Stress Tolerance and Virulence-Related Roles of Lipopolysaccharide in *Burkholderia glumae*

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The lipopolysaccharide (LPS) composed of lipid A, core, and O-antigen is the fundamental constituent of the outer membrane in gram-negative bacteria. This study was conducted to investigate the roles of LPS in *Burkholderia glumae*, the phytopathogen causing bacterial panicle blight and seedling rot in rice. To study the roles of the core oligosaccharide (OS) and the O-antigen region, mutant strains targeting the *waaC* and the *wbiFGHI* genes were generated. The LPS profile was greatly affected by disruption of the *waaC* gene and slight reductions were observed in the O-antigen region following *wbiFGHI* deletions. The results indicated that disruption in the core OS biosynthesis-related gene, *waaC*, was associated with increased sensitivity to environmental stress conditions including acidic, osmotic, saline, and detergent stress, and to polymyxin B. Moreover, significant impairment in the swimming and swarming motility and attenuation of bacterial virulence to rice were also observed in the *waaC*-defective mutant. The motility and virulence of O-antigen mutants defective in any gene of the *wbiFGHI* operon, were not significantly different from the wild-type except in

slight decrease in swimming and swarming motility with *wbiH* deletion. Altogether, the results of present study indicated that the LPS, particularly the core OS region, is required for tolerance to environmental stress and full virulence in *B. glumae*. To our knowledge, this is the first functional study of LPS in a plant pathogenic *Burkholderia* sp. and presents a step forward toward full understanding of *B. glumae* pathogenesis.

Keywords : *Burkholderia glumae*, lipopolysaccharide, stress tolerance, virulence

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The outer membrane of gram-negative bacteria is composed mainly of lipopolysaccharide (LPS) with attached integral proteins that together orchestrate the flux of hydrophilic and hydrophobic molecules across the membrane. As the fundamental component of cellular outer membrane, accounting for around three-quarters of the surface of most gram-negative bacteria, the LPS is responsible for many functions as a barrier to environmental stress and signaling machinery (Sperandeo et al., 2017).

LPS of gram-negative bacteria is composed of three parts: lipid A, core oligosaccharide (OS), and O-antigen (Dong et al., 2017). Lipid A, the most structurally conserved region of LPS is the hydrophobic moiety, which anchors LPS to the outer membrane. The core OS is composed of non-repeating sugars that link lipid A to the O-antigen portion. The core OS is divided into two parts, an outer-core OS with variable structure that attaches to the O-antigen and an inner-core OS with a more conserved structure, that attaches to lipid A. The inner-core OS is mainly composed of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo)

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and L-glycero-D-manno-heptose (heptose) (Holst, 2007). The O-antigen is the outermost portion of LPS and is composed of a variable number of repeated OS units, greatly varying between different bacterial strains (Sperandeo et al., 2017).

LPS not only provides cellular stability and protection from harsh environments but is also responsible for numerous interactions and exerts multiple signaling-related roles that are critical for pathogenicity to mammalian and plant cells (Kutschera and Ranf, 2018). The induction of cellular responses upon the recognition of bacterial LPS by mammalian cells has been described in previous studies (Park and Lee, 2013). However, the recognition of bacterial LPS by plant cells and the subsequent cell responses remain comparatively elusive (Vilakazi et al., 2017). Previous studies characterized the action of bacterial LPS as an elicitor of the plant innate immune system, triggering the production of antimicrobial compounds, reactive oxygen species, or pathogenesis-related proteins (Finnegan et al., 2016; Newman et al., 2013; Zeidler et al., 2004).

Studies involving the generation of mutant strains defective in LPS biosynthesis-related genes have indicated the requirement of LPS for bacterial virulence and resistance to various stress conditions. In particular, mutations resulting in alterations of the LPS structure were found to be associated with changes in biofilm formation, colonization of host tissues, and virulence of *Escherichia coli* (Møller et al., 2003; Nakao et al., 2012). *Salmonella enterica* was shown to lose its virulence as a consequence of mutations in LPS biosynthesis-related genes (Kong et al., 2011). In *Burkholderia cenocepacia*, the complete core OS was found to be required for bacterial resistance to antimicrobial peptides, as well as for virulence to model animals (Loutet et al., 2006). It was later reported that the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the LPS was very critical for the intrinsic resistance to antimicrobial peptides (Hamad et al., 2012).

Although many studies were conducted to characterize the functions of LPS in human and animal pathogenic gram-negative bacteria, only few have addressed the role of LPS in plant pathogenic bacteria. LPS-defective mutants of the wilt pathogen *Pseudomonas solanacearum* showed reduced virulence to tobacco plants (Hendrick and Sequeira, 1984). In the phytopathogen *Erwinia* sp., loss of virulence and unsuccessful invasion were reported in mutants with altered LPS structure (Schoonejans et al., 1987). In *Xanthomonas campestris*, defects in two genes involved in LPS core OS biosynthesis resulted in altered behavior and attenuation of virulence (Dow et al., 1995). More recently, the LPS of phytopathogenic *Xanthomonas oryzae* was

found to be required for the type III secretion system and, consequently, for bacterial virulence (Wang et al., 2013).

