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The Cell Wall of *Listeria monocytogenes* and its Role in Pathogenicity

M. Graciela Pucciarelli,[1*] Hélène Bierne[2],
and Francisco García-del Portillo[1*]

[1]*Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CSIC), Darwin 3, 28049 Madrid, Spain*
[*]*e-mail: mgpuccia@cnb.uam.es, fgportillo@cnb.uam.es*
[2]*Unité des Interactions Bactéries-Cellules, Institut Pasteur, INSERM U604, INRA USC2020, 28 Rue du Docteur Roux, 75724 Paris cedex 15, France*
e-mail: hbierne@pasteur.fr

5.1. Introduction

Listeria monocytogenes contains a cell wall formed by a multilayered cross-linked peptidoglycan decorated with teichoic and lipoteichoic acids. Like in all eubacteria, the cell wall of *L. monocytogenes* plays a critical role in its physiology since it ensures integrity of the cell while maintaining a high internal osmotic pressure. In addition, it also endows the cell with a specific cell shape and provides protection against mechanical stress. As in other noncapsulated gram-positive bacteria, the cell wall of *L. monocytogenes* is the outermost structure of the cell and acts as a scaffold in which different proteins anchor. *L. monocytogenes* is a highly successful pathogen that invades eukaryotic host cells, crosses several natural barriers of the host and survives to extreme environments, and its cell wall must necessarily contain molecules making possible the colonization of these niches. The role in pathogenesis of some of these surface molecules is just starting to be deciphered. Likewise, the genome sequences now known for a few *L. monocytogenes* strains reveal that this pathogen has a large number of genes encoding proteins with domains mediating interactions with cell-wall polymers. Some of these cell-wall-associated proteins are currently subjected to intense investigation. Recent studies have also revisited the structure of the peptidoglycan of *L. monocytogenes* and unravelled new modifications in its structure that may be important for pathogenicity.

In this chapter, we summarize the current knowledge of the biochemistry and enzymology of the *L. monocytogenes* cell wall. Moreover, we discuss on the

plethora of proteins that attach to cell-wall components, making emphasis in the distinct modes of protein–cell-wall association and their role in virulence. We also describe recent proteomics studies that have facilitated the identification of novel *L. monocytogenes* surface proteins predicted by the genome data. Finally, we briefly describe what is known on the role of *L. monocytogenes* cell-wall components in the modulation of the host immune response.

5.2. Biochemistry of the *Listeria monocytogenes* Cell Wall

The first descriptions on the composition and suspected structure of the *L. monocytogenes* peptidoglycan and associated polymers were made more than 30 years ago (Srivastava and Siddique 1973; Ullmann and Cameron 1969). Despite this long period, the fine structure of its peptidoglycan has not been known until very recently (Kloszewska et al. 2006). Likewise, while the biochemistry of teichoic acids (TA) and lipoteichoic acids (LTA) of *L. monocytogenes* was inferred two decades ago (Fiedler 1988; Uchikawa et al. 1986a,b), these polymers are now receiving further attention as they promote the attachment of several virulence proteins and stimulate a large variety of responses in the host.

5.2.1. The Peptidoglycan of Listeria monocytogenes

Listeria monocytogenes has a peptidoglycan formed by glycan chains containing alternating units of the disaccharide *N*-acetylmuramic acid (MurNAc)-(β-1,4)-*N*-acetyl-D-glucosamine (GlcNAc). Bound to the MurNAc residue is a stem peptide that in *L. monocytogenes* contains L-alanine-γ-D-glutamic acid-*meso*-diaminopimelic acid-D-Ala-D-Ala [L-Ala-γ-D-Glu-*m*-Dap-D-Ala-D-Ala] (Fiedler 1988; Kamisango et al. 1982). The glycan chains are cross-linked by 4→3 linkages between the D-Ala residue of one lateral peptide to the *m*-Dap residue of the other stem peptide. This peptidoglycan structure resembles the reported for many gram-negative bacteria as *Escherichia coli* (Schleifer and Kandler 1972). The biosynthesis of the *L. monocytogenes* peptidoglycan occurs essentially as described in *E. coli* (Holtje 1998), with the formation of the intermediates; lipid I (C_{55}-PP-MurNAc-L-Ala-γ-D-Glu-*m*-Dap-D-Ala-D-Ala) and lipid II [C_{55}-PP-MurNAc-(L-Ala-γ-D-Glu-*m*-Dap-D-Ala-D-Ala)-(β-1,4)-GlcNAc] (Holtje 1998; Navarre and Schneewind 1999). Lipid II is substrate of transglycosylases that bind the disaccharide-pentapeptide molecule to macromolecular peptidoglycan. This reaction leaves the undecaprenyl-pyrophosphate carrier (C_{55}-PP) free to reinitiate the first biosynthetic steps. The transglycosylation reaction is followed by transpeptidation of lateral peptides belonging to adjacent glycan chains, ensuring in this way the incorporation of nascent peptidoglycan.

The first reactions of the peptidoglycan biosynthetic pathway are catalyzed by a conserved number of essential enzymes (Holtje 1998). However, the transglycosylation and transpeptidation reactions are carried out by a variable number of enzymes known as penicillin-binding proteins (PBPs) (Goffin and Ghuysen 2002). All PBPs share the property of binding β-lactam antibiotics, irreversible inhibitors of the transpeptidation (acyl-transferase) reaction involving the rupture of the D-Ala-D-Ala bond. PBPs are multimodular enzymes carrying in their C-terminal module highly conserved SXXK, SXN, and KTG motifs essential for the transpeptidation reaction (Goffin and Ghuysen 2002). Some PBPs also have an N-terminal transglycosylation module. These bi-functional transglycosylase-transpeptidase enzymes are known as class A PBPs. Other PBPs, named as class B, carry an N-module that interacts with components of the morphogenetic apparatus. A third group of PBPs only have the C-module and are named carboxipeptidases or endopeptidases depending on whether they cleave, using water as acceptor molecule, D-Ala-D-Ala or *m*-Dap-D-Ala linkages, respectively. In most bacteria, this third group of PBPs are nonessential and play roles related to peptidoglycan maturation (Goffin and Ghuysen 2002).

Using isotopically labelled β-lactam antibiotics, several studies showed that *L. monocytogenes* has five PBPs, ranging in molecular weight from 95 kDa to 49 kDa (Gutkind et al. 1989; Hakenbeck and Hof 1991; Vicente et al. 1990b). The number of molecules of PBP per cell was estimated in ~ 100 for PBP1, PBP2, PBP3, and PBP4, and ~ 600 for PBP5 (Vicente et al. 1990a). Among these enzymes, PBP4 and PBP3 have the highest and lowest affinity, respectively, for binding of the β-lactam antibiotics (Pierre et al. 1990; Vicente et al. 1990b). The low affinity of PBP3 for β-lactam antibiotics is thought to be the basis of the high intrinsic resistance displayed by *L. monocytogenes* to monobactams and cephalosporins of broad spectrum. *L. monocytogenes* has a distinct PBP profile compared to other *Listeria* species, which is however fairly conserved among *L. monocytogenes* strains (Hakenbeck and Hof 1991). Despite the availability of the complete genome sequence of four *L. monocytogenes* strains (Glaser et al. 2001; Nelson et al. 2004), no study has reported the exact correspondence between each of the PBPs detected by biochemical analysis and their coding genes.

Two PBPs have been recently characterized in the *L. monocytogenes* EGD-e strain: PBP4, a class A PBP encoded by the *lmo2229* gene which displays transglycosylase, transpeptidase, and carboxipeptidase activities (Zawadzka et al. 2006); and, PBP5, a class B PBP with DD-carboxipeptidase activity encoded by *lmo2754* (Korsak et al. 2005). Deletion mutants have been obtained for each of these two PBPs, confirming that none of them is essential for *L. monocytogenes* growth, at least in laboratory conditions. Lack of PBP4 results in a slower growth rate and increased resistance to moenomycin, an antibiotic that inhibits transglycosylase activity (Zawadzka et al. 2006). Phenotypes described for the PBP5 mutant include an increase in the cell-wall thickness and a reduction in the growth rate (Korsak et al. 2005). Whether or not these two PBPs contribute to *L. monocytogenes* pathogenicity remains unknown.

