Insect Gut Symbiont Susceptibility to Host Antimicrobial Peptides Caused by Alteration of the Bacterial Cell Envelope*

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Background: The elucidation of molecular changes of symbionts is important for understanding symbiotic adaptation.

Results: Insect gut symbionts are highly susceptible to host immunity because of dramatic cell envelope changes.

Conclusion: Cell envelope changes in gut symbionts are required for successful symbiosis with hosts.

Significance: Biochemical analyses of intact gut symbionts revealed a novel mechanism of gut symbiosis.

The molecular characterization of symbionts is pivotal for understanding the cross-talk between symbionts and hosts. In addition to valuable knowledge obtained from symbiont genomic studies, the biochemical characterization of symbionts is important to fully understand symbiotic interactions. The bean bug (*Riptortus pedestris*) has been recognized as a useful experimental insect gut symbiosis model system because of its cultivatable *Burkholderia* symbionts. This system is greatly advantageous because it allows the acquisition of a large quantity of homogeneous symbionts from the host midgut. Using these naïve gut symbionts, it is possible to directly compare *in vivo* symbiotic cells with *in vitro* cultured cells using biochemical approaches. With the goal of understanding molecular changes that occur in *Burkholderia* cells as they adapt to the *Riptortus* gut environment, we first elucidated that symbiotic *Burkholderia* cells are highly susceptible to purified *Riptortus* antimicrobial peptides. In search of the mechanisms of the increased immunosusceptibility of symbionts, we found striking differences in cell envelope structures between cultured and symbiotic *Burkholderia* cells. The bacterial lipopolysaccharide O antigen was absent from symbiotic cells examined by gel electrophoretic and mass spectrometric analyses, and their membranes were more sensitive to detergent lysis. These changes in the cell envelope were responsible for the increased susceptibility of the *Burkholderia* symbionts to host innate immunity. Our results suggest that the symbiotic interactions between the *Riptortus* host and *Burkholderia* gut symbionts induce bacterial cell envelope changes to achieve successful gut symbiosis.

Symbiosis is prevalent in animals and plants, and the most intimate form of symbiosis is endosymbiosis, defined as a species living inside of another species (1). Endosymbiosis has been studied more extensively in insects than in any other living organism, likely because of their biodiversity (2) and the fact that they harbor many different symbiotic bacteria within the gut, tissues, and cells (1). Studies of endosymbiosis in insects have revealed that symbiotic bacteria affect various aspects of host biology, including the provisioning of essential nutrients to the host (3), defense against natural enemies, and adaptation to specific ecological conditions (4), and it also has parasitic consequences, such as negative fitness effects and reproductive aberrations (5). Although the effects of symbionts on host biology are understood, knowledge of the effects of hosts on symbiont biology is limited.

The majority of insect symbionts are transmitted from the mother to the offspring vertically and are highly adapted to the unique niches within their host insects (1). Therefore, these symbionts are not easily isolated from their hosts for laboratory culturing, resulting in difficulty studying their adaptive biology during symbiotic association (6, 7). Recently, a culture-independent genomic approach has provided valuable information regarding the essential genes and putative mechanisms involved in insect-symbiont interactions (3, 8). However, this genomic approach is powerful only for the study of certain symbionts with reduced genome size caused by coevolution with their host for a long period of time (9). To understand molecular changes other than genomic differences that occur in symbiotic bacteria, it is necessary to have a good experimental model system in which biochemical approaches can be applicable for the study of symbionts to examine their molecular adaptation during association with host insects.

The bean bug *Riptortus pedestris* (Hemiptera: Alydidae) has several advantages as an experimental insect symbiosis model. It harbors a monospecific gut symbiont of the β-proteobacterial genus *Burkholderia* in a specialized midgut section designated as the M4 region (10). Bean bugs acquire *Burkholderia* cells not vertically from their mother but horizontally from the

*This study was supported by the Global Research Laboratory Program Grant 2011-0021535 and Basic Science Research Program Grant 2014R1A1A4A01007507 of the National Research Foundation of Korea. The authors declare that they have no conflicts of interest with the contents of this article.

The amino acid sequences of these proteins can be accessed through the NCBI Protein Database under NCBI accession numbers A416695, AB842297, and AB842298.

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environment. In the laboratory, we established a gut symbiont-harboring (symbiotic) insect line and a symbiont-deficient (aposymbiotic) insect line by controlling the feeding of a solution containing \textit{in vitro}-cultured \textit{Burkholderia} cells to early \textit{Riptortus} nymphs. A comparison of these two insect lines has revealed the effects of \textit{Burkholderia} symbionts on host biology (11–13). Furthermore, \textit{Burkholderia} symbionts are cultivatable \textit{in vitro} and genetically manipulable (11–13), which has allowed the elucidation of several symbiotic factors for establishing associations using a genetically manipulated \textit{Burkholderia} symbiont (14–17). In addition to the recognized features mentioned above, a unique advantage of this \textit{Riptortus-Burkholderia} system is that a large number of naïve gut symbionts can be isolated from the \textit{Riptortus} host, enabling the direct comparison of symbiotic and cultured \textit{Burkholderia} cells using biochemical approaches. Finally, because \textit{R. pedestris} is a hemimetabolous insect and its innate immune mechanisms have been studied much less frequently than those of holometabolous insects such as \textit{Drosophila}, \textit{Manduca}, and \textit{Tenebrio} (18), this insect system is valuable for the study of host innate immune responses.

