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Endosymbiotic Bacteria Associated with the Spiralling Whitefly, *Aleurodicus dispersus* Russell (Hemiptera:Aleyrodidae)

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Abstract: *Aleurodicus dispersus* Russell, commonly known as spiralling whitefly (SWF), is a noxious polyphagous sucking pest infesting on an array of agricultural and horticultural crops, both in the glasshouse and field conditions. Bacterial endosymbionts associated with insects provide several benefits for the survival and reproduction of their host. Since endosymbionts play a vital role in the physiology of their host, identification of bacteria associated with SWF will entail the formulation of appropriate strategy to contain the noxious pest. In the present investigation bacterial strains associated with *A. dispersus* Russell were isolated and identified based on the biochemical parameters and comparative analysis of 16S rDNA sequences. Biochemical tests with VITEK 2 analyser and DNA revealed that there are four different strains of bacterium belonging to three different genera -- *Bacillus, Staphylococcus* and *Pantoea* associated with SFW.

Keywords: *Aleurodicus dispersus, Manihot esculenta* Crantz, Endosymbionts, Sucking pest

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Introduction

Aleurodicus dispersus Russell, (Spiralling whitefly), a deleterious sucking pest that originated from the Central America and the Caribbean region enjoys its distribution in the tropical and subtropical countries on a spectrum of agricultural crops, covering 481 plant species belonging to 295 genera from 90 families of vegetables, fruits and ornamental trees (Srinivasa, 2000; Boopathi *et al.*, 2012, 2013, 2014). In India it was first reported from cassava in 1993 at Thiruvananthapuram, Kerala (Palaniswami *et al.,* 1995). Cassava (*Manihot esculenta* Crantz) is the most important tropical tuber crops, but its production is severely affected by several insect pests, among them spiralling whitefly *Aleurodicus dispersus* ranks the foremost. Honey dew excreted by the spiralling whitefly encourages growth of sooty mould on the leaf surfaces that interferes with photosynthesis and affects the overall health and growth of the infested plant (Kumashiro *et al.,* 1983; John *et al.*, 2007). Infestation by spiralling whitefly on cassava causes 50-65% yield loss (Geetha, 2000; Boopathi *et al.,* 2014). Polyphagous nature of the *Aleurodicus dispersus* is the major impediment in

its management by using biocontrol agents alone (Boopathi *et al.,* 2013).

Endosymbionts are crucial for the development and survival of insects(Naik, 2016), and loss of these microorganisms often results in abnormal development and reduction in the survival of its host (Sreerag *et al*., 2014). Microbial endosymbionts are widespread in nature, particularly in arthropods(Baumann, 2005). Mycetomes of whitefly is believed to harbour bacterial endosymbionts (Buchner, 1965). The role of endosymbionts in protecting their host insect against natural antagonists such as predators and pathogens and to insecticide resistance have been documented (Zindel *et al.,* 2011). Zabalou *et al*. (2004) and Zindel *et al*. (2011) reported the detoxification of pesticides by endosymbionts. Since endosymbionts play a vital role in the physiology of their host, identification of bacteria associated with the host will give required information to make strategies to manage this noxious and polyphagous pest through the targeted manipulation of endosymbionts. Attempt was made in the present investigation for the isolation and identification of endosymbionts by physical, biochemical, and molecular approach.

Materials and Methods

Isolation of bacteria:

Adult females of *Aleurodicus dispersus* were collected from cassava leaves at the ICAR- Central Tuber Crops Research Institute, Sreekariyam and Thiruvananthapuram and surface sterilized with streptomycin solution (0.01%) for 1 min and two times in sterile distilled water. The spiralling whitefly, one each, taken in the Eppendorf tube was crushed well with 1 ml of the nutrient broth and these were kept in shaking incubator for overnight. After incubation the samples were serially diluted up to 10^{-10} and 1 ml sample from each tube were pour plated. The plates were incubated at room temperature (27 C) for 24 h, and the colonies isolated were further sub-cultured by streaking on a nutrient agar plate until obtaining pure culture. The colony morphology was recorded, and those were used for biochemical and molecular studies.

