Research in Microbiology 174 (2023) 104049

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

Original Article

Physiological consequences of inactivation of *lgmB* and *lpxL1*, two genes involved in lipid A synthesis in *Bordetella bronchiseptica*

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ARTICLE INFO

Article history:
Received 28 July 2022
Accepted 27 February 2023
Available online 3 March 2023

Keywords:
Bordetella
LPS
Glucosamine
LpxL1
Galleria mellonella
Virulence

ABSTRACT

To develop a *Bordetella bronchiseptica* vaccine with reduced endotoxicity, we previously inactivated *lpxL1*, the gene encoding the enzyme that incorporates a secondary 2-hydroxy-laurate in lipid A. The mutant showed a myriad of phenotypes. Structural analysis showed the expected loss of the acyl chain but also of glucosamine (GlcN) substituents, which decorate the phosphates in lipid A. To determine which structural change causes the various phenotypes, we inactivated here *lgmB*, which encodes the GlcN transferase, and *lpxL1* in an isogenic background and compared the phenotypes. Like the *lpxL1* mutation, the *lgmB* mutation resulted in reduced potency to activate human TLR4 and to infect macrophages and in increased susceptibility to polymyxin B. These phenotypes are therefore related to the loss of GlcN decorations. The *lpxL1* mutation had a stronger effect on hTLR4 activation and additionally resulted in reduced murine TLR4 activation, surface hydrophobicity, and biofilm formation, and in a fortified outer membrane as evidenced by increased resistance to several antimicrobials. These phenotypes, therefore, appear to be related to the loss of the acyl chain. Moreover, we determined the virulence of the mutants in the *Galleria mellonella* infection model and observed reduced virulence of the *lpxL1* mutant but not of the *lgmB* mutant.

1. Introduction

Gram-negative bacteria are covered by an outer membrane (OM) that primarily consists of lipopolysaccharide (LPS, a. k.a. endotoxin) in the outer leaflet of the bilayer. LPS is generally composed of three moieties, lipid A, a core oligosaccharide, and an O-antigen [1]. The lipid A domain is typically responsible for the endotoxicity associated with infections by Gram-negative bacteria. It interacts with a receptor composed of the Toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) on innate immune cells, thereby triggering a response, which eventually results in the release of pro-inflammatory cytokines, such as TNFα and IL-1β [1]. Lipid A consists of a β-1,6-disaccharide of glucosamine (GlcN), which is phosphorylated and acylated with β-hydroxylated acyl chains. These primary fatty acids can be substituted with secondary acyl chains. The lipid A structure varies between species and can be modified in response to environmental conditions. The final structure is defined by the specificity of the enzymes involved in lipid A biosynthesis and the regulated expression of post-synthetic modification systems, which include acylation, de-acylation, dephosphorylation, and substitution of the phosphates with groups like phosphoethanolamine, 4-amino-4-deoxy-L-arabinose (L-Ara4N), or GlcN [2]. The endotoxicity of lipid A varies and depends on the number and the length of the fatty acids as well as on the number and the substitutions of the phosphate groups [2,3].

LPS toxicity is also responsible for undesired side effects of whole-cell vaccines against Gram-negative bacteria [4,5]. This reactogenicity has been intensively studied in human vaccines and, although sometimes ignored in formulations for animals, it is also increasingly becoming a concern for the immunization of livestock and companion animals. This is the case for vaccines against *Bordetella bronchiseptica* [6,7], a Gram-negative bacterium involved in respiratory diseases in mammalian species, such as tracheobronchitis (a.k.a. kennel cough) in dogs and atrophic rhinitis in pigs

Abbreviations: LPS, Lipopolysaccharide.

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https://doi.org/10.1016/j.resmic.2023.104049

[8]. The LPS of this bacterium triggers strong inflammatory responses after TLR4 activation [9], which makes whole-cell vaccine formulations highly reactogenic.

Structural modification of lipid A can be used to reduce TLR4 activation and diminish the reactogenicity of a vaccine. Genetic engineering has been applied to reduce endotoxicity in several bacterial species [10–12], including *B. bronchiseptica* [13]. In the latter study, we showed the effect of inactivation of two genes encoding lipid A acylases, i.e. *pagP* and *lpxL1*, on LPS endotoxicity in a *B. bronchiseptica* dog isolate. PagP mediates the insertion of a secondary palmitate (C_{16}) to the primary acyl chain at the 3' position of lipid A. This enzyme is localized in the OM, and the regulation of its synthesis depends on the major virulence-regulatory system BvgAS [14]. Inactivation of *pagP* resulted in only a limited reduction of endotoxicity, possibly because the PagP-dependent acylation was found to be non-stoichiometric and present in only ~10% of the LPS molecules in the parental strain [13]. LpxL1 is responsible for the insertion of a secondary 2-hydroxy-laurate (2-OH C_{12}) at the 2 position of lipid A. In *B. bronchiseptica*, lipid A is stoichiometrically acylated by LpxL1 [13,15,16]. Although the *lpxL1* gene is also present in the closely related human pathogen *Bordetella pertussis*, the corresponding acylation of lipid A was only observed after its artificial overexpression, which resulted then in an increased TLR4-stimulating activity [17]. Accordingly, inactivation of *lpxL1* in *B. bronchiseptica* drastically reduced TLR4-stimulating activity [13].

Further phenotypic characterization of the *B. bronchiseptica lpxL1* mutant revealed many pleiotropic effects of the mutation, including decreased susceptibility to rifampicin and sodium dodecyl sulfate (SDS), increased susceptibility to polymyxin B, and decreased surface hydrophobicity, auto-aggregation, biofilm formation, and infectivity of macrophages. Structural analysis of lipid A showed that *lpxL1* inactivation did not only result in the expected loss of the 2-OH C_{12} chain but also of the GlcN residues that non-stoichiometrically decorate the phosphate groups in lipid A [13]. This result suggests that the decoration of the phosphates, which is mediated by the glycosyltransferase LgmB in bordetellae [18], is prevented in the absence of the 2-OH C_{12} chain. Since also the loss of these GlcN residues could affect TLR4 activation [19,20], it was not clear whether the decreased TLR4-stimulating activity of the *lpxL1* mutant, or any of its other phenotypes, is due to the loss of the acyl chain, the GlcN residues, or both.

