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K Abinaya
PG Scholar, Department of Agricultural Entomology, TNAU, Coimbatore, Tamil Nadu, India

JS Kennedy
Professor, Department of Agricultural Entomology TNAU, Coimbatore, Tamil Nadu, India

R Anandham
Assistant Professor, Department of Agricultural Microbiology, TNAU, Coimbatore, Tamil Nadu, India

Corresponding Author:
JS Kennedy
Professor, Department of Agricultural Entomology TNAU, Coimbatore, Tamil Nadu, India

Biochemical characterization of culturable bacterial endosymbionts of papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) from papaya plants

K Abinaya, JS Kennedy and R Anandham

Abstract
Papaya mealybug, *Paracoccus marginatus* is a polyphagous pest with wide host range and reported to cause heavy economic loss upon infestation. Use of chemicals was found to be ineffective in controlling this exotic pest. The best alternative to control the pest is by means of biocontrol management strategies. Microorganisms, mostly bacteria were found to be in symbiotic relationship with several insects and they play a key role in the growth and development of the insects. In the present study, twenty six culturable bacterial endosymbionts of papaya mealybug viz., 7, 10, 3 and 6 from LB, R_2A, NB and TSA media were isolated and characterized biochemically (carbohydrate fermentation, IMViC tests [Indole production, Methyl red, Voges-Proskauer test, and Citrate utilization test] and starch hydrolysis). Based on the results, the isolated gut bacteria were tentatively identified as *Bacillus*, *Burkholderia*, *Serratia*, *Escherichia* and *Pseudomonas*.

Keywords: Papaya mealybug, bacterial endosymbionts, biochemical characteristics

Introduction
The Central American native polyphagous pest, papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink has spread all over the world and has a wide host range of about 133 plants including both economic and weed hosts [12]. The pest was reported to cause heavy economic loss which ranged even upto 60 per cent and more [9]. Most of the insects govern microorganisms either inside or outside their body and they exhibit a symbiotic relationship with them. In insects, these microbes may be present as a gut microbe, ectosymbionts, endosymbionts, etc. Among them, endosymbionts are the most significant association as they are transmitted from generation to generation and play a key role in the lifecycle completion of the insects. These endosymbionts fall into two categories *viz.*, P-endosymbionts (which are obligatory and is essential for insect survival as they mainly aid in nutrition supplement) and S-endosymbionts (which have a facultative mutualistic relationship with insects). Among these two, only S- endosymbionts can be cultured under laboratory conditions [2].

Klebsiella pneumoniae, *Enterococcus faecalis* and *Pantoea* sp. were reported to be the most common culturable bacteria from the gut region of southern green stink bug, *Nezara viridula* [7]. The stored tubers mealybug, *Rhizoecus amorphophalli* was reported to have three species of culturable bacteria *viz*., *Staphylococcus gallinarum*, *Staphylococcus saprophyticus* and *Bacillus subtilis* [13]. Three culturable bacteria *viz*., *Bacillus endophyticus*, *Bacillus niacini*, *Roseomonas* sp. were isolated and characterized from the honeydew of whitefly, *Bemisia tabaci* [11]. *Erwinia persicinus*, *Pseudomonas plecoglossicida*, *Pseudomonas putida*, *Brevibacterium casei*, *Staphylococcu gallinarum*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Exiquobacterium acetylicum*, *Exiquobacterium undae* and *Micrococcus caseolyticus* were the species of bacteria isolated from sweet potato whitefly, *Bemisia tabaci*(1). *Cardinium* and *Arsenophonus* species were identified to be the S-endosymbionts of Cassava whitefly species, *Aleurotrachelus socialis* and *Trialeurodes variabilis* [6].

This present study was aimed to throw light upon diversity of culturable bacterial endosymbionts in papaya mealybug, *P. marginatus* and their biochemical characteristics.

