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Identification and Characterization of Gut Associated Bacteria in *Epilachna vigintioctopunctata* Fab. (Coleoptera : Coccinellidae)

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Abstract

Epilachna vigintioctopunctata Fab. is the most important agricultural crop pest causing destruction to many cultivated crops of the Solanaceae and Cucurbitaceae families, wild crops and medicinal plants. Microorganisms that inhabit the intestinal tract of the host pest play important roles in its growth, development and adaptation. The diversity of the gut microbiota revealed 10 bacteria based on their morphological, biochemical, physiological and molecular characteristics. Out of ten isolates, three bacteria were sent for 16S rRNA partial gene sequencing and revealed the presence of *Enterobacter bugandensis* (LSBU1EV1), *Stenotrophomonas sp.* (LSBU2EV1) and *Achromobacter xylosoxidans* (LSBU4EV1).

***Key words*:** *Epilachna vigintioctopunctata*, Gut associated bacteria, *Enterobacter bugandensis, Stenotrophomonas sp., Achromobactker xylosoxidans*

Insects are the most successful group of animals not only because of their rapid rate of reproduction, short life span and ability to develop resistance to conventional insecticides [1] but also because of the presence of bacteria which share a symbiotic relationship with the insects affecting its nutrition, development, reproduction, immunity, defense against natural enemies and speciation thereby playing a crucial role in its adaptability [2-5]. Insects are economically important organisms, some being beneficial to human being and used in the production of food, silk, medicines and so on. They also serve as pollinators, while some are pests causing harm to human beings leading to damage of crops, timber, forests etc. and also cause health hazards to human beings as well as live stocks [6].

One such insect pest is the spotted leaf beetle or Hadda beetle, *Epilachna vigintioctopunctata* Fab. (Kingdom: Animalia, Phylum: Arthropoda, Class: Insecta, Order: Coleoptera, Family: Coccinillidae) which is the most important agricultural crop pest causing destruction to many cultivated crops (*Solonum melongena*, *Solonum lycopersicum*, *Solonum tuberosum*, *Nicotiana tabacum*, *Cucumis melo*, *Cucumis sativus* and Cucurbits), wild crops [7-8] and some medicinal plants (*Datura innoxia* Mill., *D. stromonium*, *Solonum nigrum* L., *Physalis minima*, *Withania sominfera* and *Amaranthus caudatus* L [9-12]. It causes considerable damage in the fields because both, the grubs as well as adults feed on the epidermal tissue of the leaves by scrapping on the leaf surfaces resulting in drying and eventually shedding of the leaves. This in turn, leads to retarded plant growth and reduction of fruit yield upto 60% [13].

Although evidence has been presented that the gut of adult *Epilachna vigintioctopunctata* contain *Bacillus* species that were able to produce enzymes which play a role in digestion and survival of the insect [14], yet an extensive study has to be carried out to obtain deeper knowledge of the bacterial communities in *E. vigintioctopunctata* and their associations in order to develop novel approaches to control insect pests. The identification and the study of gut microbiota, its interactions among members of the gut community can be useful in the development of insect control strategies [15]. *Solanum melongena* Linnaeus, is one among the major vegetable crop grown in subtropical and tropical countries [16] including India which holds the second rank in total world production of brinjal [17-18] having high nutritive values such as phosphorus, iron and vitamins especially the B complex [19]. Therefore, an attempt was made to study the diversity of the gut microbiota of larvae of *E. vigintioctopunctata* isolated from its host plant *Solanum melongena* Linnaeus based on their morphological, biochemical, physiological and molecular characteristics.

MATERIALS AND METHODS

The larvae of *E. vigintioctopunctata* were collected from NBAIR Research Farm (Attur Layout, Yelahanka New Town, Bangalore - 560 064, Karnataka) (13.1042 ° N,77.5713 °E) in the month of July 2018. Fourth instar larvae of *E. vigintioctopunctata* were used for isolation and dissection.

The collected larvae were starved overnight, so that gut remains clear of any food particle. The insect was immobilized by chloroform, sterilized in 0.1% sodium hypochlorite and 70% aqueous ethanol for 5s to remove the adhering contaminants [20]. The insect was mounted on the wax plate with water. The head capsule was properly fixed with help of a sharp pin and similarly the last segment also pinned. Care was taken not to

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damage the digestive system. After the complete incision, the entire gut was taken onto a watch glass with 10mM phosphate buffer and minced with the help of sterile micro pestle [21].

