2 x 1.1–1.2	1.8–2.1 x 0.8–1	Bipyramidal	9	0.5	a, b	c, d
	6	14	I, W, M, R, E, G, 6.1–8	4.9–5.1 x 1–1.2	2.2–2.3 x 1–1.2	Spherical	12	0.5	a, b, c, d	None
Mahe	7	26	I, BW, M, R, E, G, 6.8–6.2	4.7–4.9 x 1.1–1.2	1–1.1 x 0.6–0.7	Small spherical	12	0.5	a, b, c, d	None
	8	5	I, CW, M, R, L, G, 7.1–8	4.9–5.1 x 1.1–1.3	1.9–2.1 x 0.9–1.1	Spherical	9	0.5	a, b, c, d	None
	9	9	C, BW, M, R, E, G, 3.7–4.3	4.6–4.8 x 0.9–1	1–1.3 x 0.7–0.8	Small spherical	12	0.5	a, b, c, d	None
Mangalore	10	18	I, W, M, R, E, G, 5–6	4.8–5.3 x 0.9–1.1	2.2.5 x 1–1.2	Bipyramidal/rhomboidal	6	0.5	a, b, c, d	None
	11	21	I, W, M, R, E, G, 4–7.1	4.7–5.2 x 1.1–1.2	2.1–2.4 x 0.9–1.1	Spherical	12	0.5	a, b, c, d	None
	12	15	I, BW, M, R, U, N, 3.1–5.1	3.5–3.8 x 0.8–0.9	1–1.2 x 0.7–0.8	Small spherical	5	0.3	a, b, c, d	None

¹The isolates were obtained from 100 µl soil suspension of 10⁻³ dilutions. ²Numerical values indicate the colony size in mm. ³Rod shaped. ⁴Elliptical. ⁵Diameter is given for spherical crystals. I = Irregular. C = Circular. W = White. M = Metallic. CW = Creamish white. BW = Brownish white. R = Raised. E = Entire. U = Undulate. L = Lobate. ER = Erose. G = Gummy. N = Non-gummy. a = Vancomycin, tetracycline, kanamycin, chloramphenicol and chlorotetracycline (30 µg/disc); gentamycin, streptomycin, norfloxacin (10 µg/disc); ciprofloxacin and rifampicin (5 µg/disc); bacitracin (10 U/disc) and erythromycin (15 µg/disc). b = Penicillin G (10 U/disc). c = Ampicillin (10 µg/disc). d = Amoxicillin (10 µg/disc).

pH 7.2) and plated on 5 plates. The plates were incubated (72h, $30 \pm 0.1^\circ\text{C}$) in a BOD incubator. All colonies were checked under a phasecontrast microscope (x100) and those producing crystal were isolated, purified and maintained on

Table 2 Identification of the bacteria based on the phenotypic characters.

Character	Bacteria (BT)	Observation
Matte colony, rod shape, endospore, motile, Gram stain, crystal, facultative anaerobe/microaerobic, catalase	1–12	+
Length (μm)	1–12	3.63–5.06
Diameter $>2.5 \mu\text{m}$, cell/filament curved, cocci	1–12	–
Filament	1–4, 6–12	–
	5	+
Sporangium	1–12	Not swollen
Oxidase	1, 2, 4, 5, 7–10	–
	3, 6, 11, 12	+
Acidity from glucose	1, 2, 4, 5, 7–12	+
	3, 6	–
Nitrate reduction	1, 3–12	+
	2	–
Genus	1–12	<i>Bacillus</i>
Species	1–12	<i>thuringiensis</i>

nutrient agar (NA) slants at $4 \pm 0.1^\circ\text{C}$. The ratio of the crystal and total spore forming bacteria of each location was recorded as the Bt index. The isolates were identified up to species level based on the morphophysiological and biochemical characters^{6,7}. Readymade discs (Himedia, India) of sixteen Gram (+)ve bacteria specific and broad spectrum antibiotics were placed (3 discs/plate) on the solidified NA plates seeded separately with 10^8 bacteria/ml medium, incubated at $30 \pm 0.1^\circ\text{C}$ in a BOD incubator. The inhibition zones were measured after 24h through a HiAntibiotic zone scale (Himedia, India) and sensitivity was recorded according to the manufacturer's instructions. Plasmid profiles of all Bt isolates were analyzed following Brinboim and Doly⁸ and electrophoresed on 0.7% agarose gel along with the marker *Mlu*I digested λ DNA (Geni, India). The plasmid sizes (kbp) were calculated using the PhotoCapt software (Vilber Lourmat, France).