In this study, we aimed to elucidate the role of the absence of GlcN in the aforementioned phenotypes of the *lpxL1* mutant by constructing *lgmB* and *lpxL1* mutants in an isogenic background and comparing their phenotypes. In addition, we studied the effect of the lipid A structural changes on bacterial virulence using the *Galleria mellonella* in vivo model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All bacterial strains used in this study are described in Supplementary Table S1. *Escherichia coli* strains were grown at 37 °C in lysogeny broth (LB) while shaking or on LB agar plates. *B. bronchiseptica* strains were grown at 35 °C on Bordet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading) (BG-blood). For liquid cultures, bacteria were scraped from plate and grown in Verwey medium [21] at 35 °C while shaking at 175 rpm. For biofilm formation assays, Stainer-Scholte (SS) medium [22] supplemented with 14 g/L Bacto casamino acids (BD biosciences) and then adjusted to pH 7.6 was used. The optical density at 600 nm (OD_{600}) of the bacterial suspensions was adjusted to 0.1 with fresh medium, and cultures were grown for 17 h, unless otherwise indicated. When needed for plasmid maintenance or strain selection, the following antibiotics were included in the medium: cefotaxime (5 μg/mL), streptomycin (300 μg/mL), gentamicin (10 μg/mL), kanamycin (50 μg/mL), and ampicillin (100 μg/mL).

2.2. DNA manipulation and construction of mutants

All plasmids used in this study are listed in Supplementary Table S1. PCR fragments used for cloning were generated using the Expand High Fidelity PCR system (Roche Diagnostics GmbH), while regular PCR reactions were performed using DreamTaq DNA polymerase (Thermo Scientific). PCR products were purified using the commercial Wizard SV Gel and PCR Clean-Up System (Promega). Plasmids were isolated with the E. Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). PCR products and plasmids were digested with the appropriate restriction enzymes (Thermo Scientific) according to manufacturer's instructions, purified, and ligated using T4 DNA ligase (5 U/μL) (Thermo Scientific).

To inactivate the *lgmB* gene (locus tag BB4268 in *B. bronchiseptica* reference strain RB50), a DNA fragment was synthesized at BaseGene that included segments of 800 bp of the sequences upstream and downstream of *lgmB* in strain RB50. The entire *lgmB* gene in between, with the exception of 4 bp overlapping with the upstream gene, was replaced in the synthetic sequence by a gentamicin-resistance (gem^R) cassette flanked by Eco81I restriction sites. In addition, the entire synthetic fragment was flanked by XbaI restriction sites. The artificial sequence was introduced in pUC57-Kan by blunt-end cloning into the SmaI restriction site of the vector. The resulting plasmid was introduced in *E. coli* strain DH5α by transformation using the $CaCl_2$ method. Strain selection was performed on LB plates containing either kanamycin alone or both kanamycin and gentamicin. No colonies were obtained on the plates with both antibiotics, suggesting that the gem^R cassette was not expressed. Therefore, an alternative gem^R cassette was PCR-amplified from plasmid pYRC using forward primer Fw-Eco81I-GemR (GCGCGCCCTGAGGGACGCACACCGTGGAAA) and reverse primer Rv-GemR-Eco81I (GCGCGCCCTCAGGGCGGCG TTGTGACAATTT), which both introduced Eco81I restriction sites (underlined). Then, the amplicon was used to replace the synthetic gem^R cassette by digestion of the plasmid and PCR product with Eco81I and ligation. After transformation of strain DH5α, colonies were obtained on LB plates containing both antibiotics, and the orientation of the new gem^R cassette in the direction of transcription of the *lgm* operon was confirmed by PCR on isolated colonies. From the plasmid obtained, named pUC57-Kan Δ*lgmB*, the knockout construct was then subcloned into the suicide vector pKAS32 after digestion of both plasmids with XbaI and ligation, yielding pKAS-Δ*lgmB*. This plasmid was used to transform *E. coli* strain SM10 (λpir), which allowed for plasmid replication and subsequent transfer to *B. bronchiseptica* by conjugation. Chromosomal knockouts were obtained by allelic exchange. First, transconjugants were selected on plates containing gentamicin and cefotaxime for selection of plasmid integration and counterselection against *E. coli*, respectively. Subsequently, selection was made with streptomycin and gentamicin to ensure the loss of the plasmid backbone together with the wild-type *lgmB* gene. Transconjugants were screened by PCR.

2.3. TLR4 stimulation assays

TLR4 stimulation assays were performed as previously described [13]. Briefly, HEK-Blue TLR4 cells co-expressing either human (h-) or murine (m-)TLR4, MD-2, and CD14 genes and an NF-κB-inducible gene for a secreted embryonic alkaline phosphatase

(SEAP) reporter (Invivogen) were incubated with serial dilutions of whole bacterial cells, which were killed by incubation for 1 h at 56 °C. After 17 h of incubation at 37 °C in a 5% saturated CO_2 atmosphere, supernatants were incubated with *p*-nitrophenyl phosphate solution for 1 h, and the absorbance at 405 nm was measured in a Biotek microplate reader.

2.4. Sensitivity to SDS and antimicrobials

Sensitivity to SDS and antimicrobials was determined as described previously [13]. In short, exponentially growing bacterial cultures were adjusted to an OD_{600} of 0.1 in fresh medium and incubated with either SDS or, as a control, Milli-Q water. After 2 h incubation, 10-fold serial dilutions were prepared, and 10-μL drops of each dilution were spotted on BG-blood agar plates. Growth was evaluated after 72 h of incubation at 35 °C. Minimal inhibitory concentrations (MICs) of different antibiotics were determined by Etest (BioMerieux). Briefly, 200 μL of exponentially growing bacterial cultures were spread on BG-blood agar, and Etest strips were placed on the plates. After two days of incubation, MICs were calculated from the zone of growth inhibition.