The fine structure of the *L. monocytogenes* peptidoglycan has been recently resolved by reverse-phase high pressure liquid chromatography (HPLC) and mass spectrometry (Kloszewska et al. 2006). Besides the conventional interpeptide linkage (4→3) D-Ala-*m*-Dap, the *L. monocytogenes* peptidoglycan contains (3→3) *m*-Dap-*m*-Dap linkages. This latter interpeptide linkage was previously reported in *Mycobacteria* and *E. coli* (Glauner and Höltje 1990; Wietzerbin et al. 1974). The transpeptidation reaction resulting in the (3→3) linkage is inherently insensitive to β-lactam antibiotics (Goffin and Ghuysen 2002). So, *L. monocytogenes* must have at least one "non-PBP" enzyme responsible for the building of this concrete bridge. The physiological significance of this type of linkage remains to be explored. Other structural features of the *L. monocytogenes* peptidoglycan include the amidation of the free carboxylic group of some *m*-Dap residues and the absence of glucosamine acetylation in certain muropeptides. These types of modifications were previously reported in *Bacillus subtilis* (Atrih et al. 1999). The average cross-linkage of the *L. monocytogenes* peptidoglycan is in the range of ~ 65% (Kloszewska et al. 2006), slightly higher than that described in the peptidoglycan of *B. subtilis*, ~ 45%. Unlike most gram-positive bacteria, *L. monocytogenes* does not lyse in the presence of β-lactams, remaining the action of these antibiotics as bacteriostatic. The underlying mechanisms are unknown.

5.2.2. Other Cell-Wall Polyanionic Polymers: Teichoic Acids and Lipoteichoic Acids

As most gram-positive bacteria, *L. monocytogenes* contains two different polyanionic polymers decorating the cell wall: the teichoic acids (TA), covalently bound to the peptidoglycan and the lipoteichoic acids (LTAs), amphipathic molecules that are embedded into the plasma membrane by a diacylglycerolipid (Navarre and Schneewind 1999; Neuhaus and Baddiley 2003). These polymers represent as much as 50–60 % of the total content of isolated dry cell walls of *L. monocytogenes* (Fiedler 1988) and play important functions in metal cation homeostasis, anchoring of surface proteins, and transport of ions, nutrients, and proteins. TA and LTA, which are synthesized by noninterconnecting metabolic pathways (Neuhaus and Baddiley 2003), are main determinants of surface hydrophobicity and immunogenicity. In fact, both TA and LTA confer the basis of the serotype diversity known in *L. monocytogenes*.

Two main types of TA exist in *L. monocytogenes*. The first is formed by a polymer consisting of repeating units (~20 to ~45) of 1,5-phopshodiester-linked ribitol residues (Fiedler 1988; Uchikawa et al. 1986a). These ribitol-P units bear variable substitutions. Thus, in serotypes 3a, 3b, and 3c, a GlcNAc residue is linked at position C-4 of the ribitol-P repeating unit whereas in serotypes 1/2a, 1/2b, and 1/2c there is an additional rhamnose residue at position C-2. Remarkably, the serotype 7 has no substitutions in the ribitol-P. The second type of TA includes more complex structures in which the GlcNAc residue incorporates as a part of the poly-ribitol-P chain (Fiedler 1988; Uchikawa et al. 1986b). Thus, the C-1 of GlcNAc binds to

hydroxyl groups present at positions C-4 (serotypes 4a and 6) or C-2 (serotypes 4b, 4d, and 4f) of the ribitol-P. The C-4 position of GlcNAc then links to the phosphate of the adjacent ribitol-P unit. In addition, the GlcNAc units are decorated with glucose and/or galactose in some serotypes (case of 4b, 4d, and 4f) (Uchikawa et al. 1986a). A TA structure similar to that of *L. monocytogenes* 4b serotype has been identified in a few *L. innocua* strains. It is possible that *L. innocua* may have acquired from *L. monocytogenes* serotype 4b, the set of genes responsible for these modifications, a hypothesis supported by comparative genome analysis (Doumith et al. 2004). The genome sequences obtained from four *L. monocytogenes* strains, two of serotype 1/2a (EGD-e and F6854) and two of serotype 4b (F2365 and H7858), have revealed the presence of 1/2a serotype-specific genes involved in rhamnose biosynthetic pathway (Nelson et al. 2004). The existence of these serotype-specific genes related to TA biosynthesis was recently confirmed upon genome content analysis of 93 *L. monocytogenes* strains of diverse serotypes (Doumith et al. 2004). Thus, serotypes 1/2, 3, and 7 carry genes involved in TA biosynthesis that are absent in serotype 4. Inversely, a gene annotated with function putatively related to TA synthesis (ORF2372) is present exclusively in serotypes 4b, 4d, and 4e (Doumith et al. 2004). The exact function of ORF2372 has not been elucidated. Another gene named *gtcA* was initially claimed as a 4b serotype-specific gene involved in the decoration (glycosylation) of TA, concretely in the incorporation of galactose and glucose to the GlcNAc residues (Promadej et al. 1999). However, *gtcA* ortholog genes exist in the genome of 1/2a strains (Autret et al. 2001). In fact, insertions in the *gtcA* gene of the serotype 1/2a strain EGD-e impair virulence and its product has been proposed to mediate incorporation of rhamnose to the TA (Autret et al. 2001). Further work is required to unravel whether the role of GtcA in the decoration of TA differs in serotypes 1/2a and 4b. It was later shown that mutants in the *gltA–gltB* gene cassette, which is a truly specific locus of serotype 4b, display a severe reduction or total loss of incorporation of galactose to the GlcNAc residue of TA (Lei et al. 2001). These mutants have unaltered the amount of glucose in the TA, which suggests that GltA and GltB are specifically involved in the linkage of galactose to GlcNAc independently of the glucose substitution. The contribution of GltA and GltB to *L. monocytogenes* pathogenicity has not been yet tested.

The ribitol-P polymer of the *L. monocytogenes* TA is covalently bound to the peptidoglycan by a linkage unit formed by two disaccharides bound by a molecule of phosphoglycerol. This linkage unit has the structure Glc(β1→3)-Glc(β1→1/3)Gro-P-(3/4)ManNAc(β1→4)GlcNAc (Kaya et al. 1985). The disaccharide formed by the two molecules of Glc is bound to the ribitol-P polymer whereas that containing the acetylated amino sugars binds via a 1,6-phosphodiester linkage to the MurNAc residue of the peptidoglycan. The *L. monocytogenes* linkage unit of the TA is more complex than that of the closely related bacteria *B. subtilis* and *Staphylococcus aureus*, which contain only one disaccharide (Navarre and Schneewind 1999; Neuhaus and Baddiley 2003).

Unlike TA, the LTA polymer of *L. monocytogenes* is formed by repeating units of glycerol-P bound by 1,3 linkages. In some strains, the C-2 position of the glycerol-P is decorated with galactose (Fischer et al. 1990). The

glycerol-P polymer attaches to the C-6 position of a nonreducing sugar molecule carried by a glycolipid molecule that embeds the LTA into the membrane. In *L. monocytogenes*, the glycolipid molecule has the structure of Gal(α1$\rightarrow$2)Glu-1(3),2-diacylglycerol (Fiedler 1988; Fischer et al. 1990). A substitution of a phosphatidic acid in the C-6 position of Glu has been reported in some strains.

