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RESEARCH ARTICLE

Determination of Detoxifying Enzyme Activity of Gut Isolates of Diamondback Moth, *Plutella xylostella* (Lepidoptera: Plutellidae) and Effect of Antibiotics on Host Insect Morphogenesis

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ABSTRACT

Microorganisms present in the host insect gut contribute to nutrient digestion, detoxification, and degradation of toxic substances and produce biologically active metabolites. The present study aimed to determine the detoxifying enzyme activity of gut microbe of Diamondback moth and the effect of antibiotics on host insect morphogenesis. Gut bacterial strains isolated from *Plutella xylostella* were tested for their glutathione-S-transferase (GST) and protease activity. The GST activity varied with different bacterial strains and the maximum was recorded for *Stenotrophomonas* sp. PRGB08 (140 µmoL min^{-1} mg $protein^{-1}$) isolated from resistant population. Irrespective of the origin, the substrate preference of the GST enzymes suggested that bacterially derived enzymes contribute to the detoxification of toxic substances and may confer resistance development. N-acyl homoserine lactone is produced by the gut bacterial strain *Stenotrophomonas* sp. PRGB08 suggested that the contribution of gut bacteria to the host insect process might be variable. Furthermore, the experimental elimination of gut bacterial strains resulted in the emergence of malformed adults and adults with less fecundity. This revealed the potential for the new management strategy against the devastating, resistance-developed insect species.

Keywords: *Insect gut microbe; glutathion-S-transferase; protease; N-acyl homoserine lactone; Host biology*

INTRODUCTION

The diamondback moth, *Plutella xylostella* L., a cosmopolitan insect pest of cruciferous vegetables has an extraordinary capacity to develop resistance to insecticides deployed against it (Sarfraz *et al*., 2006). *P. xylostella* possesses versatile and multiple resistance mechanisms to overcome the toxicity of synthetic, biotic insecticides and plant allelochemicals (Mohan and Gujar, 2003a, b; Ratzka *et al*., 2002). The enhanced activity of detoxifying enzymes such as glutathione-S-transferase (GST), cytochrome P450 mono oxygenase, and carboxy esterase activity has been proven responsible for many cases of the alteration of insecticide susceptibility (Mohan and Gujar, 2003a, b). The extent to which insects catabolize harmful compounds in their digestive tracts or on their host plants including insecticides sprayed on the host plants is of considerable importance to their survival (Lauzon *et al*., 2003). Most insects detoxify chemicals with the aid of enzymes like GST, which convert the lipophilic toxic substances into more water-soluble compounds that are more easily excreted and eventually safeguard the host insect from toxic effects. However, so far the reported reason for detoxification of toxic substances is due to host insect tissue-derived enzymes (Chian and Sun, 1993; Mohan and Gujar, 2003a). Dillon and Dillon (2004) reviewed the contribution of insect gut bacteria to their host insects right from detoxification of harmful chemicals to protection from parasitoid attack. It seems that the occurrence and contribution of insect gut bacteria to the host insect are indispensable.

Furthermore, variations in the gut bacterial community between different populations especially insecticide resistance and susceptible ones have been found in literature (Xiang *et al*., 2006). The gut bacterial community would be variable according to the toxic compounds encountered by the host insects along with their diet. Recently, we documented the variation in the gut bacterial population and bacterial phylotypes present in the prothiofos-resistance,

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susceptible, and field population of DBM (Indiragandhi *et al*., 2007). Given that insect guts are illustrious for the inhabitants of different bacterial phylotypes and these contribute significantly towards host insect processes right from food digestion to protection from toxic materials (Santo-Domingo *et al*., 2006). It was evident from the work done by Costa et al. (1997) and Koga *et al*., (2003, 2007), where they observed that loss in weight, reduced growth, malformed pupae, and adults, as a consequence of the elimination of gut bacteria by antibiotic treatment. Thus, an understanding of biological responses to antibiotics is essential to evaluate the potential for management of pest populations by disrupting their gut bacteria. In the present study, the gut bacterial strains for glutathion-S-transferase and protease, the enzymes that play a vital role in detoxification of toxic substances of chemical and biological (Bt) origin, respectively have been evaluated. In addition, the effect of antibiotics treatment on DBM larval growth and development was tested.

