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Phenotypic and genotypic characterization of *B.t.LDC-391* strain that produce cytocidal proteins against human cancer cells

Kkani Poornima, Viswanathan Saranya, Periyasamy Abirami, Chandrasekaran Binuramesh, Ponnusamy Suguna, Peter Selvanayagam & Rajaiah Shenbagarathai*

Department of Biotechnology, Lady Doak College, Madurai, Tamil Nadu, India; Rajaiah Shenbagarathai - Email: shenbagarathai@rediffmail.com; *Corresponding author

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Abstract:
An indigenous *Bacillus thuringiensis* strain *B.t.*LDC-391 producing cytocidal proteins against human colon cancer cell line, HCT-116, was subjected to phenotypic and genotypic characterization to evaluate its relatedness to *B.anthracis*. The morphological features of this strain were meta-analyzed with data of other parasporin and insecticidal protein producing *Bacillus thuringiensis* strains. The conventional biochemical analysis and antibiotic sensitivity test proved it as an ampicillin resistant which is a salient feature, absent in *B.anthracis Ames*. PCR analysis showed the absence of *cyt* and parasporin related genes in the genome of *B.t.*LDC-391. But the strain was positive for cap gene. The sequencing and bio-informatic analysis of cap gene and 16S rDNA of *B.t.*LDC-391 placed it closer to *B.thuringiensis* and revealed significant divergence from that of any *B.anthracis* strain. However our strain lacked β–hemolysis on human erythrocytes which is a common feature of *B.anthracis* strains and parasporin producers.

Keywords: Parasporin, *B.thuringiensis*, cancer cell killing, antibiotic resistance

Background:
*B*acillus *cereus*, *B. thuringiensis* and *B. anthracis* are genetically closely related members of the group 1 bacilli **[1]**. All three species are readily isolated from soil environments though they show different patho-physiologies in different hosts. *B.thuringiensis* has been widely utilized to control many agricultural and medically important insect pests whereas *B. cereus* and *B. anthracis are* considered as highly pathogenic to mammals. The presence of delta-endotoxins or crystalline proteins produced during sporulation phase is the only phenotypic criteria determining the identity of *B.thuringiensis* strains **[2]**. The genes encoding these inclusion proteins are present in the plasmid which has the ability for horizontal transfer between closely related *Bacillus* strains **[1]**. Comparative analysis of conserved genes in the core genomes and pan genomes of the related *bacillus* species have led to the identification of many overlapping loci in these strains **[3].**

Hence much debate exists in the concreteness of describing *B.thuringiensis*, *B.cereus* and *B.anthracis* as individual entities. However, a few sequences of specific genes such as a*txA, capA capB, capC, pag, lef* etc have been identified to impart high specificity to *B.anthracis* **[4].** Cap genes are essential for synthesis of polyglutamic acid capsules which confer virulence in *B.anthracis* and atxA gene is a regulatory gene for anthracis toxin. Hence the presence of these genes and analysis of sequence homology could be used as a potential genetic marker in predicting the relatedness of environmental *B.thuringiensis* strains to that of pathogenic *B.anthracis* strains. The present investigation aims at identifying these gene sequences in *B.t.*LDC-391 and comparing its relatedness to *B.anthracis.*

Parasporins are a new functional category of inclusion proteins possessing cancer cell killing property, identified first in a Japanese *B.thuringiensis* isolate **[5]**. These strains are usually non haemolytic and non-motile with other phenotypic characters

similar to that of insecticidal *B.thuringiensis* strains. *B.t.*LDC-391, investigated in this study is one such soil isolates from southern part of India, showing cytotoxicity against human colon cancer cell line, HCT-116 **[6].** Albeit this strain possesses parasporal inclusions which is a common feature of *B.thuringiensis* species, the distinctness of this strain at morphological and molecular level to other related species needs to be investigated.

Methodology:

Bacterial strains and culture media

The indigenous bacterial strain *B.t.*LDC-391 and the reference type strain *Bacillus thuringiensis israelensis,* 4Q2 (Bacillus Genetic Stock center) were cultured in Nutrient broth (Hi-media, India) supplemented with 20 IU of penicillin for routine sub culturing at 37°C with 200 rpm shaking.

