

Full Paper

Molecular and biochemical characterization of three bacterial symbionts of fruit fly, *Bactrocera tau* (Tephritidae: Diptera)

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Characterization of three bacterial symbionts (BC1, BC2 and BC3) of fruit fly *Bactrocera tau* including morphological, biochemical and 16S rDNA (*rrs* gene) analysis was done to determine their taxonomic position. Morphological and biochemical characterization placed two bacteria (BC1, *Klebsiella oxytoca* and BC2, *Pantoea agglomerans*) into family Enterobacteriaceae and the third one (BC3, *Staphylococcus* sp.) into family Staphylococcaceae. 16S rDNA gene sequence comparison with the available NCBI database sequences further confirmed the characterizations of bacterial symbionts. Molecular phylogeny of *Klebsiella oxytoca* and *Pantoea agglomerans* closely related to the other free living enterobacterial members with 74 to 93% sequence homology (genetic distance 0.000 to 0.085); however, they showed only 74 to 87% similarity with other insect symbionts (genetic distance 0.090 to 0.121). *Staphylococcus* sp. showed 94% sequence homology with other members of family Staphylococcaceae with the genetic distance of 0.013. Population of these symbionts in adult fruit flies increased exponentially up to the 10th day of adult emergence and thereafter it became almost constant.

Key Words—bacterial symbionts; *Bactrocera tau*; fruit fly; *Klebsiella*; *Pantoea*; phylogeny; 16S rRNA

Introduction

Association between insects and bacteria (intracellular and intercellular) are quite common in nature and known since the last century (Petri, 1909). About 15% of all insects harbor diverse communities of endosymbionts (Brooks, 1963; Buchner, 1965; Douglas, 1998; Moran et al., 2008; Stouthamer et al., 1999). Among various pests, the tephritid genus *Bactrocera* is widely

distributed in the Asia-Pacific region and contains economically important species causing considerable losses to fruits and vegetables. Among them, the melon fly (*Bactrocera cucurbitae* Coquillett) and pumpkin fly (*B. tau* Walker) are distributed throughout South-East Asia, attacking fruits of a range of plant species (Huque, 2006; Narayanan and Batra, 1960; White and Elson-Harris, 1992). In tephritidae, symbionts provide their hosts with certain essential amino acids lacking in fruit tissues (Drew and Llyod, 1989; Drew et al., 1983; Gupta and Anand, 2003; Jang and Nishijima, 1990). These symbionts may act as a natural source of nitrogen, amino acids and vitamins. Their nitrogenase activity might be involved in nitrogen fixation as in rhizobia of legumes (Behar et al., 2005; Murphy et al.,

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1988). Moreover, symbionts have several implications in pest management strategies; e.g., bacteria were found to be involved in the degradation of the toxic substances ingested by the host insect leading to insecticide resistance (Bousch and Matsumara, 1967). Certain components of bacterial odor play a vital role in fruit fly behavior as either feeding or ovipositional stimulants (Drew and Lloyd, 1987; Lauzon et al., 1998, 2000) and are being exploited in pest management in the form of baits or traps (Robacker, 1998; Sacchetti et al., 2007).

Tephritid gut bacteria mostly belong to family Enterobacteriaceae and two species, viz. *Klebsiella* and *Enterobacter*, are the predominant ones (Behar et al., 2005; Drew and Lloyd, 1987; Zinder and Dworkin, 2000). However, a range of bacteria belonging to different genera, viz. *Pantoea*, *Pectobacterium*, *Citrobacter*, *Erwinia*, *Bacillus*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Proteus*, *Providencia*, *Hafnia*, *Serratia* and *Xanthomonas*, have been isolated and characterized from the fruit fly gut (Behar et al., 2005, 2008, 2009; Bergey et al., 2001; Capuzzo et al., 2005; Drew and Lloyd, 1987; Kounatidis et al., 2009; Kuzina et al., 2001; Lauzon et al., 1998, 2000; Marchini et al., 2002; Sacchetti et al., 2008; Sood and Nath, 2002; Zinder and Dworkin, 2000).

The identification of these symbiotic bacteria is of utmost importance before understanding their true role and utility in the management of the pest. Traditional microbiological methods (morphological, physiological and biochemical) for phenotypic characterization have several lacunae which sometimes create a blurred image about their taxonomic status as these methods are environment dependent. The same bacteria might show different phenotypic characters under different environmental conditions, hindering their reproducibility in different labs (Prakash et al., 2007). Moreover, such methods give little insight into the phylogenetic relationship among different symbiotic bacteria.

A polyphasic approach (Colwell, 1970), however, provides a natural and authentic classification system of microbes (Prakash et al., 2007). It is used to distinguish bacterial species based on morphological and biochemical data supplemented with information obtained from molecular techniques. Perusal of the literature indicated very little work on the fruit fly-bacterial symbionts phylogeny. Thus the main objective of the

study was to characterize bacterial symbionts of *Bactrocera tau* Walker and to establish their phylogenetic position.