A common modification found in TA and LTA of many gram-positive bacteria is D-alanine-esterification at carbons of their respective repeating units (Neuhaus and Baddiley 2003). This modification is accomplished by a unique D-Ala incorporation system encoded in the *dlt* operon. D-Ala esterification has a profound effect on the electromechanical properties of the cell wall since it reduces its global negative charge. This modification modulates distinct cellular functions as the activities of autolysins, the maintenance of cation-homeostasis, and the assimilation of metal cations (Neuhaus and Baddiley 2003). The existence of a *dtl* operon in the genome does not necessarily imply that the TA are D-Ala-esterified. Thus, *Streptococcus pneumoniae* strain R6 harbours an entire *dlt* operon but contains phosphorylcholine-esters instead of D-Ala-esters in both TA and LTA.

The *L. monocytogenes dlt* operon consists of four genes, *dltA, dltB, dltC*, and *dltD*, encoding all components required for D-Ala esterification (Abachin et al. 2002). D-Ala-esterification is important for *L. monocytogenes* pathogenicity. Thus, a *dltA* mutant displays enhanced sensitivity to antimicrobial cationic peptides and virulence attenuation in the mouse-infection model (Abachin et al. 2002). D-Ala-esterification occurs in laboratory conditions in ~20% of the glycerol-P residues of LTA and is not detected in LTA of the *dltA* mutant. No study has reported the rate of D-Ala-esterification in TA of *L. monocytogenes*. Interestingly, the defect in D-Ala-esterification of LTA displayed by the *dltA* mutant does not alter the relative amount of surface proteins as internalin-A (InlA), InlB, and ActA that are extracted from the cell surface. Based on this result, D-Ala-esterification was postulated to be important for certain surface proteins to reach a functional folding state (Abachin et al. 2002), although it does not discard a potential role of D-Ala-esterification in modulating the anchoring of surface proteins to the cell wall. Noteworthily, VirR, a new response regulator implicated in *L. monocytogenes* virulence, controls among other functions the expression of the *dlt* operon (Mandin et al. 2005). This observation reinforces the idea that D-Ala-esterification is a cell-wall modification playing a prominent role in the *L. monocytogenes* infection process.

5.3. *Listeria monocytogenes* Surface Proteins Anchored to the Cell Wall

One of the most remarkable features of the *L. monocytogenes* genome is the high content of genes encoding surface proteins (Glaser et al. 2001; Nelson et al. 2004). Some of these proteins are not directly associated to the cell wall as they either carry transmembrane domains or N-terminal signals recognized for insertion of a lipid molecule (lipoproteins). None of these two groups will

be discussed in this chapter. Instead, we discuss about those proteins known to interact with structures forming part of the cell wall and, therefore, entirely located outside the plasma membrane (Figure 5.1.).

5.3.1. Surface Proteins Anchored Covalently to the Peptidoglycan

5.3.1.1. The LPXTG Protein Family

Pioneering work performed with the *S. aureus* protein A demonstrated that this surface protein is anchored covalently to the peptidoglycan by an enzyme

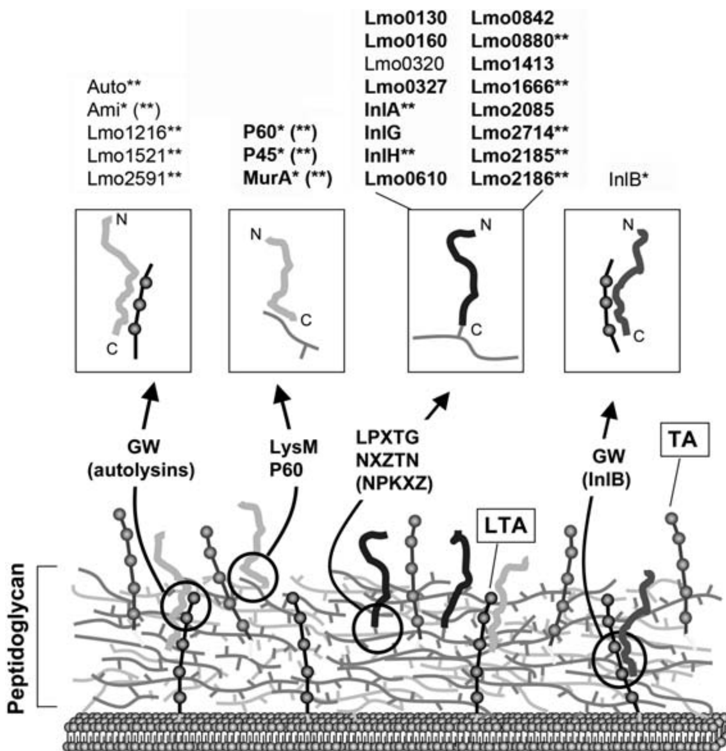

FIGURE 5.1. Global view of the distinct interactions of the *Listeria monocytogenes* surface proteins with the cell wall. Listed in the *upper* part are the proteins identified by proteomics in purified peptidoglycan material (in bold); in extracts obtained from the cell wall upon incubation of bacteria with high concentration of salts (asterisk, *); or, in the extracellular medium as components of the "secretome" (double asterisk, **). Lmo0320 (Vip), although unidentified in these studies, was detected on the cell surface by immunological assays (Cabanes et al. 2005). Other nonlisted surface proteins with mode of association to the cell wall unknown but identified in proteomic analysis include: Lmo1892 (PbpA) in purified peptidoglycan material and Lmo2504, Lmo2522, and Lmo2754 (PBP5) in the extracellular medium. TA: teichoic acid; LTA: lipoteichoic acid.

named sortase (Mazmanian et al. 1999). Protein A contains an N-terminal signal peptide and a C-terminal domain consisting of an LPXTG motif followed by a hydrophobic region of about 20 amino acids that ends in a tail of mostly positively charged residues (Ton-That et al. 2004). This C-terminus configuration, named "sorting signal", is conserved in many surface proteins of gram-positive bacteria (Navarre and Schneewind 1999; Ton-That et al. 2004).

The *in-silico* genome analysis of the *L. monocytogenes* strain EGD-e unravelled 41 genes-encoding surface proteins bearing an LPXTG motif (Glaser et al. 2001). To date, this number of LPXTG proteins is the highest among all the gram-positive bacteria with genome sequence known. A high number of genes-encoding LPXTG proteins, in the average of 45, were also identified in the genome of other three *L. monocytogenes* strains (Nelson et al. 2004). Despite this bulk of information, very few of these proteins have been characterized at the biochemical and/or functional level. The LPXTG protein most extensively studied is InlA, which promotes entry of *L. monocytogenes* into epithelial cells. InlA harbours an LPTTG motif and is anchored covalently by the sortase SrtA to *m*-Dap residues of the peptidoglycan (Bierne et al. 2002; Dhar et al. 2000; Garandeau et al. 2002). In *S. aureus*, the primary acceptor molecule in the anchoring reaction catalyzed by SrtA is the lipid-II precursor (Perry et al. 2002). Whether the *L. monocytogenes* sortase SrtA uses the same mechanism has not yet been formally demonstrated.

The large set of LPXTG proteins of *L. monocytogenes* clusters in two subfamilies that differentiate by the presence in their N-terminal half of a variable number of leucine-rich repeats (LRR) containing 20–22 amino acids each (Cabanes et al. 2002). This domain is a feature shared by all proteins belonging to the "internalin family". The LRR domain is thought to mediate protein–protein interactions, and in the case of InlA is necessary and sufficient to promote bacterial uptake. The *L. monocytogenes* strain EGD-e has 19 LPXTG proteins containing the LRR domain (Cabanes et al. 2002). Of these, only eight are present in the nonpathogenic species *L. innocua.* A similar number of internalins bearing LPXTG motifs (from 14 to 17 proteins) has been reported in the other three *L. monocytogenes* strains with genome sequence known (Nelson et al. 2004). Besides InlA, a few LPXTG proteins of the internalin family have been recently characterized in the EGD-e strain. These include InlH (Lmo0263), InlI (Lmo0333), and InlJ (Lmo2821) (Sabet et al. 2005; Schubert et al. 2001) (see Section 5.4.1).

