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Contributed Paper

Bacterial Communities in Larval Diapause and Pupal Guts In the Bamboo Borer, *Omphisa fuscidentalis* Hampson

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ABSTRACT

This study investigated bacterial diversity in the gut of diapausing larvae and pupae of bamboo borer (*Omphisa fuscidentalis*) in non-feeding stages by using culture dependent methods. The densities of culturable bacteria in guts of diapausing larvae were high in the foregut and hindgut and it was significantly higher than that from pupae ($P < 0.05$). Genetic analysis of cultured, isolated bacteria revealed 33 sequences of 16S rRNA genes, from which the bacteria could be divided into three phyla groups, namely *Gammaproteobacteria*, *Firmicutes* and *Bacteriodes*. Current result therefore is the first report of gut bacteria in diapausing larval and pupal stages of *O. fuscidentalis*. The results indicated that density of bacteria decrease metamorphosis from larvae to pupae and the diversity of bacteria in *O. fuscidentalis* is similar to many insects gut. Thus, gut bacteria in *O. fuscidaentalis* may provide larvae digestion and contribute nutrition.

Keywords: *Omphisa fuscidentalis*, gut bacteria, larval diapause, culture-dependent

1. INTRODUCTION

Omphisa fuscidentalis, the bamboo borer, is a moth in the order Lepidoptera. It is complete metamorphosis insect which the larvae have five instars. The early instar larvae (1^{st}-4^{th} instar) feed on the bamboo shoots until become to 5^{th} instar larvae. The 5^{th} instar larvae are usually found in bamboo from September to the following June annually. This is a period of developmental arrest and low metabolic activity. Moreover, in response to the depletion of food supply, at this stage the larvae do not consume nutrients, therefore they enter the stage of larval diapause. Pupae appear in the beginning of June. They become motionless and stay in this stage for one month before adult development. This is interesting to display how the diapause larvae and pupae can survive without food supply for a long period of time (nine months) [1]. Previously, evidence has been presented that guts of

lepidopterans contain microorganism that produce digestive enzymes and help the host digestion [2] and they play important and often essential roles in the growth and development of many insect species [3]. Due to the bacteria are known to contribute to insect nutrition, protection from parasites and pathogens, and modulation of immune responses [2]. These possible the gut bacterial might contribute nutrition to *O. fuscidentalis* during non-feeding stage. Although, the gut content of *O. fuscidentalis* during larval diapause was studied and the gut length decreased and shortened in the pupal stage [1]. However, information of the microbial community in the gut of *O. fuscidentalis* is still lacking.

2. MATERIALS AND METHODS

2.1 Sample Collection

O. fuscidentalis diapausing larva specimens were collected from a bamboo forest in Maewang District, Chiang Mai Province, Thailand in March, 2015 and pupa specimens were collected in June, 2015.

2.2 Dissection of *O. fuscidentalis*, Culturing and Enumeration of Gut Bacteria

Individuals of larvae were aseptically dissected in laminar flow under a stereomicroscope. The different regions of each larval gut were cut and separated. All pupae (n=5) were dissected to remove the whole guts. The guts were weighed. 30 µl of sterile ice-cold NaCl (0.85% w/v) was added and homogenized. The supernatant (10 µl) was taken and were diluted 1000 times for cultivation. The experiments were carried out in triplicates.The diluted supernatant was incubated for 30 minutes at 37 °C and 100 µl of supernatant spread in three replicates on plate count agar (PCA) for the total bacteria, eosin methylene blue (EMB) plates for Gram-negative bacteria and de Man, Rogosa and Sharpe (MRS) plates for Gram-positive bacteria. The plates were incubated at 37 °C and then the colonies with each designated morphology were counted after 24 and 48 hours. Isolates of representatives of each colony morphology were purified for individual colonies. DNA was extracted with the EasyDNA purification kit (GeneMark, GMbiolab Co., Ltd.) from 18-h-old cultures of each isolate.