Materials and Methods

Raising stock culture of fruit fly. Parental stock culture of the fruit fly (*Bactrocera tau* Walker) was raised from field collected (Entomological Research Farm, CSK HPKV, Palampur situated at 32°6' N latitude and 76°3' E longitude and at an elevation of 1,290.8 m above mean sea level in North Western Himalayas) infested fruits of cucumber in fruit fly rearing cages (40 × 40 × 45 cm³) at room temperature (25 ± 2°C). The cage was filled with 5 cm layer of sterile fine sand and mixed with saw dust for pupation. The adults were provided with their natural host (cucumber) for oviposition. The feeding was also supplemented with a mixture of dry glucose and protein hydrolyzate (Protinex®, Dumex Sciences, India) in the ratio of 1 : 1 (w/w) in Petri plates following the procedure of Sood and Nath (1999). The supplement was replaced weekly. Flies were also provided water ad libitum through water-soaked cotton swabs in a 50 ml beaker. Nine inbreeding generations of *B. tau* were reared in the laboratory for ensuring predominant and closely associated bacterial symbionts isolation.

Isolation of bacterial symbionts. The bacteria were isolated from adult flies, maggots, eggs and host phylloplane (cucumber) as described by Lloyd et al. (1986). Flies were cold anesthetized for 5 min and surface sterilized with alcohol (70%) for 30 s. followed by sodium hypochloride (0.25%) for 1 min and then washed three times with sterilized distilled water (SDW) to remove external contaminations. The surface-sterilized flies were dissected in physiological saline to remove different organs (crop and portion of alimentary canal) aseptically. Content from each part was streaked separately on Peptone Yeast Extract Agar (PYEA) for bacterial growth at 30 ± 1°C for 48–72 h. A single colony of each isolate was used for the establishment of pure culture and then maintained on PYEA slants and/or plates at 4–8°C.

Characterization of bacterial symbionts. Morphological and biochemical characterization: Morphological, cultural and biochemical characterization of pure culture was done by standard techniques and isolates were identified by using Bergey's Manual of Determinative Bacteriology (Holt et al., 2000).

Molecular characterization. Extraction of genomic DNA: Total genomic DNA of each isolate was extracted following the method of Sharma et al. (2005) with minor modifications. The 48 h old bacterial culture multiplied on Peptone Yeast Extract Broth (PYEB) was transferred to 1.5 ml microtubes and spun at 10,000 rpm for 12 min. After discarding the supernatant, the microtubes containing bacterial pellets (approx. 50 mg) were immersed in a liquid nitrogen container for 1 min and the pellet was ground to fine powder immediately using a micro pestle. To this, 700 µl of cetyltrimethylammonium bromide (CTAB) extraction buffer was added and incubated at 65°C for 1 h in a water bath (York Scientific Industries, Delhi).

An equal volume (700 µl) of chloroform : isoamyl alcohol (24 : 1 v/v) was added and contents were mixed thoroughly. Tubes were spun at 10,000 rpm for 12 min in a high speed refrigerated centrifuge (REMI India) at 4°C. The aqueous phase was transferred to new tubes and 450 µl pre-chilled isopropanol was added and kept at -20°C for 20-30 min to precipitate the DNA. Tubes were then spun at 10,000 rpm for 12 min and supernatant was decanted. The DNA pellet was washed with 70% ethanol (three times), dried and dissolved in 100 µl of Tris EDTA buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0). RNase @ 10 µl/ml (MBI Fermentas) was added and emulsion was incubated for half an hour at 37°C. The amount of DNA was quantified by recording the absorbance at 260 nm wavelength using a UV/VIS spectrophotometer (Bio Rad, SmartSpec 3000). DNA was stored at -20°C for further use.

PCR amplification and sequencing. Polymerase chain reaction (PCR) was performed with eubacterial primers 27F 5'-AGAGTTTGATCATGGCTCAG-3' and 1487R 5'-TACCTTGTACGACTTCACC-3' targeting the 16S rRNA gene (*rrs* gene) (Heddi et al., 1998). The PCR amplification was carried out in 0.2 ml PCR tubes with 25 µl reaction volume containing 10 ng of DNA template, 20 pmol of each primer in 25 mM MgCl₂, 10 mM of each deoxyribonucleoside triphosphate (Fermentas), 5 units of *Taq* polymerase (Life Technologies India, Pvt. Ltd.) and 10× reaction buffer. Amplifications were performed using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) with an initial denaturation step of 5 min at 94°C followed by 35 cycles at 94°C for 45 s, 53°C for 45 s, 72°C for 30 s and a final elongation step at 72°C for 5 min. The product was separated in a 1% (w/v) agarose gel

in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). PCR products of the *rrs* gene of three bacterial symbionts (BC1, BC2 and BC3) obtained through amplification with specific primers were freeze dried (CHRIST ALPHA I-2LD) and were custom sequenced (ABI PRISM 310™ Genetic Analyzer, Applied Biosystems) using the same upstream and downstream primers (Life Technologies India, Pvt. Ltd.).

