L-Rhamnosylation of wall teichoic acids promotes efficient surface association of *Listeria monocytogenes* virulence factors InlB and Ami through interaction with GW domains

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WTA promotes surface association of GW proteins

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This work provides the first evidence for the role of WTA-glycosylation in the surface association of virulence factors. This should have important implications in the development of antimicrobial strategies against Gram-positive pathogens.

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SUMMARY

Wall teichoic acids (WTAs) are important surface glycopolymers involved in various physiological processes occurring in the Gram-positive cell envelope. We previously showed that the decoration of *Listeria monocytogenes* (*Lm*) WTAs with L-rhamnose conferred resistance against antimicrobial peptides. Here we show that WTA L-rhamnosylation also contributes to physiological levels of autolysis in *Lm* through a mechanism that requires efficient association of Ami, a virulence-promoting autolysin belonging to the GW protein family, to the bacterial cell surface. Importantly, WTA L-rhamnosylation also controls the surface association of another GW protein, the invasin InlB, that promotes *Lm* invasion of host cells. Whereas WTA N-acetylglucosaminylation is not a prerequisite for GW protein surface association, lipoteichoic acids appear to also play a role in the surface anchoring of InlB. Strikingly, while the GW domains of Ami, InlB and Auto (another autolysin contributing to cell invasion and virulence) are sufficient to mediate surface association, this is not the case for the GW domains of the remaining six uncharacterized *Lm* GW proteins. Overall, we reveal WTA L-rhamnosylation as a bacterial surface modification mechanism that contributes to *Lm* physiology and pathogenesis by controlling the surface association of GW proteins involved in autolysis and infection.

INTRODUCTION

Listeria monocytogenes (*Lm*) is a Gram-positive bacterial pathogen responsible for human listeriosis, a rare but potentially fatal foodborne disease that affects mostly hosts with compromised immune systems (Swaminathan and Gerner-Smidt, 2007). Among zoonotic diseases under EU surveillance, listeriosis is the most severe (EFSA, 2015). As a facultative intracellular pathogen, *Lm* is able to invade phagocytic and non-phagocytic cells and reach their cytoplasm, where it can replicate and spread into neighboring cells, thus disseminating the infection (Cossart and Toledo-Arana, 2008). For this purpose, *Lm* is equipped with a diverse and evolutionarily optimized supply of virulence factors, whose spatio-temporal expression and activity are tightly regulated for optimal infection (Camejo *et al.*, 2011). Many of these virulence-promoting proteins are located at the *Lm* cell surface, an interface from which they can immediately engage with host cell receptors and substrates, to enable cell adhesion and invasion (Carvalho *et al.*, 2014).

In *Lm*, as in other Gram-positive bacteria, many surface proteins are associated to the cell envelope via non-covalent interactions typically mediated by protein domains containing tandem repeat sequences (Cabanes *et al.*, 2002). These domains enable protein binding either to the peptidoglycan (e.g. LysM domain) (Buist *et al.*, 2008) or to secondary cell wall polymers, such as teichoic acids (e.g. GW domain) (Cabanes *et al.*, 2004), which comprise membrane-tethered lipoteichoic acids (LTAs) and peptidoglycan-bound wall teichoic acids (WTAs) (Weidenmaier and Peschel, 2008). The pneumococcal virulence-promoting adhesin PspA and amidase LytA have similar C-terminal choline-binding repeat domains, which are necessary and sufficient for their attachment to the choline-decorated *Streptococcus pneumoniae* LTAs (Höltje and Tomasz, 1975; Yother and White, 1994). Similarly, GW domains were strongly suggested to mediate protein interaction with LTAs, as reported for the *Staphylococcus aureus* autolysin Atl (Yamada *et al.*, 1996) and the *Lm* invasin InlB and autolysin Ami (Jonquière *et al.*, 1999). Interestingly, many of these non-covalently bound surface proteins are predicted or shown to have autolytic activity (Scott and Barnett, 2006; Bierne and Cossart, 2007), suggesting that this type of labile surface interaction provides cell wall-degrading proteins with some sort of positional flexibility that is pivotal for their function.

WTAs can also regulate the localization and activity of autolytic proteins at the bacterial surface (Brown *et al.*, 2013). Characterization of *S. aureus* WTA mutants revealed anomalies in autolysis levels and in the ability to properly form septa and/or complete cell division (Vergara-Irigaray *et al.*, 2008; Schlag *et al.*, 2010; Biswas *et al.*, 2012; Qamar and Golemi-Kotra, 2012). The particular contribution of the different WTA substituents to *S. aureus* autolysis is divergent: whereas WTA D-alanylation is essential for proper autolytic activity (Peschel *et al.*, 2000), the impairment of WTA glycosylation with N-acetylglucosamine did not disturb this process (Brown *et al.*, 2012), indicating that sugar substituents are not involved in WTA-mediated regulation of autolysis. In *Lm*, D-alanylation of LTAs is required for cell adhesion and virulence *in vivo* (Abachin *et al.*, 2002), but its role in autolysis was never addressed. Also unknown is the role of *Lm* WTAs and their unique glycosidic substituents in this process (Kamisango *et al.*, 1983; Fiedler, 1988). We explored here the involvement of WTA glycosylation in the spatial and functional regulation of *Lm* autolysis.