Burkholderia glumae, a gram-negative phytopathogenic bacterium in the *Burkholderia sensu stricto* lineage, is the etiological agent of a serious disease threatening rice cultivation worldwide (Ham et al., 2011). Previous studies have investigated the disease-causing mechanisms, and several virulence factors (e.g., bacterial motility, secretion systems, exopolysaccharide [EPS], toxoflavin, and virulence-related enzymes such as metalloprotease) have been identified (Jeong et al., 2011; Jung et al., 2018; Kim et al., 2007; Lee et al., 2016; Lelis et al., 2019; Mannaa et al., 2019). The role of LPS in relation to virulence or adaptation to environmental stress has not yet been investigated in *B. glumae*. Therefore, this study was conducted to address the question whether the LPS of *B. glumae* is implicated in tolerance to harsh environment and/or virulence to rice.

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The rice pathogenic *B. glumae* BGR1 (wild-type), the generated mutant strains, and *E. coli* were grown at 37°C in Luria-Bertani (LB) media. Appropriate antibiotics were added to the culture media, according to the purpose, at the following concentrations: rifampicin, 50 µg/ml; kanamycin, 30 or 50 µg/ml; tetracycline, 10 µg/ml.

Molecular techniques for mutant strain generation.

Standard protocols were used for DNA amplification, recombinant DNA construction, and mutant strain generation (Sambrook et al., 1989). To amplify the upstream region (L fragment) and the downstream region (R fragment) of the target genes (*wbiF*, *wbiG*, *wbiH*, and *wbiI*) for deletion mutant generation, the genomic DNA (gDNA) of *B. glumae* BGR1 was extracted and polymerase chain reaction (PCR) was performed using Solgent Pfu-X DNA polymerase (Solgent, Daejeon, Korea) with primer pairs containing appropriate restriction sites (Supplementary Table 1). The amplified fragments and pK18mobsacB suicide vector were digested with the appropriate restriction enzymes and ligated using T4 DNA ligase (Schäfer et al., 1994). Competent *E. coli* DH5α λ pir cells were transformed with the recombinant plasmids for amplification. Amplified plasmids were extracted, reintroduced into *E. coli* S17-1 λ pir (donor strain), and subsequently used to transform *B. glumae* BGR1 (recipient strain) by bi-parental mating (Simon et al., 1983). After selection of transformed *B. glumae* BGR1 using appropriate antibiotics, the deletion mutants

Table 1. Bacterial strains and plasmids

Strains or plasmid	Characteristic	Reference
<i>Burkholderia glumae</i>		
BGR1	Wild-type, Rif ^r	Jeong et al. (2003)
BGS2	BGR1 <i>tofl::Ω</i>	Kim et al. (2004)
ΔwbiF	BGR1 Δ <i>wbiF</i> , Rif ^r	This study
ΔwbiG	BGR1 Δ <i>wbiG</i> , Rif ^r	This study
ΔwbiH	BGR1 Δ <i>wbiH</i> , Rif ^r	This study
ΔwbiI	BGR1 Δ <i>wbiI</i> , Rif ^r	This study
ΔwaaC	BGR1 <i>waaC::Ω</i> pVIK_ <i>waaC</i> , Rif ^r , Km ^r	This study
cΔ <i>wbiF</i>	Δ <i>wbiF</i> containing pBBR_ <i>wbiF</i> , Rif ^r	This study
cΔ <i>wbiG</i>	Δ <i>wbiG</i> containing pBBR_ <i>wbiG</i> , Rif ^r	This study
cΔ <i>wbiH</i>	Δ <i>wbiH</i> containing pBBR_ <i>wbiH</i> , Rif ^r	This study
cΔ <i>wbiI</i>	Δ <i>wbiI</i> containing pBBR_ <i>wbiI</i> , Rif ^r	This study
cΔ <i>waaC</i>	Δ <i>waaC</i> containing pRK_ <i>waaC</i> , Rif ^r , Km ^r	This study
<i>Escherichia coli</i>		
DH5α λpir	<i>E. coli</i> used for cloning and replication of plasmids	Lab collection
S17-1 λpir	<i>E. coli</i> used for transferring cloning plasmids to recipient cells	Lab collection
Plasmids		
pk18mobsacB	Allelic exchange suicide vector, <i>sacB</i> , Km ^r	Schäfer et al. (1994)
pVIK112	R6K suicide vector, <i>lacZY</i> for transcriptional fusion, Km ^r	Kalogeraki and Winans (1997)
pBBR1MCS-2	Broad host range expression vector, Km ^r	Kovach et al. (1995)
pRK415	Broad host range expression vector, Tc ^r	Keen et al. (1988)
pVIK_ <i>waaC</i>	pVIK112 vector cloning internal region of <i>waaC</i>	This study
pK18_ <i>wbiF</i>	pK18mobsacB vector cloning LR fragment of <i>wbiF</i>	This study
pK18_ <i>wbiG</i>	pK18mobsacB vector cloning LR fragment of <i>wbiG</i>	This study
pK18_ <i>wbiH</i>	pK18mobsacB vector cloning LR fragment of <i>wbiH</i>	This study
pK18_ <i>wbiI</i>	pK18mobsacB vector cloning LR fragment of <i>wbiI</i>	This study
pBBR_ <i>wbiF</i>	pBBR1MCS-2 vector including <i>wbiF</i> gene	This study
pBBR_ <i>wbiG</i>	pBBR1MCS-2 vector including <i>wbiG</i> gene	This study
pBBR_ <i>wbiH</i>	pBBR1MCS-2 vector including <i>wbiH</i> gene	This study
pBBR_ <i>wbiI</i>	pBBR1MCS-2 vector including <i>wbiI</i> gene	This study
pRK_ <i>waaC</i>	pRK415 vector including <i>waaC</i> gene	This study

