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Isolation and Identification of Mid-Gut Bacterial Community of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae)

[1]Md. Kamruzzaman Pramanik, [1]Abdullah-Al-Mahin, [2]Mahfuza Khan and [1]Abdul Bathen Miah

[1]Microbiology and Industrial Irradiation Division,

[2]Insect Biotechnology Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Ganak Bari, 1349, Savar, Bangladesh

Corresponding Author: Mahfuza Khan, Insect Biotechnology Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Ganak Bari, 1349, Savar, Bangladesh

ABSTRACT

Tephritidae fruit flies usually harbour different bacterial symbionts in their digestive system. These symbionts have been known to play significant role in different fitness parameters of flies. In the present study bacterial symbionts from the mid-gut of laboratory host reared Oriental fruit fly (*Bactrocera dorsalis*) (Hendel) were isolated and identified using conventional culture based techniques. The mid-gut of ten days old adult female was dissected and processed aseptically and initially total viable bacterial count was determined using nutrient agar medium. Different and discrete colonies on this media were isolated and subcultured. Pure culture of each isolate was subjected to a series of different morphological, cultural and biochemical tests for identification. Total viable bacterial count was revealed as 4.5×10^5 CFU mL^{-1} in the mid-gut of *B. dorsalis*. Eleven bacterial genera with thirteen species were identified. The identified bacterial gerera of *B. dorsalis* were *Listeria, Citrobacter, Moraxella, Proteus, Streptobacillus, Enterobacter, Serratia, Vibrio, Aeromonas, Klebsiella, Morganella*. Experimental result reported the presence of *Moraxella* and *Streptobacillus, Aeromonas* and *Morganella* for the first time in mid-gut of laboratory host reared *B. dorsalis*.

Key words: Oriental fruit fly, mid-gut, bacterial community, isolation, identification

INTRODUCTION

Association of microorganisms with insects (both intra and extracellular) are quite common in nature and known since the last century (Petri, 1909). Among them different species of Tephritidae harbour huge amount of bacteria in their digestive tract and some of the bacteria involved as gut microbiota with specialized morphology (Petri, 1910; Mazzini and Vita, 1981). Symbiotic association of bacterial population with Tephritidae has been shown to play significant role in insect nutrition and through their life cycle. Tephritidae symbionts provide their host with certain essential amino acids that are absent in fruit tissues (Drew and Lloyd, 1989; Drew *et al*., 1983; Gupta and Anand, 2003; Jang and Nishijima, 1990). Nitrogenase activity of some of them has been implicated to fix nitrogen like Rhizobia of legumes (Behar *et al*., 2005; Murphy *et al*., 1988). Some symbionts may act as natural source of vitamin (Prabhakar *et al*., 2009). Ingested

bacteria have also shown to meet the requirement of female for egg maturation (Pankaj and Amit, 2005). It has been reported that the gut mircrobiota can provide resistance against natural enemies and parasites (Oliver *et al.*, 2003). Symbionts can also hydrolyze protein and detoxify toxin ingested by their host insect and thus can provide protection to their host (Courtice and Drew, 1984; Prokopy, 1981). Recent study showed that gut microbiota also help to enhance social interaction (Dillon *et al.*, 2002) and may assist insect immunity by forming a persistent infection in the gut (Muniz *et al.*, 2006).

Besides, symbionts have several implications in pest management strategies as they can render the insecticide resistance to host by degrading the toxic substance ingested by their host (Boush and Matsumura, 1967). Controlling these gut microbiota may help to attribute the pest management strategy. Certain components of bacteria odor play a vital role in fruit fly behaviour as either feeding or ovipositional stimulants (Drew and Lloyd, 1987; Lauzon *et al.*, 2000). This attribute can also be exploited in pest management in the form of baits or traps (Robacker *et al.*, 1998; Sacchetti *et al.*, 2007).

As diversity exists among the gut microbiota of wild and reared flies, it is useful to know the gut bacterial species before exploiting their attribute in pest management program especially in Sterile Insect Technique (SIT). Conventional along with molecular approaches for detection and characterization of microbes in different insect species have revealed considerable bacterial diversity (Prabhakar *et al.*, 2009; Sood and Prabhakar, 2009; Thaochan *et al.*, 2010; Wang *et al.*, 2011). Symbionts of insect may also vary depending on the insect species as well as geographical location (Murphy *et al.*, 1994). Therefore it may anticipate having diverse microbial population in different fruit fly species of Bangladesh. As the microbial symbionts of local *Bactrocera* fruit flies was not investigated yet the present study was carried out on the isolation and identification of bacterial community associated with the mid-gut of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) one of the economically important pestiferous fruit flies of Bangladesh.

