



Glucose Decoration on Wall Teichoic Acid Is Required for Phage Adsorption and InlB-Mediated Virulence in *Listeria ivanovii*

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ABSTRACT *Listeria ivanovii* (*Liv*) is an intracellular Gram-positive pathogen that primarily infects ruminants but also occasionally causes enteric infections in humans. Albeit rare, this bacterium possesses the capacity to cross the intestinal epithelium of humans, similar to its more frequently pathogenic cousin, *Listeria monocytogenes* (*Lmo*). Recent studies in *Lmo* have shown that specific glycosyl modifications on the cell wall-associated glycopolymers (termed wall teichoic acid [WTA]) of *Lmo* are responsible for bacteriophage adsorption and retention of the major virulence factor internalin B (InlB). However, the relationship between InlB and WTA in *Liv* remains unclear. Here, we report the identification of the unique gene *liv1070*, which encodes a putative glucosyltransferase in the polycistronic WTA gene cluster of the *Liv* WSLC 3009 genome. We found that in-frame deletion of *liv1070* led to loss of the glucose substitution on WTA, as revealed by ultraperformance liquid chromatography–mass spectrometry (UPLC-MS) analysis. Interestingly, the glucose-deficient mutant became resistant to phage B025 infection due to an inability of the phage to adsorb to the bacterial surface, a binding process mediated by the receptor-binding protein B025_Gp17. As expected, deletion of *liv1070* led to loss of InlB retention on the bacterial cell wall, which corresponded to a drastic decrease in cellular invasion. Genetic complementation of *liv1070* restored the characteristic phenotypes, including glucose decoration, phage adsorption, and cellular invasion. Taken together, our data demonstrate that an interplay between phage, bacteria, and host cells also exists in *Listeria ivanovii*, suggesting that the trade-off between phage resistance and virulence attenuation may be a general feature in the genus *Listeria*.

IMPORTANCE *Listeria ivanovii* is a Gram-positive bacterial pathogen known to cause enteric infection in rodents and ruminants and occasionally in immunocompromised humans. Recent investigations revealed that in its better-known cousin *Listeria monocytogenes*, strains develop resistance to bacteriophage attack due to loss of glycosylated surface receptors, which subsequently results in disconnection of one of the bacterium's major virulence factors, InlB. However, the situation in *L. ivanovii* remains unclear. Here, we show that *L. ivanovii* acquires phage resistance following deletion of a unique glycosyltransferase. This deletion also leads to dysfunction of InlB, making the resulting strain unable to invade host cells. Overall, this study suggests that the interplay between phage, bacteria, and the host may be a feature common to the genus *Listeria*.

KEYWORDS *Listeria* subspecies, WTA biosynthesis, carbohydrate substitution, phage-host recognition, phage resistance, glycotyping, virulence factor, cellular invasion

The *Listeria* genus contains two species that are capable of causing disease in mammals. *Listeria monocytogenes* (*Lmo*) is by far the more common of the two; it is capable of invading and replicating within mammalian cells and has a mortality rate of up to 30% (1). While *Lmo* is capable of causing disease in both animals and humans, *Listeria ivanovii* (*Liv*) does so almost exclusively in ruminants (2) and rodents (3). *Liv* has

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on occasion demonstrated its ability to cause disease in humans (4–7), showing that while it may be less adapted to cause established infection in humans, it possesses all the necessary virulence factors and has the capability given the right patient circumstances. There are two subspecies of *Liv*, *Listeria ivanovii* subsp. *ivanovii* and *Listeria ivanovii* subsp. *londoniensis*. Both have been described and categorized based on the ability to metabolize *N*-acetyl-mannosamine and ribose. *Liv* subsp. *ivanovii* is generally sensitive to infection by many phages, while strains of *Liv* subsp. *londoniensis* appear to be quite resistant to phage attack due to the presence of a functional type II-A CRISPR-Cas system (8). To date, only *Liv* subsp. *ivanovii* has been shown to cause listeriosis in human and animals (3, 9). Like *Lmo* infections, *Liv* infections are thought to be foodborne, as bacteria have been isolated from both the feces and blood of infected human patients (4). From this, it can be hypothesized that *Liv* also possesses the capability to cross membrane barriers during infection; indeed, it has been shown in cell culture to be internalized in both human and bovine cell lines and possesses a major pathogenicity island similar to that in *Lmo* (6). *Liv* is also thought to be rarer in the environment, suggesting that host tropism and lower pathogenicity may not be the only reasons for its infrequent disease occurrence.

