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Identification and Characterization of gut associated bacteria in the larvae of *Spodoptera litura* using 16s rRNA gene

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Abstract
The aim of the present study was to identify and characterize the gut associated bacteria in *Spodoptera litura*. The bacterial flora of *S. litura* was determined by using both culture dependent and culture independent. In total, 31 bacteria were characterized based on their morphological, biochemical and molecular characteristics. Out of thirty one, six isolates were gram-negative bacteria, twenty seven isolates were catalase producers, nine isolates were lipase producers, twenty isolates were amylase producers and twenty isolates were gelatinase producers. The bacteria isolated from *S. litura* were determined as *Pseudomonas aeruginosa* (SLI03) and *Bacillus tequilensis* (SLI23) by using 16S rRNA gene partial sequencing. The sequences were submitted in NCBI GenBank and given accession numbers KY002523 and KY002524.

Keywords: *Spodoptera litura*, gut associated bacteria, 16S rRNA gene partial sequencing, *Pseudomonas aeruginosa* (SLI03) and *Bacillus tequilensis* (SLI23).

1. Introduction
Insects are the most diverse group of animals with over a million different species found almost in every habitat, except the sea [18]. Due to their wide spread distribution, insects are inevitably associated with extremely large variety of microscopic life forms, including viruses, bacteria, fungi, protozoa, nematodes and multicellular parasites. All insects live in close association with bacteria; however, bacteria are present on the integument, inhabit the digestive tract, and in some highly evolved cases, inhabit unique structures within the insect body [3]. The bacterial association in insects plays a significant role in host insect morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, pH regulation, vitamin synthesis, temperature tolerance, resistance against parasitoid development, and detoxification of noxious compounds [12].
The relationship of microbes within the gut of insect is of considerable interest. Many types of bacteria have been identified from different insects, such as the gypsy moth, migratory grasshopper, cabbage moth, and cotton bollworm [22]. *S. litura* is an extremely serious pest, the larvae of which can defoliate many economically important crops. Larvae cause significant damage to both the foliage and developing fruits of field grown tomatoes [16]. Currently the overall knowledge of the bacterial communities in *S. litura* 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.

2. Materials and Methods
2.1 Collection of Larvae and Dissection of Gut
S. litura were collected form Krishi Vigyan Kendra, Puducherry in the month of July 2016. The following day, they were surface sterilized in 70% ethanol for 1 min, rinsed in sterile water before dissection. The larvae dissected inside a sterile laminar flow hood using sterilized dissection scissors, needle and forceps. The head and last abdominal segment of each larva were severed, and larvae were dissected open through the middle. The gut isolated and homogenized in 0.86% NaCl solution [2].

2.2 Isolation of dominant bacteria
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 [13].

2.3 Identification of dominant bacteria
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, Lipase test, Gelatinase test and cellulase test and amylase test [5].

2.4 16S rRNA gene sequencing
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 56oC for 30 min and then heated 100oC 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 94oC for 45 sec, 55oC for 60 sec, and 72oC for 60 sec. DNA fragments were amplified about 1,400 bp in the case of bacteria.

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 using Montage PCR clean up kit (Millipore).
Sequencing - The purified PCR products of approximately 1,400 bp were sequenced by using the general primers (785F 5' GGATTAGATACCCTGGTA 3' and 907R 5' CCGTCAATTCCT TTRAGTTT 3'). Both this primers amplify the V5- V6 region of the 16S r RNA. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Bio Systems, USA). Sequencing products were resolved on an Applied Bio Systems model 3730XXL automated DNA sequencing system (Applied Bio Systems, USA) [21].

2.5 Phylogenetic tree construction
The culture sequence obtained were subjected to BLAST analysis, the phylogenetically similar type strains sequence and other phylogenetically related sequence were selected from the GenBank 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 (neighbor 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 neighbor joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.005 substitutions per site.