MATERIALS AND METHODS

Insect rearing: Rearing of adult *B. dorsalis* was maintained in the laboratory of Insect Biotechnology Division (IBD), Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), Savar, Dhaka, Bangladesh for more than 200 generations using both the natural hosts and artificial diets. About 5,000 adult flies were maintained in steel framed cages (76.2×66×76.2 cm) covered with wired net. The flies were supplied with protein based diets both in the dry and liquid form: (1) Casein: Yeast extract: Sugar at 1:1:2 ratio and (2) Baking yeast: Sugar: Water at 1:3:4 ratio. Water was supplied in a conical flask socked with cotton ball. The temperature and the relative humidity of the rearing room maintained at 27±1°C and 75±5% and 14:10 h dark and light cycle. The work related to isolation and identification of mid-gut bacteria of *B. dorsalis* was performed in the laboratory of Microbiology and Industrial Irradiation Division (MIID), IFRB, AERE, Savar.

Fly dissection and sample preparation: Laboratory host (banana) reared ten days old female *B. dorsalis* were collected and surface sterilized with 90% ethanol for 10 sec and then cold anesthetized in a refrigerator at 4°C for 10 min prior to dissection. Flies were then dissected aseptically using the techniques described by Drew *et al.* (1983) and Drew and Lloyd (1991) under laminar flow hood and the intestinal tract (mid gut) were collected in a 4 mL glass vial containing 2 mL of normal physiological saline.

Isolation: The collected guts were opened with forceps and homogenized with vortex for 30 sec. Homogenized suspensions were then serially diluted up to 10^{-3}. Approximately 100 µL of diluted suspension were inoculated onto nutrient agar plate using spread plate technique and incubated at 37°C for 24-48 h. On the basis of different colony characteristics (viz., size, edge, consistency) discrete colonies were selected for identification. Selected colonies were purified as pure culture through repeated subculturing onto nutrient agar plate and maintained at 4°C or maintained on agar slants.

Morphological, cultural and biochemical characterization: Morphological, cultural and biochemical characterization were carried out by standard techniques. Gram staining and microscopy were performed to study the morphology of those collected isolates e.g., size, shapes and opacity. Cultural characteristic of those bacterial isolates were determined by inoculating the colonies on nutrient agar and incubating at 30°C for 24-48 h. Different cultural characteristics including colony size, shape, pigmentation, edge, elevation, opacity and consistency were studied after incubation. Biochemical characterization of the selected isolates was accomplished by carrying out different biochemical tests e.g., catalase, oxidase, glucose fermentation, oxidative-fermentative (O/F) test. Carbohydrate fermentation tests with several sugars were carried out using phenol red carbohydrate broth media to be tested as the sole carbon source to determine the carbohydrate fermentation pattern of these isolates. Besides these, several selective and differential media (KIA, BSA) were also used to determine and confirm a particular characteristic of those isolates.

Identification: On the basis of different cultural, morphological and biochemical salient characteristics, isolates were identified comparing with standard characteristics of microorganisms as mentioned in the Cowan and Steel (1981) and Holt *et al.* (1994).

RESULTS AND DISCUSSION

Total bacterial count in the gut of *B. dorsalis* were determined to know the presumptive symbionts load in this fruit fly and that was 4.5×10^5 CFU mL^{-1}. Bacterial population present in the mid-gut of *B. dorsalis* were also identified based on different cultural, morphological and biochemical characteristics. A total of 34 predominant discrete colonies were selected and picked up from agar plate incubated with the mid gut sample of *B. dorsalis* and designated as Bd-01, Bd-02, Bd-03, Bd-04, Bd-05, Bd-06, Bd-07, Bd-08, bd-09, Bd-10, Bd-11, bd-12, bd-13, Bd-14, Bd-15, Bd-16, Bd-17, Bd-18, Bd-19, Bd-20, Bd-21, Bd-22, Bd-23, Bd-24, Bd-25, Bd-26, Bd-27, Bd-28, Bd-29, Bd-30, Bd-31, Bd-32, Bd-33, Bd-34.

All the colonies were then subject to identification as described in the material and method section. Cultural and morphological characteristics of those bacterial isolates are shown in Table 1. With few exceptions, colony colours of most of the isolates were off-white. For example isolates of Bd-01, Bd-02, Bd-03 etc. produce off-white isolates of colour while Bd-07 and Bd-24 produce pink colour colonies on nutrient agar. All of the colonies were circular with entire edge diameter ranging from 0.75-2.5 mm after 24 h incubation. Some colonies were opaque (e.g., Bd-01, Bd-07 etc.) and some were transparent (e.g., Bd-02, Bd-05 etc). Colony elevations of most of the isolates were flat though some produced raised and two isolates produced convex colonies.

