Chapter V. BACTERIAL DIVERSITY IN REDUVIIDS GUT

V.1. Introduction

Microorganism plays an important and essential role in the growth and development of many insects. Numerous investigations were available about the bacterial flora of insect guts appears to be fortuitous contamination of the insect’s surroundings and its food (Hunt and Charnley, 1981). The indigenous gut bacteria is regarded as a valuable metabolic resource to the nutrition of the host by improving the ability to live on sub-optimal diets and digestion efficiency, acquisition of digestive enzymes and provision of vitamins (Douglas, 1992; Tanada and Kaya, 1993; Biggs and Mc Grego, 1996; Bignell et al., 1997; Chen and Purcell, 1997; Braumen et al., 2001; Broderick et al., 2004).

In many groups of the Heteroptera, the posterior end of the midgut is characterised by the presence of sac-like appendages which opens in to the hindgut. These evaginations, called caeca (or) crypts, vary considerable in number and arrangement in different taxonomic groups and almost always contain specific bacteria (Miyamoto, 1961; Goodchild, 1966). Although several bacteria have been isolated from the gut of some heteropterans, the microbiological nature of symbiotic bacteria has been poorly understood. Meanwhile till today no works and hypotheses were reported related to this proposed work. Among the haemolymph sucking groups of the Heteroptera, the family Reduviidae shows the most remarkable behavioural and anatomical arrangement for transmission of the
symbiont (Gobalakrishnan, 2001). Many studies were reported about the number of intestinal microflora which closely associated with the feeding habits of insects (Jones, 1984).

Very little is known about the autochthonous gut bacterial populations associated with reduviids (Sahayaraj and Mary Joseph, 2003). Recently Sahayaraj (2006, 2007b, 2008) first isolated and identified the gut bacteria of three reduviid predators such as *Acanthaspis pedestris* (Stal.) *Haematorrhophus nigrovidaceous* (Reuter) and *Cattamierus brevipennis* (Distant). However, no information was available about the impact of temperatures on gut bacterial autochthonous bacterial population and their hydrolytic enzyme activities of *R. marginatus* and *R. fuscipes*.

V.2. Methodology

Laboratory emerged *R. marginatus* and *R. fuscipes* adults which maintained at different temperatures were used for this study. The stock categories were cultured under laboratory conditions (29 ± 1°C and 80% RM) on *C. cephalonica* following the methods of Sahayaraj (2002).

V.2.1. Dissection of insects

Ten *R. marginatus* and *R. fuscipes* each were selected randomly from all temperature regimes prior to the morning feeding when the gut was empty. Place the insects at 4°C for 15 minutes prior to use. Surface of the predators were sterilized with 0.1% Mercuric Chloride for 2 minutes and washed with sterile distilled water thrice. Under aseptic condition each insect was carefully dissected by using sterile pins, fine forceps and razors in a dissection tray filled with sterile
phosphate buffered saline (PBS) (pH 6.9). Guts were isolated individually, washed several times with fresh phosphate buffered saline to minimise the possible microbial contamination and used for the study. Wet weight of the gut was recorded for individually categorized with six temperatures for *R. marginatus* and *R. fuscipes*.

V.2.2. Enumeration of THMP of predators gut content

The isolated gut was homogenized with sterile insect ringer solution (IRB) in mortar and pestle. The homogenate was filtered through Whatmann filter paper No.1 and the pH was measured using pH meter. The filtrate was serially diluted in sterile saline and 0.1 ml of aliquot was plated on nutrient agar (NA) and Trypticase soy agar (TSA). The seeded nutrient agar plate (NA) was inoculated at 37°C for 24-48 hours whereas the Serially Dilution Agar (SDA) plates were incubated in 28°C for 48-72 hrs. Microbial colonies appeared after the incubation period was enumerated and the numbers of colony forming units were expressed as a wet weight of the gut.

V.2.3. Identification of Microflora

Different morphological microbial colonies were selected, sub-cultured and stored at 4°C on respective agar slants. Bacterial strains were identified using the criteria suggested by Cappucino and Sherman, 1999; Buchanon and Gibbons (1979) based on morphological, cultural and biochemical characterisations.
V.2.4. Hydrolytic extra cellular enzyme

The extra cellular enzymes like amylase, protease, cellulase and gelatinase activities were tested by using the nutrient media containing 0.2% (W/V) carboxymethyl cellulose (cellulase), starch (amylase) and skimmed with powder (protease) as substrates (Plate 3). Pure culture of each bacterial isolate was streaked on respective media and utilization of the substrates was determined by observing the clear zone around the colonies (Buchanon and Gibbons, 1979). All the screening experiments were replicated at thrice.