2.5. Bacterial adhesion to hydrocarbons (BATH)

BATH assays [23] were performed with previously described modifications [13]. Briefly, bacterial cultures grown for 20 h were washed and resuspended in phosphate-buffered saline (PBS), and the OD_{600} was adjusted to 1. The bacterial solution was added to test tubes with or without hexadecane and, after vigorous vortexing, the aqueous and organic phases were allowed to separate during 15 min. Samples were taken from the aqueous phase and their OD_{600} was measured. BATH (%) was expressed as $(n-h) \times 100/n$, with n being the OD_{600} in the tube without hexadecane and h the OD_{600} in the tube with hexadecane.

2.6. Biofilm formation

Biofilms were formed and quantified as previously described [13]. In short, cultures grown for 24 h in SS medium supplemented with casamino acids were adjusted to an OD_{600} of 0.5 and incubated for 24 h under static conditions at 35 °C in 24-well plates. Then, the biofilms formed were washed and stained for 2 min with 0.5% crystal violet. After two washes, the stained biofilm was resuspended in 500 μL of 33% (v/v) acetic acid, and the OD_{630} was quantified.

2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cells were mixed with sample buffer [24], boiled for 10 min, and analyzed on 8–16% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad). After electrophoresis, LPS was stained with silver [25].

2.8. Porcine bone marrow-derived macrophages (PBMMs)

PBMMs were grown as previously described [26] with modifications [13]. Briefly, bone marrow was harvested from six young adult pigs, and mononuclear cells were isolated by Ficoll (GE Healthcare) density gradient centrifugation. For M1-macrophage differentiation, cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal calf serum (Corning) for 6 days exposed to 40 ng/mL granulocyte macrophage colony-stimulating factor (Bio-Rad). Expression of M1 surface markers was verified by flow cytometry [26]. All animals were used and kept under the approval and guidelines of the animal ethical committee of Utrecht University.

Bacterial cultures were washed and resuspended in RPMI supplemented with 10% fetal calf serum. Bacterial suspensions were added to M1-macrophages at a multiplicity of infection (MOI) of 1 and incubated for 4 h. After removal of the supernatant, macrophages were lysed with 1% Triton X-100 in PBS. To measure only internalized bacteria, the cells were exposed to 150 μg/mL colistin sulfate (Sigma Aldrich) in PBS for 1 h at 37 °C and then washed before lysis. Samples from supernatant, lysate (attached + internalized bacteria), or lysate obtained after antibiotic treatment (internalized bacteria) were plated on BG-blood agar and incubated for 72 h at 37 °C, and colonies were counted. Total growth was calculated from the colony-forming units (CFU) in the supernatant plus those in the lysate. Attached bacteria were calculated from the CFU in the lysate minus those in the lysate of cells treated with antibiotics. After that, the CFU quantified for each fraction were normalized relative to CFU quantified in wells without macrophages.

2.9. G. mellonella *in vivo test of virulence*

Bacterial cultures grown for 17 h in Verwey medium were washed and resuspended in Dulbecco's PBS (DPBS) (Sigma Aldrich) to an OD_{600} of 10. Then, 10 μL of bacterial suspension were injected in the last proleg of larvae purchased at a pet shop using a NovoPen 5 insulin pen (Novo Nordisk) combined with BD Micro-Fine Ultra (4 mm) needles. Ten larvae of approximately 180–350 mg were injected per group. After injection, larvae were placed in the dark at 35 °C, and their survival was monitored every 24 h. Death was assessed by absence of response to physical stimuli. A control group was injected with DPBS to discard death due to mechanical trauma.

2.10. Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (version 6). In all cases, data was analyzed for statistical significance using either one- or two-way ANOVA (Dunnett's correction for multiple comparison).

3. Results

3.1. TLR4 activation by whole cells

To study the contribution of the loss of the GlcN decorations of lipid A to the various phenotypes of an *lpxL1* mutant, the *lpxL1* and *lgmB* genes were inactivated in strain BB-CHN-1P, a *B. bronchiseptica* isolate from pig. This strain was chosen as swine livestock is one of the main targets for *B. bronchiseptica* vaccines. Then, the TLR4-stimulating activities of heat-killed whole-cell suspensions of the wild type (WT) and the *lpxL1* and *lgmB* mutants were compared in HEK-Blue reporter cells expressing either h- or m-TLR4 (Fig. 1). As reported before in the genetic background of dog isolate BB-D09-SR [13], inactivation of *lpxL1* resulted in drastically decreased activation of both h-TLR4 (Fig. 1A) and m-TLR-4 (Fig. 1B) as compared with WT cell preparations. Also preparations of the *lgmB* mutant strain showed a statistically significant decrease in h-TLR4 activation compared to WT cells, but the difference was much smaller than in the case of the *lpxL1* mutant (Fig. 1A). In contrast, m-TLR4 stimulation was unaffected by *lgmB* inactivation (Fig. 1B). These results demonstrate that the reduced potency of the *lpxL1* mutant to activate TLR4 is mainly due to the loss of the 2-OH C_{12} chain in lipid A, whilst the GlcN decorations also contribute to h-TLR4 but not to m-TLR4 activation.

