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Primary Gut Symbiont and Secondary, *Sodalis*-Allied Symbiont of the Scutellerid Stinkbug *Cantao ocellatus*▽

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Symbiotic associations with midgut bacteria have been commonly found in diverse phytophagous heteropteran groups, where microbiological characterization of the symbiotic bacteria has been restricted to the stinkbug families Acanthosomatidae, Plataspidae, Pentatomidae, Alydidae, and Pyrrhocoridae. Here we investigated the midgut bacterial symbiont of *Cantao ocellatus*, a stinkbug of the family Scutelleridae. A specific gammaproteobacterium was consistently identified from the insects of different geographic origins. The bacterium was detected in all 116 insects collected from 9 natural host populations. Phylogenetic analyses revealed that the bacterium constitutes a distinct lineage in the *Gammaproteobacteria*, not closely related to gut symbionts of other stinkbugs. Diagnostic PCR and *in situ* hybridization demonstrated that the bacterium is extracellularly located in the midgut 4th section with crypts. Electron microscopy of the crypts revealed a peculiar histological configuration at the host-symbiont interface. Egg sterilization experiments confirmed that the bacterium is vertically transmitted to stinkbug nymphs via egg surface contamination. In addition to the gut symbiont, some individuals of *C. ocellatus* harbored another bacterial symbiont in their gonads, which was closely related to *Sodalis glossinidius*, the secondary endosymbiont of tsetse flies. Biological aspects of the primary gut symbiont and the secondary *Sodalis*-allied symbiont are discussed.

Insects are among the largest animal groups on the earth, embracing 750,000 to several millions of species (37, 52). Diverse insects are symbiotically associated with microorganisms, especially bacteria (5–7). In some insects, symbiotic bacteria are harbored in specialized host cells called bacteriocytes (or mycetocytes), constituting obligate mutualistic associations. For example, *Buchnera aphidicola* is harbored within bacteriocytes in the abdominal body cavity of almost all aphids and provides essential amino acids that are lacking in the phloem sap diet of the insects (9, 47). *Wigglesworthia glossinidia* is localized in a midgut-associated bacteriome of tsetse flies and plays pivotal roles in biosynthesis of B vitamins that are deficient in the vertebrate blood diet of the insects (2, 34). These obligate endocellular symbionts are often collectively referred to as "primary symbionts."

In contrast, there are facultative endosymbiotic microorganisms not essential for their host insects, often collectively called "secondary symbionts." For example, many aphids are known to harbor various facultative symbionts, which belong to distinct lineages in the *Gamma-* and *Alphaproteobacteria* (33, 43) and the *Mollicutes* (10). While the majority of those facultative bacteria are either parasitic or commensalistic for their hosts, some of them affect the host fitness beneficially in particular ecological contexts (29, 32, 36, 44, 51). In addition to the obligate primary symbiont *Wigglesworthia*, tsetse flies harbor the facultative secondary symbiont *Sodalis glossinidius*, whose biological function for the hosts is currently elusive (3, 8).

Members of the suborder Heteroptera, known as true bugs and consisting of over 38,000 described species, are characterized by their sucking mouthparts, half-membranous forewings, and incomplete metamorphosis (46). In the Heteroptera, symbiotic associations with bacteria are mainly found in phytophagous groups, especially in stinkbugs of the infraorder Pentatomomorpha. These stinkbugs generally possess many sacs or tubular outgrowths, called crypts or ceca, in a posterior region of the midgut, whose lumen is densely populated by a specific bacterial symbiont (7, 16). In some cases, experimental elimination of the symbiotic bacteria resulted in retarded growth and high mortality of the host insects (1, 13, 21, 26, 27, 39), indicating that these gut symbionts play important biological roles. Most of the gut symbionts are vertically transmitted through host generations by such mechanisms as egg surface contamination in the families Pentatomidae and Acanthosomatidae (1, 27, 39, 40, 42), coprophagy in the Cydnidae and Coreidae (22, 45), and capsule transmission in the Plataspidae (20), whereas a case of environmental acquisition has been reported from the Alydidae (26). Thus far, gut symbiotic bacteria of some members of the Acanthosomatidae, Plataspidae, Pentatomidae, Alydidae, and Pyrrhocoridae have been characterized using molecular techniques (21, 23, 25, 27, 38), while phylogenetic and biological aspects of gut symbiotic bacteria have been untouched in many other stinkbug groups.

These gut symbiotic bacteria are, despite their extracellular localization, regarded as "primary symbionts" of the stinkbugs. On the other hand, some stinkbugs may, in addition to the gut symbiotic bacteria, also be associated with facultative "secondary symbionts." For example, *Wolbachia* infections have been detected from diverse stinkbugs, most of which are probably of parasitic or commensalistic nature (24). Besides *Wolbachia*,