V.2.5. Statistical Analysis

Correlation analyses were made between the temperature and gut bacterial population of *R. marginatus* and *R. fuscipes* separately.

V.3. Results

V.3.1 Weight and pH of the reduviid guts

Generally, predator’s intestinal pH profile was alkaline nature (Lemeke, 2003). But the present study reveals that gut content of *R. marginatus* (pH 7.6) and *R. fuscipes* (pH 7.0) were found to be either slightly alkaline or neutral. Our results revealed that the gut of these reduviids predators was neutral to slightly alkaline.

In *R. fuscipes*, gut weight was gradually diminished up to 30°C then increased to 35°C whereas in *R. fuscipes* gut weight was gradually decreased up to 25°C then decreased from 30 to 35°C. Alimentary canal weight of *R. marginatus* was higher when compared to *R. fuscipes* (figure 11). Total heteroptrophic
bacterial populations (THBP) of the whole gut homogenate of both predators are presented in figure 12. The THMP of both reduviid predators were gradually increased from 10-30°C and then declined at 35°C. Between the two reduviids, maximum THMP was observed in *R. fuscipes* than *R. marginatus* (Figure 12). THMP was higher the control category.

V.3.2. Bacterial Composition

Thirteen and eleven isolates of bacteria were grouped based on morphological character, and biochemical tests for both *R. fuscipes* and *R. marginatus*. The bacterial species isolated from the gut of *R. marginatus* includes, *Staphylococcus aureus, Bacillus cereus, B. magaterium, Enterobacter aerogenes, Micrococcus luteus, Corynbacterium kutcherii, C. xerosis, Bacillus subtilis, Escheritia coli, Pseudomonas aeroginosa and Micrococcus variance* (see table 17). Among them *Staphylococcus aureus* was found to be the most dominant specie both at 10°C and 15°C and *Micrococcus variance* was the dominant bacteria between 20 to 30°C. *Bacillus subtilis* dominantly present at 15°C and 35°C for *R. marginatus* and *R. fuscipes*. *Micrococcus luteus, C. xerosis,* were represented in low percent at 25 and 20°C respectively. Among the observed bacterial species, *Escheritia coli* was observed only at 30°C. If average of all the temperatures were considered, *M. variance* constituted the dominant bacteria (40.56%) followed by *S. aureus* (31.78%) and *B. cereus* (24.10%). The most predominant bacteria observed in *R. fuscipes* (Table 18) was *M. variance* (47.22%) followed by *S. aureus* (30.50%) and *B. subtilis* (30.10%). All these bacterial populations were positively correlated (0.67, 0.50, 0.84 and 0.42 for *B. cerosus, M. variance*,
S. aureus and C. xerosis respectively) with different temperatures except B. subtilis ($r^2 = -0.35$) and Lactobacillus delbrucki ($r^2 = -0.02$) shows negative correlation to temperatures. It was more predominant (29%) at 10$^\circ$C. In general S. aureus and B. cereus were more or less similar population in all the temperatures in both reduviids.

V3.4. Hydrolytic extracellular enzymatic activity

Hydrolytic activity was observed in bacterial isolates of the whole gut in both predators. Of the four hydrolytic enzymes, cellulase activity was almost lower in these predators than the amylase and protease. Amylase activity was apparently higher at higher temperature for R. marginatus (65.91) and R. fuscipes (67.6). Another hydrolytic enzymes protease showed peak activity at 25$^\circ$C (55.1 and 47.8 for R. marginatus in R. fuscipes respectively). (Plate 3).

V.4. Discussion

The alimentary canal of insects provides a suitable substratum for the development of microorganisms because of concentrated nutrients and extended surface of the intestinal lumen. On the other hand, these associated microbes may play an important role in the digestion, nutrition and defense system of the host animals. It is possible that fastidious insoluble substrates including cellulose, alginate and chitin continue to be decomposed by the attached microbes in the fecal pellets discharged from the host animals, in addition to the alimentary canal (Pankaj et al., 2003).
Little is known about bacteria associated with the Reduviidae, the large group of mostly zoophagous insects comprising the haematophagous and predatory insects. In the present study we identified many novel bacterial species, which belongs to sub divisions of the Proteobacteria. Plata stingbug, *Megalopta punctatissima* also has this type of bacteria in its gut (Fukatsu and Hosokawa, 2002). The bacterial genera found in *R. marginatus* and *R. fuscipes* were *Streptococcus* spp., and *Staphylococcus* spp., and *Micrococcus* spp. In insects, there are the typical bacterial colonies found in the intestine of the all polyphagous and phytophagous insects (Brooks, 1963; Tanada and Kaya 1993). The present report was also indicating the diversity of bacteria present in the digestive system (fore, mid, hindgut) of *R. marginatus* and *R. fuscipes* (Sahayaraj and Mary Joseph 2003). Thermostable enzymes can be obtained from both mesophilic and thermophilic organisms; thermophiles represent an obvious source of thermostable enzymes. Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Santo *et al.*, 1998). It was also reported that abiotic factors alter the gut bacteria populations (Tsuchida *et al.*, 2002). It may be hypothesised that the bacterial flora degraded the some acid metabolites which might have induce the pH Gut of the lepidopteran moth, *Lymantria dispar* Linn. showed lightly alkaline nature (Broderick *et al.*, 2004).