We showed that a *Lm* strain lacking L-rhamnosylated WTAs has decreased autolytic activity. This phenotype is concurrent with reduced *Lm* surface-bound levels of the autolysin Ami, indicating that WTA L-rhamnosylation is necessary for efficient retention of Ami in the *Lm* cell envelope. Epitope tagging-based analysis of the subcellular localization of all GW module-containing proteins encoded in the *Lm* genome (Cabanès *et al.*, 2002), revealed that only Ami and InlB are anchored to the bacterial surface in a WTA L-rhamnosylation-dependent manner. Neither protein requires other WTA modifications, such as N-acetyl-glucosaminylation, to interact with the bacterial surface. Nevertheless, LTAs appear to play also a role in InlB surface association. Importantly, the observed decrease in surface-associated InlB correlates with impaired entry of WTA L-rhamnosylation-deficient *Lm* into epithelial cells. These data reveal novel roles for WTA glycosylation, and particularly L-rhamnosylation, in *Lm* biology, such as supporting autolytic processes and promoting host cell invasion, via its contribution to the efficient surface anchoring of proteins sharing a common cell wall-binding domain.

RESULTS

WTA L-rhamnosylation contributes to *Lm* autolysis by promoting surface association of Ami

Teichoic acids are involved in various processes taking place at the cell envelope of Gram-positive bacteria. Among other roles, these surface glycopolymers protect the bacterial cell from host defense peptides and were shown to regulate the localization and activity of autolytic enzymes (Weidenmaier and Peschel, 2008; Brown *et al.*, 2013). We previously showed that the decoration of *Lm* WTAs with L-rhamnose is required to confer resistance against cationic antimicrobial peptides, promoting bacterial survival inside the host during infection (Carvalho *et al.*, 2015). Here we analyzed if WTA L-rhamnosylation could be also involved in the modulation of autolysin activity. To test this hypothesis, we monitored the autolytic behavior of wild type *Lm* (WT) and of an isogenic mutant strain devoid of L-rhamnosylated WTAs ($\Delta rmlACBD$) (Carvalho *et al.*, 2015). $\Delta rmlACBD$ bacteria showed a small but significant lysis deficiency in comparison to WT bacteria (Figure 1A). This phenotype was reverted when the *rmlACBD* genes were expressed in the mutant strain from a single-copy chromosome-integrated plasmid ($\Delta rmlACBD+rmlACBD$, Figure 1A), thus confirming that WTA L-rhamnosylation contributes to *Lm* autolysis.

To determine if this impaired autolytic activity could be caused by an incorrect localization of autolysins at the *Lm* cell envelope, we analyzed the surface protein content of the three strains. Proteins associated non-covalently to the bacterial surface were extracted by a washing step with SDS-containing buffer and loaded into an SDS-PAGE gel. After Coomassie Blue staining, we detected a band of ~100 kDa prominently present in the WT surface protein extract and significantly reduced in the $\Delta rmlACBD$ extract (Figure 1B). Importantly, the amount of this protein band was restored to wild type-like levels in the complemented $\Delta rmlACBD+rmlACBD$ strain (Figure 1B), indicating that the basal surface levels of this protein are sustained by the presence of L-rhamnosylated WTAs. Peptide mass fingerprinting analysis identified this protein as Ami (Lmo2558), an autolytic amidase that was previously shown to contribute to *Lm* adhesion to and colonization of mouse hepatocytes (McLaughlan and Foster, 1998; Milohanic *et al.*, 2001; Asano *et al.*, 2011, 2012). The reduced levels of Ami observed in the $\Delta rmlACBD$ surface protein extracts were confirmed by Western blot using an anti-Ami antibody (Figure 1C). In addition,

similar analysis performed on the secreted proteins present in culture supernatants revealed significantly higher levels of Ami in culture supernatants of the $\Delta rmlACBD$ mutant as compared to those of the WT and complemented strains (Figure 1C). Interestingly, $\Delta rmlACBD$ bacteria did not exhibit any growth defect in nutrient-rich medium (Carvalho *et al.*, 2015), suggesting that the peptidoglycan-cleaving activity of Ami is not fundamental for cell wall remodeling during cell growth and division.

Altogether, these data indicate that WTA L-rhamnosylation contributes to physiological levels of autolysis in *Lm* through a mechanism that warrants efficient surface association of the autolysin Ami.

WTA L-rhamnosylation promotes InlB association to the *Lm* surface and host cell infection

Ami is one of nine *Lm* proteins predicted to contain one or more copies of a ~80 amino acid sequence motif called GW module, which mediates protein interaction with the bacterial cell envelope (Glaser *et al.*, 2001; Cabanes *et al.*, 2002). A relevant member of this protein family is internalin B (InlB), a major *Lm* virulence factor that promotes bacterial uptake in non-phagocytic cells by engaging its host cell receptor, c-Met (Shen *et al.*, 2000). Due to its crucial role in *Lm* virulence, we wondered whether the association of InlB to the bacterial surface could also be dependent on the WTA L-rhamnosylation status. Western blot analyses were performed on extracts of non-covalently associated surface proteins and culture supernatant proteins retrieved from exponential cultures of the WT, $\Delta rmlACBD$ and $\Delta rmlACBD+rmlACBD$ strains. A strong reduction in the levels of surface-bound InlB was detected in the mutant strain, in comparison to the WT and complemented strains. Concomitantly, we observed an increase in the amount of InlB in the supernatant fraction (Figure 2A). Therefore, like Ami, InlB also depends on the presence of L-rhamnosylated WTAs for optimal association to the *Lm* surface.