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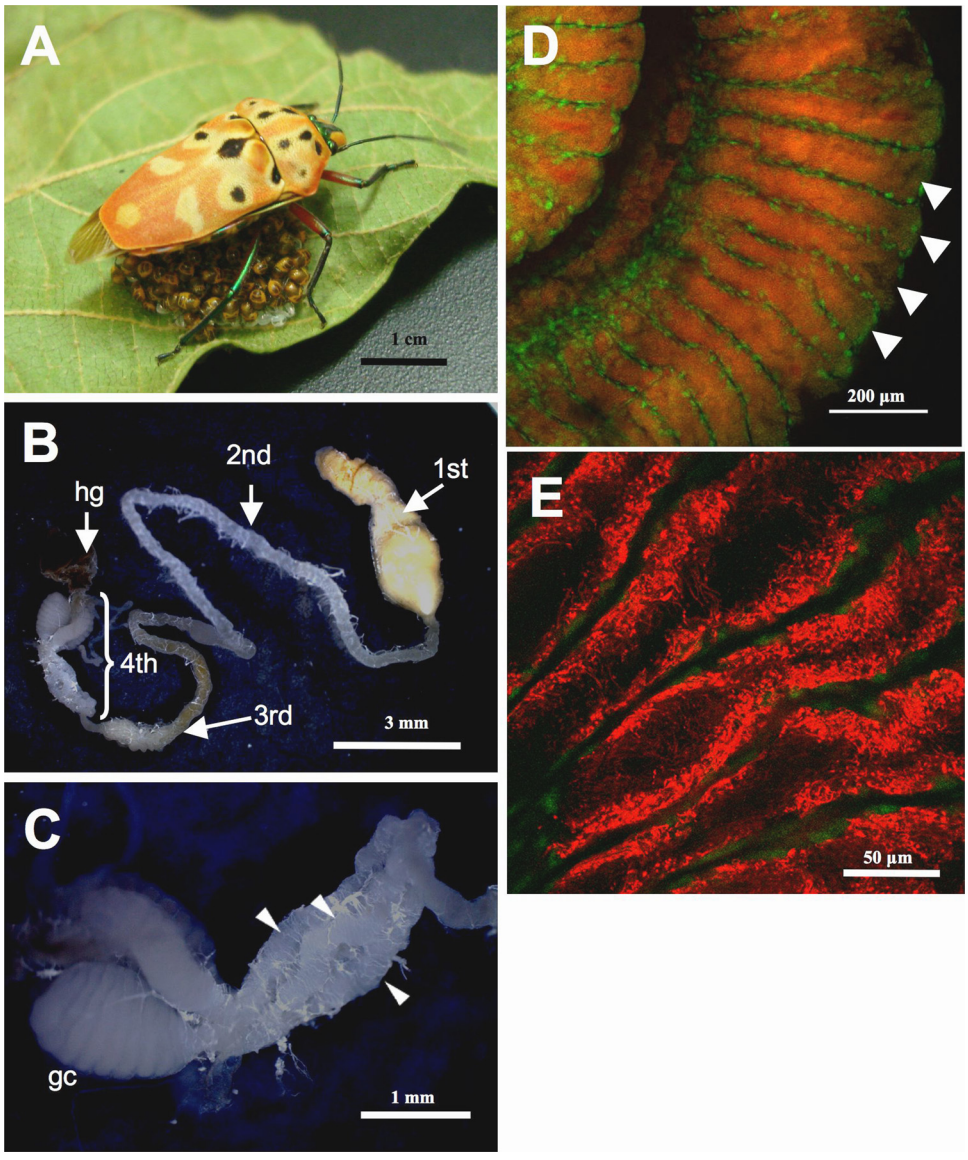

FIG. 1. (A) Adult female of *Cantao ocellatus*, guarding hatchlings under her body. (B) Dissected midgut from an adult female of *C. ocellatus*. 1st, midgut 1st section; 2nd, midgut 2nd section; 3rd, midgut 3rd section; 4th, midgut 4th section with crypts; hg, hindgut. (C) Enlarged image of the midgut 4th section with crypts. Arrowheads indicate three rows of crypts, while a fourth row is hidden behind. Glandular crypts (gc) are developed in adult females specifically, which may be involved in egg surface contamination with the symbiont. (D) An *in situ* hybridization image of the midgut 4th section, in which red and green signals indicate the gut symbiont and the host nucleus, respectively. Each arrow shows a crypt. (E) An enlarged image of the symbiotic bacteria in the crypts.

there has been no report on facultative, secondary symbionts from stinkbugs.

Members of the family Scutelleridae, often referred to as jewel bugs or shield-backed bugs, are stinkbugs characterized by their greatly enlarged convex scutellum that usually covers the entire abdomen. Some tropical species are also known for their vivid and beautiful body coloration (46). The family contains approximately 80 genera and 450 species, and in Japan, at least 7 genera and 9 species have been recorded (50). In the early 20th century, the presence of symbiotic bacteria was histologically described in midgut crypts of several scutellerid species (16, 31, 42). Since these pioneer works, however, no studies have been conducted on the symbiotic bacteria of scutellerid stinkbugs.

Here we investigated the midgut symbiont of *Cantao ocellatus*, a scutellerid stinkbug widely distributed in Asian countries, including Japan, and known to guard their eggs and newborn nymphs (Fig. 1A) (50). In addition to the gut symbiont, we also identified a *Sodalis*-allied facultative secondary symbiont from gonads of the insect.

MATERIALS AND METHODS

Insects. Collection data for the adult *C. ocellatus* insects used in this study are summarized in Table 1. Insect samples were collected from the Japanese mallotus tree, *Mallotus japonicus*, at nine localities in Japan in 2008 and 2009.

Dissection and DNA extraction. Ovaries, testes, and midgut 1st, 2nd, 3rd, and 4th sections were dissected from adult insects using a pair of fine forceps under a binocular microscope in a plastic petri dish filled with phosphate-buffered

