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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 a wide-spread key pest of the solanaceous and cucurbitaceous plants. *E. vigintioctopunctata* depends up on microorganisms that inhabit its intestinal tract. The diversity of the gut microbiota revealed 20 bacteria based on their morphological, biochemical, physiological and molecular characteristics. Out of twenty, six isolates were gram-negative bacteria. In total of 20 isolates 3 bacteria were sent to 16S rRNA partial gene sequencing and revealed the presence of *Bacillus subtilis* (EVI16), *B. vietnamensis* (EVI09) and *B. anthracis* (EVI07).

KEYWORDS: *Epilachna vigintioctopunctata*, gut associated bacteria, 16S rRNA sequencing, *Bacillus subtilis* (EVI16), *B. vietnamensis* (EVI09), *B. anthracis* (EVI07)

INTRODUCTION

Insects are extremely successful animals in view of their great adaptability to a wide range of terrestrial niches. Insect success is due to the collaboration with bacteria in term of symbiosis since bacteria play crucial roles in the biology and life cycle of most insect species, affecting nutrition, development, reproduction, immunity, defense against natural enemies and speciation (Moran and Baumann, 2000; Moran, 2001; 2006).

There has been growing interest in developing novel approaches to control insect pests through gut micro biota study. Most of the microorganisms found in nature have not yet been studied. Little is known about the composition and function of the insect gut microbiota. Moreover, most previous studies on diversity of gut microbiota of insects relied on culture-dependent methods using tra-ditional microbiological techniques to identify the gut microbiota (Dillon and Dillon, 2004). Traditional methods of bacterial isolation limit the species that can be grown and analyzed under laboratory conditions, although they can provide information about the biology and biochemical features of the isolated organism. Molecular approaches for characterization of microbes have been used in recent years. This approach based on nucleic acid sequence, par-ticularly the 16S rRNA gene, has enabled the definition of the microbial community of insects (Brauman, 2000). Knowledge of microorganisms' species can facilitate studies of the function of the gut microbiota and help to define

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interactions among members of the gut community that can lead to the development of insect control strategies (Broderick *et al.,* 2004).Moreover, the identification of microorganisms and their specific enzymatic activities can help in the understanding of the interactions between insects and provide information to control the pest.

The spotted leaf beetle or Hadda beetle, *Epilachna vigintioctopunctata* Fab. (syn. *Henosepilachna vigintioctopunctata* Fab.), is the key pest of the solanaceous and cucurbitaceous plants (Islam *et al*., 2011). The grub and adult feeds on the leaves, retarding the plant growth, which leads to loss of fruit production. Fruit reduction in yield up to 60% has been reported (Mall *et al*., 1992).Currently the overall knowledge of the bacterial communities in *E. vigintioctopunctata* and their associations with hosts is still limited and has to be studied extensively so that we may get many clues to control the pest.

MATERIALS AND METHODS

Adults of *E. vigintioctopunctata* were collected from brinjal farm, Bahour, Puducherry in the month of July 2016. The insects were surface sterilized with 70% ethanol for 1 min and rinsed in sterile water before dissection. The insect was dissected inside a sterile laminar flow using sterilized dissection scissors, needle and forceps. The head and last abdominal segment of insect were severed, and pressure was applied anterior to the crop to release the gut. The gut isolated and homogenized in 0.86% NaCl solution (Broderick *et al.,* 2004).

The stock solution was prepared by taking 1 ml of the suspension and was mixed with 9.0 ml saline. Thereby using serial dilution method seven dilutions were prepared. 1ml of each dilution was added to separate plate. Triplicates were made for each dilution. Then added 15 ml of nutrient agar medium and incubated for 24 hours at 37°C. Dominant colonies were picked out, purified three times by inoculating on the corresponding agar plates, and further transferred to agar slants (Huang, 1999).

The dominant frequently appearing gut associated bacteria were identified by bacteriological properties and16S rRNA gene sequencing. Morphological tests were done by standard procedures. The physiological-biochemical characteristics were determined on the basis of Gram stain, Catalase test, Lipolytic test, Gelatinase test and Cellulolytic test (Dong and Cai, 2001).