Morphological analysis:

Scanning Electron Microscopic analysis

Axenic cultures of *B.t.*LDC-391 were obtained by streak plate method. The sporulated (72h), cells were washed thrice with 0.5M NaCl and fixed in gradients of aqueous ethanol (30%, 50%, 70%, 90%) and finally with absolute ethanol. The SEM examination was performed at the Department of Plant Sciences, Madurai Kamaraj University, and Madurai. The samples were coated with gold to a thickness of 100 Å using the Vacuum Evaporator, (Hitachi, Model HUS 5GB), and analyzed in a Scanning Electron Microscope (Hitachi, Model S-450, Japan) operated at 15 kV and photographed.

Transmission Electron Microscopy (TEM)

Sporulated cultures of *B.t.*LDC-391 containing intact crystals were centrifuged at 4000g for 15 min. The pellets were suspended in a buffer containing 3% glutaraldehyde, 1.5% paraformaldehyde and 0.1M PBS (pH7.2) for 3h at room temperature and centrifuged at 4000 g. The pellets were resuspended in a cocktail of 1% osmic acid and 1.5% potassium ferrocyanide for 1.5 h at 4°C. After gradient fixing, the pellets were ultra cut and serially stained with uranyl acetate and lead citrate for 5 min. The sections were examined with a transmission electron microscope (Jeol, model 1010, Japan) and photographed.

Biochemical characterization

The biochemical tests for the bacterial characterization was performed according to the methods described in *Bergey's manual of Systematic Bacteriology* **[7].** All the tests were done in parallel with appropriate positive and negative controls **Table 1 (see supplementary material)**

Characterization of salt tolerance and temperature optima

Spizizen's media **[8]** containing different NaCl concentrations (0.5%-10%) were inoculated with the *B.t.*LDC-391 and its respective controls. Growth and optical density parameters were measured at 600 nm using spectrophotometer after 24 h. The sporulated cultures were checked for the presence of crystalline inclusions under phase contrast microscope. Sporulating cultures were monitored for their ability to grow at various temperatures ranging from 27°C - 50°C. The plates were incubated for 5 days to confirm optimal temperature and other physiological conditions. The sporulated colonies were analyzed under phase contrast microscope for the presence of spherical parasporal inclusion.

Antibiotic sensitivity test

Different antimicrobial agents were tested by the disc-agar diffusion method in accordance with the national committee for clinical laboratory standards (NCCLS). Cultures of *B.t.*LDC-391 were inoculated in Mueller-Hinton Agar (Difco, BBL. USA) with discs impregnated with known concentrations of antibiotics **Table 3 (see supplementary material)**.

PCR analysis

Total DNA from the strain using DNA isolation kit (Chromous Biotech, India) under investigation was titrated with the below listed primers for amplification of their respective genes in **Table 1 (see supplementary material).** PCR was performed within a volume of 25 µl containing 500 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 50 ng of forward and reverse primers of the respective gene, 10 mM of dNTP mix and 2.5U taq polymerase (Bangalore Genei, India). The samples were subjected for 30 amplification cycles at 94° C for 30s, respective annealing temperature for 40s, and 72°C for 60s in an Eppendorf Gradient Cycler. DNA from *B.thuringiensis israelensis* (BGSC 4Q2) was used as a control. The presence of the amplicon was observed using 1% agarose gel.

Analysis of 16S rRNA

The 16S rRNA gene sequences for *B.t.*LDC-391 **(EU625359.1)** obtained earlier were analyzed using the BLAST program (Basic Local Alignment Search Tool) (www.ebi.ac.uk and http://www.ncbi.nlm.nih.gov). About twenty *B.cereus* and *B.thuringiensis* sequences, ten *B.anthracis* sequences showing maximum score in the BLAST analysis and sequence of pathogenic *B.cereus* strain E33L was acquired from NCBI. These sequences were aligned using ClustalW and manually corrected. Maximum likelihood (ML) analysis was performed with Molecular Evolutionary and Genome Analysis package, MEGA 5 **[9, 10]** using the Kimura 2-parameter model with 1000 bootstraps.