3. Results
Using the isolation procedure described above, a total of 31 dominant isolates frequently appearing were successfully collected from the gut of the late instar larvae and classified based on the colony color, size, and cellular morphology and biochemical activity. All the colonies were given number from 1 to 31(SLI01 to SLI31). Table 1 summarizes the morphological characteristics of the bacterial isolates. The majority of these isolates were gram-positive bacteria (25 isolates) (Table 1). Only 10 of the isolates were rods or bacilli shaped bacteria, while the others were Cocci shaped bacteria (Table 1). Figure 1 summarizes the biochemical properties of bacterial isolates. Two bacteria were isolated using pure culture method and subjected to 16S rRNA sequencing. The sequences obtained were analyzed using BLAST and other programs and placed on the phylogenetic tree. The 2 species *Pseudomonas aeruginosa* (SLI03) and *Bacillus tequilensis* SLI23) were identified based on their similarities with other sequences (Fig.2 & 3).

Table 1: Morphological characteristics of dominant bacteria in the gut of the *Spodoptera litura*

Colony	Shape of the colony	Shape of the bacteria	Colour of the colony
SLI01	Filamentous	Cocci	Yellow
SLI02	Round	Cocci	White
SLI03	Filamentous	Cocci	White
SLI04	Lobate	Cocci	White
SLI05	Filamentous	Cocci	White
SLI06	Filamentous	Cocci	White
SLI07	Filamentous	Rod	White
SLI08	Filamentous	Cocci	White
SLI09	Round	Rod	White
SLI10	Lobate	Rod	White
SLI11	Round	Cocci	White
SLI12	Rhizoid	Rod	White
SLI13	Round	Rod	White
SLI14	Filamentous	Cocci	White
SLI15	Filamentous	Cocci	White
SLI16	Irregular	Rod	White
SLI17	Filamentous	Rod	White
SLI18	Round	Rod	Yellow
SLI19	Irregular	Cocci	Yellow
SLI20	Irregular	Cocci	White
SLI21	Filamentous	Cocci	White
SLI22	Irregular	Cocci	White
SLI23	Irregular	Cocci	White
SLI24	Filamentous	Cocci	White
SLI25	Irregular	Rod	White
SLI26	Filamentous	Cocci	White
SLI27	Filamentous	Rod	White
SLI28	Irregular	Cocci	White
SLI29	Filamentous	Cocci	White
SLI31	Lobate	Cocci	Yellow

Genbank accession numbers of bacterial isolates
The GenBank accession numbers for the partial sequence of the 16S rRNA gene of the isolates SLI03, SLI23 were KY002523 and KY002524, respectively.

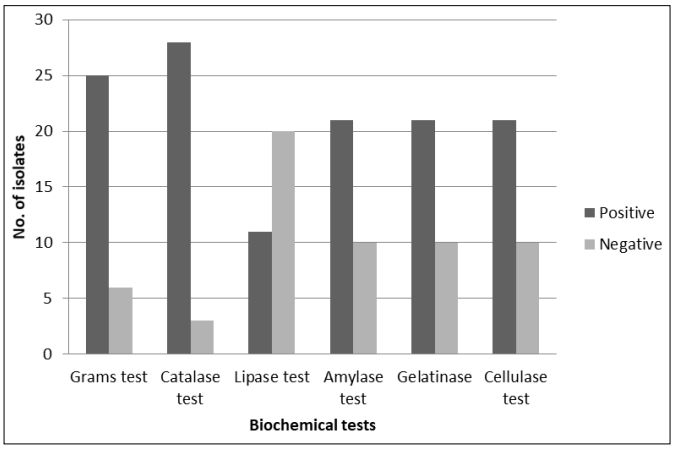

Fig 1: Biochemical characteristics of dominant bacteria in the gut of the larvae of *Spodoptera litura.*