In this study, we aimed to elucidate the molecular changes that occur in \textit{Burkholderia} cells upon symbiotic association with the host \textit{R. pedestris}. Here we found that gut symbiotic \textit{Burkholderia} were more susceptible to the purified antimicrobial peptides (AMPs)\(^2\) than cultured \textit{Burkholderia}. While searching for the mechanisms of the immunosusceptibility of \textit{Burkholderia} symbionts, we discovered dramatic molecular changes in the cell envelope of the gut symbionts: loss of lipopolysaccharide O antigen and compromised membrane integrity.

**Experimental Procedures**

\textit{Bacteria and Media—Escherichia coli} K12 and \textit{Staphylococcus aureus} RN4220 cells were cultured at 37 °C with LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). The \textit{Burkholderia} symbiont RPE75 strain, a spontaneous rifampicin-resistant strain derived from the RPE64 strain (19), was cultured at 30 °C with yeast-glucose (YG) medium (0.4% glucose, 0.5% yeast extract, and 0.1% NaCl) containing 30 \(\mu\)g/ml rifampicin unless mentioned otherwise.

\textit{Insect Rearing and Symbiont Inoculation—\textit{R. pedestris}} was maintained in our insect laboratory at 26 °C under a long day cycle of 16 h light and 8 h dark as described previously (14). Nymphal insects were reared in clean plastic containers with soybean seeds and distilled water containing 0.05% ascorbic acid. When the newborn nymphs molted to second instar, \textit{Burkholderia} inoculum solution was provided as wet cotton balls in a small Petri dish. The inoculum solution consisted of \textit{in vitro}-cultured mid-log phase \textit{Burkholderia} cells in distilled water containing 0.05% ascorbic acid at a concentration of 10\(^7\) cells/ml.

\textit{Isolation of Symbiotic \textit{Burkholderia} from the Midgut—The symbiotic organs, M4 midguts, were dissected from fifth-instar nymphs and placed in 50 \(\mu\)l of 10 mM phosphate buffer (PB) (pH 7.0). The M4 midguts were cut several times with fine scissors to break the midgut crypts. One milliliter of PB was added to the cut midguts by gentle pipetting to resuspend the \textit{Burkholderia} symbionts into the solution. The solution was then filtered through a 5-\(\mu\)m pore to remove the midgut tissues. \textit{Burkholderia} cells were then washed gently with PB and collected by centrifugation at 1800 \(\times\) g.

**Electrophoretic Analysis of LPS—** LPS was extracted from bacterial cells using a modified hot phenol method (20). \textit{Burkholderia} cells (10\(^9\) cells) were washed with PB and resuspended in 500 \(\mu\)l of PB. The same volume of hot phenol was added to the cell solution and incubated in a water bath adjusted to 65 °C. The cell solution was vortexed rigorously every 5 min. After 1 h of incubation, the solution was cooled, and 200 \(\mu\)l of chloroform was added. After vortexing, the solution was incubated at room temperature for 5 min and then centrifuged at 15,300 \(\times\) g for 15 min to separate the water and phenol phases. Next 400 \(\mu\)l of the water phase solution was transferred to a new tube, and 800 \(\mu\)l of ethanol was added to the solution. After allowing LPS precipitation at −20 °C overnight, the precipitates were collected by centrifugation at 20,400 \(\times\) g for 20 min, washed with 80% ethanol, and air-dried. The precipitates were suspended in sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.005% bromphenol blue), boiled at 95 °C for 5 min, deproteinized by incubating with 400 \(\mu\)g/ml proteinase K at 60 °C for 1 h, and boiled again prior to the electrophoretic analysis. LPS extracted from 10\(^7\) cells was loaded into a well of N-[2-hydroxy-1,1-bis(hydroxymethyl)]ethyl]glycine SDS-PAGE (21) with a 12% gel. The LPS separated in the gels was visualized using a Pro-Q Emerald 300 LPS gel stain kit (Invitrogen).

**Periodic Acid-Schiff Staining—** Cultured and symbiotic \textit{Burkholderia} cells were washed twice with distilled water and suspended with distilled water to have \(\sim\)10\(^8\) cells/\(\mu\)l. 5 \(\mu\)l of the suspensions of \textit{Burkholderia} cells was spotted separately on the same slide. Bacterial cells were heat-fixed to the slide and washed with distilled water to remove the unbound cells. After removing excess water, the heat-fixed bacteria were incubated with 1% periodic acid solution (Sigma-Aldrich) for 5 min at room temperature. The slide was rinsed gently and incubated further with Schiff’s reagent (Sigma-Aldrich) for 15 min at room temperature. After incubation, the slide was rinsed with running tap water for 5 min and air-dried. The water-mounted stained cells were covered with a coverslip and examined microscopically under an oil immersion lens (Olympus BX50).

