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Bacterial Symbionts of the Giant Jewel Stinkbug *Eucorysses grandis* (Hemiptera: Scutelleridae)

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Microbiological characterization of gut symbiotic bacteria in a limited number of stinkbugs of the families Acanthosomatidae, Plataspidae, Pentatomidae, Scutelleridae, Parastrachiidae, Alydidae and Pyrrhocoridae has shown symbiotic association with midgut bacteria to be common in phytophagous taxa of these heteropteran insects. Here we investigated the midgut bacterial symbiont of *Eucorysses grandis*, a stinkbug of the family Scutelleridae. A specific gammaproteobacterium was consistently identified in insects from five different geographic origins. The bacterium was detected in 64 of 64 insects sampled from three host populations. Phylogenetic analyses revealed that the bacterium constitutes a distinct lineage in the *Gammaproteobacteria*, neither closely related to the gut symbiont of another scutellerid stinkbug, *Cantao ocellatus*, nor to gut symbionts of other stinkbugs. Diagnostic PCR, in situ hybridization and electron microscopy demonstrated that the bacterium is located extracellularly, in the midgut fourth section, which possesses crypts. These results indicate that the primary gut symbionts have multiple evolutionary origins in the Scutelleridae. A *Sodalis*-allied facultative symbiont was also identified in some insects from natural populations. Biological aspects of the primary gut symbiont and the secondary *Sodalis*-allied symbiont are discussed.

Key words: *Eucorysses grandis*, Scutelleridae, gut symbiont, *Sodalis*, *Gammaproteobacteria*

INTRODUCTION

In the insect order Hemiptera, members of the suborder Heteroptera, commonly known as true bugs or stinkbugs, represent over 38,000 described species characterized by sucking mouthparts, half-membranous forewings, and incomplete metamorphosis (Schuh and Slater, 1995). In the Heteroptera, symbiotic associations with bacteria are common in plant-sucking taxa, particularly in stinkbugs of the infraorder Pentatomomorpha. These symbiotic species possess many sacs or tubular outgrowths, called crypts or caeca, in a posterior region of the midgut, wherein specific bacteria are harbored (Glasgow, 1914; Buchner, 1965). Aposymbiosis often results in retarded growth and high mortality in the insects (Abe et al., 1995; Fukatsu and Hosokawa, 2002; Hosokawa et al., 2006; Kikuchi et al., 2007; Kikuchi et al., 2009; Prado and Almeida, 2009b), indicating that the symbionts play a physiological role for the hosts. The symbionts are vertically transmitted across host generations by several post-hatch transmission mechanisms: (i) egg surface contamination in the Pentatomidae, Acanthosomatidae and Scutelleridae (Rosenkranz, 1939; Abe et al., 1995; Prado et al., 2006; Kikuchi et al., 2009; Prado and Almeida, 2009a; Kaiwa et al., 2010), (ii) coprophagy in the Cydnidae and Coreidae (Huber-Schneider, 1957; Schorr, 1957), or (iii) capsule transmission in the Plataspidae (Hosokawa et al., 2005), whereas a case of environmental acquisition has been reported from the Alydidae (Kikuchi et al., 2007). To date, the gut symbiotic bacteria of a limited number of the stinkbug families Acanthosomatidae, Plataspidae, Pentatomidae, Scutelleridae, Parastrachiidae, Alydidae and Pyrrhocoridae have been subjected to modern microbiological characterization (Kikuchi et al., 2005, 2009; Hosokawa et al., 2006, 2010; Kaltenpoth et al., 2009; Prado and Almeida, 2009a; Kaiwa et al., 2010).

Members of the family Scutelleridae, often referred to as jewel bugs or shield-backed bugs, are known for their vivid and beautiful body coloration (Schuh and Slater, 1995). The family includes some 80 genera and 450 species, and in Japan, at least seven genera and nine species have been recorded (Tomokuni et al., 1993). Early histological works described the presence of symbiotic bacteria in midgut...
crypts of several scutellerid species (Glasgow, 1914; Kuskop, 1923; Rosenkranz, 1939). Since that time, however, the microbial nature of scutellerid symbionts has remained obscure. Recently, a gammaproteobacterial primary gut symbiont and a Sodalis-allied facultative symbiont were identified from the scutellerid stinkbug Cantao ocellatus (Kaiwa et al., 2010).

However, it is uncertain whether these bacterial symbionts are commonly found in other scutellerid species.

In this study, we investigated the endosymbiotic microbiota of another scutellerid stinkbug, Eucorysses grandis (Fig. 1A). A gammaproteobacterial gut symbiont and a Sodalis-allied facultative symbiont were identified in E. grandis, as in C. ocellatus, but the gut symbiont was phylogenetically distinct from that of C. ocellatus, suggesting multiple evolutionary origins of the primary gut symbionts in the Scutelleridae.