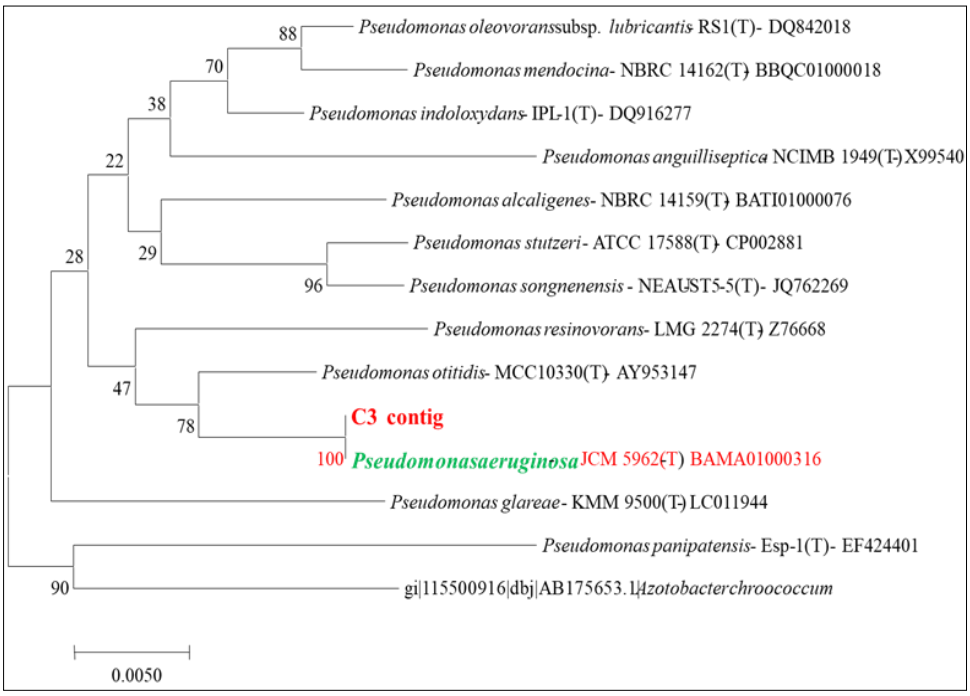

Fig 2: Phylogenetic tree of *Pseudomonas aeruginosa*

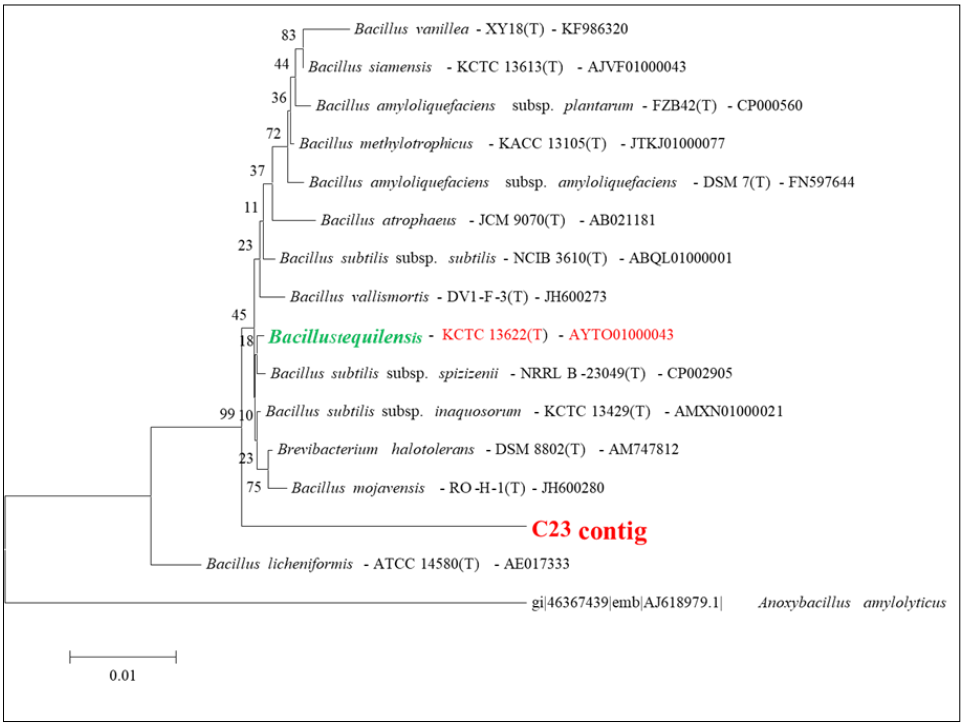

Fig 3: Phylogenetic tree of *Bacillus tequilensis*

4. Discussion

Based on microbiology theory, insects lack a complete enzyme system and thus need gut microorganisms to provide different kinds of enzymes for food digestion, nutrient absorption, and biological metabolism [20]. The larva of *S. litura* harbors a diverse community of gut bacteria. In this study, it suggested that the isolates were able to produce lipase, gelatinase, and amylase, cellulolytic and catalytic enzymes in a similar gut environment.

