



Endosymbiotic Bacteria Associated with *Aphis gossypii* Glover (Hemiptera:Aphidae) infesting taro

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Abstract

Aphids are one of the important sucking pests of many agriculturally important crops, associated with a wide variety of bacterial endosymbionts that confer many ecologically relevant traits to the host insect. Endosymbiotic bacteria (ESB) play a vital role even in the physiology of the host, hence identification of ESB associated with the aphids will help to develop important strategies for the management of this noxious pest. In the present study molecular characterization of ESB associated with the aphid, *Aphis gossypii* infesting taro was done. Morphological characters of the four strains revealed that each isolate has different colony characters. Further, the genomic DNA was isolated from each of the EPB isolates and PCR amplification of 16S rRNA gene was carried out using universal primers. The 16S rDNA gene sequences of endosymbiotic bacterial isolates were generated by sequencing the PCR product and were aligned with each other by using BioEDIT software. The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search and four genus viz., *Bacillus*, *Pseudomonas*, *Pantoea* and *Staphylococcus* were confirmed. From the aligned sequences phylogenetic tree was constructed by the Neighbor-Joining method using MEGA version X.

Key words: Endosymbiotic bacteria, aphids, 16S rDNA, molecular characterization, *Bacillus*

Introduction

Insects are the most dominating group, accounting for over 90% of known animal species, and exhibit symbiotic relationships with bacteria. The complete exclusion of endosymbionts from insects may reduce their lifespan and suppress population within few days or weeks. Sucking pests like aphids, mealybugs, and whiteflies are widespread and feed exclusively on plant sap (Sandstrom and Pettersson, 1994; Sandstrom and Moran, 1999; Douglas 2006). These insects are associated with intracellular microorganisms through which exchange of nutrients is possible and enable optimal utilization of nutrients to complete their life cycle. Aphids possess wide range of microbial symbiosis which are obligate (Primary) as well as facultative (secondary), according to the nature of the species. Nutritionally limited diet of aphids made them dependent on the primary endosymbiont *Buchnera*

aphidicola, which is present in all aphids and provides the critical amino acids to the host. Some of the aphid species are associated with several vertically transmitted facultative endosymbiotic bacteria. The five main secondary ESB associated with pea aphids (*Acyrtosiphon pisum*) are *Serratia symbiotica*, *Regiella insecticola*, *Hamiltonella defense*, *Rickettsia* and *Spiroplasma* sp. (Moran et al., 2005; Chen et al., 1996; Fukatsu et al., 2001). Among the different species of aphids *Aphis gossypii*, has a cosmopolitan distribution and is one of the most destructive pests attacking at least 64 economically important crops. Common host plants include cotton, pumpkin, cucumber, zucchini, watermelon, chilli, tomato and various flower cultivars such as *Hibiscus* (Rodriguez et al., 2009). The secondary endosymbionts can influence many ecologically important traits in aphids, including defense against pathogens and natural

enemies (Oliver et al., 2003; Oliver et al., 2005; Lukasik et al., 2013), tolerance to heat stress (Burke et al., 2009), host plant utilisation patterns (Tsuchida et al., 2004) and manipulation of host reproduction (Simon et al., 2011). Since ESB play a vital role in the physiology of their host, identifying the types of bacteria associated with aphids will give basic information, which will be useful for the development of pest management strategy.

Materials and Methods

Isolation of ESB

Adult aphids collected from the stock culture maintained at the Insect Microbiology Laboratory, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram were surface sterilized with absolute ethanol. These were homogenized in sterile 0.9% saline and plated directly on to the nutrient agar media and kept for incubation at 30°C overnight under aerobic condition.

Identification of ESB

Pure culture of each ESB was obtained by streaking the individual colony on a fresh nutrient agar plate and incubated for 24 h at 35°C. The colony characters were observed from each separated colony.

Phenotypic characterization of ESB strains

Cultural characteristics of each bacterium, which include shape, margin and elevation of the isolates of each colony type were observed using stereomicroscope (Carl Zeiss, Stemi 2000C) under 40X magnification, by using research microscope (Leica DMLB) under 100X magnification. Gram staining was done using the Hi-Media kit (Hi-Media Laboratories Pvt. Ltd., India) according to the manufacture's protocol for the identification of unknown bacterial strains collected from the nutrient broth of 24 h culture, and were observed under a compound microscope (Leica DMLB) with 100x magnification.

