The lipopolysaccharide core oligosaccharide of *Burkholderia* plays a critical role in maintaining a proper gut symbiosis with the bean bug *Riptortus pedestris*

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Lipopolysaccharide, the outer cell-wall component of Gram-negative bacteria, has been shown to be important for symbiotic associations. We recently reported that the lipopolysaccharide O-antigen of *Burkholderia* enhances the initial colonization of the midgut of the bean bug, *Riptortus pedestris*. However, the midgut-colonizing *Burkholderia* symbionts lack the O-antigen but display the core oligosaccharide on the cell surface. In this study, we investigated the role of the core oligosaccharide, which directly interacts with the host midgut, in the *Riptortus–Burkholderia* symbiosis. To this end, we generated the core oligosaccharide mutant strains, ΔwabS, ΔwabO, ΔwaaF, and ΔwaaC, and determined the chemical structures of their oligosaccharides, which exhibited different compositions. The symbiotic properties of these mutant strains were compared with those of the wild-type and O-antigen–deficient ΔwbiG strains. Upon introduction into *Riptortus* via the oral route, the core oligosaccharide mutant strains exhibited different rates of colonization of the insect midgut. The symbiont titers in fifth-instar insects revealed significantly reduced population sizes of the inner core oligosaccharide mutant strains ΔwaaF and ΔwaaC. These two strains also negatively affected host growth rate and fitness. Furthermore, *R. pedestris* individuals colonized with the ΔwaaF and ΔwaaC strains were vulnerable to septic bacterial challenge, similar to insects without a *Burkholderia* symbiont. Taken together, these results suggest that the core oligosaccharide from *Burkholderia* symbionts plays a critical role in maintaining a proper symbiont population and in supporting the beneficial effects of the symbiont on its host in the *Riptortus–Burkholderia* symbiosis.

Most, if not all, animals possess symbiotic bacteria in their bodies, and these endosymbiotic bacteria have many effects on aspects of host biology (1). To understand the symbiotic interactions between host animals and endosymbiotic bacteria, some symbiosis models, such as the aphid *Buchnera*, the tsetse fly *Wigglesworthia*, the squid *Vibrio*, and the nematode *Photorhabdus* models, have been extensively characterized (2). Recently, the bean bug *Burkholderia* symbiosis model has been accepted as a useful model to study molecular mechanisms of symbiotic interactions between host and bacteria (3). The bean bug *Riptortus pedestris*, belonging to the stinkbug family Alydidae in the insect order Hemiptera, has β-proteobacterial symbionts of the genus *Burkholderia* in their posterior midgut region, known as the M4 midgut (4). Because *Riptortus* obtains *Burkholderia* cells from the environment at an early nymphal stage, *Riptortus* and *Burkholderia* have a very short association time compared with the long association time in the aphid *Buchnera* and the tsetse fly *Wigglesworthia* symbioses. As a result, *Burkholderia* symbionts from *Riptortus* are easily culturable, and genetically manipulated *Burkholderia* strains can be symbiotically introduced into *Riptortus* (4–7). Thus, the experimental advantages of this model facilitate molecular and biochemical studies of the *Riptortus–Burkholderia* symbiosis (8, 9).

Among the many features of bacteria, the outer components of the cell wall are a reasonable target to study mechanisms of symbiotic association between hosts and bacteria. Lipopolysaccharide (LPS) is the major outer membrane component of Gram-negative bacteria, such as *Burkholderia*. It is composed of three parts: a lipid A anchored to the membrane, an outermost O-antigen of repeating oligosaccharide units, and a core oligosaccharide (OS) connecting the lipid A and O-antigen (10–12). In several symbiosis models, LPS has been shown to be important for bacterial colonization. An O-antigen–deficient

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2 To whom correspondence should be addressed. Tel.: 82-51-510-2809; Fax: 82-51-513-2801; E-mail: brlee@pusan.ac.kr.
3 The abbreviations used are: OS, oligosaccharide; Tricine, N-[2-hydroxyethyl]glycine; Hep, heptose; Hex, hexose; Kdo, 3-deoxy-o-manno-2-octulosonic acid; 3-Kdo, 3-deoxy-o-manno-oct-2-ulopyranosonic acid; 3-Ko, 3-glycer-o-talo-oct-2-ulopyranosonic acid; L-Ara4N, 4-amino-4-deoxy-L-arabinose; 4-GlcN, 2-amino-4-deoxy-D-glucose; TEM, transmission electron microscopy; ANOVA, analysis of variance; ROESY, rotating frame Overhauser enhancement spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence.
strain of *Vibrio fischeri* has a defect in motility and colonization of the light organ of the Hawaiian bobtail squid (13). A *Photorhabdus luminescens* strain lacking O-antigen fails to colonize the gut of the nematode (14). In the *Riptortus–Burkholderia* symbiosis, the LPS O-antigen is also important for bacterial colonization. An O-antigen–deficient *Burkholderia* strain exhibited a low level of colonization in the M4 midgut of *Riptortus*, which was attributed to their susceptibility to cationic antimicrobial peptides (15). However, once the *Burkholderia* cells are colonized in the M4 midgut, the symbiont titers in the later stages of symbiosis are not affected by the O-antigen deficiency (8). In fact, the *Burkholderia* symbionts lack the O-antigen during these later symbiotic stages in the M4 midgut. Despite the increased susceptibility to host immune responses, the O-antigen–deficient *Burkholderia* symbionts survived well in the M4 midgut through suppression of host antimicrobial peptides in the symbiotic midgut (16).