All species in the genus *Listeria* can be characterized by serotyping, a serological process that relies upon the structural variation of the bacterial cell surface. Of the many serovars (SVs) within the species *Lmo*, 1/2 and 4b cause the vast majority of disease in humans, suggesting that there may be a clinical relevance for the surface structures that confer serovar identity (10), which are primarily the wall teichoic acids (WTAs) (11). WTAs are complex carbohydrate molecules that make up the majority (up to ~60%) of the dry weight of the bacterial cell wall and are covalently conjugated to the peptidoglycan and extend outward (12). Beyond serving as the major antigenic determinants for serotyping, WTAs are involved in several key physiological functions, including maintaining osmotic pressure, antibiotic resistance, virulence, and interaction with host cells and bacteriophages (13–18). WTAs consist of a single glycosylated glycerol-based linkage unit and a chain of 20 to 30 repeating units which can vary in structure between individual strains (19). In *Listeria*, the WTA repeating units are generally made up of ribitol phosphate (type I WTA), but in some serovars, they contain an *N*-acetylglucosamine (GlcNAc) residue integrated into the chain (type II WTA) (20). Further structural variation stems from the carbon position at which GlcNAc is linked to the ribitol residue, or further glycosylation or other modifications, termed “decorations” (20). *Liv* strains are designated SV 5 and feature an integrated GlcNAc residue conjugated to the C-2 position of the ribitol in the primary WTA chain. This GlcNAc is further decorated with a glucose residue in most cases, but the chain can contain interspersed repeating units that are *O*-acetylated or not modified at all (21).

Work in our lab with *Lmo* showed that in the highly virulent SV 4b strains, bacteriophage predation can select for resistant strains that lack a galactose (Gal) decoration on their WTA, as certain bacteriophages specifically recognize Gal for their adsorption (22). This consequently led to an inability of the mutant strains to invade host cells and a drastic reduction in virulence, due to the fact that the major virulence factor internalin B (InIB) relies upon this Gal modification for its retention to the bacterial surface (22). InIB is well established as playing a large role in the invasion of the host liver, spleen, and placenta by recognizing the cMet receptor and inducing the endocytic pathway (23, 24). We showed that the InIB virulence factor relies exclusively on WTA and not lipoteichoic acid (a similar, membrane-anchored glycerol-based polymer), which was previously thought to be responsible for InIB's surface retention (25). Similar studies in *Lmo* SV 1/2 showed that InIB relies on the rhamnose residue on WTA for its surface retention (26), a decoration that can also function as a phage receptor (27). Because these decorations appear to serve both as receptors for bacteriophage binding and as ligands for the surface-associated virulence factor InIB, it became evident that *Listeria* faces a trade-off between maintaining virulence and developing bacteriophage resistance (18). Because *Liv* genomes also feature *inIB* (6), we set out to determine whether the glucose (Glc)

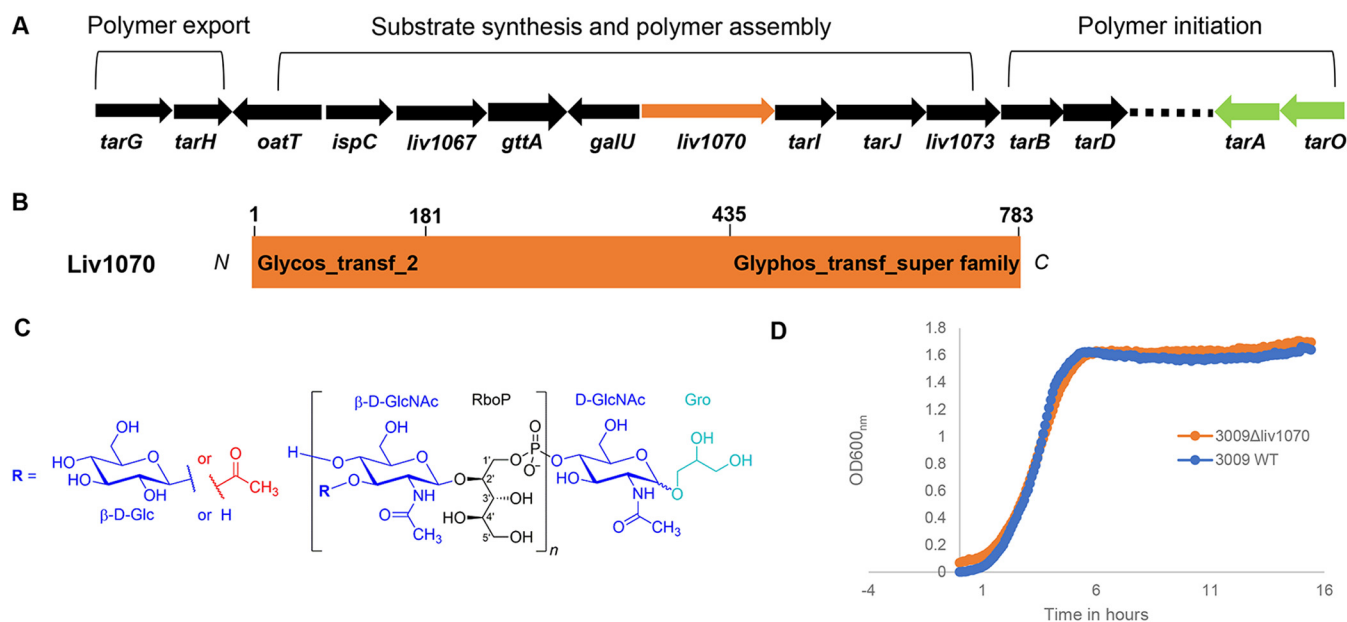


FIG 1 Genetic organization of *L. ivanovii* WSLC 3009 wall teichoic acid biosynthetic genes. (A) The ~18.8-kb WTA locus is indicated by black arrows. The dotted line indicates the gap. The annotated functions of genes are shown above the loci. (B) Domain architecture of Liv1070 based on conserved domain search. (C) Chemical structure of 3009 WTA repeating and linkage unit. Abbreviations: Glc, glucose; GlcNAc, *N*-acetylglucosamine; Rbo, ribitol; Gro, glycerol; P, phosphate. (D) Growth curves of indicated strains, as determined by measuring the OD₆₀₀ over the course of 16 h. The results from one representative experiment are shown.

decoration on the WTA of this unique species plays a role in InIB retention and function, and whether, like *Lmo*, *Liv* must also face a trade-off between being resistant to bacteriophage predation and maintaining a primary virulence function.

