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Gut Symbiotic Bacteria of the Genus *Burkholderia* in the Broad-Headed Bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae)

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The Japanese common broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* possess a number of crypts in the posterior region of the midgut, whose lumen contains a copious amount of bacterial cells. We characterized the gut symbiotic bacteria by using molecular phylogenetic analysis, light and electron microscopy, in situ hybridization, and PCR-based detection techniques. Restriction fragment length polymorphism analysis of 16S rRNA gene clones suggested that a single bacterium dominated the microbiota in the crypts of the both bug species. The predominant 16S rRNA gene sequences obtained from different individuals and species of the bugs were not identical but were very similar to each other. Homology searches in the DNA databases revealed that the sequences showed the highest levels of similarity (96% to 99%) to the sequences of *Burkholderia* spp. belonging to the β subdivision of the class *Proteobacteria*. In situ hybridization with specific oligonucleotide probes confirmed the localization of the *Burkholderia* symbiont in the lumen of the midgut crypts. Electron microscopy showed that the lumen of the crypts was filled with rod-shaped bacteria of a single morphotype. Molecular phylogenetic analysis demonstrated that the *Burkholderia* symbionts of the bugs formed a well-defined monophyletic group, although the group also contained several environmental *Burkholderia* strains. The phylogenetic relationship of the *Burkholderia* symbionts did not reflect the relationship of the host bug species at all. The sequences from *R. clavatus* and the sequences from *L. chinensis* did not form clades but were intermingled in the phylogeny, suggesting that horizontal transmission of the symbiont might have occasionally occurred between populations and species of the bugs.

Symbiosis, or "living together," generally means that there is a close association between different organisms. Of the various symbiotic associations, the most cohesive forms are found in "endosymbiosis," in which one partner called the symbiont lives inside the body of another partner called the host. Insects feeding exclusively on restricted diets, such as plant sap, vertebrate blood, or woody material, often possess symbiotic microorganisms. In many of these cases, specific microbes are localized in the lumen of the gut, in the hemocoel, or inside specialized cells called mycetocytes or bacteriocytes (6). These microorganisms are always found in the host insects, are vertically transmitted from generation to generation, and usually cannot be cultured outside the bodies of the hosts. The host insects often suffer retarded growth, sterility, and/or death when they are experimentally deprived of their symbionts (4, 6, 8).

The Heteroptera, known as the true bugs and consisting of more than 38,000 described species, is among the large groups of insects with incomplete metamorphosis (25). Heteropteran bugs are grouped into seven infraorders (25), and symbiotic bacteria have been found mainly in two of the infraorders, the Cimicomorpha and the Pentatomomorpha (6). In the Cimicomorpha, most bloodsucking species (e.g., assassin bugs [*Triatoma* spp.] and bedbugs [*Cimex* spp.]) and some sap-feeding species possess endosymbiotic bacteria that are localized in the gut or mycetocytes (6). In the pentatomomorphan bugs, the posterior end of the midgut, or the "midgut fourth section" (Fig. 1), is characterized by the presence of many sacs or tubular outgrowths. These evaginations, called crypts or ceca, are quite diverse in terms of number and arrangement in different taxonomic groups and are filled with bacteria (6, 7, 19). The symbiotic bacteria are maternally transmitted to the offspring by one of the following mechanisms: superficial bacterial contamination of eggs (egg smearing), probing of the mother's excrement (proctophagy), or deposition of bacterium-containing capsules with eggs (capsule transmission) (6). In several pentatomomorphan bugs, experimental deprivation of the symbiont resulted in retarded growth and/or nymphal mortality (1, 13, 17, 22, 24), suggesting that the symbionts may play important roles in the host bugs.

The diversity of the symbiotic organizations and the mechanisms for vertical transmission found in pentatomomorphan bugs is potentially promising for comparative studies on the evolution of the symbiotic system. However, except for old microscopic descriptions (6, 7, 15), the microbiological nature of the symbionts has been poorly characterized. The only case reported thus far is the capsule-transmitted gut symbiont of *Megacopta punctatissima* (family Plataspidae), whose phylogenetic position was shown to be the γ subdivision of the class *Proteobacteria* (13).