The biochemical tests also prove that many of these isolates may help in digestion and assimilation of nutrients of insect. Fifteen isolates shows cellulolytic activity because the larva of *Spodoptera litura* solely depends on the plant source for nutrition (Table 2).

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. In this study most of the isolates show catalase production (Table 2). Therefore it can be interpreted that these bacterial isolates which show catalase activity may be aerobes or facultative anaerobes [7].

Larva of *S. litura* depends on the plant parts, the amount of fat in such food is undoubtedly less but there may be some amount of lipids. Therefore only 50% of the isolates were shown lipolytic activity (Table 2). Lipolytic bacteria in *Bombyx mori* shown variable number when the forage is changed [9]. The diversity of lipase-producing bacteria from the gut of the *S. litura* was considerably deficient when compared with that of silkworm. This difference may relate to the different content of lipids.

Larva of *S. litura* is serious defoliator of variety of plants, these parts may contain protein content. The gut bacteria of velvetbean caterpillar produce proteolytic enzymes to digest protein content of the plant sources [19]). In the present investigation 24 isolates shows gelatinase activity (Table 2).

Amylolytic bacteria help in digestion of starch. The gut of *S. litura* is rich in starch consumption of plant parts by the larva. The present study also proves that important role played by the amylolytic bacteria in the gut of *S. litura*. The starch digestion was assisted by the amylolytic bacteria present in the insect gut [17].

Pseudomonas aeruginosa is a member of the family Pseudomonadaceae, which is gram-negative and a well-known bacterial pathogen of many insects including adult grasshoppers, *Melanoplus Bivittatus* (Say) and *Camnula Pellucida* (Scudder). *P. aeruginosa* a facultative pathogen of the red palm weevil, *Rhynchophorus ferrugineus* (Oliver) [1]. *P. aeruginosa* was the most frequently isolated bacterium from larval and pupal cadavers of the southwestern corn borer (*Diatraeagrandiosella*) and southern corn stalk borer (*Diatraeacrambidoides*) [14]. On **"**Investigating internal bacteria of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae and some *Bacillus* strains as biocontrol agents" determined that *P. aeruginosa* (SL7) had a low pathogenic effect (30%) on *S. littoralis* larvae under laboratory conditions [8].

This study proves that *Pseudomonas aeruginosa* (SLI03) present in gut of the larvae involved the activities like catalytic, cellulolytic and starch hydrolysis. Being a soil bacterium, it may enter in to insect gut through food. It is also known to be an opportunistic pathogen in insect as well as in man. It is a highly tolerant bacterium for a variety of abiotic factors. The second strain SLI23 is identified as the *Bacillus tequilensis* has been isolated from 2000 year old Mexican samples. It is a gram positive bacterium belongs to forsicutes, form endospores can survive extreme environmental conditions even for centuries. *Bacillus tequilensis* is a close relative of *Bacillus subtilis*. It form a separate clade but evolved from *Bacillus subtilis* like organisms. Being a tough organism it is mainly involved in the production of organic acids [11]. *Bacillus tequilensis* is also identified in *Toxoptera aurantii* [6].

This present study proves that when the biodiversity of microbes in the larval gut of *S. litura* maintain a symbiotic association with the host larvae. A prediction is that there will be some species of microbes that have not been identified, or if they have been identified, little is known about them.

In conclusion, we isolated and characterized different bacteria from the gut of *S. litura* larva. Identified two bacteria 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. The 2 species *Pseudomonas aeruginosa* (Fig.2) and *Bacillus tequilensis* (Fig.3*)* were identified based on their similarities with other sequences. The sequences were submitted in NCBI GenBank and given accession numbers KY002523 and KY002524.

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