Table 1: Cultural and morphological characteristics of bacterial symbionts in *Bactrocera dorsalis*

	Cultural characteristics				Morphological characteristics	
Code of isolates	Colony color	Shape	Size	Opacity	Gram staining	Shape of cell
Bd-01, Bd-18	Off-white	Circular	Large	Opaque	+	Rod
Bd-02, Bd-19	Off-white	Circular	Small	Transparent	-	Rod
Bd-03, Bd-20	Off-white	Circular	Large	Transparent	-	Rod
Bd-04, Bd-21	Off-white	Circular	Small	Slightly transparent	-	Rod
Bd-05, Bd-22	Off-white	Circular	Medium	Transparent	-	Rod
Bd-06, Bd-10, Bd-23, Bd-27	Off-white	Circular	Medium	Slightly transparent	-	Rod
Bd-07, Bd-24	Pink	Circular	Medium	Opaque	-	Short rod
Bd-08, Bd-09, Bd-17, Bd-25, Bd-26, Bd-30	Off-white	Circular	Large	Slightly transparent	-	Short rod
Bd-11, Bd-12, Bd-28, Bd-29	Off-white	Circular	Medium	Opaque	-	Rod
Bd-13, Bd-31	Off-white	Circular	Medium	Slightly transparent	-	Short rod
Bd-14, Bd-15, Bd-16, Bd-34, Bd-32, Bd-33	Off-white	Circular	Large	Slightly transparent	-	Rod

Gram staining was carried out to study shape and arrangement of the cells as well as Gram reaction. All of the isolates showed Gram negative reaction except Bd-01 and Bd-18 that showed Gram positive reaction. Some of the isolates were rods and some were short-rod. Some isolates were single and some were in chain form as observed under 100x magnification.

Major biochemical characteristics of those isolates were determined by carrying out different biochemical tests and the results are shown in Table 2. Most of the isolates showed positive result for catalase except Bd-05 and Bd-22. Some isolates showed positive and some showed negative results for oxidase test. All of the isolates could reduce nitrate to nitrite except Bd-01 and Bd-18. Some isolates showed indole and Methyl Red (MR) positive and some negative. Most of the isolates showed Voges Proskaur (VP) and citrate positive and few showed negative. Isolates Bd-02 and Bd-19 produced H_2S as shown in Triple Sugar Iron (TSI) butt. Almost all of the isolates showed fermentative energy metabolism as shown by oxidative/fermentative (O/F) test.

To observe sugar fermentation pattern of those isolates, 15 different sugars were used and the results are shown in Table 3. All of the isolates could utilize arabinose except Bd-01 and Bd-18. Most of the isolates could degrade fructose, galactose, maltose and few could not.

On the basis of overall cultural, morphological and biochemical characteristics, a total of eleven bacterial genera were identified from the mid-gut of *B. dorsalis* (Table 4). Among the identified genus, two species *Serratia liquefaciens* and *Serratia rubidaea* was found in *Serratia* genus and the genus *Vibrio* also had two species i.e., *Vibrio alginolyticus* and *Vibrio cholerae*. Rest of the bacterial genus had single species. As per overall test results, all of the identified bacteria were categorized in the family of *Enterobacteriaceae,* except *Vibrio*, *Aeromonas*, *Streptobacillus*, *Moraxella* and *Listeria*. This result is very rational with respect to the habitat or source of the sample. In the present study few bacteria genus were newly reported to inhabit in the mid-gut of laboratory host reared *B. dorsalis*, viz., *Moraxella*, *Streptobacillus*, *Aeromonas* and *Morganella*. In previous studies using the cultivable dependence techniques, members of the Enterobacteriaceae were found to be dominant microbial population in the gut of *Bacterocera* fruit flies (Drew and Lloyd, 1991; Fitt and O'Brien, 1985; Jang and Nishijima, 1990). Several bacterial

Table 2: Major biochemical characteristics of bacterial symbionts in *Bactrocera dorsalis*

