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The symbiotic role of O-antigen of *Burkholderia* symbiont in association with host *Riptortus pedestris*

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ABSTRACT

Riptortus pedestris harboring *Burkholderia* symbiont is a useful symbiosis model to study the molecular interactions between insects and bacteria. We recently reported that the lipopolysaccharide O-antigen is absent in the *Burkholderia* symbionts isolated from *Riptortus* guts. Here, we investigated the symbiotic role of O-antigen comprehensively in the *Riptortus-Burkholderia* model. Firstly, *Burkholderia* mutant strains deficient of O-antigen biosynthesis genes were generated and confirmed for their different patterns of the lipopolysaccharide by electrophoretic analysis. The O-antigen-deficient mutant strains initially exhibited a reduction of infectivity, having significantly lower level of symbiont population at the second-instar stage. However, both the wild-type and O-antigen mutant symbionts exhibited a similar level of symbiont population from the third-instar stage, indicating that the O-antigen deficiency did not affect the bacterial persistence in the host midgut. Taken together, we showed that the lipopolysaccharide O-antigen of gut symbiont plays an exclusive role in the initial symbiotic association.

1. Introduction

Bacterial lipopolysaccharide (LPS) is the major outer membrane component of Gram-negative bacteria. It consists of three different regions: lipid A, core-oligosaccharide and O-antigen (Caroff and Karibian, 2003; Raetz and Whitfield, 2002). The innermost lipid A is a hydrophobic region anchored into the membrane and generally composed of a di-glucosamine backbone linked with four to seven fatty acids. A 2-keto-3-deoxyoctonate (Kdo) unit connects the lipid A to a core-oligosaccharide. The core-oligosaccharide is linked to the outermost region of LPS called O-antigen. The O-antigen consists of repeating oligosaccharide units. Bacteria with LPS lacking O-antigen is called rough-type bacteria, and bacteria harboring LPS O-antigen is called smooth-type bacteria.

LPS O-antigen provides a protective barrier against environmental and immunological factors for bacteria (Raetz and Whitfield, 2002). In pathogenesis, O-antigen is an important virulence factor that facilitates the interaction with host tissues and provides protection from membrane-active compounds of hosts (Trent et al., 2006). Although the roles of O-antigen may vary in different bacteria, many studies on pathogenic bacteria report that rough-type bacteria are susceptible to serum complement and antimicrobial peptides, resulting in much less efficiency to invade and survive in host (Burns and Hull, 1998; Gunn and Ernst, 2007; Murray et al., 2006; Nesper et al., 2001; VanDenBosch et al., 1997). Similarly, LPS O-antigen was reported to be essential for symbiotic association in some symbiotic model systems. In legume-*Rhizobium* symbiosis, O-antigen deficient mutant *Rhizobium* impairs root colonization probably due to susceptibility to antimicrobials (Ormeno-Orrillo et al., 2008). In squid-*Vibrio* symbiosis, O-antigen deficient mutant, *waaL*, shows a motility defect and significantly delayed colonization in light organ of squid (Post et al., 2012). Also *pbgE* mutant of *Photorhabdus luminescens* exhibiting rough-type LPS is unable to colonize the gut of the nematode (Bennett and Clarke, 2005). In case of leech-*Aeromonas* symbiosis, a high-molecular weight LPS is essential for bacterial colonization by providing the complement resistance to the symbionts onto digestive tract of leech (Braschler et al., 2003). In contrast to the general notion of the importance of LPS O-antigen in pathogenesis and symbiosis, we recently found that *Burkholderia* gut symbionts exist as the rough-type bacteria in an insect-bacteria symbiosis model (Kim et al., 2015).

Riptortus pedestris (bean bug) harbors a single kind of gut symbiont, genus *Burkholderia*, in a specialized region of the

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posterior midgut (Kikuchi et al., 2005). This *Burkholderia* symbiont is not transmitted from mother to offspring, but it is orally acquired by *Riptortus* nymphs from environment. Possessing its free living ability, the symbionts isolated from the host midgut can be cultured in standard bacterial media and subjected to genetic modification (Kikuchi et al., 2007, 2011a, b). Recently we used the genetically modified *Burkholderia* symbiont strains to understand molecular cross-talks between insect and bacteria (Kim et al., 2014a, 2014b, 2013a, 2013b). Furthermore, we attempted to understand molecular changes occurring in the *Burkholderia* cells as they become gut symbionts in the *Riptortus* host. The direct comparison between the symbiotic *Burkholderia* cells and the cultured *Burkholderia* cells revealed striking differences in the cell envelope structures. The symbiotic cells isolated from *Riptortus* midgut exhibited the rough-type LPS (Kim et al., 2015).

