DOI : [http://doi.org/10.22438/jeb42/4\(SI\)/MRN-1566b](http://doi.org/10.22438/jeb42/4(SI)/MRN-1566b)

Antibiotic susceptibility of endosymbionts of papaya mealybug *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae)

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Received: 24.12.2020

Revised: 25.02.2021

Accepted: 03.03.2021

Abstract

Aim: The present study aimed to select a powerful tool to evaluate the antibiotic susceptibility of endosymbionts of papaya mealybug.

Methodology: Antibiotic Ofloxacin (0.25µg) was used to disrupt the endosymbionts of mealybug. The antibiotic susceptibility of endosymbionts was studied by conventional method; isolation of symbionts in Luria Bertani Agar and Nutrient Agar and also metagenomic analysis was conducted using nanopore sequencing of 16S rRNA gene.

Results: Molecular identification of isolates showed Gram-positive bacteria i.e., *Bacillus clausii*, *B. altitudinis*, *B. siamensis* and Gram-negative bacteria, i.e., *Serratia marcescens* and *Stenotrophomonas maltophilia*. Metagenomic analysis using 16S amplicon sequencing resulted in identification of *Candidatus Tremblaya Princeps* (55%) was more abundant followed by *Candidatus Mikella endobia* (23%) in untreated mealybug population. Their population was reduced to 7% in *Candidatus Tremblaya Princeps* and below 1% in *Candidatus Mikella endobia*.

Interpretation: The current study confirms that the metagenomic analysis acts as a power lens to evaluate the antibiotic elimination of endosymbionts, hence with this method future works on developing aposymbiotic mealybugs can be done.

Key words: Endosymbiont, Metagenomic analysis, Ofloxacin, Papaya mealybug

How to cite : Megaladevi, P. and J. S. Kennedy: Antibiotic susceptibility of endosymbionts of papaya mealybug *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae). *J. Environ. Biol.*, **42**, 1191-1194 (2021).

Introduction

The papaya mealybug (PMB) *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) was recorded for the first time in papaya during July 2008, in Coimbatore district Tamil Nadu. Severe infestation 80 to 90% was observed papaya, mulberry, tapioca, brinjal, tomato, bhendi and flower crops (Muniappan *et al.*, 2008). Throughout their life, papaya mealybug, *P. marginatus* depends on endosymbiotic microorganisms for the supply of essential amino acids and other nutrients, whereby they can live solely on the specialized food source. In the current scenario, the disruption of microbial symbiosis demanded by insect pest and symbionts-mediated manipulation of insect pest traits are the two emerging trends in insect pest control.

Researchers have been unable to isolate the endosymbionts from insect body and culture the same in laboratory conditions. Unfortunately, traditional microbiology and microbial genome sequencing and genomics rely upon cultivated clonal cultures; early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of microbial diversity in a natural sample. Hence, through traditional microbiological work, the vast majority of microbial biodiversity may miss from documenting. Metagenomics is the study of genetic material recovered directly from environmental samples and use either "shotgun" or PCR directed sequencing to get mostly unbiased samples of all genes from all the members of sampled communities. Because of its ability to reveal the previously hidden diversity of microscopic life, metagenomics offer a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world (Schloss and Handelsman, 2005). It appears that papaya mealybug houses number of secondary endosymbionts and many of them are not culturable in laboratory. These facultative secondary endosymbionts may play several roles in its host physiology viz., offers defense towards pathogens and parasites, influence insect- plant interaction, favours the host insect for adaptation to environment, impact on population dynamics and pesticide detoxification.

Materials and Methods

Isolation of culturable endosymbiotic bacteria from papaya mealybug: The culturable endosymbiotic bacteria of antibiotic treated (Ofloxacin @ 0.25µg) and untreated papaya mealybug from papaya host plant were isolated. Second and third instar nymphs (50 numbers) were taken and starved for 6–8 hr to eliminate the bacterial flora acquired through feeding the host plant. The starved nymphs were then surface sterilized with 70% ethanol followed by 0.1% sodium hypochlorite for 30 sec to remove the adhering contaminants, especially external microflora. The remnants of disinfectants used for surface sterilization were then cleared by washing thoroughly with distilled water. After final wash, washed distilled water was plated on culture media to

ensure complete elimination of external microflora. Surface-sterilized nymphs were homogenized using 1 ml of 0.1M phosphate buffer in pestle and mortar.

The homogenates were serially diluted up to 10^{-3} . A 100µl of each dilution of 10^{-1} , 10^{-2} and 10^{-3} were plated separately by pour plate method on two different sterile media such as Luria Bertani agar (10g tryptone, 5g yeast extract, 10g NaCl and 15g agar) and Nutrient Agar (5g peptone, 3g beef extract, 5g NaCl and 20g agar) and incubated at $28 \pm 2^\circ\text{C}$ for 24h – 72h (de Vries and Visser, 2001). After incubation, the colonies grown on different media were selected on the basis of morphological characteristics such as shape, color and elevation. The selected colonies were subjected to sub-culturing on their respective medium for purification. Five to six subsequent streaking was done to obtain pure bacterial cultures. The purified cultures of endosymbiotic bacteria were maintained by sub-culturing on their respective medium for every 15 days. The purified cultures were examined under a light microscope and stored at -80°C in 50% glycerol.