Proteins containing diverse non-LRR repeat regions preceding the C-terminal sorting region form the second class of LPXTG proteins. The EGD-e strain has 22 LPXTG proteins in this class, of which 14 have orthologs in *L. innocua* (Cabanes et al. 2002; our unpublished data). To date, only one protein of this group, Vip (Lmo0320), has been characterized at a functional level (Cabanes et al. 2005) (see Section 5.4.1).

Genomic comparison studies have revealed that six LPXTG proteins of *L. monocytogenes* serotypes responsible for most cases of listeriosis are absent in the rest of *Listeria* species (Doumith et al. 2004). LPXTG proteins displaying

this narrow distribution in the *Listeria* genus are currently investigated for their role in pathogenesis (see Section 5.4.1).

5.3.1.2. Non-LPXTG Proteins Bearing Cell-Wall Sorting Signals

Staphylococcus aureus has a second sortase, SrtB, which recognizes a sorting motif different than LPXTG. This sortase specifically cleaves an NPQTN motif in a surface protein involved in iron transport, IsdC (Mazmanian et al. 2002). *L. monocytogenes* has also an alternative SrtB sortase (Bierne et al. 2004). Like in *S. aureus*, the *L. monocytogenes srtB* gene maps in an operon containing two genes encoding the Lmo2185 (formerly SvpA) and Lmo2186 (SvpB) proteins, which share homology to *S. aureus* IsdC. Lmo2185 and Lmo2186 bear as putative sorting motifs NAKTN and NKVTN (NPKSS), respectively. Although a direct proof of their covalent anchoring to the peptidoglycan has not been yet shown, both proteins are detected in highly purified peptidoglycan material (Calvo et al. 2005) (see Sect. 5.5.1). Furthermore, Lmo2185 displays a unique migration on gels when extracted from peptidoglycan material (Bierne et al. 2004), suggesting that this species may correspond to the processed form covalently anchored to the peptidoglycan. Detection of Lmo2185 at the cell surface is also abolished in an *srtB* mutant (Bierne et al. 2004). Interestingly, the *L. monocytogenes* operon containing the *lmo2185, lmo2186*, and *srtB* genes is induced in iron-deficient conditions, but neither Lmo2185 nor Lmo2186 are required for haemin, haemoglobin, or ferrichrome utilization (Newton et al. 2005). The exact function of these two surface proteins, which are conserved in all *Listeria* species, remains therefore elusive.

5.3.2. Surface Proteins with Noncovalent Association to the Cell Wall

5.3.2.1. InlB, a Protein Loosely Associated to the Cell Wall by GW Modules

InlB is a surface protein required for *L. monocytogenes* entry into certain eukaryotic cell types (see Section 4.2). InlB is the only *L. monocytogenes* surface protein carrying a domain organization consisting of a N-terminal LRR domain and a C-terminal domain of three repetitions of 80 amino-acids, called GW modules (Braun et al. 1997). GW modules are also found in autolysins (see below). Domain swapping experiments revealed that the GW-modules mediate binding of InlB to lipoteichoic acids (LTA) (Jonquieres et al. 1999). This association also occur when purified InlB is added externally to intact cells of *L. monocytogenes* serotype 1/2a. InlB is efficiently extracted from the cell surface when bacteria are incubated in the presence of LTA, which indicates that the InlB-LTA association may be rather weak. Noteworthy, InlB does not bind to purified cell walls containing TA (Jonquieres et al. 1999), which supports the idea that the GW-modules specifically interacts with polymers of the LTA type. The strength of the GW domain-LTA association increases with the number

of GW-modules. Thus, an InlB variant bearing the 8-GW module region of the autolysin Ami binds more efficiently to cell surface. It has been shown that purified In1B does not attach to either the *L. innocua* surface or that of *S. pneumoniae*, the latter being decorated with LTA devoid of polyglycerol-P. This observation leaves open the possibility that other cell wall components, as TA, may also modulate the association of InlB to the cell wall. This hypothesis could be tested by determining the capacity of purified InlB for binding to *L. monocytogenes* 4b cells, which have a TA structurally different to serotype 1/2a cells.

5.3.2.2. The Autolysin Family

The peptidoglycan is a highly dynamic macromolecule whose structure is continuously modified by hydrolytic enzymes, also known as autolysins (Holtje 1998; Popowska 2004). These enzymes cleave preexisting linkages and act coordinately with biosynthetic activities during the incorporation of nascent peptidoglycan or the separation of daughter cells upon cell division. Hydrolytic enzymes are also responsible for the active release of cell-wall components (up to 30–50% per generation in some bacteria) and, as in the case of gram-negative bacteria, for the active recycling of peptidoglycan turnover products. Hydrolytic enzymes necessarily have to be subjected to tight temporal and spatial control since their indiscriminate activity may lead to cell lysis. The profile of autolytic enzymes in a given bacterium is assessed by zymogram assays using gels loaded with cell walls. Renaturated proteins displaying cell-wall degrading activities are visualized following gel staining. Zymogram assays performed with extracts of *L. monocytogenes* surface proteins have revealed a large number of hydrolytic enzymes (McLaughlan and Foster 1997, 1998; Popowska 2004). In some cases, these assays have proved to be very useful for assigning hydrolytic activity to novel proteins uncovered by genome data (Cabanes et al. 2004; Carroll et al. 2003; McLaughlan and Foster 1998; Milohanic et al. 2001). *L. monocytogenes* contains several types of autolysins, all of them harbouring domains that promote attachment of the protein to the cell wall (Cabanes et al. 2002). These include the "amidase" domain, with similarity to the MurNAc-L-Ala amidase of the Atl autolysin of *S. aureus*; the "LysM" domain; the GW modules; and, the so-called "P60-domain". Some of these enzymes are required for *L. monocytogenes* pathogenicity (see Section 5.4.2). Many of the autolysins containing an amidase domain carry a variable number of GW modules (Milohanic et al. 2001). Domains containing short repetitions are also present in autolysins of *Streptococcus pneumoniae* and *Staphylococci*. In *S. pneumoniae* these modules are responsible for binding of the protein to choline residues that decorate the TA and LTA. Seven proteins with this "amidase-GW" domain organization are known in the *L. monocytogenes* strain EGD-e (Cabanes et al. 2002). Two representative autolysins of this subfamily are Ami (Lmo2558) and Auto (Lmo1076), which contain 8- and 4-GW modules, respectively, in their C-half region (Cabanes et al. 2004; Milohanic et al. 2001). Interestingly, Ami from *L. monocytogenes* strains of serotypes 4b, 4d, and 4e carry only 6-GW modules and, in contrast

to its N-half, the C-half region displays low identity (54% at the aminoacid level) to Ami of EGD-e (serotype 1/2a) (Milohanic et al. 2004). Purified Ami protein from serotype 4b binds less efficiently to 1/2a bacterial cells than to 4b cells (Milohanic et al. 2004), suggesting that in addition to LTA, other cell-wall elements may modulate cell wall–protein association mediated by the GW modules. Such an element could be the TA molecule, which is structurally different in serotypes 1/2a and 4b (see Section 5.2.2). This hypothesis fits to the fact that Ami of serotype 1/2a does not bind to the surface of *L. innocua* serotype 6a (Milohanic et al. 2001), which has a TA structure similar to *L. monocytogenes* serotype 4.

Auto is the only autolysin of this subfamily that is absent in the nonpathogenic species *L. innocua*. The *aut* gene is flanked by *lmo1077*, a gene with function related to TA synthesis and also absent in *L. innocua*. Whether Lmo1077 is required for association of Auto to the cell wall is at present unknown.