LPS of *Burkholderia* symbionts in the M4 midgut was biochemically analyzed and was shown to have a full core OS without an O-antigen (16). The lipid A sugar backbone consisted of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and 2-amino-2-deoxy-D-glucose (D-GlcN). The core OS of the *Burkholderia* symbiont consisted of one 3-deoxy-D-manno-oct-2-ulopyranosonic acid (D-Kdo) connected to the lipid A GlcN disaccharide backbone, one D-glycero-D-talo-oct-2-ulopyranosonic acid (D-Ko), three L-glycero-D-manno-heptose (LD-Hep), one L-rhamnose (deoxyhexose), two D-glucose (D-Glc), and one D-galactose (D-Gal), which is consistent with the composition of other LPS core OS sugars previously observed within the *Burkholderia* genus (17–21). In this study, we further investigated the role of the LPS core OS of the *Burkholderia* gut symbiont in the symbiosis with *Riptortus*. To this end, we generated different core OS mutant strains and characterized the molecular structures of their LPS. Because the core OS is exposed on the surface of the *Burkholderia* symbiont during interactions with the host inside the M4 midgut, we investigated the effects of different chemical structures of the LPS core OSs on gut symbiont survival and host biology.

**Results**

**Generation of LPS core OS mutant strains of the Burkholderia symbiont**

To understand the biosynthesis of the *Burkholderia* LPS core OS, we searched for candidate genes encoding enzymes in the genome of the *Burkholderia* symbiont strain RPE64 (22). We identified at least three gene clusters related to LPS core OS biosynthesis in chromosome 1 of the *Burkholderia* symbiont, and we used the LPS biosynthesis gene names of *Burkholderia cenocepacia* (21) to annotate the homologous genes (Fig. 1A). To generate strains with different core OS chain lengths, we made mutants targeting two glycosyltransferase (Δwabs and Δwabo) and heptosyltransferase I and II (Δwac and Δwaf, respectively) genes. We included an epimerase gene mutant (ΔwbiG) that had been previously shown to express the whole-core OS without the O-antigen region (16). The pure LPS isolated from these mutant strains was examined by silver staining following SDS-PAGE and successfully exhibited different sizes of the core OSs (Fig. 1B). The core OS observed in the gel was, in order of length, ΔwbiG > Δwabo > Δwac > Δwac. The full length of LPS was restored in the most truncated core OS mutant (Δwac) by complementation using a plasmid containing the wacC gene (Δwac/Δwac) (Fig. 1B).

**Structural analysis of LPS from core OS biosynthesis mutant strains**

To determine the structure of the core OS in the *Burkholderia* symbiont mutant strains, the LPSs were extracted and purified by enzymatic digestion, ultracentrifugation, and gel-permeation chromatography. Monosaccharide compositional and linkage analysis data obtained by gas-liquid chromatography/mass spectrometry (GLC-MS) on isolated LPSs are reported in Table 1. The analysis clearly showed diverse monosaccharide compositions among the mutant strains. A structural assessment of each LPS was achieved through MALDITOF mass spectrometry (MS) analysis. As demonstrated previously (16), the negative-ion MALDITOF mass spectra of intact LPS from all *Burkholderia* symbiont mutant strains confirmed the absence of the O-antigen. Moreover, at a low mass range, all the spectra showed fragment ions related to the lipid A species (Y-type ions) due to the *in-source* rupture of the glycosidic linkage between the lipid A and OS.
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Table 1

| Assignment                      | Strains |
|---------------------------------|---------|
|                                  | ΔwabS   | ΔwabO | ΔwaaF | ΔwaaC |
| 6-substituted-δ-GlcNp            | X       | X     | X     |       |
| 4,5-disubstituted-δ-KdoP         | X       | X     |       | X     |
| 8-substituted-δ-Kop              | X       | X     | X     |       |
| terminal -1-Ara4N                | X       | X     | X     |       |
| 3,4-disubstituted-L,D-Hep        | X       |       | X     |       |
| 3-substituted-L,D-Hep            | X       |       |       | X     |
| terminal L,D-Hep                 | X       | X     |       |       |
| terminal δ-Glc                   | X       |       |       | X     |
| 7-substituted δ-Hep              | X       |       |       |       |
| 4-substituted δ-Kdo              |         |       |       | X     |

* X indicates that the sugar is present in the analysis.

a – indicates that the sugar is absent in the analysis.

