

Original Research Article

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Morphological and Biochemical Characterization of Bacteria Associated with the Developmental Stage of the Peach Fruit Fly, *Bactrocera zonata* (Diptera: Tephritidae)

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ABSTRACT

Bacterial-insects associations are very common, can be parasitic to mutualistic and reside in the gut, hemocoel and body cells of insects, which play an important role in their nutrition, digestion and development. The aim of present study was to morphological and biochemical characterization of the bacteria isolated from the different developmental stages of the peach fruit fly, *Bactrocera zonata*. The total 35 bacterial colonies on the basis of colony morphology were screened belongs to various genera different family, including rods of both gram-positive as well as gram-negative and only few gram positive coccus and rod-coccus. Out of total bacterial isolates, thirteen different bacterial species belonging to eleven genera (seven families) were identified from the developmental stage (first Instar larvae, third Instar larvae, pupa, male and female adults) of *Bactrocera zonata*. The bacteria species viz., *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter* sp., *Providencia* sp., *Pantoea* sp., *Citrobacter freundii*, *Pseudomonas* sp., *Enterococcus* sp., *Ochrobactrum* sp., *Bacillus* sp., *Microbacterium* sp., and *Rhodococcus* sp. were identified to be associated with different life stages. Genera *Klebsiella*, *Enterobacter* and *Pseudomonas* of family, Enterobacteriaceae were found in all the development stages. Therefore, the present study significantly supplements to the available information on the cultivable bacterial diversity associated with the developmental stages of *B. zonata*.

Keywords

Bactrocera zonata,
Developmental
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Introduction

Bacterial-insects associations including both intracellular and intercellular are very common in nature and known since the last century (Petri, 1909). Associations between them are close and complex ranging from

parasitism to mutualism, with a long period of association and co-evolution history (Dillon and Dillon 2004; Dale and Moran, 2006). The bacterial communities in insects mostly reside in the gut, mycetomes, hemocoel and within the cells. The associated bacteria plays an important role in host's nutrition, digestion,

protection, detoxification of insecticides, resistance to pathogens, and semiochemicals production (Dillon and Dillon 2004; Russell and Moran 2006; Oliver *et al.*, 2003; Engel and Moran 2013; Feldhaar, 2011; Douglas, 2015; Hammer and Bowers 2015; Ezenwa *et al.*, 2012; Wingfield *et al.*, 2016; Hosokawa *et al.*, 2017). The bacterial community associated with the insects is likely to be influenced by various factors such as diet and the host on which insects feed (Ferrari *et al.*, 2007; Hosokawa *et al.*, 2017; Wagner *et al.*, 2015; Medina *et al.*, 2011). The variations in the bacterial communities have been reported at different stages of insects' development (Aharon *et al.*, 2013; Malacrinò *et al.*, 2018).

The Tephritid (Dipteran : Tephritidae) fly are commonly known as “fruit flies”. The peach fruit fly, *Bactrocera zonata* (Saunders.) is a polyphagous pests, which is a menace to more than 50 species of fruit crops and wild plants (Duyck *et al.*, 2004) particularly in South Asia (including India), Southeast Asia and some parts of Africa (Kapoor, 1993, CABI/EPPO 2013). *B. zonata* is considered as one of the most destructive fruit pests species of peach (*Prunus persica* (L.) Batsch), guava (*Psidium guajava* L.), mango (*Mangifera indica* L.) in tropical and subtropical climatic countries (Choudhary *et al.*, 2015). Instead of enormous damage potential of *B. zonata*, the studies have been limited to its biology, distributions and population genetic structure (Duyck *et al.*, 2004; Choudhary *et al.*, 2015; Choudhary *et al.*, 2018).

To the best of our knowledge, very few studies have been carried out to access the bacterial communities associated with *B. zonata* adult flies using culture-dependent techniques (Reddy *et al.*, 2014; Naaz *et al.*, 2016) and culture-independent techniques across ontogeny (Naaz *et al.*, 2020). However, there are no previous reports on the characterization of the cultivable gut bacterial

communities associated with the *B. zonata* across different developmental stages. The present study was undertaken to isolate and morphologically as well as biochemically characterize the dominant bacteria associated with the different developmental stages *B. zonata*. This information will act as baseline for subsequent studies such as roles of these microorganisms in their development and management.

