PHENOTYPIC CHARACTERIZATION OF AN INDIGENOUS *Bacillus thuringiensis* STRAIN (*B.t.* LDC 501) EXPRESSING CANCER CELL KILLING PROTEIN

Abirami P¹, Poornima Kkani², Suguna P¹, Saranya V¹, Selvanayagam P¹ and Shenbagarathai R^{1,*}

¹PG and Research Department of Zoology, Lady Doak College, Madurai-2.

²Department of Zoology, Thiagarajar College, Madurai-2.

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ABSTRACT

Screening of coastal soil samples from Ramnad district, Tamil Nadu, South India, revealed the presence of a novel non-haemolytic and non-insecticidal *Bacillus thuringiensis* strain that contained spherical inclusion body and exerted cytotoxic action on cultured human cancer cells. When protoplasmic extract of the *B.t.* LDC 501 isolate digest with proteinase K exerted significant toxicity on cell lines such as U-937, HL-60 and primary leukemic cells. It also had mild influence on Jurkat (T cell lymphoma) but non-toxic to normal human leukocytes. Majority of the biochemical tests of the strain revealed similar characteristics like that of insecticidal strain *B. thuringiensis* (BGSC-4Q2) with slight variation. Moreover, antibiotic sensitivity assay discriminated the strain from *B. anthracis* due to its ability to resist ampicillin. Phylogenetic analysis revealed the distinctive nature of the strain as it forms a separate clade from other *Bacillus* strains.

* Corresponding author

E-mail: shenbagarathai@rediffmail.com (R.Shenbagarathai)

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1 Introduction

Bacillus thuringiensis was first isolated from the diseased silkworm larvae by Ishiwata (1901). During its stationary growth phase, it produces parasporal crystalline inclusions that are categorized into Cry and Cyt proteins (Van Rie & Ferre 2000). Cry proteins are solubilized and activated by the alkaline environment of the host midgut followed by protease activation. This facilitates binding of the toxin to the epithelial cells with the help of host-specific receptors like aminopeptidase N or cadherin, leading to oligomerization of the toxin monomers followed by osmotic cell lysis and death of the host (Knowles, 1994). However, Cyt proteins exert *in vitro* cytolytic and haemolytic activity on mammalian cell lines, however, without the involvement of a specific receptor (Drobniewski, 1993).

There were also reports on occurrence of non-insecticidal strains in the natural environment which elicited interest on other possible functions (Ohba & Aizawa, 1986; Hastowo et al., 1992; Maeda et al., 2000; Lee et al., 2003). Outcomes of these studies helps in the identification of another class of Cry protein named parasporin (PS) (Mizuki et al., 1999). It was non-haemolytic and exerted cytotoxic activity against human cancer cells without affecting normal cells at the same time. So far, a total of six such parasporins which kills a plethora of cultured cancer cells were discovered (Wong, 2010). In addition to that, some of the *B.t.* strains also harbors parasporin-like genes (Ammons et al., 2016). Major phenotypic characters of the eight PS1 producers, PS2, PS3 and PS4 were compared and found to be similar with the insecticidal *B. thuringiensis* (*B.t.*) strains (Ichikawa et al., 2007; Ichikawa et al., 2008). Due to the selective cytotoxic nature, this organism can be exploited as potential therapeutic agent against cancer.

With regard to classification of *B.t.* strains, H-serotyping has been widely used. But, there is lack of information about genetic relatedness and phylogenetic relationships (Joung & Cote, 2001). Thus, there is a need to differentiate the cytotoxic *B.t.* strain from other *Bacillus* species at biochemical and molecular levels. Hence, the present study focuses on the phenotypic characterization of the isolate termed as *B.t.* LDC 501 which has a unique potent cell-killing activity on human cancer cell lines.

2 Materials and Methods

2.1 Bacterial strains and culture media

*B.t.*LDC 501 was isolated and identified by screening of several coastal soil samples (Abirami et al., 2016) and compared with reference strain *B. thuringiensis israelensis* (BGSC-4Q2) and were used in this study. The bacterial strains were lyophilized for long term storage.

