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An Unusual Galactofuranose Lipopolysaccharide That Ensures the Intracellular Survival of Toxin-Producing Bacteria in Their Fungal Host**

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Bacteria are involved in a plethora of interactions with higher organisms; these interactions can be beneficial or detrimental to the host.[1] Irrespective of the type of symbiosis, in all cases the fine-tuned communication between the organisms is mediated by biomolecules. Diffusible chemical signals allow for long-range communication,[2] whereas carbohydrate structures (antigens) coating the cell surfaces enable cell–cell recognition.[3] Such antigenic components are highly specific and particularly important when bacteria have direct physical contact with the host, or even invade eukaryotic cells.[4,5] Among the most important and ubiquitously occurring surface determinants of Gram-negative bacteria are lipopolysaccharides (LPSs).[3,5,6] LPSs share a common architecture featuring a hydrophilic heteropolysaccharide moiety that is typically composed of a core oligosaccharide and an O-specific polysaccharide. This complex carbohydrate is covalently linked to the third component, a lipophilic moiety termed lipid A, which is embedded in the outer leaflet of the membrane.[3] The ability of a host organism to recognize LPSs and the consequences of this recognition in infection and symbiosis have been the subject of many ground-breaking studies and constitute a major research area.[7] However, these studies have focused on LPSs in the context of bacteria–animal or bacteria–plant interactions.[3–5] There is an evident gap of knowledge on the role of LPSs in microbe–microbe interactions, such as bacterial–fungal encounters, which are crucial in the environment.[8]

We have recently unveiled a unique symbiosis of the rice blight fungus *Rhizopus microsporus*, and intracellular bacteria (*Burkholderia rhizoxinica*)[9], which serve their fungal host as toxin factories.[10–12] The bacterial endosymbionts produce numerous antimitotic macrolides of the rhizoxin complex that efficiently stall cell division in rice plants and most other eukaryotes.[13] Notably, the fungal host itself has become resistant to the toxin as a result of a mutation in the tubulin sequence.[14] Thus, it is likely that *Rhizopus–Burkholderia* symbiosis underwent a parasitism–mutualism shift during evolution.[15] Still, it is a mystery how the endobacteria can survive within fungal host cells and how they interact with their host by means of chemical recognition and communication.[12] Herein we disclose the complete structure of an unusual LPS from *B. rhizoxinica*, elaborate its molecular basis, and provide the first evidence that the O antigen is a critical molecular determinant for the stability of the symbiosis.

To elucidate the structure of the LPS of the endofungal bacteria and its role in the interaction, we used a combination of GLC–MS, MALDI MS, and a series of 2D NMR experiments. Following hydrolysis of the LPS in an acetate buffer, the fraction containing lipid A was recovered as a sediment by centrifugation. The pure O polysaccharide (OPS), present in the supernatant, was obtained by gel permeation chromatography. Lipid A fatty acid analysis revealed the presence of (*R*)-3-hydroxyhexadecanoic acid (16:0 (3-OH)) with an amide linkage, and (*R*)-3-hydroxytetradecanoic acid (14:0 (3-OH)) and tetradecanoic acid (14:0) with ester linkages. According to chemical analysis, MALDI MS, and NMR spectroscopic data, the *B. rhizoxinica* LPS lipid A substructure has a typical *Burkholderia* lipid A architecture with a β-1→6 D-GlcN disaccharide and the nonstoichiometric presence of two L-Ara4N units (Scheme 1).[16]

The OPS was also characterized by a combination of chemical analysis and 2D NMR spectroscopy, in particular COSY, TOCSY, NOESY, HSQC (see Figures S1–S3 in the Supporting Information), and F2-coupled HSQC. The ^{1}H NMR spectrum showed a single anomeric signal indicative of a single-spin system, which was fully assigned by homonuclear 2D NMR spectroscopy, whereas from HSQC spectra it was possible to assess the furanosidic nature and anomeric configuration of the monosaccharide. These data, in conjugation with chemical analysis, revealed that the OPS is a