Phenotypic characterization of endosymbiotic bacteria:

Phenotypic characteristics of each colony were studied from the standard array of morphological characters such as form, margin, colour, elevation, pigmentation and opacity of the colony by using stereomicroscope (Carl Zeiss, Stemi 2000C, and U.S.A) under 40X magnification.

Gram Staining:

Gram staining identification of unknown bacterial strains was done as per usual protocol by using the 24 h bacterial culture, and the strains were observed under Leica DMLB compound microscope (100x).

Biochemical identification of bacteria:

Biochemical identification of the bacterial isolates was accomplished by VITEK system.

Pure culture maintenance:

Pure cultures of the isolated bacteria were maintained in nutrient agar medium by repeated subculture at 15 days intervals, and these were stored at −80 C in 20% glycerol.

Genomic DNA isolation and 16S rDNA amplification:

Total genomic DNA extracted from the isolated bacterial cultures was treated with 100 μl of RNase (Bangalore Genei, India) for eliminating the RNA contamination. The bacterial isolates were identified using 16S rDNA gene specific primers (16SF- 5′AGAGTTTGATCCTGGCTCAG3′, and 16SR-5′CGGCTACCTTGTTACGACTT3′). PCR amplify-cation was carried out in 25 μl reaction volume with standard protocol. The DNA bands were visualized under UV transilluminator and documented with Gel Doc system (Alpha Imager, Alpha Innotech, USA). Size of the amplicon was determined with a DNA ladder of 500 bp (Bangalore GeNei, India). The 16S rDNA gene

sequences were generated by sequencing the PCR product on Applied Biosystems 3500 Genetic Analyzer (Big Dye Terminator v 3.1).

Sequence Analysis:

The nucleotide sequence obtained was processed to remove low quality reads and transform into consensus sequences with Bioedit software. The resulted high-quality sequences were analysed with BLASTn (NCBI; http://www.ncbi.nlm.nih.gov) to confirm the authenticity of the isolate. The sequences of related species and genus were downloaded from the GenBank database, and a phylogenetic study was carried out with the program MEGA-X version 10.2.6. The sequences were aligned using the computer package ClustalW. The relationships among the isolates were analysed by the neighbor joining method using the Maximum Composite Likelihood model. Bootstrap values were generated using 1000 replicates to infer the robustness of the tree topology.

Phylogenetic analysis:

All sequences of 16S rDNA genes of various bacteria used in this analysis were obtained from GenBank database for the phylogenetic analysis of the endosymbionts. Phylogenetic trees were constructed using MEGA X 10.2.6 software.

Results

Phenotypic characterization of endosymbiotic bacteria:

Morphological characters of colonies were observed on nutrient agar plates for each bacterial strain, and found no pigmentation (Fig. 1). Colonies formed on NA plates were irregular, regular and circular form with convex elevation. The isolates T1, T2 and T3 were yellowish in colour, whereas T4 was white in appearance, but not transparent (Table 1). All the strains, except T3 were gram-positive.

Biochemical characteristics of endosymbiotic bacteria:

Biochemical tests were done for each bacterial isolate by VITEK 2 analyser (Table 1). According to the BLAST search test the isolates were identified T1, T2, T3 and T4*, Bacillus amyloliquefaciens, Staphylococcus hominis, Pantoea agglomerence* and *Bacillus cereus*, respectively (Table 2).

Molecular characteristics of endosymbiotic bacteria:

