Gut bacteria mediated insecticide resistance in cotton leafhopper *Amrasca biguttula biguttula*

G. Sivakumar[1,*], M. Mohan[1], K. Subaharan[1], T. Venkatesan[1], Sanjay Yelshetti[1], M. Kannan[2], R. Anandham[3], M. S. Yandigeri[1], Surabhi Kumari[1], K. Elango[4] and P. Ram Kumar[1]

[1]ICAR-National Bureau of Agricultural Insect Resources, Bengaluru 560 024, India
[2]Department of Nanoscience and Technology, Tamil Nadu Agricultural University, Coimbatore 641 003, India
[3]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641 003, India
[4]Department of Agricultural Entomology, Kumaraguru Institute of Agriculture, Erode 638 315, India

Cotton leafhopper, *Amrasca biguttula biguttula* (Ishida) (Hemiptera: Cicadellidae) is a major sucking insect pest of cotton in India. Indiscriminate use of pesticides has led to the development of resistance to most of the recommended pesticide groups. Though there are multiple mechanisms and principles of insecticide resistance development in insects, the gut bacterial-mediated degradation of insecticides is relatively less explored. In the present study, the gut bacteria of field-collected, insecticide-resistant population of *A. biguttula biguttula* were compared with a laboratory-reared susceptible population. Among the five culturable gut bacteria from the imidacloprid-resistant population, only *Enterococcus silesiacus* CLHG1a exhibited growth in the agar medium amended with 50 and 100 ppm of imidacloprid. The imidacloprid degrading capacity of *E. silesiacus* CLHG1a was further confirmed by HPLC analysis. *E. silesiacus* and *Bacillus amyloliquefaciens* CLHG2 showed higher esterolytic activity (0.348 and 0.309 μmoles/min/mg respectively). The esterase zymogram on native PAGE revealed a single major band. This study provides clear evidence that the bacterium *E. silesiacus* isolated from the gut of *A. biguttula biguttula* has the ability to degrade imidacloprid and may have played a role in the detoxification of pesticides.

Keywords: Cotton, detoxification, esterase activity, gut microflora, insecticide resistance, leafhopper.

LEAFHOPPER *Amrasca biguttula biguttula* is a major sucking insect pest of cotton in India causing significant yield loss[1]. In addition to the insecticides for bollworm control, cotton seeds of all commercial cultivars are invariably treated with either imidacloprid or acetamiprid followed by scheduled spray of insecticides, especially imidacloprid, acetamiprid and acephate throughout the season for leafhopper management[2,3]. Due to indiscriminate use of these chemicals over the years, the sucking pests, including the leafhopper, have developed resistance to insecticides through various biochemical and gut symbiont-mediated mechanisms[4–8].

There is an amazing diversity of symbiotic bacteria either as an endosymbiont in a specialized organ or as free living in the insect gut[9]. These microbes play various physiological roles, thereby imparting host-fitness attributes[10,11]. They enhance defence against natural enemies[12,13] and entomopathogens[14], aid in the development of reproductive traits[15], tolerance towards abiotic stress[16], preference in mating[15], nitrogen fixation[17], protection from heavy metals[18] and dietary components[19,20].

Recent studies have characterized the gut microflora for their possible role in metabolizing insecticides, thereby imparting survival fitness to the host insect[21–23]. In an insecticide-resistant insect species, the insecticides are usually metabolized by the metabolic system of the insect or in combination with the associated endosymbionts[24,25].

It is well known that *Bemisia tabaci* (Hemiptera; Aleyrodidae) and *A. biguttula biguttula* harbour numerous endosymbionts and gut microflora. The species of some bacterial genera such as *Serratia*, *Bacillus*, *Enterococcus*, *Enterobacter*, *Pantoea*, *Methylobacterium*, *Stenotrophomonas*, *Pseudomonas* and *Paenibacillus* were found to inhabit the gut and other organelles of *A. biguttula biguttula*[22,26,27].

The goal of this work was to isolate and characterize the gut bacteria of *A. biguttula biguttula* using molecular methods. Furthermore, the ability of the isolated gut bacteria to degrade imidacloprid has been demonstrated.