MATERIAL AND METHODS

Chemicals, insects and culture conditions

Glutathione (GSH), benzene substrates 1-Chloro-2-4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene also known as 1,2-dichloro-4-nitrobenzene (DCNB), Gelatin, and antibiotics were purchased from Sigma chemicals, St. Louis, USA. All other reagents were of commercially available analytical grade one. DBM larvae were reared with Chinese cabbage, Brassica chinensis var. jinbo in acryl cage (30 × 30 × 30 cm) maintained at 25-28°C and 50-60% RH under 16L: 8D photophase. DBM gut bacterial isolates as listed in table 1 were maintained in 50% glycerol at - 80°C and revived on nutrient agar (NA) media for experimental purposes.

Crude enzyme preparation from DBM larvae and bacterial cells for the determination of GST activity

The larval homogenate was prepared by grinding 20 each third and fourth instar larvae in ice–cold 50 mM sodium phosphate buffer (pH 6.5). The homogenates were centrifuged at 10,000 × g for 30 min at 4 ºC and the culture supernatant was used for enzymatic assays. GST activity was determined by using the standard benzene substrates CDNB and DCNB. The assay mixture consisted of 500 µL of 0.8 mM GSH and CDNB/DCNB in 95% ethanol each and 1 mL of enzyme extract in 1 mL of phosphate buffer (pH 7.5). The change in absorbance was measured at 340 and 344 nm for 5 min for CDNB and DCNB, respectively. The enzyme activity in terms of µmoL of CDNB/DCNB conjugated min^{-1} mg of $protein^{-1}$ was calculated using the extinction coefficient of 9.6/8.5 $mM^{-1}cm^{-1}$, respectively. To correlate enzyme activity with the amount of protein in each sample, protein concentration was measured by using the standard method (Lowry, 1958).

Determination of proteolytic activity for DBM gut isolates

Proteinase activity was measured spectrophotometrically using the substrate gelatin, according to the method described by Jones *et al*., (1998). To 1.5 mL of the substrate solution (1%), 500 µL of the culture supernatant extract and 500 µL of 50 mM sodium phosphate buffer, pH 5.5 were added and the mixture was incubated at 37 ºC for 2 h. One unit of enzyme activity is defined as the amount of enzyme that caused an increase of 0.01 absorbance unit at 280 nm under standard assay conditions.

Production of N-acyl homoserine lactones

Chromobacterium violaceum CV026 was used to detect the production of N-acyl homoserine lactones (AHL) in cross-streak experiments, as previously described (McClean *et al*., 1997). The quorum-sensing inducing activities of DBM gut bacterial strains were tested by streaking, side by side, the sensor strain CV026 and bacterial strains on solid Luria Bertani (LB) agar medium. Petri dishes were incubated overnight at 30°C, then examined for the stimulation of violacein synthesis (indicated by purple pigmentation of the sensor strain CV026).

Antibiotic bioassay

Antibiotics to which the DBM gut bacterial isolates of Acinetobacter, Pseudomonas, Serratia, and Stenotrophomonas were susceptible (Indiragandhi *et al*., 2007) were selected to test their effect on larval growth and development because of elimination of gut bacteria from the host larvae. Leaf discs (5 cm dia) were prepared from the Chinese cabbage leaves, which were washed with distilled water containing 0.1% Triton X-100, and dried for about 30 min. The leaf discs were dipped in antibiotic solutions for 10 min and placed in a slanting position for about 2 min over a blotting paper in a tray to drain excess solution. To dry the test solution the leaves were then flattened for about 30 min. All the procedures were carried out under aseptic conditions. Ten numbers of two days old larvae of each third and fourth instar were released on individual Petri plates containing antibiotic-treated leaf discs. Three concentrations (0.001, 0.005, and 0.01%) of each antibiotic such as doxycycline, gentamycin, kanamycin, polymyxin and spectinomycin were used with three replications for each concentration. Larvae were allowed to feed on the treated leaf disc for 48 h at 28 ºC and checked

for mortality. After that, live larvae were observed for pupation, adult emergence, and fecundity. Meanwhile, every 24 h the live larvae were fed with plain Chinese cabbage leaf discs.