Materials and Methods

Mass culturing of papaya mealybug in laboratory conditions

The papaya mealybugs were collected from papaya field, TNAU orchard ($11^0$0'86" N, 76^0 93'13" E) Coimbatore. The mealybugs were released on the papaya plants at the rate of 3 to 5 ovisacs per plant and reared for generations in insect cages (60 x 60 cm). These reared mealybugs were used for isolation of endoymbionts.

Isolation of culturable bacterial endosymbionts from papaya mealybug

The pre starved (6-8 h) second and third instar nymphs (100-150 numbers) of papaya mealybug were surface sterilized with 70 per cent ethanol followed by 5 per cent sodium hypochlorite, then washed with sterile water for 60 s for each sterilization to remove the ectosymbionts. The surface sterilized nymphs were homogenized with 1ml of 0.1M phosphate buffer (pH 7.0). The nymphal homogenates were diluted to 10^{-1}, 10^{-2} and 10^{-3} dilutions and then plated on different media *viz.,* Nutrient agar, Luria Bertani agar, Tryptone soya agar, R_2A, Endo agar, Mac Conkey agar, and MRS agar. The plates were incubated at 28 ± 2 ºC for 24 h [4]. The grown bacterial colonies were selected and purified based on their morphological characters such as shape, colour and elevation after 24 h of incubation. The purified bacterial isolates were examined through light microscope and maintained in 50 per cent glycerol at -80 ºC. The bacterial isolates were revived in nutrient broth for further experimental purposes.

Biochemical characterization

The bacterial isolates were initially Gram-stained and subjected to basic biochemical characterization including carbohydrate fermentation, IMViC tests and starch hydrolysis [3].

Carbohydrate fermentation

The fermentative tests were performed for various carbohydrates such as glucose, sucrose, lactose and starch using Durham's tubes. The nutrient broth with a specific carbohydrate @ 1 per cent (glucose/sucrose/lactose/starch) and phenol red as pH indicator was used for carbohydrate fermentation. 1 per cent of the respective bacterial isolate was inoculated and incubated for 48 h at 28 ± 2 °C along with control. The tubes were observed for colour change from red to yellow (due to production of acid) or colour change with appearance of bubbles in Durham's tube (due to production of acid and gas).

IMViC tests

The IMViC include four different tests *viz.*, indole production tests, methyl red tests, Voges-Proskauer tests and citrate utilization.

Indole Production tests

Indole production was tested by using tryptone broth (1%) to which 1 per cent of the bacterial culture was inoculated and incubated at 28 ± 2 °C for 48 h. 1 ml of Kovac's reagent was added and shaken gently for 10-15 mins at regular intervals and allowed to stand. The appearence of deep cherry red colour at the top layer was considered as a positive test (indole reacts with Kovac's reagent to produce deep cherry red Rosindole dye)

Methyl red and Voges Proskauer (VP) tests

Both the methyl red and Voges Proskauer tests were performed by using the MRVP broth. The bacterial culture (1%) was inoculated and incubated for 24-48 h at 28 ± 2 °C. In methyl red test, after incubation, five to six drops of methyl red indicator was added. The retention of original red colour was considered to be positive while, colour change from red to yellow was considered as negative. In case of VP tests, 1-2 drops of naphthol solution (VP reagent I) followed by 2-3 drops of 40 per cent potassium hydroxide (VP reagent II) was added and the tubes were shaken gently with their caps open to expose the media to oxygen. The reaction was allowed to complete for 15-30 min. The positive reaction was indicated by the development of crimson to ruby pink colour to red colour.

Citrate utilization

Citrate utilization test was done by inoculating the bacterial cultures into Simon's citrate agar slants with bromothymol blue as an indicator. The inoculated slants were incubated at room temperature for 24-48 h. The positive test was the change of colour from green to blue.

Starch hydrolysis (Amylase production test)

The isolated bacterial cultures were tested for starch hydrolysis by streaking and incubating them on the starch agar medium for 24-48 h at 28 ± 2 °C. After incubation, the surface of the plates was flooded with iodine solution for 30 sec. The plates were examined for the formation of a clear zone around streak line which was considered as positive reaction.

