Screening and testing the potentiality of entomopathogenic strains of *Bacillus* isolated from *Caloptilia theivora* (Lepidoptera:Gracillariidae)

Damayanti De and Ananda Mukhopadhyay

ABSTRACT

Four strains of bacteria were isolated from the diseased caterpillars of leaf roller, *Caloptilia theivora* infesting the tea. Analysis of the bacteria based on polyphasic approach such as, growth phase, biochemical tests, whole body protein, crystal protein profiles along with bioassay (i.e. LC_{50} and LT_{50} values) established them as different strains of *Bacillus* (*Bacillus* sp., CT01, CT02, CT03 and CT04) that were close to *Bacillus thuringiensis kurstaki* (*Btk*). Biochemical characteristics of CT01 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and oxidase tests and in utilization of trehalose and glucose. However, CT01 differed from *Btk* in ONPG, urease, nitrate and oxidase tests; showed difference in utilization tests of arabinose, xylose, cellobiose, mellibiose, saccharose and lactose too. Strain CT02 showed difference with *Btk* in ONPG, urease and nitrate tests, and in utilization tests of citrate, arabinose, xylose, cellobiose, melibiose and lactose. CT03 strain exhibited difference with *Btk* in urease and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, raffinose and lactose. CT04 strain showed difference with *Btk* in urease and esculin hydrolysis tests and in utilization of citrate, malonate, cellobiose, glucose and lactose. The doubling time was higher for the strains compard to *Btk*. When protein composition was analyzed by SDS-PAGE, crystals of CT01 showed one major protein band having the molecular weight 51 kDa while *Btk* showed the band as 52 kDa. CT02 had two protein bands having molecular weight 37 kDa and 31 kDa. A major protein band of 118 kDa was found in CT03 which was absent in all the three strains and *Btk*. 38 kDa and 29 kDa protein bands were found in CT04 strain. SDS-PAGE profiles of whole cell protein of CT01, CT03 and CT04 strains as well as that of *Btk* were similar. However, in CT02 an additional protein band of 34 kDa was found. *In vitro* studies revealed that among the four strains of *Bacillus* sp., CT04 was more pathogenic compared to the other three strains and *Btk*. Low LC_{50} and LT_{50} values qualify the strain CT04 more promising for biocontrol.

Key words: *Caloptilia theivora*, *Bacillus* strains CT01, CT02, CT03, CT04, C*amellia sinensis*, Darjeeling

INTRODUCTION

Caloptilia theivora Walsingham, commonly called 'leaf roller caterpillar' often cause substantial loss of tea crop (Anonymous, 1994). *Caloptilia theivora* its nest by folding the corner of a tender leaf and by depositing excreta inside the nest which when mixed during processing of tea leaf deteriorates the quality of made tea. Epizootics due to bacriria regularly occurr which naturally spread in the field populations. In the present study characterization and evaluation of pathogenicity of the bacteria, isolated from cada vers of leaf rolling caterpillar, *C.theivora* was undertaken.

MATERIALS AND METHODS

Isolation and Characterization of Bacterial Strain

Bacterial strains were isolated adopting the method of Lacey and Brooks (1997) and stored at -20°C for further analysis. After centrifuging at 3000 rpm for 30 min, the precipitate mainly containing bacteria was taken for pure culture isolation by 'dilution streak method' in nutrient agar medium. The infectivity of the isolated bacterial strains was determined following Koch's postulates with first instar larvae of *C.theivora*. Cell, spore shape and structure of crystal protein were observed in the isolated bacteria under phase contrast microscope (1000X) (Olympus, CX31) and the same were compared with *Bacillus thuringiensis kurstaki* (*Btk*).The colony texture and motility of the bacteria were also determined. Biochemical analyses like, indole, Voges-proskour, methyl red, citrate utilization, esculin hydrolysis, lysine decarboxylase, ornithin decarboxylase, H_2S production, nitrate reduction, fermentation of different carbohydrates, urease tests were performed using Biochemical testing kit (KB003) (Himedia) with *Btk* as reference which was obtained from a reliable Institutional source. Growth of the isolated bacterial strains were measured by

turbidimetric method (Cappuccino and Sherman, 1996). The OD value was taken at 540 nm in spectrophotometer at 30 minutes interval. Doubling time of the strains were determined and compared with *Btk*.

SDS - PAGE Profile of Crystal Protein

Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. They were grown up to sporulation phase. The crystals were harvested in high pH buffer of sodium carbonate and 2-marcaptoethanol following the method described by Kranthi (2005) with slight modification. The isolated crystal proteins were taken for SDS-PAGE analysis using gel documentation system (Spectroline, model no. TVD-1000R/F). SDS-PAGE analysis of vegetative proteins of the Gram positive bacteria was done following Costas (1992). The bacteria were cultured on Luria-Bertani (LB) agar for 24h at 37°C. The proteins were extracted using 1% lysozyme solution and lysis buffer containing 4% SDS, 20% glycerol, 2% 2-marcaptoethanol, 70% Tris-HCL, P^H 6.8, and 4% deionized water. The protein was subjected to PAGE analysis.