Nucleotide sequence analysis. The sequences of different bacterial isolates were blasted using the on-line NCBI Blastn program <http://www.ncbi.nih.gov/blast>. Therefore twenty-six sequences of 16S rRNA of different bacteria (free living and insect symbionts including fruit fly symbionts) of high sequence similarity were selected for sequence comparison from the GenBank Nucleotide Database, NCBI. The selected sequences along with three submitted sequences were aligned by the ClustalW program using website <http://www.ebi.ac.uk/clustalw/> and pair-wise per cent nucleotide sequence homology in BC1, BC2 and BC3 isolates of bacterial symbionts and other selected bacterial sequence was determined.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) with *Burkholderia pseudomallei* kept as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) with the Maximum Composite Likelihood method to compute evolutionary distances (Tamura et al., 2004) and in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 298 positions in the final dataset. Phylogenetic analysis was conducted in MEGA 4.1 Software program (Tamura et al., 2007).

Population kinetics of bacterial symbionts within the fruit fly. The bacterial flora of adult fruit flies as affected by their age was studied by bacterial count of whole fruit flies in different age groups by the serial dilution method. Three randomly trapped adult flies were anesthetized using cold treatment at 4°C for 10 min. The flies were surface sterilized in sequential washings of 70% (v/v) alcohol for 30 s, 0.25% (v/v) sodium hypochloride for 1 min and sterile distilled water (SDW) for 30 s. The sterilized flies were crushed individually in a test tube with a sterile glass rod in 1 ml SDW. The final volume was made to 10 ml by adding SDW (stock solution). The bacterial population in the whole fruit fly

was enumerated on PYEA plates using the serial dilution method. A known quantity (0.1 ml) of bacterial suspension from different dilutions was spread on the PYEA plates with the help of glass spreader. The plates were incubated at 30°C for 48 h. The process was repeated three times each for 1-, 2-, 3-, 4-, 5-, 10-, 15-, 20- and 25-day-old flies (after emergence). Data obtained were expressed in colony forming units per day after emergence (cfu/dae) of adult fruit flies.

Results

Characterization of bacterial symbionts

Morphological and biochemical characterization: Based on the morphological, cultural and biochemical characteristics (Table 1), the three bacterial isolates (BC1, BC2 and BC3) from *B. tau* were identified as *Klebsiella oxytoca*, *Pantoea agglomerans* and *Staphylococcus* sp., respectively by comparing the results with Bergey's Manual of Determinative Bacteriology (Holt et al., 2000).

Molecular characterization: 16S rRNA sequencing of three bacterial isolates, viz. *K. oxytoca*, *P. agglomer-*

Table 1. Morphological and biochemical characteristics of predominant bacterial symbionts of fruit fly (*Bactrocera tau*).

Characteristics	BC1	BC2	BC3
Morphological			
Shape	Rod Shape	Rod Shape	Spherical
Motility	–	+	–
Gram's reaction	–	–	+
Pigment production	–	Y	–
Growth in broth medium	Sediment	Sediment	Pellicle formation
Biochemical			
Citrate test	+	+	–
Methyl red test	–	–	–
V.P. test	+	+	–
Indole test	+	–	–
TSI test	–	–	+
Urease test	+	–	+
Catalase test	+	+	+
Oxidase test	–	–	–
Growth in 10% NaCl	+	+	+
Carbohydrate fermentation			
D-Glucose	+	+	+
D-Glucose (Gas production)	+	–	–
Sucrose	+	+	+
Lactose	+	–	+
Maltose	+	+	–
L- Arabinose	+	–	–
Raffinose	+	–	–
D- Mannose	+	+	+
Trehalose	+	+	+
Celliobiose	+	–	–
Melibiose	+	–	–
D- Mannitol	+	+	±
D- Sorbitol	+	–	–
Inositol	+	±	–
D- Xylose	+	+	+
Characterized as	<i>Klebsiella oxytoca</i>	<i>Pantoea agglomerans</i>	<i>Staphylococcus</i> sp.

+, positive reaction; –, negative reaction; ±, doubtful result; Y, yellow.