units, whereas deprotonated molecules related to intact LPS species were also present at higher masses. The mass spectrum of the LPS from the ΔwabS mutant strain showed peaks in the mass range of m/z 2400–3200 relative to the whole-LPS molecule (Fig. 2A), whereas in the mass range of m/z 1400–2000, peaks corresponding to a mixture of tetra- and penta-acylated lipid A species were also identified, consistent with previously reported data on lipid A from the Burkholderia genus (10, 19–21). Briefly, the peak at m/z 1444.0 matched with a tetra-acylated lipid A species carrying one 14:0 (3-OH) and one 14:0 and two 16:0 (3-OH) acyl chains. The relative lipid A species decorated by one Ara4N residue was also detected at m/z 1575.0. The peak at m/z 1670.2 corresponded to a penta-acylated lipid A species carrying an additional 14:0 (3-OH) unit, whereas at m/z 1801.2, the above species decorated by one Ara4N was also identified. Peaks corresponding to intact LPS deprotonated molecules, composed of lipid A and OS moieties, allowed for the inference, by difference, of the molecular mass of the core OS, and thus its sugar composition, which corresponded to three heptoses (Hep), one xheose (Hex), one Kdo, one Ko, and one Ara4N (Fig. 2A). Similarly, the MALDI-TOF mass spectra obtained from the LPS sample of the ΔwabO mutant strain showed the same pattern of tetra- and penta-acylated lipid A species, and at higher masses, LPS-deprotonated molecules revealed that the core OS was made up of two Hep, one Kdo, one Ko, and one Ara4N (Fig. 2A). No Hex were detected, in agreement with the chemical analysis (Table 1). The mass spectra of the ΔwaaF and ΔwaaC mutants (Figs. 2, C and D) showed, as expected, very short core OS chains for both symbiotic strains. Briefly, the ΔwaaF revealed a core OS composed of one Kdo, one Ko, one Hep, and one Ara4N, whereas the ΔwaaC expressed LPS with the same sugar composition minus the Hep unit, in full agreement with compositional analysis (Table 1).

To further confirm the core OS structures, an aliquot of each pure LPS sample was subjected to a mild acid hydrolysis to isolate the water-soluble saccharide constituent from the lipid A part. After several purification steps, each core OS fraction was analyzed by 1D and 2D NMR spectroscopy (DQF-COSY, TOCSY, ROESY, NOESY, 1H, 13C HSQC, 1H, 13C HSQC-TOCSY, and 1H, 13C HMBC), which corroborated the MALDI-TOF MS data. For example, in Fig. 2E the 1H NMR spectra of the less truncated core OS chains are shown, specifically those isolated from the ΔwabS and ΔwabO mutant strains. Corroborating the MS data, the NMR spectra clearly showed a lack of both the terminal Hep and the terminal Hex (specifically the β-glucose unit) in the ΔwabO strain.

Thus, the combined data from the MALDI-TOF MS and NMR spectroscopy investigations allowed the LPS core OS structures of the mutant strains to be defined as depicted in Fig. 3.

Examination of colonization efficiency of the core OS mutant strains

To understand the role of the core OS of the Burkholderia symbiont in the Riptortus midgut, we investigated the colonization rates of the core OS mutant strains. Colonization rates were measured by examining the presence of Burkholderia cells in the symbiotic organ, the M4 midgut. To accurately demonstrate the in vivo effect of the core OS on the host biology, inefficiency of colonization of the core OS mutant strains should be corrected to exclude aposymbiotic (without symbiont) insects in the core OS mutant strain test groups. We previously showed that the O-antigen-deficient mutant strains were less efficient at colonizing in the M4 midgut because they were more susceptible to cationic antimicrobial peptides (15). In this study, insects were orally infected with different concentrations (105–109 cfu/ml) of the core OS mutant strains at the second instar stage and were examined in the presence of the Burkholderia cells in the M4 midgut at the third instar stage. Interestingly, the infectivity was not correlated with the length of core OS. Rates of colonization of strains were observed in the following order: ΔwaaF > ΔwbiG and ΔwabS > ΔwabO and ΔwaaC (Table 2). The O-antigen–deficient ΔwbiG and the outer core OS mutant ΔwabS exhibited a similar colonization efficiency, achieving 100% colonization with 108 cfu/ml. Using a 10-fold higher bacterial concentration, the ΔwabO, which has a core OS composed of two heptoses, Kdo and Ko, reached a 100% colonization rate. However, the ΔwaaF, which has only one heptose, in addition to Kdo and Ko, was able to infect insects better than the other mutant strains. To understand the inconsistency between the core OS length and infection rate, we attempted to identify factors affecting the colonization rates (Fig. 4). Based on the colonization rate results (Table 2), we determined the bacterial inoculum concentration for each strain used to ensure the complete symbiotic colonization of Burkholderia cells in the M4 midguts for further experiments.