RESULTS

Organization of the WTA biosynthesis gene cluster in *Liv* WSLC 3009. The genome of *Liv* strain WSLC 3009 was recently sequenced. Since much is known about the genetic characteristics and function of WTA in rod-shaped bacteria, we identified and annotated the WTA gene cluster in WSLC 3009 using the prototype polyribitol phosphate WTA-producing strain *Bacillus subtilis* W23 (28). The majority of polycistronic WTA genes are in a single locus (Fig. 1A), in addition to the monocistronic genes *tarO* and *tarA*, whose transcribed products are known to initiate WTA biosynthesis (29). In this locus, the function of most genes can be predicted based on sequence similarity to their homologs in *B. subtilis*, *Staphylococcus aureus*, and *L. monocytogenes*. The sequential action of TarO, TarA, and TarB enzymes produce the conserved GlcNAc-ManNAc-Gro linkage unit (28). TarI, TarJ, and TarD produce and transfer the CDP-ribitol substrate involved in the WTA glycosylation process (30). Liv1073 possesses a GT-2 (type 2 glycosyltransferase) domain, and it is conserved across *Listeria* subspecies (see Fig. S1 in the supplemental material) featuring type II WTA with an integrated GlcNAc in the primary WTA chain (20). In our hands, previous attempts to delete the Liv1073 homolog in 4b *Lmo* did not yield a viable mutant, which led us to speculate that this gene is involved in the addition of GlcNAc onto the polymer chain, a process required for cell growth and development. Other members of this gene cluster are annotated OatT (22) (which has 47% amino acid sequence identity), IspC (31) (which has limited sequence identity but contains conserved glycine-tryptophan modules and the amidase motif), GttA (25) (which has 34% amino acid identity), and GalU (which has 89% amino acid identity), which have recently been described in *Lmo* SV 4b (22). Domain homology searches did not reveal any functional domains in Liv1067, yet Liv1070 was found to be conserved in *L. ivanovii* and some other *Listeria* subspecies (Table S1). The encoded protein bears an *N*-terminal GT-2 domain (pfam00535) and a glycerol phosphotransferase domain at its C terminus (Fig. 1B). We therefore hypothesized that