Because InlB needs to be at the *Lm* surface to efficiently promote bacterial uptake by host cells (Braun *et al.*, 1998), we investigated the capacity of $\Delta rmlACBD$ bacteria to invade cells. We used human epithelial HeLa cells shown to express c-Met at their surface and thus appropriate for the study of InlB-dependent cell invasion (Pizarro-Cerdá *et al.*, 2012). As shown in Figure 2B, gentamicin protection assays revealed that $\Delta rmlACBD$ bacteria are strongly impaired in their ability to infect HeLa cells (20–30% of WT levels). Again, this phenotype is attributed to the absence of L-rhamnosylated WTAs on the *Lm* surface since it is rescued in the complemented strain (Figure 2B). Moreover, we showed that this defect is not a

consequence of attenuated host cell-adhering properties, because $\Delta rmlACBD$ bacteria were able to bind to HeLa cells as well as the WT strain (Figure 2C).

Together, these data suggest that WTA L-rhamnosylation contributes to *Lm* internalization by promoting proper association of InlB to the bacterial cell envelope. This hints at a common mechanism of *Lm* surface association for GW module-containing proteins that relies on WTA L-rhamnosylation.

WTA L-rhamnosylation does not promote surface association of all *Lm* GW proteins

In addition to Ami and InlB, *Lm* encodes seven other proteins with predicted GW modules: Auto (Lmo1076), Lmo1215, Lmo1216, Lmo1521, Lmo2203, Lmo2591 and Lmo2713 (Cabanès *et al.*, 2002, 2004) (Figure 3A). Except for Lmo2713, the remaining proteins are predicted to have peptidoglycan hydrolase activity, important for cell wall turnover (Cabanès *et al.*, 2002, 2004; Bierne and Cossart, 2007) (Figure 3A). From these six potential autolysins, only Auto has been functionally characterized and shown not only to possess autolytic activity but also to contribute to cell invasion and virulence (Cabanès *et al.*, 2004).

To assess whether WTA L-rhamnosylation is a general requirement for the association of proteins containing GW modules to the *Lm* surface, we investigated the subcellular localization of the full set of *Lm* GW proteins in the WT, $\Delta rmlACBD$ and $\Delta rmlACBD+rmlACBD$ strains. We performed Western blots on extracts of non-covalently associated bacterial surface proteins or secreted proteins. Due to the lack of antibodies recognizing GW proteins other than Ami and InlB, we employed an epitope-tagging approach to enable their immunodetection. The GW module-containing domain of each protein (including Ami and InlB) was translationally fused at its N-terminus with a FLAG-tag preceded by the signal peptide region of Ami, and placed under the transcriptional control of the *ami* promoter in the *Listeria* integrative vector pPL2 (Lauer *et al.*, 2002) (Supplementary Figure 1). The resulting plasmid constructs were introduced into the WT and $\Delta rmlACBD$ strains, and FLAG-tagged proteins were detected in bacterial extracts using an anti-FLAG antibody. We were able to detect each of the nine recombinant GW domain proteins (Figure 3B), despite the weak signal obtained from those with the lowest molecular weight, such as Lmo1215, Lmo2713 (both 10 kDa) and Lmo1216 (17 kDa). In WT bacteria, only the GW domains of Ami (Ami_{GW}), InlB (InlB_{GW}) and Auto (Auto_{GW}) appeared associated with the *Lm* cell surface, while the

others were exclusively found in the culture supernatant fraction (Figure 3B). InlB_{GW} was also detected in supernatants, in agreement with its previously acknowledged dual localization (Braun *et al.*, 1997). These results reproduce those already observed with the native full-length proteins, both in this study (Figures 1C and 1D) and in previous reports suggesting that the GW modules of these three proteins are responsible and sufficient for their association to *Lm* surface (Braun *et al.*, 1997; Cabanes *et al.*, 2004). In a $\Delta rmlACBD$ background, Ami_{GW}, InlB_{GW} and Auto_{GW} showed distinct localizations. While Auto_{GW} remained fully associated with the bacterial surface, Ami_{GW} was completely delocalized to the culture supernatant fraction (Figure 3B). InlB_{GW} was still detected at the *Lm* surface, but at lower levels than when expressed in the WT background. Concomitantly, we observed an increase in the amount of secreted InlB_{GW} (Figure 3B). As expected, similar results were obtained with the $\Delta rmlT$ mutant strain (Figure 4), which is still capable synthesizing L-rhamnose but lacks the glycosyltransferase responsible for its attachment to *Lm* WTAs (Carvalho *et al.*, 2015), demonstrating that it is the presence of L-rhamnose on WTAs that is required for the proper surface association of these GW proteins.