Chen and Purcell (1997) suggested that environmental condition mainly affect the growth of the microorganism in the digestive system of adult reduviid predators. The gut microflora represent all the aspects of microbial relationship from pathogenic to obligate mutualism (Dillon and Dillon, 2004). Numerous
investigations have been available about the microbial flora of insect gut. Some studies have been presented the incidence of entomopathogens (Lysenko, 1985). The contradiction of gut microbiota to nutrition and disease suppression was reported extensively (Hagen, 1966; Dillon and Charnely, 1986, 1988). Moreover, the study of the microflora associated with the insect predators may lead to the isolation of possible pathogens which may help to design the biological control agents (says Pankaj et al., 2003). The present study deals with the gut microflora associated with six different temperatures reveals that indefinite growth of microbial organisms mainly depends upon the abiotic factors, such as favored temperatures at 25 and 30°C.

We utilised the dilution plate method to recover the microorganism. This is the conventional technique used to isolate microorganisms in most of the microbiology related studies (Santo et al., 1998). Eventhough microorganisms are capable of grow in the SDA media, another major limitation factor was the dilution. Plate method is that rare occurring or poor growing isolates will most likely go undeducted.

Small bacteria population comprises Shigella spp. like L. casei, and L. delbruckii were recovered from the alimentary canal of these to reduviid predators. Others bacteria species from the family of Streptococcus, Bacillus and Micrococcus were also identified from these reduviids. From these genus common species like Bacillus cercus, Bacillus subtilus, Micrococcus variance, Micrococcus leutus, Enterobater aerogenes were isolated from reduviids. Many members of Enterobacteriace, Microcococeca, Bacillacea are common in freshwater, soil,
sewage, plants, vegetables and animals including insect guts (Koch, 1967; McKillip et al., 1997).

From the results, it was hypothesised that the bacterium is a mutualistic gut symbiont of *R. marginatus* which is vertically transmitted through the egg capsule or food and is essential for normal development and growth of the host insect. Previously the morphology of symbiotic bacteria of plant sucking insect species has been described (Buchner, 1965, Tustomu et al., 2006). Therefore, this study is the first report of phylogenetic position of a gut symbiont in accord with various higher and lower temperatures from zoophagous reduviid bugs.

*E. aerogenes* was the dominantly present in the gut of *Chrysoperla rufilabris* (Burmeistre) (Mecoptera) an important biological control agent throughout the world (Harklein, 2003). In *R. marginatus*, *E aerogenes* was reported at 10°C. It was a common bacterium in plants, vegetables and animals including insect guts (Dash et al., 1984). Temperatures alter the clima or mature bacterial community within the gut. Both lower (10 and 15°C) and higher temperatures (20, 25, 30 and RT) were in favour for the colonization of *S. aureus* and *M. variance* in both reduviids. These may be due to inter specific competition among autochthonous gut bacteria of the reduviids.
V.5. Conclusion

THMB of both the species increased upto 30°C for *R. marginatus* (12.37x10⁴) and *R. fuscipes* (9.4 x 10⁴ CFU/gm). From this result it was very clear that predators maintained at room temperature had maximum gut weight with higher bacterial density. Between the two predators *R. fuscipes* has minimum bacterial density than *R. marginatus*. In lower temperatures (10,15 and 20°C) microbial colony forming tendency was low in both reduviids. Bacterial strains like *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus variance* were increased when temperature increased. Though both predators have similar kind of bacterial populations such as *K. pneumonsae*, *Lactobacillus delbruckii* and *Lactobacillus casei* were considered as autochthons bacteria of these reduviids and their populations on present only in *R. fuscipes* implies the species specificity of bacterial populations. *Staphylococcus aureus* was present dominantly between 10-20°C whereas *Micrococcus variance* was higher < 30°C. More than 12 bacterial isolates were identified in *R. marginatus* and *R. fuscipes* when fed with *S. litura*. Isolated bacterial strains were able to produce cellulase and amylase (C+A) followed by xylenase. Maximum hydrolytic activity was observed in cellulase and amylase producing isolates belonging to *M. variance* and *S. aureus*. 