TABLE 1. Samples of *Cantao ocellatus* used in this study

Collection locality	Collection date	Collector	Accession no. for:	
			Midgut symbiont	*Sodalis*-allied symbiont
Onna, Okinawa Island, Okinawa Prefecture, Japan	April 2008	Masaaki Kimura	AB541001 (16S rRNA) AB541011 (*groEL*)	
Kumejima Island, Okinawa Prefecture, Japan	April 2008	Takahiro Hosokawa	AB541002 (16S rRNA)	
Kumejima Island, Okinawa Prefecture, Japan	May 2008	Suguru Ohno		
Kunigami, Okinawa Island, Okinawa Prefecture, Japan	June 2008	Takahiro Hosokawa	AB541003 (16S rRNA)	
Ishigaki Island, Okinawa Prefecture, Japan	July 2008	Takahiro Hosokawa	AB541004 (16S rRNA)	AB541010 (16S rRNA) AB541012 (*groEL*)
Ishigaki Island, Okinawa Prefecture, Japan	September 2008	Takahiro Hosokawa		
Ishigaki Island, Okinawa Prefecture, Japan	September 2009	Takahiro Hosokawa		
Iriomote Island, Okinawa Prefecture, Japan	August 2009	Hiromi Mukai	AB541005 (16S rRNA)	
Muroto, Kochi Prefecture, Japan	October 2008	Katsura Ito	AB541006 (16S rRNA)	
Tosa-Shimizu, Kochi Prefecture, Japan	November 2008	Mikio Takai	AB541007 (16S rRNA)	
Naze, Amami-Oshima Island, Kagoshima Prefecture, Japan	June 2008	Yuki G. Baba	AB541008 (16S rRNA)	
Naze, Amami-Oshima Island, Kagoshima Prefecture, Japan	July 2009	Takahiro Hosokawa		
Nishino-Omote, Tanagashima Island, Kagoshima Prefecture, Japan	July 2009	Takahiro Hosokawa	AB541009 (16S rRNA)	

saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 [pH 7.5]). These insect tissues were immediately subjected to DNA extraction using Nucleo Spin tissue kit (Macherey-Nagel).

DNA cloning and sequencing. A 1.5-kb segment of bacterial 16S rRNA gene was amplified with the primers 16SA1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16SB1 (5′-TACGGYTACCTTGTTACGACTT-3′) (12). A 1.7-kb fragment of bacterial *groEL* gene was amplified with the primers Gro-F1 (5′-ATGGCAG CWAAAGACGTAAATTYGG-3′) and Gro-R1 (5′-TTACATCATKCCGCCC ATGC-3′). PCR was conducted with Ampli *Taq* DNA polymerase (Applied Biosystems) and the supplemented buffer system, under the following temperature profile: 95°C for 10 min, followed by 35 cycles consisting of 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The PCR products were subjected to the cloning using TA cloning vector pT7Blue (Takara Bio) and *Escherichia coli* DH5α competent cells (Takara Bio) with a blue-white selection system, using ampicillin and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal). In order to check the length of the inserted DNA fragment, white colonies, which were expected to contain inserted plasmids, were directly subjected to PCR with the primers Univ19 (5′-GTTTTCCCAGTCACGACGT-3′) and Rev20 (5′-AG CTATGACCATGATTACGC-3′). When a PCR product of expected size (1.5 kb and 1.7 kb, respectively) was obtained, the product was subjected to restriction fragment length polymorphism (RFLP) genotyping. Three or more clones for each of the RFLP genotypes were cultured at 37°C in LB liquid medium overnight and subjected to plasmid extraction using a QIAprep-Spin miniprep kit (Qiagen). The purified plasmids were subjected to DNA sequencing with the primers Univ19 and Rev20 and the internal primers Eub925r (5′-CCGYCAAT TCCTTTGAGTTT-3′) and Eub1405r (5′-GACGGGCGGTGTGTRCA-3′) for the 16S rRNA gene, groE-GS (5′-TAATGCTGATGAAAGCGT-3′) for the *groEL* gene of the midgut symbiont, and groE-SD (5′-ACTATCTCCGCCAAC TC-3′) for the *groEL* genes of the *Sodalis*-allied symbiont, under a temperature profile of 96°C for 1 min, followed by 26 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, using a Genetic Analyzer (Applied Biosystems 3130x1).

Molecular phylogenetic analysis. Bacterial 16S rRNA and *groEL* gene sequences were subjected to molecular phylogenetic analysis together with those of gammaproteobacterial representatives. Multiple alignments were generated using the program ClustalW (48). Aligned nucleotide sites containing a gap were removed from the data set using the program SeaView (15), and the final alignment was corrected manually. Phylogenetic analyses were conducted by maximum likelihood (ML) and maximum parsimony (MP) methods. The best-fit substitution models were selected by using the program MrModeltest v2.1 (J. A. Nylander, 2004; http://www.ebc.uu.se/systzoo/staff/nylander.html). ML phylogenies, with 100 bootstrap resamplings, were estimated using the program phyML 3.0 (18) with the GTR model for both 16S rRNA and *groEL* phylogenies. MP phylogenies, with 1,000 bootstrap resamplings, were estimated using the program phylo_win (15).

Relative rate test. A relative rate test, based on genetic distances estimated under the Kimura's two-parameter model (28), was performed using the program RRTree (41). For 16S rRNA gene sequences, 1,426 unambiguously aligned nucleotide sites were subjected to the analysis. For *groEL* gene sequences, 1,040 unambiguously aligned nucleotide sites at the first- and second-codon positions were analyzed, while nucleotide sites at the third-codon positions were omitted from the analysis due to saturated nucleotide substitutions.

Diagnostic PCR. The following specific primer sets were used for diagnostic PCR detection of 16S rRNA gene of the symbiotic bacteria: 16SA2 (5′-GTGC CAGCAGCCGCGGTAATAC-3′) and akagi16S780R (5′-GCTACGGAGGCT ACTTATT-3′) for a 0.16-kb segment from the midgut symbiont; and sodalis370F (5′-CGRTRGCGTTAAYAGCGC-3′) and 16SB2 (5′-CGAGCTGACGACAR CCATGC-3′) for a 0.62-kb segment from the *Sodalis*-allied symbiont. The temperature profile was 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. To check the quality of the DNA samples, a 1.5-kb segment of the insect mitochondrial 16S rRNA gene was amplified with the primers MtrA1 (5′-AAWAAACTAGGATTAGATACCCTA-3′) and MtrB1 (5′-TCTTAATYCAACATCGAGGTCGCAA-3′) (11).