The bacterial 16S rRNA gene was amplified by PCR from total DNA by using primers 28F and 519R [4]. PCR products were purified by using Wizard SV Gel and PCR Clean-Up system (Promega, Madison, Wiz) and was sequenced by using either 28F or 519R at the Biomaterials Analysis Division, Technical Department at the Tokyo Institute of Technology. All 16S rRNA gene sequence (500 to 600 bp) were compiled by using ATGC software (GENETYX, JAPAN) and were compared to available databases by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine approximate phylogenetic affiliations. Individual sequence of isolates that were ≥98% identical to each other were considered the same phylotype and were combined for analysis. Determined 16S rRNA encoding gene sequences were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

2.3 Statistical Analysis and Programs

Bacteria density was statistically analyzed with one way ANOVA using SPSS Statistics version 17. Phylogenetic tree was performed on MEGA version 6 [5].

3. RESULTS AND DISCUSSION

We first studied the bacterial density in the guts of diapausing larvae and pupae in non-feeding stages. Cultivatable bacteria counts from different parts of *O. fuscidentalis*

gut during larval diapause were obtained by using three different medium plates (Table 1). Total bacteria counts from foregut on PCA plates was no significant difference when compared to the total bacteria population from hindgut. However, the bacterial population decreased in the midgut. Similar trends were shown in the other two medium plates. The result was somewhat different from the previous observation on the variegated grasshopper, *Zonocerus variegatus* which showed the highest bacterial population in the midgut [3]. This is possibly due to the midgut length of *O. fuscidentalis*, which is longer than the other part and contains no solid food [1], contributing to low density of bacteria observed in the midgut. In contrast, the foregut is a short tube and the bacteria might be acquired from an outside environment in the bamboo when the diapausing larvae lick on individual skin or feces. The high density of gut bacteria was also found in hindgut containing fibrous materials [1]. When comparing the density of gut bacteria of the diapausing larvae and pupae (Figure 1), the results showed that fewer bacteria were present in pupal guts, which might be due to the fact that the pupal guts contained no solid materials and there had been no feeding for a long period of time [1]. This result consistent with previous study which report that the reduction of bacteria occurs prior to pupation by voiding of the gut and secretion of antibacterial proteins into the pupal gut lumen [6]. Moreover, we did not find any pupal gut bacteria in MRS plate which MRS medium suitable for Gram-positive bacteria. This showed that Gram-positive bacteria might not present in the pupal gut. This indicates that the bacterial community changes during metamorphosis in which the overall body organization of the larva changes completely. This result was supported by previous studies in insect the herbivorous, *Spodoptera littoralis* [7]. However, the members of bacteria in the diapause larvae are associated to pupa probably because the bacteria are able to transit across the insect developmental stages [8]. When compared with previous studies amongst members of Lepidoptera [9, 10], the bacterial diversity in *O. fuscidentalis* gut displayed much higher.

Table 1. Bacterial density in the different region of larval diapause gut in *O. fuscidentalis* determined on different medium. Each value is the mean ± SD, * indicate significant differences at $P < 0.05$.

Medium	Bacterial density (log CFU/g) ± SD		
	Foregut	Midgut	Hindgut
PCA	8.36±0.22	7.36±0.30*	8.12±0.49
EMB	8.19±0.22	7.17±0.41*	8.00±0.59
MRS	8.07±0.17	7.18±0.37*	7.99±0.56

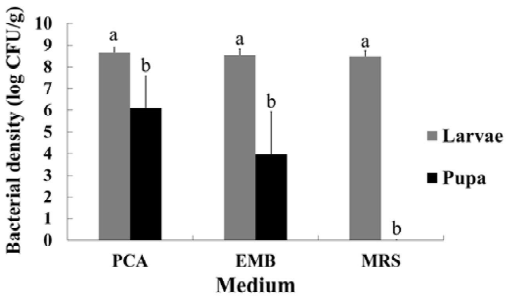

Figure 2. Bacterial Density in the gut of diapause larvae (a) and pupae (b) in *O. fuscidentalis* in the PCA, EMB and MRS plate. Each value is the mean±SD. Means with different letters indicate significant differences (ANOVA, n=5, P<0.05).