These data reveal that, contrarily to what occurs with Ami, InlB and Auto, the GW domains of the other six uncharacterized *Lm* GW proteins are not capable of promoting an association to the bacterial surface, suggesting that these proteins are mainly secreted to the extracellular medium in a WTA L-rhamnosylation-independent manner. Furthermore, these results confirm that WTA L-rhamnosylation is required for the surface association of Ami and InlB, but surprisingly not of Auto. This suggests that, in addition to the number of modules, their sequence/structure/organization may contribute to the interaction of GW domains with the *Lm* surface.

A careful analysis of the GW domain sequences of the nine predicted GW proteins revealed that, although they all contain the signature GW dipeptide, they appear rather heterogeneous (Supplementary Figure 1A). The GW modules of Lmo1521 are shorter than all the other ones and appear to have only in common the presence of the GW dipeptide. The other GW modules appeared to cluster into different groups (Supplementary Figure 2A). As previously described (Milohanic *et al.*, 2004), Ami and InlB contain a C-terminal domain composed of four and one hetero-repeats respectively, each made up of two GW modules in tandem (Supplementary Figure 2B). The second GW module of each tandem seems to be highly conserved and specific of Ami and InlB (Supplementary Figure 2A and 2C). This suggests that this

specific GW module could be responsible for the WTA L-rhamnosylation-dependent association of Ami and InlB with the *Lm* surface.

GW domain surface association depends on their intrinsic sequence and on the cell wall structure.

Besides L-rhamnose, each *Lm* WTA subunit is also modified with an N-acetyl-glucosamine residue (Kamisango *et al.*, 1983). Having revealed the importance of WTA L-rhamnosylation for the *Lm* surface binding of Ami and InlB, we speculated whether WTA N-acetyl-glucosaminylation could also play a role in their surface association. To address this hypothesis, we generated an *Lm* mutant strain lacking *gtcA*, which encodes a predicted integral membrane protein involved in the mechanism of N-acetyl-glucosamine appendage to *Lm* WTA subunits (Eugster *et al.*, 2011), and transformed it to express FLAG-tagged Ami_{GW} or InlB_{GW}. Exponential cultures of these strains were then processed to obtain surface-associated and secreted protein extracts, which were analyzed by Western blot. As shown in Figure 4, when expressed in a $\Delta gtcA$ background FLAG-tagged proteins maintained their WT-like localization, indicating that N-acetyl-glucosaminylation of WTAs is dispensable for the *Lm* surface binding of Ami_{GW} and InlB_{GW}. This result thus highlights the unique role of WTA L-rhamnosylation in the association of these proteins to the *Lm* surface.

Subcellular fractionation and *in vitro* binding assays previously allowed the identification of LTAs as the major *Lm* surface molecular anchor for Ami and InlB, *via* interaction with their GW domains (Jonquière *et al.*, 1999). To genetically confirm the key role of LTAs, we analyzed the binding of Ami_{GW} and InlB_{GW} to the surface of an *Lm* $\Delta lafAB$ strain, which cannot express the first two enzymes of the LTA biosynthetic pathway and is thus unable to assemble LTAs (Webb *et al.*, 2009). Western blot results showed that LTAs are not required to sustain the interaction of Ami_{GW} with the *Lm* surface, but appeared to partly contribute to the surface association of InlB_{GW} (Figure 4).

A report characterizing Ami orthologues produced by *Lm* strains of serotypes 1/2a and 4b, which have distinct WTA structures and glycosidic substituents (Fiedler, 1988), showed that each protein only bound efficiently to the surface of bacteria from its own serotype, and that the GW domain sequences are homologous within serotypes with similar WTA structures (Milohanic *et al.*, 2004). To analyze if this binding specificity could be related to WTA glycosylation, we studied the binding of Ami_{GW} to the surface

of a *Lm* 4b strain and reversely the binding of Ami_{GW} from a *Lm* 4b strain (Ami_{GW} *Lm* 4b) to the *Lm* EGDe 1/2a strain. Our results confirmed that Ami_{GW} bound more efficiently to the surface of bacteria from its own serotype (Figure 4). However, whereas the surface binding of Ami_{GW} was independent of WTA N-acetyl-glucosaminylation, Ami_{GW} *Lm* 4b appeared to be entirely dependent on this WTA modification to bind the surface of the *Lm* EGDe 1/2a strain (Figure 4). In addition, InlB_{GW} seemed to bind the surface of *Lm* 4b as efficiently as the surface of its own serovar (Figure 4). Altogether, these data indicate that, unlike L-rhamnosylation, N-acetyl-glucosaminylation of *Lm* WTAs is not a prerequisite for the interaction of GW proteins with the bacterial surface. In addition, they confirm the involvement of LTAs in the surface association of InlB_{GW}. Our data also reveal the high specificity of binding of the GW domains probably depending on the structure of the cell wall as well as on the internal GW module sequences.

DISCUSSION

This study revealed the existence of a link between *Lm* WTA L-rhamnosylation and autolytic activity. In particular, it showed that this WTA tailoring mechanism supports basal levels of autolysis by promoting *Lm* surface association of Ami, an autolysin belonging to the GW protein family. In addition, it revealed the impaired surface association of another GW protein, InlB, in *Lm* strains lacking L-rhamnosylated WTAs. Importantly, being a major host cell invasion-promoting factor of *Lm*, the decreased level of surface-associated InlB observed in absence of WTA L-rhamnosylation was correlated with a significant impairment of their host cell invasion ability.

