

Listeria monocytogenes Surface Proteins: from Genome Predictions to Function

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INTRODUCTION

Bacterial surface proteins constitute a diverse group of molecules involved in various important processes, such as bacterial growth, sensing of and protection from environmental stresses, adhesion, invasion of host cells, signaling, and interaction with the immune system. Because of these various functions, an in-depth characterization of the surface protein repertoire is required to better understand the factors that contribute to the success of a bacterial pathogen in colonizing different environmental niches and its mammalian host. This is especially the case for the food-borne pathogen *Listeria monocytogenes*, whose ability to survive in diverse environments, including food and the cytosol of eukaryotic cells, relies in part on its complex surface proteome. Annotation of the first *L. monocytogenes* genome (strain EGDe) (67) predicted a total of 2,853 proteins, among which were 133 surface proteins that were precisely classified according to their different anchoring systems and potential structural domains (30). Accumulation of genome sequence information, new predictive bioinformatics approaches, comparative genomics, proteomics, X-ray three-dimensional structure determination of specific domains, and the recent characterization of mutants in animal models has since then provided new insight into the *L. monocytogenes* "surfaceome." We present here an updated characterization of listerial surface proteins present in the *L. monocytogenes* model strain EGDe (67), focusing on their highly modular nature. We have performed an in-depth annotation of surface proteins and combined these in silico analyses with recently published experimental data. This review emphasizes the diversity of modules dedicated to cell envelope association and that of other modules involved in more specific functions, such as cell wall metabolism or host-pathogen interactions. Many of these modules are structurally related to domains found in eukaryotic proteins and have evolved to bind a variety of bacterial or host receptors.

To annotate protein domains, we have combined sequence information from the database dedicated to the analysis of the genomes of *L. monocytogenes* (strain EGDe) and of its non-pathogenic relative *Listeria innocua* (strain CLIP 11262) (<http://genolist.pasteur.fr/ListiList> [67]) with that from the Pfam database (<http://www.sanger.ac.uk/Software/Pfam> [11]). We also used information from the NCBI Conserved Domain database (194) and the Interpro database (98). Sequence comparisons were performed using a PSI-BLAST analysis.

THE *L. MONOCYTOGENES* CELLULAR ENVELOPE

A prerequisite to understand how proteins remain attached to the bacterial envelope is a good knowledge of the biochemical characteristics of this compartment. Most of the descriptions of the composition and suspected structure of the *L. monocytogenes* membrane, peptidoglycan, and associated poly-

mers were made about 20 to 40 years ago (57, 65, 171, 180, 181). However, this topic now is receiving further attention, which should help to refine the overall structure (for a review, see reference 145).

Membrane

The listerial membrane is ~90 Å thick and is composed of 55 to 60% protein, 30 to 35% lipid, and 1.3 to 2.3% carbohydrate (glucose, galactose, ribose, and arabinose) (65). Lipids include 80 to 85% phospholipids, such as phosphatidylglycerol, diphosphatidylglycerol, and phosphoglycolipid (185). The *L. monocytogenes* membrane fatty acid composition is dominated to an unusual extent by branched-chain fatty acids (>90% of the total fatty acid content) (6) and is modulated upon temperature variation. For instance, upon cold shock the anteiso-C_{15:0} content rises to maintain optimal membrane fluidity (6, 51, 132). However, nothing is yet known about the variation of the plasma membrane composition during *Listeria* cellular infection and how it affects anchoring of membrane proteins.

Peptidoglycan

A striking characteristic of the *Listeria* peptidoglycan is its similarity with that of gram-negative bacteria, such as *Escherichia coli* (82, 162). It is a polymer of alternating units of the disaccharide *N*-acetylmuramic acid (MurNAc)-(β-1,4)-*N*-acetylglucosamine (GlcNAc), cross-linked by peptidic bridges. Muropeptides, which in *L. monocytogenes* are L-alanyl-γ-D-glutamyl-meso-diaminopimelyl-D-alanine-D-alanine, are bound to the MurNAc residue and are connected by a direct link between the D-Ala residue of one lateral peptide and the meso-diaminopimelyl residue of the other stem peptide (44, 57, 89, 162). Partial deacetylation of GlcNAc residues by PgdA enzyme is another characteristic of *Listeria* peptidoglycan (21, 89) (see below).

Cell Wall Secondary Polymers: Teichoic Acids and Lipoteichoic Acids

L. monocytogenes contains two different polyanionic polymers decorating the cell wall: the teichoic acids (TAs), which are covalently bound to the peptidoglycan, and the lipoteichoic acids (LTAs), which are embedded into the plasma membrane by a diacylglycerolipid (128, 130). These polymers play important functions in metal cation homeostasis, anchoring of surface proteins, and transport of ions, nutrients, and proteins and are main determinants of surface immunogenicity, conferring most of the basis of the serotype diversity known in *L. monocytogenes*. Of note, D-Ala esterification of TAs and LTAs is important for *L. monocytogenes* pathogenicity (1, 118).