PCR amplification of 16S rDNA of ESB

PCR amplification of 16S rDNA gene by universal primers: forward primer fD1 5'AGAGTTTGATCCTG GCTCAG3' (corresponding to 8–27 of *Escherichia coli*) and reverse primer RP2 5'CGGCTACCTTGTTACGA CTT3' (corresponding to 1492–1510 of *E. coli*) (Weisburg et al., 1991) were used. The PCR was

performed in a 25 µl reaction mixture having 2.5 µl of 10X Taq buffer A (containing 15 mM MgCl₂, mM each), 1.0 µl of each primer (20 ng), 2 µl of template DNA and 0.25 µl of (1U) Taq DNA polymerase and 17.75 µl of sterile distilled water. The reaction was carried out in a Biorad thermal cycler with the thermal cycle programme of 92°C for 2min 10 s (initial denaturation), 30 cycles at 94°C for 1min 10 s (denaturation), at 49°C for 30 s (annealing), at 72°C for 2 min (extension) and final extension at 72°C for 10 min. The amplified products were resolved on a 1.2% agarose gel. DNA ladder of 500 bp (Bangalore GeNei, India) was used for determining the size of the amplicon. The DNA bands were visualised under UV transilluminator and the purified PCR products of 1500 bp were sequenced at SciGenom Labs, Ernakulam, Kerala.

Phylogenetic analysis

The sequences obtained for the EPB isolates were aligned with each other by using Clustal alignment programme of MEGA X software (Kumar et al., 2018). The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov/BLAST>). From the aligned sequences phylogenetic tree was constructed by the Neighbor - Joining method using MEGA X software.

Results and Discussion

Phenotypic characterization of EPB strains

A total of four EPB strains were isolated from the *A. gossypii* and were assigned code numbers as isolates CA1, CA2, CA3 and CA4. Morphological variations were observed for each endosymbiotic bacterial strains, but no pigmentation was observed. Colonies formed on nutrient agar were circular, raised, convex, flat, entire white in colour, with margins entire or undulate.

Table 1. Morphological characteristics of the bacterial isolates

Bacterial isolate	Gram staining	Colony morphology
CA 1	+	Circular, Flat, Entire
CA 2	–	Circular, Raised, Undulate
CA 3	+	Circular, Convex, Entire
CA 4	–	Circular, Flat, Entire

Molecular characterization of bacterial strains

The PCR amplification of the 16S rDNA of the EPB with the primers 16SF and 16SR at an annealing temperature of 49°C yielded a fragment of approximately 1500 bp. The PCR amplification of 16S rDNA of five EPB strains with universal primer are shown in Fig 1. Comparison of sequences tested in the NCBI Gen Bank database revealed that most of them have 97 to 100% sequence similarities to sequences of known species. Sequences with > 98% similarity to their nearest phylogenetic neighbor were identified to the species level. The four different EPB strains were identified as four different genera by comparative analysis of 16S rDNA sequences. The isolates were identified as CA1-*Bacillus cereus*, CA2-*Pseudomonas* sp., CA3-*Staphylococcus hominis*, CA4-*Pantoea* sp.. Sequencing of 16S rDNA of seven EPB isolates indicate that all the EPB strains were different (Table 1). Sequence similarity analysis of CA1 strain shows that they were closely related to *Bacillus cereus* with more than 98% similarity. Sequencing of 16S rDNA of all the EPB isolates indicate that all the strains were different from each other and are belonging to the following.

Table 2. Identification of bacterial species associated with aphids based on sequencing of 16S rDNA

Isolate	Identification	Similarity (%)
CA 1	<i>Bacillus cereus</i> strain SVP4 16S ribosomal RNA gene, partial sequence EU 161996	98.00
CA 2	<i>Pseudomonas</i> sp. SCAU7 16S ribosomal RNA gene, partial sequence KF 772885	97.66
CA 3	<i>Staphylococcus hominis</i> strain LH-Ka4 16S ribosomal RNA gene, partial sequence MG 996858	97.77
CA 4	<i>Pantoea</i> sp. strain JZ108 16S ribosomal RNA gene, partial sequence KR 190247	97.00