ans and *Staphylococcus* sp., using eubacterial specific primers showed sequences of 1,057, 912 and 859 bp, respectively. These sequences were submitted to the NCBI GenBank nucleotide database with accession number EF 560611 (*K. oxytoca*), EF 569226 (*P. agglomerans*) and EF 600797 (*Staphylococcus* sp.). This constitutes the first record of 16S rRNA sequence of bacterial symbionts associated with *B. tau*. Nucleotide sequence analysis of test symbionts using the clustalW programme revealed that *K. oxytoca* and *P. agglomerans* showed maximum homology with members of the family Enterobacteriaceae, whereas *Staphylococcus* sp. resembled Staphylococcaceae. Homology analysis with the NCBI GenBank Blastn program revealed that *Klebsiella oxytoca* showed maximum homology with uncultured bacteria (EU464477, E-value 0.0, Total score 1,310, Maximum score 1,310 and Maximum identity 94%), *Pantoea agglomerans* with *Pantoea* sp. (EF522820, E-value 0.0, Total score 1,380, Maximum score 1,380 and Maximum identity 94%) and *Staphylococcus* sp. with *Staphylococcus* sp. (EF061904, E-value 0.0, Total score 1,312, Maximum score 1,312 and Maximum identity 96%). The multiple sequence alignment analysis of 16S rRNA genes (*rrs* gene) of the three bacterial isolates with twenty-six 16S rRNA sequences available in the GenBank Database (NCBI) revealed per cent pair-wise similarity ranging from 58 to 93% and genetic distance from 0.000 to 0.401. *K. oxytoca* showed maximum homology (93%) with *K. oxytoca* (EJ 424514) followed by *P. agglomerans* (EF 569226) and *Erwinia* sp. (EF 522135). However, *P. agglomerans* showed maximum homology of 92% with *K. oxytoca* (FJ 424514 and EF 560611) followed by *Erwinia billingiae* (AM 117486), *Erwinia* sp. (EF 522135) and *Pantoea* spp. (AF 373198, EF 522820 and EF522135), all belonging to the family Enterobacteriaceae. *Staphylococcus* sp. showed maximum homology (94%) with *Staphylococcus* sp. (EF 061904 and AF 467429) and *Staphylococcus succinus* (AY 748916, AF 004219 and AJ 320272), thus further confirming their identity.

The genetic distance estimates and phylogenetic tree also confirmed a similar relationship pattern of the three symbionts. The phylogenetic tree (Fig. 1) analyzed with *Burkholderia pseudomallei*, an α -proteobacteria as an outgroup placed *K. oxytoca* and *P. agglomerans* within the γ -proteobacteria group. The genetic distance of *K. oxytoca* was much less with the free living enterobacterial species (0.013 to 0.062) as

compared to endosymbionts of *Sitophilus zeamais* (Motschulsky), *Sitophilus oryzae* (Linnaeus) and *Bemisia tabaci* (Gennadius) i.e. 0.090, 0.069 and 0.121, respectively. *P. agglomerans* showed minimum genetic distance with other *Pantoea* spp. and *Erwinia* spp. (0.000 to 0.026) and showed a similar genetic relationship with free living and endosymbionts as in *K. oxytoca*. Pair-wise genetic distance estimates revealed that *K. oxytoca* and *P. agglomerans* were genetically different with *K. oxytoca* (DQ533884) and *Pantoea* sp. (EF645649) present in the gut of the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann with genetic distance 0.033 and 0.027, respectively. Irrespective of their free living or symbiotic nature, all the bacteria of Enterobacteriaceae clustered together. *Staphylococcus* sp. had a genetic distance of 0.013 and clustered with other members of the family Staphylococcaceae.

Population kinetics of bacterial symbionts of the fruit fly

Symbiotic bacteria were present in the newly emerged adult flies and the total bacterial count in one-day-old flies was 0.85×10^6 cfu. The bacterial population in adult flies significantly increased thereafter with the increase in age up to the 10th day (406.77×10^6 cfu). However, after the 10th day, the bacterial population did not vary significantly and fluctuated around 406.77×10^6 cfu. A characteristic sigmoid growth curve was obtained for population kinetics of bacterial symbionts with respect to the age of adult flies (Fig. 2).

The populations of *K. oxytoca*, *P. agglomerans* and *Staphylococcus* sp. increased significantly up to the 10th day and thereafter fluctuated between 161.89×10^6 to 179.11×10^6 , 138.78×10^6 to 149.00×10^6 and 83.99×10^6 to 88.66×10^6 cfu up to the 25th day, respectively. When the populations of different bacteria were plotted with respect to the age of fruit flies, all these showed the characteristic sigmoid curves (Fig. 2).

Discussion

Bacterial symbionts were similar to *Klebsiella oxytoca*, *Pantoea agglomerans* and *Staphylococcus* sp. on the basis of morphological and biochemical characteristics. Out of the three bacterial symbionts, two were gram-negative and the third one was gram-positive. The gram-negative bacterial symbionts were members of family Enterobacteriaceae and the gram positive was of family Staphylococcaceae.

