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Remodeling of the *Listeria monocytogenes* cell wall inside eukaryotic cells

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Listeria monocytogenes is an intracellular Gram-positive bacterial pathogen that produces many types of surface proteins. To get insights into its intracellular lifestyle, we used high-resolution mass spectrometry to characterize the cell wall proteome of bacteria proliferating within the eukaryotic cell. The relative amount of a few surface proteins was found notoriously different in intracellular bacteria. Internalin A (InlA), which is covalently bound to the peptidoglycan and plays a central role in bacterial entry into non-phagocytic eukaryotic cells, was present in high amounts in the cell wall of intracellular bacteria. Our study also revealed that the actin assembly-inducing protein ActA co-purified with peptidoglycan isolated from intracellular bacteria. Growth of *L. monocytogenes* in minimal media reproduced the predominance of InlA in the cell wall and the association of ActA with peptidoglycan. Intriguingly, bacteria grown in this condition used ActA for efficient invasion of host cells. These findings suggest that the adaptation of *L. monocytogenes* to the intracellular lifestyle involves changes in the relative abundance of certain surface proteins and in their mode of association to the peptidoglycan. These alterations, probably promoted by yet-unknown changes in the cell wall architecture, may instruct these proteins to perform different functions outside and inside the host cell.

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Gram-positive bacteria contain a thick multilayered peptidoglycan macromolecule decorated with different types of proteins, teichoic and lipoteichoic acids.[1] In infections caused by these bacteria, surface proteins contribute to escape from the host immune attack, to biofilm formation and to promote adhesion/invasion of host cells.[2-4] *Listeria monocytogenes* is a Gram-positive intracellular bacterial pathogen in which surface proteins have been intensively studied.[5] This pathogen encodes a large family of surface proteins covalently bound to the peptidoglycan upon recognition of their LPXTG motif.[5-7] An important LPXTG surface protein of *L. monocytogenes* is Internalin A (InlA), an invasin that interacts with E-cadherin to promote bacterial entry into non-phagocytic eukaryotic cells.[8]

Despite many studies focused on surface proteins, the function of the numerous LPXTG proteins of the genus Listeria remains in most cases unknown. Only seven *L. monocytogenes* LPXTG proteins have a function assigned,[8-14] which contrasts with the more than 40 genes encoding this type of proteins that are found in every Listeria genome sequenced to date. We are also missing a global view of how the entire LPXTG protein family is regulated as a consequence of remodeling in the peptidoglycan structure. In this respect, several lines of evidence support the existence of changes in the peptidoglycan of *L. monocytogenes* when bacteria are located inside eukaryotic cells. Increased expression of genes encoding different cell wall-associated proteins and enzymes that modify peptidoglycan chemistry has been shown in intracellular bacteria.[15,16] Similar findings were obtained in vivo in bacteria collected from mouse organs.[17] Cell wall remodeling and host cell colonization by *L. monocytogenes* seem therefore related events. Modifications in the peptidoglycan structure involving N-deacetylation or O-acetylation reactions are also known to

impair *L. monocytogenes* recognition by the host immune system.[18,19] Whether such structural changes occur in the peptidoglycan of intracellular *L. monocytogenes* has not been addressed yet. Considering that the peptidoglycan is the platform to which all LPXTG proteins anchor, changes in their relative amount or distribution are also conceivable in bacteria residing within the host cell.

Our first gel-less proteomic studies identified 13 LPXTG proteins in cell wall of *L. monocytogenes* growing in laboratory media.[20,21] Using mass spectrometry equipment of higher resolution, we have extended that work to define the LPXTG protein content in bacteria proliferating inside eukaryotic cells.[22] Different numbers of peptides for concrete LPXTG proteins were identified in the cell wall of extra- and intracellular bacteria, which denoted changes in their relative abundance. Two examples were Lmo0514, identified in intracellular bacteria but barely detected in extracellular bacteria and Lmo2085, which displayed an opposite trend.

Another novel finding of our study involved the association of the actin-assembly protein ActA with the peptidoglycan. Up to now, ActA was believed to be tethered to the envelope only by its C-terminal transmembrane region. These diverse modes of association could provide functional versatility to ActA and explain why this protein is involved in so varied processes as the formation of actin-tails that propel intracellular bacteria to neighbor cells, the resistance to autophagy, or the invasion of host cells.[23-25] Our proteomic data support the idea of ActA adopting these distinct conformations in response to alterations in the cell wall architecture, as it may occur inside the eukaryotic cell. The significance of these hypothetical conformational changes is still unknown, but they could be linked to the 'exposure' outside the peptidoglycan lattice that this protein demands to interact with host cell cytoskeletal proteins such as VASP and the Arp2/3 complex. Interestingly, ActA is also associated with peptidoglycan in bacteria grown in minimal defined medium. In this condition, bacteria uses ActA for efficient invasion of epithelial cells together with InlA and InlB.[22] An early study reported that ActA could interact with a heparan-sulfate proteoglycan (HSPG) receptor and that such interaction promoted bacterial entry into epithelial cells.[26] Based on our current data, we favor a model of invasion in which ActA could play a relevant role in attachment of the bacteria to the host cell surface that could further facilitate the binding of InlA and/or InlB to their respective receptors (**Fig. 1**). Such hypothetical role in adhesion might rely on the association of ActA with the peptidoglycan, which could be promoted by either a particular structure in this scaffold or by interacting proteins. Importantly, changes in cell wall architecture, as those caused by the absence of the peptidoglycan hydrolase IspC, have been shown to decrease ActA exposure on the cell surface.[27]