Materials and Methods

Sample collection and rearing

Fruit flies infested wood apple (*Aegle marmelos* L.) fruits were collected from Research farm of ICAR Research Complex for Eastern Region, Research Centre (ICAR-RCER, RC), Ranchi, India (23° 45'N latitude, 85° 30' E longitude, elevation 620 m AMSL) in May, 2018. Subsequent rearing (up to 6 generations) was carried out in the laboratory at ambient room conditions (25±1°C temperature; 65±5% RH; 12:12 h LD photoperiod). The infested fruits were kept individually in 20 × 15 cm cage with 5 cm of thick sterile fine sand until emergence of the adults. The adults pair (male and female) of *B. zonata* was released into a smaller rearing cage (30×30×30 cm) provided with their natural host (Bael) for oviposition. The feeding was supplemented with adult diet [(glucose and protein hydrolyzate (Protinex®, Pfizer Ltd., India) in the ratio of 1:1 in Petri plates)] and water ad-libitum through soaked cotton swabs in a 50 ml beaker. The food supplements were replaced weekly. After oviposition, the 1st instar larvae were directly collected by cutting the infested fruits of wood apple with fruit fly. Different stages of *B. zonata* were reared on same host fruits in the laboratory conditions according to protocol described by Choudhary *et al.*, (2020) and collected for further experiments. Larvae that emerged from the fruits and

moved out to the sand for pupation were collected for 3rd instar larval stage. A fair number of 3rd instar larvae were left in the soil to pupate in order to obtain pupal stage samples and other pupae were left for the emergence of adult stage. Five days old adult male and females were collected to study the microbiota associated with the adult stage.

Dissection and isolation of bacteria

The bacteria were isolated from gut of first instar larvae and third instar larvae, mid-aged pupae (whole-body) and 5-day-old male and female adults of *B. zonata*, following the protocol described by Liu *et al.*, (2016). Before being dissected, adults were anaesthetized at -18°C for 5 min. Samples (larvae, pupae and adults) were washed with ethanol (75%) for 30 s, followed by sodium hypochlorite (1%) for one minute and finally rinsed with sterilized water for three times to remove surface contamination. Five individuals of larvae, five male and five female flies were dissected aseptically in a petri dish under a stereoscope, and then all the dissected tissues and five pupae were placed in a 1.5 mL centrifuge tube containing 400 μL of sterile water, respectively. The tissue suspension was blended under a laminar air flow hood. There were three biological replications for each treatment. To isolate the cultivable gut bacteria, the blended samples were diluted with four concentrations (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}). One hundred microliters of diluent was separately spread onto PYEA (Peptone yeast extract agar) and NA (Nutrient Agar) medium and incubated at $37\pm 1^{\circ}\text{C}$ for 24–48 h for bacterial growth. Single colony of each of the bacterial isolates was separated with the inoculation loop and streaked onto respective PYEA and NA plates for their growth. Predominant bacterial isolates were obtained through repeated sub-culturing to ensure their purity. The purified bacterial isolates with respective medium were

maintained on PYEA slants and/or plates at $4-8^{\circ}\text{C}$ for further use.

Morphological and biochemical characterization of gut bacteria

Morphological (Shape, Gram's staining), cultural (Pigment production, growth in broth medium) and biochemical (citrate, methyl red, Voges–Proskauer (V.P.), triple sugar iron (TSI), catalase, oxidase and carbohydrate fermentation tests) characterization of the pure culture was done by standard techniques and isolates characteristics were compared with Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 2000).

Results and Discussion

Isolation of bacterial isolates associated with the developmental stage of the peach fruit fly, *Bactrocera zonata*

Bacteria associated with the different developmental stage (First Instar larvae, Third Instar larvae, Pupa, male and female Adult) of the peach fruit fly, *Bactrocera zonata* were isolated on two culture media *viz.* Peptone Yeast Extract agar (PYEA) and nutrient agar (NA) media (enriched culture media). It was found that bacteria were associated with all the developmental stage of the *B. zonata*. A total of 35 different bacterial colonies were observed on two different enriched culture media (Table 1).