Riptortus clavatus and *Leptocorisa chinensis*, which belong to

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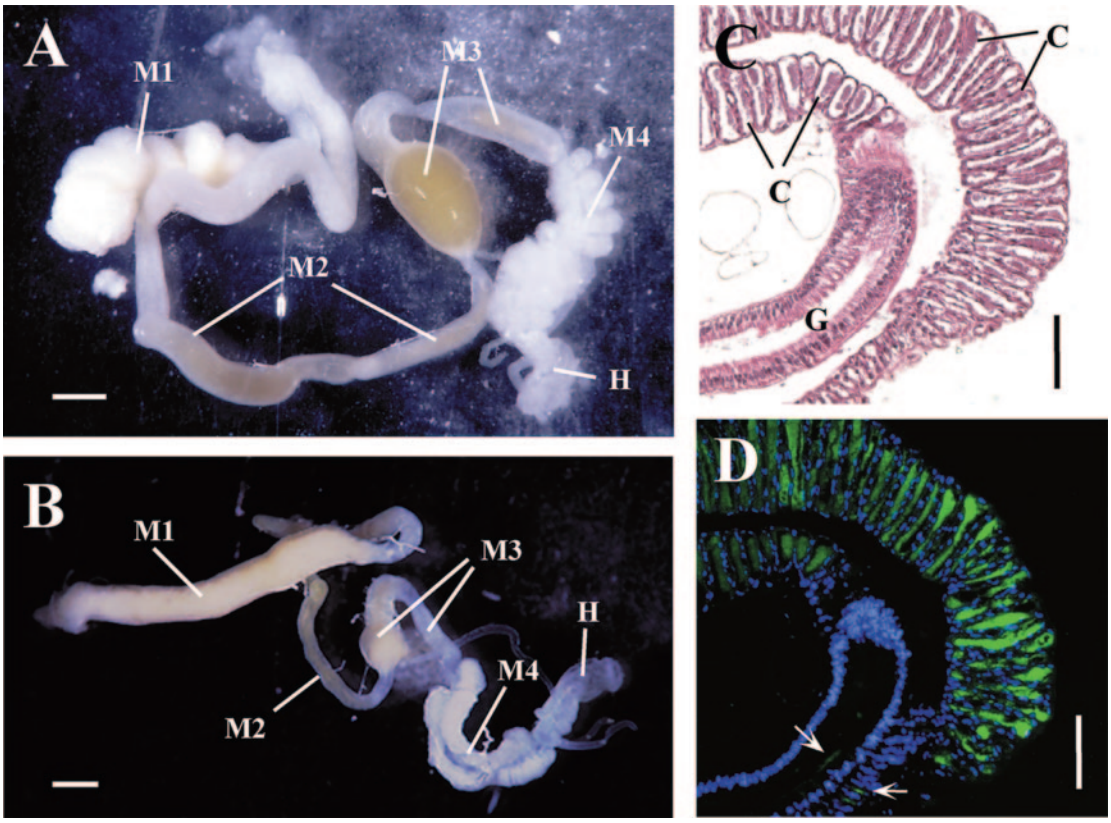

FIG. 1. Midgut organization of *R. clavatus* and *L. chinensis*. (A) Dissected midgut of *R. clavatus*. (B) Dissected midgut of *L. chinensis*. (C) Tissue section of midgut crypts of *R. clavatus*, stained with hematoxylin and eosin. (D) Fluorescent in situ hybridization of a tissue section of midgut crypts of *R. clavatus*, targeting 16S rRNA of the *Burkholderia* symbiont. Green signals are due to the *Burkholderia*-specific probe Cy3-Alsym16S. Blue signals are nuclei of the host cells visualized by DAPI. The arrows indicate the *Burkholderia* signals in the main tract of the midgut and signals in the ducts connecting the crypts with the main tract. (A) Bar = 0.5 mm. (B) Bar = 0.2 mm. (C and D) Bars = 100 μm. Abbreviations: C, crypt: G, gut; M1, first midgut section; M2, second midgut section; M3, third midgut section; M4, fourth midgut section (symbiotic organ); H, hindgut.

the broad-headed bug family Alydidae, are serious pests of soybean and rice, respectively, in Japan (33). In this study, we investigated the phylogenetic position, in vivo localization, ultrastructure, and prevalence in host populations of gut symbiotic bacteria of the alydid bugs by using molecular phylogenetic analysis, light and electron microscopy, in situ hybridization, and PCR-based detection techniques.

MATERIALS AND METHODS

Materials. *R. clavatus* and *L. chinensis* adults were collected in soybean fields and grassy plains, respectively, in Japan (Table 1). For histology and diagnostic PCR of tissues, the insects were transported alive to the laboratory. For other molecular analyses, the insects were preserved in acetone immediately after collection (12). Adult insects belonging to the other alydid species listed in Table 1 were also preserved in acetone immediately after collection.

DNA extraction. DNA was extracted from dissected midguts with crypts or other tissues. The tissue was homogenized in 200 μl of lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, 0.2 mg/ml proteinase K) and incubated at 56°C for 2 h. The lysate was extracted with 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1). DNA was recovered from the purified lysate by ethanol precipitation and dissolved in 200 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA).

Cloning, RFLP typing, and sequencing of the 16S rRNA gene. A 1.5-kb segment of the eubacterial 16S rRNA gene was amplified by using primers 16SA1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16SB1 (5′-TACGGYTACCTTGTTACGACTT-3′) (14), whose Oligonucleotide Probe Database (OPD) designations are S-D-Bact-0007-a-S-20 and S-D-Bact-1492-a-A-22, respectively (3). A PCR was conducted by using Ampli*Taq* DNA polymerase (Roche, Basel, Switzerland) and the supplemented buffer system with a temperature profile of 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The PCR product was cloned with the TA cloning vector pT7Blue (Takara) and *Escherichia coli* DH5α competent cells (Takara). To check the length of the inserted DNA fragment, white colonies expected to contain an inserted plasmid were directly subjected to PCR by using primers Univ19 (5′-GTTTTCCCAGTCACGACGT-3′) and Rev20 (5′-AGCTATGACCATGATTACGC-3′). When a PCR product of the expected size (1.5 kb) was obtained, the product was digested with restriction endonucleases HaeIII and RsaI and electrophoresed in Tris-acetate-EDTA agarose gels. More than three clones for every restriction fragment length polymorphism (RFLP) type were cultured and subjected to plasmid extraction using a QIAprep-Spin Miniprep kit (QIAGEN). The purified plasmids were eluted with 50 μl distilled water and used for DNA sequencing. A dye terminator-labeled cycle sequencing reaction was conducted with an FS DNA sequencing kit (Perkin-Elmer) and four sequencing primers, Univ19, Rev20, Eub925 (5′-AGCTATGACCATGATTACGC-3′; OPD designation, S-D-Bact-0925-a-A-20), and Eub1405 (5′-AGCTATGACCATGATTACGC-3′; OPD designation, S-D-Bact-1405-a-A-20), with a temperature profile of 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 50°C for 1 min, and 60°C for 4 min. The products were analyzed with an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

Histology. The insects were dissected in 80% ethanol, and the tissues were transferred to Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1). After overnight fixation, the tissues were dehydrated and cleared using an ethanol-xylene series and embedded in paraffin. Serial tissue sections (thickness, 5 μm) were cut with a rotary microtome and mounted on silane-coated glass slides. The sections were dewaxed using a xylene-ethanol series and air dried prior to in situ hybridization.