Another important group of *L. monocytogenes* autolysins is formed by the P60-subfamily. All the members of this subfamily share an NPLC/P60 domain in their C-terminal region. Four proteins of the EGD-e strain have been classified in this subfamily: Spl (P45), Lmo0394, Lmo1104, and P60 itself. Lmo1104 is the only autolysin of the group that is absent in *L. innocua*. In addition to the P60 domain, the P60 protein carries two LysM domains and a bacterial Src-homology 3 (SH3) domain which promote protein association to the cell wall (Cabanes et al. 2002). P60-defective mutants display abnormal morphology, characterized by the presence of filamented cells containing fully formed septa (Gutekunst et al. 1992; Wuenscher et al. 1993). This phenotype links the function of P60 to cell division.

A third type of domain organization is found in a recently characterized autolysin named MurA (Lmo2691). This protein contains an amidase domain in the N-half of the protein followed by 4 LysM domains (Carroll et al. 2003). As in P60-deficient mutants, strains lacking MurA display elongated morphology. The defect in MurA also correlates with increased resistance to detergent-mediated lysis (Carroll et al. 2003), which suggests that MurA could be involved in generalized remodelling of the peptidoglycan. MurA and P60 are secreted, together with other proteins, by a specialized secretion machinery dependent on the SecA2 protein (Lenz et al. 2003). Defects in the secretion of these two autolysins have been linked to the transition to a rough phenotype observed in some *L. monocytogenes* isolates (Machata et al. 2005).

The *L. monocytogenes* EGD-e strain has another two putative autolysins with a unique domain organization. The first, Lmo0849, contains an amidase domain in the middle part of the protein and a transmembrane domain in its C-terminal region. This protein, which would be the only *L. monocytogenes* autolysin embedded in the plasma membrane, has not been characterized yet. The second is Lmo0327, a LPXTG protein containing five LRR domains in the N-half of the protein and 15 repeat regions specific of this protein in its C-half region (Popowska and Markiewicz 2006). The autolytic activity of Lmo0327 was inferred in a screening of autolytic activity using a library of *L. monocytogenes*

EGD-e cloned in *E.coli*. The *L. monocytogenes lmo0327* mutant lacks several bands in zymogram assays, one of them with the expected molecular weight of Lmo0327. This mutant displays an elongated shape, defects in cell separation, and slightly higher resistance to Triton X100-stimulated lysis. Further work is required to confirm whether Lmo0327, which does not contain any domain related to peptidoglycan hydrolysis, is a bona-fide autolysin. In vitro assays with purified protein could provide such evidence. It is worth to mention that to date no study has provided a direct proof of the specific linkage(s) of the peptidoglycan cleaved by any autolysin of *L. monocytogenes*. Attempts made with purified P60 on peptidoglycan were unsuccessful due to the inherent property of this protein to aggregate (Wuenscher et al. 1993).

5.4. Role of Cell-Wall-Associated Proteins in *Listeria monocytogenes* Virulence

This section summarizes those studies in which the contribution of *L. monocytogenes* surface proteins to pathogenicity was tested using diverse infection models. For additional information, the reader is referred to Chap. 8 by Pizarro and Cossart.

5.4.1. LPXTG Proteins and Sortases

5.4.1.1. Species-Specific Role of InlA in Crossing of Intestinal and Placental Epithelia

Despite the critical role of InlA as an *L. monocytogenes* invasion protein, it was for long impossible to associate InlA with virulence in mouse models (Gaillard et al. 1996; Gregory et al. 1996; Pron et al. 1998). This unexpected result was explained by the discovery of a species specificity of InlA interaction with its host receptor, the cell adhesion molecule E-cadherin (Ecad). Thus, InlA interacts with human or guinea pig Ecad but does not recognize mouse or rat Ecad. This specificity is due to a single amino acid, a proline at position 16 in the binding site of human Ecad, which is a glutamic acid residue in the mouse or rat Ecad (Lecuit et al. 1999). This change leads to structural modifications that prevent mEcad–InlA interaction (Schubert et al. 2002). The usage of transgenic mice expressing hEcad in the intestine revealed a prominent role of the InlA–hEcad interaction in *L. monocytogenes* invasion of enterocytes (Lecuit et al. 2001). This conclusion was also established upon oral infection of guinea pigs, which naturally possess a permissive Ecad. Altogether, these results demonstrate that in permissive species InlA plays a role in the crossing of the intestinal barrier. Recent data indicate that InlA is also critical for *L. monocytogenes* fetoplacental tropism (Lecuit et al. 2004). Thus, the ability of *L. monocytogenes* to target the placental villi and cross the placental barrier is dependent upon InlA interaction with trophoblast E-cadherin. This observation correlates with epidemiological data showing that

100% of *L. monocytogenes* isolates are obtained from pregnancy-associated listeriosis but only 65% of food isolates express a functional InlA (Jacquet et al. 2004).

5.4.1.2. Inactivation of Sortases to Assess the Role in Virulence of Cell-Wall Bound Proteins

Inactivation of *L. monocytogenes* SrtA abolishes anchoring of many cell-wall bound proteins to peptidoglycan (Bierne et al. 2002). Interestingly, an *srtA* mutant displays lower organ colonization in mice than an isogenic *inlA* mutant when used by both oral or intravenous routes (Bierne et al. 2002; Garandeau et al. 2002). These data indicate that besides InlA, other LPXTG proteins are required for full virulence. This conclusion was further confirmed in guinea pigs and h-Ecad transgenic mice, in which *L. monocytogenes* efficiently crosses the intestinal barrier in an InlA-dependent-manner. In these models, the *srtA* mutant is also more attenuated for virulence than the *inlA* mutant. These results point to the critical role of SrtA substrates, besides InlA, in bacterial invasion and/or persistence in deeper organs in listeriosis, from the crossing of the intestinal barrier to the hepatic phase of infection (Sabet et al. 2005).

The second *L. monocytogenes* sortase, SrtB, does not play any detectable role in virulence in the mouse model (Bierne et al. 2004). SrtB is also dispensable for virulence following oral infection of guinea pigs (Sabet et al. 2005). Therefore, the two substrates recognized by this sortase, Lmo2185 and Lmo2186, are unlikely to play a major role in food-borne listeriosis. Consistently, an *lmo2185* mutant is not attenuated in virulence in the mouse model (Newton et al. 2005). The inactivation of the *svpA-srtB* operon, containing *srtB*, *lmo2185(svpA)*, *lmo2186*, and genes encoding an iron transporter system, has however a moderate effect on persistence of *L. monocytogenes* in mouse organs (Newton et al. 2005).

5.4.1.3. New LPXTG proteins as virulence factors

5.4.1.3.1. InlH

Searching for *inlA*-related genes by southern hybridization using an *inlA* probe revealed the presence of the *inlC2-inlD-inlE* locus in strain EGD (Dramsi et al. 1997). The inactivation of this locus did not result in decreased virulence in the murine model. Surprisingly, three related genes, *inlG-inlH-inlE*, are found at the same locus in strain EGD-e (Raffelsbauer et al. 1998). Multiple deletions of the *inlG-inlH-inlE* genes or a single deletion of *inlH* decreases *L. monocytogenes* virulence in the mouse (Schubert et al. 2001). These results implicate at least *inlH* in pathogenicity although further work is required to discern its exact role in virulence.

5.4.1.3.2. Vip

The *vip* gene (*lmo0320*), identified as one of the 8 genes encoding LPXTG proteins present in *L. monocytogenes* EGD-e and absent from *L. innocua*

is positively regulated by PrfA, the transcriptional activator of the major *L. monocytogenes* virulence factors. Vip is required for bacterial entry into some eukaryotic cells as well as for the infectious process in vivo at both the intestinal level and the later stages of organ colonization (Cabanes et al. 2005). Vip may contribute to pathogenesis by interacting with the host endoplasmic reticulum chaperone Gp96 (Li et al. 2002), inducing either cell invasion and/or signaling events that interfere with the host immune response (Cabanes et al. 2005).