Material and methods

Collection and culturing of leafhoppers

The nymphs and adults of *A. biguttula biguttula* were collected with the help of an aspirator/sweep net from cotton crop in Guntur district (16.18°N and 80.26°E), Andhra Pradesh, India, where excessive spraying of insecticides on cotton crop is being practised. The collected insects were mass-reared under net-house conditions (5 × 10 m) at the ICAR-National Bureau of Agricultural Insect

*For correspondence. (e-mail: sivakumarg.nbaii@gmail.com)

Resources (NBAIR) farm campus, Bengaluru (13°06′N, 77°33′E) on caged cotton plants. The laboratory-reared iso-female-derived *A. biguttula biguttula* (NBAIR-GR-CIC-01) population maintained at ICAR-NBAIR served as an insecticide susceptible control.

Leaf-dip bioassay for determining insecticide resistance in the Guntur population

The commercial formulation of imidacloprid (trade name: confidor, 17.8% SL) was serially diluted with water to obtain the desired concentrations (10–100 ppm), according to the preliminary dose-bracketing bioassays. Leaf-dip bioassays were conducted according to the standard Insecticide Resistance Action Committee (IRAC) (Method No. 8). Plastic insect-breeding dishes with ventilated lids (HiMedia, India) were used in the bioassays.

Cotton leaves with a petiole were selected and washed, the surface was decontaminated with sodium hypochlorite (0.1%) for 5 min, then rinsed 3–4 times with distilled water and dried in the shade.

Then the leaves were dipped in appropriate concentrations of imidacloprid (10 sec) and shade-dried. The petioles were wrapped with wet cotton and placed in a plastic container (3 cm diameter × 3 cm height). In each container ten second nymphal stages of *A. biguttula biguttula* were released. Leaves dipped in sterile distilled water for 10 sec served as control. The experiment was replicated four times and insect mortality was observed at 48 and 72 h after the exposure to insecticide, as reported by Saranya *et al.*[27]. The assays were repeated twice on different days. Corrected per cent mortality was calculated using Abbot's formula. Pooled mortality data were analysed using POLO PLUS software to determine LC_{50}, slope and fiducial limits[28].

Isolation and characterization of culturable gut bacteria

Field-collected second nymphal stages of *A. biguttula biguttula* were used for the isolation of endosymbionts. Bacteria were isolated based on the standard protocol described by Feng *et al.*[29]. The surface-sterilized nymphs were macerated with 100 mM sodium phosphate buffer (pH 7.2) under aseptic conditions with the help of a sterile mini pestle and mortar. Homogenates were centrifuged and the supernatant was plated onto nutrient agar (NA) media (HiMedia, India). The petri plates were incubated at 30°C for 48 h and observed at every 24 h interval for bacterial growth. The number of colony-forming units was observed and counted. The colonies were differentiated based on their size, colour, and morphology, and a single representative isolate of each morphotype was transferred to a new plate. After 5–6 successive streakings, the pure culture was obtained. Further, the culture purity was ascertained by examination under a light microscope and the purified bacterial isolates were maintained in 50% glycerol at –80°C till further use. Morphological characters of these bacterial isolates were determined according to the procedure described by Harley and Prescott[30].

Molecular characterization

DNA extraction of all the five bacterial isolates was carried out with HiPurA™ Bacterial and Yeast Genomic DNA Purification Spin Kit (HiMedia, India). Isolated genomic DNA was amplified using the forward primer pA-5′AGAGTTTGATCCTGGCTCAG3′ and reverse primer pH-5′AAGGAGGTGATCCAGCCGCA3′. Sequencing of PCR products was done directly with the *Taq*-mediated dideoxy chain terminator cycle sequencing in ABI 3130xl automated genetic analyser (Applied Biosystem, UK), according to the manufacturer's instructions. Forward and reverse sequences were used to develop contiguous sequences employing the on-line CAP3 program. Homology search of 16S rDNA sequences was done based on contiguous sequences using the sequences deposited in public databases (GenBank) and employing BlastN. The identity was based on percentage similarity with the NCBI public database sequences using BLAST homology. The sequences obtained were submitted to the NCBI database and the respective accession numbers were obtained.