The bacterial isolates were identified based on 16S rDNA sequencing. PCR amplification yielded ~1500 bp amplicon in all the isolates. BLAST analysis of isolates T1, showed 100% similarity to *Bacillus amyloliquefaciens* available in the GenBank database and thus the bacterium was identified as *Bacillus amyloliquefaciens*. Whereas, isolate T2 showed 100% similarity to *Staphylococcus hominis* sequence, and T3 and T4 were 100% similarity to *Pantoea agglomerence* and *Bacillus cereus*, respectively. The sequence data generated from the study was deposited in the GenBank nucleotide database (NCBI) with accession numbers for the isolates T1, T2, T3 and T4 as OK135940, OK135975, OK136104, and OK148146, respectively. The bacterial isolates were successfully grouped to their respective reference strains obtained from the GenBank database, confirming the authenticity of the isolate. Phylogenetic analysis of 10 sequences of *Bacillus amyloliquefaciens* from gene bank along with the sequences of bacterial isolate T1 indicated that the *Bacillus amyloliquefaciens* was amonophyletic group (100% bootstrap value) (Fig. 1). Similarly of bacterial isolate T2, *Staphylococcus hominis* sequence along with 10 similar sequences from GenBank were used for the construction of phylogenetic tree (Fig. 2). Phylogenetic trees of bacterial isolate T3, *Pantoea agglomerence* (Fig. 3) and bacterial isolate T4, *Bacillus cereus* (Fig. 4) showed similar monophyletic group.

Discussion

Phenotypic, biochemical and 16S rDNA sequence analysis of endosymbiotic isolates prove the presence of cultivable bacteria namely *Bacillus amyloliquefaciences*, *Staphylococcus hominis,*

Table 1: Colony morphology of each isolate obtained from nutrient agar plates

Bacteria	Colour	Form	Margin	Elevation	Gram rection	Opacity
T1	Yellowish	Irregular	Entire	Convex	Positive	Not transparent
T2	Yellowish	Circular	Undulate	Convex	Positive	Not transparent
T3	Yellowish	Circular	Entire	Convex	Negative	Not transparent
T4	White	Regular	Entire	Convex	Positive	Not transparent

Table 2: Biochemical characteristics of bacterial isolates from *Aleurodicus dispersus*

S. No.	Biochemical parameters	Bacterial isolates			
		T1	T2	T3	T4
1	Beta- Xylosidase (BXYL)	+	-	-	-
2	L- Lysine Arylamidase (LysA)	-	-	-	-
3	L- Aspartate Arylamidase (AspA)	-	-	-	-
4	Leucine Arylamidase (leuA)	+	-	+	-
5	Phenylalanine Arylamidase (PheA)	+		+	-
6	L- Proline Arylamidase (ProA)	-	-	-	-
7	Beta- Galactosidase (BGAL)	-	-	+	-
8	L- Pyrrolydonyl Arylamidase (PyrA)	+	+	+	-
9	Alpha- Galactosidase (AGAL)	+	-	-	-
10	Alanine ARYLAMIDASE (AlaA)	-	-	-	-
11	Tyrosine Arylamidase (TyrA)	+	-	-	-
12	Beta- N- Acetyle Glucosaminidase (BNAG)	-	-	-	-
13	Ala- Phe- Pro Arylamidase (APPA)	+	-	-	-
14	Cyclodextrin (CDEX)	-	-	-	-
15	D- Galactose (DGAL)	-	+	-	-
16	Glycogen (GLYG)	-	-	-	-
17	Myo- Inositol (INO)	-	-	-	-
18	Methyl A- D- Glicopyranoside (MdG)	+	-	-	-
19	Ellman (ELLM)	-	-	+	-
20	Methyl- D- Xyloside (MdX)	-	-	-	-
21	Alpha Mannosidase (AMAN)	-	-	-	-
22	Maltotroice (MTE)	+	-	+	-
23	Glycine Arylamidase (GlyA)	+	+	-	-
24	D- Mannitol (dMAN)	+	-	-	+
25	D- Mannose (dMNE)	+	+	-	-
26	D- Melezitose (dMLZ)	-	-	-	-
27	N- Acetyl D- Glucosamine (NAG)	-	-	+	-
28	Palatinose (PLE)	-	-	-	-