Results

Gut bacteria isolation and biochemical characterization

On isolation of culturable bacterial endosymbionts from papaya mealybug, *Paracoccus marginatus*, a total of 26 bacterial isolates were obtained. The number of bacterial isolates were 7, 10, 3, and 6 in the LB, R2A, NB, and TSA media, respectively. They were assigned the code PMB (Papaya Mealybug Bacteria) in sequence from 1 to 26 (PMB1 to PMB26) for easy identification and differentiation purposes.

The results of biochemical tests of all the isolates were listed in Table 1. Bacterial isolates from papaya mealybug were mostly Gram-negative and showed positive results for carbohydrate fermentation and IMViC tests.

Carbohydrate fermentation

Acid production from glucose and sucrose was observed in almost all the isolates except 7 (PMB 1, 3, 5, 8, 15, 17 and 19) and 3 isolates (PMB 4, 5 and 16), respectively. Lactose was fermented only by 10 isolates (PMB 3, 7, 8, 9, 11, 13, 17, 18, 20 and 23). Seven isolates (PMB 2, 11, 13, 15, 18, 23 and 26) showed positive results for starch fermentation.

IMViC tests

Citrate was utilized by almost all the bacterial isolates as carbon source except five isolates (PMB 8, PMB 16, PMB 18, PMB 19 and PMB 20). In the present study, all the 26 isolates failed to produce indole and hydrolyze the starch.

Discussion

Based on the basic biochemical characterization, the isolates PMB 1, PMB 2, PMB 12, PMB 14, PMB 18 and PMB 23

were tentatively identified as *Bacillus* sp. as they are Gram-positive strain and reacted positive to methyl red test, citrate utilization and sucrose fermentation and showed negative results towards lactose fermentation, indole production and VP tests. Similar results were shown when five bacterial isolates (Rhizo 1, Rhizo 2, Rhizo 3, Rhizo 4, Rhizo 5) were isolated from stored tuber mealybug, *Rhizoecus amorphopalli*. The biochemical tests of the isolates revealed that the isolates Rhizo 1, Rhizo 2, Rhizo 3 were identified as *Bacillus* sp. as they showed positive results for citrate utilization and negative for indole production and Voges- Proskauer tests [13].

The four isolates *viz.*, PMB 8, PMB 16, PMB 19 and PMB 20 showed negative results for citrate utilization and Voges-Proskauer. These results are in line with biochemical characters of *Escherichia* sp. (10). PMB 4, PMB 5, PMB 6, PMB 9, PMB 15, PMB 17, PMB 21, PMB 22, PMB 24, PMB 25 and PMB 26 may probably be *Pseudomonas* sp. since they gave negative results for Voges- Proskauer tests as reported earlier from the gut bacterial isolates of diamondback moth, *Plutella xylostella* [8]. The remaining isolates *viz.*, PMB 3, PMB 7, PMB 10, PMB 11 and PMB 13 may be either *Serratia* sp. or *Burkholderia* as they were positive for Voges-Proskauer tests [8, 5]. Since biochemical characterization was used for preliminary identification of bacteria, the molecular identification of these isolates is yet to be done by using the 16S rRNA sequencing for confirmation upto species level.

Table 1: Biochemical characterization of bacterial isolates of papaya mealybug from papaya