 Determination of factor(s) affecting the colonization efficiency of the core OS mutant strains

To determine the factors affecting the colonization rates of the core OS mutants, the in vitro properties of these strains were examined. For the assessment of growth in nutrient rich (YG) and minimal media, all the mutant strains exhibited growth rates similar to the wild-type strain except for the complemented ΔwaaC/waaC strain (Fig. 4, A and B). Burkholderia cells possessing complementation plasmids have been shown to grow more slowly than Burkholderia cells without complementation plasmids, probably due to the expense of maintaining the plasmids.
Next, we tested the susceptibility of the core OS mutant strains to the lysate of salivary glands of host insects. When Burkholderia cells are orally delivered to Riptortus, they first encounter antimicrobial peptides in the saliva (23). We speculated that the survival of cells in saliva might be an important factor in determining the efficiency of colonization in the host midgut. The bacterial survival results showed that the mutant strains were more susceptible to the antimicrobial activity of the salivary gland lysates than the wild-type and /H9004 waaC/waaC strains. However, there were no significant differences in susceptibility among the core OS mutant strains (Fig. 4C). Therefore, the susceptibility to the antimicrobial peptides in the saliva did not elicit the differential colonization rates of the core OS mutant strains.

Finally, the motility of the core OS mutant strains was examined. Bacterial motility has been shown to be one of the key factors for establishing the Riptortus–Burkholderia symbiotic association (24, 25). The swimming radius of bacteria strains was measured in soft agar. The resulting motility of the strains was as follows: /H9004 wabG and /H9004 wabS > /H11022/H9004 waaF > /H11022/H9004 wabO (Fig. 4D). The loss of the outer core OS in the /H9004 wabS cells seemed to have no effect on cell motility. However, the loss of heptoses in the inner core OS significantly influenced the motility of Burkholderia cells. The strain with the most truncated core OS (ΔwaaC), which has only Ko and Kdo, exhibited the lowest motility. Surprisingly, the /H9004 waaF, which has one heptose in addition to Ko and Kdo, exhibited dramatically increased motility that was better than the /H9004 wabO possessing two heptoses. Unfortunately, we were unable to ascertain why Burkholderia cells with one heptose in the core OS have better motility than cells with two heptoses. However, the superior motility of the ΔwaaF strain seems to explain why it exhibited the higher colonization rate than the ΔwabO and ΔwaaC, as shown in Table 2. In the other mutant strains,
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ΔwbiG, ΔwabS, ΔwabO, and ΔwaaC, the motility levels also seemed to correlate to the level of colonization. These results indicate that the major factor determining the efficiency of colonization in the core OS mutant strains may be their motility.

In vivo symbiont titers of the core OS mutant strains in the host midgut

We next asked whether the core OS plays a role in maintaining the symbiont titer in the M4 midgut. To address this question, we examined the symbiont titers of 5th instar insects colonized with the core OS strains. The titers of the ΔwbiG and ΔwabS strains were similar to that of the wild-type strain, and the titers of ΔwabO and ΔwbiG were not significantly different. However, the inner core mutant strains, ΔwaaF and ΔwaaC, had significantly reduced populations compared with the wild-type and ΔwbiG strains (Fig. 5A). In particular, the symbiont titer of the ΔwaaC strain (2.4 × 10^6 cfu/insect) was ~20-fold less than that of the wild-type strain (5.1 × 10^7 cfu/insect). When we compared the transmission electron microscopic...
images of the M4 midgut colonized with the wild-type and \( /H9004\) \(waaC\) cells, the dramatic reduction in the number of \( /H9004\) \(waaC\) symbionts was confirmed, and we observed some \( /H9004\) \(waaC\) cells with different, expanded cocci morphology (Fig. 5, B and C). These findings indicate that the first two heptoses of core OS are critical for the survival of the \(Burkholderia\) symbiont in the \(Riptortus\) M4 midgut.

Effects of core OS mutant strains on host growth and fitness

To determine whether the core OS plays a role in controlling host biology, we assessed the growth rate of the \(Riptortus\) host by measuring how many days were required for \(Riptortus\) to reach adulthood. As shown in Fig. 6A, the adult emergence rates of the \(\Delta waaF\) and \(\Delta waaC\)-colonized insects were similar to that of apsymbiotic insects, taking more than a day longer than the wild-type-, \(\Delta wbiG\), \(\Delta wabS\), and \(\Delta wabO\)-colonized insects. With respect to the adult emergence rate of the \(\Delta waaC\)-colonized insects, the range of emerging days was broad in comparison with that of the apsymbiotic insects. Approximately 65% of \(\Delta waaC\)-colonized insects had a faster growth rate than the apsymbiotic insects. However, the remaining 35% of insects exhibited more delayed growth than the apsymbiotic insects (Fig. 6A).

The fitness of the \(Riptortus\) hosts was accessed by measuring their lengths and weights at the young adult stage. Among the core OS mutant strains, \(\Delta waaC\)-colonized insects exhibited significantly decreased fitness, similar to insects without \(Burkholderia\) symbionts (Fig. 6B). The complemented \(\Delta waaC/\Delta waaC\) strain was restored for growth and fitness of the host insects. Our growth and fitness results of \(Riptortus\) hosts suggest that severe truncation of the inner core OS of the \(Burkholderia\) symbiont dramatically reduces host growth rate and fitness.