Morphological characterization

The bacterial isolates isolated from the different developmental stages of *B. zonata* were mostly rod shaped except two bacterial isolates namely BL1G and B13D were coccus and BM5 was Rod-coccus shaped isolated from larval stage and male adult flies. All the bacterial isolated were Gram-negative but few isolates such as BL1E and BL1G in first

instar larvae, BL3B and BL3H in third instar larvae, BM4 and BM5 in male adult flies and only BF5 in female adult flies were Gram-positive in nature (Table 2). Most of the isolates were found non-motile, except few isolate in each developmental stage i.e. BL1B and BL1E in first instar larvae, BL3E, BL3F and BL3G in third instar larvae, BP2, BP3 and BP6 in pupa, BM1 and BM4 in male adult flies and BF2 and BF4 in female adult flies were motile. On the basis of growth in broth, all the bacterial isolates of different stages have sediment growth while only bacterial isolate BM5 showed pellicular formation in broth medium.

Biochemical characterization

In first instar larvae, Isolate BL1A and BL1C gave a positive reaction for citrate, VP, Indole, catalase and carbohydrate fermentation whereas, a negative reaction for MR, oxidase and TSI test. However, isolate BL1D have similar results for all the biochemical tests except indole negative. Isolate BL1B was citrate, catalase, oxidase and D-glucose positive while MR, VP, Indole, TSI, and gas production in glucose medium negative. A positive reaction for catalase, VP and D-glucose was observed for isolate BL1E and it was negative for MR, Indole and gas production in glucose medium while doubtful for oxidase, citrate and TSI tests. BL1F gave a positive reaction for citrate, MR, Indole, catalase and D-glucose whereas, a negative reaction for VP, oxidase and TSI test. The only cocci isolate BL1G gave positive reaction for VP, D-glucose and few other carbohydrates whereas, a negative reaction for citrate, Indole, catalase, oxidase and TSI test (Table 2).

In third instar larvae, BL3A isolate showed positive reaction for citrate, VP, catalase and D-glucose whereas, a negative reaction for MR, indole, oxidase and TSI test. Isolate BL3D and BL3I gave a positive reaction for

citrate, VP, indole, catalase and carbohydrate fermentation whereas, a negative reaction for MR, oxidase and TSI test. However, isolate BL3C have similar results for all the biochemical tests except indole negative. A positive reaction for catalase, VP and D-glucose was observed for isolate BL3B and it was negative for MR, indole and gas production in glucose medium while doubtful for oxidase, citrate and TSI tests. BL3E gave a positive reaction for citrate, MR, indole, catalase and D-glucose whereas, a negative reaction for VP, oxidase and TSI test. Isolate BL3F were citrate, catalase, oxidase and D-glucose positive while MR, VP, indole, TSI, and gas production in glucose medium negative. Isolate BL3G gave a positive reaction for citrate, MR, catalase and carbohydrate fermentation whereas, a negative reaction for, VP, Indole, oxidase and TSI test. Lastly the isolate BL3H have similar results as BL1G isolated from first instar larvae (Table 2).

In pupal stage, Isolate BP1 and BP7 gave a positive reaction for citrate, VP, indole, catalase and carbohydrate fermentation whereas, a negative reaction for MR, oxidase and TSI test. However, isolate BP5 have similar results for all the biochemical tests except indole negative. The isolate BP2, BP4 and BP6 gave similar results for different biochemical tests as BL3F, BL3G and BL3E isolated from third instar larvae, respectively. Finally, BP3 gave a positive reaction for citrate, MR, Indole, catalase and D-glucose whereas, a negative reaction for VP, oxidase and TSI test (Table 2).