5.4.1.3.3. InlJ

The DNA content and genomic biodiversity of *Listeria* strains of different species and serotypes analyzed by DNA arrays identified *L. monocytogenes*-specific marker genes (Doumith et al. 2004). Among these markers there are five LPXTG proteins of the internalin family, InlA, InlH, InlE, InlI, and InlJ. The contribution to pathogenesis of InlJ (Lmo2821) and InlI (Lmo0333) was recently evaluated. An *inlJ* mutant displays a virulence defect in mice and transgenic hEcad mice after intravenous and oral infection, respectively. In contrast, deletion of the inlI gene has no effect on virulence (Sabet et al. 2005). No phenotype could be attributed to the *inlJ* mutant in tissue culture cells, making its function elusive. Noteworthily, InlJ is not detected in the cell-wall proteome of bacteria grown in brain-heart-infusion (BHI) medium (Pucciarelli et al. 2005) (Section 5.5.1), raising the possibility that expression of *inlJ* might be tightly regulated. InlJ is structurally related to InlA, bearing a new type of cysteine-rich LRR motifs and repetitions related to MucBP domains in the C-terminal region (Sabet et al. 2005). A future challenge will be to assess when and where *inlJ* is expressed and to identify the InlJ eukaryotic binding partner as well as its signaling pathways.

5.4.1.3.4. L. monocytogenes *serotype 4b-specific LPXTG proteins*

The biodiversity DNA array data revealed two genes encoding LPXTG proteins, *ORF29* and *ORF2568*, present exclusively in serotypes 1/2b, 4a, 4b, and 4c (Doumith et al. 2004). Since *L. monocytogenes* serotype 4b strains are responsible for the majority of epidemic cases of listeriosis, these genes were investigated for their role in infection. Inactivation of these genes does not however alter either infection in vitro or virulence following oral infection of hEcad mice (Sabet et al. 2005). However, the bacterial load of the *ORF2568* deletion mutant in organs, especially spleens, increases compared to the wild type 4b strain. Inactivation of *ORF2568* may somehow affect expression or function of other virulence factors and enhance bacterial fitness in organs.

5.4.2. Other NonCovalently Bound Proteins

5.4.2.1. InlB: A Second Species Specificity

The invasion protein InlB is a functional homologue of the human hepatocyte growth factor, h-HGF, acting as an agonist of the hepatocyte growth factor receptor (HGF-R/Met). Met is a widely expressed receptor tyrosine kinase

involved in complex cellular processes such as cell proliferation, dissociation, migration, and differentiation (Shen et al. 2000). InlB also interacts with proteoglycans and gC1q-R, a ubiquitous glycoprotein (Braun et al. 2000; Jonquieres et al. 2001). InlB–Met interaction is required for *L. monocytogenes* invasion in a variety of cell types in which InlA plays no role, such as hepatocytes, endothelial cells, and fibroblasts (Dramsi et al. 1995; Gregory et al. 1997; Greiffenberg et al. 1998; Lingnau et al. 1995; Parida et al. 1998) (see also Chap. 8). However, despite in-depth knowledge on InlB in vitro activities, its role in vivo was not extensively explored for long. InlB was first shown to be involved in mouse liver and spleen colonization using a double *inlA-inlB* deletion mutant (Gaillard et al. 1996), or in competition index experiments using wild-type and *inlB* strains (Dramsi et al. 2004). A recent study confirmed that InlB is required for liver and spleen colonization in mice (Khelef et al. 2006). InlB is not however required for the invasion of the intestinal epithelium of transgenic hEcad mice and does not cooperate with InlA for this function. Furthermore, InlB is not involved in *L. monocytogenes* infection of guinea pigs or rabbits due to its inability to stimulate the Met receptors of these two hosts (Khelef et al. 2006). Thus, similar to the InlA–Ecad interaction, InlB mediates an *L. monocytogenes* species-specificity critical for the pathophysiology of listeriosis. These results also emphasize the need for developing new animal models to dissect the exact contribution to pathogenicity of cell wall-associated proteins.

5.4.2.2. AUTOLYSINS

5.4.2.2.1. Ami

Inactivation of Ami alone does not affect *L. monocytogenes* pathogenicity. However, inactivation of Ami in mutants lacking InlA, InlB, or both internalins results in strong reduction of adhesion to hepatocytes and enterocyte-like cell lines (Milohanic et al. 2001). Like in InlB, the GW modules appear to promote Ami adhesion to host cells (Milohanic et al. 2001). Since InlB GW modules bind cellular matrix proteoglycans (Jonquieres et al. 2001), it is possible that Ami GW modules exert a similar function. Thus, Ami may act as a complementary adhesin during infection. This conclusion is supported by the fact that an *ami* mutant is slightly attenuated in the liver of mice infected intravenously (Milohanic et al. 2001).

5.4.2.2.2. Auto

The morphology of an *aut* deletion mutant has been reported similar to those of the wild-type strain, with no defect in septation and cell division (Cabanes et al. 2004). Auto is however required for entry of *L. monocytogenes* into different nonphagocytic eukaryotic cell lines although it is dispensable for efficient adhesion, formation of comet tails, or cell-to-cell spreading. An *aut* mutant displays reduced virulence following intravenous inoculation of mice and oral infection of guinea pigs, which correlates with its low invasiveness (Cabanes et al. 2004). How Auto contributes to pathogenicity is unknown, although its

contribution to pathogenicity was tentatively linked to maintenance of the cell surface architecture and/or release of immunologically active cell-wall components (Cabanes et al. 2004).

5.4.2.2.3. P60

The autolysin P60, encoded by the invasion-associated protein (*iap*) gene, is both secreted and associated with the bacterial cell wall (Kuhn and Goebel 1989; Ruhland et al. 1993; Wuenscher et al. 1993). On the basis of its similarity to the autolysin LytF from *Bacillus subtilis*, P60 is predicted to have a D-*i*Glu-*m*Dap endopeptidase activity (Lenz et al. 2003; Smith et al. 2000). The role of P60 in pathogenicity was first evaluated in rough mutants expressing lower levels of this protein (Kuhn and Goebel 1989). These rough mutants are less virulent and enter less efficiently in certain eukaryotic cells, suggesting a role for P60 in invasion (Gutekunst et al. 1992; Hess et al. 1995; Kuhn and Goebel 1989). A P60-deficient mutant displays similar phenotypes, including virulence attenuation after intravenous infection of mice. This Δ*iap* mutant is also impaired in its intracellular motility process due to mis-localization of the actin-polymerizing factor ActA (Lenz et al. 2003; Pilgrim et al. 2003). P60 plays an important role in the immune response against *L. monocytogenes*. P60-specific antibodies act as opsonins and might play a role in preventing systemic infections in immunocompetent individuals (Kolb-Maurer et al. 2001). P60 is also a major protective antigen that induces both T-CD8 and Th1 protective immune responses (Bouwer and Hinrichs 1996; Geginat et al. 1998; Geginat et al. 1999; Harty and Pamer 1995).

5.5. Proteomics of the *Listeria monocytogenes* Cell Wall

Listeria monocytogenes surface proteins have been traditionally characterized using extraction methods involving acid treatment, LiCl or detergents like SDS. These methods allow the extraction of surface proteins non-strongly attached to peptidoglycan, although they do not solubilize proteins covalently bound to the peptidoglycan. Unless peptidoglycan-hydrolytic enzymes are used, proteins bound covalently to the peptidoglycan, as those of the LPXTG family, are obtained in minute amounts (Navarre and Schneewind 1999). This is probably one of the reasons of why in *L. monocytogenes*, as in other gram-positive bacteria, relatively few proteins covalently bound to the peptidoglycan have been characterized.