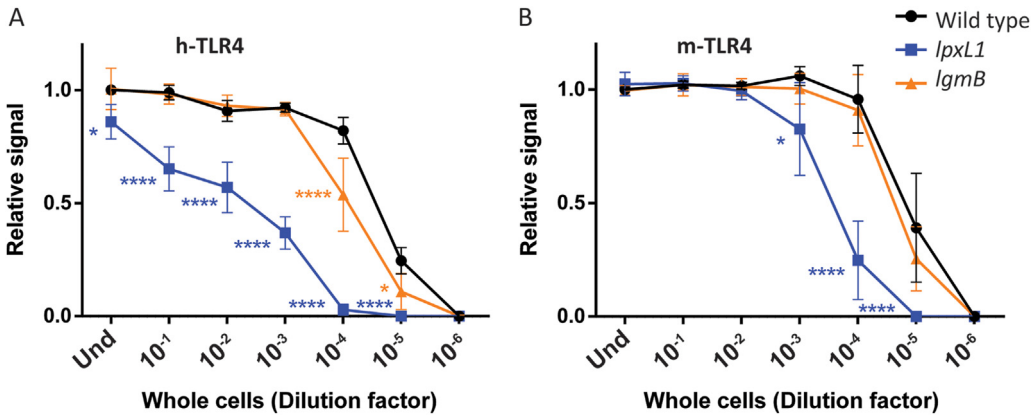

Fig. 1. TLR4 activation by whole-cell preparations of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives. HEK-Blue cells expressing either h-TLR4 **(A)** or m-TLR4 **(B)** were incubated for 17 h with 10-fold serial dilutions of heat-inactivated whole bacterial cells. The OD_{600} of the undiluted bacterial suspensions (Und) was 0.04. Graphs show means and standard deviations of relative SEAP activity calculated as the ratio between the signal measured for each dilution of each strain and the signal measured for the undiluted wild-type sample. Three independent experiments were performed in duplicate. Dilutions of the mutants with activities statistically different from those of the wild type are indicated with asterisks (*, $p \leq 0.05$; ****, $p \leq 0.0001$).

3.2. OM barrier function

To test the integrity of the OM barrier function, SDS-sensitivity assays were performed. Consistent with previous results [13], the *lpxL1* mutant of strain BB-CHN-1P survived exposure to SDS much better than the WT did (Fig. 2). On the other hand, the *lgmB* mutant strain showed similar sensitivity to SDS as the WT, demonstrating that the higher SDS sensitivity of the WT compared with the *lpxL1* mutant is due to the presence of the 2-OH C_{12} chain in lipid A and not to the GlcN decorations.

Furthermore, we compared the susceptibility of the WT and the mutants to the cationic antimicrobial peptides (CAMPs) polymyxin B and colistin (Table 1). Like the *lpxL1* mutant, the *lgmB* mutant strain showed a two-fold lower MIC for polymyxin B than the parental strain, indicating that the GlcN substitutions protect the WT against polymyxin B. In contrast, whilst the *lpxL1* mutant is slightly more sensitive to colistin than the WT, inactivation of *lgmB* did not affect colistin susceptibility (Table 1). Similarly, the susceptibility of the mutants to other antimicrobials was tested. Whilst susceptibility to the amphipathic antibiotic ciprofloxacin was hardly or not affected in the *lpxL1* or *lgmB* mutants, the *lpxL1* mutant was resistant to the hydrophobic antibiotic rifampicin in contrast to the *lgmB* mutant whose susceptibility to rifampicin was barely affected as compared with the WT (Table 1). Thus, the loss of the 2-OH C_{12} chain protects the *lpxL1* mutant against rifampicin.

Table 1
Antimicrobial susceptibility of strain BB-CHN-1P and its mutant derivatives[a].

	Wild type	*lpxL1*	*lgmB*
Polymyxin B	0.38	0.19	0.19
Colistin	0.125	0.094	0.125
Ciprofloxacin	0.38	0.50	0.38
Rifampicin	3	>32	4

[a] MICs reported are in μg/mL. Results were obtained in at least three independent experiments.

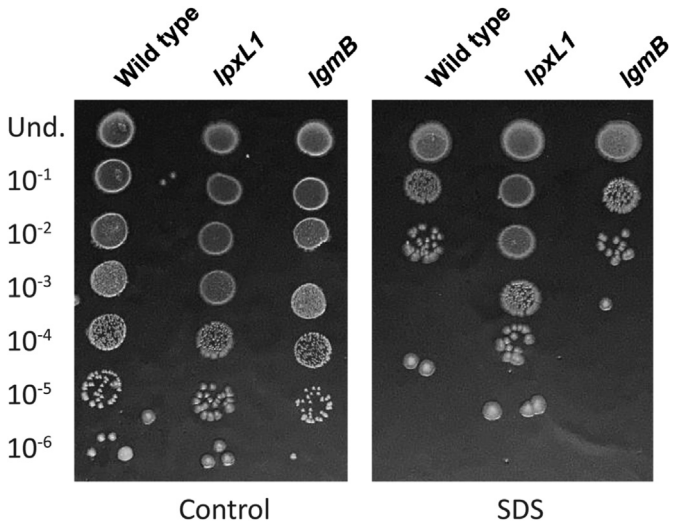

Fig. 2. Bacterial sensitivity to SDS. Cultures of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were incubated with 1% SDS. After 2 h incubation, 10-μL drops of 10-fold serial dilutions from these cultures were plated on BG-blood agar and incubated for 72 h. The control (left panel) shows the results for bacteria not exposed to SDS. A representative result of three independent experiments is shown.

3.3. Cell-surface hydrophobicity and biofilm formation

Effects of the mutations on cell-surface hydrophobicity were tested by employing the BATH method. In concord with previous results [13], *lpxL1* inactivation in strain BB-CHN-1P strongly reduced cell-surface hydrophobicity, which dropped from 91% for the WT to ~32% for the mutant (Fig. 3A). A statistically significant reduction in surface hydrophobicity was also observed for the *lgmB* mutant, but, as it dropped only to 85%, this reduction in hydrophobicity is negligible compared to the effect of *lpxL1* inactivation (Fig. 3A).

Since changes in cell-surface hydrophobicity can affect biofilm formation [27], also biofilm formation was measured. As compared to the WT, no significant reduction in biofilm formation was observed in the *lgmB* mutant strain, in contrast to the *lpxL1* mutant strain, which showed much lower production of biofilm (Fig. 3B), consistent with previous results [13]. These results suggest that the reduction in surface hydrophobicity and in biofilm formation of the *lpxL1* mutant is mainly a consequence of the loss of the 2-OH C_{12} chain.