Rif, rifampicin; Km, kanamycin; Tc, tetracycline.

were obtained after a second homologous recombination by sub-culturing in LB containing 30% sucrose (w/v).

To generate mutant strain defective in *waaC* gene, the target gene was amplified by Pfu-X DNA polymerase and the appropriate restriction sites were added to the 5' end region of the target gene PCR primers, *waaC_F* and *waaC_R* (Supplementary Table 1). The amplified fragments and the suicide vector pVIK112 were digested using appropriate restriction enzymes and ligated using T4 DNA ligase (Kalogeraki and Winans, 1997). Amplification of the recombinant plasmid and transformation of the *B. glumae* BGR1 were performed as described above. Mutant BGR1 strain was selected on LB plates containing kanamycin (100 μg/ml) and rifampicin (100 μg/ml), then confirmed by PCR with the specific primers, Lacfuse_R and *waaC_*

up_F designed to bind outside the *EcoRI* and *KpnI* sites of pVIK112 (Supplementary Table 1). A schematic diagram for the generation of mutants is shown in Supplementary Fig. 1.

To generate the complemented strains for each deleted or disrupted gene, the complete open reading frame was amplified using appropriate primers (Supplementary Table 1) and inserted into pRK415 or pBBR1MCS-2 expression vectors (Keen et al., 1988; Kovach et al., 1995). Recombinant vectors were introduced into each mutant by conjugation. All generated mutants and complemented strains were confirmed by PCR assays. The growth rate of the generated mutant strains was compared to that of the wild-type by measuring the optical density (OD₆₀₀) in freshly inoculated LB broth every 6 h.

LPS electrophoretic analysis. Total LPS from *B. glumae* BGR1 wild-type, the generated mutants, and complemented mutants were extracted using the modified hot phenol method (Kim et al., 2017; Westphal, 1965). Briefly, bacterial cultures in LB were grown to $OD_{600} = 1.0$, harvested, washed, and resuspended in 4 ml of 10 mM phosphate buffer. An equal (4 ml) volume of hot phenol was added to the cell suspensions and incubated for 1 h in a water bath at 65°C with vigorous vortexing every 5 min. The suspension was then cooled, and 1.6 ml of chloroform was added. The aqueous layer was then separated by centrifugation at $8,500 \times g$ for 15 min and transferred to a new tube. The LPS was precipitated overnight using 10-ml isopropyl alcohol at -20°C. The precipitate was washed twice in 80% ethanol, and air-dried.

For the electrophoretic analysis, the extracted LPS was suspended and heated at 95°C for 5 min in sample buffer (80 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.0006% bromophenol blue). LPS was then separated on 15% acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized according to the manufacturer's instructions using the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen, Carlsbad, CA, USA).

EPS quantification assay. Quantification of EPS was performed as previously described by He et al. (2010). Briefly, bacterial cultures in Casamino acid–Peptone–Glucose medium (10 g peptone, 5 g glucose, 1 g casamino acids, 1 g yeast per liter) were adjusted to $OD_{600} = 0.4$. The supernatant was collected from 10 ml of bacterial suspension by centrifugation at $10,000 \times g$ for 20 min. For EPS precipitation, 2 volumes of absolute ethanol were added to the collected supernatant and incubated for 30 min at -20°C. The precipitated EPS was filtered using a vacuum pump through a membrane filter (Hyundai Micro, Anseong, Korea) and dried overnight at 55°C. The dried EPS on the membrane filter were then quantified and expressed as mg per milliliter of the culture supernatant.

