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ROLE OF GUT MICROFLORA IN TOXICITY OF *BACILLUS THURINGIENSIS* SUBSPECIES *GALLERIAE* TO THE LARVAE OF *SPODOPTERA LITURA* (NOCTUIDAE: LEPIDOPTERA)

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Abstract

It is well known understands that synergistic and additive effects both between *Bt* toxins and other compounds do occur. Mode of action *of B. thuringiensis (Bt)* Cry toxins are very specific. Different strains of *Bt* are specific to different receptors in insect gut wall. *Bt* toxicity depends on recognizing receptors, damage to the gut by the toxin occurs upon binding to a receptor. Each insect species have different types of receptors. Comparatively less consideration has been devoted to other important research aspects, such as the compound network of molecular interactions underpinning the host killing mechanism and the role of factors other than the pore-forming toxins that contribute to *B. thuringiensis* pathogenicity and virulence. The presence of symbiotic gut flora in the insect midgut also plays a major role. We examined the interaction between *Bacillus thuringiensis* subsp. *galleriae* and larval gut flora in the lepidopteran pest, *Spodoptera litura.* Dose-mortality bioassays of *Bt* galleriae were conducted on normal and gut flora eliminated larvae of *S. litura*. The results showed that the gut microflora play a major role in determining the toxicity of *B. thuringiensis.*

Keywords: Bacterial diversity, insect gut microbes, Elimination of bacteria, microbial interactions

Introduction

The entomopathogenic bacterium, *Bacillus thuringiensis* (*Bt*) produces parasporal crystals that are toxic to wide array of insects and other related organisms. The targeted mode of action in the insect gut includes solubilization of the crystals upon ingestion by the susceptible insect and its activation by midgut proteases then binding to the pore forming toxin at specific receptors present on the midgut brush border membrane vesicle (Soberon *et al*., 2016). However, comparatively less interest has been shown to the other factors such as the function of gut microflora in modulating *Bt* toxicity. Insects harbor a different range of gut bacteria that aids in host insect nourishment and protection from pathogenic microbes (Engel and Moran, 2013). However, compared to mammalian gut, the insect gut harbors only few microbial species, but specialized in their function. DGGE analysis of the field population of *Spodoptera litura* reportedly contain more than 15 gut bacterial OTUs (Gadad *et al*., 2017).

It is well understood that the *S. litura* is relatively less susceptible to many *Bt* strains including the widely used *Bt kurstaki* HD-1. *S. litura* (Noctuidae: Lepidoptera) is a polyphagous pest and cosmopolitan in distribution. The various experimental evidences indicate that the gut microflora play an additive role in increasing the toxicity of *Bt* in few insect species (Broderick *et al*., 2009). However, in other insects the gut microfloras were linked to degradation/ inactivation of toxic effect of *Bt* (Heckel *et al*., 2018; Xia *et al*., 2018).

Bt is considered as feasible alternative way to manage the pests under organic agriculture as well as to eliminate the pesticide resistant individuals. The current work explored the effective role of gut microflora on toxicity of *Bt* to the larvae of *S. litura*.

Materials and Methods

Collection and rearing of test insect

The egg masses and larvae of *S. litura* were collected from cabbage field at ICAR-NBAIR farm, Bengaluru, Karnataka, India (13°05' N, 77°33' E). The *S.litura* larvae were reared on fresh castor leaves till pupation. During ovipositions of the adult moths were provided with 10% honey solution fortified with multivitamins. Eggs were surface sterilized in 0.2% sodium hypochlorite solution and the larvae from F1 generation were maintained on chickpea based artificial diet at 28± 2 C, 60% relative humidity and 14:10h photoperiod.

Elimination of gut microflora

The elimination of gut micro flora was determined by feeding the larvae of *S. litura* with a range of antibiotic concentrations in combination. The neonate larvae of *S. litura* was fed with a combination of streptomycin and rifampicin (each 50µg/ml) amended diet till molting to second instar was found to inhibit >99% of the culturable midgut bacteria in the larvae and hence in subsequent gut curing experiments the same combination and dose were used. The freshly molted second instar larvae reared on antibiotic amended diet and normal diet were used in dose-mortality bioassays.

Culturing and formulation of *Bacillus thuringiensis* (*Bt*)

The locally isolated *Bt* subsp. *galleriae* (MTCC 8977) was grown in Luria Bertani broth at laboratory conditions 150 rpm for 72 hours at 30°C. The sporulated culture was centrifuged at 8000 rpm and the pellet was washed twice with 0.5M NaCl solution followed by washing thrice with sterile distilled water. The resultant pellet was subjected to lactose-acetone based co-precipitation method (Dulmage *et al*., 1970). The precipitate containing spore crystal mixture was vacuum dried, powdered and used for bioassay studies.