Our proteomic study also revealed an unexpected predominance of the invasin InlA in the cell wall of intracellular

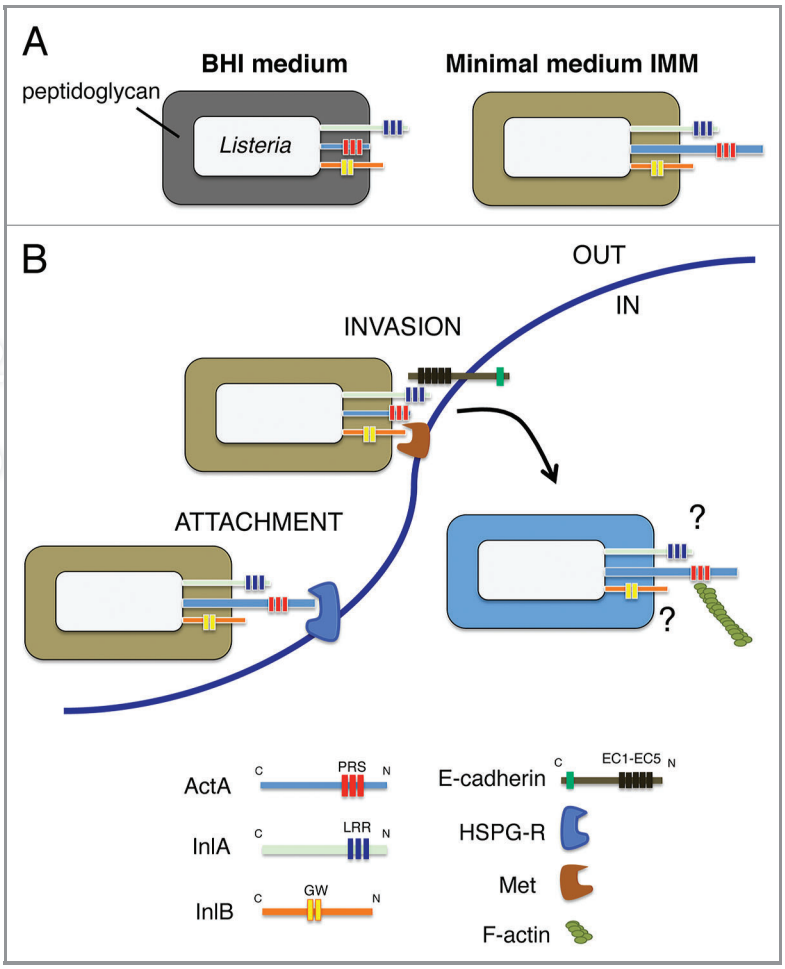

Figure 1. Remodeling of the cell wall architecture in *L. monocytogenes* when growing extracellularly in two different broth media or inside eukaryotic cells may affect exposure and function of ActA and the invasins InlA and InlB. (A) The different structure of the cell wall in bacteria grown in BHI or minimal media influences ActA association with the peptidoglycan and also probably its degree of exposure on the cell surface; (B) Based on data collected with *L. monocytogenes* grown in minimal medium, which show that ActA, InlA and InlB are all required for bacterial invasion of epithelial cells,[22] a model is proposed in which ActA could promote bacterial early attachment via its interaction with heparan-sulfate proteoglycan receptor (HSPG-R). This stage would be followed by InlA/E-Cadherin and/or InlB/Met interactions ultimately responsible for mediating bacterial entry. Different colors in the peptidoglycan denote changes in the cell wall architecture. Abbreviations: PRS: proline-rich sequences; LRR: leucine-rich repeats; GW: GW-rich domain; EC1-EC5: extracellular immunoglobulin-like domains reported for E-Cadherin. The putative role(s) played by InlA and InlB in intracellular *L. monocytogenes* remain unknown.

L. monocytogenes several hours after entry. The other well-known invasin of this pathogen, Internalin B (InlB), was also identified by proteomics in membrane fractions of intracellular bacteria. Early studies claimed low expression of the *inlAB* locus in intracellular bacteria compared with those grown in broth media.[28] However, two subsequent transcriptomic studies listed *inlA* and *inlB* as genes induced inside macrophages and epithelial cells.[15,16] Interestingly, one of these studies described a 'late-induction' of *inlA* since it seems to be upregulated at 6 h post-infection.[16] These observations are in concordance with our cell wall proteome data. However, none of these studies explain why intracellular bacteria anchor to the cell wall so large amounts of invasins used by extracellular bacteria to bind to plasma membrane receptors. InlA interacts via its leucine-rich repeat (LRR) domains with the 'extracellular' EC1 immunoglobulin-like domain of the E-cadherin molecule[8,29] but this EC1 domain should be 'invisible' to intracellular bacteria. A similar rationale could be made for InlB. So, it is tempting to speculate on yet-unknown interaction (s) of bacteria-associated InlA and InlB with alternate host molecule(s) inside the infected cell. Release of InlA and/or InlB from the bacterial surface as a result of the cell wall turnover could also potentially signal the infected cell from the 'inside' based on interactions with host molecules located either in the cytosol or internal membranes facing to this compartment. Future work is clearly needed to dissect whether InlA and/or InlB could play different roles outside and inside the host cell, as it occurs with ActA.

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