Effects of core OS mutant strains on host survival in response to a bacterial septic challenge

Because the growth and fitness parameters of the \(\Delta waaC\)-colonized insects were similar to those of apsymbiotic insects, we questioned whether the presence of the \(\Delta waaC\) cells in the M4 midgut has a debilitating effect to the host that was worse than having no symbiont. To address this question, we examined the ability of the host to defend and survive a bacterial septic challenge.
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Figure 5. In vivo symbiotic properties of Burkholderia core OS mutant strains. A, symbiont titers of Burkholderia strains at the fifth-instar stage. Means and S.E. (n = 20) are shown as columns and error bars, respectively. Different letters (a–d) at the top of the columns indicate statistically significant differences (p < 0.05; unpaired t test with Bonferroni correction). B and C, transmission electron microscopic images of the M4 midguts of fifth-instar Riptortus colonized with the wild-type (B) and ΔwaaC (C) strains. Scale bars of main images, 5 μm. Scale bars of zoomed images, 1 μm.

Figure 6. Effects of Burkholderia core OS mutant strains to Riptortus host. A, growth rates of Riptortus colonized with Burkholderia core OS mutant strains indicated by adult emergence days. B and C, fitness of Riptortus. Body weight (B) and length (C) of young adult insects were measured. Numbers of insects used in the experiments are written next to the strain names. Mean and S.E. are indicated as columns and error bars, respectively. Different letters (a–d) at the top of the columns indicate statistically significant differences (p < 0.05; one-way ANOVA with Tukey correction).
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Figure 7. Riptortus survival rate after bacterial septic challenges. Adult insects colonized with Burkholderia strains were injected with E. coli cells and monitored for their survival every 12 h. For each sample group, 13–16 insects were examined.

Discussion

In this study, we investigated multiple aspects of the core OS of the Burkholderia symbiont. First, we identified the genes involved in the biosynthesis of core OS and defined the chemical structures of core OS produced by strains mutated for those genes. Second, we demonstrated the roles of the core OS in the Riptortus–Burkholderia symbiosis. The inner core OS, composed of Kdo, Ko, and two heptoses, is especially important in maintaining a symbiont titer in the M4 midgut and supporting host growth, fitness, and defense against bacterial challenge.

The detailed chemical and structural analyses of the lipid A–core OS of the LPS expressed by the mutant strains suggested that the waaC gene encodes a heptosyltransferase I involved in the glycosylation of the Kdo unit at its O-5 position. Indeed, the structure of the core OS for this mutant is identical to the one previously determined for the B. cenocepacia K56-2 mutant strain that lacks heptose (21, 27). The presence of only the first heptose unit linked to the Kdo confirmed the functional assignment of WaaF as a heptosyltransferase II enzyme. The lack of a β-glucose unit confirmed that the wabO gene encodes a glucosyltransferase responsible for the linkage of this monosaccharide to the first heptose unit of the Burkholderia core OS. Similarly, mutation of the wabS gene resulted in the absence of the α-glucose unit linked to the second heptose unit, thus confirming that this gene encodes for a second glucosyltransferase enzyme.

In our previous study, we observed that Burkholderia symbionts exhibit only core OSs on their surface when they are colonized in the host midgut (16). Therefore, we questioned the importance of the core OS for the Burkholderia symbiont inside of the host M4 midgut. To address this question, we first ensured that the core OS mutants colonized every experimental insect by testing different concentrations of bacterial inocula to identify the optimal concentration to achieve 100% colonization rates. Interestingly, we found that the concentration required for complete colonization of the ΔwaaF (10⁷ cfu/ml) was similar to that of the wild-type strain (Table 2). However, using a 10⁶ cfu/ml inoculum, the ΔwaaF colonization rate was lower than the other three strains assayed, ΔwibiG, ΔwabS, and ΔwaaC/waaC, for which complete colonization was achieved at 10⁵ cfu/ml. Therefore, we used a 10⁷ cfu/ml inoculum rather than 10⁶ cfu/ml to ensure the complete colonization of the ΔwaaF strain. Using an experimental inoculum of 10⁷ cfu/ml, the ΔwaaF exhibited a higher colonization rate than the ΔwaaO (100% colonization with 10⁵ cfu/ml inoculum), which has a core OS containing one more heptose than that of the ΔwaaF (Table 2). We further investigated the reason for why the ΔwaaF exhibited a better colonization rate than that of the ΔwabO, and we found that the ΔwaaF has better motility than the ΔwaaO (Fig. 4D). An interesting question worth exploring further is why Burkholderia cells with a core OS containing one heptose are more motile than those with two heptoses.