Whole-mount FISH. The Alexa 555-labeled oligonucleotide probe Al555-AKG447 (5′-GTGTATTATTTTTCATCACC-3′) was used for whole-mount fluorescent *in situ* hybridization (FISH) targeting 16S rRNA of the midgut symbiont. Midgut sections with crypts were dissected from adult females and fixed in Carnoy's solution (6:3:1 ethanol-chloroform-acetic acid) overnight. The fixed samples were incubated in ethanolic 6% H_2O_2 solution for a week to quench the autofluorescence of the tissues (30). The insects 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 specific probe, SYTOX green and DAPI (4′,6-diamidino-2-phenylindole) were added at final concentrations of 0.1 μM, 0.5 μM, and 0.1 μg/ml, respectively. After an overnight incubation, the samples were thoroughly washed in PBSTx (PBS containing 0.3% Triton X-100) and observed under an epifluorescence microscope (Axiophoto; Carl Zeiss) and a laser confocal microscope (PASCAL5; Carl Zeiss). To confirm the specificity of the detection, the following control experiments were conducted: a no-probe control experiment, a no-SYTOX control experiment, and a competitive suppression control experiment with excess unlabeled probe.

Transmission electron microscopy. The midgut crypts were dissected from adult females in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde, prefixed in the fixative at 4°C overnight, and postfixed in the phosphate

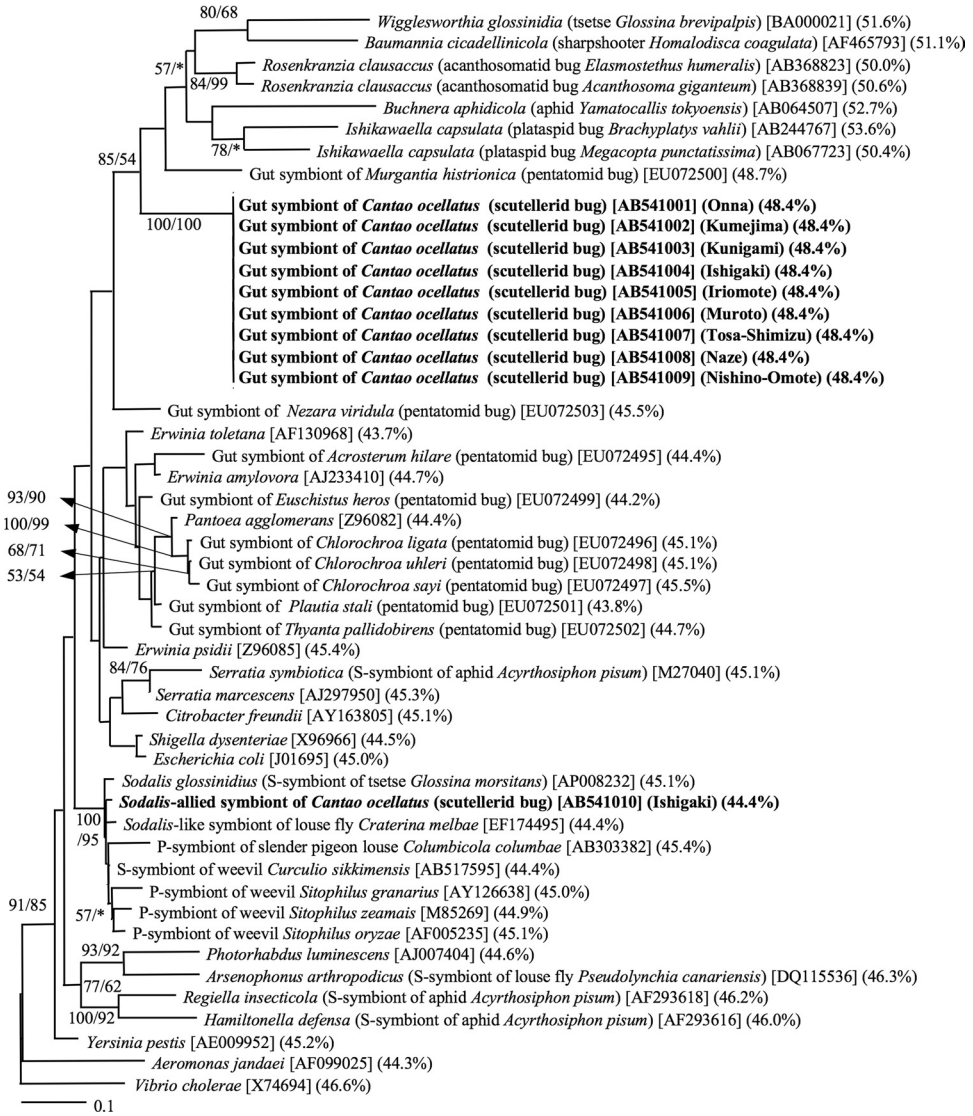

FIG. 2. Phylogenetic placement of the symbiotic bacteria of *C. ocellatus* on the basis of 16S rRNA gene sequences. A maximum likelihood (ML) tree inferred from a total of 1,243 aligned nucleotide sites is shown, whereas a maximum parsimony (MP) analysis gave substantially the same topology (data not shown). Bootstrap values higher than 50% are indicated at the nodes in the order of ML/MP. Asterisks indicate support values lower than 50%. Sequence accession numbers and AT contents of the nucleotide 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.

buffer containing 2% osmium tetroxide at 4°C for 60 min. After dehydration through an ethanol series, the materials were embedded in Spurr resin (Nisshin-EM). Ultrathin sections (thickness, 80 nm) were made on an ultramicrotome (Ultracat-N; Leichert-Nissei), mounted on collodion-coated copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (model H-7000; Hitachi).