We previously reported that the *lpxL1* mutant of dog isolate BB-D09-SR produced increased amounts of O-antigen-containing LPS, which could potentially explain the reduction in cell-surface hydrophobicity [13]. To examine O-antigen production in strain BB-

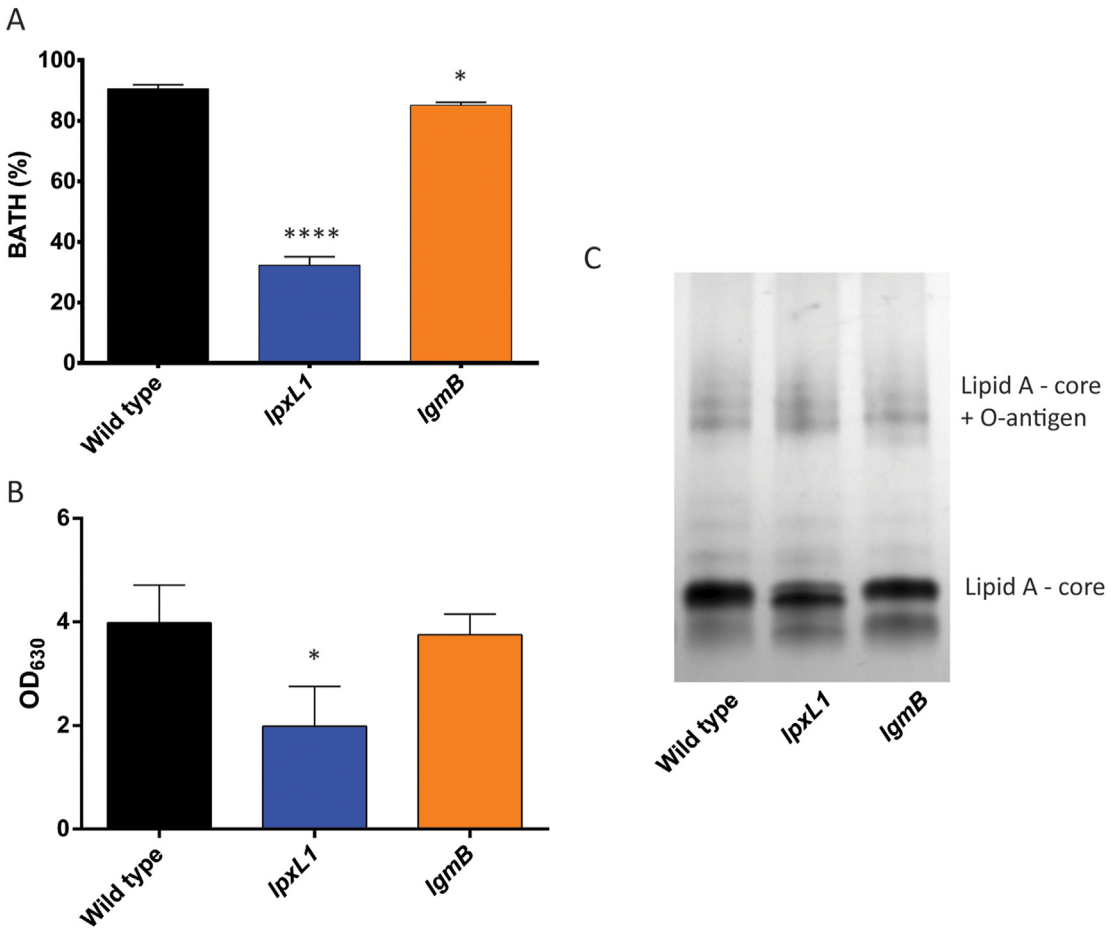

Fig. 3. Effect of the inactivation of *lgmB* on surface hydrophobicity, biofilm formation, and LPS structure. **(A)** Surface hydrophobicity was assessed using the BATH method. Bacterial suspensions of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were mixed with hexadecane, and the percentage of hydrophobicity was calculated from OD_{600} measurements of samples from the hydrophilic phase. Data represent means and standard deviations from three independent experiments performed in duplicate. **(B)** Biofilm formation. Biofilms were grown for 24 h under static conditions in SS medium supplemented with casamino acids, stained with crystal violet, and quantified by measuring the OD_{630}. Data represent means and standard deviations from three experiments performed in triplicate. In both panels A and B, statistically significant differences compared to the wild type are indicated with asterisks (*, $P \leq 0.05$; ****, $p \leq 0.0001$). **(C)** Analysis of LPS modifications by SDS-PAGE. Whole-cell lysates of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were analyzed by SDS-PAGE, and LPS was stained with silver. O-antigen-containing LPS appears as diffuse smear with lower electrophoretic mobility than the lipid A-core forms.

CHN-1P and its mutant derivatives, whole-cell lysates were treated with proteinase K and analyzed by SDS-PAGE. The majority of the LPS detected does not contain O-antigen and consists of lipid A and the core oligosaccharide (Fig. 3C). Consistent with previous results in strain BB-D09-SR [13], a slightly increased electrophoretic mobility of this form of the LPS was observed in the *lpxL1* mutant (Fig. 3C). Since a similar increase in electrophoretic mobility was not observed in the *lgmB* mutant, it is mainly due to the loss of the 2-OH C_{12} chain and not of the GlcN residues. In all three strains, similar amounts of O-antigen-containing LPS were detected (Fig. 3C). This suggests that the increased amount of O-antigen previously observed in the *lpxL1* mutant of strain BB-D09-SR is a strain-specific consequence of the *lpxL1* mutation and not (solely) responsible for the observed reduction in surface hydrophobicity and biofilm formation.

3.4. Infection of macrophages

To determine whether the mutations affect the viability of *B. bronchiseptica* in the presence of macrophages, the bacteria were incubated for 4 h with porcine macrophages, and bacterial survival was quantified. The *lpxL1* mutant of BB-CHN-P1 showed a slightly increased viability in the presence of macrophages, which is in agreement with previous results [13], but the *lgmB* mutant showed a slightly reduced viability relative to the WT (Fig. 4A). These differences were reflected in the supernatant (Fig. 4B), but not in the bacterial adhesion to macrophages (Fig. 4C). The *lgmB* mutant showed increased ability to attach to the immune cells compared with the WT and *lpxL1* mutant strains. However, none of these differences was statistically significant. Interestingly, however, the amount of both the *lgmB* mutant and of the *lpxL1* mutant detected inside the macrophages was significantly lower than that of the WT (Fig. 4D). Thus, the reduced infectivity of macrophages of the *lpxL1* mutant, also previously reported in strain BB-D09-SR [13], appears to be related to the loss of the GlcN decorations of lipid A, rather than to the loss of the 2-OH C_{12} chain.