Results and Discussion

CDNB, DCNB/GST activities of DBM gut bacterial strains

The insect gut provides a habitat for supporting a diverse microbial community and the gut microbes were involved in the conversion of complex organic materials and toxic substances in the diet into nutrients of utilizable and nontoxic form for the host insects (Dillon and Charnley, 2002; Lauzon *et al*., 2003; Dillon and Dillon, 2004). GST was the foremost enzyme responsible for the detoxification of organophosphate insecticides used against pest insect species (Mohan and Gujar, 2003a). Dichloromethane dehalogenase gene from *Methylobacterium sp*. belongs to the GST supergene family, which suggested that GST-like enzymes may be widely distributed in bacteria xenobiotic degradation pathway (La Roche and Leisinger, 1990). A bacterial strain present in the DBM gut environment is variable with respect to different populations i.e. an organophosphate insecticide prothiofos-resistant, susceptible, and field-caught population (Indiragandhi *et al*., 2007). As expected, the DBM gut bacterial strains showed different levels of GST activity with respect to different substrates, DBM population, and growth stages. Stenotrophomonas sp. PRGB08 from the resistant population showed the highest GST activity (140 µmoL min^{-1}mg $protein^{-1}$) with CDNB substrate (Table 1). There was a wide range of specific activities observed among different strains of the same species. Acinetobacter species strains (PRGB16 and 15) from resistance population showed 44.5 to 100 µmoL min^{-1} mg $protein^{-1}$ and Serratia species (PSGB18, PSGB13, PSGB12) from susceptible population showed the activity ranging between 1.45 - 11 µmoL min^{-1} mg $protein^{-1}$. *Serratia marcescens* strains from the field-caught population showed GST activity of 0.9 to 76.76 µmoL min^{-1} mg $protein^{-1}$. CDNB/GST activity was undetectable in Gram-positive bacterial strain *Brachybacterium sp*. PSGB10 and Gram-negative bacterial strain Serratia marcescens FLGB16 (Table 1) and showed 85.73 and 61.92 µmoL min^{-1} mg $protein^{-1}$ with DCNB. In general, the DBM gut bacterial GST activity in conjugation with DCNB was higher than that of with CDNB. Results of the present study suggested that the type of GST present in the DBM gut bacterial strain might be the GST-3 type according to the findings of Chiang and Sun, (1993).

Different types of GST enzymes present in an organism could be identified through their substrate preference. Of the four GST isozymes present in the DBM, GST-1, -2, and -3 recognize 1-chloro-2,4-dinitrobenzene (CDNB) and GST-3 and -4 recognize 1,2-dichloro-4-nitrobenzene (DCNB) (Ku *et al*., 1994; Chiang and Sun, 1993).

GST enzyme of an organophosphate insecticide resistant DBM, which showed high preference for DCNB substrate has been purified and characterized as GST-3 (Chiang and Sun, 1993). An increase in the activity (2 to 4 fold) of GST toward DCNB in comparison to CDNB has been reported (Takeda *et al*., 2006). This suggested that the elevated GST-3 or GST-4 activity proceeded with the parasitization and led to the detoxification of organophosphate insecticide diazinon. Thus, it could be possible that the gut bacterial strains in the host insect contribute to the detoxification processes of the host insects (Boush and Matsumura, 1967; Santos and Domingo *et al*., 2006). Saranya *et al*., (2021) documented the CDNB activity of bacterial endosymbionts isolated from rugose spiraling whitefly. *Stenotrophomonas* sp. PRGB08 was the only strain, which showed growth response in different insecticides including prothiofos tested. Hence, it may contribute to the resistance development in host insect species. Concurrent with this finding, the insecticide-resistant cotton bollworm was also reported for its gut bacterial community with *Stenotrophomonas* sp. (Xiang *et al*., 2007). *Stenotrophomonas* sp. PRGB08 was previously been reported as negative for chitinase, siderophore production in chrome-azural S (CAS) assay, and antagonistic activity against entomopathogenic fungi (Indiragandhi *et al*., 2007). Arunkumar *et al*., (2021) reported chitinase, protease, and siderophore production by the bacterial isolates from *Thrips palmi*.