Altogether 185 crystal forming bacteria viz. Port Blair 51, Srinagar 66, Mahe 14 and Mangalore 54 were isolated (Table 1) having the indices 0.002 for Mahe but 0.006 for the other three places. The organisms were characterized by cultural, morphological, physiological and biochemical characters, and place wise divided into 12 groups viz. BT1–12 (Table 1). The organisms produced different types of colonies, the bacteria were Gram (+)ve motile rods ($3.5\text{--}5.3 \times 0.8\text{--}1.3 \mu\text{m}$) with elliptical spores ($0.8\text{--}2.5 \times 0.6\text{--}1.3 \mu\text{m}$) (Table 1). The crystals were monotypic viz. bipyramidal ($1.06\text{--}1.13 \times 0.80\text{--}0.93 \mu\text{m}$), spherical

Table 3 Physiological and biochemical differentiation of different groups of bacteria.

Characters	Bacteria (BT)				
	1	2, 10	3, 7, 9, 11, 12	4, 5, 8	6
Acetylmethyl carbinol	–	+	–	+	+
Urease	+	+	–	–	–
Arginine dihydrolase	+	±	–	–	+
Pellicle	+	+	±	+	+
Cellobiose utilization	–	–	–	–	–
Mannose utilization	–	–	+	–	+
Starch hydrolysis	+	+	+	±	+
Sucrose fermentation	+	±	+	+	+
Tween esterase	–	±	–	+	+
Citrate utilization	–	–	±	–	+
Salicin fermentation	+	–	–	–	–
Lecithinase	–	+	±	+	+
Gelatinase	+	+	+	+	+
Casein	+	+	+	+	+
Esculin fermentation	–	–	–	–	–
Chitin hydrolysis	–	–	–	–	–

+ = Positive result. – = Negative result.

Fig. 1 Plasmid profile of the Bt strains on agarose gel. The plasmid bands of BT10, 4, 5, 8, 1 and 2 are indicated by arrows. The lane M shows the profile of the marker DNA fragments (kbp).

Fig. 2 Plasmid profile of the Bt strains on agarose gel. The plasmid bands of BT11, 6, 12, 7, 9 and 3 are indicated by arrows. The lane M shows the profile of the marker DNA fragments (kbp).

(1.10–1.26 μm) and small spherical (0.66–0.80 μm) or heterotypic (mixed type) viz. polymorphic/bipyramidal (1.21–1.30 $\times$ 0.81–0.90 μm) and rhomboidal-bipyramidal (1.11–1.50 $\times$ 0.81–1.40 μm) (Table 1). The organisms tolerated 5–12% NaCl that was not recorded to date and all but BT12 tolerated 0.5M Na-acetate (Table 1). Phenotypically, the bacteria were identified (Table 2) as the genus

Bacillus (Group I) and species *thuringiensis* i.e. *Bacillus thuringiensis*^{6,7}. All isolates were sensitive to twelve different Gram (+)ve specific and broad spectrum antibiotics (Table 1). Broadly, the isolates were resistant to the penicillin group but strictly, the BT1 and 2 were resistant to penicillin, ampicillin and amoxicillin; BT4 was resistant to penicillin and ampicillin and BT5 is resistant to ampicillin and amoxicillin (Table 1) which was in agreement with other Bt^{4,9}. The biochemical and physiological characters revealed that the BT3, 7, 9, 11 and 12 were similar i.e. same strain and habited in all places, whereas, the BT1 and 6 were different strains and location specific (Table 3). The soils possessed 2–4 groups of Bt each comprising 5–26 isolates with different degrees of salt/acetate tolerance and antibiotic sensitivity (Tables 1–3). The results corroborated the proposition that Bt is a ubiquitous, abundant, diverse bacteria and may infect more than 50% different insects in rice fields^{1–3}. The Bt index (0.002–0.006) of the rice field soils was comparable to that (0.005–0.5) of several other soils but lower than the phyllosphere (0.3–1)^{2–4}. However, endurance of 0.5M sodium acetate by the Bt isolates (Table 1) contradicted the report that it was inhibitory to 37 different bacteria including 26 Bt¹⁰.

The BT1–12 had 3 (9.69–46.27 kbp), 5 (5.78–47.44 kbp), 3 (8.06–28.75 kbp), 2 (43.92, 38.04 kbp), 2 (41.56, 12.52 kbp), 4 (17.39–39.04 kbp), 3 (20.09–37.39 kbp), 1 (38.63 kbp), 3 (5.17–32.04 kbp), 4 (6.10–58.61 kbp), 2 (17.83–36.16 kbp) and 4 (0.89–38.21 kbp) plasmids, respectively, including 1 or 2 megaplasms and varied within the same group (Figs. 1, 2). The results were in agreement with the records of 2–12 plasmids including the cryptic and megaplasms in Bt which might be inconsistent in the same subspecies/serovar/serotype and have no relation with crystal morphotype¹¹.

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