3.5. Virulence of B. bronchiseptica mutants in the G. mellonella infection model

To determine the effect of different lipid A structural modifications on virulence, we injected equivalent amounts of bacterial suspensions of the WT strain BB-CHN-P1 and its *lpxL1* and *lgmB* mutant derivatives into *G. mellonella* larvae and monitored larval

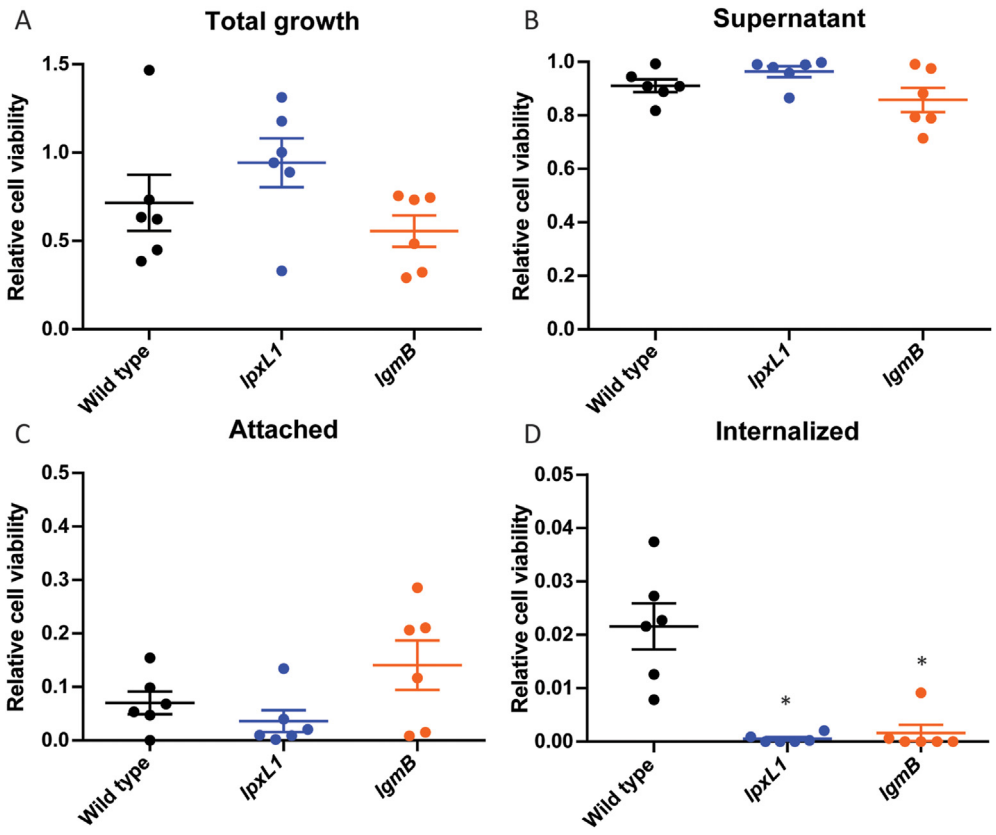

Fig. 4. Bacterial survival with porcine macrophages. Suspensions of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were incubated for 4 h with M1 macrophages at an MOI of 1. Data represent cell viability calculated from the CFU in different fractions expressed relative to CFU after incubation in the absence of macrophages. **(A)** Total relative bacterial count (i.e. both inside and outside of the macrophages). **(B)** Relative bacterial count in the supernatant. **(C)** Relative count of bacteria attached at the macrophage surface. **(D)** Relative bacterial count inside macrophages. Symbols correspond to PBMMs of six separate pigs. Statistical significance compared to the wild type was found for the internalized bacteria and is indicated with asterisks (*, $P \leq 0.05$).

mortality over time. Independent experiments consistently showed increased survival of the larvae infected with the *lpxL1* mutant compared to those infected with the WT or the *lgmB* mutant (see representative example in Fig. S1A). However, combination of the data from all replicate experiments resulted in large standard deviations and, consequently, differences were not statistically significant. This is probably due to the use of larvae from a pet shop, which are known to present large batch-to-batch variation due to differences in breeding and storage conditions. Such variation may affect the status of the immune system of the model at the time of the assay [28]. Therefore, we decided to express the combined results of the experiments as the difference in survival of mutant-infected larvae relative to that of WT-infected larvae per day in percentage points. In this way, we observed a small but significant effect on virulence by the *lpxL1* mutation (Fig. 5A). To verify this result, we also tested the effect of *lpxL1* inactivation in the genetic background of strain BB-D09-SR. Of this strain, we also had a *pagP* mutant at our disposal, which we also tested. Again, the *lpxL1* mutation resulted in reduced virulence compared to the WT and the *pagP* mutant (see representative example in Fig. S1B). When data from all replicate experiments were combined (Fig. 5B), the effect on virulence of the *lpxL1* mutation was significant and even more prominent than in the genetic background of strain BB-CHN-1P (compare Fig. 5A and B). As the virulence of the *lgmB* mutant was unaffected (Fig. 5A and Fig. S1A), the reduced virulence of the *lpxL1* mutants appears to be due to the loss of the 2-OH C_{12} chain, rather than to the loss of the GlcN modifications. Interestingly, the *pagP* mutant showed slightly increased virulence (Fig. 5B and Fig. S1B), but this difference was not statistically significant.

4. Discussion

Inactivation of the *lpxL1* gene in *B. bronchiseptica* strain BB-D09-SR resulted in many pleiotropic effects [13]. Structural analysis of lipid A showed the expected loss of the 2-OH C_{12} acyl chain and, unexpectedly, also of the GlcN residues that decorate the phosphates in lipid A. In this study, we wanted to determine which structural alteration is responsible for the different phenotypes observed. Therefore, we constructed an *lgmB* mutant in strain BB-CHN-P1 and compared its phenotypes with those of an isogenic *lpxL1* mutant. A caveat of this study is that we didn't perform mass spectrometric analysis of lipid A to confirm that the *lpxL1* mutation also leads to the loss of the GlcN decorations in strain BB-CHN-P1. However, the myriad of phenotypes described for the *lpxL1* mutant of strain BB-D-09-SR was also found in that of strain BB-CHN-P1, suggesting the presence of identical structural changes in the LPS. Moreover, some of these phenotypes were also found in the *lgmB* mutant demonstrating that they can be attributed to the loss of the GlcN residues. In addition, it has been reported that mutation of *lpxM*, the gene encoding an acyltransferase responsible for the attachment of a secondary acyl chain to lipid A, in *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) prevents the decoration of the phosphates with L-Ara4N [29]. Thus, a complete acylation pattern appears to be a common prerequisite for the glycosylation of the phosphates of lipid A, and, therefore, strain-specific differences in *B. bronchiseptica* in this respect seem to be very unlikely.