Egg sterilization experiments. In total, 17 egg masses laid by different females collected at Ishigaki Island in 2008 and 2009 were subjected to the experiments. Nine randomly selected egg masses were treated with 70% ethanol for 5 min and 4% formalin for 15 min, rinsed with 70% ethanol, and dried in air. For a control, five egg masses were treated with water for 20 min instead of the surface sterilization. The other three egg masses were kept untreated. Each of the 17 egg masses was separately placed in a plastic petri dish (5 cm in diameter and 1 cm in depth) with a wet cotton ball at 25°C under a long-day condition (16 h of light and 8 h of dark [16L/8D]) until hatching. The hatchlings were fed with raw peanuts, dry soybeans, and distilled water at 25°C (16L/8D) until they reached the 3rd instar. Ten to 20 nymphs were randomly collected from each of the egg masses and preserved in acetone and were subjected to DNA extraction and diagnostic PCR.

RESULTS

General observation of digestive tract. Digestive organs were dissected from adult females of *C. ocellatus* (Fig. 1B). The enlarged midgut 1st section was filled with liquid, followed by the elongated midgut 2nd and 3rd sections. The midgut 4th section bore a number of well-developed crypts, which were white, arranged in four rows, and fused to each other into a loose helical assemblage (Fig. 1C).

Bacterial gene sequences from midgut section with crypts. Insects collected at nine localities in Japan were examined.

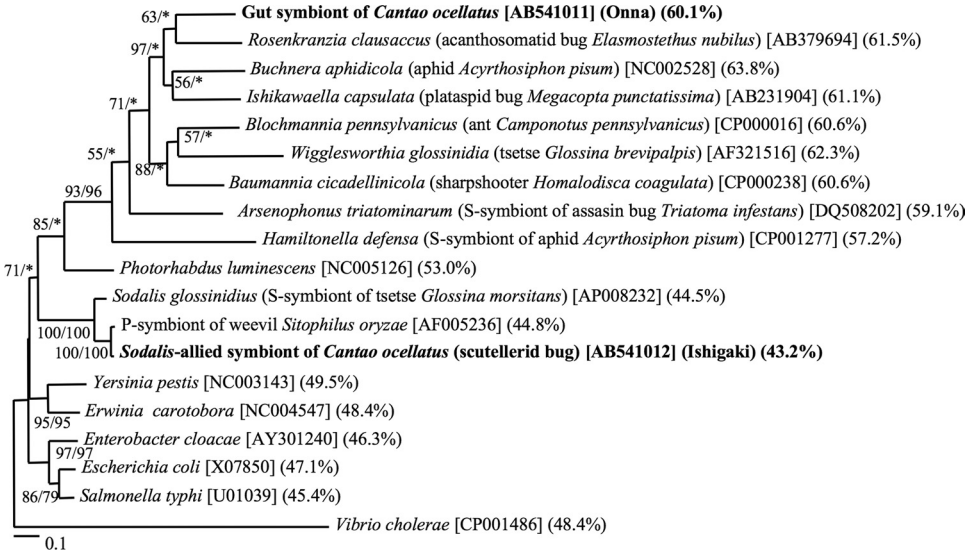

FIG. 3. Phylogenetic placement of the symbiotic bacteria from *C. ocellatus* on the basis of *groEL* gene sequences. A total of 1,104 aligned nucleotide sites at first and second codon positions were subjected to the analysis. The third codon positions were not used because of saturated nucleotide substitutions. Analysis of deduced amino acid sequences gave substantially the same results (data not shown). A maximum likelihood tree is shown. Support values for the nodes are indicated as in Fig. 2. Sequence accession numbers and AT contents of the nucleotide sequences are in brackets and parentheses, respectively. Note that the AT content values are based on the data of all codon positions.

One to three insect samples from each of the localities were dissected, and their midgut 4th sections were subjected to DNA extraction and PCR amplification of the bacterial 16S rRNA gene. The PCR products were cloned, and three to eight clones per insect were sequenced. All of the bacterial sequences obtained were identical without geographic and populational variations. The bacterial *groEL* gene was also amplified, cloned, and sequenced from an insect collected at Onna, Okinawa Island.

Phylogenetic placement of the gut symbiont. Phylogenetic analyses of the 16S rRNA gene sequences revealed that the gut symbiont constituted a distinct lineage in the *Gammaproteobacteria*, not closely related to gut symbionts of other stinkbugs. Meanwhile, the lineage was placed in a cluster of diverse insect symbionts, including endocellular symbionts of aphids, sharpshooters, and tsetse flies, and also gut symbionts of acanthosomatids, plataspids, and pentatomids, although the phylogenetic assemblage was not conclusive, with relatively low bootstrap supports (Fig. 2). Phylogenetic analyses on the basis of *groEL* gene sequences yielded a similar phylogenetic relationship: the gut symbiont was placed in a cluster of insect symbionts consisting of endocellular symbionts of aphids, sharpshooters, and tsetse flies, and also gut symbionts of acanthosomatids and plataspids (Fig. 3).

Localization and morphology of the gut symbiont. Diagnostic PCR analysis of dissected tissues detected the symbiont DNA sequence preferentially in the midgut 4th section with crypts, while the midgut 2nd and 3rd sections of females also exhibited the symbiont infection (Fig. 4). *In situ* hybridization targeting 16S rRNA of the gut symbiont identified strong and specific signals in the lumen of the crypts in the midgut 4th section (Fig. 1D). In each of the crypts, tubular symbiont cells were distributed not uniformly but concentrated near the epithelial cell layer (Fig. 1E).