In adult stage, Bacterial isolates BM2 and BM6 (isolated from male adult flies) and BF1, BF3 and BF6 (isolated from female adult flies) gave a positive reaction for citrate, VP, Indole, catalase and carbohydrate fermentation whereas, a negative reaction for MR, oxidase and TSI test. However, isolate BM3 of male adult flies and BF5 of male

adult flies have similar results as above isolates for all the biochemical tests except indole negative. The isolate BM1 (isolated from male adult flies) and BF4 (isolated from female adult flies) gave similar results for different biochemical tests as BP2 isolated from pupal stage. Isolate BM4 of male adult flies and BF2 of male adult flies have similar results for different biochemical tests. Finally, BM5 gave a positive reaction for catalase, D-glucose and sucrose whereas, a negative reaction for citrate, MR, Indole, VP, oxidase and TSI test were observed (Table 2).

Identification of bacterial isolates associated with the developmental stage of the peach fruit fly, *Bactrocera zonata*

The bacterial community associated with the developmental stage of the peach fruit fly isolated by culture-dependent technique were morphologically and biochemically characterized and belongs to various genera different family. The total 35 different bacterial colonies on the basis of colony morphology were screened and out of which, thirteen different bacterial species belonging to eleven genera (seven families) were identified from the developmental stage (first Instar larvae, third Instar larvae, pupa, male and female adults) of *Bactrocera zonata* (Table 3). The bacteria species viz., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter* sp., *Providencia* sp., *Pantoea* sp., *Citrobacter freundii*, *Pseudomonas* sp., *Enterococcus* sp., *Ochrobactrum* sp., *Bacillus* sp., *Microbacterium* sp., and *Rhodococcus* sp. were identified to be associated with different life stages. Out of total bacterial isolates, seven bacterial species from first instar larvae, nine from third instar larvae, seven from pupae and six each from male and female adult flies were identified. Genera *Klebsiella*, *Enterobacter* and *Pseudomonas* were found in all the development stages.

To the best of our knowledge, this study is the first of its kind to explore the cultivable bacterial community in the different life stages (larvae, pupae, male and female adults) of *B. zonata* by conventional method. The gut of tephritid fruit flies in general (Behar *et al.*, 2009; Lloyd *et al.*, 1986; Wang *et al.*, 2011) and *B. zonata* in particular (Reddy *et al.*, 2014; Naaz *et al.*, 2016; Naaz *et al.*, 2020) is a storehouse of bacterial community. In the present study, 35 different bacterial colonies were designated to thirteen different bacterial species. The bacteria species viz., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter* sp., *Providencia* sp., *Pantoea* sp., *Citrobacter freundii*, *Pseudomonas* sp., *Enterococcus* sp., *Ochrobactrum* sp., *Bacillus* sp., *Microbacterium* sp., and *Rhodococcus* sp. were identified to be associated with different life stages. Enterobacteriaceae was found to be the predominant family in all the developmental stages. Moreover, Enterobacteriaceae have frequently been identified as the dominant species in the gut of several other tephritids, such as *Dacus* (Drew and Lloyd 1987), *Bactrocera* (Capuzzo *et al.*, 2005; Prabhakar *et al.*, 2009; Shi *et al.*, 2012; Wang *et al.*, 2011, 2013), *Anastrepha* (Kuzina *et al.*, 2001) and *Ceratitis* (Behar *et al.*, 2008). These findings show that such bacteria are widespread in tephritids, suggesting a stable association with these fruit flies (Jang and Nishijima 1990; Lauzon *et al.*, 2009; Wang *et al.*, 2011).

The bacterial species *Enterobacter* sp., *K. oxytoca*, *K. pneumoniae* and *Pseudomonas* sp., were common across all the developmental stages of *B. zonata* suggest the possibility of vertical transmission of these bacterial communities. Vertical transition is common for some of the bacterial communities in tephritid flies (Andongma *et al.*, 2015; Lauzon *et al.*, 2009; Naaz *et al.*, 2020).