Burkholderia symbionts with a truncated core OS of one Kdo and one Ko without heptose (ΔwaaC) or with one heptose (ΔwaaF) exhibited impaired adaptation in the M4 midgut. The titers of the ΔwaaC and ΔwaaF were dramatically reduced. The TEM images of the ΔwaaC in the M4 midgut revealed that the integrity of the ΔwaaC cells was greatly challenged (Fig. 5). These findings suggest that the core OS, especially the first two heptoses of the core OS, may provide a protective shield to Burkholderia symbionts and promote survival in the midgut. Because symbiotic bacteria and pathogenic bacteria share some bacterial adaptive mechanisms to survive in their hosts, the function of heptosyltransferase can be vital for the pathogen of chronic infection as well. In that respect, inhibitors such as sugar analogues targeting heptosyltransferase (28, 29) or heptose synthesis (30) may be a new way to design antibacterial drugs. After observing the distorted morphology of the ΔwaaC cells and cell debris in the TEM images (Fig. 5C), we questioned whether the Riptortus M4 midgut secretes antimicrobial peptides into the lumen by recognizing ΔwaaC cells as pathogens. However, when we assessed the expression of antimicrobial peptide-encoding genes of Riptortus, including riptocin, rip-defensin, and rip-thanatin, as well as lysozymes, there was no difference between the wild-type and ΔwaaC-colonized M4 midguts (data not shown). The impaired adaptation of the ΔwaaC and ΔwaaF cells in the M4 midgut may be due to the bacterial physiological changes caused by the inner core OS truncation.

The colonization of Burkholderia cells with truncated inner core OSs (ΔwaaC and ΔwaaF) negatively affects host growth.
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Table 3
Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Characteristics | Refs. |
|----------------------------|-----------------|-------|
| **Burkholderia symbiont**  |                 |       |
| RPE75                      | Burkholderia symbiont (RPE64); Rif<sup>e</sup> | 44    |
| BBL014                     | RPE75 ΔwaaC; Rif<sup>e</sup> | 16    |
| BBL015                     | RPE75 ΔwabS; Rif<sup>e</sup> | This study |
| BBL016                     | RPE75 ΔwabO; Rif<sup>e</sup> | This study |
| BBL017                     | RPE75 ΔwaaF; Rif<sup>e</sup> | This study |
| BBL018                     | RPE75 ΔwaaC; Rif<sup>e</sup> | This study |
| BBL018                     | BBL018/pBR122 containing waaC gene; Rif<sup>e</sup>, Km<sup>R</sup> | This study |
| **E. coli**                |                 |       |
| DH5α                       | F<sup>−</sup>ΔlacZAM15Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (K<sup>R</sup>, mK<sup>R</sup>) phoA supE44 λ<sup>−</sup> thi-1 gyrA96 relA1 | Invitrogen |
| PIR1                       | F<sup>−</sup>Δlac169 proph (am) robA1 creC510 hsdR514 endA recA1 u169Δ (ΔM159) lacY1 pir-116 | Invitrogen |
| HBL1                       | PIR1 carrying pEV104; Cm<sup>R</sup>, Km<sup>R</sup> | 31    |
| **Plasmid**                |                 |       |
| pEV104                     | oriR6K helper plasmid containing conjugal tra and trb; Km<sup>R</sup> | 45    |
| pK18mobsacB                | pMB1ori allelic exchange vector containing oriT; Km<sup>R</sup> | 46    |

and fitness (Fig. 6). The development and fitness of *Riptortus* have been shown to be affected by the symbiont titer (4, 31, 32). In fact, the ΔwaaC and ΔwaaF strains exhibited reduced symbiont titers of 2.4 × 10<sup>6</sup> and 5.5 × 10<sup>6</sup> cfu/insect, respectively, compared with the wild-type titer of 5.1 × 10<sup>7</sup> cfu/insect. In addition, ΔwaaC-colonized insects had somewhat lower growth rate and fitness values than ΔwaaF-colonized insects. However, it was puzzling to us that, despite possessing 2.4 × 10<sup>6</sup> *Burkholderia* symbionts, ΔwaaC-colonized insects were similar to aposymbiotic insects in terms of growth rates and fitness. It could be that 2.4 × 10<sup>6</sup> symbionts is too small a number to adversely affect the host, weakening them similar to aposymbiotic insects. In our bacterial septic challenge tests, the survival rates of ΔwaaC-colonized insects were not significantly different from that of aposymbiotic insects. However, ΔwaaC-colonized insects died faster than aposymbiotic insects (median survival time of 36 and 72 h, respectively) (Fig. 7). This suggests that ΔwaaC-colonized insects may be more susceptible to bacterial challenge, indicating that ΔwaaC-colonized insects may be in worse physical condition than aposymbiotic insects. If that is true, it would be the first report of a *Burkholderia* mutant strain that has a debilitating effect to the host *Riptortus*.

Our results show that *Burkholderia* symbionts with truncated core OSs lose the ability to adapt in the symbiotic midgut, negatively affecting the biology of the *Riptortus* host. In other words, the full-length core OS promotes the beneficial and harmonious association between *Riptortus* and *Burkholderia*. At this moment, there is a lack of cellular studies of the M4 midgut and molecular studies of the interaction between M4 midgut cells and *Burkholderia* symbionts. Elucidating host proteins recognizing core OS of *Burkholderia* symbionts may give us important clues to answer why core OS is critical in the *Riptortus–Burkholderia* gut symbiosis and help us to fully understand the mechanisms of gut homeostasis.