Because the O-antigen is important for the pathogenic bacteria to escape from the host immunological factors (Burns and Hull, 1998; Gunn and Ernst, 2007; Murray et al., 2006; Nesper et al., 2001; Trent et al., 2006; VanDenBosch et al., 1997) and for the several symbiotic bacteria to successfully colonize the host (Bennett and Clarke, 2005; Ormeno-Orrillo et al., 2008; Post et al., 2012), it was quite unexpected to find the loss of the O-antigen in the *Burkholderia* symbiont. Therefore, in this study, we investigated the role of the *Burkholderia* O-antigen in the symbiotic association with the host *Riptortus* host. By using the O-antigen mutant *Burkholderia* strains, we addressed the importance of the O-antigen in the initial stage as well as in the later stage of the symbiosis.

2. Materials and methods

2.1. Bacteria and media

List of bacteria used in this study is shown in Table 1. *Escherichia coli* cells were cultured at 37 °C with LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). *Burkholderia* symbiont RPE75 cells were cultured at 30 °C with YG medium (0.4% glucose, 0.5% yeast extract, 0.1% NaCl) containing 30 μg/ml rifampicin (Kikuchi et al., 2011b).

2.2. Isolation of symbiotic Burkholderia from midgut

The symbiotic midguts, M4s, were dissected from fifth instar nymphs and placed in 50 μl of 10 mM phosphate buffer (PB, pH7.0). The M4 midguts were cut into pieces with fine scissors to break the crypts. One ml of PB was added to the M4 midgut pieces and gently pipetted to resuspend *Burkholderia* symbionts into the solution. The solution was then filtered through 5 μm pore to remove the gut tissues. *Burkholderia* cells were further washed with PB to remove host molecules.

2.3. Generation of deletion mutant strains

Chromosomal deletion mutant stains of the O-antigen biosynthesis genes were generated as described (Kim et al., 2013b). Briefly, allelic exchanges of target genes were accomplished by utilizing the suicide vector pK18mobsacB harboring 5′ region and 3′ regions of the genes. Primers used to amplify 5′ region and 3′ regions of the gene from the *Burkholderia* symbiont RPE75 are indicated in Table S1. After transforming *E. coli* DH5α cells with pK18mobsacB vector containing 5′ and 3′ regions of the target genes, they were mixed with *Burkholderia* RPE75 cells along with helper cells HBL1 for the conjugal transfer of the cloned vector to the *Burkholderia* RPE75 (Table 1). *Burkholderia* cells with the first crossover were selected on the YG-agar plates containing rifampicin and kanamycin and cultured in YG medium for allowing the second crossover. The in-frame deletion mutant cells by double crossover were isolated on the YG agar plates containing rifampicin and 20% (w/v) sucrose.

2.4. Electrophoretic analysis of LPS

LPS were extracted from full grown bacterial cells using modified hot-phenol method (Westphal and Jann, 1965). In brief, full grown *Burkholderia* 10^9 cells were washed with PB and resuspended in 500 μl of PB. The same volume of hot phenol was added to the cell solution and incubated in water bath adjusted to 65 °C. The cell solution was vortexed rigorously every 5 min. After 1 h of incubation, the solutions were cooled and 200 μl of chloroform was added. After vortexing, the solution was incubated at room temperature for 5 min and then centrifuged at 15,300× g for 15 min to separate the water and phenol phases. Four hundred μl of the water phase solution were transferred to a new tube and 800 μl of ethanol were added to the solution. After allowing LPS precipitation at −20 °C overnight, the precipitates were collected by centrifugation at 20, 400× 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, 0.005% bromophenol blue), boiled at 95 °C for 5 min, de-proteinated by incubating with 400 μg/ml proteinase K at 60 °C for 1 h, and boiled again prior to the electrophoretic analysis. The LPS extracted from 10^9 cells were loaded to a well of Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Schägger, 2006) with 12% gels. LPS separated in the gels were visualized using the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen) by following manufacturer's instruction.

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Characteristics	References
Burkholderia symbiont		
RPE75	*Burkholderia* symbiont (RPE64); Rif^R	(Kikuchi et al., 2011b)
BBL011	RPE75 Δ*wbxA*; Rif^R	This study
BBL012	RPE75 Δ*wbxB*; Rif^R	This study
BBL013	RPE75 Δ*wbiF*; Rif^R	This study
BBL014	RPE75 Δ*wbiG*; Rif^R	(Kim et al., 2015)
Escherichia coli		
DH5α	F–Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ–*thi*-1 *gyr*A96 *rel*A1	Invitrogen
PIR1	F–Δ*lac*169 *rpoS*(am) *rob*A1 *cre*C510 *hsd*R514 *end*A *rec*A1 *uidA*(Δ*Mlu I*)::*pir-116*	Invitrogen
HBL1	PIR1 carrying pEVS104; Cm^R, Km^R	(Kim et al., 2013b)
Plasmid		
pEVS104	oriR6K helper plasmid containing conjugal tra and trb; Km^R	(Stabb and Ruby, 2002)
pK18mobsacB	pMB1ori allelic exchange vector containing oriT; Km^R	(Schäfer et al., 1994)