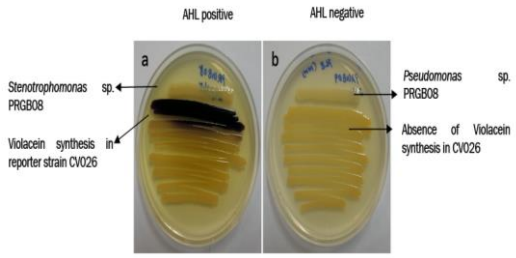

Figure 1. Agar plate assay for the AHL production by DBM gut bacterial isolates using the reporter strain CV026. Strain CV026 was streaked parallel to the DBM gut bacterial strains on LB agar plates. Diffusible AHLs produced by AHL-producing bacteria (a) Stenotrophomonas sp. PRGB08 induce the reporter (CV026) to produce violacein and (b) lack of violacein production in reporter strain indicates the absence of AHL production by Pseudomonas sp. PRGB06.

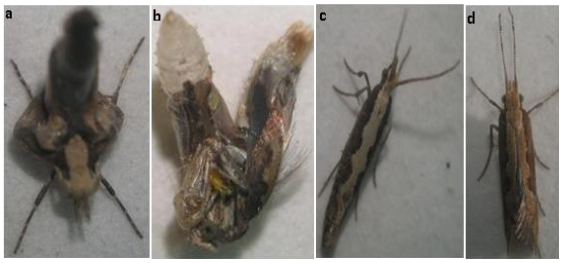

Figure 2. Effect of antibiotics treatments on host insect morphogenesis. Emergence of (a-b) malformed adult from antibiotic (0.01%) treated leaf fed larvae (c-d) successful DBM adults from untreated control.

Protease Activity

All the gut bacterial strains of DBM showed a substantial amount of protease activity (Table 1). Proteolytic activity of the host insect gut would decide the efficiency of the biopesticide, *Bacillus thuringiensis* (Mohan and Gujar, 2003b). Bacterial strains from the DBM gut which were able to produce protease may contribute to the digestion of toxic proteins ingested by the host insect. Reports suggested that the gut protease activity contributes to the development of resistance against Bt in the host insects, especially from Lepidoptera. Also, the growth and development of Bt were negatively influenced by the gut bacteria of host insects (Oppert *et al*., 1997; Takatsuka and Kunimi, 2000). Several workers also reported the diversity of bacterial strains and their related functional significance in the host insect (Dillon and Dillon, 2004). It seems that the gut bacteria from DBM may also do similar functions for its host insects either by modifying the host gut pH (Broderick *et al.*, 2004) or digesting the protoxin into toxin of low or high molecular weight protein, which would not harm the target insect (Oppert *et al*., 1997). Some of the DBM gut bacterial strains cross-feeds the siderophores, a growth determining, iron chelating, low molecular weight compounds produced by *Bt* (Indiragandhi *et al*., 2008).

QS Signal production by gut isolates

One more interesting finding with the DBM gut bacterial strains is the production of N- acyl homoserine lactone (AHL). AHLs are signal molecules mediating the quorum sensing (QS) mechanism in gram negative bacteria. QS regulates the gene expression that detects bacterial cell density, enabling bacteria to coordinate diverse biological functions (Pliego *et al*., 2007). Under the tested conditions, none of the DBM gut bacterial strains, except *Stenotrophomonas* sp. PRGB08 was able to induce biosynthesis of violacein in *C. violaceum* CV026 (Figure 1). Many previous findings are consistent with these results indicating the AHL production by insect gut bacterial strains may coordinate diverse biological functions in response to different stress conditions such as scarcity of nutrients, and elevated concentration of toxic substances (Pliego *et al*., 2007; Guan *et al*., 2007). AHL molecules produced by the gypsy moth gut bacterial strains have been reported as biologically active indole derivatives (Guan *et al*., 2007). DBM gut bacterial strains were reported for their indole production and antimicrobial activity (Indiragandhi *et al*., 2007, 2008). Signal mimics from uncultured bacteria associated with the midguts of gypsy moth larvae that induce QS have also been identified by a metagenomic analysis (Guan *et al*., 2007). Furthermore, the importance of QS signals in the alkaline environment of the lepidopteran midgut and its activity in the same in the microbial community of the CWB larval gut was reported by Bradeley *et al*., (2008).