**Generation of Deletion Mutant Strains—** The O antigen-deficient mutant strain was generated by deleting the \textit{wbiG} gene (accession no. BAN23935) as described previously (16). Briefly, allelic exchange of the \textit{wbiG} gene was accomplished by utilizing the suicide vector pK18mobsacB harboring a 5′ region and 3′ regions of the \textit{wbiG} gene. A primer set of \textit{wbiG-L-P1} (5′-CCC GGA TCC GTC ATC ACG CTC AGC ATC TC-3′) and \textit{wbiG-P1-P2} (5′-CCC TCT AGA GAC GCC CTC ATA GCC ACT-3′) was used to amplify the 5′ region of the gene, and a primer set of \textit{wbiG-R-P1} (5′-CCC TCT AGA GCT CGA GAC GCC TCA TAT C-3′) and \textit{wbiG-R-P2} (5′-CCC AAG CTT GTC TTC GCC ATC GGT CAC-3′) was used to amplify the 3′ region of the gene from the \textit{Burkholderia} symbiont RPE75. After transforming \textit{E. coli} DH5\(\alpha\) cells with the pK18mobsacB vector con-
AMP Susceptibility Assay—Symbiotic and cultured *Burkholderia* cells were washed with PB and suspended in PB at 10^4 cfu/200 μL. To 200 μL of the cell solution, 200 μL of purified AMP solutions with a concentration of 0.2, 0.4, 1, or 2 μg/mL was added and incubated for 2 h at room temperature. After incubation, the mixtures were diluted and spread on YG agar plates containing rifampicin. The colonies were counted after 2 days of incubation at 30 °C.

**Measurement of Expression of AMP Genes**—The expression of AMP genes was measured in the fat body and M4 midgut. The fat body or M4 midgut was dissected and subjected to RNA extraction using RiboEx (GeneAll, South Korea). The RNA samples were reverse-transcribed using TOPscript RT DryMix containing oligo(dT) primers (Enzymomics, South Korea) to synthesize cDNA. cDNA was subjected to real-time quantitative PCR after mixed with a TOPreal qPCR 2× Premix with SYBR Green (Enzymomics, South Korea) and 0.25 μM each of the following primers: rip-defensin primer set, 5′-TCG GTC GGA CTG AGA AA-3′ and 5′-TTG CCG CCT TTG TAT CCC TT-3′; ripocin primer set, 5′-TCC GAA GCT GAG GGT CTT CCC G-3′ and 5′-TCC GCA TCC AAC TGG CCG TCC-3′; rip-thanatin primer set, 5′-GTC TGG CTG CGT TGA AGA CG-3′ and 5′-ATT CGG TTG AAA ACG CCG-3′; and elongation factor 1 α (EF1α) gene of *R. pedestris* as an endogenous control gene.

**MALDI-TOF Analysis**—MALDI-TOF analysis was performed in linear mode using a Perseptive (Framingham, MA) Voyager STR instrument equipped with delayed extraction technology. The ions formed by a pulsed UV laser beam (nitrogen laser, 337 nm) were accelerated at 24 kV. The mass spectra reported are the result generated from the data derived from several single laser shots in 200-shot steps from different positions of the sample spot. High-resolution mass spectra were acquired in reflector mode on a 4800 Proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). In this case, mass spectra resulting from the sum of 1250 laser shots were obtained with a resolution higher than 10,000 (as the ratio between the mass of the peak and its full width at half-maximum intensity) and with a mass accuracy below 100 ppm. The sample preparations were performed by adopting a laborious “lipooligosaccharide thin layer procedure” as described previously (22). Briefly, a small amount of the intact LPS was suspended in a mixture of methanol/water (1:1) containing 5 mM EDTA and dissolved by ultrasonication. When converted in the ammonium form and desalted, the sample was deposited on top of a thin layer of a 2,4,6-trihydroxyacetophenone/nitrocellulose matrix together with the same volume of 20 mM dimannoside citrate.

**NMR Spectroscopy**—For structural assignments of LPS oligosaccharide, one-dimensional and two-dimensional 1H NMR spectra were recorded in D_2O at 298 K at δD = 7 with a Bruker 600 DRX spectrometer equipped with a cryoprobe. The spectra
were calibrated with internal acetone (δH, 2.225; δC, 31.45). The 2D-double quantum filter (DQF)-correlation spectroscopy (COSY) spectra were acquired with 4096 × 512 data points in both F2 and F1 dimensions. The coupling constants were determined on a first-order basis from 2D phase-sensitive DQF-COSY (23, 24). The total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 100 ms using data sets (t1 × t2) of 4096 × 256 points. The NOESY and ROESY experiments were performed by using datasets (t1 × t2) of 4096 × 256 points with mixing times between 200–400 ms.