We found that *lgmB* deletion reduces the h-TLR4-stimulating activity but to a much lesser extent than *lpxL1* inactivation did.

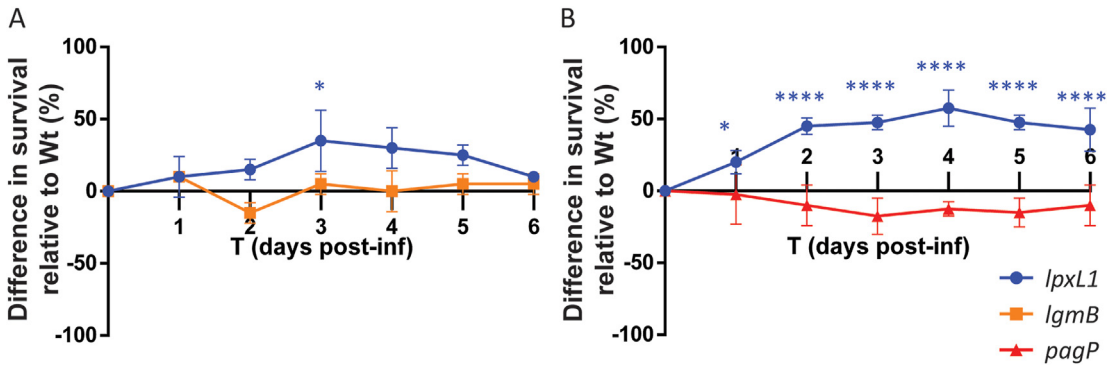

Fig. 5. Survival of *G. mellonella* larvae infected with *B. bronchiseptica* WT strains and *lgmB*-, *lpxL1*-, and *pagP*-mutant derivatives. The *G. mellonella* larvae were infected with OD-adjusted bacterial suspensions of strains BB-CHN-1P **(A)** or BB-D09-SR **(B)** and their mutant derivatives. Survival was monitored every 24 h over 6 days post infection. Graphs show the difference in survival of larvae injected with mutant derivatives relative to those infected with the corresponding parental strain in percentage points. Data represent means and standard deviations from three independent experiments. For the mutants, statistically significant differences compared to the WT infections are indicated with asterisks (*, $p \leq 0.05$; ****, $p \leq 0.0001$).

Furthermore, in contrast to *lpxL1* inactivation, it had no effect on m-TLR4-stimulating activity. It is also noteworthy that the *lpxL1* mutation has a considerably stronger effect on h-TLR4 activation than on m-TLR4 activation (Fig. 1, compare panels A and B). This data points to an additive effect of the loss of the GlcN residues and of the acyl chain on h-TLR4 activation, while m-TLR4 activation is only affected by the loss of the acyl chain. With respect to the relevance of the GlcN residues, our results are in agreement with previous observations in *B. pertussis*, where it was demonstrated that *lgmB* inactivation resulted in drastic decline of h-TLR4 stimulation but had no effect on m-TLR4 activity [19]. In another study, reduced production of pro-inflammatory cytokine IL-6 was observed after stimulation of human monocytes with *lgmB* mutants of *B. pertussis* or *Bordetella parapertussis* [20]. In addition, it has been reported that the production of the cytokine TNFα in murine macrophages upon stimulation with *B. bronchiseptica* cells was not affected by *lgmB* inactivation [30]. Altogether, it appears that the loss of the secondary 2-OH C_{12} acyl chain in the *lpxL1* mutant strongly reduces TLR4 activation, whereas the effect of the loss of GlcN residues on TLR4 stimulation is smaller and species specific as it additively reduces the activation of h-TLR4 but not of m-TLR4.

The OM of the *lpxL1* mutant is fortified as appears from the reduced susceptibility to hydrophobic (rifampicin) and amphipathic (SDS) antimicrobial compounds (Table 1 and Fig. 2). We expected this to be related to the loss of the GlcN residues [13], since it should enable the phosphates in lipid A to bind divalent cations, which in turn should promote cross linking between LPS molecules and boost OM integrity [31]. However, inactivation of *lgmB* hardly affected the susceptibility to these antimicrobials. Only a slight decrease in susceptibility to rifampicin was observed, which, indeed, could be due to improved cross linking between LPS molecules. Thus, the loss of the 2-OH C_{12} chain appears to be much more relevant for the decrease in OM permeability of the *lpxL1* mutant. Probably the loss of this hydroxylated acyl chain improves the packing of the remaining acyl chains in the outer leaflet of the OM, thus preventing the penetration of the antimicrobial compounds into the hydrophobic interior of the bilayer.

It is well established that the decoration of the phosphate groups of lipid A can reduce bacterial susceptibility to CAMPs [2], but also the insertion of acyl chains can have a similar effect, as has been demonstrated for example in *E. coli* and *Klebsiella pneumoniae* [32,33]. Inactivation of *lgmB* indeed resulted in increased susceptibility to the CAMP polymyxin B (Table 1), which matches previous observations [30]. However, this increment was similar to that of the *lpxL1* mutant, suggesting that the reduced lipid A acylation in the latter had no additional impact on polymyxin B sensitivity. Although CAMPs are supposed to have a common mechanism of action, no effect of *lgmB* inactivation was observed on the susceptibility to colistin (a.k.a. polymyxin E), which has a chemical structure very similar to that of polymyxin B. Such contrasting results have also been reported for the susceptibility of an *lgmB* mutant to the CAMPs porcine β-defensin 1 (pBD1) and the closely related mouse β-defensin 3 (mBD3) [30]. While the mutant showed increased susceptibility to pBD1, no differences in susceptibility were detected for mBD3. Interestingly, the *lpxL1* mutant did show slightly increased sensitivity to colistin (Table 1) indicating that the loss of the acyl chain facilitates the activity of colistin but not of polymyxin B. Again, this discrepancy suggests that the two polymyxins do not have an identical mechanism of action.