	Biochemical tests													
Code of isolates	Catalase	Oxidase	Nitrate reductase	Indole	MR	VP	Citrate utilization	H_2S(TSI)	Gas from glucose	Gelatin hydrolysis	Urea hydrolysis	Lysine decarbox	Malonate	O/F
Bd-01, Bd-18	+	+	-	+	-	+	-	-	-	-	-	-	-	F
Bd-02, Bd-19	+	-	+	+	+	+	+	+	+	-	-	-	-	F
Bd-03, Bd-20	+	+	+	+	-	+	-	-	-	-	-	-	-	-
Bd-04, Bd-21	+	-	+	+	+	-	+	-	-	-	+	-	-	F
Bd-05, Bd-22	-	-	+	-	-	+	+	-	-	-	-	-	-	F
Bd-06, Bd-10, Bd-23, Bd-27	+	-	+	-	-	+	+	-	+	+	-	+	+	F
Bd-07, Bd-24	+	-	+	-	-	+	+	-	-	+	-	+	+	F
Bd-08, Bd-25	+	+	+	-	-	+	+	-	+	+	-	+	-	F
Bd-09, Bd-17, Bd-26, Bd-30	+	+	+	+	+	-	-	-	+	+	+	-	+	F
Bd-11, Bd-12, Bd-28, Bd-29	+	+	+	+	-	+	+	-	+	+	-	+	-	F
Bd-13, Bd-31	+	-	+	+	+	-	+	-	-	-	+	+	+	F
Bd-14, Bd-15, Bd-32, Bd-33	+	-	+	+	-	-	-	-	+	-	+	-	-	F
Bd-16, Bd-34	+	-	+	-	+	+	+	-	+	+	-	+	+	F

Table 3: Sugar fermentation pattern of the bacterial isolates of *Bactrocera dorsalis*

	Sugar fermentation														
Code of isolates	Arabinose	Cellobiose	Dulcitol	Fructose	Galactose	Glucose	Inositol	Lactose	Maltose	Manitol	Rhamnose	Sucrose	Sorbitol	Salicin	Xylose
Bd-01, Bd-18	-	-	-	+	-	+	-	+	+	-	-	+	-	+	-
Bd-02, Bd-19	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+
Bd-03, Bd-20	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bd-04, Bd-21	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+
Bd-05, Bd-22	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+
Bd-06, Bd-10, Bd-23, Bd-27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bd-07, Bd-24	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Bd-08, Bd-25	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Bd-09, Bd-17, Bd-26, Bd-30	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Bd-11, Bd-12, Bd-28, Bd-29	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Bd-13, Bd-31	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+
Bd-14, Bd-15, Bd-32, Bd-33	+	-	-	-	+	+	-	+	-	-	-	-	+	-	-
Bd-16, Bd-34	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+

Table 4: Identified bacteria species from the mid-gut of *B. dorsalis*

Code of isolates	Bacteria
Bd-01, Bd-18	*Listeria monocytogenes*
Bd-02, Bd-19	*Citrobacter freundii*
Bd-03, Bd-20	*Moraxella phenylpyruvica*
Bd-04, Bd-21	*Proteus rettgeri*
Bd-05, Bd-22	*Streptobacillus moniliformis*
Bd-06, Bd-10, Bd-23, Bd-27	*Enterobacter aerogenes*
Bd-07, Bd-24	*Serratia rubidaea*
Bd-08, Bd-25	*Vibrio alginolyticus*
Bd-09, Bd-17, Bd-26, Bd-30	*Vibrio cholerae*
Bd-11, Bd-12, Bd-28, Bd-29	*Aeromonas hydrophila*
Bd-13, Bd-31	*Klebsiella pneumonia*
Bd-14, Bd-15, BD-32, Bd-33	*Morganella morganii*
Bd-16, Bd-34	*Serratia liquefaciens*

genera viz, *Listeria*, *Citrobacter*, *Enterobacter*, *Serratia*, *Vibrio*, *Proteous* and *Klebsiella* identified from gut of *B. dorsalis* in the present study were quiet common for other Tephtriitd fruit flies like, *Bactrocera tau* (Walker), *Bactrocera tryoni* (Frogatt) *Ceratitis capiata* (Wiedemann) (Behar *et al*., 2008; Khan *et al*., 2014; Kuzina *et al*., 2001; Wang *et al*., 2011). Lauzon (2003) stated that the gut microbiota of these flies is relatively conserved in terms of species composition and comprised mainly Enterobacteriaceae. The uniformity across genera in morphology and in the composition of the microbiota suggested that bacteria are intimately associated with the life cycle of these flies (Ben-Yosef *et al*., 2008). Wang *et al*. (2011) reported that intestinal tract of *B. dorsalis* adult contains a diverse bacterial community, some of which are stable and different environmental conditions and food supply could influence the diversity of the harboured bacterial communities and increase community variations.

Therefore, the diversified bacterial population present in the mid-gut of *B. dorsalis* is expected to facilitate new research in the different aspect of gut-microbial symbionts. Further molecular study of microbial community of different *Bactrocera* species may be carried out on different temporal and spatial basis.

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