29	L- Rhamnose (lRHA)	-	-	-	-
30	Beta Glucosidase (BGLU)	+	-	-	-
31	Beta Mannosidase (BMAN)	-	-	-	-
32	Phosphoryl Choline (PHC)	-	-	-	-
33	Pyruvate (PVATE)	+	-	+	-
34	Alpha Glucosidase (AGLU)	+	-	+	-
35	D- Tagatose (dTAG)	-	-	-	-
36	D- Trehalose(dTRE)	-	+	+	+
37	Inulin (INU)	-	-	-	-
38	D- Glucose (dGLU)	+	-	+	-
39	D- Ribose (dRIB)	+	+	+	+
40	Putrescine (PSCNa)	-	-	-	-
41	Growth in NaCl6.5% (NC6.5)	+	-	+	-
42	Kanamycine resistance (KAN)	-	-	+	-
43	Oleandomycine resistance (OLD)	-	-	-	-
44	Esculinhydrolysis (ESC)	+	-	+	-
45	Tetrazolium Red (TTZ)	-	-	-	-
46	Polymixin_B Resistance (POLYB_R)	-	-	+	-
47	Amygdalin (AMY)	-	-	-	-
48	Phosphatidylinositol Phospholipase C (PIPLC)	-	-	-	-
49	D- Xylose (dXLY)	-	-	-	-
50	Arginine Dihydrolase1 (ADH1)	-	+	-	-
51	Beta Galactopyranosidase (BGAR)	-	-	-	-
52	Phosphatase (PHOS)	-	+	-	-
53	Beta Glucuronidase (BGURr)	-	-	-	-
54	Beta Glucuronidase (BGUR)	-	-	-	-
55	D- Sorbitol (dSOR)	-	-	-	-
56	Urease (URE)	-	-	-	-
57	L- Lactate Alkalinization (ILATk)	-	+	-	-
58	Lactose (LAC)	-	+	-	-
59	D- Maltose (dMAL)	-	+	-	+
60	Bacitracinresistance (BACI)	-	-	-	-
61	Novobiocinresistance (NOVO)	-	+	-	+
62	Methyl B- D- Glycopyranoside (MBdG)	-	-	-	-
63	Pullulan (PUL)	-	-	-	-
64	D- Raffinose (dRAF)	-	+	-	-
65	O/129 resistance Vibrio. (O129R)	-	+	-	-
66	Salicin (SAL)	-	-	-	-

67	Saccharose (SAC)	-	-	-	+
68	Arginine Dihydrolase-2 (ADH2s)	-	-	-	-
69	Optochin resistance (OPTO)	-	+	-	+

Fig. 1: Endosymbiotic bacteria associated with *Aleurodicus dispersus.*

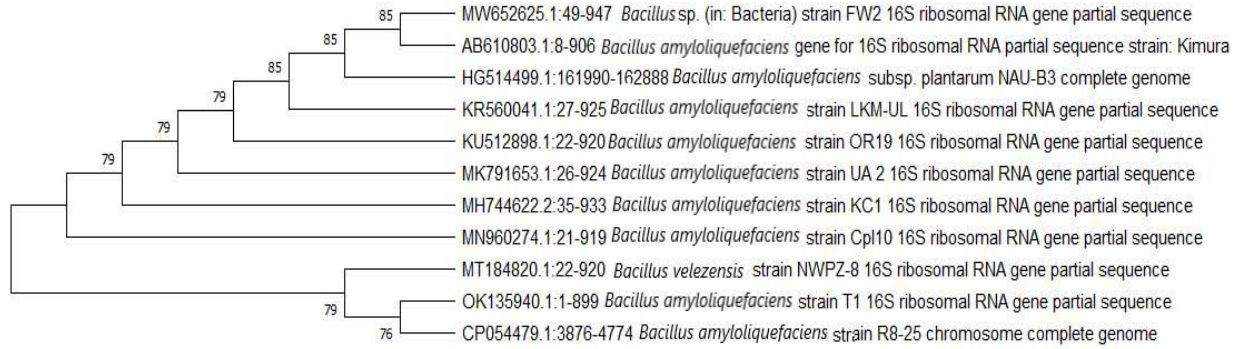

Fig. 2: Neighbor-joining (NJ) tree showing the evolutionary relationship of *Bacillus amyloliquefaciens* (T1) from the current study.