In all homonuclear experiments, the data matrix was zero-filled in the F1 dimension to provide a matrix of 4096 × 2048 points and resolution-enhanced in both dimensions by a 90° shifted Qsine function before Fourier transformation. Heteronuclear single quantum coherence (HSQC), HSQC-TOCSY, and heteronuclear multiple bond correlation experiments were measured in the 1H-detected mode via single quantum coherence with proton decoupling in the 13C domain using data sets of 2048 × 256 points. A 60-ms delay was used for the evolution of Qsine function before Fourier transformation. In all heteronuclear experiments, the data matrix was extended to 2048 × 1024 points using a forward linear prediction extrapolation.

Transmission Electron Microscopy Analysis—Burkholderia cells were washed with PB three times and prefixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 18 h. After washing with sodium cacodylate buffer three times at room temperature for 15 min each, the cells were post-fixed with 1% osmium tetroxide in sodium cacodylate buffer at room temperature for 1 h. After washing three times with sodium cacodylate buffer, the samples were dehydrated and cleared through an ethanol and propylene oxide series and embedded in Epon 812 resin. The embedded samples were trimmed and sectioned on an ultramicrotome (Richert Super Nova, Leica). The sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (Hitachi H-7600).

SDS Sensitivity Assay—The Burkholderia cells were washed and diluted with PB to 500–1000 cfu/50 µl for each sample. To the 50 µl of the bacterial samples, 50 µl of SDS solution was added to have 0.0015, 0.005, 0.015, or 0.075% as final concentrations and incubated at room temperature for 30 min. After incubation, the samples were spread onto YG agar plates containing 30 µg/ml rifampicin. After 2 days, the plates were subjected to colony counting.

M4 Lysate Preparation—The lysate of M4 midguts of symbiotic and asymbiotic insects was prepared as described previously with some modifications (25). Twenty samples of the M4 region were dissected from fifth-instar nymphs and homogenized in 100 µl of PB with a pestle. The M4 homogenates were centrifuged at 2300 × g for 10 min to remove tissue and cell debris and centrifuged further at 20,000 × g for 5 min. The supernatants were heat-treated at 55°C for 3 min to kill symbiotic Burkholderia that might be present in solution and centrifuged at 20,000 × g for 10 min. For the heat-untreated M4 lysate, the M4 lysate samples of asymbiotic insects were prepared in the same way, with the exception of skipping the heat treatment step. The final M4 lysates collected from 20 insects were diluted up to 1 ml with PB (20 M4s/ml).

To test the M4 lysate effect on the SDS sensitivity of the cultured Burkholderia cells, the M4 lysates were serially diluted, and 25 µl of the lysates at concentrations of 0.5, 1, 1.5, or 2 M4/ml were incubated with 25 µl of the cultured Burkholderia cell suspension. After 20 min of incubation at room temperature, the samples were incubated with 50 µl of SDS solutions (final concentrations of 0.05% and 0.07%) for 30 min. The samples were spread onto YG agar plates containing rifampicin, cultured for 2 days, and subjected to colony counting.

AMP Susceptibility Assay—Each sample consisted of Burkholderia cells (~1000 cfu) and either riptocin or rip-defensin with a final concentration of 1, 3, 10, 20, or 30 µg/ml. For samples with M4 lysate treatment, M4 lysate with a final concentration of 2 M4s/ml was included in the sample. The combined samples were incubated for 30 min at room temperature and spread onto YG agar plates containing rifampicin. After 2 days of incubation at 30°C, the colony-forming units were counted.

In Vivo Clearance Assay—Burkholderia cells were washed with PB and resuspended with Grace’s insect medium to 10⁷ cells/ml. Burkholderia cells treated with M4 lysate were incubated with M4 lysates (2 M4s/ml) at room temperature for 30 min prior to washing with PB. 2 µl of the bacterial cell solution was injected into asymbiotic fifth-instar nymphs. 1, 2, or 3 h after injection, three insects of the same injection group were cut into small pieces in 500 µl of PB with fine scissors. After vigorous vortexing, the solution was collected with a syringe and transferred to a new tube. The cut insects were further rinsed with 1 ml of PB twice, and the rinsed solution was combined with the solution collected previously. The collected solutions were filtered (5-µm pore size) and centrifuged at 1800 × g for 10 min. The bacterial cell pellets were washed and resuspended with PB. The serially diluted cell solutions were spread on YG agar plates containing rifampicin and subjected to colony counting after 2 days of incubation at 30°C. The data of three independent experiments, where each experiment used samples from three insects, were used for statistical analysis.

Statistical Analyses—The statistical significance of data was determined using unpaired Student’s t test or two-way analysis of variance with Sidak’s or Turkey’s post hoc test as provided in GraphPad Prism software.