TAs are electronegative polymers of ribitol-phosphates substituted with D-Ala residues and diverse sugars, which vary

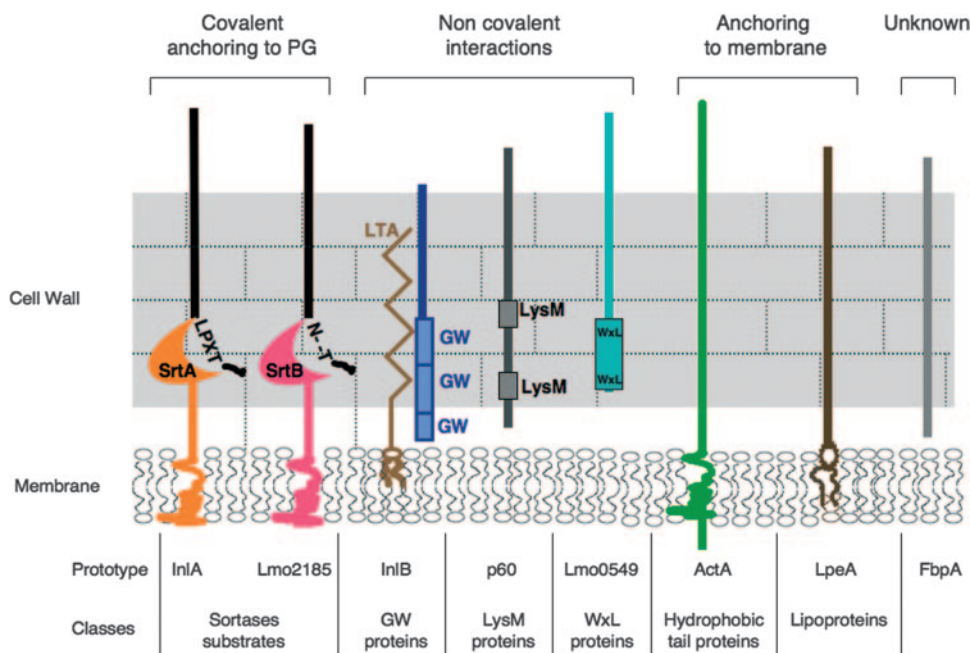


FIG. 1. The different types of surface proteins found in *L. monocytogenes*. A prototype of each family is given.

depending on the serotype (57–59, 145, 155). For instance, in serovar 1/2 (e.g., serotypes 1/2a and 1/2b), GlcNAc and rhamnose are present as substituents on the ribitol, whereas in serovar 4, GlcNAc is integral to the TA chains. Interestingly, serotype 4b strains are unique in bearing both galactose and glucose substituents on the GlcNAc of TA. This particularity is related to the presence in these strains of specific genes, such as *gtcA*, *gliA*, and *gliB* (106, 144).

The distinct glycosylations of TAs are probably of importance for their specific recognition by listerial proteins or phage endolysins (113) (see below). LTAs are polymers of glycerophosphate substituted with Ala, galactose, and lipid residues. The glycerophosphate chain of LTAs is uncovalently anchored to the outer leaflet of the plasma membrane through a lipid anchor, composed of galactose bound to glycerol and substituted with fatty acids (69, 79, 145). The LTAs are both membrane associated and secreted in the growth medium (58). Their X-ray structure suggests that they adopt a compact, organized micellar form with a length of 10 to 20 nm (101).

CLASSIFICATION OF LISTERIAL SURFACE PROTEINS ACCORDING TO THEIR ANCHORING MECHANISMS

To be specifically localized at the cell surface, proteins from gram-positive bacteria need to translocate across the membrane and then associate with a cell surface component. Surface localization of proteins relies on the presence of specific domains or motifs known to mediate secretion and attachment to the cell envelope. The first step of transport across the membrane occurs by two main pathways, both of which require a specific N-terminal secretion signal: the general secretory Sec pathway and, to a lesser extent, the Tat pathway (176). However several alternative pathways have been discovered, such as the SecA2 pathway (27, 108) or specialized pathways for se-

cretion of specific proteins, such as the pseudopilin export pathway, various ABC transporter pathways, the holin systems (176), and the ESAT-6/WXG100 secretion system (135, 136). The presence of these secretion systems in *L. monocytogenes* has been extensively reviewed recently by Desvaux and Hebraud (43). In this review, we focus only on proteins exported through the Sec and SecA2 pathways, as well as flagellin, which is secreted by the specialized flagellar export machinery. The numerous nonsecreted integral membrane proteins that presumably also expose specific domains on the outside of the membrane bilayer are not discussed in this review.