2.2 Processing of the parasporal inclusions

2.2.1 Solubilization and activation of spore crystal complex

The isolated *B.t.* LDC 501 strain was inoculated in Arret and Kirshbaum Medium (sporulating agar) (Himedia, India) and incubated at 28°C for 3-4 days. After observing the maximum parasporal inclusions, the spore crystal mixture was harvested using 1 M NaCl followed by centrifugation (5810 R, Eppendorf, Germany) at 12000×g for 10 min at 4°C. The pellet washed twice with distilled water supplemented 1 mM PMSF (phenyl methyl sulphonyl fluoride). The spore crystal pellet was solubilized in 50 mM Na₂CO₃ buffer of pH 10.5 containing 10 mM DTT and 1 mM EDTA for 1 h at 37 °C (Mizuki et al., 1999). The solubilized protein in the supernatant was separated by centrifugation at 12,000×g for 20 min at 4 °C. It was then digested with 10 µg/ml of proteinase K (Merck-Genei, Bangalore, India) for 1.5h at 37 °C and 1 mM PMSF (Himedia, India) was added to stop the proteolytic reaction.

2.2.2 Estimation of protein concentration

Protein concentration of the parasporal inclusion was estimated by Bradford's method using BSA (Bovine Serum Albumin) as a standard (Bradford, 1976). Both the standard and test protein of increasing concentration was added to the test tubes and made up to 0.1 mL with sodium carbonate buffer. Tube without protein sample was kept as blank. About 5 mL of Bradford reagent (Coomassie Brilliant Blue G-250 -100 mg, Ethanol (95%) - 50 mL, Phosphoric acid (85%) - 100 mL) was added to each tube and incubated at room temperature for 5 min. Then absorbance was read at 595 nm and the values were plotted with that of standard.

2.3 Maintenance of the cell lines

The cell lines U-937 (histiocytic lymphoma), HL-60 (myelogenous leukemia), Jurkat (T cell lymphoma), primary leukemic cells from a patient and peripheral blood leukocytes were cultured in media prescribed by the supplier with 10% FBS and 1% antibiotic at 37°C with 5% CO₂ in humidified incubator (SCO10W, Sheldon Manufacturing Inc, USA).

2.4 One dose cytotoxic assay

The proteinase K activated parasporal protein of the potential strain *B.t.* LDC 501 was tested against the suspension cancer cell lines U-937, HL-60, Jurkat and primary leukemic cells for cytotoxic activity (Mizuki et al., 1999). Aliquots of 90 µl containing 2 x 10⁵ cells were added to wells in a microtitre plate in triplicates. About 100 µl of the parasporal proteins (100 µg/ml) was added to each of the experimental wells and incubated at 37°C with 5 % CO₂ for 16 h. The solubilising buffer containing Proteinase K and PMSF was used as mock control. The cyto-morphological changes on these cells were monitored under inverted phase contrast microscope (Olympus, CKX41, Tokyo, Japan).

2.5 MTT-assay

Proteinase K activated parasporal toxin (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) was added to the cancer cells ($\sim 2 \times 10^5$) in a 96-well plate for 16 h. After incubation, 10 µl of MTT solution (from 5 mg/ml stock) was added to the cells and kept in dark for 4 h. After centrifugation at 1100 ×g for 5 min, the cells were solubilised in 100 µl of DMSO. The absorbance was measured at 570 nm in the ELISA plate reader (VersaMax, Molecular Devices, CA, USA). The viability percentage was calculated from the cells treated with buffer alone. The protein concentration inflicting 50% cell death was considered as median lethal dose (LC₅₀).