Results
The Susceptibility of Symbiotic Burkholderia Cells to Purified Riptortus AMPs—We hypothesized that the changes occurring in Burkholderia symbionts must be essential for symbiotic association with their hosts and strongly associated with the responses of the symbiont to host innate immunity. Therefore, we examined these responses to understand the molecular changes of Burkholderia cells in the transition from in vitro cultured cells to in vivo symbiotic cells. To test our hypothesis, AMPs, the major components of insect humoral immunity, were purified from the host Riptortus. Three different types of AMPs were purified from E. coli-challenged Riptortus hemolymph using a series of chromatographic procedures (Fig. 1, A and B). The molecular masses of the purified AMPs were deter-
mined using a MALDI-TOF mass spectrometer (Fig. 1C), and their N-terminal sequences were identified by Edman degradation (Fig. 1D, underlined sequences). The deduced amino acid sequences, termed riptocin, rip-thanatin 1 and 2, and rip-defensin, are shown in Fig. 1D.

Evaluation of the responses of cultured and symbiotic Burkholderia cells to these AMPs revealed that the symbiotic cells were highly susceptible to riptocin (Fig. 2A) and rip-defensin (Fig. 2B) compared with the cultured cells but not to the rip-thanatins (Fig. 2C and D). The AMP susceptibility result for symbiotic Burkholderia was unexpected because we speculated that the symbionts would be resistant to host AMPs to enable survival in the host midgut. To understand how the symbiotic cells were able to survive inside of the host gut despite their high level of susceptibility to host AMPs, the expression levels of the AMPs in the symbiotic organ (M4 midgut) were examined. Compared with that in the midgut of aposymbiotic insects, no significant differences were observed (Fig. 3A). Comparison of the AMP expression levels in the M4 midgut with the basal expression levels in the fat body showed that the expression of rip-defensin was significantly lower in the M4 midgut (Fig. 3B). These results suggest that the gut-colonizing Burkholderia symbionts did not induce AMP expression in the M4 midgut and that AMP expression in the M4 midgut was suppressed compared with expression in the fat body. Therefore, the immunosusceptible Burkholderia symbionts were able to establish a symbiotic association with the host midgut.

The Loss of LPS O Antigen in Symbiotic Burkholderia Cells—Next we asked how symbiotic Burkholderia cells exhibit high susceptibility against Riptortus AMPs. To answer this question, we focused on the bacterial cell envelope because it is the frontline of bacterial defense. The cell envelope of Burkholderia, a Gram-negative bacterium, consists of inner and outer membranes separated by a periplasmic space that contains a peptidoglycan layer (26, 27). Among these features, the most unique characteristic of the envelope of Gram-negative bacterial cells

FIGURE 1. Purification and characterization of Riptortus AMPs. A, schematic for the purification of AMPs from the E. coli-injected hemolymph of Riptortus insects. B, the final HPLC chromatograms. AU, arbitrary units. C, MALDI MS spectra of riptocin (i), rip-defensin (ii), rip-thanatin 1 (iii), and rip-thanatin 2 (iv). D, amino acid sequences of the AMPs purified from the Riptortus hemolymph.
compared with that of Gram-positive bacterial cells is the presence of LPS in the outer leaflet of the outer membrane (28, 29). Because LPS interacts directly with the host environment, we first compared the LPS patterns in symbiotic *Burkholderia* cells isolated from the M4 midgut with those in cultured *Burkholderia* cells grown in YG medium. This examination revealed strikingly different LPS patterns, as determined using carbohydrate-stained SDS-PAGE gels (Fig. 4A). The LPS extracted from cultured *Burkholderia* cells showed the typical migration pattern of smooth-type LPS with the following three bands: a lower band representing the lipid A plus core oligosaccharide, middle ladder-like bands representing low molecular weight O antigens, and an upper band representing high molecular weight O antigen (Fig. 4A, lane 1). In contrast, the LPS extracted from symbiotic cells exhibited typical rough-type LPS, showing a strong band near the lipid A plus core oligosaccharide while lacking the O antigen ladder-like bands (Fig. 4A, lane 2). Culturing of symbiotic cells for 1 day in YG medium resulted in restoration of the low molecular weight and high molecular weight O antigens (Fig. 4A, lane 3). To confirm that the symbiotic *Burkholderia* cells lost O antigens during colonization in the insect gut, we generated an O antigen-deficient mutant strain by mutating an O antigen biosynthesis gene, *wbiG*, that encodes NAD-dependent epimerase. When the LPS pattern of this mutant was compared with that of symbiotic *Burkholderia* cells, the mutant cells were found to exhibit a rough-type LPS pattern by SDS-PAGE (Fig. 4B, lane 4). Next we stained these cells with periodic acid-Schiff reagent to detect saccharides. The staining of symbiotic cells and Δ*wbiG* Burkholderia mutant cells was much less intense compared with...
that of cultured cells (Fig. 4, D and F), indicating that the symbiotic and mutant cells contained fewer saccharide residues on their cell surfaces than the cultured cells (Fig. 4C) and symbiotic cells with 1 day of in vitro cultivation (Fig. 4E). These results suggest that symbiotic Burkholderia cells have a phenotype similar to that of O antigen-deficient ΔwbiG Burkholderia mutant cells.