The stock solution was prepared by taking 1 mL of the suspension and was mixed with 9.0 mL saline. Using serial dilution method, seven dilutions were prepared. 1mL of each dilution was added to separate plates. The serially diluted contents were plated on different nutrient media- Nutrient agar, Luria-Bertani medium, Potato dextrose agar and Yeast peptone dextrose agar (HiMedia Laboratories Pvt. Ltd.). The plates were incubated for 24 to 48 h at 37°C. PDA plates were kept for 5 to 7 days. Bacterial colonies were selected based on difference in colony morphology and selected colonies were sub cultured on Tryptic Soy Agar for further use. Bacterial cultures were also preserved in glycerol stock at -20°C.

Morphological and biochemical characterization

The bacterial isolates were identified using morphological and biochemical techniques. Morphological tests were done based on the colony characters involving colony size, shape, colour, elevation and margin of the isolates colonies. The physiological-biochemical characteristics were determined on the basis of Gram stain, IMViC test – Indole test, methyl red test, Voges-Proskauer test, citrate utilization test. Glucose fermentation, catalase activity, lipase and cellulolytic tests were also conducted [22].

Molecular characterization

DNA extraction

DNA isolation was done for both gram positive and gram-negative bacteria according to manufacturer's protocol (HiPurA^{TM} Bacterial Genomics DNA Purification Kit). In case of gram-negative bacteria, the bacterial cultures were grown overnight in Luria-Bertani medium. From this 1.5mL of bacterial sample was taken and centrifuged for 1 to 2 min at 13,000 rpm and supernatant was discarded. Pellet was treated with lysis solution as well as proteinase K and incubated in water bath at 55°C for 30 min. To this 20µl of RNase solution was added, mixed well and incubated at room temperature for 5 min.

For gram positive bacteria the pellets were treated with lysozyme solution and incubated for 30 min at 37°C. To this 20µL of proteinase and RNase was added and mixed thoroughly, samples were incubated for 5 min. After incubation both gram positive as well as negative cultures were processed together. Lysis solution was added, vortexed thoroughly for 15 s and incubated at 55°C for 10 min, followed by ethanol treatment. The lysate obtained was added to HiElute Miniprep Spin Column provided with kit and centrifuged at 10,000 rpm for 1 min at room temperature. The flow through liquid was discarded and column was kept in same collection tube. To this pre-wash solution was added and centrifuged again, flow through liquid was discarded. The column was transferred to new Eppendorf tube. The DNA was eluted with elution buffer and kept in the refrigerator for further use.

Polymerase chain reaction (PCR) study

For identification of bacterial isolates universal primer fd1 (5'-GAGTTTGATCCTGGCTCA) and rp2 (5'-ACGGCTACCTTGTTACGACTT) were used for 16S rRNA PCR [23]. The PCR reaction conditions consisted of an initial denaturation step at 94°C for 3 min followed by a 35 cycle reaction which included denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 2 min and a final extension cycle at 72°C for 10 min. The generated sequences were compared with sequences available in GenBank by using the BLASTn program (http://www.ncbi.nih.gov) [24]. The sequences were submitted to GenBank and accession numbers were obtained.

Agarose gel electrophoresis

The PCR amplified DNA products were examined using gel electrophoresis on a 1% (w/v) agarose gel in 1 X TAE buffer, stained with Ethidium bromide, visualized under U.V. light in gel documentation system (DNR minilumi, Biotron) and photographed according to the procedure by Sambrook and Russell [25]. A 1kb DNA ladder (Sigma-Aldrich) was used as a marker.

Phylogenetic study

The culture sequences obtained were subjected to BLAST analysis and were compared with sequences available in GenBank by using the BLASTn program (http://www.ncbi.nih.gov). The phylogenetically similar type strain sequences and other phylogenetically related sequences were selected from the GeneBank, subjected to multiple sequence alignment, trimmed to similar length and a phylogenetic tree (neighbour joining) was constructed using Molecular Evolutionary Genetics Analysis version 6 (MEGA 6) with 1000 replicates of bootstrap values. In the tree the numbers at the nodes indicate the levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support].

RESULTS AND DISCUSSION

Identification of the isolates

Using the isolation procedure, a total of 10 dominant isolates were successfully isolated from the gut of the fourth instar larva of *E. vigintioctopunctata* and were morphologically classified based on colony shape, colour and size, margin, elevation and pigmentation (Table 1). Among the 10 bacteria isolated from *E. vigintioctopunctata* larva, 7 isolates were gram positive while three were gram-negative rod shaped, catalase positive bacteria (Table 2-3) and were further subjected to biochemical characterization (Table 2).