Fine structure of the midgut crypt and the gut symbiont. Electron microscopy revealed a peculiar histological configuration inside the midgut crypts. At the central region of the crypts, the symbiont cells were found in the extracellular space in a relatively dispersed manner (Fig. 5A). Meanwhile, at the peripheral zone of the crypts, the symbiont cells and the host epithelial cells were closely associated with each other in an intermingled manner (Fig. 5A and B). Some of the symbiont cells were seen in the epithelial cell layer, next to mitochondria and endoplasmic reticula, as if they were located in the host cytoplasm (Fig. 5C). However, close examination of a number of electron microscopic images, including those from serial sections, revealed that those symbiont cells were actually located in extracellular spaces. It was postulated that the symbiont cells were entrapped in narrow extracellular spaces consti-

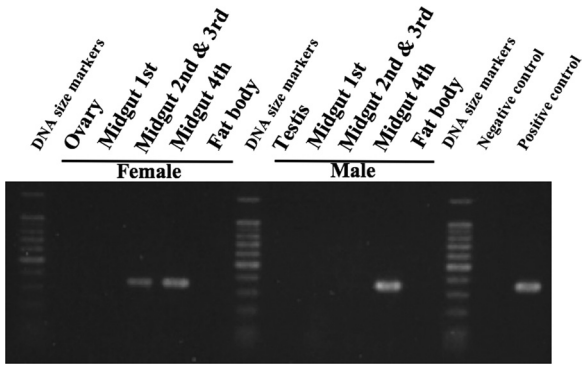

FIG. 4. Diagnostic PCR detection of the midgut symbiont in dissected tissues of adult *C. ocellatus*. Negative control, distilled water; positive control, DNA from midgut crypts of *C. ocellatus*; lane M, DNA size markers (from bottom to top) from 100 bp to 1,000 bp in 100-bp increments and 1,500 bp.

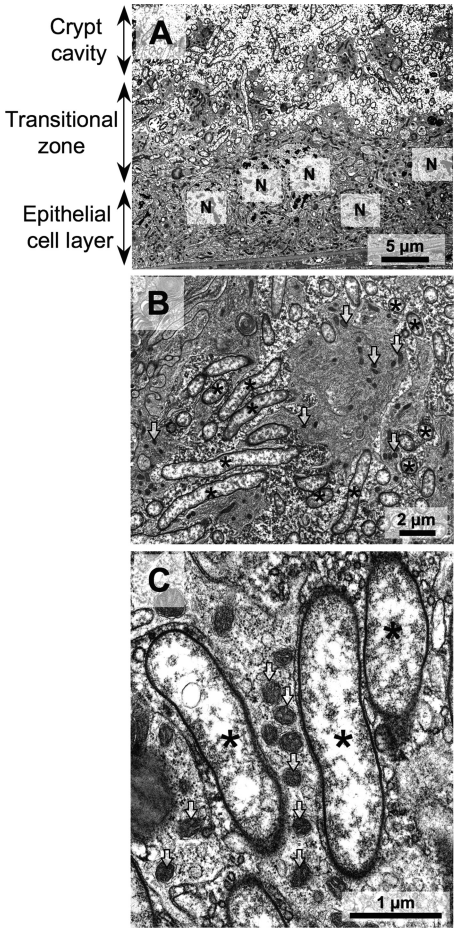

FIG. 5. Transmission electron microscopy of the midgut crypts in adult females of *C. ocellatus*. (A) Image of the interface between the crypt cavity and the epithelial cell layer; (B) enlarged image of the transitional zone; (C) enlarged image of the symbiont cells indicating an extracellular, rather than endocellular location. Asterisks and arrows indicate the symbiont cells and mitochondria, respectively. N, nucleus of the epithelial cell of the midgut crypt.

tuted by convoluted projections from the epithelial cell layer. Of course, we cannot rule out the possibility that, while the majority of the symbiont cells were found extracellularly, a small population of the symbiont cells might be nearly or entirely engulfed by the gut epithelial cells.

Detection and phylogenetic placement of the *Sodalis*-allied symbiont. From ovarial DNA of an adult female collected at Ishigaki Island, bacterial 16S rRNA and *groEL* genes were detected. The PCR products were cloned, and eight clones were sequenced. All of the sequences were identical to each other. Phylogenetic analyses of the sequences revealed that the ovarial symbiont belongs to the *Gammaproteobacteria*, forming a well-supported clade with the secondary symbiont of tsetse flies *Sodalis glossanidius*, the bacteriome-associated symbiont of grain weevils, the symbiont of a louse fly, the bacteriocyte-associated symbiont of a pigeon louse, and the secondary symbiont of a *Curculio* weevil (Fig. 2 and 3).

AT richness of the gut symbiont genes. The 16S rRNA gene sequence of the gut symbiont exhibited an AT content of 48.4%, which was slightly higher than the AT contents of free-living gammaproteobacteria around 45% (Fig. 2). The *groEL* gene sequence of the gut symbiont was 60.1% AT, which was drastically higher than the values of free-living gammaproteobacteria of around 45% (Fig. 3). On the other hand, the 16S rRNA gene sequence and the *groEL* gene sequence of the *Sodalis*-allied symbiont were not AT biased: 44.4% and 43.2% AT, respectively (Fig. 2 and 3).

Accelerated molecular evolution of the gut symbiont genes. The evolutionary rates of the 16S rRNA and *groEL* gene sequences of the gut symbiont of *C. ocellatus* were significantly higher than those of free-living gammaproteobacteria, respectively. Meanwhile, the evolutionary rates in the *Sodalis*-allied symbiont of *C. ocellatus* were not significantly different from those of free-living gammaproteobacteria (Table 2).