Screening of the isolates against imidacloprid degradation

All the bacterial isolates were screened using poisoned food technique to test their ability to degrade the imidacloprid molecule. Bacterial isolates were streaked on nutrient agar medium amended with various concentrations of imidacloprid (25, 50 and 100 ppm). The bacterium *Enterococcus silesiacus* CLHG1a which was able to grow on the poisoned nutrient agar was selected for further degradation studies.

HPLC analysis of imidacloprid degradation

Imidacloprid degradation assay was conducted in a minimal medium in which 25, 50 and 100 ppm of imidacloprid was added as the sole carbon source. Next, 1 ml seed culture of *E. silesiacus* CLHG1a (1×10^8 cfu ml^{-1}) was inoculated and incubated in a incubator shaker at 120 rpm at 37°C for 48 h. Minimal medium either devoid of inoculum or imidacloprid was used as control. Growth of bacterial cells was measured by recording the absorbance at 660 nm using a spectrophotometer (Hitachi U-2910, Japan). Growth rate was measured by the slope of the line representing the linear fit of the increase in absorbance over time, during the exponential phase of bacterial growth.

The concentration of imidacloprid in each treatment was determined using HPLC analysis. For this, 500 µl of sample was taken in 2 ml of microcentrifuge tube and mixed with 500 µl of HPLC-grade acetonitrile (SD Fine Chemicals, Mumbai, India) and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to sterile, small, amber HPLC vials and stored in a refrigerator. Next, 10 ml of each sample was injected into the HPLC system equipped with a diode array detector and Chem Station software for data acquisition and processing. Reverse-phase C18 analytical column (150 × 4.6 mm ID, 5 µm particle size) was used to separate the sample. The column operation at 27°C was isocratical at a steady flow rate of 1 ml min^{-1} using 60 : 40 (v/v) Milli-Q water/acetonitrile. A UV–VIS detector operating at 254 nm was also used. Identification of imidacloprid degradation was done based on retention time and compared with pure imidacloprid (Sigma Aldrich Laborchemikalien GmbH). The quantification of imidacloprid was performed using linear regression analysis.

Detection and quantification of esterase activity

E. silisiacus CLHG1a and other endosymbiotic bacteria were cultured in liquid nutrient medium at 37°C and 200 rpm for 24 h for isolation of intracellular esterase enzyme. The bacterial cell pellet was obtained through centrifugation for 10 min at 8000 rpm and suspended in phosphate buffer (100 mM; pH 7.4), and further subjected to sonication. The supernatant collected after sonication was concentrated and used as an intracellular enzyme source. The activity of esterase was determined using α-naphthyl acetate as a substrate, according to the method of Meghji *et al.*[31]. Enzyme extract (10 µl) was added in the 96-well microplate and initiation of the reaction was done by addition of 200 µl of 0.3 mM α-naphthyl acetate (substrate) to the wells. The microplate was covered with parafilm and kept under incubation in the dark at 30°C for 30 min. Then 50 µl of staining solution (two parts of 1% fast blue solution in five parts of 5% SDS) was added to stop the reaction and incubated for 20 min at room temperature to develop colour. The absorbance was read at 595 nm. The α-naphthol formed after the reaction was quantified using α-naphthol standard curve. The esterase enzyme activity was expressed as µmol of α-naphthol formed/min/mg of protein.

The enzyme extracts were run on native PAGE (polyacrylamide gel electrophoresis) with 8.0% acrylamide concentration at 4°C (BioRad Mini Protean), until the dye front reached the bottom of the gel. The gel was initially pre-incubated in 100 ml of phosphate buffer (40 mM, pH 6.5) containing 0.02% α-napthyl acetate. The solution was prepared using 1% α-napthyl acetate (20 mg of α-napthyl acetate in 2 ml of acetone) and 100 ml of sodium phosphate buffer (40 mM, pH 6.5). After pre-incubation, the gel was transferred to 100 ml phosphate buffer (40 mM, pH 6.5) containing 0.02% α-napthyl acetate and 0.1% Fast Blue BB salt. The gel was incubated in the dark at normal room temperature with occasional mild shaking until dark green–black-coloured bands appeared[32,33].