**Experimental procedures**

**Bacteria and media**

The bacterial strains used in this study are shown in Table 3. *E. coli* K12 cells were cultured at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). The *Burkholderia* symbiont RPE75 strain, spontaneous rifampicin-resistant mutant strain from RPE64 strain, was cultured at 30 °C in YG medium (0.4% glucose, 0.5% yeast extract, and 0.1% NaCl) containing 30 µg/ml of rifampicin.

**Generation of deletion mutant strains**

Chromosomal deletion mutant strains of the core biosynthesis genes were generated as described previously (31). Briefly, the suicide vector pK18mobsacB harboring 5’- and 3’-regions of the gene of interest was used for the allelic exchange of the gene of interest. After transforming *E. coli* DH5α cells with the pK18mobsacB vectors that contained the 5’- and 3’-regions of the gene of interest, the triparental mating was performed using the transformed *E. coli* DH5α, *Burkholderia* RPE75, and HBL1 helper cells to transfer the cloned vector into *Burkholderia* RPE75 (Table 3). *Burkholderia* cells with the first crossover were selected on YG-agar plates containing rifampicin and kanamycin (50 µg/ml). The selected cells were allowed to undergo the second crossover by cultivating in YG medium overnight. The deletion mutant cells were finally isolated on YG agar plates containing rifampicin and sucrose (200 mg/ml).

**Extraction of lipopolysaccharide (LPS) from Burkholderia cells for Tricine/SDS-PAGE analysis**

LPS was extracted from bacterial cells using a modified hot-phenol method (33). The *Burkholderia* cells were washed and resuspended in 4 ml of 10 mM phosphate buffer, pH 7.0 (PB). The same volume of hot phenol was added to the *Burkholderia* cell suspension and incubated in a water bath adjusted to 65 °C with rigorous vortexing performed every 5 min. After 1 h of incubation, the solutions were cooled, and 1.6 ml of chloroform was added and vortexed. Next, the water and phenol phases were separated by centrifugation at 8500 × g for 15 min. The water phase was transferred to a new tube. To precipitate LPS, 10 ml of isopropyl alcohol was added to the water phase and stored at −20 °C overnight. The precipitates were washed with 80% ethanol and air-dried.

**Silver staining of LPS**

The extracted LPS samples were suspended in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.005% bromphenol blue) and boiled at 95 °C for 5 min. Next, LPS samples were separated by Tricine/SDS-PAGE in 15% gels (34).
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The LPS that separated in the gels was stained by the conventional silver staining method of Tsai and Frasch (35). Briefly, LPS was fixed in the gel with 40% ethanol containing 5% acetic acid and was oxidized with 0.07% sodium metaperiodate. Then, the gel was stained with freshly prepared staining solution for 20 min. The staining solution was prepared by the addition of 2 ml of ammonium hydroxide added to 28 ml of 0.1 m sodium hydroxide. After the addition of 100 ml of the staining solution, 2 ml of 20% silver nitrate was added dropwise with stirring. The color of the LPS bands was developed by reduction in 150 ml of developing solution (8 mg of sodium citrate, 80 ml of 37% formaldehyde, and distilled water to 150 ml). The gel was either photographed immediately or the color reaction was stopped by the addition of 10% acetic acid followed by repeated washings in distilled water.

Large-scale LPS extraction, compositional analysis, and isolation of the core OS fractions

Dried cells were extracted by the hot phenol/water method (33). After dialysis against distilled water, the extracted material was subjected to enzymatic treatment to remove cell contaminants. Additional purification steps, including a ultracentrifugation (100,000 × g, 4 °C, 16 h) and gel-filtration chromatography, were performed. Monosaccharide composition and linkage analyses by GLC-MS analysis were carried out as described elsewhere (36, 37).

Isolation of the core OS fractions was achieved by mild acid hydrolysis in acetate buffer, pH 4.4. After lipid A removal by centrifugation (10,000 × g, 30 min), the water-soluble products were purified by gel-filtration chromatography on a Bio-Gel P-6 column (Bio-Rad).

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra of intact LPS were recorded in linear and reflectron mode and negative ion polarity on a Voyager STR (Applied Biosystems) instrument and on a 4800 Proteomic analyzer (Sciex). LPS MALDI preparations were performed as reported previously (38, 39).

NMR spectroscopy

1D and 2D 1H NMR spectra were recorded in D2O (300 K, pD 7) with a 600 DRX spectrometer (Bruker) equipped with a cryo-probe. The spectrometer was internally calibrated with acetone (δH = 2.225 ppm; δC = 31.45 ppm). ROESY and NOESY experiments were performed using data sets (1 × 12) of 4096 × 256 points with mixing times between 100 and 300 ms. Double quantum-filtered phase-sensitive COSY experiments were executed with sets of 4096 × 512 points. TOCSY experiments were performed with spin-lock times of 100 ms with data sets (1 × 12) of 4096 × 256 points. The data matrix in all the homonuclear experiments was zero-filled in both dimensions to give a matrix of 4 K × 2 K points, and it was resolution-enhanced in both dimensions using a cosine-bell function before Fourier transformation. Coupling constants were determined by 2D phase-sensitive DQF-COSY (40, 41). HSQC and HMBC experiments were performed in 1H-detection mode by single-quantum coherence with proton decoupling in the 13C domain using data sets of 2048 × 256 points. Experiments were carried out in the phase-sensitive mode (42). A 60-ms delay was used for the evolution of long-range correlations in the HMBC experiment. The data matrix in all the heteronuclear experiments was extended to 2048 × 1024 points by using forward linear prediction extrapolation.