Environmental stress and antibiotics tolerance assays. The wild-type *B. glumae* BGR1 and the generated mutants were tested for tolerance to different environmental stresses (*i.e.*, acidic, osmotic, saline, and oxidative stress), as previously described by Li and Wang (2011) with some modifications. Bacterial cultures in LB were grown to early exponential level ($OD_{600} > 1$) and OD_{600} values were adjusted to 0.1. Bacterial cells were then harvested and suspended in phosphate-buffered saline. For acidity and salt stress tolerance, cells were cultured in acidic LB agar plates

(pH = 4.5) and saline media (1.5% NaCl), respectively. Tolerance to osmotic pressure was tested by adding 20% of D-sorbitol to the cell suspension and incubating for 40 min. Tolerance to oxidative stress was evaluated based on the sensitivity to H_2O_2 , using disk diffusion assays on agar plates, as described in previous studies (Kim et al., 2018; King et al., 2000). In brief, LB agar plates were seeded by the tested strains and sterile paper disks pre-soaked in 30% H_2O_2 were placed onto the center of the seeded agar plates. After a 1-day incubation at 37°C, the clear zones around the H_2O_2 disks were measured. Bacterial sensitivity to detergent stress and antibiotics was also tested. Detergent stress tolerance was tested by adding 0.01% SDS to the cell suspension and incubating for 10 min. The bacterial sensitivity to the antimicrobial peptide, polymyxin B, was tested using the method described by Loutet et al. (2006). Polymyxin B (Merck, Darmstadt, Germany) was added to the bacterial suspensions at a final concentration of 5 µg/ml and incubated for 30 min. Except for the H_2O_2 sensitivity assay, the survival rate of the tested strains was evaluated by counting the viable cells before (T0) and after (T1) exposure to each stress condition.

Bacterial motility assays. The swimming motility assay was conducted as described elsewhere (Castelli et al., 2008). Briefly, LB plates with 0.25% agar were prepared and single colonies from each strain were inoculated using sterile toothpick into the center of the swimming plates. After incubation for 24 h at 37°C, the radius of the swimming zones around the inoculation points were measured. To perform the swarming motility assay, bacterial cells from 1 ml of LB cultures were harvested by centrifugation and resuspended in 100 µl of fresh LB broth. Five microliters from each suspension were spotted onto the center of LB soft agar plates (0.4% [w/v] agar). The plates were then incubated for 24 h at 37°C and photographed, and the radius of the swarms were measured based on the average dendrite lengths.

Virulence assay on rice plants. Virulence assays were performed with wild-type BGR1 and generated mutants, as follows. Rice plants (*Oryza sativa* L. cv. Saeilmi) were grown until flowering stage under greenhouse conditions. To prepare the inocula, cells were harvested in sterile distilled water (SDW) from overnight LB cultures and OD_{600} values were adjusted. Rice panicles were inoculated by dipping in the prepared suspensions or control SDW for 1 min. Six days after inoculation, disease severity was assessed on panicle grains based on a 0-5 scale, as follows: 0, clear panicle; 1, 0-20% discoloration; 2, 20-40% discoloration; 3,

40-60% discoloration; 4, 60-80% discoloration; and 5, 80-100% discoloration (Kim et al., 2018). After counting the number of grains at each rank, the following formula was used for disease severity:

Disease severity = $\sum$ (number of grains at each rank $\times$ rank value)/(the total number of panicles).

A disease distribution pattern was also used to further visualize the differences between treatments (Chun et al., 2009).

Statistical analysis. Each experiment was conducted at least twice with three replicates. Statistical analysis of the data was performed using the Statistical Analysis Systems (SAS Institute, Cary, NC, USA). Data from repeated experiments were tested for equality of variances using the Levene's test (Levene, 1960). Analysis of variance was performed using the GLM procedure in SAS, and the separation between means was assessed using the Tukey's honestly significant difference test, where a *P*-value of 0.05 was considered statistically significant.

Results

The *wbiFGHI* and *waaC* genes are involved in the biosynthesis of O-antigen and core OS. The targeted genes, *wbiFGHI* (852, 984, 1,044, and 1,884 nt), and *waaC* (996 nt), are located on chromosome 1 in the *B. glumae* BGR1 genome (Fig. 1A). There were no obvious differences in growth rate between the wild-type (BGR1) and the generated mutant strains ($\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, and $\Delta wbiI$), except for a slight reduction in the $\Delta waaC$ mutant, which was recovered in the complemented strain (Supplementary Fig. 2).

To test the effect of the constructed mutants on the LPS profile, electrophoretic analysis was performed. The electrophoretic analysis of LPS from the generated mutants ($\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, and $\Delta waaC$), their complemented strains (*c* $\Delta wbiF$, *c* $\Delta wbiG$, *c* $\Delta wbiH$, *c* $\Delta wbiI$, and *c* $\Delta waaC$), and the wild-type (BGR1) revealed the involvement of the *wbiFGHI* operon and the *waaC* gene in the biosynthesis of the O-antigen and the core OS, respec-