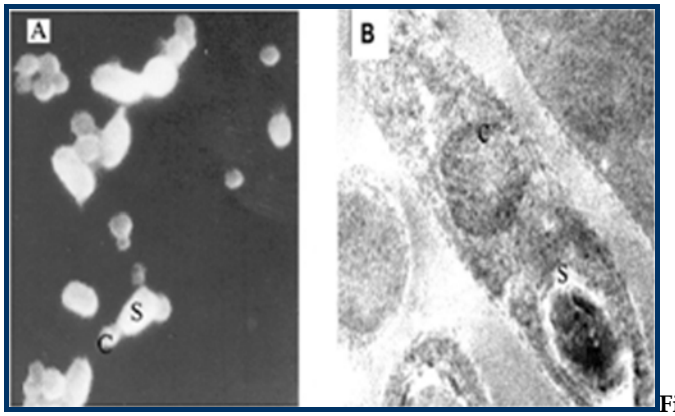

Figure 1: A) SEM analysis of *B.t. LDC*-391; B) TEM analysis of *B.t. LDC-391*; C-Crystalline inclusions and S-Spores

Discussion:

SEM and TEM analysis

Scanning and Transmission Electron microscopic analysis of *B.t.*LDC-391 spore crystal complex showed spherical crystal morphology and were smaller than spores in comparison with the established parasporin producers **[11].** The size of the inclusions varied from 0.5 µm to 1µm in width. There was an envelope surrounding the sporangia and they were found as

detached entities from the crystalline inclusions at the fully developed stage **(Figure 1A & Figure 1B)**.

Biochemical characterization

The biochemical and physiological characterization of *B.t.*LDC-391 done in triplicates showed all the typical biochemical features exhibited by *Bacillus thuringiensis* strains **Table 2** (**see supplementary material**). In sharp contrast to all the parasporin producers reported so far, *B.t.*LDC-391 is the only strain showing motility. Observing the motility of the strain is of special interest among group 1 Bacilli, because it is usually associated with the vegetative strains of *B.thuringiensis* and *B.cereus* but not *B.anthracis* **[12].**

Although *B.t.*LDC-391 was able to grow in high saline (10%) and temperature (above 35°C) conditions, the number of crystalline inclusions was reduced three to five fold when observed under phase contrast microscope. Carbon utilization potential profile of *B.t.*LDC-391 was similar to that of earlier observations that all parasporin producers were able to produce acid from all the monosaccharides except from arabinose **[13].** *B.thuringiensis* strains are not capable of utilizing non-reducing sugars, lactose and galactose which is also a common feature found in *B.t.*LDC-391.

Antibiotic sensitivity

*B.t.*LDC-391 showed resistance to ampicillin, penicillin and bacitracin but sensitive to kanamycin. Apart from the absence of δ-endotoxins, the only biochemical parameter reported to be varying from *B.thuringiensis* and *B.anthracis* is the tolerance to ampicillin as reported elsewhere **[14]**. The tolerance to β-lactam group of antibiotics place *B.t.*LDC-391 close to *B.thuringiensis* **Table 3 (see supplementary material).**