Estimation of toxin protein content

The spore-crystal formulation was dissolved in solubilizing buffer (50mM sodium carbonate containing 10mM dithiothreitol, pH 10.5) and incubated at 37°C at slow shaking for four hours. The content was centrifuged at 10000 rpm at 4°C for 10 minutes. The protein content of supernatant containing the protoxin was predicted by Bradford's method (Bradford., 1976).

Dose-mortality bioassays

The serially diluted *Bt galleriae* toxin *viz*., 150, 75, 37.5, 18.75, 9.375, 4.688, 2.344 ppm and sterile water control were incorporated into artificial diet prepared aseptic lab conditions. Experiments were conducted in two sets, one with gut microflora eliminated larvae and another with normal diet fed larvae in 16 cells bioassay tray (C-D International, Pitman, NJ). The larval mortality was assessed after eight days of feeding on the *Bt* treated diets. The pooled larval mortality data was subjected to probit analysis using the software POLO (LeOra, 1994) and the lethal concentration to kill 50% of the test larvae (LC_{50}) was calculated for each population.

16S rDNA sequencing analysis

The culturable gut microflora estimation in the neonate larvae of *S. litura* was done by plating a known amount of midgut homogenate on different media. The dead larvae were first washed with ethanol 70% (×4) and then with sterile water (×4) for surface sterilization. The larvae were dissected aseptically by removing anterior and posterior part of larvae in 1ml of saline phosphate buffer (pH 8.0). The gut was carefully transferred using sterile forceps to 1ml phosphate buffer solution and homogenised using micropestle and mortar. The bacterial colonies with different morphologies were subcultured, purified and used for isolation of DNA. The total number of colony forming units (CFUs) for each microbial species were was estimated. The homogenate was serially diluted up to 10^{-6} and then 100µl of 10^{-6} dilution was spread plated on different laboratory media (Luria Bertani and Nutrient agar) in triplicates. The control plate was also maintained for confirmation of efficiency of surface sterilization using 100 µl final wash elute from surface sterilization step. The plates were incubated in BOD incubator at 28±2°C and the colony forming units (CFU) count after incubation was enumerated after 24h. The quadrant streak method followed to purify representative bacterial isolates on plates with respective media. The purified bacterial isolates were inoculated in Luria broth medium and incubated overnight and DNA was extracted using HiPura DNA extraction kit (HiMedia India Pvt. Ltd., India) as per manufacturers' instructions. 16S rRNA gene was amplified using universal 16S rRNA primers (forward primer pA 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer pH 5' AAGGAGGTGATCCAGCCGCA 3'). The final volume of reaction mixture of 25 µl contained 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1 mM of each primer, 1.0U of *Taq* polymerase and 100 ng of template DNA. The amplification of DNA was carried out in thermal cycler (Qantarus, UK) (initial denaturation step at 95°C for 3 min, followed by 35 amplification cycles of 94°C-1 min, 55°C-1 min, 72°C-1 min and 72°C-10 min for final elongation). DNA template replaced with known DNA served as control. The amplified PCR sample products were purified with PCR purification kit (Qiagen GmbH, Hilden, Germany) and sequenced directly with the *Taq*-mediated dideoxy chain terminator cycle sequencing in ABI 3130*xl* automated genetic analyzer (Applied Biosystems, UK) according to manufacturer's instructions (Yalashetti, 2017).

Phylogentic analysis of gut microflora

The inference on evolutionary history was accounted using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree is depicted with the highest log likelihood (-4129.5988). The percentage of trees is shown next to the branches in which the associated taxa clustered together. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The evolution tree is set to scale, along with branch lengths evaluated in the number of substitutions every site. The analysis is having 10 nucleotide sequences. All positions related to consisting gaps and missing data were removed. There were a totally of 1163 positions in the final dataset (Fig.1). MEGA7 software was used for evolutionary analyses (Kumar *et al*., 2016).

Results

Neonate larvae in groups were exposed to the diet amended with combination of streptomycin and rifamycin (each 50µg/ml) suppressed the midgut culturable microflora load by >99%. Based on 16S rRNA sequencing, the major gut bacteria associated with gut untreated normal larvae of *S. litura* identified were *Bacillus subtilis, B. oleronius, B. licheniformis, B. megaterium, Bacillus* sps., *Enterococcus mundtii, Pseudoclavibacter faecalis, Lysinibacillus macroides, Pseudomonas stutzeri, Staphylococcus sciuri, S. saprophyticus* and *Enterobacter cloacae* (Table 1). These bacterial species altogether accounted for 3×10^4 CFUs per gut of neonate larvae. The dose-mortality bioassays using normal and gut microflora eliminated larvae with *Bt galleriae* and subsequent probit regression analysis revealed that gut flora-eliminated *S. litura* larvae were more susceptible (LC_{50} 1.73 ppm) as compared to normal larvae (LC_{50} 1.44 ppm) to *Bt galleriae* (Table 2). However, there was no extensive difference in toxicity based on overlapping of 95% fiducial limit values.