2.6 Biochemical characterization

Biochemical characterization of the *B.t.* LDC 501 strain was carried out using HiBacillus™ Identification Kit (HiMedia, India) for the Voges-Proskauer, citrate utilization, malonate utilization, arginine utilization, catalase, nitrate reduction, ortho-Nitrophenyl-β-galactoside (ONPG), carbohydrate fermentation tests (Jung et al., 2007). *B. thuringiensis israelensis* (BGSC-4Q2) and *E. coli* (DH5 α) were used as positive and negative control respectively. These tests were performed by inoculating a loopful of culture on each well and incubated for 24 h at 37 °C. After incubation, Voges-Proskauer test was performed by adding 1-2 drops of Baritt Reagent A and B. For the nitrate reduction test, 1-2 drops of sulfanilic acid and one drop of α-naphthylamine was added and observed for its change in color. To determine the catalase action, 3% hydrogen peroxide solution was added and production of effervescence was observed. All the biochemical tests were performed as described in Bergey's manual of Systematic Bacteriology (Garrrity & Holt, 2001).

2.6.1 Gelatin hydrolysis

The cultures were inoculated in the medium containing nutrient gelatin stab and incubated at 25°C for 48 h. The tubes were then kept at 4°C for 30 min to check gelatin liquefaction.

2.6.2 Starch hydrolysis

The cultures were streaked as a single line on the starch agar and incubated for 24 h. A solution of iodine was added to find out the starch utilization.

2.6.3 Casein hydrolysis

To the milk agar plate, cultures were inoculated to check the casein hydrolyzing property. The formation of clear zone around the growth of the organism was observed.

2.6.4 Indole production

containing SIM agar. After 24-48 h of incubation at 37 °C, Kovac's reagent was added and observed for the color change.

2.6.5 Urease test

This test was performed to check the production of urease enzyme by the organism. The urea broth was prepared and cultures were inoculated aseptically. The tubes were then kept for 24-48 h incubation at 37 °C and observed for the change in colour from phenol red to deep pink.

2.6.6 Hydrogen sulphide

Cultures were inoculated by stab method to the SIM agar deep tubes and kept for 24-48 h incubation. The tubes were observed for the presence of black precipitate due to the production of H₂S gas.

2.6.7 Carbohydrate Fermentation

2.6.7.1 Triple Sugar Iron

The TSI agar slants containing 1% each of lactose and sucrose as well as 0.1% glucose with phenol red indicator. The culture inoculated by stab and streak method was incubated for 24 h and observed for the change in colour from red to yellow.

2.6.8 Phenotypic characterization

The sporulated cells of *B.t.* LDC 501 strain were smeared on a glass slide and heat fixed. It was stained with Coomassie Brilliant Blue R-250 (Appendix-8) for 3 min (Rampersad & Ammons, 2005). Excess stain was removed by washing with tap water, dried and observed under bright field microscope using 100X oil immersion (Olympus-DP 12, Japan).

2.7 Antibiotic sensitivity test

Antibiotic sensitivity test was carried out using disc diffusion method (Doern, 2011). *B.t.* LDC 501 was spread on to the plates containing Muller Hinton agar in triplicates. Antibiotic diffusion discs such as ampicillin (10 mcg/disc), bacitracin (10 mcg/disc), chloramphenical (30 mcg/disc), kanamycin (10 mcg/disc), penicillin (20 U/disc), polymixin (300 U/disc), streptomycin (10 mcg/disc), tetracycline (30 mcg/disc) and rifampicin (5 mcg/disc) were kept in each corner as three per plate. The plates were incubated for 24 h at 37 °C.

2.8 Phylogenetic analysis

The genomic DNA from *B.t.* was extracted by the method of Ceron et al., (1994) with slight modifications. 16S rDNA sequence analysis was performed to confirm the strain belongs to *Bacillus thuringiensis*. Bacteria-specific forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primers (5'-ACGGCTACCTTGTTACGACTT-3') (27F/1492R) were used for PCR amplification (Poornima et al., 2010).

2.9 Statistical Analysis

Data obtained for MTT values were represented as mean $\pm$ standard error of the mean and the significant differences between the groups were tested using one-way ANOVA (SigmaStat 2.0, Systat Software Inc, USA). When the level of significance was $p \leq 0.05$, then a posteriori post hoc pairwise comparison was performed using Tukey test.