Table.1 Isolation of gut bacteria from different development stages of *Bactrocera zonata*

Development stages of <i>B. zonata</i> isolates used for bacterial isolation	Culture media used			
	Peptone Yeast Extract Agar (PYEA)		Nutrient Agar (NA)	
	Bacterial colonies isolated	Bacterial isolate number	Bacterial colonies isolated	Bacterial isolate number
First Instar larvae	5	BL1A, BL1B, BL1C, BL1D, BL1E	2	BL1F, BL1G
Third Instar larvae	7	BL3A, BL3B, BL3C, BL3D, BL3E, BL3F, BL3I	2	BL3G, BL3H
Pupa	6	BP1, BP2, BP3, BP4, BP5, BP7	1	BP6
Adult Male	5	BM1, BM3, BM4, BM5, BM6	1	BM2
Adult Female	5	BF1, BF2, BF3, BF4, BF5	1	BF6

Table.2 Morphological and biochemical characteristics of bacteria associated with the developmental stages of the peach fruit fly, *Bactrocera zonata*

Characteristics	Bacterial isolates												
	BL1E, BL3B	BL3G, BP4	BL1C, BL3D, BP1, RM2, BF6	BL1G, BL3H	BL1A, BL3I, BP7, EM6	BL1D, BL3C, BP5, BM3, BM4, BF2	BP3	BL3A	BL1F, BL3E, BP6	BL1B, BL3F, BP2, BM1, BM5			
Morphological													
Shape	Rod shape	Rod shape	Rod shape	Rod shape	Cocci shape	Rod shape	Rod shape	Rod shape	Rod shape	Rod shape	Rod shape	Rod shape	Rod-coccus shape
Motility	-	+	-	-	-	-	-	+	+	+	+	+	-
Gram's reaction	+	-	-	-	+	-	-	+	-	-	-	-	+
Colonies Colour	Creamy white	Opaque, mucoid	White & opaque	White & opaque	White	White	Creamy White	Yellow	White	Yellow	Dull grey	Light	Orange
Growth in broth medium	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Sediment	Pellicular formation

Biochemical													
Citrate test	+	+	+	+	-	+	+	+	+	+	+	+	-
Methyl red test	-	+	-	-	±	-	-	-	-	-	+	-	-
V.P test	+	-	+	+	+	+	+	-	-	+	-	-	-
Indole test	-	-	+	+	-	+	-	-	-	-	+	-	-
TSI test	±		-	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	-	+	+	+	+	+	+	+	+
Oxidase test	±	-	-	-	-	-	-	-	+	-	-	+	-
Growth in 10% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	-
Carbohydrate fermentation													
D-Glucose	+	+	+	+	+	+	+	+	-	+	+	+	+
D- Glucose (Gas production)	-	-	+	+	-	+	+	-	-	-	-	-	-
Sucrose	±	+	+	+	±	+	+	+	+	+	±	-	+
Lactose	-	+	+	+	+	+	+	+	+	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	-	+	-
Raffinose	+	±	+	+	-	+	+	-	-	-	-	±	-
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	-	-
Trehalose	+	+	+	+	+	+	+	-	-	+	+	-	+
Celliobiose	+	+	+	+	+	+	+	-	-	-	-	-	-
D-Sorbitol	+	+	+	+	-	+	+	+	-	-	-	-	+
Inositol	+	-	+	+	-	+	+	-	-	±	±	-	+
D-Xylose	+	+	+	+	-	+	+	+	-	+	±	-	±
Identified as	<i>Bacillus</i> sp.	<i>Citrobacter freundii</i>	<i>Enterobacter</i> sp.	<i>Enterobacter cloacae</i>	<i>Enterococcus</i> sp.	<i>Klebsiella oxytoca</i>	<i>Klebsiella pneumonia</i>	<i>Microbacterium</i> sp.	<i>Ochrobactrum</i> sp.	<i>Pantoea</i> sp.	<i>Providencia</i> sp.	<i>Pseudomonas</i> sp.	<i>Rhodococcus</i> sp.