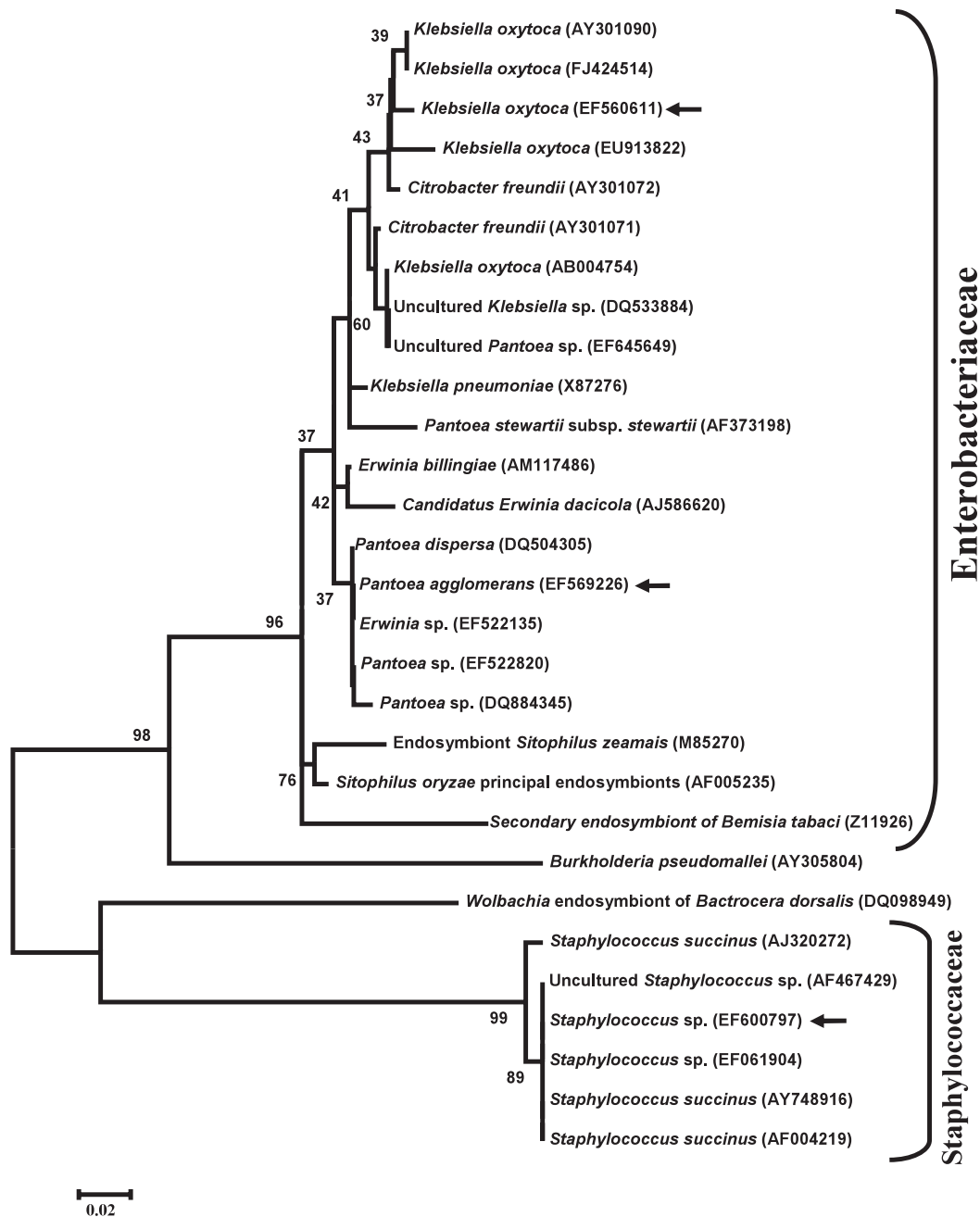


Fig. 1. Neighbor-joining tree of bacterial symbionts of *Bactrocera tau* based on 16S rRNA sequences. GenBank accession numbers are followed by names of bacteria or host insect species. *Burkholderia pseudomallei* was taken as an outgroup. The arrows indicate the position of symbionts of *B. tau*.

Bacterial association with tephritidae in general and *Bactrocera* in particular is well known and has been confirmed by many workers. Gupta et al. (1982a) isolated bacteria from different organs of *B. cucurbitae*, but could record only one type of bacteria from the fruit flies. Sood and Nath (2002) isolated 11 types of bacteria from *B. tau* and *B. cucurbitae* and established the association of fruit fly type bacteria with *Bactrocera*

tau which is in conformity with the present findings. The presence of bacteria in the alimentary track of *B. tryoni* Froggatt in Australia has been well documented (Drew and Lloyd, 1987). Unlike Gupta et al. (1982b), who reported only one species (*Pseudomonas pseudomallei*) in *B. cucurbitae*, Drew and Lloyd (1987) reported six types of bacteria in *B. tryoni*. The variation in number and types of bacteria associated with *Bac-*