5.5.1. The Listeria monocytogenes *Cell-Wall Proteome*

Based on genome data, a recent study developed a preliminary proteome reference map of the *L. monocytogenes* EGD-e strain (Ramnath et al. 2003). Using total cell extracts, 261 spots were differentiated on two-dimensional (2D) gels. Of these, only 33 distinct proteins were identified, most of

them corresponding to abundant proteins (chaperons, translation factors, and enzymes of central metabolism). Noteworthy, no cell-wall-associated protein was identified in this study. A further study, focused on the characterization of the *L. monocytogenes* "secretome" using 2D gels and non-gel proteomics, revealed the presence of numerous cell-wall-associated proteins in the extracellular medium (Trost et al. 2005). These proteins could be released from the cell either as complexes containing fragments of cell-wall polymers or as a result of proteolytic processing. The secretome of the EGD-e strain contains the LPXTG proteins InlA, InlH, Lmo0880, Lmo1666, and Lmo2714; the two NXZTN proteins Lmo2185 and Lmo2186; several autolysins containing GW modules (Auto, Ami, Lmo1216, Lmo1521, and Lmo2591); the autolysin Ami; two members of the P-60 subfamily (P60, P45); two PBPs (Lmo2039 and Lmo2754); and InlB. Two other proteins of unknown function, Lmo2504 and Lmo2522, annotated as "hypothetical-cell-wall binding proteins" were also identified (Trost et al. 2005). Despite the relevant information provided by this study, it did not address the analysis of the protein profile in purified cell-wall material. This was recently made applying a non-gel proteomic approach to peptide mixtures obtained upon incubation of peptidoglycan with trypsin (Calvo et al. 2005). Since peptidoglycan is inherently insensitive to trypsin, this peptide mixture was an ideal source for identifying novel proteins strongly attached to the peptidoglycan. This study performed in *L. monocytogenes* was the first in providing a detailed profile of proteins remaining bound to cell wall upon exhaustive purification of the peptidoglycan employing ionic detergents. Nineteen proteins of the EGD-e strain associated to the peptidoglycan were identified in bacteria growing in BHI medium. All of these proteins correspond to enzymes involved in peptidoglycan metabolism or surface proteins expected to be anchored to this cell-wall component (Calvo et al. 2005). Among these proteins, 13 were LPXTG proteins (including InlA). In comparison with the secretome, a higher number of LPXTG proteins were detected when analyzing purified peptidoglycan material. This result can be explained by the difficulty for detecting in the extracellular medium certain LPXTG proteins that are present in the cell surface only in scarce amounts. It is also possible that some LPXTG proteins locate in surface regions not undergoing massive release of cell-wall fragments, i.e. the polar caps. LPXTG proteins identified in the peptidoglcyan but not in the extracellular medium include Lmo0130, Lmo0160, InlG, Lmo0327, Lmo0610, Lmo0842, Lmo1413, and Lmo2085. Besides LPXTG proteins, the non-gel proteomic analysis performed on peptidoglycan material identified Lmo2185 and Lmo2186 (the two proteins bearing the NXZTN motif); several autolysins (P60, P45, and MurA); and the PBP Lmo1982 (Calvo et al. 2005)(Figure 5.1). No autolysins attaching to the cell wall via GW modules were identified, supporting the idea that the protein–cell wall association mediated by LysM or P60 domains is probably stronger than that mediated by GW modules. This non-gel proteomic study was the first in reporting the identification of a large number of *L. monocytogenes* LPXTG proteins; however, it only covered one-third of LPXTG proteins predicted by the EGD-e genome sequence. Tight regulation of the biosynthesis

of concrete LPXTG proteins is conceivable. It is also possible that some LPXTG proteins might be used by *L. monocytogenes* exclusively during the intracellular phase of the infection. In fact, two recent transcriptome analyses have shown up-regulation of a few genes encoding LPXTG proteins in intracellular *L. monocytogenes* growing within epithelial cells (Chatterjee et al. 2006; Joseph et al. 2006). Some of them have not been identified yet in extracellular bacteria. Lastly, in laboratory conditions, some LPXTG proteins may be present on the *L. monocytogenes* cell surface in extremely scarce amounts below the sensitivity threshold of the non-gel proteomics technology.

A proteomic analysis of the *L. monocytogenes* cell wall based on 2D gels has also recently been reported (Schaumburg et al. 2004). In this case, surface proteins were serially extracted from the cell wall with high concentration of salts (Tris and KSCN) and resolved on gels. This work led to the identification of 55 proteins (Schaumburg et al. 2004), but none of them were of the LPXTG family. InlB and the autolysins P60, P45, MurA, and Ami were efficiently extracted from the cell wall with high salt. It becomes clear from these results that the identification of *L. monocytogenes* surface proteins covalently bound to peptidoglycan requires methods involving digestion of either the peptidoglycan itself or the proteins that copurify with it.

5.5.2. Identification of Listeria monocytogenes *Sortase Substrates by Proteomics*

The sortase SrtA of *S. aureus* cleaves the T–G linkage of the LPXTG motif (Ton-That et al. 1999, 2000), whereas SrtB was recently shown to cleave the T–N linkage in the NPQTN sorting motif of IsdC (Marraffini and Schneewind 2005). Sortases contain a conserved TLXTC motif and an H residue involved in catalysis (Ilangovan et al. 2001; Mazmanian et al. 2001). These features have allowed the identification of new putative sortase genes in the genome sequences available in databases. In fact, many gram-positive bacteria contain several sortases to which recognition of distinct sorting motifs has been assigned (Boekhorst et al. 2005; Comfort and Clubb 2004; Dramsi et al. 2005). Like *S. aureus*, *L. monocytogenes* contains two genes encoding sortases, *srtA* (*lmo0929*) and *srtB* (*lmo2181*).

Most of the sortase substrates have been predicted by *in silico* analysis based on the presence of a sorting-signal domain in the C-terminus (Boekhorst et al. 2005; Comfort and Clubb 2004; Dramsi et al. 2005; Ton-That et al. 2004). However, biochemical evidence for recognition of surface proteins by sortases has been reported only in few cases. SrtA and SrtB of *S. aureus* anchor different set of proteins to the cell wall with a specificity that correlates to the presence in the substrate of either a LPXTG or NPQTN motif, respectively (Mazmanian et al. 2001; Ton-That et al. 2004). Sortase substrates have also been identified by 2D SDS-PAGE using cell-wall extracts of sortase-deficient mutants (Osaki et al. 2002). The specificity of the sortases SrtA and SrtB of *L. monocytogenes* strain EGD-e was recently assessed by non-gel proteomics using mutants deficient in these sortases (Pucciarelli et al. 2005). Like in *S.*

aureus, each of the *L. monocytogenes* sortases anchors a distinct subset of proteins to the peptidoglycan. Thus, the LPXTG proteins detected in peptidoglycan of the wild-type strain were all present in the Δ*srtB* mutant but missing in the peptidoglycan of Δ*srtA* or Δ*srtA*Δ*srtB* strains (Pucciarelli et al. 2005). An exception was Lmo0842, which was barely detected in the Δ*srtB* mutant. This result suggests that efficient anchoring of Lmo0842 to the cell wall may require a functional SrtB sortase. Lmo2185 and Lmo2186, both carrying NXZTN sorting motifs, were identified only in strains having a functional StrB sortase. Interestingly, an Lmo2186 peptide covering the first of the two putative sorting motifs predicted in this protein, SDSS*NKVTN*PK, was identified in the peptidoglycan material. This observation supports the hypothesis that SrtB may not cleave the motif NKVTN in certain growth conditions. This hypothesis contemplates that the overlapping motif NPKSS, similar to those described in other gram-positive bacteria (Dramsi et al. 2005), would be preferentially recognized. If demonstrated, Lmo2186 would be the first case of a surface protein covalently anchored to peptidoglycan by a sortase-mediated recognition of two alternative sorting motifs.