**MATERIALS AND METHODS**

**Insect materials**

Table 1 shows collection data of E. grandis used in this study. Adult insects were collected at five localities in areas of southern Japan.

**Dissection and DNA extraction**

The first, second, third, and fourth sections of midgut, as well as ovaries and testes, were dissected from adult insects using a pair of fine forceps under a dissection microscope in a glass Petri dish filled with phosphate buffered saline (PBS; 137 mM NaCl/8.1 mM Na₂HPO₄/2.7 mM KCl/1.5 mM KH₂PO₄ [pH 7.5]). The dissected tissues were either immediately subjected to DNA extraction using NucleoSpin® Tissue kit (Macherey-Nagel), or preserved in acetone until use (Fukatsu, 1999).

**DNA cloning and sequencing**

A 1.5 kb segment of bacterial 16S rRNA gene was amplified with the primers 16SA1 (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16SB1 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Fukatsu and Nikoh, 1998). Polymerase chain reaction (PCR) was conducted with AmpliTaq GoldTaq DNA polymerase (Applied Biosystems) and the supplemented buffer system, following a temperature profile of 95°C for 10 min, followed by 35 cycles consisting of 95°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. Cloning and sequencing of the amplified products were performed as previously described (Kaiwa et al., 2010).

**Diagnostic PCR**

The following specific primer sets were used for diagnostic PCR detection of 16S rRNA gene of the symbiotic bacteria: ookin162F (5′-GCT AAT ACC GCA TAA CGT CTT-3′) and

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**Table 1.** Samples of Eucorysses grandis used in this study.

<table>
<thead>
<tr>
<th>Collection locality</th>
<th>Collection date</th>
<th>Collector</th>
<th>Host plant</th>
<th>Gut symbiont</th>
<th>Sodalis symbiont</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yakushima,</td>
<td>November</td>
<td>Takema</td>
<td>Schefflera octophylla</td>
<td>AB571334</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kagoshima, Japan</td>
<td>2008</td>
<td>Fukatsu</td>
<td>Vernicia cordata</td>
<td>AB571333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nishino-Omote,</td>
<td>July</td>
<td>Takahiro</td>
<td>Unknown</td>
<td>AB571332</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kagoshima, Japan</td>
<td>2009</td>
<td>Hosokawa</td>
<td>Unknown</td>
<td>AB571331</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kashima,</td>
<td>September</td>
<td>Fumitaka</td>
<td>tobira</td>
<td>AB571335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saga, Japan</td>
<td>2009</td>
<td>Kuchiki</td>
<td>Unknown</td>
<td>AB571333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minami-Boso,</td>
<td>March</td>
<td>Takahiro</td>
<td>Pittosporum</td>
<td>AB571331</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chiba, Japan</td>
<td>2009</td>
<td>Hosokawa</td>
<td>tobira</td>
<td>AB571335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tosa-Shimizu, Kochi, Japan</td>
<td>November</td>
<td>Takuya</td>
<td>Unknown</td>
<td>AB571332</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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ookin616R (5′-TCT ACG AGA CTC AAG CCT GT-3′) for specific
detection of the Sodalis-allied symbiont. The PCR temperature profile was 95°C for 10 min, fol-
lowed by 35 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. To confirm the quality of the DNA samples, a 1.5 kb seg-
ment of the insect mitochondrial 16S rRNA gene was amplified with

In situ hybridization

An Alex555-labeled oligonucleotide probe EUB338 (5′-GCT GCC TCC CTA AGG ACT GC-3′) (Amann et al., 1990) was used for
whole mount fluorescent in situ hybridization targeting eubacterial 16S rRNA. Midgut preparations with crypts were dissected from adult females and were fixed in Carnoy’s solution (ethanol: chloroform: acetic acid = 6:3:1) overnight. The fixed samples were incubated
in ethanolic 6% H2O2 solution for a week to quench the autofluo-
rescence of the tissues (Koga et al., 2009). The samples were thoroughly washed and equilibrated with a hybridization buffer (20 mM Tris HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide), to which the probe and SYTOX green were
added at final concentrations of 0.1 μM and 0.5 μM, respectively. After overnight incubation, the samples were thoroughly washed in PBSTx (PBS containing 0.3% Triton X-100) and observed under an epifluorescence microscope (Axioptico, Carl Zeiss) and a laser confocal microscope (PASCAL5, Carl Zeiss).

Electron microscopy

Midgut crypts were dis-
sected from adult females in
0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaral-
dehyde, prefixed in the fixative at 4°C overnight, and post-
fixed in the phosphate buffer containing 2% osmium tetrox-
ide at 4°C for 60 min. After dehydration through an etha-

Table 2. Infection frequencies of the gut symbiont and the Sodalis-
allied symbiont in natural populations of Eucorysses grandis.