Impact of gut bacterial elimination on host morphogenesis

Bacterial strains present in the insect gut environment help the host insects in different ways right from nutrition to protection (Dillon and Dillon, 2004; Indiragandhi *et al*., 2007, 2008). The exploitation of gut bacteria of insects may serve as novel approach to insect pest management. For example, great progress has been made in understanding and altering gut bacteria of different insect pest species (Costa *et al*., 1997; Gil *et al*., 2004; Aksoy and Rio, 2005; Koga *et al*., 2007). Hence, elimination of the gut bacterial strains would give fruitful results for resistance developing, devastating insect pest species like DBM. In the present investigation, except for adult emergence, there is no significant difference in larval and pupal mortality of third instar larvae with respect to different concentrations of antibiotics tested (Table 2). In the case of fourth instar larvae, the larval and pupal mortality and a number of successive adults emerged varied significantly according to the antibiotics concentrations. Statistical analysis showed that adults developed from third instar larvae fed with antibiotics at 0.001% fecund lesser than that of adults from control (Table 2). The trend of lower survival rates in DBM larvae of either instar indicates that the loss of gut bacterial strains due to antibiotic treatment might influence the development processes of host insects. Antibiotics concentration at 0.005% and 0.01% level significantly influenced the number of eggs laid by adult females (d.f = 2, 3; $p < 0.0001$; $r^2 = 0.99$). Adults developed from the fourth instar larvae showed significant difference among different treatments (d.f = 2, 3; $p < 0.0001$; $r^2 = 0.99$). The tested antibiotics were able to inhibit the protein synthesis in bacteria and damage the cytoplasmic

Table 1. Glutathione -S-transferase (GST) and proteolytic activity of DBM gut bacteria

DBM Population	DBM gut bacterial strains	GST specific activity (μmoL min^{-1}mg protein^{-1})		Protease activity (U mg protein^{-1})
		CDNB	DCNB	
Prothiofos - resistant population				
III instar	*Pseudomonas* sp. PRGB06	53.0±1.1	75.4±0.8	10.3±1.1
	Stenotrophomonas sp. PRGB08	140.0±2.1	102.3±0.8	10.4±1.2
	S. marcescens PRGB09	97.0±1.4	106.0±3.4	6.3±0.2
	Serratia sp. PRGB11	130.7±1.2	113.0±2.0	22.3±0.7
IV instar	*Acinetobacter* sp. PRGB15	100.0±1.4	96.2±0.6	6.0±0.1
	Acinetobacter sp. PRGB16	44.5±1.4	51.7±1.0	0.8±0.1
Prothiofos-susceptible population				
III instar	*Acinetobacter* sp. PSGB03	98.07±1.0	110.7±2.5	37.0 ± 1.4
	Acinetobacter sp. PSGB04	135.6±4.1	148.6±2.4	7.9 ± 1.3
	Acinetobacter sp. PSGB05	132.4±1.3	296.2±1.4	1.7 ± 0.3
IV instar	*Serratia* sp. PSGB07	78.6±1.3	84.6±1.6	55.5 ± 2.4
	Brachybacterium sp. PSGB10	-20.1±1.1	85.7±0.8	109.3 ± 6.8
	Serratia sp. PSGB12	11.0±0.7	92.9±2.0	51.4 ± 1.3
	Serratia sp. PSGB13	10.0±0.7	128.6±1.0	4.2 ± 0.3
	Serratia sp. PSGB18	1.4±0.2	19.6±0.8	7.6 ± 0.5
	Serratia marcescens PSGB15	34.6±1.2	109.2±2.8	29.1± 0.6
Field caught population				
III instar	*S. marcescens* FLGB02	76.76±1.50	102.75±1.8	57.54 ± 2.4
	S. marcescens FLGB11	3.74±0.65	121.97±1.2	49.50 ± 1.5
IV instar	*S. marcescens* FLGB16	4.68±0.68	65.06±1.3	13.72 ± 1.8
	S. marcescens FLGB17	0.90±0.15	13.57±0.8	21.30 ± 1.2
	S. marcescens FLGB20	-4.06±0.57	61.92±1.8	1.78 ± 0.1

Values are mean ± SE of three replications.