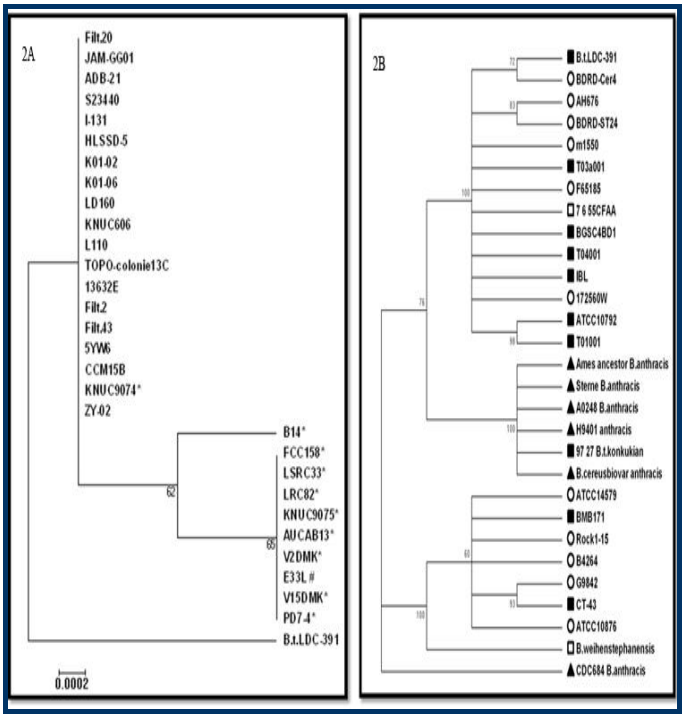

Figure 2: A) Phylogram analysis using using Maximum likelihood for 16s rDNA sequence of *B.t.LDC-391*. **B.anthricis* strains. #pathogenic *B.cereus* strain. Bootstrap values indicated at the nodes. Scale bar indicates no.of substittions per site; **B)** Phylogram analysis using UPGMA for cap gene sequence of *B.t.LDC-391* along with representive strains from *B.cereus* group. *B.cereus* ungrouped bacillus *B.thuringiensis*, *B.anthracis*. Bootsrap values are indicated at the nodes of the phylogram.

16S rDNA sequence analysis

About 30 representative strains from *B.thuringiensis, B.cereus* and *B.anthracis* showing maximum score on the BLAST analysis of 16S rDNA sequence for *B.t.*LDC-391 were used for the phylogenetic tree construction.No anthracis strains were obtained within the first 50 hits. The dendrogram did not represent the strain *B.t.*LDC-391 in the cluster of the *B.anthracis* which included the pathogenic *B.cereus* strain E33L, with a significant bootstrap value. It is in accordance to the study reported elsewhere **[15]**. The strain *B.t.*LDC 391, was represented at a different node although it was not statistically significant **(Figure 2A)** implying the divergence from the pathological strains within the *B.cereus* group. Its exclusion from the pathogenic cluster of *B.cereus* group deserves special mention and confirms that the strain under investigation does not belong to the virulence causing bacillus strains.

PCR analysis

PCR analysis of total DNA using *B. thuringiensis israelensis* (4Q2) as positive control confirmed that the strain *B.t.*LDC-391 under investigation did not possess any *cyt I* and *cyt II* related genes. *B.thuringiensis israelensis* (4Q2) showed the expected 480 base pairs amplicon for *cyt I* and a 350 base pairs amplicon for *cyt II* primers. Surprisingly, the strain *B.t.*LDC-391 was positive for the presence of cap gene, while the other primers mentioned in **Table 1 (see supplementary material)** did not generate amplicons, implying the absence of *atxA*, a regulatory gene of anthracis toxin and previously reported parasporin related genes**.** It is to be noted that very permissible annealing conditions were provided for the amplification of *atxA*, PS-1, PS-4 and PS-6 genes. Unfortunately, no reference strains are available as positive control for these reactions. Genomic analysis using cyt I, cyt II specific primers confirmed that there is no cyt related genes present in strain *B.t.*LDC-391. This result confirms that the protein exerting cytolytic action on cancer cells might not be due to the *cyt* gene. The information on the sequence of novel parasporin gene in *B.t.*LDC391 is yet under study.

DNA sequencing of the amplified PCR product using cap A primers revealed a high degree of sequence similarity to the gene for capsule biosynthesis protein. Cap genes are responsible for producing a protective capsule around the organism during adverse conditions, so that it can escape from host immune response **[4]**. The amplicon sequences obtained by the use of *capA* primers was submitted in GenBank and the accession number **JQ319804** was obtained. The orf fragment from the amplicon revealed 98% sequence identity to the cap A genes with e-values ranging from 6^{e-178} to 3^{e-175}. There was no *B.anthracis* hits obtained in BLAST analysis using cap sequences of *B.t.*LDC-391. UPGMA based analysis with 1000 boot strap using jukes cantor method was utilized to generate the phylogram. Cap sequences of representative *B.anthracis* strains for the tree generation were acquired from NCBI, non redundant nucleotide data base. The phylogram revealed that the cap gene sequences were placed in a different node which

included only *B.cereus* and *B.thuringiensis* strains with 100% boot strap values **(Figure 2B).**