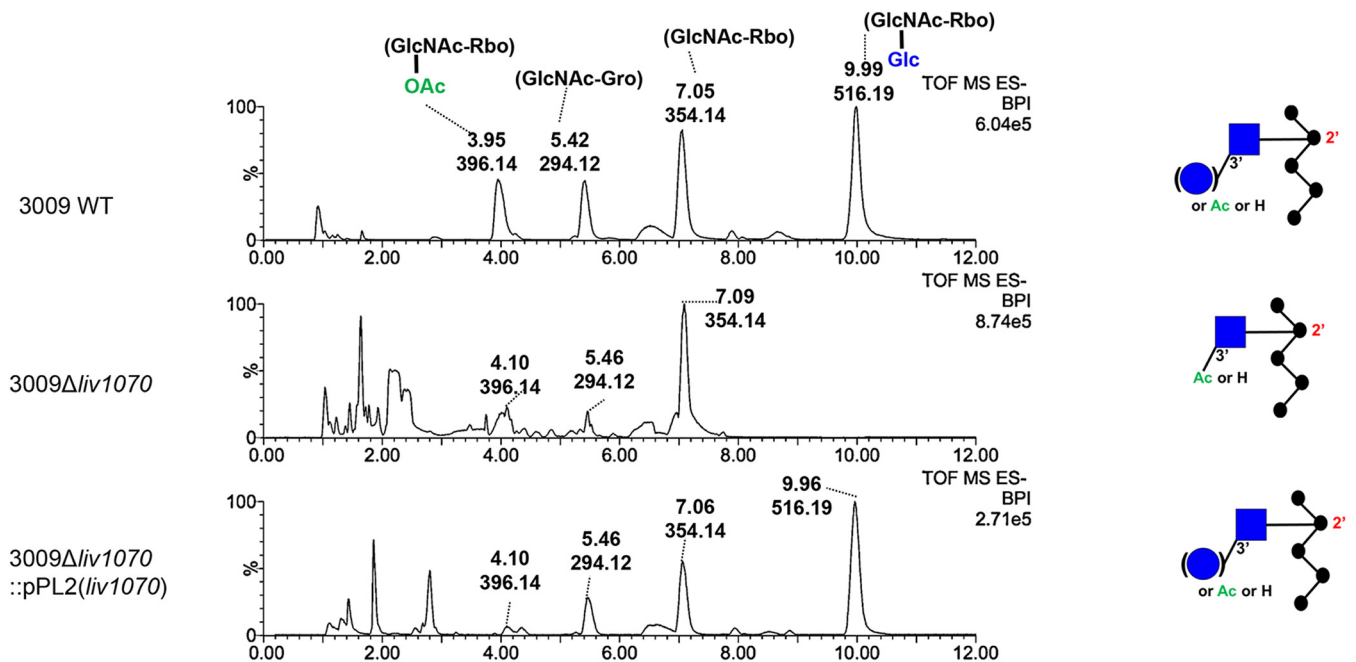


FIG 2 Liquid chromatographic separation and mass spectrometry identification of the WTA repeating unit of the indicated strains, as determined by UPLC-MS. Major peaks are labeled with their respective retention times (in minutes) and base peak ion $[M-H]^-$ (m/z). The deduced structures of the respective WTA type with a C-2 *N*-acetyl substituent are shown on the right in CFG representation (Consortium for Functional Glycomics). Abbreviations: Glc, glucose (blue circles); GlcNAc, *N*-acetylglucosamine (blue squares); Rbo, ribitol; Gro, glycerol; P, phosphate; OAc, *O*-acetyl group.

Liv1070 is responsible for the glucose decoration on WTA (Fig. 1C). To test this hypothesis, we first generated the knockout mutant 3009Δ*liv1070* and found that this mutant strain did not show any growth defects relative to the wild-type (WT) strain (Fig. 1D).

In-frame deletion of *liv1070* results in loss of glucose decoration on WTA. To further verify that *liv1070* confers WTA glucosylation in the parent strain 3009, WTA was purified from the WT and mutant strains using a previously described analytical technique (20). The structure of the WTA repeating unit was determined using ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) (Fig. 2). The structure of the WT strain showed two major peaks, one with m/z 354 (representing the GlcNAc-Rbo fragment) and the other at 516 (with the Glc decoration) (Fig. 2). Consistent with previous findings, this shows that in the WT 3009 strain, only a portion of WTA repeating unit structures are glycosylated, in this case with glucose. In the 3009Δ*liv1070* strain, however, the peak with m/z 516 is completely missing, indicating that no Glc decoration exists on the WTA of this strain. As expected, the chromatogram of the 3009Δ*liv1070*::pPL2(*liv1070*) complemented strain (see Materials and Methods) appears identical to that of the WT strain, indicating that the phenotype is fully restored.

Loss of glycosylated WTA renders *Liv* 3009 cells insensitive to phage adsorption. Bacteriophage B025 is thought to utilize the Glc decoration on the WTA for binding and recognition, as it exhibits specificity for SV 5 *Liv* strains. To determine whether deletion of *liv1070* confers phage resistance, a pulldown assay was performed. As expected, far fewer B025 phage particles adsorbed to the surface of the 3009Δ*liv1070* strain (Fig. 3A). To further verify that the Glc moiety was missing from the WTA in strain 3009Δ*liv1070*, the fluorescently labeled receptor binding protein B025_Gp18-GFP was utilized for glycotyping (32, 33). We previously demonstrated that this protein binds and recognizes *Listeria* strains possessing WTA monomers with the GlcNAc moiety linked to the C2 position of ribitol and decorated with Glc or Gal (33). Thus, loss of Glc decoration from the WTA of strain 3009 would lead to an inability of the protein to recognize the bacterial cell surface. Indeed, incubation of 3009Δ*liv1070* with the B025_Gp18-GFP fusion protein showed no fluorescence signal relative to that of the 3009 WT strain (Fig. 3B). The complemented

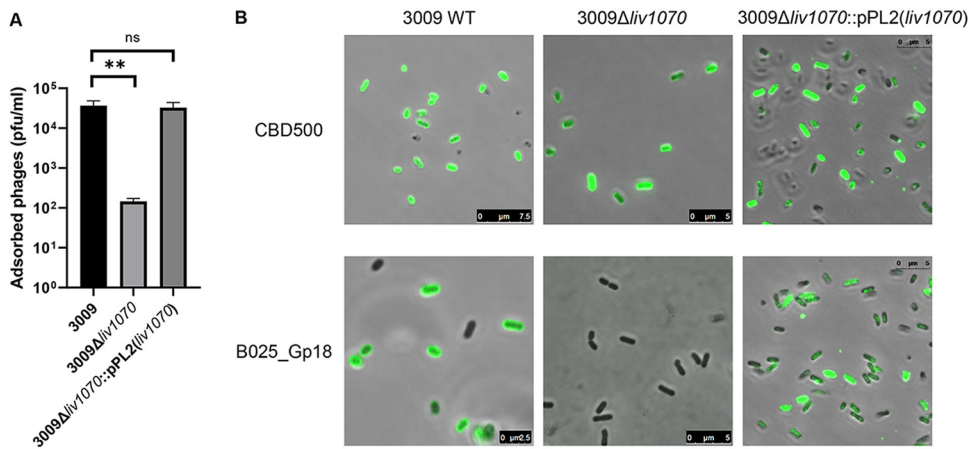


FIG 3 WTA glucosylation in *L. ivanovii* determines phage adsorption via its interaction with the receptor-binding protein. (A) Total adsorbed B025 bacteriophages, as determined by a pull-down assay and quantification by PFU counting (means and standard errors of the means [SEM]). (B) Staining of the indicated *Liv* strains with the phage-derived recombinant *O*-acetylated WTA-binding protein CBD500 and the receptor-binding protein B025_Gp18, both fused to GFP. Visualization was performed using fluorescence confocal microscopy to detect the GFP signal. For both assays, experiments were performed in triplicate. **, $P < 0.01$; ns, not significant.

strain showed a restored B025_gp18-GFP protein binding and phage adsorption, demonstrating that *lmo1070* is sufficient to confer this phenotype. Together, these data strongly suggest that the gene *lmo1070* is responsible for WTA glucosylation in the *Liv* strain 3009, a structure which mediates bacteriophage adsorption and susceptibility.