Bioassay

Crude spore crystal mixture (100, 300, 500, 750 and 1000 ìg/ml) of the bacteria isolated from *C. theivora* was used for bioassay adopting the method of Unnamalai and Sekar (1995). Tea leaves dipped in different concentrations of the mixtures were offered as food to the second instar larvae (n=100) of *C. theivora*. Leaves dipped in sterile distilled water were used as control. The mortality was observed at 24h interval after exposing them. Median lethal concentration (LC_{50}) was determined by probit analysis (Finney, 1971). Median lethal time (LT_{50}) was also determined following standard method.

Field Experiment

Field experiment was conducted using the varieties TV-25 and TV-26 with four concentrations viz., 4000 µg/ml, 3000 µg/ml, 2000 µg/ml and 1000 µg/ml of the most pathogenic strain CT04. Three replications were maintained. Bacterial formulations were sprayed in the tea plantation infested with leaf rollers and mortality was recorded up to seven days. One-way analysis of variance test (ANOVA) of the resulting mortality was performed. The field level LC_{50} value was also determined.

RESULTS

Morphological Characteristics

All the morphological characteristics of the isolated bacteria (CT01, CT02, CT03 and CT04), such as, vegetative body structure, spore-shape, motility, colony texture, were found to be similar to that of *Bacillus thuringiensis kurstaki* except crystal protein shape. The isolated strains revealed characteristics of genus *Bacillus* such as rod shaped vegetative body, endospore formation, Gram positivity, facultative anaerobe in nature, catalase positive, acid production from glucose and motility (Sneath, 1986).

Biochemical Characteristics

CT01 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and oxidase tests, and in utilization of trehalose and glucose. CT01 exhibited difference with *Btk* in ONPG, urease, nitrate and oxidase tests. In utilization tests difference in arabinose, xylose, cellobiose, mellibiose, saccharose and lactose was observed for CT 01. Strain CT02 positive reaction was evident for lysine decarboxylase, ornithin decarboxylase, Voges-Proskaur, and urease tests, and in utilization of citrate, saccharose, trehalose and glucose. It showed difference with *Btk* in ONPG, urease and nitrate tests and in utilization of citrate, arabinose, xylose, cellobiose, melibiose and lactose. On the other hand, CT03 strain showed positive reaction in ONPG, lysine decarboxylase, ornithin decarboxylase, urease, esculin hydrolysis and Voges-Proskaur tests, and in utilization of citrate, malonate, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose and glucose. Difference with *Btk* was evident in urease and esculin hydrolysis tests and in utilization tests it showed difference in citrate, malonate, arabinose, raffinose and lactose. CT04 showed difference with *Btk* in urease and esculin hydrolysis tests; and in citrate, malonate, celobiose, glucose and lactose utilization. Hence, CT04 is biochemically different from *Btk*.

Growth Phase or Determination of Doubling Time

The doubling time was 132, 78, 42 and 66 min in CT01, CT02, CT03 amd CT04 respectively compared to 42 min in *Btk*.

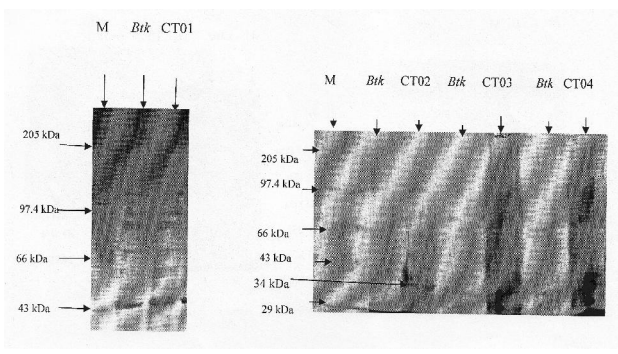

Figure 1. SDS-PAGE analysis of vegetative of *Bacillus* sp. CT01, *Bacillus* sp, CT 02, CT 03 and CT 04 compared with *Btk*.

Table 1. Bioassay of four strains of *Bacillus* on *C. theivora*

Strain	Btk and CT04 Concentration (µg/ml)	Corrected mortality (%)	LC_{50}	Lower Fiducial limit (µg/ml)	Upper Fiducial limit (µg/ml)	Regression	LT_{50}	X^2
Btk	1000	78	436.5 µg/ml	402.014	470.986	Y=2.067X-2.2	4.96	93.8312
	750	67					7.307	68.6094
	500	53					8.238	42.8456
	300	31						13.5297
	100	30						12.5000
Bacillus sp. CT01	1000	77	95.50 µg/ml	66.729	124.271	Y=3.33X-21.3	3.75	77.3752
	750	77					4.11	77.3752
	500	63					4.63	52.0833
	300	62					6.23	46.6477
	100	61					8.41	44.9066