Molecular identification of bacterial isolates: The bacterial genomic DNA of bacterial cultures obtained from papaya mealybug was isolated using EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt. Ltd., India as per the protocol described in the instruction manual. The isolated DNA was then amplified through PCR (Polymerase Chain Reaction) targeting the 16s ribosomal RNA (rRNA) gene using 27F (forward primer): 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R (reverse primer): 5'-GGTACCTGTACGACTT-3'. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using primers. Sequencing reactions were performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Bio-systems).

Metagenomic analysis of papaya mealybug: About 50, 3rd instar nymphs were taken from each PMB clone and used for metagenomic analysis. Genomic DNA was isolated from the surface sterilized mealybugs using c-TAB and phenol: chloroform extraction method. The isolated DNA was quantified using nanodrop by determining the A260/280 ratio. The endosymbiotic profile of papaya mealybug were studied using Nanopore sequencing platform (Instrument make, Company, Location) with primers 27F 5' AGAGTTTGATCMTG and 1492R 5'AAGGAGGTGATCCAGCCGCA.

Results and Discussion

Molecular identification of isolates showed Gram-positive bacteria, i.e., *Bacillus clausii*, *B. altitudinis*, *B. siamensis* and Gram-negative bacteria, i.e., *Serratia marcescens* and *Stenotrophomonas maltophilia* (Table 1). Through conventional isolation and identification only few endosymbionts were isolated and cultured under laboratory conditions. It was not impossible to evaluate the antibiotic

Table 1: Molecular characterization of culturable endosymbiotic bacteria isolated from papaya mealybug

Isolates	Colony morphology	Gram test	Closest match	Genbank accession number
TNAUPS1	White, irregular	+	<i>Bacillus altitudinis</i>	Mn907690
TNAUPS2	Cream white, filamentous	+	<i>Bacillus clausii</i>	Mn907691
TNAUPS3	Creamy white, translucent	+	<i>Bacillus siamensis</i>	Mn907692
TNAUPS4	Red elevated, entire margin	-	<i>Serratia marcescens</i>	Mn907693
TNAUPS5	White convex, smooth	-	<i>Stenotrophomonas maltophilia</i>	Mn907694

Fig. 1: Per cent abundance of bacterial endosymbionts from papaya mealybugs: (A) At genus level in control mealybug population; (B) At species level in control mealybug population; (C) At genus level in aposymbiotic mealybug population and (D) At species level in aposymbiotic mealybug population.

susceptibility as all the endosymbionts were not cultivable. As there are number uncultivable endosymbionts in insects, hence, metagenomic analysis was performed to explore the whole endosymbiotic profile of Papaya mealybug. Metagenomic analysis indicated that *Candidatus Tremblaya Princeps* (55%) was more abundant followed by *Candidatus Mikella endobia* (23%) in untreated population. *Candidatus Tremblaya Princeps* was reported as the primary endosymbiont of papaya mealybug and its presence is important for the survival and reproduction of its host insect. Their population reduced to 7% in *Candidatus Tremblaya Princeps* and below one per cent in *Candidatus Mikella endobia*.

This clearly confirms the influence of antibiotic ofloxacin on the abundance of endosymbionts of papaya mealybug (Fig. 1). While experimenting the isolation of bacterial endosymbiont associated with mealy bug, *Rhizococcus amorphophalli* (Hemiptera: Pseudococcidae), three culturable bacteria, namely, *Bacillus subtilis*, *Staphylococcus gallinarum* and *S. saprophyticus* were reported by Sreerag *et al.* (2014). Sreerag *et al.* (2014) reported three culturable bacteria, namely, *Bacillus subtilis*, *Staphylococcus gallinarum* and *S. saprophyticus*. The protective role of *Serratia marcescens* as an extracellular endosymbiont of *Rhynchophorus ferrugineus* was first reported by Scrase *et al.* (2016). *Stenotrophomonas* sp. has been

recognized to be associated with insects like Collembola. Indiragandhi *et al.* (2007) isolated *Pseudomonas* sp. and *Stenotrophomonas* sp. from the guts of larvae and adults of diamond back moth. Previous studies revealed that different lineages of mealybugs are associated with distinct lineages of bacterial endosymbionts. For example, many species of subfamily Pseudococcinae harbour a beta-proteobacterial endosymbiont *Tremblaya princeps* and an additional gamma-proteobacterial endosymbiont (McCutcheon and Von Dohlen, 2011; Oliver, *et al.*, 2005; Tamura *et al.*, 2011; Thao, *et al.*, 2002). The primary endosymbionts *T. princeps* are found in almost all Pseudococcine species and exhibit host-symbiont co-speciation (Muniappan *et al.*, 2008; Von Dohlen, *et al.*, 2001). In this study, metagenomics offers access to genetic diversity of uncultivable microbial communities. In future there is a need to explore their metabolic diversity.

In summary, metagenomic data have been useful in understanding the endosymbiotic profile in papaya mealybug with the establishment of both primary and secondary endosymbionts.

Acknowledgments

This study was conducted in partial requirement for PhD at the Tamil Nadu Agricultural University, Coimbatore. We duly Yaazh Xenomics, Coimbatore for assistance in the metagenomic analysis.

Add-on Information

Authors' contribution: P. Megaladevi: Sample collection, DNA extraction, Molecular Biology, Bioinformatics, Data interpretation, and Manuscript writing; J.S. Kennedy: Conceptualization of study, Bioinformatics, Data interpretation, and Manuscript writing.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: NotApplicable

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: NotApplicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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