The molecular characterization of four ESB strains isolated from aphids based on 16S rDNA sequence analysis and the isolates were identified as three different classes namely γ -proteobacteria (*Pantoea*, *Pseudomonas*) and *Bacilli* (*Staphylococcus bacillus*). Aphids infected by a wide variety of bacterial endosymbionts can alter many

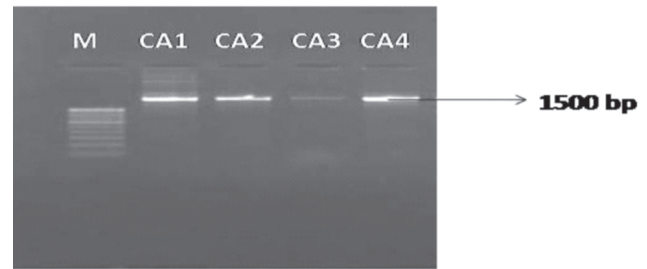


Fig. 1. PCR amplification of 16S rDNA of EPB with universal primer (M-1kb plus DNA ladder)

ecologically relevant traits related to the life cycle of insect host. Previous studies have reported that the sequencing the V4 region of the 16S rDNA of the cotton aphids *A. gossypii* associated with Bt cotton in northern China revealed that the bacterial communities were generally dominated by the primary endosymbiont *Buchnera*, together with the facultative endosymbionts *Arsenophonus* and *Hamiltonella* (Glare and Callagan, 2000). Previous studies have also reported the association of *Bacillus cereus* with many insect hosts and they can secrete a large array of proteinaceous and non proteinaceous toxins acting on other insects and mammals (Perchat et al., 2005). In our study one of the ESB strains (CA1) was identified as *B. cereus*. *Bacillus* spp. have been commercially used as biological control agents against pathogens and pests (Weller 1988; Stabb et al., 1994; Backman et al., 1994; Paulitz and Belanger, 2001; Berger et al., 1996). The biocontrol mechanisms of *Bacillus* spp. include production of antibiotics and extracellular hydrolytic enzymes such as chitinase, laminarinase, lipase, and protease. These hydrolases contribute to degradation of fungal cell walls (Korsten et al., 1993; Paulitz and Belanger, 2001; Helisto et al., 2001). It was also reported that *B. cereus* strains were known to secrete insecticidal proteins named Vip during the vegetative phase (Warren et al., 1996) and were found pathogenic to insects belonging to Lepidoptera and Diptera (To et al., 1975; Kaaya and Darji, 1989). Moreover, some of the studies have reported the production of bioactive metabolites by *B. cereus* associated with entomopathogenic nematodes with antimicrobial property (Kumar et al., 2014).

Gauthier et al., (2015) by deep sequencing of 16S ribosomal DNA have reported *Pantoea* as gut associated bacteria of pea aphid *Acyrtosiphon pisum*. In the present study *Pantoea* was observed as one of the bacterial isolates (CA4). *Pantoea agglomerans* like bacteria have been identified in phylloxera inducing species where its

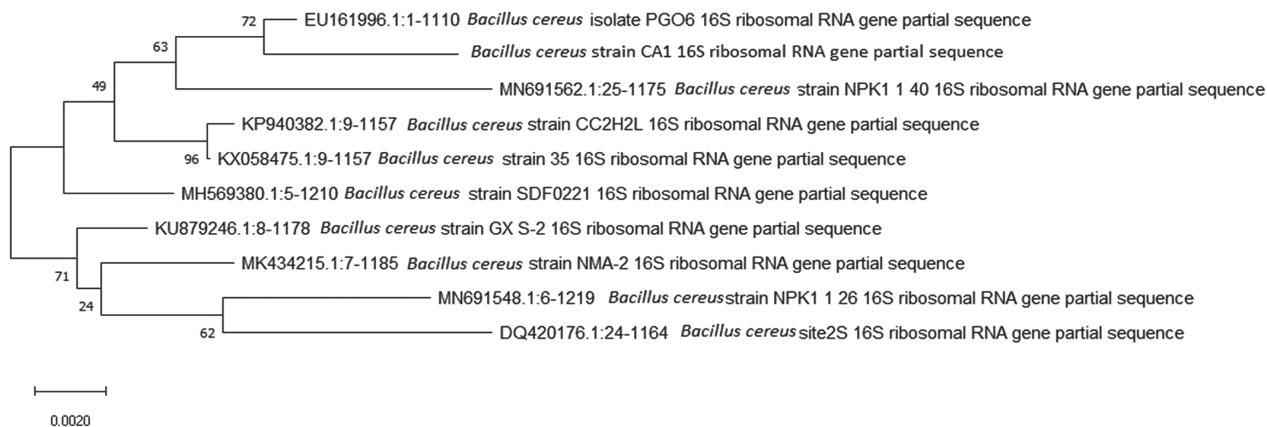


Fig. 2. Phylogenetic tree inferred from 16S rDNA sequences analysis showing the relationships of *B. cereus*