Preparation of template DNA – Pure cultured bacterium was used for gene sequencing. Colonies were picked up with a sterilized toothpick, and suspended in 0.5 ml of sterilized saline in a 1.5 ml centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of Insta Gene Matrix (Bio-Rad, USA). Incubated 56^{o}C for 30 min and then heated 100^{o}C for 10 min. After heating, supernatant was used for PCR.

PCR - 1µl of template DNA was added in 20 µl of PCR reaction mix. 518F/800R primers were used and then performed 35 amplification cycles at 94^{o}C for 45 sec, 55^{o}C for 60 sec, and 72^{o}C for 60 sec. DNA fragments were amplified about 1,400 bp in the case of bacteria including a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

518F	5'CCAGCAGCCGCGGTAATACG 3'
800R	5' TACCAGGGTATCTAATCC 3'

Purification - Purification of PCR products of approximately 1,400 bp were sequenced by using the primers and dNTPs from PCR products by using Montage PCR clean up kit (Millipore).

Sequencing - The purified PCR products of approximately 1,400 bp were sequenced by using general primers (785 F 5' GGA TTA GAT ACC CTG GTA 3' and 907 R 5' CCG TCA ATT CCT TTR AGT TT 3'). Both this primers amplify the V5- V6 region of the 16S r RNA gene. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied BioSystems model 3730XXL automated DNA sequencing system (Applied Bio Systems, USA) (Weisburg *et al.,* 1991).

The culture sequence obtained were subjected to BLAST analysis, the phylogenetically similar type strains sequence and other phylogenetic related sequence were selected from the GeneBank and they were subjected to multiple sequence alignment and then align sequence were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6. In the tree the numbers at the nodes indicates the levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbour joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.005 substitutions per site.

RESULTS AND DISCUSSION

Using the isolation procedure, a total of 20 dominant isolates were successfully collected from the gut of the adult and classified based on the colony color, size, and cellular morphology and biochemical activity. Among the 20 bacteria isolated from *E. vigintioctopunctata* adult, majority were gram-positive bacteria (14 isolates) and only six were gram-negative bacteria.

Three bacteria identified were isolated using pure culture method and subjected to 16S rRNA partial gene sequencing. The sequences obtained were analyzed using BLAST and other programs and placed on the phylogenetic tree. *B. subtilis* (EVI16), *B. vietnamensis* (EVI09) and *B. anthracis* (EVI07) species were identified based on their similarities with other sequences (Table 1).

Genbank accession numbers of bacterial isolates:

The GenBank accession numbers for the partial sequence of the 16S rRNA gene sequences for the isolates EVI09 (*B. vietnamensis)*, EVI07 (*B. anthracis*) and EVI16 (*B. subtilis*) were KY002646, KY002647 and KY002648 respectively.

The bacteria isolated from the gut of *E. vigintioctopunctata* were able to produce lipase, protease, cellulase and catalase enzymes in a similar

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

Colony	Shape	Colour
EVI01	Filamentous	Yellow
EVI02	Rhizoid	White
EVI03	Filamentous	White
EVI04	Lobate	White
EVI05	Lobate	White
EVI06	Filamentous	White
EVI07	Filamentous	White
EVI08	Filamentous	White
EVI09	Round	Cream
EVI10	Round	White
EVI11	Filamentous	White
EVI12	Filamentous	Cream
EVI13	Lobate	Cream
EVI14	Lobate	Cream
EVI15	Filamentous	White
EVI16	Lobate	White
EVI17	Filamentous	White
EVI18	Round	Yellow
EVI19	Irregular	Yellow
EVI20	Irregular	White

gut environment. The insect gut microbiota is considered to be a complex ecosystem containing over a hundred bacterial species including anaerobes and facultative anaerobes (Brauman, 2000). Microbial colonization depends on the physicochemical conditions in the lumen of different gut compartments, and these can display extreme variation in both pH and oxygen availability (Appel and Martin, 1990; Harrison, 2001). The gut of the insect is anaerobic in nature. It is conducive for the aerobic bacteria to live in. Therefore bacteria produce catalase to generate oxygen so that the bacteria can use oxygen for respiration. These bacteria may be pure aerobes or facultative anaerobic organisms (Engel and Moran, 2012). In this study 50% of the bacteria are involved in catalase activity (Table 2).