**Structural Analysis of LPS from Symbiotic and Cultured Burkholderia Cells**—To elucidate the primary structures of LPS from cultured, symbiotic, and ΔwbiG mutant Burkholderia cells, the LPSs were isolated and further purified by enzymatic digestion and gel filtration chromatography. The LPS samples were then analyzed by MALDI-MS. The MALDI-MS spectra confirmed the absence of O antigen in the symbiotic cells (Fig. 5B) and symbiotic cells (Fig. 5C) cells because no ion signals were observed in the very high mass region, unlike what was observed in the cultured cells (Fig. 5A). Furthermore, as shown in Fig. 5, D–F, the analysis of the MALDI-MS spectra revealed the peaks belonging to lipid A and core oligosaccharide moieties observed at low masses in addition to the lipooligosaccharide (lipid A plus core) molecular ions that appeared at higher masses. The lipid A moieties from the cultured wild-type and mutant cells were made up of a heterogeneous mixture of tetra- and penta-acylated species, whereas the MALDI spectrum of the symbiotic wild-type lipooligosaccharide basically showed penta-acylated species. In detail, the ion at the average m/z 1444.6 (monoisotopic ion at m/z 1443.9) corresponded to a bis-phosphorylated tetra-acylated backbone disaccharide carrying one residue of arabinosamine. L	etra1 represents the lipid A species carrying a further primary fatty acid on glucosamine 1 and an additional residue of arabinosamine. The most representative lipid A plus core oligosaccharide (L + OS) molecular ions are labeled in the spectra. G, 1H NMR spectrum of the core oligosaccharide (OS) fraction. H, the lipooligosaccharide structure of the Burkholderia symbiont as derived by MALDI MS and NMR spectroscopic data.
structure of the core oligosaccharide, a mild hydrolysis was performed to cleave lipid A from the core oligosaccharide portion, and a combination of homo- and heteronuclear 2D NMR experiments (DQF-COSY, TOCSY, ROESY, NOESY, $^1$H – $^13$C HSQC, $^1$H – $^13$C HSQC-TOCSY, and $^1$H – $^13$C heteronuclear multiple bond correlation) were executed to assign the spin systems and the monosaccharide sequence. All proton resonances were deduced by DQF-COSY and TOCSY spectra and were used to assign the carbon signals in the HSQC spectrum. The $^1$H proton spectrum of oligosaccharide is shown in Fig. 5G. Seven anomic signals were identified in the anomic region. The values of $^3J_{	ext{H,H}}$ coupling constants and the intraresidual NOE contacts were used to identify the anomeric configuration of each residue, whereas the relative configuration of each monosaccharide unit was obtained by the values of the vicinal $^3J_{	ext{H,H}}$ coupling constants. The NMR spectroscopic data showed remarkable heterogeneity, indicating a mixture of oligosaccharides with a different length of carbohydrate backbone. This heterogeneity caused the splitting of anomic signals of some residues. The substitution pattern of all residues was established by interresidual NOE contacts and heteronuclear multiple bond correlation long-range correlations together with the downfield shift of the carbon resonances of the glycosylated positions. Therefore, in summary, NMR spectroscopic data with the MALDI-MS results allowed us to determine the primary structure of the lipooligosaccharide of the *Burkholderia* symbiont (structure shown in Fig. 5H).

The Effects of LPS Alterations on the Susceptibility of *Burkholderia* Symbionts to AMPs—We used ΔwbiG mutant *Burkholderia* cells to examine whether there is a direct relationship between the loss of LPS O antigen and the susceptibility of symbiotic *Burkholderia* cells to host AMPs. Although ΔwbiG *Burkholderia* cells do not seem to have the same lipid A structure as symbiotic *Burkholderia* cells (Fig. 5, E and F), they share the same phenotype of a core oligosaccharide without O antigen (Figs. 4 and 5, B and C). When the wbiG mutant cells were treated with riptocin and rip-defensin, they exhibited AMP resistance like cultured wild-type *Burkholderia* cells (Fig. 8, A and B). These results indicate that the loss of O antigen in symbiotic *Burkholderia* cells is not likely to be responsible for their increased susceptibility to host AMPs. Because the immune responses of insects involve not only AMPs but also other immune components, we examined the effects of O antigen loss in *Burkholderia* symbiont cells in an *in vivo* bacterial clearance experiment, which involved whole innate immunity of the host *Riptortus*. O antigen-deficient ΔwbiG cells and symbiotic cells exhibited significantly faster clearance than cultured *Burkholderia* cells (Fig. 8C), suggesting that the loss of LPS O antigen in gut symbionts renders them more susceptible to host innate immune responses in *in vivo*.