Glucosylated WTA is required for InIB cell wall association and Caco-2 and HeLa cell invasion. Because *Liv* is an invasive species causing disease in certain animals, their cells likely harbor functional InIB on the surface. InIB has been shown to rely upon WTA rhamnose decorations for its surface retention in SV 1/2 strains in *Lmo* (26). In SV 4b strains, it is also known that InIB relies upon the WTA galactose decoration for its surface retention but not the glucose decoration (25). *Liv* possesses a WTA structure similar to that of the *Lmo* SV 4b, but it differs in its GlcNAc connectivity and features only Glc decoration instead of both Glc and Gal as in SV 4b. We thus hypothesized that Glc alone may be responsible for InIB retention via *Liv* type II WTA.

To evaluate whether the loss of the Glc decoration on the WTA of strain 3009 affects InIB surface retention, Western blot assays were performed using whole-cell protein extracts and precipitated supernatant and tested with anti-InIB antibody. As can be seen, the 3009Δ*liv1070* strain seems to lose the surface-associated InIB protein (Fig. 4A), similar to what has been described for the *gtaA* mutant in SV 4b *Lmo* (22). The phenotype was restored in the complemented 3009Δ*liv1070*::pPL2(*liv1070*) strain. Overall, it appears that 3009 expresses lower levels of InIB protein than *Lmo* strain 1042 (Fig. 4A), which may be consistent with *Liv* being a somewhat less virulent species. To determine whether the loss of InIB in this Glc-deficient mutant has an effect on the strain's ability to invade host cells, a gentamicin protection assay was performed in both HeLa cells and the Caco-2 epithelial cell line (Fig. 4B). Because the 3009Δ*liv1070* strain is severely deficient in its invasive abilities, it can be assumed that InIB function is lost. This is supported by the observation that invasion into HeLa cells was almost abolished. As HeLa cells do not express E-cadherin, invasion in this cell line is known to be entirely InIB dependent (34), and the observed complete loss of HeLa invasion by 3009Δ*liv1070* strongly suggests a loss of InIB function. The low-level invasion of 3009Δ*liv1070* in Caco-2 cells, which do express E-cadherin, is presumably mediated via InIA and possibly other, yet-unknown factors. Invasion by the complemented strain 3009Δ*liv1070*::pPL2(*liv1070*) is insignificantly different from that by the WT strain, demonstrating that the Glc decoration on WTA is sufficient to maintain proper invasion levels via its ability to retain InIB on the cell surface. Together, these data clearly show that glucosylation of the WTA in SV 5 *L.*

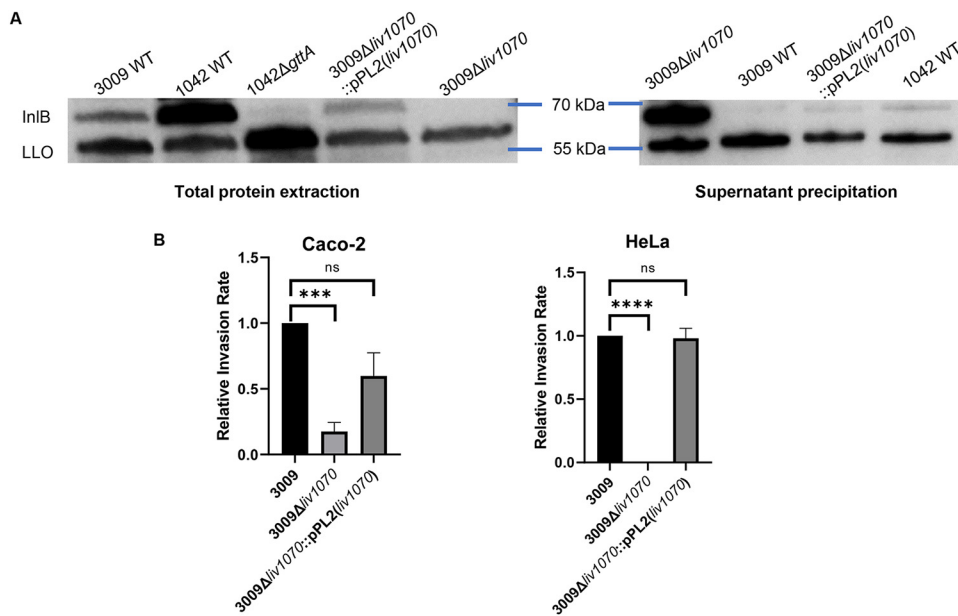


FIG 4 *L. ivanovii* requires glucosylated wall teichoic acids for InIB-mediated invasion. (A) Western blot of *Liv* total and surface proteins of indicated strains, detected using an anti-InIB antibody with anti-LLO as a loading control. *L. monocytogenes* serovar 4b strain WSLC 1042 and its *ΔgttA* knockout derivative were compared to demonstrate the relative or decreased amount of surface-associated InIB. (B) Relative invasiveness of the indicated strains compared to that of WT *Liv* 3009, in both Caco-2 and HeLa cells for 3 h (mean normalized to WT 3009 and SEM, as determined by a gentamicin protection assay). For both assays, $n = 4$. ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant relative to 3009.

ivanovii mediates phage resistance and maintains the function of one of the major *Listeria* virulence factors.