Bacteria associate with insects in providing essential nutrients. Insects belonging to the order Hemiptera like aphids, white fly, mealy bug, plant hoppers feed

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

Colony	Grams Stain	Catalase	Lipolytic	Gelatinase	Cellulolytic activity
EVI01	+	-	+	+	+
EVI02	+	+	+	+	-
EVI03	-	+	+	+	+
EVI04	+	-	+	-	-
EVI05	+	-	-	+	-
EVI06	+	-	-	+	-
EVI07	+	+	+	+	-
EVI08	+	+	+	+	-
EVI09	-	+	+	+	-
EVI10	-	-	+	+	+
EVI11	-	+	+	-	+
EVI12	+	-	+	+	+
EVI13	+	-	+	+	+
EVI14	+	-	-	+	-
EVI15	-	+	+	+	-
EVI16	+	+	-	-	-
EVI17	+	+	-	-	+
EVI18	+	+	+	+	+
EVI19	-	+	+	+	+
EVI20	+	+	+	+	+

exclusively on the plant sap – a freely available sugar rich diet. Since plant sap is very poor in nitrogen and amino acids, these insects have developed obligate symbiosis with bacteria like *Buchenra* (aphid) or *Portiera* (white fly), where in the bacteria supply all the essential amino acids required by the insect, while the insect accommodate these bacteria in specialized structures in their gut - mycetomes / bacteriocytes (Douglas, 2006). Among 20 bacteria isolated from gut of *E. vigintioctopunctata*, 16 isolates including *B. anthracis* and *B. vietnamensis* were involved in proteolytic activity (Table 2). A recent study reported that a part of velvet bean caterpillar gut protease was secreted by their gut bacteria (Visotto *et al*., 2009).

Several bacteria isolated from the soil have been reported as cellulase producer such as *F. johnsoniae, Pseudomonas mendocina* (Lednicka *et al.*, 2000) and also from the gut of scrab *H. parallela* (*P. nitroreducens*) (Huang *et al.*, 2012). Cellulase activity was also reported in *Acinetobacter anitratus* (Ekperigin, 2007). *Acinetobacter lwoffi* and *Microbacterium paraoxydans* were found in the gut of *Ostrinia nubilalis* (*Lepidoptera*). Subodh *et al.* (2012) isolated bacteria from the gut of termite showing cellulolytic activity. They were identified as *Citrobacter, Enterobacter* and *Cellulomonas*. Among 20 bacteria isolated from gut of *Epilachna vigintioctopunctata*, 10 isolates were involved in cellulolytic activity (Table 2). The bacterial strain EVI09 was identified as *B. vietnamensis* which had 98% of sequence similarity with said sequence *B. vietnamensis* is a common soil bacterium that belongs to *Firmicutes*. It has a thick cell wall, forms endospore, highly resistant to extreme environments. This bacterium (isolate EVI09) has much similarity and requires further verification.

This may act as endosymbiont that helps in digestion and assimilation of food by the insect.

The isolate EVI07 has been identified as *B. anthracis* (Fig.1). This bacterium is known to cause anthrax in mammals. The nucleotide similarity has 99% with *Bacillus anthracis.* In this study it has been noted that this bacterium is found in the insect gut. These bacteria are found in many environments, notably in the soil and on plants where they can be associated with invertebrates (Schuch *et al.*, 2010). The isolates EVI16 and EVI09 were identified as *B. subtilis* (Fig.2) and *B. vietnamensis*. In future studies, it may possible to analyse the presence of *B.anthracis* associated with insects. *B. subtilis* is aerobic, endospore-