TABLE 1. Alydid stinkbug samples used in this study

Species	Sample	Locality	Collection date	Collector
Riptortus clavatus[a]	IB1	Mito, Ibaraki	30 June 2001	Y. Kikuchi
	IB2	Tomobe, Ibaraki	22 August 2002	M. Ishizaki
	IB3	Tsukuba, ibaraki	5 May 2001	Y. Kikuchi
	GN	Numata, Gunma	15 July 2003	Y. Kikuchi
	NG	Joetsu, Nigata	6 October 2003[c, d]	H. Higuchi
	KY	Kyoto, Kyoto	29 September 2003[d]	C. Himuro
	NR	Nara, Nara	16 October 2003[d]	C. Himuro
	KC	Nakamura, Kochi	26 September 2003	M. Takai
	SG	Kanzaki, Saga	7 May 2002	T. Kashima
	KM	Kikuchi, Kumamoto	3 October 2003[d]	Y. Kikuchi
	OK	Ishigaki, Okinawa	25 July 2002	K. Kohno
Leptocorisa chinensis[a]	FK	Hanawa, Fukushima	14 August 2002	Y. Kikuchi
	IB1	Mito, Ibaraki	29 August 2002	Y. Kikuchi
	IB2	Tsukuba, Ibaraki	10 August 2002[d]	Y. Kikuchi
			10 October 2003[c]	Y. Kikuchi
	GN	Kiryu, Gunma	18 November 2003	E. Hara
	CB	Maruyama, Chiba	8 September 2002	Y. Kikuchi
	KY	Kyoto, Kyoto	29 September 2003[d]	C. Himuro
	NR	Nara, Nara	16 October 2003[d]	C. Himuro
	SG	Saga, Saga	3 June 2002	T. Kashima
	KM	Kikuchi, Kumamoto	3 October 2003[d]	Y. Kikuchi
Riptortus linearis[b]		Naha, Okinawa	20 June 2002	T. Fukatsu
Leptocorisa acuta[b]		Iriomote, Okinawa	26 May 2004	T. Yasuda
Leptocorisa oratoria[b]		Iriomote, Okinawa	26 May 2004	T. Yasuda
Megalotomus costalis[b]		Sapporo, Hokkaido	26 August 2002	K. Ito
Daclera levana[b]		Ishigaki, Okinawa	23 July 2002	K. Kohno
Paraplesius unicolor[b]		Monobe, Kochi	27 July 2002	M. Takai

[a] One individual from each population was subjected to cloning and sequencing.
[b] One individual was subjected to diagnostic PCR analysis.
[c] Subjected to diagnostic PCR analysis to examine the localization in a body.
[d] Subjected to diagnostic PCR analysis to survey the infection rate in a natural population.

In situ hybridization. Probe Cy3-Alsym16S (5′-ACACTCAAAGCCTGCCA GT-3′; OPD designation, S-G-Burk-0642-a-A-19), whose 5′ end was labeled with the fluorescent cyanine dye Cy3, was designed to specifically detect the gut symbiotic bacteria of *R. clavatus* and *L. chinensis*. The specificity of the probe was confirmed by a homology search performed with the DNA databases, although several 16S rRNA gene sequences from environmental *Burkholderia* strains, including NF100, S4.9, and AK-5 (see Fig. 2), showed 100% identity to the probe sequence. About 150 μl of hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide) containing 50 pmol of probe and 4 nmol of DAPI (4′,6-diamidino-2-phenylindole) per ml was applied to a glass slide, covered with a coverslip, and incubated in a humidified chamber at room temperature overnight. To eliminate nonspecifically bound probe, the preparation was washed in 1× TBS buffer (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl) for 5 min at room temperature. The specificity of hybridization was confirmed by performing the following control experiments: no-probe control, RNase digestion control, and competitive suppression control with excess unlabeled probe (14). The fluorescent signals were observed with an epifluorescence microscope (Axiophot; Zeiss) and were recorded with a digital camera (Axiocam; Zeiss).

Transmission electron microscopy. The tissues of crypts were dissected in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The tissues were prefixed with the fixative at 4°C overnight and were postfixed in 2% osmium tetroxide for 60 min at 4°C. After dehydration using an ethanol series, the materials were embedded in LR white acrylic resin (Sigma) and sectioned (thickness, 80 nm). The ultrathin sections were double stained with uranyl acetate and lead citrate and were observed with a transmission electron microscope (H-7000; Hitachi).

Diagnostic PCR. Specific primers Burk16SF (5′-TTTTGGACAATGGGGG CAAC-3′; OPD designation, S-G-Burk-0364-a-S-20) and Burk16SR (5′-GCTC TTGCGTAGCAACTAAG-3′; OPD designation, S-G-Burk-1120-a-A-20) were used for diagnostic PCR detection of the 16S rRNA gene of *Burkholderia* spp. with a temperature profile of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The primers yielded a 0.75-kb amplified segment. The specificity of the primers was checked by performing a homology search with the DNA databases and was also confirmed by cloning and sequencing of the amplified products. To check the quality of template DNA samples, a 0.65-kb segment of the insect mitochondrial cytochrome oxidase I gene was amplified by using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (10).