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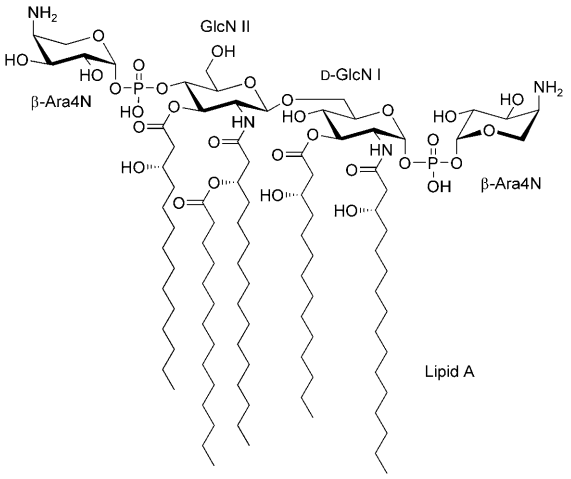


Scheme 1. Structure of lipid A of endosymbiotic *B. rhizoxinic*a. Ara4N = 4-amino-4-deoxy-L-arabinose, GlcN = 2-amino-2-deoxy-D-glucose.

homopolymer of 2-substituted D-galactofuranose, [→2)-β-D-Gal*f*-(1→]$_n$. This unusual type of polysaccharide is fully unprecedented for *Burkholderia* spp. and related bacteria. Furthermore, the existence of [→2)-β-D-Gal*f*-(1→]$_n$ in nature has only been implicated once, on the basis of a limited data set.[17] The discovery of an antigenic poly-D-galactofuranose chain in an endofungal bacterium is particularly intriguing, since Gal*f* conjugates have been found to be especially abundant in filamentous fungi.[18,19] Furthermore, structurally related galactofuranan bioactive antigens have been identified in fungi.[20,21] Thus, it is well conceivable that the O antigen mimics structural components of the host cell.

To test this hypothesis and gain insight into the molecular basis of LPS formation, we analyzed shotgun-sequence data of the *B. rhizoxinica* genome and searched for candidate genes for LPS biosynthesis. Automated annotation revealed a gene cluster comprising 29 open reading frames, 22 of which code for proteins similar to known enzymes involved in LPS biosynthesis (Figure 1a; see also the Supporting Information). The majority of the genes in this locus code for the biosynthesis and transfer of outer-core and O-antigen building blocks, including the pathway for dTDP-L-rhamnose (*rml*A-F; dTDP = deoxythymidine diphosphate). More importantly, the finding of genes that code for a UDP-galactopyranose mutase (Glf; UDP = uridine diphosphate)[22] and a UDP-glucose-4-epimerase (GalE) is fully in line with the elucidated LPS O-antigen structure, since these enzymes are well known to be involved in the formation of UDP-D-galactofuranose (Figure 1b).[23] Furthermore, the gene cluster contains several glycosyl-transferase genes, and two genes (*wzm* and *wzt*) for an ABC transporter system that shuttles membrane-anchored O-antigen chains from the cytosol to the periplasm prior to ligation to the core oligosacharide.[24] Finally, we found a gene coding for an O-antigen ligase (WaaL). WaaL is essential for the transfer of the O antigen to the outer core to finish the LPS-assembly process.[24,25] Consequently, mutants devoid of the O-antigen ligase gene