Infection frequencies of the gut symbiont and the *Sodalis*-allied symbiont in natural host populations. Table 3 shows infection frequencies of the gut symbiont and the *Sodalis*-allied symbiont in natural populations of *C. ocellatus*. The gut sym-

TABLE 2. Relative-rate tests for comparing the molecular evolutionary rates of 16S rRNA and *groEL* gene sequences between the lineages of the gut symbiont and the *Sodalis*-allied symbiont of *Cantao ocellatus* and their free-living relatives

Gene	Lineage 1	Lineage 2	Outgroup	K_1[a]	K_2[b]	Difference of distances[c]	Rate ratio[d]	*P* value[e]
16S rRNA	Gut symbiont of *C. ocellatus* (AB541001)	*Escherichia coli* (J01695) and *Shigella dysenteriae* (X96966)	*Vibrio cholerae* (X74694)	0.052	0.027	0.025	1.9	0.0031*
groEL	Gut symbiont of *C. ocellatus* (AB541011)	*Escherichia coli* (X07850) and *Salmonella enterica* serovar Typhi (U01039)	*Vibrio cholerae* (CP001486)	0.060	0.032	0.028	1.9	0.013*
16S rRNA	*Sodalis*-allied symbiont of *C. ocellatus* (AB541010)	*Escherichia coli* (J01695) and *Shigella dysenteriae* (X96966)	*Vibrio cholerae* (X74694)	0.030	0.026	0.004	1.2	0.53
groEL	*Sodalis*-allied symbiont of *C. ocellatus* (AB541012)	*Escherichia coli* (X07850) and *Salmonella enterica* serovar Typhi (U01039)	*Vibrio cholerae* (CP001486)	0.028	0.018	0.010	1.6	0.19

[a] K_1, estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.
[b] K_2, estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
[c] $K_1 - K_2$.
[d] K_1/K_2 ratio.
[e] *P* values were generated using the program RRTree (41). *, statistically significant difference.

TABLE 3. Infection frequencies of the gut symbiont and the *Sodalis*-allied symbiont in natural populations of *Cantao ocellatus*

Locality	Infection frequency (%)[a]					
	Gut symbiont[b]			*Sodalis*-allied symbiont[c]		
	Male	Female	Total	Male	Female	Total
Onna	100 (3/3)	100 (3/3)	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/6)
Kumejima	100 (4/4)	100 (8/8)	100 (12/12)	0 (0/8)	0 (0/5)	0 (0/13)
Kunigami	100 (7/7)	100 (12/12)	100 (19/19)	0 (0/3)	0 (0/1)	0 (0/4)
Ishigaki	100 (9/9)	100 (2/2)	100 (11/11)	17 (2/12)	10 (4/41)	11 (6/53)
Iriomote	100 (9/9)	No data	100 (9/9)	0 (0/9)	No data	0 (0/9)
Naze	100 (5/5)	100 (8/8)	100 (13/13)	0 (0/3)	0 (0/10)	0 (0/13)
Muroto	100 (1/1)	100 (1/1)	100 (2/2)	0 (0/1)	0 (0/1)	0 (0/2)
Tosa-Shimizu	100 (15/15)	100 (15/15)	100 (30/30)	0 (0/15)	0 (0/15)	0 (0/30)
Nishino-Omote	100 (5/5)	100 (9/9)	100 (14/14)	0 (0/5)	0 (0/9)	0 (0/14)
Total	100 (58/58)	100 (58/58)	100 (116/116)	3 (2/59)	5 (4/85)	4 (6/144)

[a] Values in parentheses represent the number infected/number tested.
[b] Dissected midgut 4th section with crypts was subjected to DNA extraction and diagnostic PCR.
[c] Dissected testis/ovary was subjected to DNA extraction and diagnostic PCR.

biont was detected from all 116 insects representing 9 populations examined. Meanwhile, the *Sodalis*-allied symbiont was detected only from 6 of 144 insects examined. All of the infected insects were collected at Ishigaki Island, where the infection frequency with the *Sodalis*-allied symbiont was 11% (6/53).

Egg surface sterilization blocks vertical transmission of the gut symbiont. Table 4 summarizes the results of the egg surface sterilization experiments. The formalin and ethanol sterilization procedure did not affect the hatching rates of the treated eggs, indicating no detrimental effects of the treatment on the embryonic development. All of the nymphs from the sterilized eggs were free of the gut symbiont, whereas most of the nymphs from the water-treated eggs and untreated eggs were infected with the gut symbiont.

DISCUSSION

Here we identified a specific gammaproteobacterial midgut symbiont from the scutellerid stinkbug *C. ocellatus*. Previous studies have identified gammaproteobacterial midgut symbionts from stinkbugs of the families Acanthosomatidae, Plataspidae, and Pentatomidae. Although these symbionts are commonly harbored in the same symbiotic organ, midgut 4th section with crypts, irrespective of the host systematics, their microbiological affiliations are different: *Rosenkranzia clausaccus* with acanthosomatids, *Ishikawaella capsulata* with plataspids, and unnamed gammaproteobacterial lineages with pentatomids (21, 27, 38). Here we demonstrated that the gut symbiont of *C. ocellatus* is phylogenetically distinct from those stinkbug symbionts, indicating that the evolutionary origin of the gut symbiont in the Scutelleridae is independent of those of the gut symbionts in the Acanthosomatidae, Plataspidae, and Pentatomidae.