Molecular phylogenetic analysis. The 16S rRNA gene sequences determined were subjected to molecular phylogenetic analysis together with 16S rRNA gene sequences of β-proteobacterial representatives retrieved from the DDBJ nucleotide sequence database. A multiple alignment of the sequences was generated with the Clustal W program package (28) and then manually realigned. A total of 1,403 unambiguously aligned nucleotide sites were subjected to analyses. Neighbor-joining (NJ) trees were constructed by using Clustal W (28) with Kimura's two-parameter model (18). Maximum-parsimony (MP) trees were constructed by using the PAUP 4.0b10 program package (26), in which insertions, deletions, transitions, and transversions were equally weighted. In the MP analysis, 50% majority-rule consensus trees were produced. Bootstrap tests were performed with 1,000 and 100 replicates in the NJ and MP analyses, respectively. A similarity matrix of the sequences was generated by using Clustal W (28) with Kimura's two-parameter model (18).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the symbionts of *R. clavatus* and *L. chinensis* determined in this study have been deposited in the DDBJ nucleotide sequence database under the accession numbers shown in Fig. 2.

RESULTS

General observation of the midgut. In both *R. clavatus* and *L. chinensis*, crypts were observed in the fourth section of the midgut (Fig. 1A and B). The crypts were white, arranged in two rows, and associated with branched tracheae. Copious amounts of rod-shaped bacteria were found in the contents of the crypts by light microscopy.

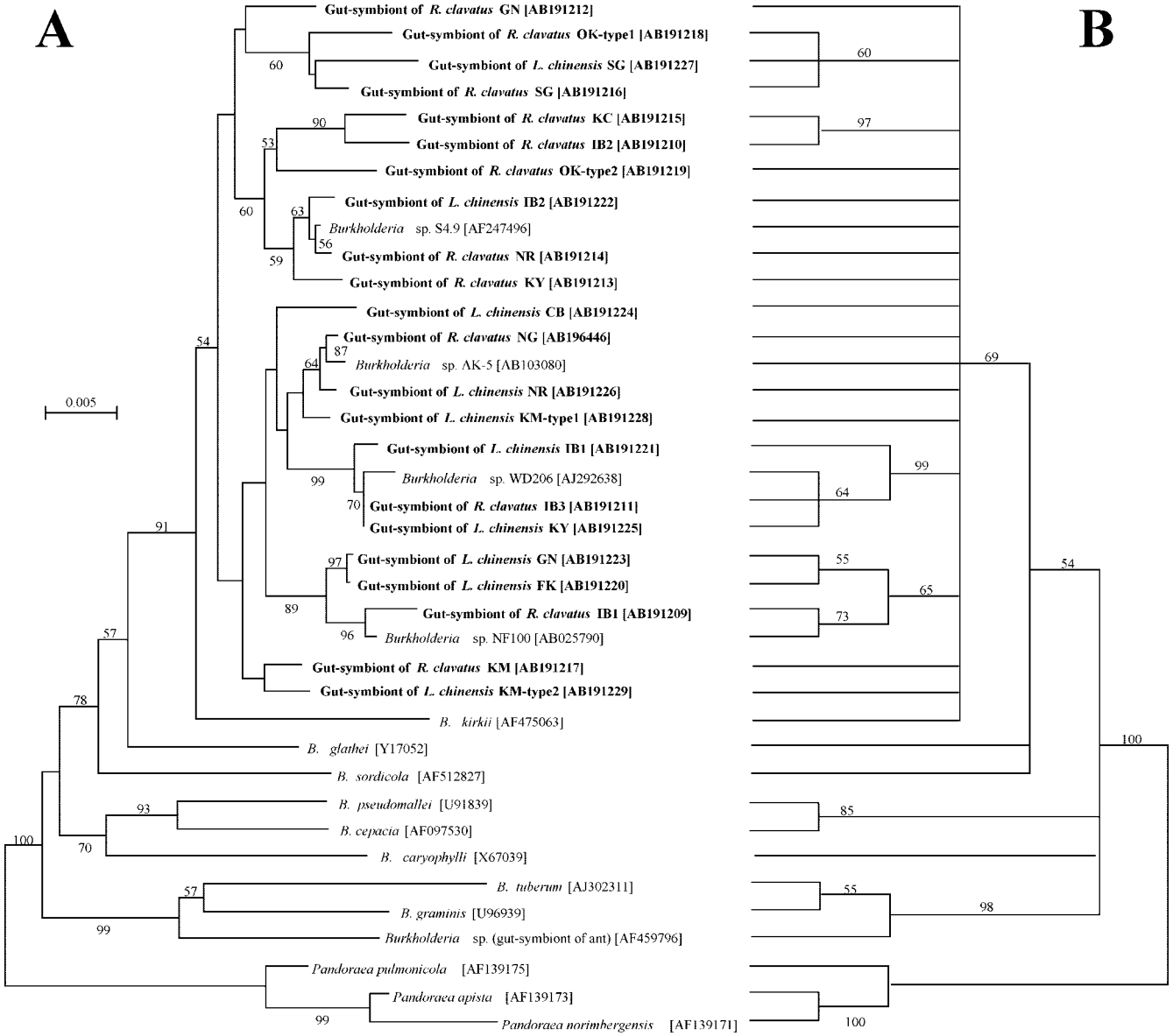

FIG. 2. Phylogenetic relationship of the *Burkholderia* symbionts from *R. clavatus* and *L. chinensis* on the basis of 16S rRNA gene sequences. (A) Neighbor-joining tree. (B) Maximum-parsimony tree based on 50% majority-rule consensus. *Pandoraea* spp. were used as outgroup taxa for the genus *Burkholderia*. Bootstrap values greater than 50% are shown at the nodes. The numbers in brackets are nucleotide sequence accession numbers.