Table 1 Morphological characteristics of dominant bacteria in the gut of the larva of *E. vigintioctopunctata*

Isolate	Colony shape	Margin	Elevation	Pigmentation	Colour	No. of colonies	Size of colonies
LSBU1EV1	Circular	Entire	Raised	Translucent	Cream	>300	~1 mm
LSBU2EV1	Irregular	Lobate	Flat	Opaque	Yellowish white	8	4 cm
LSBU4EV1	Circular	Entire	Raised	Translucent	Cream	>200	1.3 cm

Genbank accession numbers of bacterial isolates

The three dominant gram-negative bacteria were identified through 16S rRNA sequences with the available bacterial sequences (closest representatives) in the public database (GenBank, NCBI). The nucleotide sequences of the collected bacterial strains were subjected to homology searches in DNA databases, which revealed that the sequences of *E. bugandensis*, *Stenotrophomonas sp.* and *Achromobacter xylosoxidans* showed 100% similarity with the 16S rRNA gene sequences of the respective identified organism.

Table 2 Biochemical characteristics of dominant bacteria in the gut of the larva of *E. vigintioctopunctata*

Isolate	Gram staining		Biochemical tests [IMViC]			
	Gram reaction	Bacteria shape	Indole test	Methyl red	Voges Proskaeur's	Citrate utilization
LSBU1EV1	-	Rod	-	-	+	+
LSBU2EV1	-	Rod	-	+	-	+
LSBU4EV1	-	Rod	-	-	-	+

Table 3 Activity of enzymes of dominant bacteria in the gut of the larva of *E. vigintioctopunctata*

Isolate	Activity of enzymes			
	Catalase	Glucose fermentation	Lipase	Cellulase
LSBU1EV1	+	+	+	-
LSBU2EV1	+	-	+	+
LSBU4EV1	+	-	+	+

Table 4 Accession numbers obtained from GenBank for the dominant bacteria isolated from the larva of *E. vigintioctopunctata*

Isolate	Accession	E value	Identity	Bacteria determined from blast
LSBU1EV1	MN394114	0.0	100.00%	*Enterobacter bugandensis*
LSBU2EV1	MT417182	0.0	100.00%	*Stenotrophomonas sp.*
LSBU4EV1	MN394127	0.0	100.00%	*Achromobacter xylosoxidans*

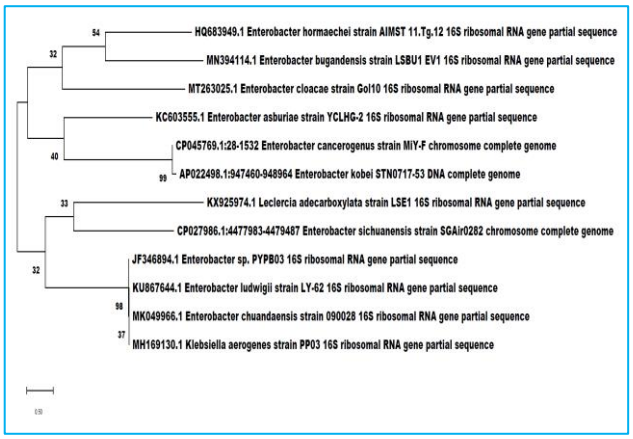

Fig 1 Phylogenetic tree using Neighbour-Joining method from sequences deposited in GenBank showing closely related species of *Enterobacter bugandensis*

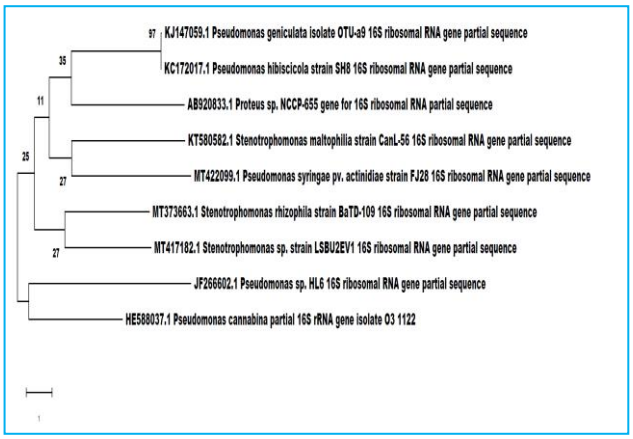

Fig 2 Phylogenetic tree using Neighbour-Joining method from sequences deposited in GenBank showing closely related species of *Stenotrophomonas sp.*