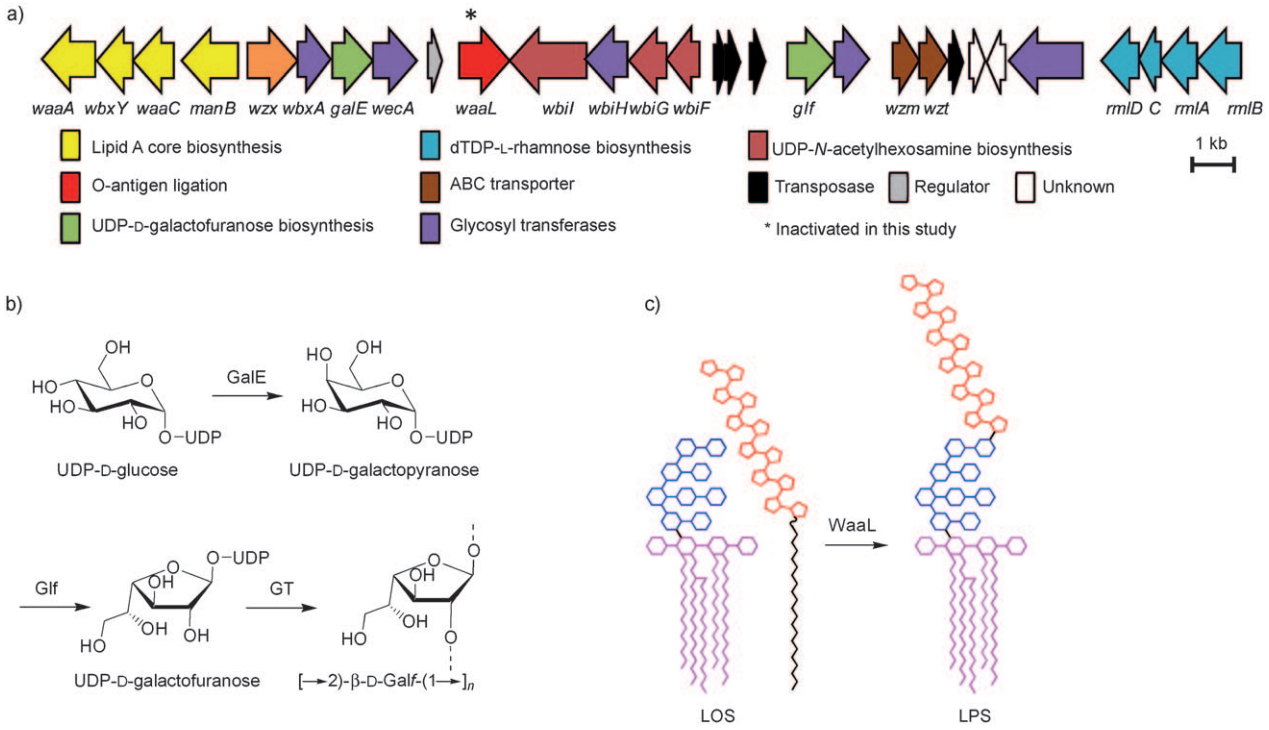
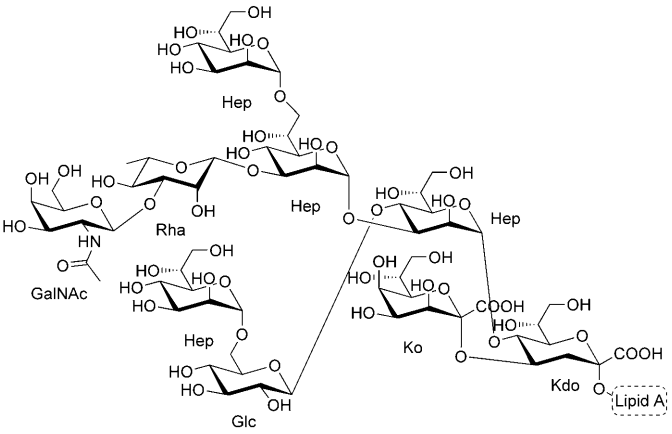


Figure 1. a) Schematic illustration of the LPS biosynthesis gene cluster of *B. rhizoxinic*a. Protein-coding genes are indicated as arrows. Colors represent deduced functional categories of gene products. See Table S3 in the Supporting Information for details. b) Model for the biosynthesis of UDP-D-galactofuranose, the O-antigen building block of *B. rhizoxinica* (GT = glycosyl transferase). c) Attachment of the O antigen to the LOS by the ligase WaaL.

(Δ*waaL* mutants) would lack the O-antigenic chain on the cell surface (rough phenotype). To study the role of the O antigen in vivo, we created an O-antigen ligase mutant by replacing the *waaL* gene with a kanamycin-resistance cassette (Δ*waaL*::Kan^r). Thus, we created a suicide vector containing a counter-selectable marker: a mutated phenylalanyl-tRNA synthetase gene *pheS*.[26] After various selection rounds, we succeeded in generating the desired mutant with a rough colony phenotype indicative of the presence of a lipooligosaccharide (LOS); that is, with the core oligosaccharide region attached to lipid A, but with no O antigen.