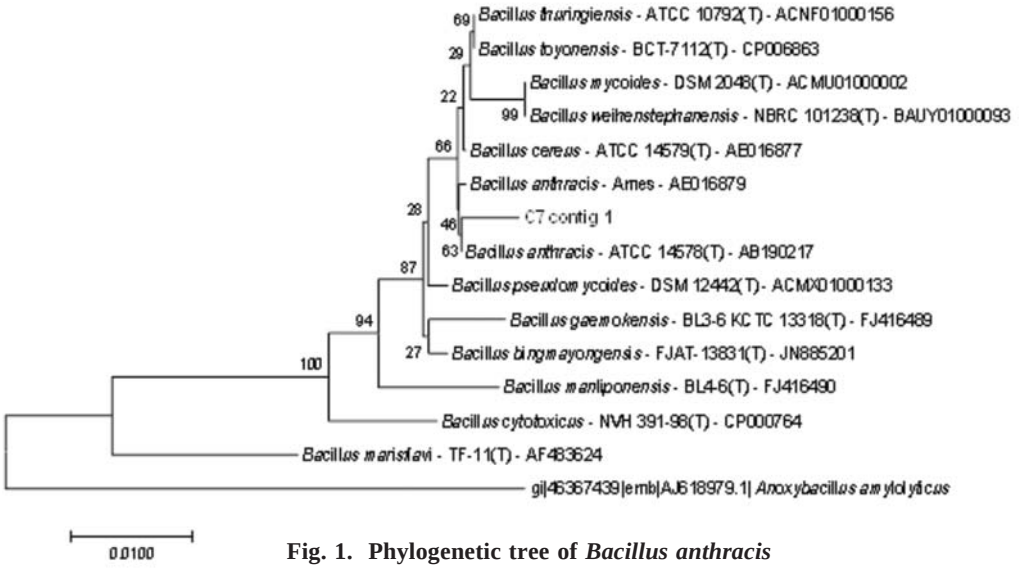

Fig. 1. Phylogenetic tree of *Bacillus anthracis*

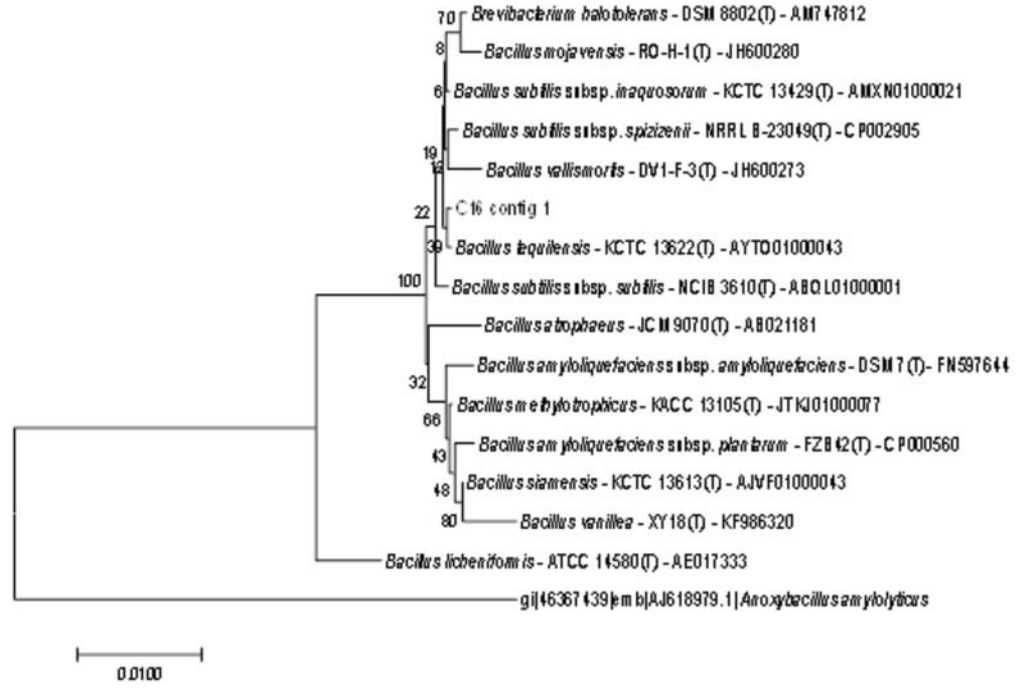

Fig. 2. Phylogenetic tree of *Bacillus subtilis* sub sp. *inaquosorum*

forming, gram positive bacteria and opportunistic pathogen. Mandla Rajashekhar *et al*., in 2017 studied the potential of *B. subtilis* as microbial insecticide for effective management of insect pests.

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