In the Acanthosomatidae and Plataspidae, respectively, the gut symbionts are monophyletic, cospeciate with the host insects, and exhibit AT-biased nucleotide composition and accelerated molecular evolution, indicating strict host-symbiont coevolution within each of the families over evolutionary time (21, 27). In the Pentatomidae, in contrast, the gut symbionts are polyphyletic: the gut symbionts of *Acrosternum hilare*, *Murgantia histrionica*, and *Nezara viridula*, the gut symbionts of *Chlorochroa* spp., the gut symbiont of *Euschistus heros*, and the gut symbionts of *Plautia stali* and *Thyanta pallidovirens* constitute distinct lineages in the *Gammaproteobacteria*, respectively

TABLE 4. Effects of egg surface sterilization on vertical transmission of the gut symbiont in *Cantao ocellatus*

Treatment group and egg mass no.	Clutch size	Hatching rate (%)	Nymphal infection rate (%)[a]
Formalin and ethanol treatment[b]			
FE1	162	98 (159/162)	0 (0/20)
FE2	82	97 (80/82)	0 (0/14)
FE3	164	98 (161/164)	0 (0/20)
FE4	196	86 (169/196)	0 (0/11)
FE5	150	84 (126/150)	0 (0/20)
FE6	175	97 (169/175)	0 (0/18)
FE7	120	95 (114/120)	0 (0/20)
FE8	98	99 (97/98)	0 (0/20)
FE9	133	98 (130/133)	0 (0/20)
Total	1,280	94 (1,205/1,280)	0 (0/163)
Water treatment control[c]			
WT1	209	99 (207/209)	100 (14/14)
WT2	172	91 (157/172)	100 (15/15)
WT3	148	100 (148/148)	100 (10/10)
WT4	55	24 (13/55)	80 (8/10)
WT5	192	98 (189/192)	100 (13/13)
Total	776	92 (714/776)	97 (60/62)
No-treatment control[d]			
UT1	110	90 (99/110)	100 (14/14)
UT2	128	98 (125/128)	100 (16/16)
UT3	100	96 (96/100)	95 (19/20)
Total	338	95 (320/338)	98 (49/50)

[a] Inspected at 3rd instar.
[b] Egg masses were treated with 70% ethanol for 5 min and 4% formalin for 15 min and rinsed with 70% ethanol for 10 s.
[c] Egg masses were treated with water for 20 min.
[d] Egg masses were untreated.

(38). Their 16S rRNA gene sequences generally exhibit no remarkable AT bias (Fig. 2). Hence, although the gut symbionts are vertically transmitted and play biological roles in the Pentatomidae (1, 39, 40), host-symbiont coevolution is not so strict but occasional lateral transfers and/or symbiont replacements must have taken place. Since this study is the first to characterize a scutellerid gut symbiont, it is currently obscure whether the host-symbiont relationship in the Scutelleridae is similar to the situation in the Acanthosomatidae/Plataspidae or the situation in the Pentatomidae. AT-biased nucleotide composition (Fig. 2 and 3) and accelerated molecular evolution (Table 2) in the gut symbiont seem suggestive of an intimate host-symbiont relationship in *C. ocellatus*.

In the midgut crypts of *C. ocellatus*, the gut symbiont was not distributed uniformly but was concentrated in the peripheral zone (Fig. 1E). Electron microscopy revealed a peculiar histological configuration at the host-symbiont interface: convoluted projections from the epithelial cell layer form an intermingled structure, where the symbiont cells are entrapped in narrow extracellular spaces in close association with the host cells (Fig. 5). In acanthosomatids and plataspids, electron microscopy detected no such cellular configuration: their midgut crypts were lined with thin and flat epithelial cells, and the crypt cavities were densely packed with symbiont cells (20, 27). The structure is probably the reason why the symbiont cells are concentrated near the epithelial cell layer in the midgut crypts (Fig. 1E).

The biological roles of the gut symbiont for *C. ocellatus* are currently unknown. Considering the essential roles of the gut symbionts in other stinkbug groups (1, 13, 21, 26, 27, 39) and the 100% infection frequencies in natural host populations (Table 3), it appears plausible that the gut symbiont plays some important roles for *C. ocellatus*. As is the case of scutellerid species, *C. ocellatus* is large with beautiful coloration (Fig. 1A). The gut symbiont might be involved in formation of these costly host traits, possibly via nutritional provisioning or other metabolic supplementation. The pericarp of *Mallotus* fruits, the main food source for *C. ocellatus* nymphs, contains rottlerin-like phloroglucinol derivatives and other cytotoxic compounds (4). Although speculative, the gut symbiont might be involved in detoxification of such plant defense substances.

In addition to the gut symbiont, we identified another bacterial associate of *C. ocellatus*. The bacterium was phylogenetically related to *Sodalis glossinidius*, the facultative symbiont of tsetse flies (Fig. 2 and 3), and detected only from a local host population at an infection frequency around 10% (Table 3). In this study, because of infrequent occurrences in our samples, we could not inspect biological aspects of the *Sodalis*-allied symbiont such as vertical transmission mechanism, effects on host fitness, etc. For a long time, *Sodalis* and allied symbionts have been known only from tsetse flies and grain weevils (8, 19). Recently, however, *Sodalis*-allied symbionts have been detected from a louse fly (35), a pigeon louse (14), a chestnut weevil (49), and a longicorn beetle (17). It has turned out that the *Sodalis*-allied symbionts are associated with a wider array of insects than previously envisioned.

In conventional model systems for insect symbiosis studies, like aphids and tsetse flies, the obligate primary symbiont is harbored in specialized host cells called bacteriocytes, while facultative secondary symbionts reside in either different cells, the body cavity, or the alimentary tract (5–7). The case of *C. ocellatus* is unique in that the obligate primary symbiont is harbored extracellularly in the midgut cavity, whereas the facultative secondary symbiont is associated with gonads of the host insect. How the extracellular primary symbiont and the secondary endosymbiont interact in the same host body is of interest, deserving future studies.

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