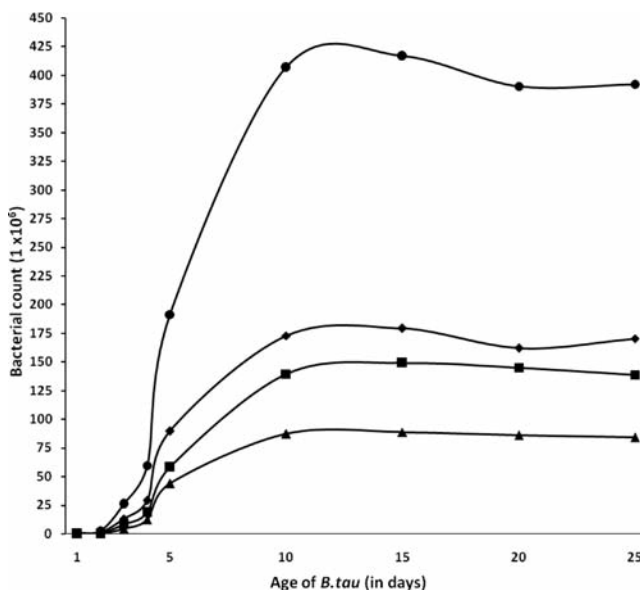


Fig. 2. Growth curve of three bacterial symbionts and overall bacterial population within *B. tau* adult against age of *B. tau*. ◆, *K. oxytoca*; ■, *P. agglomerans*; ▲, *Staphylococcus* sp.; ●, overall bacterial population.

trocera by different workers from different species of fruit fly and from different areas might be due to variation in species and geographical locations. In spite of this variation, all bacterial spp. associated with *Bactrocera tau* belonged to families Enterobacteriaceae and Staphylococcaceae.

K. oxytoca has been found to be associated with *B. tau* for the first time, though it has been reported to be common in other species of fruit flies (Behar et al., 2005, 2008; Bergey et al., 2001; Capuzzo et al., 2005; Lloyd et al., 1986; Sacchetti et al., 2008). The most common bacteria associated with *Bactrocera* flies are *Citrobacter freundii*, *Enterobacter agglomerans*, *E. cloacae*, *K. oxytoca* and *Kluyvera* spp. (Behar et al., 2009; Drew and Lloyd, 1991; Jang and Nishijima, 1990; Lauzon, 2003; Lloyd et al., 1986). *K. oxytoca*, a diazotrophic bacterium was reported to be widely distributed within the host population and found at high levels in fruit flies in different developmental stages (Behar et al., 2005, 2009; Bergey et al., 2001; Zinder and Dworkin, 2000). It helps in fulfilling the nitrogen requirements of the host insects. *Pantoea agglomerans* is also a free-living diazotrophic bacterium of family Enterobacteriaceae and was consistently associated with different organs and stages of *B. tau* and *B. cucurbitae* and reported as common gut bacteria of fruit flies across species and locations in Himachal

Pradesh, India (Sood and Nath, 2002). Thus the present findings get substantial support from the observations of other workers, who consistently observed the association of *K. oxytoca* and *P. agglomerans* with different organs and developmental stages of *B. tau*.

Kuzina et al. (2001) have reported occurrence of members of genus *Staphylococcus* in the gut of the Mexican fruit fly, *Anastrepha ludens* Loew, but no report pertaining to its association with genus *Bactrocera* was found. The bacterium, however, could not be identified up to the species level, hence vertebrate pathogenicity of this bacterium cannot be denied as some species of *Staphylococcus* have been reported as human pathogens (Matthews et al., 1997; Ogston, 1984; Ryan and Ray, 2004), which need further investigation.

The total bacterial population within the adult fruit fly increased significantly up to the 10th day (406.77×10^6 cfu) and did not differ significantly thereafter. The bacterial population reached its maximum on the 10th day, coinciding with the reproductive maturity of fruit flies (gonad development), indicating a possible role in meeting the protein requirements of the females. Earlier *Erwinia dacicola*, a predominant species in the esophageal bulb of *B. oleae* (emerging in sterile conditions), had a vital role in adult survival (Capuzzo et al., 2005). It was involved in nitrogen fixation and/or in pectin degradation like in case of gut bacteria (Enterobacteriaceae) of *Ceratitis capitata* (Behar et al., 2005, 2008). In the case of young larvae also, pectinolytic activity could be an important metabolic function when the fruits have low protein. Bacterial cells may also be consumed as food, providing amino acids, nitrogen compounds and other nutrients which are scarce in fruit (Sacchetti et al., 2008). The true role of gut bacteria in the life cycle of different fruit fly species, however, needs further study. A lack of sufficient available nucleotide sequences and population kinetics of the fruit fly bacterial symbionts also necessitate increasing such information on fruit fly-bacteria symbiotic relationships, which in turn may increase our understanding of these bacteria for eco-friendly management of fruit flies in the near future.