The full structure of the LOS core region of the mutant was deduced by a combination of chemical analysis, MALDI mass spectrometry, and 2D NMR spectroscopy. Seven anomeric signals were identified in the ^{1}H NMR spectrum. Furthermore, the upfield-shifted signals were identified as the 3-H methylene hydrogen atoms of the 3-deoxy-D-*manno*-oct-2-ulosonic (Kdo) residue (see the Supporting Information). The proton resonances of all spin systems were identified from DQF-COSY and TOCSY spectra and were used to assign the carbon resonances in the HSQC spectrum. The anomeric configuration of each monosaccharide was assigned on the basis of the $^3J_{1\text{-H},2\text{-H}}$ coupling constants observed by DQF-COSY and the intraresidual NOE contacts observed in the ROESY and NOESY spectra, whereas the values of the vicinal $^3J_{\text{H,H}}$ coupling constants enabled the determination of the relative configuration of each sugar residue. The absence of chemical-shift values above 80 ppm for ring carbon atoms confirmed that all monosaccharides were in the pyranose form. The relative intensities of the anomeric signals suggested the existence of a mixture of oligosaccharides with different carbohydrate-chain lengths owing to the presence of the Kdo reducing end as multiple ring isomers or as lactone forms. Once ^{1}H and ^{13}C resonances had been attributed to each spin system, it was possible to assign the oligosaccharide-chain resonances from the interglycosidic contacts found in the NOESY and ROESY spectra (see Table S1 in the Supporting Information for complete structural assignment). The full structure was confirmed by mass spectrometry (see Table S2 and Figures S7 and S8 in the Supporting Information). The core of the LPS from *B. rhizoxinica* consists of a nonasaccharide backbone comprising four heptose (Hep) residues as well as a GalNAc, a Glc, a rhamnose (Rha), a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), and a D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) unit (Scheme 2). The core oligosaccaride structure of the *B. rhizoxinica* LOS resembles that of *B. multivorans*,[27] especially in terms of the inner core and the presence of a further heptose residue attached to the β-Glc unit. The outer core is different from but still coherent with LOS structures from *Burkholderia* spp.[16] Finally, the structure of the truncated LPS clearly showed that the mutant lacks the O antigen.

To test whether this mutant is capable of initiating a stable symbiosis with the host fungus, we mixed pure cultures of *B. rhizoxinica* (wild-type and mutant) with endosymbiont-free (cured) *R. microsporus* cultures. Usually, wild-type bacteria readily reinfect the fungus, reestablish the symbiosis, and elicit sporulation of the host. Cured fungi, however, are unable to sporulate.[28] Consequently, we considered host



Scheme 2. Core oligosaccharide structure of the R-LPS (LOS) from the O-antigen-ligase mutant (Δ*waaL*::Kan^r). GalNAc = 2-amino-2-deoxy-D-galactose, Glc = D-glucose, Hep = L-*glycero*-D-*manno*-heptose, Rha = L-rhamnose.

sporulation as an indicator for the reestablishment of symbiosis. Thus, we monitored sporulation behavior over time both on agar plates and in liquid culture by using 48-well plates. Cocultivation of the cured fungus with wild-type bacteria typically resulted in high levels of successful reinfection, which is mirrored by about 90% host sporulation. In stark contrast, the Δ*waaL* mutant showed significantly reduced reinfection/sporulation rates (Figure 2). In cases in which reinfection with Δ*waaL*::Kan^r mutants gave a positive response, the intensity of host sporulation was much lower than with the wild type. When the fungus was reinfected with Δ*waaL*::Kan^r mutants, intracellular bacteria could be detected by microscopic examination, albeit in greatly reduced number ($< 10\%$) relative to the wild type. Furthermore, after the subcultivation of reinfected fungi, we observed persistently low host sporulation or even a complete lack of spore formation.