Bacterial 16S rRNA gene sequences identified from the cryptic midgut. For *R. clavatus* and *L. chinensis* samples representing 20 localities (Table 1), the cryptic midgut sections were dissected from adult insects and subjected to DNA extraction. For the 20 DNA samples, a 1.5-kb segment of a eubacterial 16S rRNA gene was amplified by PCR and cloned, and 10 clones for each of the samples were subjected to RFLP analysis. Almost all 200 clones exhibited identical RFLP patterns; the only exceptions were two clones from an *L. chinensis* sample (sample CB) (Table 1).

Three or more clones were sequenced for each of the 20 samples. For 18 samples, all sequences derived from a single insect were identical. For two samples, two different sequences were detected in a single insect. In an *R. clavatus* individual (sample OK) (Table 1), two sequences, designated OK type 1 (two clones) and OK type 2 (one clone), which showed 98.5% sequence similarity to each other, were identified. In an *L. chinensis* individual (sample KM) (Table 1), two sequences, designated KM type 1 (eight clones) and KM type 2 (two clones), which showed 99.6% sequence similarity, were detected. The 22 sequences obtained from the 20 insect samples were not identical but were very similar, exhibiting 97 to 100% similarity to each other (Table 2). Homology searches performed with the DNA databases revealed that all the sequences exhibited the highest levels of similarity (96 to 99%) to 16S rRNA gene sequences of species of *Burkholderia*, a genus of soil bacteria in the β subdivision of the class *Proteobacteria*.

The two RFLP types identified from *L. chinensis* sample CB

TABLE 2. Levels of similarity for the *Burkholderia* symbionts detected in local samples of *R. clavatus* and *L. chinensis* and other *Burkholderia* strains based on 16S rRNA gene sequences[a]

Sample	*R. clavatus*											*L. chinensis*										*Burkholderia* sp.								
	IB1	IB2	IB3	GN	KY	NR	KC	SG	KM	OK type 1	OK type 2	FK	IB1	IB2	GN	CB	KY	NR	SG	KM type 1	KM type 2	S4.9	AK-5	WD206	NF100	*B. kurkii*	*B. glathei*	*B. sordidicola*	*B. capacia*	*B. graminis*
R. clavatus																														
IB1	1	0.98	0.983	0.974	0.98	0.978	0.977	0.974	0.984	0.971	0.969	0.991	0.981	0.978	0.992	0.983	0.983	0.983	0.98	0.985	0.983	0.978	0.979	0.981	0.996	0.972	0.971	0.972	0.968	0.954
IB2		1	0.974	0.981	0.985	0.983	0.991	0.976	0.982	0.982	0.977	0.98	0.973	0.981	0.98	0.977	0.974	0.974	0.976	0.974	0.977	0.983	0.974	0.973	0.981	0.969	0.969	0.965	0.959	0.957
IB3			1	0.982	0.98	0.983	0.977	0.985	0.991	0.974	0.977	0.987	0.998	0.983	0.988	0.988	1	0.992	0.977	0.991	0.987	0.984	0.992	0.998	0.985	0.969	0.974	0.971	0.961	0.952
GN				1	0.986	0.991	0.982	0.985	0.988	0.986	0.988	0.982	0.981	0.989	0.981	0.983	0.982	0.987	0.98	0.987	0.989	0.991	0.988	0.98	0.979	0.976	0.974	0.961	0.958	0.953
KY					1	0.993	0.99	0.98	0.985	0.985	0.979	0.981	0.979	0.994	0.98	0.98	0.98	0.98	0.976	0.98	0.983	0.995	0.98	0.977	0.984	0.976	0.979	0.968	0.962	0.96
NR						1	0.986	0.983	0.989	0.986	0.982	0.985	0.983	0.996	0.985	0.983	0.983	0.984	0.979	0.984	0.986	0.999	0.984	0.981	0.983	0.973	0.974	0.965	0.96	0.959
KC							1	0.976	0.981	0.986	0.977	0.976	0.974	0.986	0.977	0.976	0.977	0.974	0.971	0.974	0.976	0.987	0.974	0.975	0.977	0.971	0.971	0.961	0.956	0.957
SG								1	0.983	0.983	0.991	0.983	0.984	0.982	0.983	0.99	0.985	0.989	0.991	0.989	0.985	0.983	0.989	0.982	0.979	0.973	0.971	0.963	0.959	0.958
KM									1	0.98	0.98	0.988	0.989	0.988	0.988	0.987	0.991	0.988	0.976	0.991	0.994	0.99	0.988	0.988	0.986	0.974	0.977	0.967	0.965	0.953
OK type 1										1	0.985	0.976	0.973	0.988	0.975	0.977	0.974	0.981	0.979	0.981	0.983	0.988	0.978	0.973	0.971	0.971	0.966	0.955	0.949	0.953
OK type 2											1	0.978	0.977	0.98	0.977	0.983	0.977	0.982	0.987	0.982	0.983	0.983	0.982	0.976	0.974	0.973	0.97	0.959	0.955	0.957
L. chinensis																														
FK												1	0.986	0.984	0.999	0.99	0.987	0.987	0.988	0.988	0.986	0.985	0.987	0.985	0.996	0.966	0.972	0.972	0.967	0.955
IB1													1	0.982	0.987	0.988	0.998	0.991	0.976	0.99	0.986	0.983	0.991	0.996	0.984	0.668	0.973	0.971	0.96	0.949
IB2														1	0.983	0.983	0.983	0.985	0.977	0.985	0.987	0.998	0.983	0.98	0.981	0.973	0.974	0.963	0.959	0.956
GN															1	0.991	0.988	0.986	0.988	0.988	0.985	0.985	0.986	0.985	0.995	0.967	0.971	0.971	0.966	0.955
CB																1	0.988	0.99	0.985	0.991	0.987	0.984	0.99	0.986	0.985	0.971	0.97	0.966	0.965	0.952
KY																	1	0.992	0.977	0.991	0.987	0.984	0.992	0.998	0.985	0.969	0.974	0.971	0.961	0.952
NR																		1	0.981	0.997	0.993	0.985	0.997	0.99	0.984	0.974	0.974	0.966	0.96	0.95
SG																			1	0.981	0.979	0.979	0.981	0.974	0.984	0.967	0.965	0.966	0.959	0.956
KM type 1																				1	0.996	0.985	0.994	0.988	0.987	0.975	0.973	0.965	0.963	0.95
KM type 2																					1	0.987	0.991	0.985	0.985	0.977	0.975	0.965	0.964	0.951
Burkholderia sp.																														
S4.9																						1	0.985	0.982	0.983	0.974	0.975	0.965	0.961	0.959
AK-5																							1	0.99	0.984	0.972	0.974	0.966	0.961	0.951
WD206																								1	0.982	0.967	0.971	0.969	0.958	0.949
NF100																									1	0.971	0.975	0.976	0.971	0.957
B. kirkii																										1	0.968	0.955	0.959	0.952
B. glathei																											1	0.972	0.96	0.956
B. sordidicola																												1	0.967	0.952
B. cepacia																													1	0.948
B. graminis																														1