References

- Behar, A., Ben-Yosef, M., Lauzon, C. R., Yuval, B., and Jurkevich, E. (2009) Structure and function of the bacterial community associated with the Mediterranean fruit fly. *In* Insect

- Symbiosis, Vol. 3, ed. by Bourtzis, K. and Miller, T., CRC, Boca Raton, pp. 251–271.
- Behar, A., Jurkevitch, E., and Yuval, B. (2008) Bringing back the fruit into fruit fly-bacteria interactions. *Mol. Ecol.*, **17**, 1375–1386.
- Behar, A., Yuval, B., and Jurkevitch, E. (2005) Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly, *Ceratitis capitata*. *Mol. Ecol.*, **14**, 2637–2643.
- Bergey, D. H., Holt, J. G., and Krieg, N. R. (2001) *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins, Baltimore, 964 pp.
- Bousch, G. M. and Matsumura, F. (1967) Insecticidal degradation by *Pseudomonas melophthora*, the bacterial symbiont of the apple maggot. *J. Econ. Entomol.*, **60**, 918–920.
- Brooks, M. A. (1963) The microorganisms of healthy insects. In *Insect Pathology: An Advanced Treatise*, ed. by Steinhause, E. A., Academic Press, London, pp. 215–250.
- Buchner, P. (1965) Symbiosis in animals which suck plant juices. In *Endosymbiosis of Animals with Plant Microorganisms*, Wiley Interscience, New York, USA, pp. 210–432.
- Capuzzo, C., Firrao, G., Mazzon, L., Squartini, A., and Girolami, V. (2005) '*Candidatus Erwinia dacicola*,' a coevolved symbiotic bacterium of the olive fly *Bactrocera oleae* (Gmelin). *Int. J. Syst. Evol. Microbiol.*, **55**, 1641–1647.
- Colwell, R. R. (1970) Polyphasic taxonomy of the genus *Vibrio*: Numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus* and related *Vibrio* species. *J. Bacteriol.*, **104**, 410–433.
- Dougals, A. E. (1998) Nutritional interactions in insects—microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.*, **43**, 17–37.
- Drew, R. A. I., Courtice, A. C., and Teakle, D. S. (1983) Bacteria as a natural source of food for fruit flies (Diptera: Tephritidae). *Oecologia*, **60**, 279–284.
- Drew, R. A. I. and Lloyd, A. C. (1987) Relationship of fruit flies (Diptera: Tephritidae) and their bacteria to host plants. *Ann. Entomol. Soc. Am.*, **80**, 629–636.
- Drew, R. A. I. and Lloyd, A. C. (1989) Bacteria associated with fruit flies and their host plants. In *World Crop Pests: Fruit Flies, Their Biology, Natural Enemies and Control*, Vol. 3A, ed. by Robinson, A. S. and Hopper, G., Elsevier Science Publishers, Amsterdam, Netherlands, pp. 131–140.
- Drew, R. A. I. and Lloyd, A. C. (1991) Bacteria in the life cycle of tephritid fruit flies. In *Microbial Mediation of Plant-herbivore Interactions*, ed. by Barbarosa, P., Krischik, V. A., and Jones, C.G., John Wiley & Sons, New York, pp. 441–465.
- Gupta, K. and Anand, M. (2003) In vitro vitamin synthesis by the symbionts of *Bactrocera cucurbitae*. *Indian J. Entomol.*, **65**, 518–528.
- Gupta, M., Pant, N. C., and Lal, B. S. (1982a) Symbionts of *Dacus cucurbitae* (Coquillette). I. Location and nature of association. *Indian J. Entomol.*, **44**, 325–330.
- Gupta, M., Pant, N. C., and Lal, B. S. (1982b) Symbionts of *Dacus cucurbitae* (Coquillette). II. Cultivation and identification. *Indian J. Entomol.*, **44**, 331–336.
- Heddi, A., Charles, H., Khatchadourian, C., Bonnot, G., and Nardon, P. (1998) Molecular characterization of the principal symbiotic bacteria of the weevil *Sitophilus oryzae*: A peculiar G + C content of an endocytobiotic DNA. *J. Mol. Evol.*, **47**, 52–61.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (2000) *Bergey's Manual of Determinative Bacteriology*, Lippincott Williams and Wilkins, New York, pp. 175–533.
- Huque, R. (2006) Comparative studies on the susceptibility of various vegetables to *Bactrocera tau* (Diptera: Tephritidae). *Pakistan J. Biol. Sci.*, **9**, 93–95.
- Jang, E. B. and Nishijima, K. A. (1990) Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). *Environ. Entomol.*, **19**, 1726–1731.
- Kounatidis, I., Crotti, E., Sapountzis, P., Sacchi, L., Rizzi, A., Chouaia, B., Bandi, C., Alma, A., Daffonchio, D., Mavragani-Tsipidou, P., and Bourtzis, K. (2009) *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). *Appl. Environ. Microbiol.*, **75**, 3281–3288.
- Kuzina, L. V., Peloquin, J. J., Vacek, D. C., and Miller, T. A. (2001) Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Curr. Microbiol.*, **42**, 290–294.
- Lauzon, C. R. (2003) Symbiotic relationships of tephritids. In *Insect Symbiosis*, Vol. 2, ed. by Bourtzis, K. and Miller, T., CRC, Boca Raton, pp. 115–129.
- Lauzon, C. R., Sjogren, R. E., and Prokopy, R. J. (2000) Enzymatic capabilities of bacteria associated with apple maggot flies, a postulated role in attraction. *J. Chem. Ecol.*, **26**, 953–967.
- Lauzon, C. R., Sjogren, R. E., Wright, S. E., and Prokopy, R. J. (1998) Attraction of *Rhagoletis pomonella* (Diptera: Tephritidae) flies to odour of bacteria: Apparent confinement of specialized members of Enterobacteriaceae. *Environ. Entomol.*, **27**, 853–857.
- Lloyd, A. C., Drew, R. A. I., Teakle, D. S., and Hayward, A. C. (1986) Bacteria associated with some *Dacus* species (Diptera: Tephritidae) and their host fruits in Queensland. *Aust. J. Biol. Sci.*, **39**, 361–368.
- Marchini, D., Rosetto, M., Dallai, R., and Marri, L. (2002) Bacteria associated with the oesophageal bulb of the medfly *Ceratitis capitata* (Diptera: Tephritidae). *Curr. Microbiol.*, **44**, 120–124.
- Matthews, K. R., Roberson, J., Gillespie, B. E., Luther, D. A., and Oliver, S. P. (1997) Identification and differentiation of coagulase-negative *Staphylococcus aureus* by polymerase chain reaction. *J. Food Prot.*, **60**, 686–688.
- Moran, N. A., McCutcheon, J. P., and Nakabachi, A. (2008) Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.*, **42**, 165–190.
- Murphy, K. M., MacRae, I. C., and Teakle, D. S. (1988) Nitrogenase activity in the Queensland fruit fly, *Dacus tryoni*. *Aust. J. Biol. Sci.*, **41**, 447–451.