Fig. 1. Genetic organization of lipopolysaccharide (LPS) biosynthesis-related genes in *Burkholderia glumae* BGR1, and LPS electrophoretic analysis in wild-type and mutant strains. (A) The genes targeted for mutagenesis are involved in O-antigen (*wbiF*, *wbiG*, *wbiH*, and *wbiI*) or core oligosaccharide (OS) (*waaC*) biosynthesis. The 5 targeted genes, indicated by dark arrows, are located on chromosome 1 of *B. glumae* BGR1, and are 852 nt, 984 nt, 1,044 nt, 1,884 nt, and 996 nt in length, respectively. (B) The LPS profiles of the tested strains were visualized following extraction and separation on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The typical bands of the O-antigen and lipid A + core OS regions are clearly observable in the wild-type BGR1, lane 1. Apparently incomplete O-antigen regions and highly intense core OS + lipid A were observed in the $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, and $\Delta wbiI$ mutants, lanes 2-5. The O-antigen synthesis was restored in the complementary strains, *c* $\Delta wbiF$, *c* $\Delta wbiG$, *c* $\Delta wbiH$, and *c* $\Delta wbiI$, lanes 6-9. Both O-antigen and core OS + lipid A were greatly affected in the core OS biosynthesis-related mutant $\Delta waaC$, lane 10. Both regions were restored in the complementary strain, *c* $\Delta waaC$, lane 11.

Fig. 2. Quantification of exopolysaccharide (EPS) in *Burkholderia glumae* BGR1 wild-type, *wbiFGHI*, and *waaC*-defective mutants. EPS extracted from wild-type *B. glumae* (BGR1) and the mutants $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, $\Delta waaC$, and $c\Delta waaC$ was quantified and expressed as mg/ml of culture supernatant. No significant differences were observed in any of the mutants with respect to the wild-type. The $\Delta waaC$ mutant showed a significant EPS increase compared to both $\Delta wbiH$ and $\Delta wbiI$ mutants. The complementary strain, $c\Delta waaC$, exhibited a significantly lower EPS production compared to the $\Delta waaC$ mutant. The different letters on the error bars represent statistically significant differences at $P < 0.05$, according to Tukey's honestly significant difference test.

tively. The mutants deficient in each gene of the *wbiFGHI* operon showed reduced O-antigen region, with thicker core OS- and lipid A-specific bands, whereas the *waaC*-deficient mutant gene showed almost complete loss of both O-antigen and core OS regions, which were restored in the complemented strains (Fig. 1B). This result indicated that the *wbiFGHI* operon was associated with the biosynthesis of the O-antigen, whereas the *waaC* gene was essentially involved in core OS biosynthesis and also affected O-antigen assembly.

EPS production is not affected by disruption of LPS biosynthesis-related genes in *B. glumae* BGR1. As one of the known virulence factors of *B. glumae*, the EPS production was assessed as response to disruption in the LPS biosynthesis-related genes. The production of EPS by the mutants $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, and $\Delta waaC$ was not significantly different from *B. glumae* BGR1 wild-type. The $\Delta waaC$ mutant produced significantly more EPS than the $\Delta wbiH$ and $\Delta wbiI$ mutants. The complemented strain, $c\Delta waaC$, showed reduced EPS production compared to the $\Delta waaC$ mutant (Fig. 2). This result suggested that EPS

production was not significantly affected by mutations in the target genes with slight effect of the *waaC* gene when compared to the other O-antigen-related mutants, $\Delta wbiH$ and $\Delta wbiI$.

The loss of the WaaC protein is involved in tolerance to antibiotic and environmental stress in *B. glumae* BGR1. As one of the potential roles of LPS bacterial cells, tolerance of the generated mutants to environmental stress was assessed. The tolerance to various stress conditions was compared between the mutant strains ($\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, and $\Delta waaC$) and the wild-type (BGR1). The $\Delta waaC$ mutant, showed a significant reduction in the survival rate under acidic, osmotic, and saline stress conditions (Fig. 3A, B, and C). The complemented strain $c\Delta waaC$ restored normal survival to acidic and saline stress but not to osmotic stress. The sensitivity to H_2O_2 was used to assess tolerance to oxidative stress. There was no difference in bacterial sensitivity to H_2O_2 between the wild-type and all the tested mutants ($\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, and $\Delta waaC$). Moreover, both mutants and wild-type BGR1 were significantly more tolerant to H_2O_2 stress compared to the quorum sensing (QS)-defective mutant BGS2, which was used as a negative control (Fig. 3D).

The mutants were also compared for their tolerance to detergent (SDS) stress and to the antimicrobial peptide, polymyxin B. Among the tested mutants, a significant reduction in the survival rate was only observed with the core OS defective mutant, $\Delta waaC$. The tolerance to both SDS and polymyxin B was completely restored in the complemented strain, $c\Delta waaC$ (Fig. 4).

These results suggested that the loss of the WaaC protein causing the formation of a heptoseless LPS, resulted in more sensitivity of *B. glumae* BGR1 to acidic, osmotic, saline, and detergent stress conditions, and to polymyxin B exposure. The results also indicated that neither the O-antigen-related operon *wbiFGHI* nor the core OS-related *waaC* gene were related to tolerance to oxidative stress in *B. glumae* BGR1.