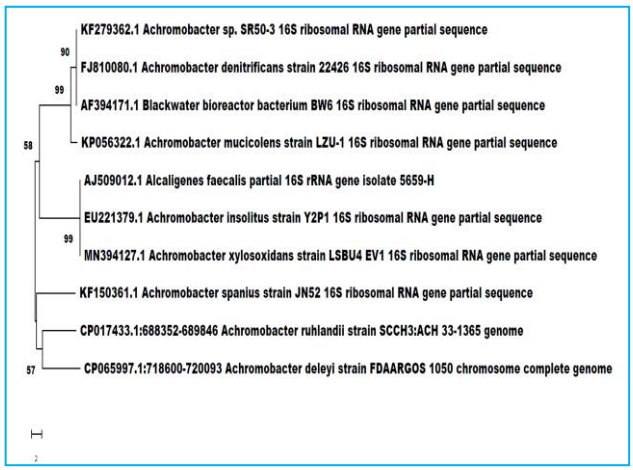

Fig 3 Phylogenetic tree using Neighbour-Joining method from sequences deposited in GenBank showing closely related species of *Achromobacter xylosoxidans*

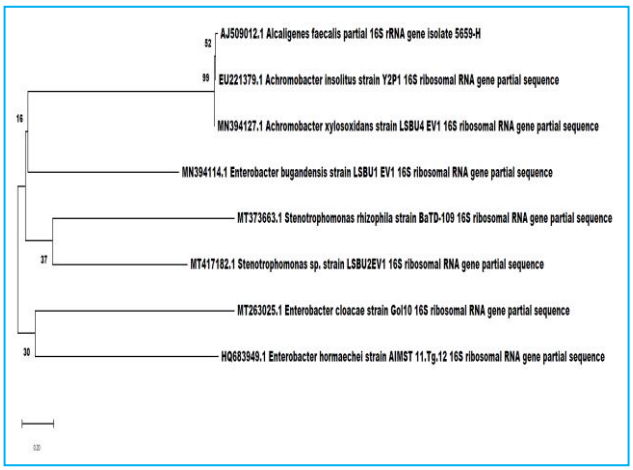

Fig 4 Phylogenetic tree using Neighbour-Joining method showing the relationship of the three bacterial isolates – *Enterobacter bugandensis, Stenotrophomonas sp.* and *Achromobacter xylosoxidans*

The GenBank accession numbers for the partial sequence of the 16S rRNA gene for the isolates LSBU1EV1 (*Enterobacter bugandensis*), LSBU2EV1 (*Stenotrophomonas sp.)* and LSBU4EV1 (*Achromobacter xylosoxidans)* were MN394114, MT417182 and MN394127 respectively (Table 4).

Molecular phylogenetic analysis of bacterial isolates

The Molecular phylogenetic analysis was done for each isolate using Neighbour-Joining method from sequences deposited in GenBank which showed the evolutionary relationships of the respective bacteria. (Fig 1) showed that *Enterobacter bugandensis* was closely related to *Enterobacter hormaechei* and *Enterobacter cloacae* while *Stenotrophomonas sp.* was closely related to *Stenotrophomonas rhizophila* (Fig 2).

Similarly, *Achromobacter xylosoxidans*, *Alcaligenes faecalis* and *Achromobacter insolitus* were closely related (Fig 3). From the analysis done for each isolate, a comprehensive phylogenetic tree was then constructed concluding that *Enterobacter bugandensis* showed a close relationship with *Stenotrophomonas sp.* and hence both these bacteria shared a common ancestor as *Achromobacter xylosoxidans* (Fig 4). This clearly determines that the three isolates are inter related. The numbers at branch points of the tree designate the bootstrap values.

Gut microflora is shown to play a major role on the growth and development of insects as well as their resistance to major insecticides [26]. Insect gut is anaerobic in nature therefore bacteria survive by the production of catalase which generates oxygen in order to use it for respiration, hence bacteria can either be pure aerobes or facultative anaerobes [27]. In the present study all the three isolates, namely *Enterobacter bugandensis*, *Stenotrophomonas sp.* and *Achromobacter xylosoxidas* were catalase positive interpreting that these bacteria are either aerobic or facultative anaerobic in nature.