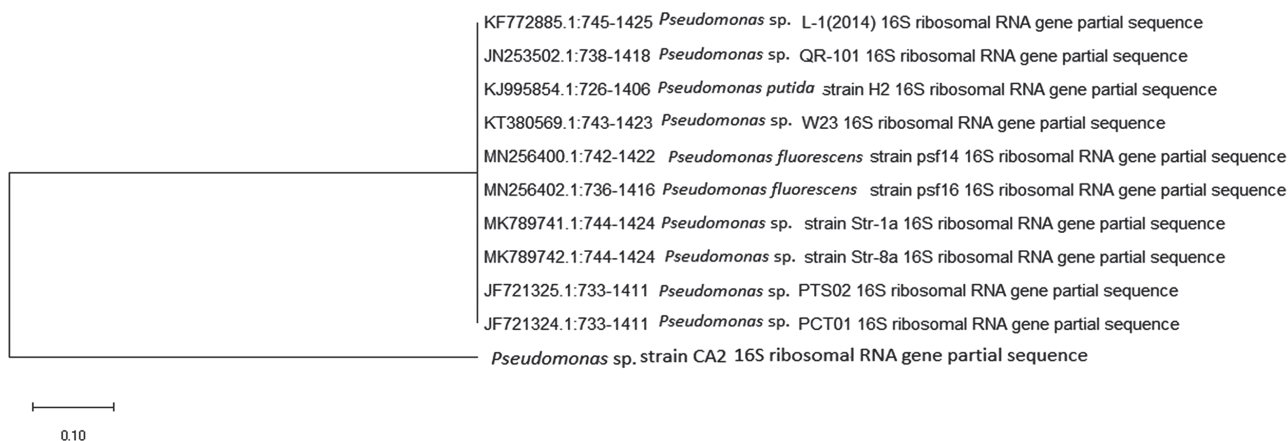


Fig. 3. Phylogenetic tree inferred from 16S rDNA sequences analysis showing the relationships of *Pseudomonas* sp.

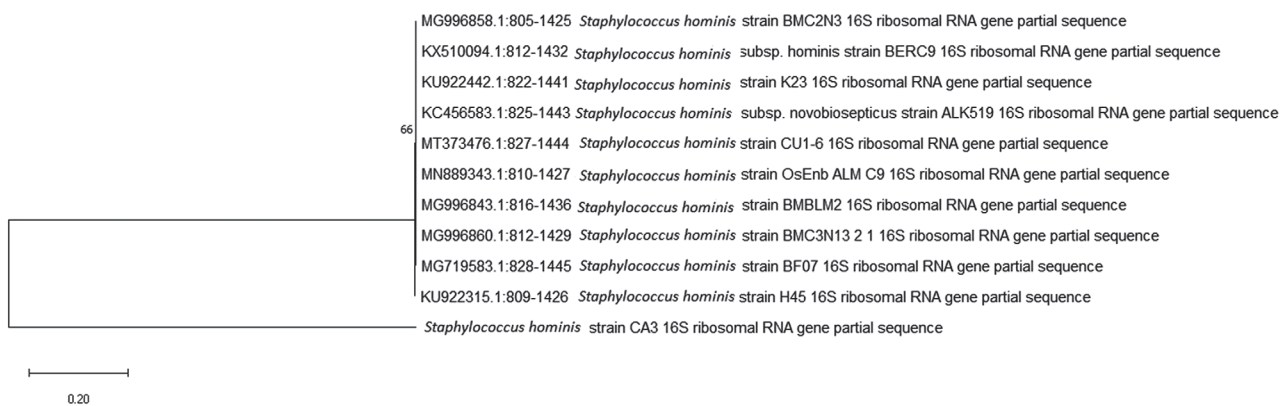


Fig. 4. Phylogenetic tree inferred from 16S rDNA sequences analysis showing the relationships of *Staphylococcus hominis*