3 Results

3.1 Cytotoxicity assay

The activated proteins of *B.t.* LDC 501 exerted significant cytotoxic action on primary leukemic cells followed by U-937 and HL-60. The cells readily collapsed after toxin exposure that resulted in clump formation and finally cell death (Figure.1a). Among the cell lines tested, the T cell lymphoma cell line Jurkat was resistant to the *B.t.* LDC 501 protein action. The activated protein of *B.t.* LDC 501 did not show any

of the toxic effects on the normal human leukocytes serving as control, when observed under similar time intervals (Figure.1c).

3.2 MTT assay

The activated proteins of *B.t.* LDC 501 exhibited dose-dependent action on primary leukemic cells, U-937 and HL-60 with LC_{50} value of 0.05 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ respectively (Figure. 1b). The resistant Jurkat cell line showed cell death at 100 $\mu\text{g/ml}$ only whereas normal human leukocytes remain intact at all concentrations of the protein.

3.3 Biochemical characterization

The results of biochemical characterization for the strain *B.t.*LDC 501 was illustrated in Figure 2 & Table 1. However, tests such as arginine utilization, triple sugar iron and sucrose utilization were positive for *B.t.* LDC 501 strain which is in contrast to the negative values obtained with BGSC-4Q2.

Figure1a: Cytopathic effect of activated *B.t.*LDC 501 proteins on HL-60, U-937, primary leukemic cells and Jurkat under phase contrast microscope at 16 h postinoculation.

Figure1C: Non-toxic effect of *B.t.*LDC 501 proteins on normal human leukocytes at regular time intervals

Figure 1B: Dose response curve of *B.t.*LDC 501 activated proteins on HL-60, U-937, primary leukemic cells and Jurkat under phase contrast microscope at 16 h postinoculation. The level of cytotoxicity was assessed with MTT assay after 16 h post inoculation; Symbols: *black circle shaded*, HL 60; *black circle without shade*, U-937; *black triangle shaded*, Human Leukemic cells; *black triangle without shade*, Jurkat; respectively. Each point represents mean $\pm$ standard error of triplicates. Different alphabets represent statistical significance $p \leq 0.05$, between the groups tested by Tukey test.

3.4 Phenotypic characterization

The Coomassie stained crystals appeared better than unstained crystals. As observed in Figure 3, the crystals were spherical in shape and smaller than spores.

3.5 Antibiotic sensitivity test

Antibiotic sensitivity test revealed that *B.t.* LDC 501 strain was resistant to ampicillin, penicillin and bacitracin but highly sensitive to kanamycin and streptomycin (Figure 4 & Table 2).

3.6 Phylogenetic analysis

The amplified product (1400 bp) of 16S rDNA of *B.t.*LDC 501 strain was sequenced (Figure 5a). BLAST analysis of 16S rDNA sequence showed maximum score for *B. thuringiensis* followed by *B. cereus* and *B. anthracis*. The sequence was submitted to GenBank and accession number: JQ988062 was obtained. Phylogenetic analysis revealed that the strain originates from *B. cereus* group and then forms separate clade with *B. thuringiensis* (Figure 5b).

Figure 2: Biochemical test performed using Bacillus Identification kit. Arrow head in the 8th well inoculated with *B.t.*LDC 501 culture denotes the ability to ferment the carbohydrate whereas it is not observed in BGSC-4Q2 strain.