The phylogenetic positions of the gut bacteria of diapausing larvae and pupae in *O. fuscidentalis* are shown in Figure 2. The 16S rRNA genes from 33 cultured bacteria can amplified. The sequence were aligned with

23 sequences identified as closely related references in MAGA6 using the 16S rRNA gene sequence of *Methanococcus aeolicus* (Genbank accession DQ195165) as an out group. Phylogenetic analysis with 1000 bootstrap re-samplings identified three groups, namely *Gammaproteobacteria* (42.42%), *Firmicutes* (48.48%) and *Bacteriodes* (9.1%). This results were similar in many insects gut [10]. Members of *Gammaproteobacteria* and *Firmicutes* were found to be abundant in the

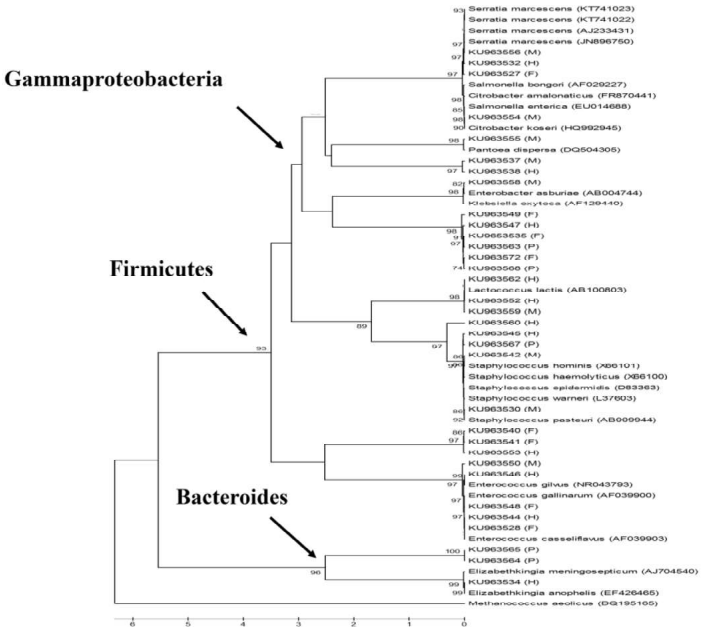

Figure 3. Phylogenetic tree of bacteria 16S rRNA gene sequences isolated from diapause larval and pupal gut *O. fuscidentalis* and closely related references sequences. Bootstrap values (based on 1000 resampling) higher than 50 are indicated at the nodes. Tip label includes GenBank Accession number and gut parts ((F) foregut, (M) midgut, and (H) hindgut gut).

guts of moths, honey bees and termites [10-12].

Our study showed that, the bacterial sequences from three gut regions including pupal gut were closely related to *Serratia marcescens*. This bacterium has been described in many insects [9, 13]. *Serratia* also produce digestive enzymes in the gut of the silkworm, *Bombyx mori* [14]. *Enterococcus gallinarum* and *Enterococcus casseliflavus* were also found in all three gut parts; these species could also be isolated from the larval gut of the tobacco hornworm, *Manduca sexta* [10]. The member of genus *Enterococcus* are amongst the most common gut bacteria detected in the larval guts across a diversity of insect orders including Lepidoptera [9]. In addition, *Citrobacter* sp was detected from larval foreguts and midguts in this study. It has been previously found in many insect guts [10]. It is, in fact, a metabolically active gut inhabitant and assists nitrogen fixation in termite guts [15]. *P. dispersa* was detected only in midgut. This bacterium was previously isolated from guts of the gypsy moth, *Lymantria diaper* [9]. The important role of *P. dispersa* in the insect gut is nitrogen fixation and also nutrient support and digestion in insects. *L. lactis* was detected in both larval midguts and hinguts. *L. lactis* is a lactic acid bacterium

that is frequently found in the gut of termite species [16]. The DNA sequences matched to members of *Staphylococcus* were found in midguts, hindguts and pupal guts. *Staphylococcus* are the most common gut bacteria and were found in many insects such as the variegated grasshopper, *Z. variegatus* [3]. Our result suggested that density of bacteria in *O. fuscidentalis* decreased during metamorphosis from larvae to pupae and the diversity of bacteria was similar to many insects gut. Thus, gut bacteria in *O. fuscidaentalis* may provide larvae digestion and contribute nutrition. However, the roles of these bacteria in diapausing larvae and pupae are still unknown.

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