Table.3 Identified cultured bacteria at different development stages of *Bactrocera zonata*

Sl No.	Family	Bacteria	First Instar larvae	Third Instar larvae	Pupa	Adult Male	Adult Female
1.	Enterobacteriaceae	<i>Klebsiella pneumonia</i>	+	+	+	+	+
2.	Enterobacteriaceae	<i>Enterobacter cloacae</i>	-	-	-	-	+
3.	Enterobacteriaceae	<i>Enterobacter</i> sp.	+	+	+	+	+
4.	Enterobacteriaceae	<i>Klebsiella oxytoca</i>	+	+	+	+	+
5.	Enterobacteriaceae	<i>Providencia</i> sp.	+	+	+	-	-
6.	Pseudomonadaceae	<i>Pseudomonas</i> sp.	+	+	+	+	+
7.	Enterobacteriaceae	<i>Citrobacter freundii</i>	-	+	+	-	-
8.	Microbacteriaceae	<i>Microbacterium</i> sp.	-	-	-	+	+
9.	Nocardiaceae	<i>Rhodococcus</i> sp.	-	-	-	+	-
10.	Enterococcaceae	<i>Enterococcus</i> sp.	+	+	-	-	-
11.	Brucellaceae	<i>Ochrobactrum</i> sp.	-	-	+	-	-
12.	Bacillaceae	<i>Bacillus</i> sp.	+	+	-	-	-
13.	Enterobacteriaceae	<i>Pantoea</i> sp.	-	+	-	-	-
		Total bacterial species	7	9	7	6	6

Recently, It has been also postulated that gut enterobacteria are dispersed into the female reproductive system, where they are subsequently transferred to the eggs, then to fruit during oviposition and finally passed to the fly offspring (Behar *et al.*, 2008; Shi *et al.*, 2012). Among all the common bacterial species across developmental stages, *Pseudomonas* sp. was not reported earlier to be associated with *B. zonata*. However, genus *Pseudomonas* have been reported from many insect species including fruit flies such as *Bactrocera dorsalis*, *Zeugodacus tau* (Brinkmann *et al.*, 2008; Sood and Nath, 2002; Prabhakar *et al.*, 2013, Noman *et al.*, 2020).

Earlier, *K. oxytoca*, *K. pneumonia*, *Enterobacter cloacae*, *Enterobacter* sp., *Bacillus* sp., *Microbacterium* sp., and *Rhodococcus* sp. were also reported from the gut of *B. zonata* (Reddy *et al.*, 2014; Naaz *et al.*, 2016) whereas, *Citrobacter freundii*, *Providencia* sp., *Pantoea* sp., *Pseudomonas* sp., *Enterococcus* sp., and *Ochrobactrum* sp. were newly added in the list of bacterial community associated with the *B. zonata*. However, genera *Citrobacter* and *Providencia* were identified from four *Bactrocera* species (Lloyd *et al.*, 1986) and *B. dorsalis* (Gujjar *et al.*, 2017). The bacterial species of genus *Pantoea*, is a free-living diazotrophic bacterium of family Enterobacteriaceae and was consistently reported from the different organs and stages of *Zeugodacus cucurbitae* and *Z.tau* (Sood and Nath 2002; Prabhakar *et al.*, 2009). *Ochrobactrum* sp. have been reported from many insect species including fruit flies such as *B. dorsalis* and *Z. tau* (Brinkmann *et al.*, 2008; Sood and Nath, 2002; Prabhakar *et al.*, 2013, Noman *et al.*, 2020). Thus the present findings get substantial support from the observations of other workers, who consistently observed the association of phylum, Proteobacteria with different organs and developmental stages of

other *Bactrocera* species (Sood and Nath, 2002; Prabhakar *et al.*, 2013, Gujjar *et al.*, 2017, Noman *et al.*, 2020) including *B. zonata* (Reddy *et al.*, 2014; Naaz *et al.*, 2016; Naaz *et al.*, 2020).

The present finding added a few more genera to the list of bacterial diversity of the *B. zonata*, but a vast range of gut bacterial diversity exists in the *B. zonata* system is still unknown. Therefore, an extensive study to explore the gut bacterial diversity across all the developmental stages of the fly and to understand the host behavior in relation to gut bacterial community are needed using both culture-dependent and -independent techniques (with large number of replicates). However, our study significantly supplements to the available information on the cultivable bacterial diversity associated with the developmental stages of *B. zonata*.

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