Results and discussion

The bioassay data indicated that the *A. biguttula biguttula* population collected from Guntur was 6.6–7.0-fold more resistant to imidacloprid compared to the susceptible laboratory population (Table 1). High level of insecticide resistance was developed in leafhopper against several synthetic insecticides, especially to the neonicotinoid group of insecticides, including imidacloprid, thiamethoxam and acetamiprid, due to their continuous and indiscriminate application[34].

In the present study five culturable gut bacteria associated with the imidacloprid-resistant population of leafhopper were identified by both morphological and molecular methods (Table 2). Full-length sequences of 16S rRNA genes were subjected to homology searches with the NCBI sequence database. The 16S rRNA gene sequencing results revealed that the leafhopper harboured with *Bacillus amyloliquefaciens*, *Staphylococcus pasteuri*, *Enterococcus silesiacus*, *Enterobacter asburiae* and *Bacillus pumilus* (Table 2). These microflorae are more commonly housed in many insect species[22,23,25,35,36]. Primary endosymbionts are maternally inherited and generally located in specialized cell structures called bacteriocytes within the insects. On the contrary, secondary endosymbionts could reside in various host tissues, including hemolymph, gut, malphigian tubules, salivary glands and ovaries, and influence various biological and ecological characteristics[37,38]. The bacteria isolated in this study probably belong to secondary endosymbionts and with the use of selective media, recurrent isolation of these bacteria can be achieved.

Among the microflora screened, only *E. silesiacus* CLHG1a grown in the minimal medium amended up to 100 ppm of imidacloprid and hence was taken for further degradation study. *E. silesiacus* grew in a minimal carbon-deficient medium, supplemented with imidacloprid as a carbon source, and showed an increase in growth rate (OD value of 0.81 ± 0.01) (Figure 1).

Bacterial species belonging to the genus *Enterococcus* are commonly found in both lepidopteran and hemipteran insects, and perform a relatively wide range of conserved functions, including the degradation of pesticides[11,23,39,40]. *Enterococcus termitis* SL-2, *E. rotai* SL-4, *Enterococcus moraviensis* SL-5 and *Enterococcus* sp. SL-10 were isolated and characterized from the larval gut of *S. litura*[41–43]. *Enterococcus* sp. isolated from the gut of *Plutella xylostella* enhanced resistance to a wide range of insecticides, including chlorpyriphos[23].

Table 1. Susceptibility status of *Amrasca biguttula biguttula* to imidacloprid

Duration (h)	Imidacloprid 17.8 SL	LC_{50} (ppm)	Slope ± SE	Fiducial limits		χ^2 value (3DF)
				Lower	Upper	
48	Bengaluru (control)	1.06	0.85 ± 0.14	0.59	1.87	14.4
	Guntur	6.61	0.85 ± 0.21	3.467	24.365	4.7
72	Bengaluru (control)	0.41	0.79 ± 0.13	0.198	0.723	15.2
	Guntur	2.91	0.82 ± 0.19	1.601	6.736	7.19

Table 2. Identification of gut bacteria associated with *A. biguttula biguttula* through 16S rRNA sequencing

Geographical location	Population(s) collected (Insecticide usage pattern)	Number of rounds of spray	Isolate code	Identified organism	Identity match (%)	GenBank accession no.
Guntur 16.18°N, 80.26°E	3 (Ap + Dm; Ap + Imi; Ap + Am)*	5	CLHG1	*Staphylococcus pasteuri*	100	KC425474
			CLHG1a	*Enterococcus silesiacus*	99	KC427093
			CLHG2	*Bacillus amyloliquefaciens*	100	KC428704
			YCLHG2	*Enterobacter asburiae*	97	KC603555
			CLHA	*Bacillus pumilus*	99	KF958277

*Ap, Acephate; Am, Acetamiprid; Imi, Imidacloprid, Dm, Dimethoate.