Differences in Cell Membranes between Cultured and Symbiotic *Burkholderia* Cells—On the basis of the striking differences in the LPS structures of cultured and symbiotic *Burkholderia* cells, we postulated that the bacterial cell envelopes of *Burkholderia* cells undergo drastic changes during colonization in the M4 midgut of the *Riptortus* host. To gain additional information regarding these changes in the bacterial cell envelope, we examined the morphologies of cultured and symbiotic cells by transmission electron microscopy. The transmission electron microscopy images revealed that the symbiotic cells had distorted cell membranes, with dissociation of the inner and outer membranes (Fig. 6B) compared with the normal cell membranes of the cultured cells (Fig. 6A). Both symbiotic cells cultured 1 day in *in vitro* (Fig. 6C) and O antigen-deficient ΔwbiG mutant cells (Fig. 6D) also exhibited cell membrane morphologies similar to that of the cultured wild-type cells (Fig. 6A). The different morphology of the symbiotic *Burkholderia* cells suggests that their cell membrane integrity may be compromised under symbiotic conditions.

To further assess the structural differences between these bacterial cell membranes, we examined the detergent sensitivities of cultured and symbiotic *Burkholderia* cells. Upon treatment with different concentrations of SDS, an anionic surfactant, the number of colony-forming units of the symbiotic cells decreased significantly (Fig. 6F) but cultured cells (Fig. 6E), symbiotic cells cultured for 1 day in *in vitro* (Fig. 6G) and ΔwbiG mutant cells (Fig. 6H) were minimally affected. These results indicate the following. The cell membranes of symbiotic *Burkholderia* cells were more sensitive to the detergent than those of cultured cells, the detergent sensitivity of the symbiotic cells might not have been due to the loss of O antigen, and *in vitro* cultivation of symbiotic cells restored detergent resistance.
Host factor-induced changes in cell membranes of Burkholderia symbionts—Because the detergent resistance of symbiotic cells was restored by in vitro cultivation, we questioned whether the membrane integrity changes of Burkholderia symbionts were induced by host factor(s) that are present in the M4 midgut. To answer this question, a lysate of the M4 midgut was examined for its ability to induce detergent sensitivity in cultured Burkholderia cells. When M4 lysates from gut symbiont–harboring insects were heat-treated (55 °C) to inactivate intrinsic host factor(s), cultured cells were incubated with M4 lysates and SDS-untreated colony-forming units were set to 100% prior to the detergent-sensitivity assay. Colony-forming units were normalized by M4 lysate, and SDS-untreated colony-forming units were set to 100%. Data are mean ± S.D. are shown (n = 3).

FIGURE 7. Detergent sensitivity induced by M4 lysate treatment. The different doses of heat-treated M4 lysates obtained from symbiotic insects (A) and heat-treated aposymbiotic insects (B) as well as heat-untreated M4 lysate of aposymbiotic insects (C) were incubated with cultured Burkholderia cells prior to the detergent-sensitivity assay. Colony-forming units were normalized by M4 lysate, and SDS-untreated colony-forming units were set to 100%. Data are mean ± S.D. are shown (n = 3).

The Effects of Cell Membrane Alterations in Burkholderia Symbionts on Their Susceptibility to Host AMPs—Because we generated cultured Burkholderia cells exhibiting altered membrane integrity by exposing them to M4 lysate (Fig. 7), we then examined the susceptibility of these cells to riptocin and rip-defensin (Fig. 8, A and B). The susceptibility of the symbiotic cell to riptocin was low, with an IC_{50} of 3.86 μg/ml, and cultured and ΔwbiG cells were not affected by even 30 μg/ml of riptocin. However, M4 lysate-treated cultured and ΔwbiG cells demonstrated increased riptocin susceptibility with IC_{50} values of 21.7 μg/ml and 17.9 μg/ml, respectively (Fig. 8A). In contrast, M4 lysate-treated Burkholderia cells were still resistant to the antimicrobial activity of rip-defensin (Fig. 8B). The difference between the antimicrobial effects of riptocin and rip-defensin may be due to their distinct modes of action (33). Riptocin, a pyrrhocoricin-like, monosaccharide-modified and proline-rich AMP, most likely acts on internal targets such as DnaK (34), whereas rip-defensin targets the cell membrane and disrupts the permeability barrier (35).

When we further examined in vivo bacterial clearance of M4 lysate-treated cultured cells, they did not show faster clearance than untreated cells (Fig. 8C), suggesting that the membrane integrity change induced by the lysate in the cultured cells was minimal compared with that in symbiotic cells. However, this slight membrane alteration was able to enhance bacterial susceptibility to the antimicrobial activity of riptocin, suggesting that the immunosusceptibility of this gut symbiont is attributable to cell membrane alterations.