- Narayanan, E. S. and Batra, H. N. (1960) Fruit Flies and Their Control, ICAR, New Delhi, India, p. 68.
- Ogston, A. (1984) On abscesses: Classics in infectious diseases. *Rev. Infect. Dis.*, **6**, 122–128.
- Petri, L. (1909) Ricerche sopra i batteri intestinali della mosca olearia. *Mem. St. Pat. Veg. Roma*, 1–130.
- Prakash, O., Verma, M., Sharma, P., Kumar, M., Kumari, K., Singh, A., Kumari, H., Jit, S., Gupta, S. K., Khanna, M., and Lal, R. (2007) Polyphasic approach of bacterial classification — An overview of recent advances. *Indian J. Microbiol.*, **47**, 98–108.
- Robacker, D. C., Martinez, A. J., Garcia, J. A., and Bartelt, R. J. (1998) Volatiles attractive to the Mexican fruit fly (Diptera: Tephritidae) from eleven bacteria taxa. *Florida Entomol.*, **81**, 497–509.
- Ryan, K. J. and Ray, C. G. (2004) Sherris Medical Microbiology, McGraw Hill Book Company, New York, USA.
- Sacchetti, P., Granchietti, A., Landini, S., Viti, L., Giovannetti, L., and Belcari, A. (2008) Relationships between the olive fly and bacteria. *J. Appl. Entomol.*, **132**, 682–689.
- Sacchetti, P., Landini, S., Granchietti, A., Cama, A., Rosi, M. C., and Belcari, A. (2007) Attractiveness to the olive fly of *Pseudomonas putida* isolated from the foregut of *Bactrocera oleae*. Integrated protection of olive crops. *IOBC/WPRS Bull.*, **30**, 37–42.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.
- Sharma, P. N., Kaur, M., Sharma, O. P., Sharma, P., and Pathania, A. (2005) Morphological, pathological and molecular variability in *Colletotrichum capsici*, the cause of fruit rot of chillies in the subtropical region of North-Western India. *J. Phytopathol.*, **153**, 141–148.
- Sood, P. and Nath, A. (1999) Fruit flies associated with cucurbits in Himachal Pradesh. *J. Hill Res.*, **12**, 52–54.
- Sood, P. and Nath, A. (2002) Bacteria associated with *Bactrocera* sp. (Diptera: Tephritidae) — Isolation and identification. *Pest Manag. Econ. Zool.*, **10**, 1–9.
- Stouthamer, R., Breeuwer, J. A. J., and Hurst, G. D. D. (1999) *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.*, **53**, 71–102.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, **24**, 1596–1599.
- Tamura, K., Nei, M., and Kumar, S. (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA*, **101**, 11030–11035.
- White, I. M. and Elson-Harris, M. M. (1992) Fruit Flies of Economic Significance: Their Identification and Bionomics, Centre for Agriculture and Biosciences International, Wallingford, UK, 601 pp.
- Zinder, D. E. and Dworkin, M. (2000) Morphological and physiological diversity. In *The Prokaryotes*, ed. by Dworkin, M., Rosenberg, E., Schleifer, K. H., and Stackebrandt, E., Springer Verlag, New York, USA. URL <http://www.prokaryotes.com>