SDS-PAGE of crystal protein

Protein composition analyis by SDS-PAGE, revealed one major protein band having the molecular weight 51 kDa in crystals of CT 01 compared to 52 kDa protein band in *Btk*. So, a narrow difference in banding pattern was found between CT01 and *Btk* . In CT02 two protein bands having molecular weight 37 kDa and 31 kDa were found. A major protein band 118 kDa was found in CT 03 which was absent in all the other three strains and *Btk*. Protein bands in CT04 were 38 kDa and 29 kDa (Fig 1). No differences were found in whole cell protein profile of CT01, CT03 and CT04 strains. A protein band having molecular weight 34 kDa was found in CT02 strain only, which was absent in other three strains and in *Btk* (Fig 2).

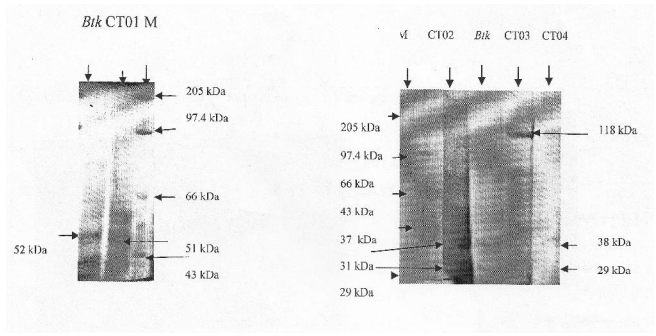

Figure 2. SDS-PAGE analysis of crystal protein of four *Bacillus* strains isolated from *Caloptelia theivora*

Bioassay

Bioassay with four strains *viz.*, CT01, CT02. CT03 and CT 04 showed that LC_{50} values were, 95.50, 117.5, 104.7 and 87.10 µg/ml for CT01, CT02, CT03 and CT04 strains, respectively. *Btk* was found to be less toxic with higher LC_{50} values of 436.5 µg/ml.

LT_{50} values of CT01, CT02 and CT04 were lower compard to CT 03 (Table 1). Multivoltine variety of silkworm larvae (*Bombyx mori* nistari) when treated with the *Caloptilia theivora Bacillus* strains did not showed any mortality due to entomopathogenicity.

Field Experiment

Among the four isolated strains of *Bacillus* from *C. theivora* the most pathogenic was CT04. The dose, 4000 µg/ml inflicted high mortality (86.74%) followed by other three concentrations (66.76, 16.08 and 7.17% for 3000, 2000 and 1000 µg/ml, respectively) and 2.28% within seven days of spraying. One-way analysis of variance test (ANOVA) revealed, a significant difference among the treatment. The field level LC_{50} was 2759.49 µg/ml with fiducial limits of 2564.318 and 2969.509 µg/ml for CT04.

DISCUSSION

The strains of bacteria isolated from *C. theivora* exhibited typical characteristics of *Bacillus thuringiensis*, especially in their vegetative body structure and crystal production. As crystals are the typical distinguishing characteristics of *Bt* (Heimple and Angus, 1958; Bai *et al*., 2002), the new strains were identified as *Bt* strains on the basis of the spore structure and crystal formation (Brussock and Currier, 1990). However, the *Bacillus* strains showed a significant difference with *Bacillus thuringiensis kurstaki* on the basis of biochemical testing and generation time. Even the crystal protein profiles on PAGE and the vegetative protein profiles of these strains were found to be different from that of *Btk*.

Bioassay revealed that the new natural strains (CT01, CT02, CT03 and CT04) of *Bacillus* were found to be highly pathogenic and comparable to *Btk*. *Btk* has a wide application as microbial pesticide against lepidopteran

pests. As the LT_{50} values of the new strains were lower than the commercially used *Btk*, these appeared to have better killing efficacy. There strains inflected no mortality in silkworm (*Bombyx mori*) indicating their bio safety to the local sericulture industry.

As no naturally occurring *Bacillus* strains has so far been reported from *C. theivora* of sub Himalayan tea plantations, the newly isolated and charaterized strains were designated as, *Bacillus* sp. CT01, *Bacillus* sp. CT02, *Bacillus* sp. CT03 and *Bacillus* sp. CT04. Report on development of insect resistance to *Btk* has stimulated new research to find additional *Bt* strains and other microbes that have specific activity spectrum against certain insect pests (Bai *et al*., 2002; Mc. Gaughey, 1985; Monnerat *et al*., 2000; Salama and Abdel-Razek, 2000). In this context these newly reported strains of *Bacillus*, CT01, CT02, CT03 and CT04 with their appreciable entomopathogenicity appeared to be promising.

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Damayanti De and Ananda Mukhopadhyay*

Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, 734 013, India.

*Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, 734 013, India, Phone : 91-0353-2776353 (O), 91-0353-2581830 (R), Fax : 0353-2699001, E-mail: am_nbu@yahoo.co.in