Discussion

Here we report that Burkholderia cells exhibit increased susceptibility to host AMPs when they become gut symbionts. We further demonstrate that the increased susceptibility of the symbionts is promoted by drastic bacterial cell envelope changes in their LPS O antigen and cell membrane integrity. Interestingly, host M4 lysates induced changes in cell membrane integrity in cultured Burkholderia cells. These lysates increased the susceptibility of the bacteria to detergent and to an AMP (Figs. 7 and 8A). These results suggest that an unidentified host factor(s) in the M4 midgut may actively participate in altering the membrane integrity of the gut symbiont. Additionally, it is conceivable, although speculative, that the loss of LPS
O antigen in the gut symbiont may be attributed to host factors, e.g. a host-encoded glycosyl hydrolase that is capable of cleaving the linkage between the O antigen and core oligosaccharide or a molecule that inhibits the biosynthesis and/or transport of O antigen. These putative host factors could play roles in novel mechanisms to establish successful gut symbiosis, and, therefore, they warrant further study.

Our findings raise the following question: how do these cell envelope changes of gut symbiotic bacteria benefit the symbiotic association between the Riptortus host and the Burkholderia gut symbionts? Although we cannot rule out the possibility that the cell envelope changes can benefit Burkholderia symbionts, such as facilitating nutrient exchange, we will primarily address the symbiotic benefit to host insects on the basis of the following two findings: host factors are involved in the cell envelope changes of Burkholderia cells (Figs. 7 and 8A), and symbiotic Burkholderia cells are highly susceptible to host AMPs (Figs. 2 and 8). In view of symbionts, the cell envelope alterations that increase vulnerability to host immune responses would be a disadvantage for their survival inside the host. However, from the host perspective, the cell envelope changes would allow the host to easily manage its gut symbionts.

Previous studies have shown that the effects of the symbiont on host biology tend to be related to the population of symbionts within the host. The level of symbiont population affects host fitness, the fidelity of vertical transmission, and the intensity of reproductive aberrations (36–39). Therefore, host insects have to control and maintain the symbiont population within an optimal range. Several mechanisms for controlling the population of intracellular symbionts have been reported (40–43), and, recently, two mechanisms to control the population of extracellular gut symbionts have been proposed by our group (25, 44). In Riptortus-Burkholderia symbiosis, the symbiont population appears to be controlled by up-regulated immune responses during the molting periods of the insect (25) and by a specified midgut region (the M4B region) that exhibits potent antimicrobial activity against the Burkholderia symbiont (44). Both controlling mechanisms are effective only if Burkholderia symbionts are susceptible to host immune responses.

It should be noted that members of the genus Burkholderia are inherently resistant to antimicrobial agents (45). The resistance of various Burkholderia species to AMPs has been investigated, and many resistance mechanisms have been proposed (46). The Burkholderia symbiont strain RPE75 used in this study also exhibited a high level of resistance to the bacteria-challenged hemolymph (data not shown) and purified host AMPs (Figs. 2, and 8, A and B). Therefore, unless the cell envelopes of Burkholderia symbionts were altered to increase susceptibility to host immune responses, the host would have difficulty in controlling the Burkholderia gut symbionts, which can reach \( \sim 10^8 \) cells/M4 midgut. Taken together, the data in our study suggest that, through bacterial cell envelope changes, the Riptortus host can effectively manage its Burkholderia symbiont and successfully establish gut symbiosis.

**FIGURE 8. The effect of cell envelope changes on susceptibility against host immune responses.** A and B, comparison of the susceptibility against riptocin (A) and rip-defensin (B) among the cultured wild-type, M4 lysate-treated cultured, \( \Delta wbg \) mutant, M4 lysate-treated \( \Delta wbg \) mutant, and symbiotic cells. Colony-forming units were normalized by AMP-untreated colony-forming units set to 100%. Data are mean ± S.D. (n = 3). These are representative data of three independent repeats. C, comparison of the in vivo clearance. Injected colony-forming units were set to 100%. Data are mean ± S.D. (n = 3). For each post-injection time point, columns with different letters (a, b, and c) indicate statistically significant differences between the experimental groups (p < 0.05), and columns with at least one identical letter indicate no significant differences between the experimental groups (p > 0.05), as determined by two-way analysis of variance with Tukey’s multiple comparison test.
Cell Envelope Changes of Gut Symbiont

Finally, gut-microbe symbiosis is pivotal to human life, and a molecular understanding of gut-microbe cross-talk and homeostasis is important for improving human health. Along with other gut symbiosis studies using mice, nematodes, leeches, and fruit flies, the *Riptortus-Burkholderia* model certainly provides new insights into gut-microbe symbiosis through *in vivo* determination of the molecular characteristics of naïve gut symbionts. Our study shows that insect gut symbionts exhibit drastic cell envelope changes and differential susceptibility to host innate immunity, suggesting a possible mechanism of gut-microbe homeostasis achieved through alterations in gut symbiont characteristics.

**Author Contributions**—J. K. K., B. L. L., A. M., and T. F. conceived and coordinated the study and wrote the paper. D. W. S., C. H. K., Y. R. H., and H. N. designed, performed, and analyzed the experiments shown in Fig. J. K. K. and D. W. S. designed, performed, and analyzed the experiments shown in Figs. 6 and 7. All authors reviewed the results and approved the final version of the manuscript.

**References**