DISCUSSION

In this investigation, we sought to determine whether the InIB virulence factor also relies upon WTA decoration, and more specifically, glucosylation in *L. ivanovii*. Previous investigations in *L. monocytogenes* showed that InIB requires the presence of galactose on the WTA polymer for its surface retention, and this decoration is also essential for phage recognition and binding (22, 25, 26). However, *L. ivanovii* possesses a somewhat different WTA structure, with a different type of glycosylation, a structure which confers the unique SV 5 designation (20). Here, we show that the gene *liv1070* is necessary for glucosylation of the WTA monomer. Its deletion led to the loss of glucosylation, which was determined both structurally and by glycotyping with specific WTA-binding phage proteins (33). As expected, the glucose moiety is utilized by phage B025, which specifically infects strains possessing glycosylated WTA with an integrated GlcNAc linked to the ribitol backbone at the C-2 position. Loss of glucosylation confers phage resistance and leads to a loss of surface InIB, together showing that like *Lmo*, *Liv* must also face an evolutionary trade-off: to maintain an important virulence factor that mediates invasion of certain cell types or to be resistant to predation by bacteriophages.

The WTA of *Lmo* SV 4b cells possesses both glucose and galactose decorations, but it was previously shown that only galactose, not glucose, decoration was responsible for InIB surface retention (25). The data shown here suggest that the C-terminal GW domain of InIB, which is responsible for WTA association, apparently features a certain degree of binding specificity (22, 26). It may be the position and orientation of the sugar moiety on the WTA that govern binding to InIB, as it was shown that glucosylated GlcNAc decoration at the C-4 position of the ribitol backbone (in SV 4b WTA) or galactosylated GlcNAc substitution at the C-2 position (in SV 4c WTA) failed to retain InIB on the bacterial surface (22). However, in the *Liv* strain investigated here, the GlcNAc is C-2 linked to the ribitol, but the Glc modification is at a different position on

the GlcNAc than the Gal in SV 4c WTA (20). These findings suggest that the difference in configuration/stereochemistry is key for InIB binding. How InIB interacts with different types of WTA and the extent of this specificity are still clearly not understood, but these will be interesting to experimentally test in future specificity studies.

Some evidence suggests that the InIB protein is associated with the membrane fraction, possibly due to its affinity for the membrane-tethered lipoteichoic acid (LTA) (35). Nevertheless, we have shown that retention and functional surface display of InIB are dependent on the (abundant and exposed) WTA polymers but not on the short length of LTA, which is likely buried within the thick, multilayered *Listeria* cell wall (25, 36). Activation of the host cMet receptor has been shown to require both the C-terminal GW domain and the N-terminal LRRs of InIB (37). Nevertheless, how InIB activates the host cell receptor on the bacterial surface remains unclear and requires further investigation.

InIB functions by recognizing the host cell receptor cMet and inducing a receptor-mediated endocytosis pathway. Data presented here show that the phage-resistant strain lacking a WTA glycosyltransferase does not express InIB on its cell surface and is deficient in its ability to invade certain host cells. InIB is involved in the invasion of the liver, spleen, and placenta (23, 38–40), meaning that its loss leads to a virulence attenuation. *Lmo* strains deficient in WTA glycosylation lack the function of other proteins, namely, ActA (41) and the autolysins (26). Whether this is also the case in *Liv* remains to be determined. Of note, a previous report suggested that *Liv* can heavily colonize the liver (42). Further *in vivo* studies are thus needed to reveal whether the glucose-deficient 3009 Δ *liv1070* would show a corresponding virulence attenuation.

We have identified a putative enzyme (Liv1070) required for the glucosylation of WTA in *Liv*. Based on *in silico* predictions and the previous findings obtained in *Lmo* (25), we propose a model (Fig. 5) for the WTA glucosylation process in *Liv*. In previous work, GttA was shown to be involved in catalyzing the addition of Gal onto the undecaprenyl lipid carrier (C₅₅-P). Due to its close homology to GttA, Liv1068 is predicted to be a cytoplasmic glycosyltransferase that transfers UDP-Glc to the lipid carrier. In addition, we presume that Liv2596 functions as a putative flippase (84% identity to GtcA) that transports the C₅₅-P-Glc lipid intermediate across the membrane (43). We show that 3009 Δ *liv1070* lacks Glc decorations on WTA, suggesting that *liv1070* might encode a GT-B fold glycosyltransferase responsible for the transfer of the Glc residues onto the WTA chain. Since Liv1070 is predicted to feature 2 transmembrane helices (44) (amino acids [aa] 121 to 141 and aa 585 to 713), we speculate that this glucosylation process may also occur on the outside of the cell, as revealed in *Lmo* (45). Nevertheless, further biochemical evidence is required to elucidate this process.