Table 1: Results of various biochemical tests performed for *B.t.LDC 501* strain

S.No	Name of the test	<i>B.t.LDC 501</i>	<i>B.t.israelensis</i> 4Q2 (Positive control)	<i>E.coli</i> (Negative control)
1.	Gram's stain	Gram positive	Gram positive	Gram negative
2.	Spore forming	Positive	Positive	Negative
3	Cell shape	Long rod	Long rod	Small rod
4	Density	Opaque	Opaque	Translucent
5	Elevation	Convex	Convex	Raised
6	Margin	Irregular	Irregular	Entire
7	Motility	Positive	Positive	Positive
8	Malonate utilization	Negative	Negative	Negative
9	Catalase	Positive	Positive	Negative
10	Voges Proskauer	Negative	Negative	Negative
11	Citrate utilization	Negative	Negative	Negative
12	ONPG	Negative	Negative	Positive
13	Nitrate reduction	Positive	Positive	Positive
14	Arginine utilization	Positive	Negative	Negative
15	Triple Sugar Iron	Positive	Negative	Negative
16	Hydrogen sulfide	Negative	Negative	Negative
17	Indole	Negative	Negative	Positive
18	Urease	Positive	Positive	Positive
19	Starch hydrolysis	Positive	Positive	Negative
20	Casein hydrolysis	Negative	Negative	Negative
21	Carbohydrate utilization			
	Sucrose	Positive	Negative	Positive
	Mannitol	Negative	Negative	Negative
	Glucose	Positive	Positive	Positive
	Arabinose	Negative	Negative	Negative
	Trehalose	Positive	Positive	Negative

4 Discussion

Since *B. thuringiensis* strains are more similar to *B.cereus* taxonomically, the production of crystalline inclusions is the key phenotypic feature to distinguish both the types. *B. cereus* is a well-known food-borne pathogen which produces diarrhoeal type of enterotoxin. *B.anthraxis* is a causative agent

for the disease anthrax in animals and humans (Ombui et al., 1997). Thus, there is a need to differentiate the cytotoxic *B.t* strain from other *Bacillus* species in biochemical and molecular levels. In addition to that, number of phenotypic tests also required to separate single species (Drobniewski, 1993).

Figure 3 Microscopic images of *B.t.LDC 501* parasporal inclusions under bright field; S-Spores, C -Crystals.

Figure 4 Plates showing antibiotic sensitivity of *B.t.*LDC 501 against nine different antibiotics discs. Amp-Ampicillin, B-Bacitracin, C-Chloramphenicol, K-Kanamycin, P-Penicillin, PB-Polymyxin, S-Streptomycin, TE-Tetracycline, RF-Rifampicin.

The spherical crystalline inclusion of the potential *B.t.*LDC 501 strain was smaller than the spores which is formed during sporulation stage (Figure 3). Generally, spherical shaped crystals are associated with Dipteran toxicity. Interestingly, *B.t.*LDC 501 strain was non-toxic against two Dipteran mosquito species (*Culex quinquefasciatus* and *Aedes aegypti*) (Abirami et al., 2016). The ability to act specifically on cancer cells by discriminating normal cells is the distinguished characteristic of parasporins (Mizuki et al., 1999). This action differs from broad cytolytic *Cyt* proteins that kill both normal and cancer cells (Thomas & Ellar, 1983). This is correlated with the earlier studies that denoted the association of *cyt* genes with haemolysis and mosquitocidal action (Guerchicoff et al., 2001).

The specific cytotoxic action of *B.t.* LDC 501 proteins on colon cancer cell lines was reported (Abirami et al., 2016). In this study, it showed significant toxicity towards suspension cell lines such as U- 937, HL-60 and primary leukemic cell lines. The only exception was the T-lymphoma cell line Jurkat which was completely resistant with LC_{50} of 110 $\mu\text{g/mL}$ (Figure 1a & 1b). The normal peripheral blood leukocytes (Figure 1c) were not susceptible to the cytotoxic action of *B.t.*LDC 501 proteins when tested at different time intervals. This is in contrast to an earlier reported parasporin protein PS-2 with broad cell-killing action on various human cancer cell lines and also on normal T lymphocytes (Ito et al., 2004). The other parasporins reported acted only on a few cell types, not ubiquitously, which is also a striking feature of *B.t.*LDC 501 protein.