[a] The levels of similarity were calculated by using Kimura's two-parameter model.

TABLE 3. Diagnostic PCR detection of the *Burkholderia* symbionts in the tissues of *R. clavatus* and *L. chinensis*

Species	Sex	No. positive[a]							
		Foreleg	Proboscis	Foregut	Midgut section 1	Midgut sections 2 and 3	Midgut section 4 (crypts)	Hindgut	Testis or ovary
R. clavatus	Male	0	0	0	0	2	10	4	1
	Female	0	0	0	2	2	10	1	0
L. chinensis	Male	0	0	0	0	0	10	0	0
	Female	0	0	1	0	1	10	1	0

[a] Ten individuals were examined for each species and each sex.

were also sequenced, and they exhibited the highest levels of similarity to 16S rRNA gene sequences of *Enterococcus faecalis* (accession no. AE016957) (99.9%) and *Lactococcus lactis* (accession no. AE006288) (99.6%).

Molecular phylogenetic analysis. The 22 sequences of the 16S rRNA gene identified from the cryptic midguts of the alydid bugs were subjected to molecular phylogenetic analyses together with β-proteobacterial sequences retrieved from the DNA databases, including 13 sequences of *Burkholderia* spp. and three sequences of *Pandoraea* spp. (Fig. 2). In both the NJ and MP trees, the sequences from the bugs formed a clade in the genus *Burkholderia* together with several environmental *Burkholderia* strains, such as S4.9, AK-5, WD206, and NF100. The most basal branch of the clade was the sequence of *Burkholderia kirkii*, which was reported to be associated with leaf galls of the plant *Psychotria kirkii* (30). Notably, the phylogenetic relationship of the 16S rRNA gene sequences did not reflect the systematics of the host bugs at all. The sequences from *R. clavatus* and the sequences from *L. chinensis* did not form separate clades but were intermingled in the phylogeny.

Localization of the symbiont in adult bugs. Dissected tissues of *R. clavatus* and *L. chinensis* were subjected to diagnostic PCR detection of the *Burkholderia* symbiont (Table 3). In all individuals of both species examined, the symbiont was detected in midgut section 4 with crypts. Occasionally, the symbiont was also detected in other tissues, such as the foregut, midgut sections 1 to 3, and the testis, although the bands of the PCR products were generally faint.

In situ hybridization of the symbiont in the cryptic midgut. For specific detection of the *Burkholderia* symbionts in *R. clavatus* and *L. chinensis*, a fluorescence-labeled oligonucleotide probe, Cy3-Alsym16S, was synthesized. In situ hybridization with the probe revealed dense signals in the contents of the midgut crypts of *R. clavatus* (Fig. 1D). A few sparse signals were also detected in the main tract of the midgut and in the ducts connecting the crypts with the main tract (Fig. 1D). No signals were detected in the no-probe control, the RNase digestion control, and the competitive suppression control (data not shown). In *L. chinensis*, in situ hybridization similarly localized the symbiont in the midgut crypts (data not shown).

Electron microscopy of the crypt. Ultrathin sections of the midgut crypts of *R. clavatus* and *L. chinensis* were observed by electron microscopy (Fig. 3). In *R. clavatus*, the lumen of the crypts was full of rod-shaped bacteria (Fig. 3A). The cytoplasm of the crypt epithelial cells contained a nucleus and mitochondria but no bacteria (Fig. 3A and B). The cell wall of the bacteria was well developed (Fig. 3C). Some bacterial cells contained electron-dense particles, although it is not clear whether the particles were some biological structure, such as a chromosomal region, or they were merely an artifact. In *L. chinensis*, similar localization and ultrastructure of the symbiont were observed (Fig. 3D).

Prevalence of the symbionts in natural populations of *R. clavatus* and *L. chinensis*. A diagnostic PCR survey of field-collected individuals of *R. clavatus* and *L. chinensis* revealed a very high prevalence (95 to 100%) of the *Burkholderia* symbionts in all populations examined (Table 4).