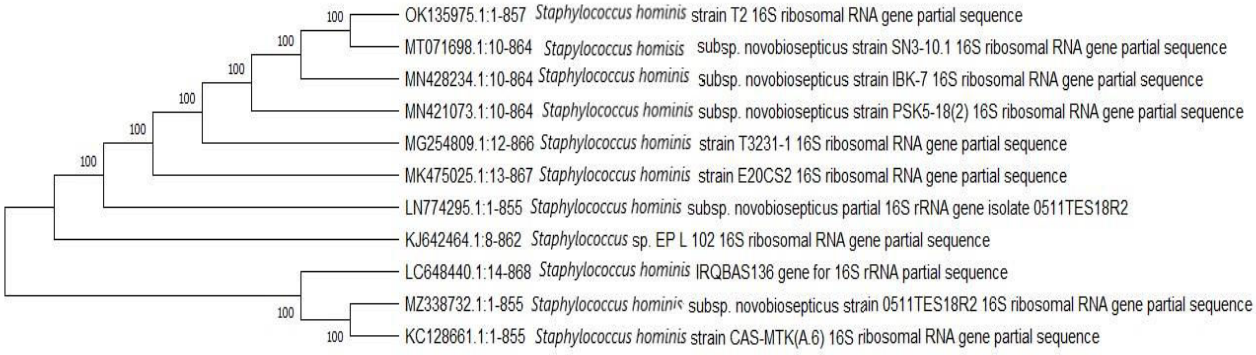

Fig. 3: Neighbor-joining (NJ) tree showing the evolutionary relationship of *Staphylococcus hominis* (T2) from the current study.

Fig. 4: Neighbor-joining (NJ) tree showing the evolutionary relationship of *Pantoea agglomerans* (T3) from the current study.

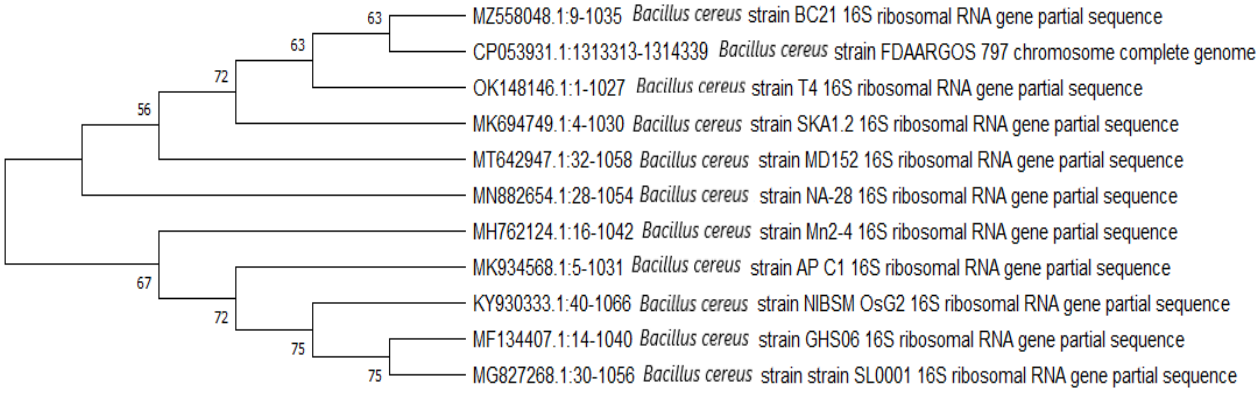

Fig. 5: Neighbor-joining (NJ) tree showing the evolutionary relationship of *Bacillus cereus* (T4) from the current study.