Through extensive in silico analysis of the genome of *L. monocytogenes* EGDe, Trost et al. (179) were able to predict a total of 525 sequences coding for proteins carrying a signal peptide, which corresponds to 18.4% of the total number of protein-coding genes. Among them, the “surface proteins” discussed in this review are those carrying motifs enabling strong or weak interactions with at least one component of the bacterial envelope (Fig. 1).

Proteins Covalently Linked to the Peptidoglycan: Sortase Substrates

LPXTG sorting signal. The best known class of *L. monocytogenes* surface proteins is that of the LPXTG proteins, which in strain EGDe comprises 41 members. They are characterized by a short C-terminal sorting signal predicted to direct covalent attachment to the peptidoglycan (Fig. 2) (Pfam PF00746, <http://www.sanger.ac.uk/Software/Pfam>). Originally characterized in *Streptococcus pyogenes* protein M and *Staphylococcus aureus* protein A, this signal consists of an LPXTG motif, followed by an hydrophobic domain of about 20 amino acids and a tail of positively charged residues (60, 128). The sorting signal is the substrate of sortase, a membrane-bound transpeptidase, which

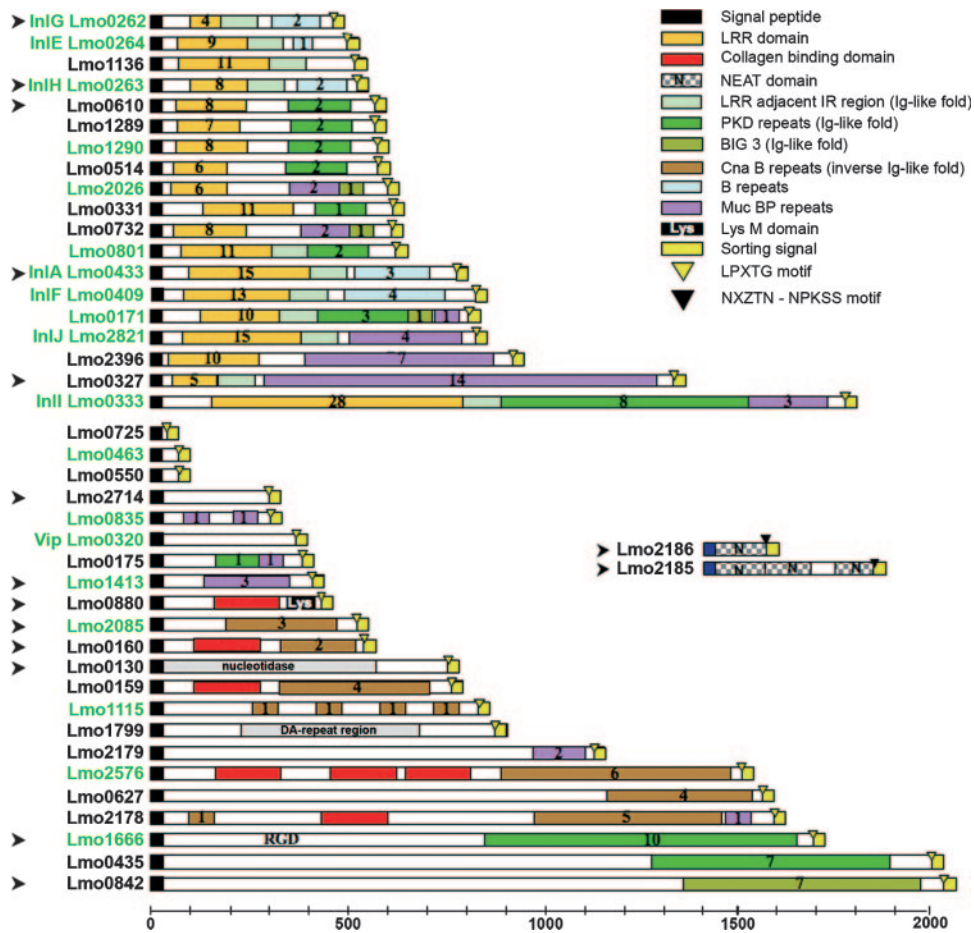


FIG. 2. Sortase substrates. A schematic representation of *L. monocytogenes* LPXTG and NXXTX proteins is shown. The numbers within domains indicate the number of repeats. Nineteen proteins are absent in *L. innocua* and are indicated in green letters. Proteins detected in the *L. monocytogenes* cell wall fraction (146) are indicated by an arrow. The two sortase B substrates, Lmo2185 and Lmo2186, are drawn on the right of LPXTG proteins. AA, amino acids. (Adapted from reference 30 with permission from Elsevier.)