We first showed that the autolytic rate was lower in bacterial mutants deficient for WTA L-rhamnosylation. A similar observation was made with *S. aureus dltA* mutants, which are unable to perform D-alanylation of LTAs/WTAs (Peschel *et al.*, 2000). In this case, it was suggested that the increased electronegativity of D-alanine ester-free teichoic acids promotes a stronger binding and entrapment of positively charged autolysins (Fischer *et al.*, 1981), thereby spatially restricting their activity. However, a parallel study using a *dltC* mutant of another *S. aureus* strain and slightly different

experimental conditions provided an opposite phenotype, *i.e.* enhanced cell lysis (Nakao *et al.*, 2000). This was also reported in D-alanylation mutant strains of *L. lactis* and *L. plantarum* (Steen *et al.*, 2005; Palumbo *et al.*, 2006), where deregulated activity of a major autolysin was pointed as the reason for the mutant phenotypes. In the *L. lactis* mutant, this was attributed in part to a reduced HtrA-mediated degradation of the AcmA autolysin (Steen *et al.*, 2005), while the LTA polymers of the *L. plantarum* *dltA* mutant were found to be longer and heavily glucosylated (Palumbo *et al.*, 2006). The conflicting phenotypes observed in different bacterial species deficient for similar systems highlight the complexity of the mechanisms linking teichoic acids and autolytic activity.

The reduced *Lm* surface-associated levels of Ami in absence of WTA L-rhamnosylation are correlated with a significant release of this protein into the extracellular medium. This suggests that Ami cannot be correctly retained in the *Lm* cell wall and thus escapes to the extracellular environment, resulting in an impaired autolysis. To our knowledge, this reveals a previously unknown role for WTA glycosylation in the non-covalent association of surface proteins with the Gram-positive cell envelope. Importantly, Ami remained associated to the *Lm* surface in a mutant strain unable to modify its WTAs with N-acetylglucosamine (Eugster *et al.*, 2011), demonstrating that WTA L-rhamnosylation is not only required but also sufficient to anchor this protein to the bacterial cell surface. Results observed with the endogenous full-length Ami were reproduced with only its GW domain (Ami_{GW}), demonstrating that this region is sufficient for *Lm* surface anchoring of Ami, and strengthening the crucial role of L-rhamnosylated WTAs in this process.

Previous reports suggested that Ami binds to LTAs through its GW domain, in a way similar to InlB (Braun *et al.*, 1997; Jonquière *et al.*, 1999; Asano *et al.*, 2012). However, we observed that the *Lm* surface-associated levels of Ami were not perturbed in a LTA-deficient mutant strain, therefore ruling out these membrane-bound teichoic acids as players in the surface anchoring of Ami. Recently, Percy *et al.* corroborated this result by showing that neither LTA substitution nor the LTA polymers themselves are required for *Lm* cell wall anchoring of an InlB chimera carrying the GW domain of Ami in its C-terminal region (Percy *et al.*, 2016). As previously suggested (Milohanic *et al.*, 2004), we demonstrated that the binding of GW domains to the bacterial surface depends not only on their intrinsic sequence, but

also on the structure of the bacterial cell wall, and in particular the WTAs and their specific sugar substituents.

We also evaluated the contribution of WTA L-rhamnosylation to the *Lm* envelope association of InlB, the only member of the *Lm* GW protein family that does not possess a catalytic domain involved in peptidoglycan turnover (Cabanès *et al.*, 2002), and more importantly, a major determinant of *Lm* entry into host cells (Dramsi *et al.*, 1995; Lingnau *et al.*, 1995; Ireton *et al.*, 1996; Parida *et al.*, 1998). Despite already partly secreted in normal conditions (Braun *et al.*, 1997), we observed that surface-bound InlB levels also depend on the decoration of WTAs with L-rhamnose, but not with N-acetyl-glucosamine, again highlighting the unique role of WTA L-rhamnosylation. Unlike Ami, InlB seems to require the presence of LTAs to retain its surface localization. These observations contrast with the findings of Jonquière *et al.* showing that LTAs are the major *Lm* surface anchor of InlB *in vitro* and that other cell wall-associated components, such as WTAs, are not involved in this process (Jonquière *et al.*, 1999). The reduced surface-bound InlB levels in WTA L-rhamnosylation-deficient bacteria impact *Lm* entry into host cells, but not bacterial adhesion. Functional characterization of Ami demonstrated its contribution towards *Lm* adherence to target cells, but this role appears to be secondary as it only becomes relevant in a $\Delta inlAB$ background (Milohanic *et al.*, 2001), and varies with host cell type (Milohanic *et al.*, 2001; Asano *et al.*, 2012). In this context, one could expect a decreased host cell adhesion of WTA L-rhamnosylation mutants, since surface Ami and InlB levels are reduced. However, it is possible that the presence of other surface adhesins (Camejo *et al.*, 2011) is sufficient to sustain optimal levels of adherence to target host cells. Although InlB occurs as both surface-attached and secreted forms, the first is preponderant for triggering host cell uptake (Braun *et al.*, 1998), which could explain the attenuated invasive phenotype of bacteria deficient for WTA L-rhamnosylation. Moreover, InlB is known to interact with other eukaryotic cell surface components, such as glycosaminoglycans, to further promote bacterial invasion (Braun *et al.*, 2000; Jonquière *et al.*, 2001). In this case, this interaction takes place through the InlB GW domain (Jonquière *et al.*, 2001). One could speculate that excessive amounts of soluble InlB close to the site of *Lm* interaction with the host cell surface may also saturate these InlB-binding polysaccharides hindering subsequent bacterial internalization.