Liv is thought to occur seldom in the environment. In the dairy industry, a study from Ireland showed that *Liv* isolates exist at a low prevalence of 1.4%, but these strains were as capable of invading Caco-2 cells as (or even more capable than) *Lmo* EGDe (46). While the prevalence is lower than what is typically found for *Lmo* (47), it seems significant nonetheless. This also suggests that *Liv* may be more prevalent in the environment and in food processing plants than previously assumed, promoting a need for further research into approaches for its containment. Bacteriophages have evolved as a viable option for biocontrol of *Listeria*, although as with classical antimicrobials, the occurrence of resistant strains may present a significant hurdle. However, evidence from this study and other recent studies has shown that resistance to bacteriophages may often be accompanied by physiological and virulence defects, which could present benefits to the host and decrease pathogenicity (48, 49). This could further the argument for bacteriophage use in biocontrol measures, if it holds true that resistant strains are less harmful.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and growth conditions. All bacterial strains, plasmids and phages used in this study are listed in Table S2. *Escherichia coli* XL1-Blue (Stargene) used for cloning and plasmid construction was routinely cultured in Luria-Bertani (LB) broth at 37°C. The *E. coli* BL21-Gold strain was used for protein expression. *L. monocytogenes* strains were grown in 1/2-strength brain heart infusion (BHI) at 30°C with shaking for work with phages or at 37°C when the cells were used for infection studies. For a list of strains, plasmids, and primers utilized in the study, see Table S2. Propagation and purification of bacteriophage B025 were performed using *L. ivanovii* strain 3009 as previously

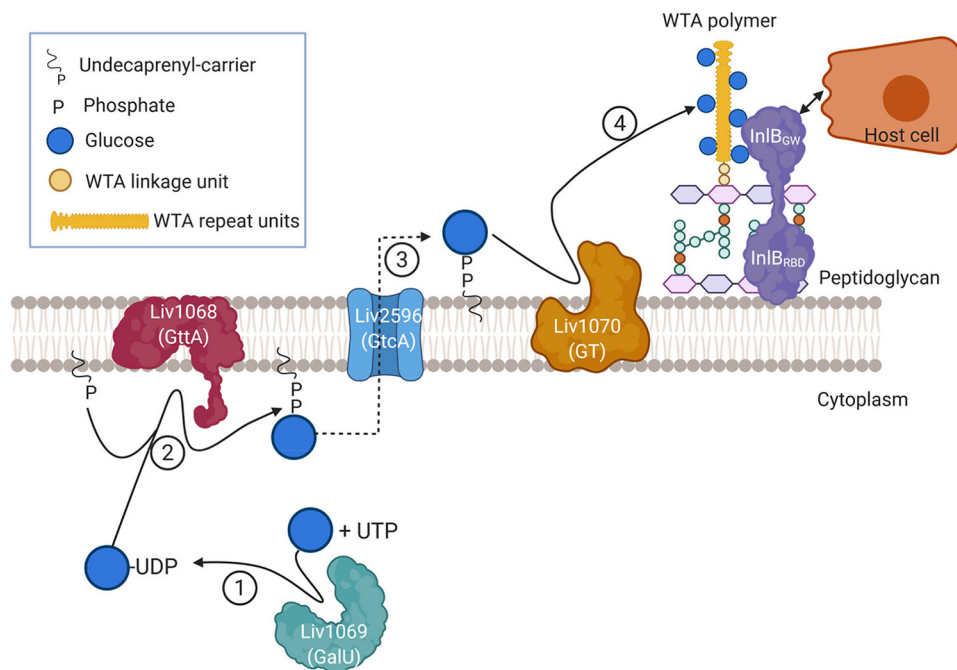


FIG 5 Proposed model for the WTA glucosylation process in *L. ivanovii*. Based on the bioinformatic and genetic data presented in this study, we propose that WTA in *L. ivanovii* is glucosylated with the aid of cytoplasmic GT Liv1069 (GalU), glucose-1-phosphate uridylyltransferase, which we predict produces UDP-Glc from UTP and glucose-1-phosphate (step 1). Next, we hypothesize that Liv1068 catalyzes the addition of UDP-Glc residues onto the undecaprenyl lipid carrier (C₅₅-P) on the inner leaflet of the cell membrane due to its high homology to GttA (step 2), as previously described for serovar 4b *L. monocytogenes*. The C-55-P-glucose intermediate is then transported across the membrane by Liv1069 (step 3), which is 84% identical to the *L. monocytogenes* GtcA protein reported in a previous study. The glucose residues are subsequently transferred onto the growing WTA chain outside the cell (step 4), and we suggest that this step is catalyzed by glucosyl transferase Liv1070. The WTA chain is conjugated by a TarTUV homologue to the MurNAc of peptidoglycan following export by an ABC transporter TarGH. The glucosylated WTA confers the retention of the InlB on the *Listeria* surface, which interacts with the host receptor cMet to activate receptor-mediated endocytosis, facilitating entry into host cells. (Created using BioRender.com.)

described (25). For growth curve determination, overnight cultures were diluted in full BHI to an optical density (OD) of 0.05 in triplicate in a 96-well plate, and the OD₆₀₀ was measured for 16 h in a plate reader set to 37°C.