The loss of the WaaC protein is associated with impaired swimming and swarming motility in *B. glumae* BGR1. Bacterial motility is known to be linked to virulence by facilitating movements to infection sites and colonization of the host tissues. The swimming and swarming motility tested on soft agar plates showed that the *waaC*-defective mutant, $\Delta waaC$, exhibited a significantly declined motility compared to the wild-type BGR1. This was clearly indicated by the reduced swimming motility diameters and swarm diameters around the inoculation spot, compared

Fig. 3. *Burkholderia glumae* lipopolysaccharide role in adaptation to environmental stress. The wild-type BGR1 and mutant strains $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, $\Delta waaC$, and $c\Delta waaC$ were tested for tolerance to various environmental stresses; acidic media (pH = 4.5) (A), osmotic stress (20% D-sorbitol) (B), saline stress (1.5% NaCl) (C), and oxidative stress (30% H₂O₂) (D). The bacterial survival rate was estimated based on the difference between the bacterial population before and after exposure to the environmental stress. Tolerance to oxidative stress was evaluated as the sensitivity to H₂O₂ exposure by measuring the clear zones around paper disks previously immersed in 30% H₂O₂, on agar plates seeded with each tested strain. The quorum sensing-defective mutant BGS2 was used as a positive control in the H₂O₂ sensitivity test. The different letters on the error bars indicate significant differences at $P < 0.05$ according to Tukey's honestly significant difference test.

Fig. 4. *Burkholderia glumae* lipopolysaccharide role in resistance to antibiotic and detergent stress. The wild-type BGR1 and the mutant strains $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, $\Delta waaC$, and $c\Delta waaC$ were tested for resistance to the antimicrobial peptide polymyxin B and to detergent stress from sodium dodecyl sulfate (SDS). Cultures of each strain were treated with 5 μ g/ml polymyxin B or 0.01% SDS and incubated for 30 or 10 min, respectively, then survival rates were estimated. The survival rate of the core oligosaccharide biosynthesis-related $\Delta waaC$ mutant was significantly lower compared to the other strains. The complementary strain, $c\Delta waaC$, restored resistance, which was comparable to that of wild-type BGR1. The different uppercase and lowercase letters on error bars represent significant differences in bacterial survival rate in the presence of polymyxin B and SDS, respectively, according to Tukey's honestly significant difference test at $P < 0.05$.

Fig. 5. The role of lipopolysaccharide in *Burkholderia glumae* motility. (A) The swimming motility of the wild-type BGR1 and mutant strains $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbil$, $\Delta waaC$, and $c\Delta waaC$ was tested on Luria-Bertani (LB) plates containing 0.25% agar. (B) The swarming motility was tested on LB plates containing 0.4% agar. The swimming and swarm radius (cm) for each strain was estimated 24 h after incubation as an average of the dendrite measurements from each plate (a replicate). The swimming and swarming motility of the four O-antigen mutants was not significantly different compared to that of the wild-type BGR1, except for $\Delta wbiH$, which showed a slightly reduced motility. The strongest effect on motility was observed in the core oligosaccharide-related mutant $\Delta waaC$, displaying a great reduction compared to the other strains. The complementary strain, $c\Delta waaC$, partially restored the swarming motility. The quorum sensing-defective mutant BGS2 was used as a negative control in both assays. The different letters following the mean radius $\pm$ standard deviation, indicate significant differences according to Tukey's honestly significant difference test at $P < 0.05$.

Fig. 6. The role of lipopolysaccharide in *Burkholderia glumae* virulence to rice. Disease severity on rice plants was assessed 5 days after inoculation by dipping in sterile distilled water (SDW, control) or in cultures of the O-antigen mutants $\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, $\Delta wbiI$, the core oligosaccharide (OS) biosynthesis-related $\Delta waaC$ mutant, or its complemented strain, $c\Delta waaC$. (A) Photographs of the rice panicles at the time of disease severity assessment. The mean values of disease severity rank (0-5) are shown, followed by letters representing the significant differences according to Tukey's honestly significant difference test at $P < 0.05$. (B) Stacked bar graph representing the distribution patterns of disease severity for each treatment. Disease severity was significantly reduced only with the core OS biosynthesis-related $\Delta waaC$ mutant treatment and was restored by the complementary strain, $c\Delta waaC$.

to the other mutants ($\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, and $\Delta wbiI$) and the wild-type strain (BGR1). A partial recovery of the swimming and swarming motility were observed in the complemented strain, $c\Delta waaC$. The $\Delta wbiH$ mutant showed slightly reduced swimming and swarming motility compared to the wild-type, with significantly reduced swimming and swarm diameters. The negative control, *i.e.*, the QS-defective mutant BGS2, showed almost complete loss

of the swimming and swarming motility (Fig. 5). These results indicated the requirement of functional WaaC protein for swarming motility in *B. glumae* BGR1.