Table 1 : Prominent native gut microflora of 1st and 2nd instar larvae of *S. litura.*

Strain name	Gut microflora	CFU per gut x 10^4 (before gut curing)	CFU per gut (after antibiotic treatment)	NCBI Accession No.
SL 1-1	*Bacillus subtilis*	0.53 ±0.05	4.2 ± 0.32	MK312473
SL 1-2	*Bacillus oleronius*	0.49±0.04	5.2 ± 0.12	MK312474
SL1-3	*Bacillus licheniformis*	0.69±0.05	2.2 ± 0.21	MK312475
SL 1-4	*Bacillus megaterium*	0.89 ±0.03	5.3 ± 0.11	MK312476

SL 2-1	*Enterococcus mundtii*	0. 66 ±0.03	3.2 ± 0.12	MK312477
SL2-2	*Pseudoclavibacter faecalis*	0.48 ± 0.03	3.4±0.07	MK312478
SL2-3	*Bacillus* sp.	0.54 ±0.05	5.0±0.04	MK312479
SPL-1*	*Lysinibacillus macroides*	0.65 ±0.03	4.0±0.11	KT818804
SPL-2*	*Pseudomonas stutzeri*	0.59±0.05	6.1±0.07	KT818805
SPLN-1*	*Staphylococcus sciuri*	0.71±0.04	4.1±0.09	KT818800
SPLN-2*	*Staphylococcus saprophyticus*	0.46±0.04	6.5±0.06	KT818801
SPLN-3*	*Enterobacter cloacae*	0.61±0.04	4.7±0.05	KT818802

*Reisolated from midgut of larval *S. litura* and same were reported earlier (Yalashetti *et al*., 2017)

Table 2 : Toxicity of *Bt galleriae* against the normal and gut microflora eliminated 2nd instar larvae of *S. litura*

LC_{50} (ppm, 5th day)	Slope+SE	Fiducial limits		Chi square heterogeneity value	Degrees of freedom
		Lower	Upper		
Normal larvae					
31.9	2.44+0.71	16.36	46.66	5.4	3
Gut microflora eliminated larvae					
19.9	1.73+0.38	11.30	31.32	4.02	3