Previously, we reported a strong decrease in surface hydrophobicity of an *lpxL1* mutant of strain BB-D09-SR [13]. We hypothesized this decrease to be related to the increase in the amount of LPS containing O-antigen, which was observed in this mutant. However, this increase in O-antigen seems to be strain specific, inasmuch as it was not observed in the genetic background of strain BB-CHN-1P, which was used in the present study (Fig. 3C). Nevertheless, reduced surface hydrophobicity and a concomitant decrease in biofilm production were also observed in the *lpxL1* mutant of this strain, but not in the *lgmB* mutant. Thus, apparently, the decreased hydrophobicity observed in the *lpxL1* mutant is related to the loss of the acyl chain, but how this structural change in lipid A exactly affects this phenotype is not immediately clear.

The capacity of *B. bronchiseptica* to invade macrophages was reduced upon inactivation of *lpxL1* [13]. As *lgmB* inactivation similarly affected macrophage invasion (Fig. 4D), the loss of the GlcN residues seems to be responsible for this defect. Previously, we suggested that the absence of the secondary 2-OH C_{12} chain was likely responsible for this phenotype [13]. This hypothesis was based on earlier observations in an *lpxL1* mutant of *B. pertussis*, whose capacity to invade macrophages was similarly affected as was the *lpxL1* mutant of *B. bronchiseptica* [17]. However, GlcN decorations can also be found in *B. pertussis* lipid A [18,20], and it is likely that inactivation of *lpxL1* in *B. pertussis* hinders lipid A glycosylation similarly as in *B. bronchiseptica*. Thus, the loss of the GlcN residues in lipid A is probably also responsible for the reported defect in macrophage invasion in *B. pertussis* [17]. Interestingly, it was recently reported that also the decoration of lipid A with L-Ara4N in a colistin-resistant *K. pneumoniae* strain enhances the survival of the bacteria in human monocytes [34]. Defense mechanisms of macrophages against intracellular bacteria include the

production of CAMPs [35]. Since the susceptibility of the *lgmB* mutant to polymyxin B is increased (Table 1), the CAMPs produced by the macrophages could be responsible of the reduced presence of intracellular bacteria lacking the GlcN decorations in lipid A.

The *G. mellonella* infection model has been established as a suitable in vivo model to study host−pathogen interactions due to its similarity to mammalian models with respect to the innate immune response, including the presence of a cellular response (i.e. hemocytes) and a humoral response (i.e. antimicrobial peptides, opsonins, and melanization). This model is easy to manipulate and reduces the use of mammalian models and costs [28]. Unfortunately, the commercial *G. mellonella* TruLarv, which was specifically bred for scientific purposes, is not available anymore, as the producer has gone out of business because of trading problems due to the Brexit and the COVID-19 pandemic. Since another supplier of similar larvae could not be found, we had to rely on larvae obtained from a pet shop, which are known to yield considerable variation between experiments [28]. Nevertheless, we were able to show that inactivation of *lpxL1* significantly reduces the virulence of *B. bronchiseptica*, which could be related to an increased susceptibility of the mutant to the antimicrobial peptides produced by the larvae. Similarly, a mutant of *S.* Typhimurium with a defective *phoQ* gene, which is part of the two-component virulence regulatory system PhoPQ and regulates, amongst others, the decoration of the phosphate groups in lipid A with L-Ara4N, showed reduced virulence in the *G. mellonella* model and was less resistant to polymyxins [36]. However, the *lgmB* mutant did not show reduced virulence despite being as susceptible to polymyxin B as the *lpxL1* mutant (Fig. 5 and Table 1). As the *lgmB* mutant, in contrast to the *lpxL1* mutant, is not affected in colistin susceptibility, the mutants might also respond differently to the antimicrobial peptides produced by the larvae, and the presence of the secondary 2-OH C_{12} chain in lipid A might confer protection against these peptides. Alternatively, the LPS of *B. bronchiseptica* might be toxic to the *G. mellonella* larvae as has been demonstrated for LPS from *Caulobacter* spp [37]. If that is the case, the loss of the 2-OH C_{12} chain might result in reduced LPS toxicity.

Taken together, our results indicate that the loss of the secondary 2-OH C_{12} chain in *B. bronchiseptica* lipid A strongly reduces TLR4-stimulating activity, cell-surface hydrophobicity, and biofilm formation, and it fortifies the OM barrier function as evidenced by increased resistance to SDS and rifampicin. All these modifications have been observed in two different genetic backgrounds, discarding accidental secondary mutations as the reason of these changes. The GlcN decorations also contribute to the h-TLR4-stimulating activity but much less so than the secondary acyl chain, and they do not contribute to the m-TLR4-stimulating activity. Therefore, *lpxL1* mutants of *B. bronchiseptica* might be more valuable in the development of new whole-cell- or OMV-based vaccines with reduced endotoxicity than *lgmB* mutants. Furthermore, the GlcN residues confer protection against polymyxin B and improve macrophage invasion. We also demonstrate that *G. mellonella* can be a valuable model to investigate the effect of genetic modifications on the virulence of *B. bronchiseptica*, and the results obtained indicate that the secondary 2-OH C_{12} chain in lipid A contributes to the virulence of the bacteria. However, breeding conditions of the larvae should be meticulously controlled to avoid large differences between experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Esther M. Keizer for support with larvae experimental setup. This work received funding from the domain Applied and Engineering Sciences (TTW) of The Netherlands Organization for Scientific Research (NWO) (TTW Perspectief grant numbers 14921 and 14924), which received financial contributions for this grant from GlaxoSmithKline Biologicals SA and PULIKE Biological Engineering Inc.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2023.104049.

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