Pantoea agglomerence, and *Bacillus cereus* from *Aleurodicus dispersus.* Bacteria of genus *Bacillus* and *Staphylococcus* were previously reported from many insect species, but scanty reports are available on *Pantoea* as endosymbiont. Endosymbiotic bacteria are known to be involved in protecting their host insect against natural antagonists such as predators and pathogens or even implicated in insecticide resistance mechanisms (Zindel *et al.,* 2011). Such bacteria have an interesting target to detoxify pesticide by direct or indirect manipulation (Zabalou *et al.,* 2004; Zindel *et al.,* 2011). Exploration of potent endosymbiotic organisms is an important avenue in the insect pest management strategies. Bacteria of genus *Bacillus* and *Staphylococcus* were previously reported from many insect species as endosymbionts (Sreerag *et al*., 2014). Reports are also available for the presence of *Bacillus* and *Staphylococcus* in the gut of *Aedes aegypti* and *Bombyx mori,* respectively (Gusmao *et al.,* 2010; Feng *et al.,* 2011). *Bacillus* and *Staphylococcus* strains are well known for their potential to produce sucrose for sticky honeydew secreted by the host insect (Indiragandhi *et al.,* 2010). The dominant endosymbiotic species in *Phlebotomus papatasi* are reported to be *Bacillus cereus* and *Bacillus subtilis* (Mukhopadhyay *et al.,* 2012). *Bacillus* strains are capable to produce amylase enzyme which is involved in the initial breakdown of starch into simple sugars (Amund and Ogunsina, 1987; Oyewole and Odunfa, 1992). Molecular studies on the gut microbiota of ladybird beetle, *Coccinella septempunctata,*

revealed the association of *Lysinibacillus fusiformis* and *Bacillus cereus* with this insect, and it is said to be crucial for the survival and evolution of insect (Gadhave *et al*., 2016). *Bacillus cereus* can secrete a large array of proteinaceous and non-proteinaceous toxins acting on insects and mammals (Perchat *et al*.2005). Traits of *Bacillus* spp. associated with biocontrol mechanisms include production of antibiotics and extracellular hydrolytic enzymes such as chitinase, laminarinase, lipase and protease, and those contribute to the degradation of fungal cell walls (Korsten *et al*., 1993; Helisto *et al.*, 2001; Paulitz and Belanger, 2001). Species of *Bacillus* have been shown to be effective and are commercially applied as biological control agents against fungal and nematode pathogens and pests (Weller, 1988; Paulitz and Be langer, 2001; Jiang *et al.*, 2006).

Bacillus amyloliquefaciens is one of the most common bacterial endophytes infecting many plants, and that improvise plant disease resistance (Sun *et al*., 2006; Chen *et al*., 2009; Li *et al.*, 2013). Lipopeptides are among the main metabolites responsible for anti-pathogen activity in *Bacillus amyloliquefaciens* (Sun *et al.*, 2006; Ongena and Jacques, 2008; Alfonzo *et al*., 2012), and stimulate 'induced systemic resistance' (ISR) in plants (Ongena and Jacques, 2008; Choudhary and Johri, 2009). Koumoutsi *et al*. (2004) reported that endophytic *Bacillus amyloliquefaciens* can be used as an ideal biocontrol agent against plant pathogens.

Staphylococcus hominis, subsequently renamed as *S. hominis* subsp. *novobiosepticus* (SHN) is associated with nosocomial outbreaks (Kloos *et al*., 1998). Multidrug resistance, including resistance to novobiocin and oxacillin, is particularly important feature of SHN. In the current investigation, one of the bacterial strains associated with *Aleurodicus dispersus* was identified as *Staphylococcus hominis.*

Pantoea, recognized as a multi host pathogen, colonizing various hosts, including insects, plants, and humans and are often isolated from aquatic and terrestrial environments, and also from insects, animals and humans (Muraschi *et al.*, 1965; Volksch *et al.*, 2009; Nadarasah and Stavrinides, 2014). Certain species of *Pantoea* are also recognized as plant pathogens causing galls, wilting, soft rot and necrosis in a variety of agriculturally relevant plants. Some isolates of the bacteria are antibiotic producers and have been developed into biocontrol agents for the management of plant diseases. *Pantoea* is frequently isolated from the nosocomial environment; nevertheless, its role in human disease is controversial (Walterson and Stavrinides, 2015). Much of the research surrounding the association of *Pantoea* with insects has stemmed from the specific adaptations of *Pantoea stewartii* subsp *stewartii* DC283 (DC283) to its flea beetle vector (Roper, 2011).

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