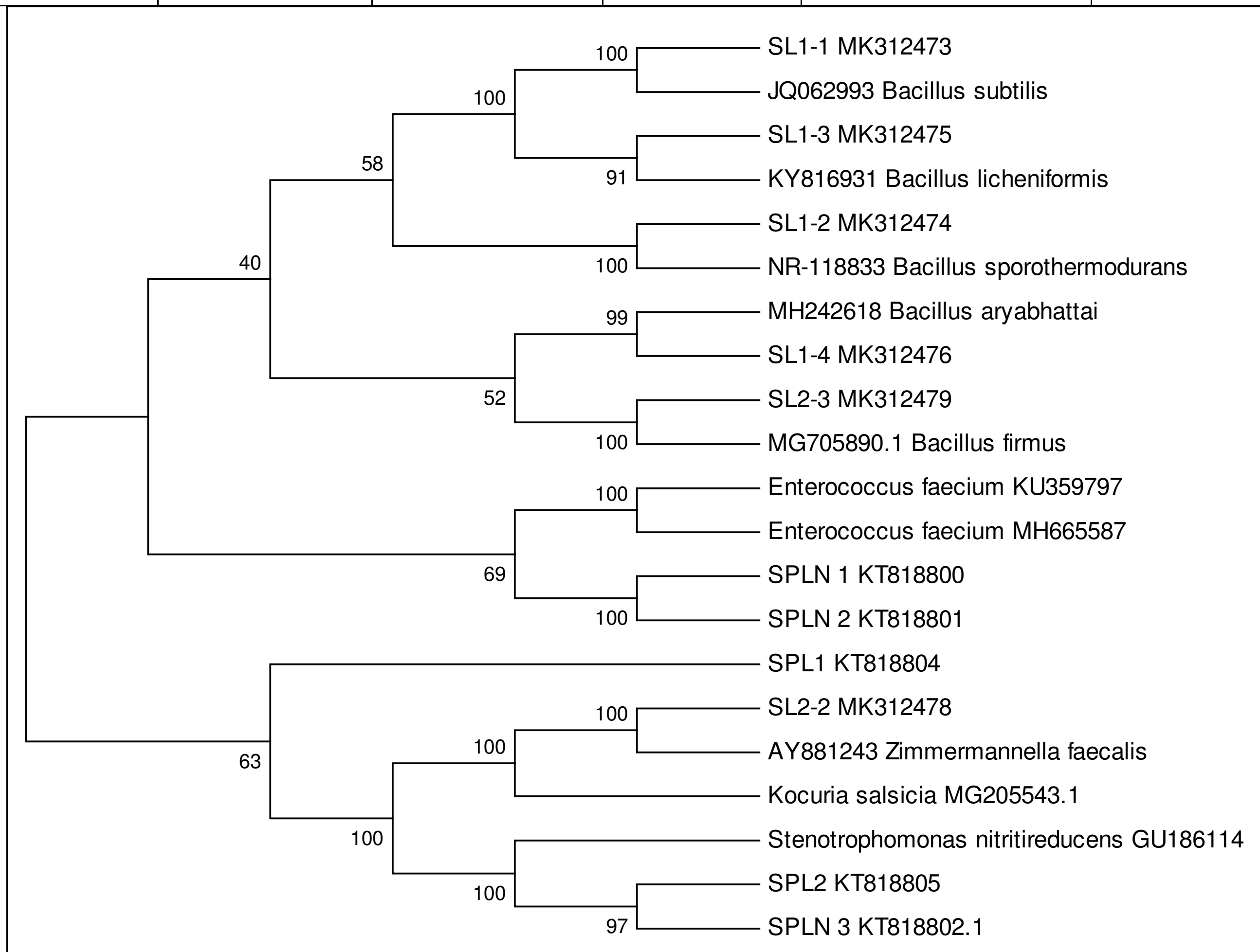

Fig. 1 : Phylogenetic analysis of prominent gut microflora

Discussion

S. litura is a major serious pest of crops that attacks many legumes, oil seeds, cereals, ornamentals and fruit crops. The larvae of *S. litura* are in general less susceptible to various subspecies of *B. thuringiensis* including *B. thuringiensis* subspecies *kurstaki*. However, it is relatively more susceptible to *Bt* subspecies *galleriae* (Mohan *et al*., 2014). Hence, *Bt galleriae* was chosen to study the role played by midgut micro flora on *Bt* toxicity. The larvae did not exhibit significant difference in mortality to *Bt* in presence or absence of gut microflora. Gut microflora of insects are necessary for host insect digestion and defensive mechanism of gut against pathogenic microbes. Many of studies have been investigated the role of midgut microflora on *Bt* toxicity by using antibiotics to eliminate bacteria from gut.

The presence of indigenous gut microflora is necessary to induce mortality in few insects (Broderick *et al*., 2006). Essentially not all gut microbes play a key role in insecticidal resistance development; (Johnston *et al*., 2009), lethality is reduced in the larvae that are continuously exposed to antibiotics before bioassay for *B. thuringiensis* insecticidal activity towards *Manduca sexta.* The gut microflora contributions towards *Bt* induced mortality may vary across

the range of Lepidoptera (Broderick *et al*., 2009) and gut microflora have a role to play in degradation of organophosphate fenitrothion insecticide such as *Burkholderia* sps. developing symbiont mediated resistance within a generation (Kikuchi *et al*., 2012), meanwhile gut eliminated insect would have more susceptibility to *Bt* toxins (Patil *et al*., 2013). Gut microflora show many roles apart from insecticidal degradation, contributing beneficial roles in nourishment and defense from pathogens and inflict on insect immune system (Engel and Moran, 2013). Presence of microbiota was reported to provide an additive effect along with activated Cry1Ac and *Bt* formulation in midgut of *Helicoverpa armigera* by inhibiting the protease activity normally inducing resistance thus, substantiating the nature of gut microbiome with Cry1Ac and *Bt* formulation (Visweshwar *et al*., 2015). Three bacterial strains of gut of *S. litura* in enhanced counts in the gut of same host revealed increased mortality of host. Among three bacterial strains, *Enterobacter cloacae* showed an increased mortality upto 30-70% in *S. litura* and proved as promising biological agent (Thakur *et al*., 2015). The gut microbe *Serratia marcescens* facilitated the entomopathogenic fungi *B. bassiana* promoting mortality of mosquitoes by reaction of the toxin oosporin, via down-regulation of antimicrobial peptides and dual oxidase in the midgut (Wei *et al*., 2015).

Conclusion

Our results showed that midgut microflora does not play a major important role in synergizing the toxicity of *Bt galleriae*. It is likely that the *Bt* pathogen could able to breach the protection offered by native midgut immunity and native microflora to bind to receptor present in the brush border membrane vesicle.

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