The UPGMA and Neighbor Joining method based analysis of cap gene sequence from *B.t.*LDC-391 has shown that it is not grouped with any of the pathogenic or *B.anthracis* strains. It is also note worthy that several reports have shown that strains of *B.cereus* and *B.thuringiensis* contained similar capA sequences obtained by PCR and DNA hydridization **[16, 17].** However, the functionality of the detected capA gene in *B.t.*LDC-391 is in question because no capsule formation was observed in both phase contrast and electron microscopic analysis. This may be perhaps due to the absence of atxA gene which regulates the capABCDE operon **[18].** This gives us the notion that the cap genes may have undergone evolutionary changes resulting in divergence from *B.anthracis* strains.

Conclusion:

In view of their natural competence, horizontal spreading of plasmids may take place and has in fact has been demonstrated for *B. cereus* group. The above results suggest that the strain *B.t.*LDC-391 might have evolved as a result of interaction with neighboring species, probably by the horizontal transfer of extraneous plasmid genes from *B.cereus* and *B.thuringiensis* as evinced by the presence of a non functional capA gene closer to *B.cereus*. The stand alone nature of the 16S rDNA sequence in the phylogenetic tree and the presence of the divergent cap gene along with the cancer cell killing proteins throw light into the possible permutations and combinations of nature. As hypothesized by earlier workers, what seems to be a minor problem of taxonomy may result in evolution of virulence, pathogenicity **[1]** and even beneficial characteristics such as novel cancer cell killing quality as in our strain *B.t.*LDC-391.

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Reference:

[1] Helgason E *et al. Appl Environ Microbiol. 2000* **66**: 2627 [PMID: 10831447].
[2] Porcar M & Juárez-Pérez V, *FEMS Microbiol Rev*. 2003 **26**: 419 [PMID: 12586389]
[3] Burton JE *et al. J Appl Microbiol.* 2006 **101**: 754 [PMID: 16968287].
[4] Adone R *et al. J Appl Microbiol.* 2002 **93**: 117 [PMID: 12067380].
[5] Mizuki E *et al. J Appl Microbiol.* 1999 **86***:* 477 [PMID: 10196753]
[6] Poornima Kkani *et al. J Appl Microbiol.* 2010 **109**: 348 [PMID: 20337765]
[7] Jung YC *et al. J Appl Microbiol*. 2007 **103**: 65 [PMID: 17584453].
[8] Spizizen J, *Proc Natl Acad Sci USA*. 1958 **1072**: 1078 [PMID: 16590310]
[9] Kimura M, *J Mol Evol.* 1980 **16:** 111 [PMID: 7463489]
[10] Tamura K *et al. Mol Bio Evol.* 2011 **28**: 2731 [PMID: 21546353].
[11] Uemori A *et al. Naturwissenschaften.* 2007 **94**: 34 [PMID: 16957922]
[12] Martson C *et al. BMC Microbiol*. 2006 **6**: 22 [PMID: 16515693]
[13] Ichikawa M *et al. J Fac Agric Kyushu Univ*. 2007 **52**: 307
[14] Ichikawa *et al. Appl Entomol Zool*. 2008 **43**: 421
[15] Han CS *et al. J Bacteriol*. 2006 **188:** 3382 [PMID: 16621833]
[16] Sergeev N *et al. J Microbiol Methods.* 2006 **65**: 488 [PMID: 16242802]
[17] Sue D *et al. J Clin Microbiol.* 2006 **44**: 3426 [PMID: 16954292].
[18] Ibarra JE *et al. Appl Environ Microbiol.* 2003 **69:** 5269 [PMID: 12957913]
[19] Uemori A *et al. Naturwissenschaften.* 2007 **94**: 34 [PMID: 16957922].
[20] Okumura S *et al. J Agric Food Chem.* 2005 **53:** 6313 [PMID: 16076112]
[21] Nagamatsu Y *et al. Biosci Biotechnol Biochem.* 2010 **74**: 494 [PMID: 20208360]