Table 2. Fecundity (eggs/female), adult emergence from the pupae developed from different concentration of antibiotic treated Chinese cabbage leaf fed third and fourth instars larvae of DBM

Treatments	III instar				IV instar			
	Dead Larva	Dead Pupae	Adult emergence	Fecundity*	Dead Larva	Dead Pupae	Adult emergence	Fecundity*
Control	4.0±0.5a	2.0±0.1b	3.3±0.2a	4.5±0.1a	2.4±0.4a	5.0±0.5a	2.1±0.1a	36.8±1.8a
0.001%	5.0±0.5a	3.3±0.4a	2.0±0.1b	4.0±0.2b	1.2±0.1d	1.8±0.2c	5.8±0.3b	36.2±1.2a
0.005%	5.0±0.3a	3.3±0.3a	1.3±0.1d	0.7±0.1c	1.6±0.2c	1.8±0.1c	5.0±0.3c	25.0±1.1 b
0.01%	4.0±0.1a	3.7±0.1a	1.7±0.1c	0.7±0.2c	2±0.02 b	3.2±0.1b	4.0±0.7d	21.8±1.2c
LSD(P≤0.05)	1.2	0.5	0.2	0.2	0.4	0.6	0.6	0.8

*Fecundity: Egg/Female value is square root of X+0.5 transformed values. The values indicate the mean ± SE of five replications. In the same column, significant differences according to the LSD at 0.05% levels are indicated by different letters.

membranes (Costa *et al*., 1997). Though the larvae were able to pupate, they could not emerge as successful adults (Figure 2) indicating that the elimination of gut bacteria certainly affected the pest population. Elimination of gut symbionts through antibiotics altered the host insect physiology and indirectly affected the oviposition of rugose spiralling whitefly, *Aleurodicus rugioperculatus* (Saranya *et al*., 2021). It could be explained that the chitinase enzymes produced by the gut bacterial strains are important for adult emergence by digesting the chitinous sheath in the pupa (Iverson *et al*., 1984). The gut bacterial strains of DBM were able to produce chitinase and accelerated the quantity of food taken by the host insects (Indiragandhi *et al*., 2007).

CONCLUSION

It has been demonstrated that the presence of potential detoxification enzyme GST, protease activity, and production of AHLs would decide the production of biologically active molecules by the bacterial strains in the gut of DBM and suggested that the gut bacterial strains might contribute to the host insect's detoxification processes. It seems that gut bacterial communities are essential for host insect processes. Loss of gut bacterial strains due to treatments negatively influenced the host insect's growth and development. The findings of the present investigation showed the importance of gut bacteria to the survival of host insects and potential avenues in the development of novel pest management strategies.

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Ethics statement

No specific permits were required for the described studies because no human or animal subjects were involved in this research.

Originality and plagiarism

It is ensured that the entire MS contains original works and others works are cited appropriately in the text. In addition, authors declared that there is no plagiarism in this content.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There are no conflict of interest in the publication of this content

Conflict of Interest

The authors P. Indiragandhi, R. Anandham and Tong Min Sa of the research article entitled Determination of detoxifying enzyme activity of gut isolates of diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae) and effect of antibiotics on host insect morphogenesis declared that they have no conflict of interest.

Data availability

All the data of this manuscript are included in the MS. No separate external data source is required. If anything is required from the MS, certainly, this will be extended by communicating with the corresponding author through corresponding official mail

Author contributions

Research grant-PI, Idea conceptualization-PI, Experiments- PI, RA; Guidance –Sa TM; Writing original draft - PI, Writing- reviewing & editing - PI, RA

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