Besides Ami and InlB, *Lm* encodes seven other proteins containing one or more GW modules (Cabanès *et al.*, 2002). None of these proteins showed WTA L-rhamnosylation-dependent cellular localization, being exclusively detected either at the *Lm* surface (Auto_{GW}) or in the extracellular medium (all others). Interestingly, despite having equal number of GW module repeats, Auto_{GW} shows strong association to the bacterial surface, while Lmo2591_{GW} is only detected in the culture supernatant. This strongly advocates that other factors besides the number of GW repeats, such as the amino acid sequence of these modules, may also determine the localization of GW proteins. In particular, the presence of a hetero-tandem containing a GW module specific of Ami and InlB could be responsible for their WTA L-rhamnosylation-dependent association to the *Lm* surface.

We recently showed that WTA L-rhamnosylation is crucial for the resistance of *Lm* against antimicrobial peptides (Carvalho *et al.*, 2015). We demonstrate here that this specific WTA glycosylation is also critical for the proper surface anchoring of major *Lm* virulence factors. Importantly, while *Lm* WTA L-rhamnosylation appeared pivotal for *in vivo* virulence, it is dispensable for *Lm* growth. Given that *Lm* WTA L-rhamnosylation is mediated by a unique glycosyltransferase RmlT (Carvalho *et al.*, 2015), this enzyme appears thus as a promising target for anti-virulence drugs diminishing pathogenicity and promoting antimicrobial peptide sensitivity of Gram-positive bacteria.

This work has brought to light the contribution of WTAs – and WTA L-rhamnosylation in particular – to important physiological and virulence-promoting processes of *Lm*, through a newly identified role in the anchoring and stabilization of non-covalently surface-bound virulence proteins sharing a common cell surface-binding motif.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. *Lm* and *E. coli* strains were routinely cultured aerobically at 37 °C in brain heart infusion (BHI, Difco) and Luria-Bertani (LB) media, respectively. When appropriate, the following antibiotics were added: ampicillin (Amp), 100 µg/mL; chloramphenicol (Cm), 7 µg/mL (for *Lm*) or 20 µg/mL (for *E. coli*); erythromycin (Ery), 5 µg/mL. For selection of clones with chromosomally integrated plasmids colistin sulfate (Col) and nalidixic acid (Nax) were used at 10 and 50 µg/mL, respectively.

Construction of deletion mutant strains

Lm deletion mutant strains were constructed in the EGD-e background through a process of double homologous recombination mediated by the suicide plasmid pMAD (Arnaud *et al.*, 2004). DNA fragments corresponding to the 5'- and 3'-flanking regions of *gtcA* (*lmo2549*) were amplified by PCR from *Lm* EGD-e chromosomal DNA using respectively primer pairs 23–24 and 25–26 (Table S2), and cloned in tandem between the *SalI*–*MluI* and *MluI*–*NcoI* sites of pMAD, to produce pFC61. Similarly, DNA fragments corresponding to the 5'- and 3'-flanking regions of the *lafAB* (*lmo2555*–*lmo2554*) locus were amplified using respectively primer pairs 29–30 and 31–32 (Table S2), and cloned in tandem between the *SalI*–*NcoI* and *NcoI*–*BglII* sites of pMAD, to produce pDC658. The plasmid constructs were introduced in *Lm* EGD-e by electroporation and transformants selected at 30 °C in BHI–Ery. Positive clones were re-isolated in the same medium and grown overnight at 43 °C. Integrant clones were inoculated in BHI broth and grown overnight at 30 °C, after which the cultures were serially diluted, plated in BHI agar and incubated overnight at 37 °C. Individual colonies were tested for growth in BHI–Ery at 30 °C and antibiotic-sensitive clones were screened by PCR for deletion of *gtcA* (primers 27 and 28, Table S2) and *lafAB* genes (primer pairs 33–34 and 35–36, Table S2). Plasmid constructs and gene deletions were confirmed by DNA sequencing.