<table>
<thead>
<tr>
<th>Population1</th>
<th>Gut symbiont2</th>
<th>Sodalis-allied symbiont3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Yakushima</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>(15/15)</td>
<td>(15/15)</td>
<td>(30/30)</td>
</tr>
<tr>
<td>Kashima</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>(2/2)</td>
<td>(10/10)</td>
<td>(12/12)</td>
</tr>
<tr>
<td>Minami-Boso</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>(10/10)</td>
<td>(12/12)</td>
<td>(22/22)</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>(27/27)</td>
<td>(37/37)</td>
<td>(64/64)</td>
</tr>
</tbody>
</table>

1Nishino-Ofote and Tosa-Shimizu populations were excluded
because only a few insects were obtained from each population.
2DNA samples from dissected midgut were analyzed.
3DNA samples from dissected gonads were analyzed.

Molecular phylogenetic and evolutionary analyses

A multiple alignment of
nucleotide sequences was generated using the program Clustal W (Thompson et al., 1994), and the final alignment was corrected manually. Phylogenetic analyses were conducted by maximum likeli-
hood and maximum parsimony methods. The best-fit

Fig. 2. Phylogenetic placement of the symbiotic bacteria from E. grandis on the basis of 16S rRNA gene sequences. A maximum likelihood (ML) tree inferred from a total of 1,005 unambiguously aligned nucleo-
otide sites is shown, whereas a maximum parsimony (MP) analysis gave substantially the same topology (data not shown). Bootstrap values higher than 50% are indicated at the nodes in the order ML/MP. Aster-
isks indicate support values lower than 50%. Sequence accession numbers and AT contents of the nucleo-
tide sequences are in brackets and parentheses, respectively. As for insect endosymbionts, the name of the host insect is also indicated in parentheses. P-symbiont, primary symbiont; S-symbiont, secondary symbiont.
Detection of bacterial symbiont in the midgut fourth section of E. grandis

Digestive organs were dissected from adult females of E. grandis (Fig. 1B). The midgut first section was determined to be a large stomach-like structure filled with liquid, followed by tubular midgut second and third sections. The midgut fourth section was equipped with a number of crypts, which were white in color, arranged in four rows, and fused to each other into a loose helical assemblage (Fig. 1C). In situ hybridization detected strong bacterial signals in the crypts of the midgut fourth section (Fig. 1D). Electron microscopy identified tubular bacterial cells of a single morphotype densely packed in the cavity of the midgut crypts (Fig. 1E). A 16S rRNA gene sequence (1,465 bp) was cloned and determined from the midgut fourth section of a female insect of Yakushima population, to which the sequences obtained from insects of Minami-Boso, Tosa-Shimizu, Kashima and Nishino-Omote populations were identical. BLAST searches using this sequence as the query retrieved gammaproteobacterial sequences, of which the top hit was a 16S rRNA gene sequence of uncultured bacterium isolated from mucosal biopsy from human intestinal pouch (98.5% [1,443/1,465] identity; accession number GQ157226).

Detection of another bacterial symbiont in the gonads of E. grandis

A bacterial 16S rRNA gene was detected by PCR on ovarian DNA of a female insect from the Yakushima population. Cloning and sequencing of the PCR product yielded a 1,464 bp sequence. BLAST searches using this sequence as the query retrieved gammaproteobacterial sequences, of which the top hit was 16S rRNA gene sequence of the Sodalis-allied symbiont of Cantao ocellatus (Hemiptera; Scutelleridae) (99.7% [1,459/1,464] identity; accession number AB571330).

Prevalence of the bacterial symbionts in natural populations of E. grandis

Diagnostic PCR surveys showed the prevalence of the gut symbiont in natural populations of E. grandis; of 64 insects from three populations examined, all were PCR-positive. By contrast, the Sodalis-allied symbiont was detected in only a subset of the insects: 13% (4/30), 0% (0/12) and 0% (0/22) in Yakushima, Kashima and Minami-Boso populations, respectively (Table 2).

Phylogenetic placement of the bacterial symbionts from E. grandis

Figure 2 shows the phylogenetic placement of the bacterial symbionts detected from E. grandis on the basis of 16S rRNA gene sequences. None of the gut symbionts of other stinkbugs, including that of the scutellerid C. ocellatus (Kaiwa et al., 2010), was related to the gut symbiont of E. grandis. Meanwhile, the Sodalis-allied symbionts of E. grandis and C. ocellatus formed a well-supported gammaproteobacterial clade with the secondary symbiont of the tsetse fly Sodalis glossinidius, the bacteriome-associated symbiont of grain weevils, the symbiont of a louse fly, the bacteriocyte-associated symbiont of a pigeon louse, and a secondary symbiont of a chestnut weevil.