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Supplementary material:

Table 1: Details of the primers used in this study

Primer Pair	Annealing temp in C	Primer Sequence	Expected Amplicon Length	Ref
AtxA	50	5′–GACATGCTAACACCGATATCC–3′, 5′–TGCATTCACCGTTCTTTCC–3′	1500 bp	[4]
cap A	50	5′GTGCTTTTAACCGCTTGTGGTAATAC-3′ 5′TAGTCTTTATACCAATTTCCCAACAC-3′	1178 bp	Designed in this study
cyt1gral	52	5′ CCTCAATCAACAGCAAGGGTTATT -3′ 5′TGCAAACAGGACATTGTATGTGTAATT-3′	450 bp	[18]
cyt2gral	50	5′ ATTACAAATTGCAAATGGTATTCC 5′TTTCAACATCCACAGTAATTTCAAATGC	350 bp	[18]
ps1	48	5′-ATCAAGAATTTTCCGATAATC-3′ 5′-CCAAAGTGCCAGAATG-3′	1000 bp	[19]
ps-4	48	5'-TTTTTGCAAAGGTAGGT-3' 5'-AAATAGGAATATCTAATC-3'	471 bp	[20]
ps-6	48	5′ GGATCTGTTACCGCATTTCA-3′ 5′ TGGGATTGATGGGATTATGT-3,	1000 bp	[21]

Table 2: Morphological and Biochemical characteristics

Sl.No	Name of the parameter	Observations
1	Gram Staining	Positive
2	Spore staining	Positive
3	Cell shape	Long rod
4	Density	Opaque
5	Elevation	Convex
6	Margin	Irregular
7	Configuration	Entire
8	Pigmentation	Nil
9	H_2S production	Negative
10	Fluorescence	Negative
11	Motility	Positive
12	Haemolytic activity	Negative
13	Catalase	Positive
14	Methyl Red	Positive
15	Voges Proskaeur	Positive
16	Indole	Positive
17	Citrate utilization	Positive
18	Starch utilization	Positive
19	Caesin	Positive
20	Gelatin	Negative
21	Oxidation Fermentation	Positive
22	Nitrate reduction	Positive
23	Anaerobic growth	Negative
	Acid production from carbohydrates	
	Glucose	Positive
	Fructose	Positive
	Arabinose	Negative
	D-xylose,	Negative
24	D-mannitol,	Negative
	Lactose,	Negative
	Maltose	Positive
	Mannitol	Negative
	Sucrose	Mild
	Galactose	Negative
Sl No	**Growth parameter**	**Observation**
	Growth on NaCl	
	5%	Positive
1	7%	Positive
	10%	Positive
	12%	Weak
	Temperature	
	Growth at 4^0 C	Negative
	Growth at 15^0 C	Negative
2	Growth at 25^0 C	Positive
	Growth at 37^0 C	Positive
	Growth at 42^0 C	Weak
	Growth at 50^0 C	Negative

Table 3: Antibiotic sensitivity test on *B.t.*LDC-391

Antibiotics tested	Concentration per disc	Zone of clearance	Remarks
Ampicillin	10µg	0mm	Resistant
Bacitracin	10µg	0mm	Resistant
Amoxicillin	50µg	0mm	Resistant
Penicillin	20 U	0mm	Resistant
Polymyxin B	300 U	0mm	Resistant
Rifampicin	5 µg	5 mm	Intermediate
Streptomycin	10µg	8 mm	Sensitive
Tetracyclin	30µg	10 mm	Sensitive
Chloramphenicol	30µg	13 mm	Sensitive
Kanamycin	10µg	12 mm	Sensitive
Azithromycin	10µg	15 mm	Sensitive

(0 mm clearance: Resistant; 0-5 mm clearance: Intermediate; >5 mm clearance: Sensitive).