Construction of strains expressing FLAG-tagged GW domain proteins

The *Listeria* integrative plasmid pPL2 (Lauer *et al.*, 2002) was used to construct another plasmid (pDC426) allowing the expression and secretion of N-terminally FLAG-tagged proteins in *Lm* strains

from chromosome-integrated, single-copy locus. A 270-bp DNA fragment comprising the *ami* promoter (*P_{ami}*) sequence, the Ami signal peptide (residues 1–30)-encoding sequence, and the FLAG epitope-encoding sequence was amplified by PCR from *Lm* EGD-e genomic DNA using primers 1 and 2 (Table S2). The PCR product was cloned between the *SalI* and *PstI* sites of pPL2 to produce pDC426. This master plasmid was then used to generate other plasmids, each containing the GW repeat domain of one of the nine GW proteins encoded in the *Lm* genome (Cabanes *et al.*, 2002). DNA fragments comprising the GW repeat domain of InlB (InlB_{GW}), Auto (Auto_{GW}), Lmo1215 (Lmo1215_{GW}), Lmo1216 (Lmo1216_{GW}), Lmo1521 (Lmo1521_{GW}), Lmo2203 (Lmo2203_{GW}), Ami (Ami_{GW}), Lmo2591 (Lmo2591_{GW}) and Lmo2713 (Lmo2713_{GW}) were amplified by PCR from *Lm* EGD-e genomic DNA using primer pairs 3–4, 5–6, 7–8, 9–10, 11–12, 13–14, 15–16, 17–18 and 19–20, respectively (Table S2). The PCR products were then cloned between the *PstI* and *NotI* sites of pDC426, to generate plasmids pDC481, pDC459, pDC460, pDC461, pDC480, pDC462, pDC440, pDC463 and pDC464. Each pDC426-derivative plasmid was introduced into *E. coli* S17-1 and transferred to *Lm* strains by conjugation on BHI agar. Transconjugant clones were selected in BHI–Cm/Col/Nax and chromosomal integration of the plasmids confirmed by PCR with primers 21 and 22 (Table S2). Plasmid constructs were confirmed by DNA sequencing.

Autolysis assay

Lm cultures grown to the exponential phase (OD₆₀₀=1.0) were centrifuged and the pelleted cells washed with ice-cold distilled water before resuspension in 50 mM glycine buffer, pH 8.0 to a final OD₆₀₀ of 1.0. Bacterial suspensions were shaken at 37 °C and the autolytic activity was monitored through time as the percentage of OD₆₀₀ decrease relative to the initial value. For each time point, values were calculated as mean ± standard deviation of three independent experiments.

Extraction of non-covalently surface-associated and secreted *Lm* proteins

Extraction of non-covalently surface-associated and secreted *Lm* proteins was performed as described before (Braun *et al.*, 1997; Cabanes *et al.*, 2004), with minor changes. Samples (20 mL) of *Lm* cultures grown to the exponential phase (OD₆₀₀ 0.8) were centrifuged (3,800 g, 15 min, 4 °C) and the cell pellet and culture supernatant fractions collected for further processing. Bacteria were washed with ice-cold

PBS, resuspended in 200 µL of PBS + 2% (v/v) SDS, and incubated for 30 min at 37 °C. After centrifugation (21,100 g, 1 min), the supernatant containing solubilized non-covalently associated surface proteins was analyzed by Western blot. Culture supernatants were filtered (0.22 µm) and proteins precipitated by treatment with 0.2 mg/mL of sodium deoxycholate (30 min, 4 °C) and 6% (v/v) trichloroacetic acid (2 h, 4 °C). Proteins were collected by centrifugation (13,400 g, 15 min, 4 °C) and washed twice with cold acetone. The pellet was air-dried and resuspended in 200 µl of 20 mM Tris-HCl, pH 7.4 for analysis by Western blot.

SDS-PAGE and Western blot analysis of *Lm* protein extracts

Lm protein extracts were analyzed by SDS-PAGE in 8 or 10% polyacrylamide gels and subsequently stained with Coomassie Brilliant Blue or transferred (Trans-Blot Turbo Transfer System, Bio-Rad Laboratories) onto a nitrocellulose membrane for total protein staining with Ponceau S followed by immunoblotting. Blotted proteins were detected with the following antibodies: mouse monoclonal anti-FLAG (clone M2, Sigma-Aldrich) at a 1:250 dilution; mouse monoclonal anti-InlB (H15.1, (Braun *et al.*, 1999)) at a 1:1000 dilution; rabbit polyclonal anti-Ami antiserum (R5, kind gift from Pascale Cossart) at a 1:5000 dilution; rabbit polyclonal anti-*Lm* GAPDH (GAPDH_{*Lm*}, Abgent) at a 1:1000 dilution, and HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (P.A.R.I.S Biotech) at 1:2000 or 1:10,000 dilutions, respectively. Immunolabeled proteins were detected by enhanced chemiluminescence using Western Blotting Substrate kit (Pierce) for signal development and ChemiDoc XRS+ equipment (Bio-Rad Laboratories) for signal capture.

Cell infection assays

HeLa (ATCC CCL-2™) cell lines were propagated at 37 °C (7% CO₂) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Lonza). To assess bacterial adhesion to cells, confluent monolayers (~2×10⁵/well) were inoculated for 30 min at 37 °C (7% CO₂) with log-phase bacteria (OD₆₀₀ 0.6) at a multiplicity of infection (MOI) of 75 bacteria/cell in cell culture medium. After removing the inoculum, cells were washed three times with warm medium to remove weakly associated bacteria, and were lysed with 1 mL of cold 0.2% (v/v) Triton X-100. Ten-fold serial dilutions were plated in BHI agar and incubated overnight at 37 °C for quantification of cell-adhered bacteria. To assess bacterial invasion

of cells, confluent monolayers were inoculated for 1 h at 37 °C (7% CO₂) with log-phase bacteria (OD₆₀₀ 0.6) at a MOI of 75. After removing the inoculum, cells were incubated for 1.5 h at 37 °C (7% CO₂) with 20 µg/mL gentamicin to kill extracellular bacteria. Cells were then washed and lysed as above for quantification of intracellular viable bacteria. Each condition was assayed in triplicate in at least three independent experiments. Statistical analysis (one-way ANOVA with Tukey's test) was performed with Prism 6 (GraphPad Software).