The LPS core OS is required for full virulence of *B. glumae* to rice plants. To finally investigate the effect of disruption in the targeted LPS-related genes on *B. glumae* BGR1 virulence to rice, rice assays were performed using

the constructed mutants compared to wild-type. The observation of inoculated rice panicles indicated that a clear reduction in the blight symptoms, compared to the wild-type BGR1, only occurred upon inoculation of the $\Delta waaC$ mutant, whereas the other mutants ($\Delta wbiF$, $\Delta wbiG$, $\Delta wbiH$, and $\Delta wbiI$) caused similar symptoms as the wild-type strain (Fig. 6A). This observation was confirmed by the assessment of disease severity and distribution pattern, showing a significant reduction in the disease severity rank after treatment with the $\Delta waaC$ strain (Fig. 6A and B). The complemented strain, $c\Delta waaC$, showed complete virulence recovery. The negative control rice plants treated with SDW did not display any blight symptoms. These results indicated that the core OS-related gene, *waaC*, was required for the full virulence of *B. glumae* BGR1 to rice plants.

Discussion

To study the functions of LPS in *B. glumae*, mutants targeting the O-antigen and the core OS biosynthesis-related genes were generated, and their phenotype was characterized. A study in *Burkholderia cenocepacia* proposed the following functions for the genes belonging to the *wbiFGHI* operon involved in O-antigen biosynthesis: *wbiF*, glycosyltransferase; *wbiG*, nucleotide sugar epimerase-dehydratase; *wbiH*, UDP-UDP-N-acetylglucosamine 1-P transferase; and *wbiI*, nucleotide sugar epimerase-dehydratase (Ortega et al., 2005). Although other genes are involved in the O-antigen biosynthesis, the *wbiFGHI* operon is a fundamental part of the O-antigen gene cluster, encoding enzymes that are essential for O-antigen biosynthesis and assembly (Ortega et al., 2005). The targeted gene for core OS was *waaC*, which is part of the core OS gene locus, the most conserved across the *Burkholderia* genus. The *waaC* encodes heptosyltransferase I, which functions by adding heptose to the inner core in an early stage of the core OS biosynthesis pathway (Gronow et al., 2000; Ortega et al., 2005; Wang et al., 2015).

The effect of the induced mutations was verified by comparing the associated LPS profiles to that of the wild-type strain. The *wbiFGHI* mutants exhibited a reduced O-antigen region and an increased production of the core and lipid A, whereas the *waaC* mutant exerted a clear detrimental effect on the LPS synthesis, mainly a truncated lipid A core. Chemical alteration of the core OS structure and reduced length of the O-antigen region were also previously reported as a consequence of *waaC* gene mutations in the insect symbiont *Burkholderia* sp. (Kim et al., 2017). In our study on *B. glumae*, the impact of *waaC* disruption on LPS structure was stronger compared to that of O-antigen-

affecting *wbiFGHI* mutations. The phenotypic characterization showed that *waaC* disruption was associated with impaired bacterial resistance to environmental stress conditions, loss of motility, and attenuation of virulence to rice plants. On the other hand, the *wbiFGHI* mutants did not display significant phenotypic effects, except for a partial inhibition of swarming motility caused by *wbiH* deletion. Bacteria were apparently able to recover from, or tolerate, partial O-antigen losses due to single gene disruption without significant impairment of cellular functions related to adaptation to harsh environment and virulence.

EPS is generally considered as a bacterial virulence factor particularly in wilt-causing xylem-colonizing phytopathogens (Denny, 1995). *B. glumae* was previously reported to cause wilting symptoms in many other crops in addition to rice, and EPS could be the virulence factor responsible for occlusion of the plant vascular system in these cases (Ham et al., 2011). Wang et al. (2013) reported that mutation in one enzyme involved in LPS synthesis resulted in moderate impairment of EPS accumulation, thus reducing the virulence of *X. oryzae* pv. *oryzicola*. On the contrary, our results showed no significant differences in EPS production between the generated *B. glumae* mutants and the wild-type strain.

One of the best-known functions of the bacterial outer membrane is the tolerance to environmental stress and antibiotics. Our results showed that the core OS mutant, deficient in the *waaC* gene, was particularly sensitive to different stress conditions, unlike the O-antigen-deficient mutants defective in *wbiFGHI* genes, which did not exhibit significant changes in their sensitivity to such environmental stress conditions nor to the antimicrobial peptide. These results came in accordance with previous studies reporting a significant increase in the bacterial sensitivity to different environmental stress condition and antibiotics as a consequence of mutations in genes for LPS synthesis, particularly the core OS biosynthesis-related genes (Berry et al., 2009; Kong et al., 2011; Loutet et al., 2006; Møller et al., 2003).