Autolysins and PBPs (P60, P45, MurA, and Lmo1892) were identified in the peptidoglycan of all the sortase mutants, an observation that validates the experimental approach and demonstrates that these surface proteins attach to the cell wall by sortase-independent mechanisms.

5.6. The *Listeria monocytogenes* Cell Wall and Inflammation

5.6.1. *Immunogenicity of the* Listeria monocytogenes *Cell Wall*

Bacterial cell-wall components are potent biological effectors of a large variety of stimulatory activities in eukaryotic cells and responsible for severe pathologies like septic shock (Boneca 2005). Early studies performed with distinct cell-wall preparations of *L. monocytogenes* (crude material, peptidoglycan, or LTA) revealed that besides its capacity to activate macrophages, the peptidoglycan has potent adjuvant and antitumor activities (Hether et al. 1983; Paquet et al. 1986; Saiki et al. 1982). Purified peptidoglycan alone is not sufficient to confer protection against *Listeria* infection, requiring priming with crude cell-wall preparations (Hether et al. 1983). Some of the stimulatory effects, such as the adjuvant and mitogenic activities, require both peptidoglycan and TA, whereas others, such as the antitumoral and natural killer activities, are triggered by samples devoid of TA (Hether et al. 1983). These observations indicate that all distinct cell-wall components contribute to stimulate host responses.

Host immunity is also modulated by *L. monocytogenes* cell-wall components. Thus, muropeptide structures found in the peptidoglycan of *L. monocytogenes*

are representative of the molecular patterns known to be recognized by Nod1 and Nod2, two members of the intracellular eukaryotic sensor family (Chamaillard et al. 2003a; Inohara et al. 2005; Murray 2005). Nod1 specifically recognizes the disaccharide-tripeptide GlcNAc-MurNAc-L-Ala-γ-D-Glu-*m*-Dap molecule, whereas Nod2 recognizes the disaccharide-dipeptide GlcNAc-MurNAc-L-Ala-γ-D-Glu (Chamaillard et al. 2003a; Inohara et al. 2005; Murray 2005). Nod1 also recognizes the dipeptide γ-D-Glu-*m*-Dap (Chamaillard et al. 2003b). Nod1 has been proposed to be involved in sensing of gram-negative bacteria due to the requirement of *m*-Dap for peptidoglycan recognition and Nod2 would act as a general sensor of bacterial peptidoglycan. Nods have a cytosolic location, which suggests that they have evolved as sensors to respond to infections caused by intracellular bacterial pathogens. Nod2-deficient mice are highly susceptible to bacterial infections initiated at the intestine, including *L. monocytogenes* (Kobayashi et al. 2005). Noteworthy, this increased susceptibility correlates with an impaired secretion of antimicrobial peptides known as cryptdins, an observation that directly links Nod2 function to innate immunity (Kobayashi et al. 2005). Evidence for sensing of *L. monocytogenes* by Nod1 has also been recently found (Opitz et al. 2006). Thus, Nod1 activity is required for both NFκβ-activation and induced secretion of IL-8 in *L. monocytogenes*-infected endothelial cells. Sensing of *L. monocytogenes* by Nod1 also induces the MAPK p38 signaling pathway. Activation of both NFκβ and p38 was shown to mediate IL-8 secretion (Opitz et al. 2006).

5.6.2. *Recognition of* Listeria monocytogenes *Cell-Wall Components by Eukaryotic Molecules*

Teichoic acids have been implicated in adhesion of *L. monocytogenes* to host cells. Thus, purified galactose-containing TA, but not those lacking this modification, adhere to HepG-2 epithelial cells (Cowart et al. 1990). Consistently, a *gtcA* mutant deficient in TA glycosylation (see Section 5.2.2) displays a partial defect for adhesion and entry into HepG-2 cells (Autret et al. 2001). These observations favor the hypothesis that TA and/or LTA could act as bacterial “lectins” promoting the interaction of *L. monocytogenes* with membrane-located glycoproteins of nonphagocytic cells. The α-D-galactose receptor present in HepG-2 cells was proposed as the molecule recognizing TA (Cowart et al. 1990), although this interaction has not been formally demonstrated.

In addition to lipopolysaccharide from gram-negative bacteria, the macrophage-scavenger receptor class A (MSR-A) recognizes LTA purified from several gram-positive bacteria, including *L. monocytogenes* (Dunne et al. 1994; Greenberg et al. 1996). MSR-A recognizes preferentially LTA molecules negatively charged since modifications such as D-Ala esterification or positive-charged sugar residues (as those carried by the *S. pneumoniae* LTA) notably reduce LTA–MSRA interaction (Greenberg et al. 1996). MSR-A-deficient mice are highly susceptible to *L. monocytogenes* infection (Ishiguro et al. 2001), which indicates that this receptor is a major effector of the host immune response

against *L. monocytogenes*. Toll-like receptors (TLRs), involved in recognition of pathogen-associated molecular patterns (PAMPs) (Takeda and Akira 2005), have also been linked to sensing of *L. monocytogenes*. Thus, mice deficient in TLR2, a receptor that recognizes LTA but not peptidoglycan, are highly susceptible to *L. monocytogenes* infection (Torres et al. 2004). A deficiency in MyD88, an adaptor required for signal transduction from the surface-located TLR receptor, also increases the susceptibility to *L. monocytogenes* (Torres et al. 2004). These data implicate LTA as an important modulator of the host immune response. LTA induce the maturation of dendritic cells and are responsible for the stimulation of IL-18 release observed in *L. monocytogenes*-infected dendritic cells (Kolb-Maurer et al. 2003). *L. monocytogenes* LTA also activate NFκβ in epithelial and macrophage cells (Hauf et al. 1997, 1999).

A recent study showed that *L. monocytogenes dltA* mutants defective in D-Ala esterification (see Section 5.2.2) are less virulent in the mouse model and adhere poorly to phagocytic and non-phagocytic cells (Abachin et al. 2002). It would be of interest to test whether esterification of the TA with D-Ala might alter recognition of LTA by MSR-A or other type of receptor on the surface of epithelial cells.

5.7. Future Challenges

The *L. monocytogenes* cell wall has revealed as an intricate platform in which several polyanionic polymers, the peptidoglycan and TA precisely dictate the anchoring of a plethora of surface proteins. While some modes of cell wall–protein association have been examined in-depth in a few cases, some unresolved aspects remain. Thus, the association promoted by GW modules, involving the interaction with LTA in the case of InlB, is not yet completely understood for autolysins bearing these modules. The potential role of the TA polymer in modulating anchoring of surface proteins has not been explored, although some observations suggest that it might be the case. Another intriguing issue is the large number of autolysins expressed by *L. monocytogenes* in laboratory conditions. Insights on this phenomenon could be now obtained by determining the specific linkage of the peptidoglycan cleaved by these autolysins. It is expected that these activities do not overlap given the simultaneous presence of these autolysins on the cell surface. These goals can also now be addressed using proteomic techniques. Lastly, an exciting but basically unexplored field deals with the cell-wall physiology of *L. monocytogenes* during the infection process and, more specifically, during the intracellular phase. The changes in expression of genes encoding surface proteins registered when *L. monocytogenes* proliferates inside eukaryotic cells are predictive of significant alterations in the cell wall occurring in intracellular bacteria. These challenges open a new avenue of research that undoubtedly will increase our yet limited knowledge of the *L. monocytogenes* cell wall and its contribution to pathogenicity.

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Note

While this chapter was in the editing process, a study was published by Guinane et al. describing the contribution of distinct pencillin-binding protiens (PBPs) of *Listeria monocytogenes* to antibiotic resistance and virulence (Guinane et al. 2006).

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