Insect rearing and symbiont inoculation

R. pedestris was reared at 26 °C under a day cycle of 16 h light and 8 h dark as described previously (43). Insects were raised in clear plastic containers with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). When the nymphs were at early second instar, water was removed from the cages for 6 h, and nymphs were provided with a Burkholderia inoculum solution for 1 day. The Burkholderia inoculum solution was composed of mid-log phase Burkholderia cells in DWA at a concentration of 106−109 cfu/ml.

Measurement of bacterial growth in liquid media

Growth curves of the Burkholderia symbiont strains were examined in either YG or minimal medium (0.2% glucose, 0.6% Na2HPO4, 0.3% KH2PO4, 0.1% NH4Cl, 0.05% NaCl, 0.1 mM CaCl2, and 1 mM MgSO4). The starting cell suspensions (OD600 0.05) were prepared with a sterile air phase cells in either YG or minimal medium. The cells were cultured on a rotator shaker at 180 rpm speed and 30 °C for 36 h. The OD600 values were recorded every 3 h using a spectrophotometer (Mecasy, Korea).

Measurement of bacterial motility

Three microliters of Burkholderia cultures (OD600 of 0.3) were used to inoculate soft agar plates (YG medium containing rifampicin with 0.3% agar). After incubation for 26 h at 30 °C, the swimming radius for each strain was measured.

Susceptibility assay against salivary gland lysate

The salivary glands of Riptortus insects were dissected from fifth-instar aposymbiotic nymphs and homogenized in 200 μl of PB with a pestle. The salivary gland homogenates were centrifuged at 20,000 × g for 15 min, and supernatants were collected. After measuring the protein concentration of the supernatant via the Bradford assay, 50 μl of salivary gland lysate (50 μg/ml) was incubated with 50 μl of a Burkholderia cell suspension (5 × 104 cfu/ml) for 2 h at 30 °C. Next, 50 μl of the incubated solution was spread onto YG agar plates containing rifampicin, cultured for 2 days, and subjected to colony counting.

Measurement of symbiont titers

Each Riptortus M4 midgut was dissected and collected in 100 μl of PB. The solution containing the midgut was homogenized with a plastic pestle and serially diluted with PB. The diluted solution was spread onto YG agar plates containing rifampicin. After 2 days of incubation at 30 °C, colonies on the plates were counted. The colony-forming units (CFUs) per insect were calculated by colony counts × dilution factor.

Measurement of Riptortus growth rate, weight, and height

To access the growth rate of insects, adult emergence was monitored by daily inspections of late fifth-instar nymphs and...
by counting the number of newly molted adult insects. Young adult insects were examined for their body lengths and dry body weights. For weight measurements, the insects were immersed in acetone for 5 min and then completely dried in a 70 °C oven.

Transmission electron microscopy analysis of M4 midguts

The M4 midguts of wild-type or ΔwaaC-colonized insects were dissected from fifth-instar nymphs, pre-fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate trihydrate, pH 7.4 (SCB), at 4 °C for 18 h, washed three times with SCB, and post-fixed with 1% osmium tetroxide in SCB for 1 h at room temperature. After three washes with SCB, the samples were dehydrated with an ethanol/propylene oxide series and embedded in Epon 812 resin. The embedded samples were trimmed and sectioned on an ultramicrotome (Reichert Super-Nova, Leica). Finally, the sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (HITACHI H-7600).

Insect survival rate against bacterial septic challenge

*E. coli* K12 cells were washed and suspended in PB at 5 × 10⁸ cfu/ml. Two microliters of the bacterial cell suspensions were injected to young male *Riptortus* 3 days after having molted into adults. The survival rate was monitored every 12 h after bacterial septic injections.

Statistical analyses

We used Prism GraphPad software for statistical analysis of data. An unpaired t test with Bonferroni correction or one-way analysis of variance (ANOVA) with Tukey correction was used for group comparisons. p < 0.05 was considered significant.

Author contributions—J. K. K. and B. L. L. conceived and coordinated the study. J. K. K. and H. A. J. wrote the paper. H. A. J. and J. B. L. designed, performed, and analyzed the experiments shown in Fig. 7. J. K. K. and M. S. K. designed, performed, and analyzed the experiments shown in Figs. 1, 4, and 6. A. M., A. S., F. D. L., and L. S. designed, performed, and analyzed the experiments shown in Figs. 2 and 3. J. H. C. designed, performed, and analyzed the experiments shown in Fig. 5. All authors reviewed the results and approved the final version of the manuscript.

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