Production of mutant and complemented strains. Deletion knockouts were produced via allelic exchange. Flanking regions (500 bp) of the gene *liv1070* from strain 3009 were produced by PCR, along with the pHoss1 plasmid backbone. The pHoss1 plasmid contains a temperature-sensitive origin of replication, which cannot replicate at higher temperatures. The primers used for this are listed in Table S2 and contained homologous overlap regions to allow for assembly. The three fragments were assembled together using Gibson assembly, followed by transformation into *E. coli* XL1-Blue. The pHoss1 plasmid was extracted from a single colony of XL1-Blue containing the two 500-bp flanking regions of *liv1070* and transformed into *Liv* strain 3009. This transformant was grown at permissive temperatures, allowing gene deletion to proceed via allelic exchange. The complete deletion of *liv1070* was confirmed by PCR and Sanger sequencing. Complementation of 3009Δ*liv1070* with a functional copy of *liv1070* was performed by using the chromosome-integrating vector pPL2 (50). The *liv1070* gene was inserted by Gibson assembly into plasmid pPL2 under the control of the native promoter.

Phage pulldown assay. A pulldown assay using bacteriophage B025 was performed using a multiplicity of infection (MOI) of 0.01 as previously described (22), using WSLC 3009 as the propagation strain. Following serial dilution of the phage, the number of phages adsorbed to the bacterial surface was evaluated by phage overlays and expressed as the total PFU of phage adsorbed to WT, mutant, and complemented strains.

Listeria glycotyping assay. The abilities of green fluorescent protein (GFP)-tagged CBD500 and B025_Gp18 to bind to *Listeria* cell surface were tested using a fluorescence binding assay as previously reported (33). Briefly, *Listeria* cells from log-phase cultures were harvested by centrifugation and resuspended in 1/5 volume of phosphate-buffered saline (PBS) (pH 7.4). One hundred microliters of cells was incubated with 5 μl of 1 mg/ml of GFP-tagged cell wall-binding domains (CBDs) and incubated for 5 min at room temperature. The cells were spun down, washed three times, and finally resuspended in PBS buffer. The samples were then subjected to confocal laser scanning microscopy.

WTA extractions and structural analysis. WTA was purified from the indicated *L. ivanovii* strains as previously described (20). Purified WTA polymers were depolymerized into monomeric repeating units

by hydrolysis of the phosphodiester bonds using 48% hydrofluoric acid for 20 h at 0°C. The purified WTA monomers were lyophilized and subjected to UPLC-MS/MS for compositional and structural analysis.

Gentamicin protection assay. The Caco-2 and HeLa cell lines used for *in vitro* assays were cultured at 37°C with 5% CO₂ in Dulbecco's minimal essential medium (DMEM) with GlutaMAX (Gibco), supplemented with sodium pyruvate, 1% nonessential amino acids, and 10% fetal bovine serum (FBS). Before infection, human cells were diluted to a concentration of 2 × 10⁵ to 4 × 10⁵ cells/ml and seeded onto 96-well plates in triplicate the day before the experiment. On the day of infection, bacteria were grown at 37°C to an OD₆₀₀ of 0.8 to 1.0, before being washed twice in PBS and diluted in DMEM lacking FBS to an OD₆₀₀ of 0.01, which corresponds to an MOI of ~100. Human cells were washed twice with Dulbecco's PBS (DPBS), and bacterial suspensions were added on top. The coculture was incubated for 2 h at 37°C. Cells were washed twice with DPBS and incubated for a further 1 h in normal growth medium (with FBS) supplemented with 40 μg/ml gentamicin. Cells were lysed with 0.5% Triton X-100 and serially diluted, and 10 μl was spot-plated onto BHI agar plates. CFU counts were determined the following day. The number of bacteria that had adhered or invaded was expressed as a fraction relative to the invasion rate of strain WSLC 1042, which was normalized to 1 for each replicate.

Western blotting. To detect total InlB, total cell extracts were used instead of surface protein extracts in order to obtain a better visualization for the loading control. One milliliter of overnight culture grown in 1/2 BHI medium was mixed with 0.5 ml of 0.5-mm glass beads in a 2-ml tube and shaken on a vortex mixer at maximum speed for 30 min. The tubes were spun down for 1 min at 200 × g, and 500 μl of supernatant was transferred to a 1.5-ml Eppendorf tube and spun again for 15 min at maximum speed. The supernatant was discarded, and the pellet was resuspended in 50 μl SDS sample buffer, at an initial OD of 2 (volume was adjusted accordingly if the initial OD varied), containing 5% β-mercaptoethanol and boiled for 5 min. Samples were loaded onto an SDS-PAGE gel, and Western blotting was performed as previously described (22), using a custom anti-InlB rabbit polyclonal antibody (1:5,000), with a listeriolysin O (LLO) antibody as the loading control (1:5,000; Abcam, Inc.). Supernatant extracts were produced via a trichloroacetic acid (TCA) precipitation method using the supernatant from a 5-ml culture, as previously described.

Statistics. All experiments were performed with a value of *n* of ≥3, unless otherwise stated. For the phage pulldown and bacterial invasion assays, comparisons between mutant and WT and between WT and complementation strains were evaluated for statistical significance using an unpaired *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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