Molecular evolutionary aspects of the bacterial symbionts from E. grandis

The 16S rRNA sequences of the gut symbiont and the Sodalis-allied symbiont exhibited AT contents of 45.3% and 44.7%, respectively, which were not different from AT contents of other phylogenetically-related free-living gammaproteobacteria at around 45% (Fig. 2). The evolutionary rates of the 16S rRNA gene sequences in the gut symbiont and the Sodalis-allied symbiont were not significantly different from the evolutionary rates in phylogenetically-related free-living gammaproteobacteria (Table 3).

DISCUSSION

We demonstrated that E. grandis harbors a specific gammaproteobacterial symbiont in the cavity of the midgut crypts (Fig. 1; Fig. 2). The 100% infection frequencies in natural populations (Table 2) suggest that the gut symbiont is the primary obligate symbiont of E. grandis, although the biological role of the symbiont for the host, if any, is unknown. In addition to the gut symbiont, a Sodalis-allied symbiont was identified from gonad of E. grandis. The partial infection frequencies in natural populations (Table 2) indicate that the Sodalis-allied symbiont is a secondary, facultative symbiont of E. grandis.

A recent study reported that the scutellerid stinkbug C. ocellatus possesses a gammaproteobacterial primary gut symbiont and a Sodalis-allied secondary symbiont (Kaiwa et al., 2010). This symbiotic configuration may appear similar to that of E. grandis. However, molecular phylogenetic analysis clearly showed that the gut symbiont of E. grandis is phylogenetically distinct from that of C. ocellatus (Fig. 2), indicating that the primary gut symbionts of scutellerid stink-
bugs are not monophyletic, but of multiple evolutionary ori-
gins.

Previous studies have identified gammaproteobacterial
midgut symbionts from diverse stinkbugs of the pentatomo-
morphan families Acanthosomatidae, Plataspidae, and
Pentatomidae. Although these symbionts are commonly
localized in the same symbiotic organ, the midgut fourth
section with crypts, their microbiological affiliations are dif-
ferent: Rosenkranzia clausaccus in the Acanthosomatidae
(Kikuchi et al., 2009), Ishikawaella capsulata in the Platasp-
idae (Hosokawa et al., 2006), and unnamed gammapro-
teobacterial lineages in the Pentatomidae (Prado and
Almeida, 2009a). In the Acanthosomatidae and Plataspidae,
the gut symbionts are respectively monophyletic and co-
speciate with the host insects, reflecting strict host-symbiont
co-evolution over evolutionary time (Hosokawa et al., 2006;
Kikuchi et al., 2009). By contrast, the gut symbionts are
polyphylectic in the Pentatomidae, suggesting occasional lat-
eral transfers and/or replacements of the symbionts (Prado
and Almeida, 2009a). The situation in the Scutelleridae
turned out to be similar to the situation in the Pentatomidae,
and not to that in the Acanthosomatidae/Plataspidae.

With limited information on only two species, C. ocellatus
and E. grandis, the diversity of the gut symbionts in the
Scutelleridae warrants future study. It should be noted that
the gut symbiont of C. ocellatus exhibits AT-biased nucle-
ootide composition and accelerated molecular evolution
(Kaiwa et al., 2010), whereas the gut symbiont of E. grandis
does not (Fig. 2; Table 3). Given that these peculiar genetic
traits are often associated with long-lasting intimate symbi-
otic associations (Wernegreen, 2002), it may be that the
developmental role of the gut symbiont of E. grandis is more
recent than that of C. ocellatus.

It is notable that, although symbiotic bacteria have been
examined in only two scutellerid stinkbugs, Sodalis-allied
secondary symbionts were identified in both cases. For a
long time, Sodalis glossinidius and allied symbionts have
been known only from tsetse flies and grain weevils (Dale
and Maudlin, 1999; Heddi and Nardon, 2005). However,
recent studies have uncovered an unexpected prevalence of
Sodalis-allied symbionts among diverse insects including a
louse fly (Novakova and Hypsa, 2007), a pigeon louse
(Fukatsu et al., 2007), a chestnut weevil (Touj et al., 2010),
and a longicorn beetle (Grünwald et al., 2010). Infection
prevalence and biological roles of the Sodalis-allied symbionts in scutellerid and other stinkbugs are of interest for
future study. The scutellerid stinkbugs are unique in that the
obligate primary symbiont is extracellular in the midgut
cavity, whereas the facultative secondary symbiont is asso-
ciated with the host gonads. The primary extracellular symbiont and the secondary endosymbiont coexisting in the
same host body may unveil a previously unknown aspect of
microbe-microbe interactions in insect symbiosis.

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