Our findings provide strong evidence that the O antigen plays a crucial role in the bacterial–fungal symbiosis. Since the carbohydrate coating supports the processes of host infection and triggering of sporulation, it seems to serve as a key determinant in chemical-recognition processes during infection and colonization of the host. Furthermore, it is a prerequisite for the long-term intracellular survival of the endosymbiont and for the formation of a stable bacterial–fungal association. A plausible explanation for these observations is given by a model in which the polygalactofuranose O antigen protects endobacteria against as yet unknown fungal defense mechanisms. Such strategies are only known from associations of bacteria with higher organisms. For example, beneficial bacteria inhabiting mammalian intestines decorate their surface with fucose, which is an abundant surface molecule of intestinal epithelial cells.[29] It is also well-known that some other Gram-negative pathogens, such as *Neisseria* and *Helicobacter*, decorate their surface with blood-group antigens.[30]

In summary, we have fully elucidated the first LPS structure of a bacterium living within a fungus and revealed

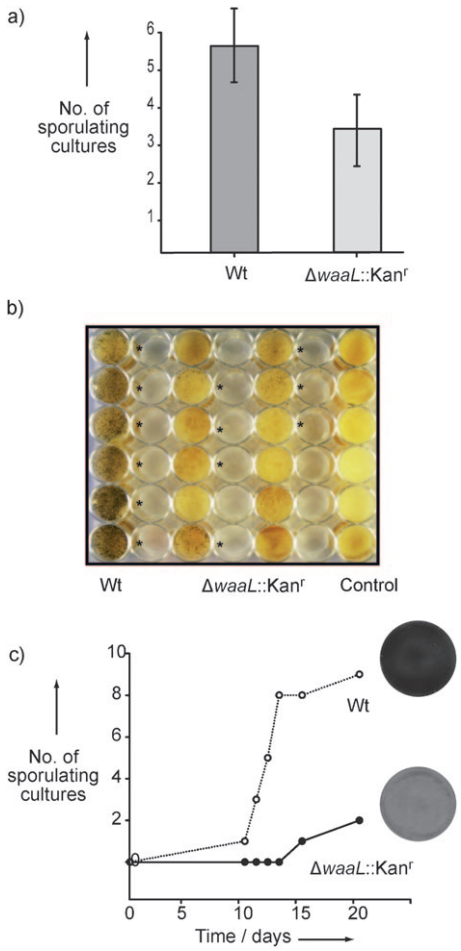


Figure 2. Testing of O-antigen mutants for defects in the symbiont–host interaction by the monitoring of reinfection/sporulation rates. a) Bar chart showing reinfection/sporulation rates in liquid cultures. Six cultures were observed in parallel, and each experiment was repeated four times. Mean values were calculated from the results of all experiments; error bars show the standard deviation. Wild-type (Wt) bacteria reinfected the host with significantly higher rates than those observed for O-antigen-ligase (Δ*waaL*:Kan^r) mutants. b) Photograph of a completed reinfection/sporulation experiment (liquid culture); sporulating cultures are marked with an asterisk. Wild-type controls (left) showed strong sporulation; O-antigen mutants (middle) showed decreased sporulation; negative controls (endosymbiont-free *R. microsporus*) did not sporulate. c) Reinfection experiment on agar plates, as observed over time.

the presence of a unique 1,2-β-D-galactofuranose glycoconjugate. To gain insight into the biological function of the O antigen, we sequenced an LPS-biosynthesis gene locus, which fully supported the structure elucidation. Furthermore, we succeeded in generating a targeted O-antigen-ligase mutant, which was incapable of producing the polygalactofuranoside conjugate, and confirmed the absence of the O-antigenic chain by chemical analysis. A sporulation assay and microscopic investigation finally revealed that the intracellular survival of the mutant was critically impaired, and that the O antigen is a crucial component for stable bacterial–fungal symbiosis to be established and maintained. Since galactofuranose units are particularly abundant in filamentous fungi, one may conclude that the galactofuranosyl O antigen serves as mimicry to put the bacterium into "stealth mode". To our knowledge, this study is the first to shed light on the role of surface carbohydrates in an interaction between endobacteria and fungi. Our results disclose the role of a glycoconjugate in a novel biological context and thus fill an evident gap in the current knowledge on LPS-mediated communication.

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