Cellulose is one of the most important polysaccharides and forms the major component of plant cell walls. Although previous studies from the gut of adult *E. vigintioctopunctata* revealed the absence of cellulose activity by the bacteria- *B. anthracis*, *B. vietnamensis* and *B. subtilis* [28], the present study from the gut of larva of *E. vigintioctopunctata* showed very little cellulose activity by *Stenotrophomonas sp.* and *Achromobacter xylosoxidas*. This is because bacteria with CM Case activity were abundant in the hindgut but seldom in the midgut of *H. parallela* [29]. Similar results were also seen in another scarab beetle, *P. marginata* wherein a large number of bacteria showing CM Case and xylanase activities were present in the hindgut but not detected in the midgut indicating that the bacteria in the scarab midgut does not show cellulolytic activity and hence has a predigestive function for lignocellulose rather than microbial degradation of cellulose and hemicellulose [30]. The *Stenotrophomonas* genus comprises of a wide range of species, one such *S. maltophilia* strain from the mesophilic microbial community has also been reported to be cellulolytic [31-32].

A previous study on the fourth instar larva of *E. vigintioctopunctata* on two host plants namely *Solanum melongena* and *Solanum nigrum* showed the bacterial communities were considerably influenced by the host plants hence, the main factors that influence the formation of insect gut bacterial communities are life stage, diet, and environmental factors. It was also found that Proteobacteria and *Enterobacteriaceae* were the predominant phylum and family, respectively [33]. Larva of *E. vigintioctopunctata* depends on the leaves of *S. melongena* for their food which contains some amount of sugars (glusose, sucrose, lactose and mannitol) and is very low in fat and calories. Therefore, in this current study it was found that *E. bugandensis* is capable of fermenting glucose, sucrose, lactose and mannitol while both *A. xylosoxidans* and *Strenotrophomonas sp.* are non-fermentative. Similarly, both *Enterobacter bugandensis* and *Achromobacter xylosoxidans* showed positive lipolytic activity.

Based on our previous knowledge of the microorganisms found within the gut of ground beetles, these gut bacteria could be serving many functions, such as causing or preventing diseases, degrading insecticides, and directly or indirectly contributing to food digestion [34]. A previous study by Cheng *et al*. [35] isolated *Enterobacter aerogenes* from the alimentary canal of the southern pine beetle [36] which belongs to the same Genus as that of Isolate LSBU1EV1 identified as *Enterobacter bugandensis* which is a Gram-negative, facultative anaerobic, rod-shaped, non-spore forming ubiquitous bacteria of the family Enterobacteriaceae, Order Enterobacterales. They are widely spread in nature and are present in the intestinal gut of both humans and animals. They were also found to be nosocomial opportunistic pathogens that are highly virulent and possibly the most pathogenic species of the genus *Enterobacter* currently known [37].

Iolate LSBU2EV1 was identified as *Strenotrophomonas sp.*, an aerobic, gram-negative bacilli found in the soil and can be isolated from the rhizosphere. It is also closely associated with plants as it can be isolated from the vascular tissues of the root and stem. The *Stenotrophomonas* genus ranges from common soil organisms (*Stenotrophomonas nitritireducens*) to opportunistic human pathogens with multidrug resistant profile like *S. maltophilia*. They are versatile and the most common species used in agriculture as biocontrol with promising applications in bioremediation and phytoremediation strategies with high potential in the field of production of biomolecules of economic value [38].

Isolate LSBU4EV1 was identified as *Achromobacter xylosoxidans*, an aerobic, gram negative, non-spore forming, motile bacilli with peritrichous flagella found in wet environments and usually resistant to a variety of antibiotics including penicillins. It has been found to cause bacteremia in immune-compromised patients [39].

CONCLUSION

In conclusion the present study shows that *Strenotrophomonas sp.* and *Achromobacter xylosoxidans* have cellulase activity while *Enterobacter bugandensis* ferment glucose. These dominant microbes found in the larval gut of *E. vigintioctopunctata* also show lipolytic activity thus proving that they maintain a symbiotic association with the host. Though there are many reports on the distribution and diversity of gut bacteria in several species of insects, exploratory studies on the functional role of gut microbiota in insects are limited. Therefore, understanding the insect gut microbial diversity, their roles in insect growth and development and their contribution in insecticide degradation would offer significant information for designing future pest management strategies.

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