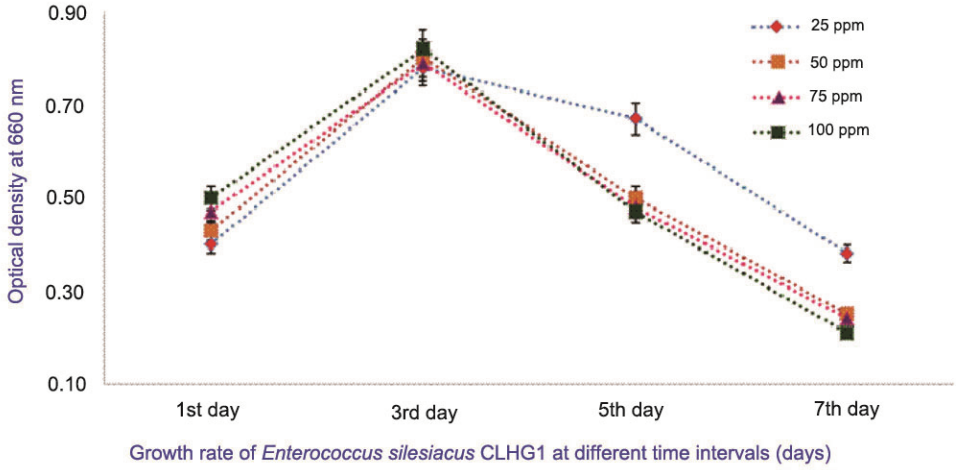

Figure 1. Effect of imidacloprid on growth rate of *Enterococcus silesiacus* CLHG1a.

HPLC analysis for imidacloprid degradation by *E. silesiacus* CLHG1a revealed a peak area of 169.896 at retention time of 3.098 min, corresponding to 8.4% utilization of imidacloprid as the sole carbon source (Figure 2). The oxidative and nitro-reduced metabolism of imidacloprid was analysed in *Drosophila melanogaster*. A study of the axenic maggots of *D. melanogaster* indicated that the endosymbiotic bacteria were responsible for the production of the nitro-reduced metabolites of imidacloprid[24,44].

Bacteria *Bacillus* sp., *Brevibacterium* sp., *Pseudomonas putida*, *Bacillus subtilis* and *Rhizobium* sp., isolated from the soil, were reportedly involved in the degradation of imidacloprid[45–48]. The degradation of imidacloprid by a gut-associated microflora, *Lysinibacillus macroides* SPL-1 was previously demonstrated through *in vitro* degradation assays[43,49–51]. In the present study, *E. silesiacus* CLHG1a probably hydrolysed imidacloprid to dechlorinated 6-chloronicotinic acid and then 6-hydroxynicotinic acid, which was then metabolized by the nicotinic acid pathway. However, LC-MS analysis is yet to establish this.

The esterolytic activities of the four endosymbiotic bacteria ranged from 0.132 to 0.348 µmol/min/mg of protein (Table 3). *E. silesiacus* exhibited the highest activity (0.348 µmol/min/mg of protein), followed by *Bacillus amyloliquefaciens* (0.309 µmol/min/mg of protein). The esterase activity was visualized on native PAGE as a single band in all the bacterial samples analysed (Figure 3). Three groups of bacterial enzymes have been reported to degrade organophosphate compounds, namely phosphotriesterases (EC 3.1.8.1), methyl parathion hydrolases (MPH, E.C.3.1.8.1) and organophosphorous acid anhydrolases (EC 3.1.8.2)[52–54]. Degradation of insecticides by extracellular esterases produced by bacterial species has

been reported earlier. The extracellular esterase B1 of marine *Bacillus subtilis* strain C5 was involved in the degradation of methyl parathion[55]. Bacteria that can produce parathion-methyl hydrolase include *Acinetobacter*, *Pseudomonas*, *Burkholderia*, *Arthrobacter* and *Ochrobactrum*[55–58]. In the present study, extracellular esterase activity was detected in *E. silesiacus* CLHG1a which could have aided in the partial hydrolysis of imidacloprid.

Table 3. Carboxylesterase activity of culturable gut bacteria

Bacteria	Specific activity (μmol/mg protein)
E. silesiacus CLHG1a	$0.348^{a} \pm 0.002$
B. amyloliquefaciens CLHG2	$0.309^{b} \pm 0.006$
E. asburiae YCLHG2	$0.204^{c} \pm 0.010$
S. pasteuri CLHG1	$0.123^{d} \pm 0.003$
B. pumilus CLHA	$0.112^{e} \pm 0.001$
Standard error of difference	0.003
Critical difference (0.05)	0.008

Values are mean of three replications ± error. Means in column with the same letter are not significantly different at 0.05 level.