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CONFLICT OF INTEREST

None declared.

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587

588

FIGURE LEGENDS

Figure 1 – WTA L-rhamnosylation supports *Lm* autolysis by promoting cell surface association of Ami.

(A) Log-phase bacteria (OD₆₀₀ 1.0) were resuspended in 50 mM glycine, pH 8.0 and agitated at 37 °C until clarification (i.e. complete cell lysis). For each time point, measured OD₆₀₀ values are given as percentage of initial OD₆₀₀ values are represented as the mean $\pm$ SD of three independent experiments.

(B) Proteins non-covalently associated to the *Lm* surface were extracted with PBS + 2% SDS (30 min, 37 °C), separated by SDS-PAGE and analyzed by Coomassie staining. Arrow indicates protein band identified as Ami by peptide mass fingerprinting. Numbers indicate molecular weight (in kDa) of protein ladder bands. **(C)** Western blot of non-covalently surface-associated and secreted *Lm* protein extracts obtained from wild-type (WT), WTA L-rhamnosylation-deficient ($\Delta rmlACBD$) and complemented ($\Delta rmlACBD+rmlACBD$) strains. *Lm* GAPDH (GAPDH_{*Lm*}) protein levels were used as sample loading control. Image representative of at least two independent experiments.

Figure 2 – WTA L-rhamnosylation promotes InlB association to the *Lm* surface and host cell infection.

(A) Western blot of non-covalently surface-associated and secreted *Lm* protein extracts obtained from wild-type (WT), WTA L-rhamnosylation-deficient ($\Delta rmlACBD$) and complemented ($\Delta rmlACBD+rmlACBD$) strains. *Lm* GAPDH (GAPDH_{*Lm*}) protein levels were used as sample loading control. Image representative of at least two independent experiments. **(B, C)** Quantification of intracellular bacteria in HeLa cells after 2.5 h of infection **(B)** or bacteria adhered to the surface of HeLa cells after 30 min of infection **(C)**. Values for each condition are shown as percentage relative to HeLa cells infected with wild-type bacteria, and represent the mean $\pm$ SD of three independent experiments. Statistical significance determined with unpaired two-tailed t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 3 – Subcellular localization of *Lm* GW proteins.

(A) Schematic representation of the putative GW proteins identified in the genome of *Lm* EGDe and their predicted functional domains. **(B)** Western blot of non-covalently surface-associated and secreted *Lm* proteins extracted from log-phase wild-type (WT) and WTA L-rhamnosylation mutant ($\Delta rmlACBD$) strains expressing FLAG-tag fusions of the GW

domains from all nine *Lm* GW proteins. Immunoblotting (IB) was performed with indicated antibodies (right). *Lm* GAPDH (GAPDH_{*Lm*}) protein levels were used as sample loading control.

Figure 4 – GW domain surface association depends on their internal sequence and on the cell wall structure. Western blot of non-covalently surface-associated and secreted *Lm* proteins extracted from log-phase wild-type EGDe serovar 1/2a (WT), their isogenic WTA L-rhamnosylation ($\Delta rmlACBD$, $\Delta rmlT$), WTA N-acetyl-glucosaminylation ($\Delta gtcA$) and LTA ($\Delta lafAB$) mutant strains, and a wild-type *Lm* serovar 4b, all expressing FLAG-tagged GW domains of Ami (FLAG-Ami_{GW}), InlB (FLAG-InlB_{GW}) or Ami from the 4b strain (FLAG-Ami_{GW} *Lm* 4b). Immunoblotting was performed with an anti-FLAG antibody (FLAG). Images are representative of at least two independent experiments.

Supplementary Figure 1 – Expression of FLAG-tagged GW domains. Map of the pDC426 plasmid used to express FLAG-tagged GW domains of the *Lm* GW proteins. In this plasmid, derived from the *Listeria* site-specific integrative vector pPL2 (Lauer *et al.*, 2002), transcription is driven by the *ami* promoter (P_{ami}) and N-terminally FLAG-tagged proteins are targeted for secretion by the Ami signal peptide.

Supplementary Figure 2 – Alignments of the GW modules. Amino acid sequence alignments of (A) the GW modules of all nine predicted *Lm* GW proteins, (B) the GW modules of Ami and InlB in tandem, and (C) the second highly conserved GW module of each tandem of Ami and InlB. Residues present in at least 30% of the sequences are shown in blue while residues conserved in at least 80% of the sequences are shown in red.

A

B

C

Figure 1

Figure 2

A **B** **Figure 3**

Figure 4

Supplementary Figure 1

A

B

C

Supplementary Figure 2