SI No.	Isolate	Gram staining	Carbohydrate fermentation				IMViC tests				Starch hydrolysis
			Glucose	Sucrose	Lactose	Starch	Indole production	Methyl red test	Voges Proskauer test	Citrate utilization	
1	PMB 1	+	-	+	-	-	-	+	-	+	-
2	PMB 2	+	+	+	-	+	-	+	-	+	-
3	PMB 3	-	-	+	+	-	-	+	-	+	-
4	PMB 4	-	+	-	-	-	-	+	-	+	-
5	PMB 5	-	-	-	-	-	-	+	-	+	-
6	PMB 6	-	+	+	-	-	-	+	-	+	-
7	PMB 7	-	+	+	+	-	-	-	+	+	-
8	PMB 8	-	-	+	+	-	-	+	-	-	-
9	PMB 9	-	+	+	+	-	-	+	-	+	-
10	PMB 10	-	+	+	-	-	-	-	+	+	-
12	PMB 11	-	+	+	+	+	-	-	+	+	-
13	PMB 12	+	+	+	-	-	-	-	+	+	-
14	PMB 13	-	+	+	+	+	-	-	+	+	-
15	PMB 14	+	+	+	-	-	-	+	-	+	-
16	PMB 15	-	-	+	-	+	-	+	-	+	-
17	PMB 16	-	+	-	-	-	-	+	-	-	-
18	PMB 17	-	-	+	+	-	-	+	-	+	-
19	PMB 18	+	+	+	+	+	-	+	-	-	-
20	PMB 19	-	-	+	-	-	-	+	-	-	-
21	PMB 20	-	+	+	+	-	-	+	-	-	-
22	PMB 21	-	+	+	-	-	-	+	-	+	-
23	PMB 22	-	+	+	-	-	-	+	-	+	-
24	PMB 23	+	+	+	+	+	-	+	-	+	-
25	PMB 24	-	+	+	-	-	-	+	-	+	-
26	PMB 25	-	+	+	-	-	-	-	-	+	-
27	PMB 26	-	+	+	-	+	-	-	-	+	-

*PMB-Papaya mealybug

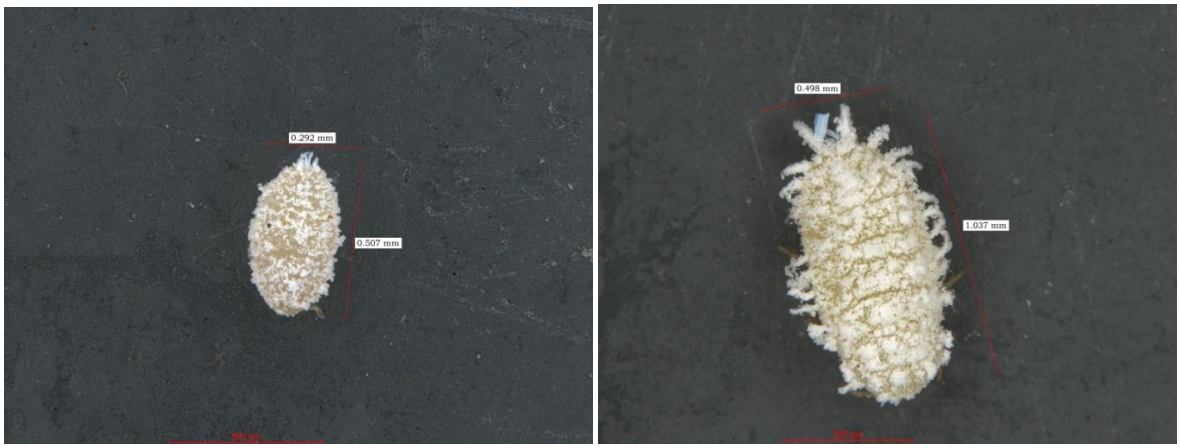

Fig 1: Nymphs of Papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink, (a) 2nd instar (b) 3rd instar

Conclusion

Based on the above results, the obtained culturable bacterial isolates can be identified as *Bacillus* sp. or *Escherichia* or *Pseudomonas* or *Serratia* or *Burkholderia* sp.

Endosymbionts of insects have several functions to mediate that are essential for survival and development of insects [14].

These endosymbionts were reported to coevolve with their respective hosts for the successful maintenance of their symbiotic relationship [15]. Therefore, complete understanding on papaya mealybug gut bacteria can be used as a new biocontrol tool in pest management strategy.

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