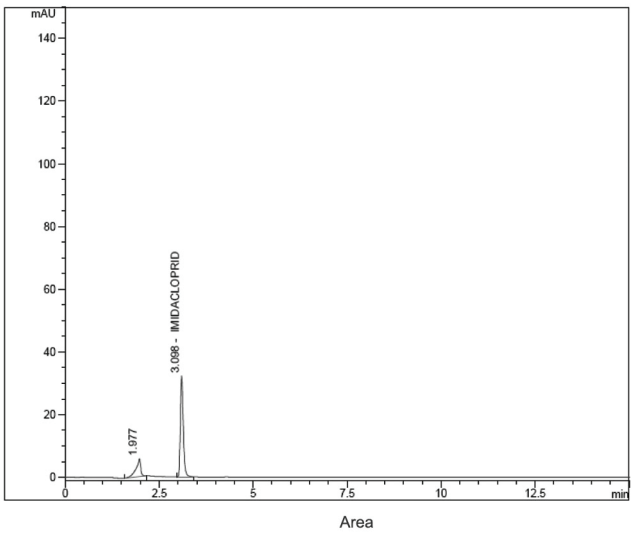

Figure 2. HPLC analysis for imidacloprid degradation by *E. silesiacus* CLHG1a.

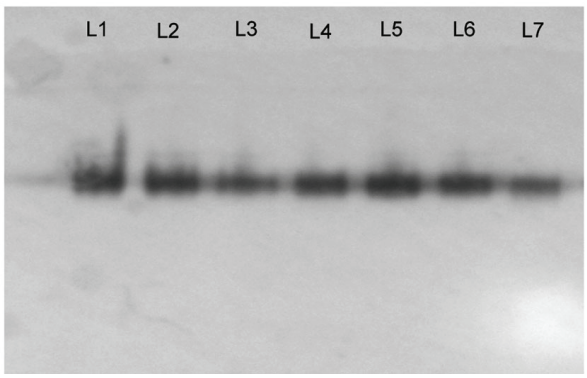

Figure 3. Native PAGE for esterase bands from gut bacteria. Lane 1, Positive control; lane 2, *E. silesiacus*; lane 3, *Bacillus pumilus*; lane 4, *Enterobacter asburiae*; lane 5, *Staphylococcus pasteuri*; lane 6, *Enterobacter hormaechei*; lane 7, *Bacillus amyloliquefaciens*.

In this study, imidacloprid was partially hydrolysed by *E. silesiacus* CLHG1a extracellular esterases in order to adapt and live in the host insect system. *Hymenobacter latericoloratus* CGMCC 16346, an oligotrophic bacterium, was identified from a water environment by Guo *et al.*[59]. This bacterium can survive in apotrophic surface water for a long period on a 1/10,000 diluted nutrition media and breakdown imidacloprid. Detoxifying enzymes, including glutathione sulphur transferase, esterase and P450s, can catabolize pesticides and toxins in insects[60]. The gut bacteria linked with the host contribute to the detoxifying enzymes in the insects[61]. Krishnamoorthy *et al.*[61] found that *Bacillus* sp. in papaya mealybug might have a role in the detoxification of profenophos and chlorpyrifos OP compounds that were used in the management of the mealybug complex. Recently, Saranya *et al.*[62] documented glutathione sulphur transferase in the gut bacteria isolated from a field-caught population of rugosa spiralling whitefly.

Conclusion

In summary, five gut bacteria were isolated from a pesticide-resistant field population of *A. biguttula biguttula* and characterized using 16S rDNA sequencing techniques. Further, it was discovered that the degradation of imidacloprid by *E. silesiacus* is possible by the esterase enzyme. The results of the present study have clearly shown that the interruption of *E. silesiacus* mediated imidacloprid resistance in *A. biguttula biguttula* will pave the way to develop a sustainable pest management strategy.

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