prevalence reach 100% in some *Daktulosphaira vitifoliae* populations. (Medina et al., 2011; Vorwerk et al., 2007). Gauthier et al., (2015) reported that some of the pea aphids infected by phytopathogenic bacteria can colonize insect gut (Gauthier et al., 2015). Aphids may acquire ESB bacteria from honeydew excreted from plant surface

or may be ingested from plant sap, which circulate in the phloem (Stavrinos et al., 2009; Sabri et al., 2013; Fluger et al., 2012; Gauthier et al., 2015). Fakhour et al., (2018) reported the symbiotic association of facultative ESB viz. *Pseudomonas*, *Acinetobacter*, *Pantoea*, *Erwinia* and *Staphylococcus* from five cereal aphid species

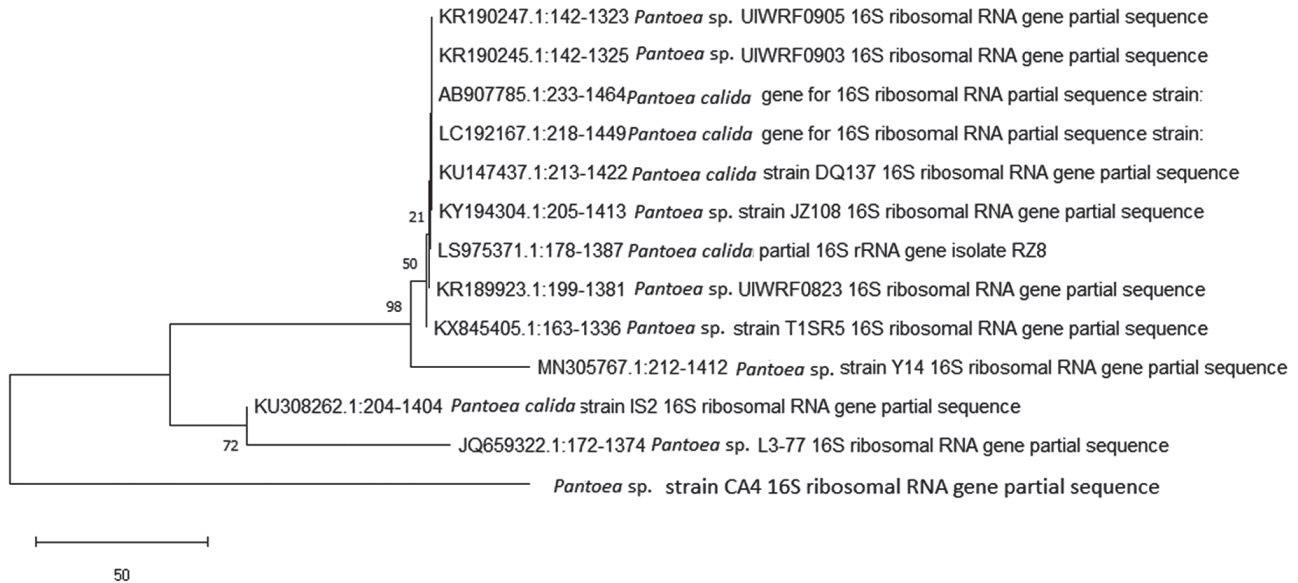


Fig. 5. Phylogenetic tree inferred from 16S rDNA sequences analysis showing the relationships of *Pantoea* sp.

namely, *Sitobion avenae*, *Rhopalosiphum padi*, *R. maidis*, *Sipha maydis* and *Diuraphis noxia*. The abundance of *Pseudomonas* was significantly higher in *Rhopalosiphum* genus than in *S. avenae*. These studies also concluded the occurrence of aphid endosymbiont combinations are mainly host specific and have significant ecological and evolutionary impacts on their hosts. Our study also confirmed the isolates CA2 and CA3 were *Pseudomonas* and *Staphylococcus* sp.

Conclusion

Insect symbionts always serve as an untapped source of bioactive molecules and digestive enzymes which are significantly important for the completion of insect host life cycle. With the advances in culturing techniques and genomic tools for the identification of expression of gene of interest, these symbionts can be exploited for the insect pest management strategies. The increasing research in insect-symbiosis and ecology will uncover new symbiotic microbial association and this will be the new sources of biotechnologically important bioactive molecules and enzymes for mankind.

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