Mutations in the core OS biosynthesis-related genes displayed a hypersensitivity to SDS detergent and salt stress in *E. coli* (Møller et al., 2003). Similarly, in *Salmonella enterica*, mutations in LPS-related genes resulted in higher sensitivity to detergent stress and polymyxin B (Kong et al., 2011). In addition, mutations in two genes involved in core OS biosynthesis in a related strain of *B. cenocepacia* resulted in increased sensitivity to polymyxin B, as well as to structurally distinct antimicrobial peptides (Loutet et al., 2006; Ortega et al., 2009).

Previous studies have reported a link between the core

OS biosynthesis-related *waaC* and expression of the outer membrane protein A (OmpA) (Beher and Schnaitman, 1981). OmpA is an abundant protein of the outer membrane, suggested to be important for membrane barrier properties and structural integrity, which in turn are required for protection against stress conditions such as acidity (Rowbury, 2004; Vivijis et al., 2016). This finding could explain, in part, the increased sensitivity to acidic stress that we observed in the *waaC*-defective mutant. Nevertheless, unlike the other tested stress conditions, the bacterial sensitivity to H₂O₂ was not affected by any of the mutations generated in the current study. However, increased sensitivity to oxidative stress was previously reported in an *Erwinia amylovora* mutant defective in LPS biosynthesis (Berry et al., 2009). Thus, LPS might have different functions in distinct bacterial genera.

Bacterial motility is another key factor for virulence to host plants, as it facilitates the migration of bacteria, allowing them to reach infection sites. In a non-motile *B. glumae* mutant that normally produces toxoflavin, the major virulence factor, bacterial virulence was found to be compromised, highlighting the importance of motility in this process (Kim et al., 2007). Bacterial motility is highly sensitive to the surrounding environment, which affects the development of flagella (Jang et al., 2014; McCarter, 2006). Moreover, LPS structural deficiency, especially in the core OS, was reported to alter the regulation of gene expression through the Rcs phosphorelay system. The latter is a cell stress-response system allowing bacterial cells to sense stress conditions via changes in the outer membrane. It consists of a cascade of reactions initially involving a membrane lipoprotein and ultimately regulating the expression of specific genes (Huang et al., 2006; Ren et al., 2016). Several studies reported that activation of the Rcs signaling system inhibits the transcription of the *flhDC* master operon, which is involved in the assembly of flagella and, thus, is crucial for swimming and swarming motility (Francez-Charlot et al., 2003; Wang and Harshey, 2009). In light of these findings, the structural changes associated with the *waaC* mutation in the current study could have affected the expression of genes involved in the development of flagella, resulting in impaired motility. Consistently, the insect symbiont *waaC*-defective mutant was previously shown to display reduced motility (Kim et al., 2017).

Finally, the impact of gene disruption affecting O-antigen and core OS biosynthesis on bacterial virulence to rice plants was tested. The impact of defective core OS synthesis on *B. glumae* virulence could be attributed to several factors, including inhibition of bacterial motility, as well as impaired tolerance to environmental stress. Tolerance

to such stress conditions is particularly important for the survival of *B. glumae* both in its natural environment, the soil, and inside the host plants, which produce defense-related compounds in response to pathogen infection. In rice plants, the recognition of LPS from pathogenic and non-pathogenic bacteria was shown to trigger the production of reactive oxygen species and the expression of defense-related genes (Desaki et al., 2006). Furthermore, a previous study has reported the role of an LPS-related gene, *wzyB*, as a potential genetic signaling component independent from the quorum sensing, in the production of toxoflavin, the major virulence factor of *B. glumae* (Chen et al., 2015).

The complemented strain *cA_{waaC}*, have shown complete recovery of bacterial virulence to rice plants, although the same strain showed only partial recovery of bacterial motility compared to the *A_{waaC}* strain. These results indicate that although *waaC* is important for bacterial motility, other factors could be involved. For the bacterial virulence, bacterial motility plays a role in full virulence along with the bacterial ability to tolerate the host defense and other environmental stress. Therefore, full virulence was recovered in the complemented strain *cA_{waaC}*, not only because of the recovery of motility but also the recovery of bacterial tolerance to different stress conditions.

In conclusion, to our knowledge, this is the first study to investigate the roles of LPS in *B. glumae*. The results revealed the requirement of the core OS for environmental stress resistance, swimming and swarming motility, and virulence. However, the deletion of any gene of the O-antigen biosynthesis-related *wbiFGHI* operon did not affect the bacterial sensitivity to environmental stress nor virulence to rice. This could be due to the bacterial ability to overcome the partial loss of repeating units in the O-antigen region, or to structural changes that prevent or revert outer membrane instability. The association of the core OS-related gene *waaC* with the outer membrane protein or the Rcs phosphorelay system could be linked to the observed impact on bacterial motility, tolerance to environmental stress and consequently bacterial virulence to rice. Further studies are required to elucidate the molecular mechanisms by which rice plant cells recognize and respond to *B. glumae* LPS, thus enhancing our comprehension of *B. glumae* pathogenesis.

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Electronic Supplementary Material

Supplementary materials are available at Plant Pathology Journal website (<http://www.ppjonline.org/>).

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