Detection of the symbionts in other species of the family Alydidae. In addition to *R. clavatus* and *L. chinensis*, six other species of alydid bugs (Table 1) were subjected to PCR analysis. The cryptic organs of the bug samples were subjected to DNA extraction and PCR as described above. *Burkolderia* symbionts were detected in all the alydid species examined (data not shown).

DISCUSSION

Both *R. clavatus* and *L. chinensis* had midgut crypts which were white and arranged in two rows (Fig. 1) and which were densely populated by bacterial cells having a single morphotype (Fig. 3). RFLP analysis and sequencing of the 16S rRNA gene obtained from the midgut crypts revealed that a single bacterial species dominated the gut microbiota of the insects. Molecular phylogenetic analysis demonstrated that although the 16S rRNA gene sequences were slightly different in different insect individuals, the gut bacteria were very closely related to each other and belonged to the genus *Burkholderia* in the β subdivision of the *Proteobacteria* (Fig. 2). The prevalence of *Burkholderia* infection was consistently high (95 to 100%) in natural populations of the insects (Table 4). These results indicated that *R. clavatus* and *L. chinensis* harbor a β-proteobacterial symbiont belonging to the genus *Burkholderia* in the lumen of the midgut crypts. Of course, the possibility that some minor microbial associates are present in addition to the *Burkholderia* symbionts cannot be excluded.

In addition to *R. clavatus* and *L. chinensis*, *Burkolderia* symbionts were also detected in the cryptic midguts of all six other species of alydid bugs examined. It appears to be plausible that bugs belonging to the family Alydidae are generally associated with *Burkholderia* symbionts in midgut crypts, although additional surveys are needed to confirm this idea.

Although symbiotic bacteria in midgut crypts have been microscopically described for a wide array of pentatomomorphan bugs (6, 7, 15), the microbial identities and biological functions have been very poorly characterized. The only case reported thus far is the capsule-transmitted gut symbiont of a plataspid