Table.2 Zone of inhibition exhibited by *B.t.*LDC 501 against various antibiotics

S.No	Name of the antibiotic diffusion disc	Zone of inhibition (in cm)	Susceptibility
1.	Ampicillin	0.0	Resistant
2.	Bacitracin	0.0	Resistant
3.	Chloramphenicol	0.9	Sensitive
4.	Kanamycin	1.0	Sensitive
5.	Penicillin	0.0	Resistant
6.	Polymyxin	0.4	Mild Resistant
7.	Streptomycin	1.0	Sensitive
8.	Tetracycline	0.8	Sensitive
9.	Rifampicin	0.7	Mild Sensitive

Biochemical analysis was performed to assess the ability to utilize, reduce some compounds, and to ferment specific carbon sources. It is also useful to characterize and distinguish *B. thuringiensis* strains from other *Bacillus* species. *B.t.LDC* 501 isolate exhibits similar biochemical characteristics like that of the reference strain BGSC-4Q2 (Figure 2).

However, it differs in the following tests such as arginine utilization, triple sugar iron and sucrose utilization (Table 1). This might be due to the variation in speciation process as described by Martin & Travers (1989). Carbohydrate utilization and spore aspect ratio of PS2, PS3 and PS4 producers were similar to that of insecticidal *B.t.* strains (Ichikawa et al., 2008). It is imperative to note that the cytotoxic Canadian isolate (M15) was unable to ferment starch like *B.cereus* (Jung et al., 2007).

Figure 5a: PCR analysis of 16S rDNA of *B.t.LDC* 501 strain. Lane1-100 bp -3000bp DNA marker, Lane2- BGSC-4Q2, Lane3- *B.t.LDC* 501

Bacillus species were also identified based on the level of susceptibility against different groups of antibiotics (Reva et al., 1995). The result of antibiotic sensitivity test (Table 2) was similar to that of earlier reports (Luna et al., 2007). Penicillin resistance of *B.t.LDC* 501 is comparable with that of *B.cereus* and *B.thuringiensis* strains but not with *B.anthraxis* (Logan,

2005). Like *B.t.LDC* 501, parasporin (PS2, PS3 and PS4) producing strains and other insecticidal strains were also highly resistant to ampicillin (Figure 4). Further resistance to bacitracin exhibited by *B.t. LDC* 501 strain is in contradiction to the PS1, PS2, PS3 and PS4 producers (Ichikawa et al., 2007; Ichikawa et al., 2008).

These results illustrated that there is no major dissimilarity in the phenotypic characteristics of the non-insecticidal strains from that of insecticidal ones (Ichikawa et al., 2008). Thus, biochemical analysis must be accompanied with molecular characterization in order to classify the microorganism. The unique cytotoxic South Indian isolate, *B.t. LDC* 391 was also found to be similar in phenotypic and genotypic characteristics like that of insecticidal strains (Poornima et al., 2012).

The diversity and phylogenetic relationships between the organisms was decoded by the gene sequences (Zuckerandl & Pauling, 1965). The phylogenetic analysis of the potential strain *B.t.LDC* 501 was done by 16S rDNA analysis. The sequence showed high similarity to that of *B. thuringiensis* (Figure.5a & b). *Bacillus* species such as *B. cereus*, *B. thuringiensis* and *B. anthracis* shared high levels of 16S rDNA sequence similarity (Thorne, 1993).

Conclusion

In general, the existing parasporins react on only a limited set of cancer cell types, each with its own specificity. Protein of this study is also specific against certain cells, thus showing a tendency similar to that of other parasporins. The newly identified cytotoxic strain *B.t.LDC* 501 was phenotypically characterized and found to be different from other parasporin producers. Thus, further cytotoxic characterization of the parasporin-like protein enumerates its mode of action and uniqueness among the reported parasporins.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

Figure 5b: Phylogenetic analysis of 16S rDNA of *B.t.*LDC 501. Among the 11 different *Bacillus* strains analyzed using ClustalW, *B.t.*LDC 501 strain formed separate clade with the *Bacillus thuringiensis* strain.

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