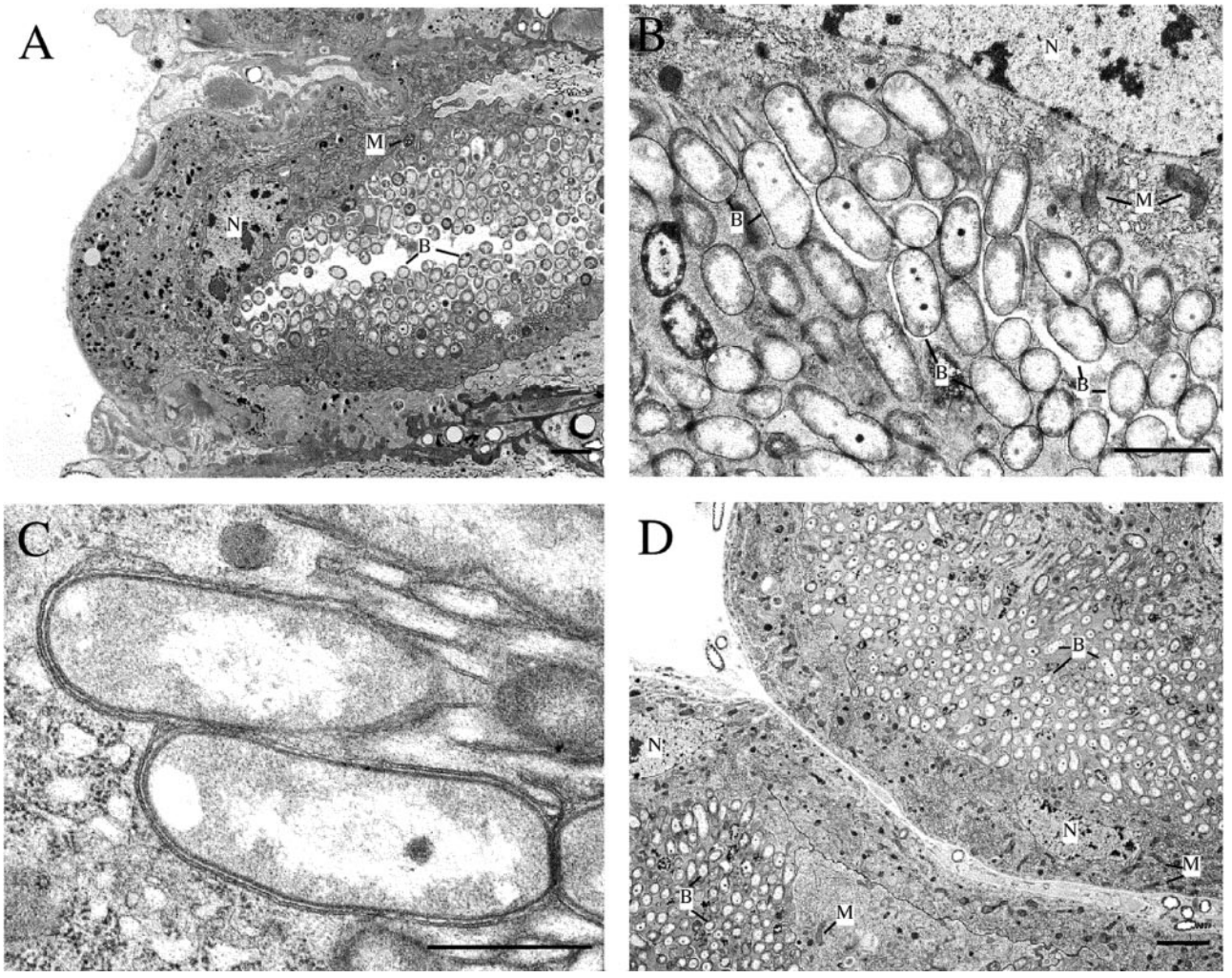

FIG. 3. Transmission electron microscopy of the midgut crypts of *R. clavatus* and *L. chinensis*. (A) Crypt of *R. clavatus* harboring many rod-shaped bacteria. Bar = 2 μm. (B) Enlarged image of the interface between the lumen and the epithelium of the crypt in *R. clavatus*. The bacteria are present only in the lumen. Bar = 1 μm. (C) Enlarged image of the rod-shaped bacteria in *R. clavatus*. A well-developed cell wall and a particle-like structure are present. Bar = 300 nm. (D) Crypt of *L. chinensis* harboring many rod-shaped bacteria. Bar = 2 μm. Abbreviations: B, symbiotic bacterium; M, mitochondrion; N, nucleus.

bug, *Megacopta punctatissima*, which belongs to the γ subdivision of the *Proteobacteria* (13). The discovery of β-proteobacterial symbionts in the alydid bugs indicates that gut symbiotic bacteria have been acquired repeatedly in the evolutionary course of pentatomomorphan bugs.

In obligate endosymbiotic associations like the aphid-*Buchnera* and tsetse fly-*Wigglesworthia* systems, the symbionts are transmitted strictly vertically through host generations, and consequently, the phylogeny of the symbionts generally reflects the phylogeny of the host insects (2, 20). The *Burkholderia* symbionts identified in natural populations of *R. clavatus* and *L. chinensis* constituted a well-defined monophyletic group in the molecular phylogeny of the genus *Burkholderia*. However, the phylogenetic relationship was far from a pattern of cocladogenesis or cospeciation and reflected neither the host systematics nor the geographic distribution of the hosts (Fig. 2). The intermingled phylogenetic pattern suggests the possibility that there was horizontal transmission of the symbiont between populations and species of the alydid bugs. Occasional coinfections with two strains of *Burkholderia* (cf. *R. clavatus* sample OK and *L. chinensis* sample KM) (Fig. 2) also suggest that such horizontal transmission occurs.

To evaluate the fidelity of vertical transmission, as well as the probability of horizontal transmission, information concerning the mechanism of transmission of the symbiont is pivotal. Although no information is available for alydid bugs, the following mechanisms for symbiont transmission have been reported for other groups of pentatomomorphan bugs: (i) egg smearing, in which excrement containing symbiotic bacteria is smeared by the mother on the surface of deposited eggs, and hatchlings orally acquire the symbiont on the eggshell (Pentatomidae, Acanthosomatidae, Urostylidae, and others); (ii) proctophagy, in which hatchlings suck feces of the mother or other insects to acquire the symbiont (Coreidae and Cydnidae); and (iii) capsule transmission, in which symbiont-containing capsules are deposited with eggs by the mother, and

TABLE 4. Detection of the *Burkholderia* symbionts in midgut crypts in natural populations of *R. clavatus* and *L. chinensis*

Species	Sample	% Detection (no. positive/total no.)
R. clavatus	NG	100 (26/26)
	KY	100 (11/11)
	NR	100 (10/10)
	KM	95 (40/42)
	Total	98 (87/89)
L. chinensis	IB2	97 (70/72)
	KY	100 (12/12)
	NR	100 (11/11)
	KM	100 (21/21)
	Total	98 (114/116)

hatchlings probe the contents of the capsules to acquire the symbiont (Plataspidae) (6). In any of the types of transmission, symbiont exchange between conspecific and heterospecific individuals can accidentally occur under certain conditions. Future studies should focus on the mechanism of transmission of the *Burkholderia* symbiont in the Alydidae, and we should be able to understand why and how horizontal transmission of the symbiont occurs.

It is notable that *Burkholderia* isolates obtained from soil environments, such as strains S4.9, AK-5, WD206, and NF100 (11, 16, 23, 27), were also included in the well-defined monophyletic group containing the *Burkholderia* symbionts from the alydid bugs (Fig. 2). The phylogenetic data suggest that the *Burkholderia* symbiont might have a free-living phase outside the host body, possibly in the soil environment. If so, a horizontal transmission route involving free-living bacteria should also be taken into account.

In a number of pentatomomorphan bugs, symbiont-free insects exhibit retarded growth, nymphal mortality, and/or sterility (1, 13, 17, 22, 24), suggesting that their symbionts play important physiological roles. Because of the dense and specific colonization in the midgut crypts (Fig. 1 and 3 and Table 3) and the prevalence in natural populations of *R. clavatus* and *L. chinensis* (Table 4), it appears likely that the *Burkholderia* symbionts play important roles in the host bugs. Members of the genus *Burkholderia* are major soil bacteria that are most commonly found on plant roots, in adjacent areas, and in other moist environments (32). Notably, a wide variety of biological activities have been reported for this bacterial group. A number of strains possess N_2-fixing ability (9); some strains nodulate the roots of leguminous plants (21, 29); one strain is associated with plant leaf galls (30); and some members of the *Burkholderia cepacia* complex promote plant growth and suppress plant diseases (5) and are thus utilized as biofertilizing agents (31). The biological roles of the *Burkholderia* symbionts in the alydid bugs might be relevant to one of these biological activities. On the other hand, considering the genetic diversity of the symbionts, the incongruence with the host systematics, and the putatively frequent horizontal transfers (Fig. 2), it also seems plausible that the *Burkholderia* symbionts may be commensal or even parasitic associates, which simply adapt to the intestinal environment of the alydid bugs. Biological effects of the symbionts should be evaluated by using experimentally generated symbiotic and aposymbiotic bugs.

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