cleaves the LPXTG motif between the threonine and glycine residues and catalyzes the formation of an amide link between the carboxyl group of the threonine and cell wall precursors (123, 177). For a thorough analysis of the sorting mechanism, we refer the reader to an excellent recent review on sortases (121). The most studied LPXTG protein in *L. monocytogenes* is internalin (InlA), which promotes bacterial entry into epithelial cells (61, 73). InlA harbors an LPXTG motif and is anchored covalently by the sortase SrtA to *meso*-diaminopimelic acid residues of the peptidoglycan (17, 44, 62). The recently discovered virulence factor LPXTG protein Vip is also localized on the bacterial surface by an SrtA-dependent pathway (32). Using a novel nongel proteomic method, Pucciarelli et al. (146) were able to identify 13 SrtA substrate proteins (Fig. 2), through comparison of the peptidoglycan contents from wild-type EGDe and sortase-defective mutants grown in rich medium. The remaining LPXTG proteins predicted by the genome may not be produced under the growth conditions used in that study.

The *L. monocytogenes* genome encodes the highest number of LPXTG proteins among all gram-positive bacteria whose genome sequences are known. InlA and 18 other family mem-

bers remarkably display an N-terminal leucine-rich repeat (LRR) domain and belong to the so-called "internalin family" (Fig. 2) (30, 73), which also includes the GW protein InlB, the WxL protein Lmo0549 (see below), and four proteins without any obvious surface anchoring motif, among which is InlC (73). The LRR domain consists of tandemly arranged repeats of 22 amino acids each. In InlA, InlB, and 10 other surface internalins, the LRR domain is flanked at its C terminus by a conserved LRR-adjacent domain (Fig. 2; also see below). InlA, InlB, InlG, InlH, InlE, and InlF also possess a region of B repeats. The crystal structures of the LRR domains of InlA, InlB, InlH, and InlE have been solved, revealing a curved structure ideally shaped for protein-protein interaction (120, 133, 164). However, while the LRR domains of InlA and InlB are structurally related, they bind to very different mammalian receptors, namely, E-cadherin (Ecad) and Met (125, 167). Therefore, one cannot assume that members of the internalin family share similar functions solely on the basis of their structural classification. Of the internalin-like proteins, only InlA and InlB have so far been identified as invasins (see below).

NXXTX sorting signal. A second sortase type, sortase B (SrtB), was originally identified in *S. aureus* and specifically

TABLE 1. *L. monocytogenes* EGDe proteins predicted to carry motifs promoting noncovalent association to the cell wall

Protein ^a	Name	Cell wall association domain(s)	Predicted function and/or domain
Lmo0434	InlB	GW modules	Bacterial invasion into host cells, LRR
Lmo1076	Auto	GW modules	Autolysin, <i>N</i> -acetylglucosaminidase ^b
Lmo1215		GW modules	Autolysin, <i>N</i> -acetylglucosaminidase ^b
Lmo1216		GW modules	Autolysin, <i>N</i> -acetylglucosaminidase ^b
Lmo1521		GW modules	Autolysin, <i>N</i> -acetylmuramoyl-L-alanine amidase ^b
Lmo2203		GW modules	Autolysin, <i>N</i> -acetylglucosaminidase ^b
Lmo2713		GW modules	Unknown
Lmo2558	Ami	GW modules	Autolysin, <i>N</i> -acetylmuramoyl-L-alanine amidase ^b
Lmo2591		GW modules	Autolysin, <i>N</i> -acetylglucosaminidase ^b
Lmo0582	P60 (Iap)	LysM	γ -D-Glutamyl-L- <i>m</i> -Dpm peptidase ^b
Lmo0880		LysM and LPXTG	Unknown, collagen binding domain ^c
Lmo1303		LysM	Unknown
Lmo1941		LysM	Unknown
Lmo2522		LysM	Unknown, C-terminal domain with similarities to lysozyme ^b
Lmo2691	MurA	LysM	Autolysin, <i>N</i> -acetylglucosaminidase ^b
Lmo549		WxL	Unknown, CscC like, LRR ^d
Lmo551		WxL	Unknown, CscB like ^d
Lmo585		WxL	Unknown, CscB like ^d
Lmo587		WxL	Unknown, CscC like ^d
Lmo1851		Peptidoglycan binding domain	Unknown, peptidase domain ^e

^a Proteins absent from *L. innocua* are indicated in boldface.

^b See Fig. 3. *m*-Dpm, *meso*-diaminopimelic acid.

^c See Fig. 2.

^d See Fig. 4.

^e See Fig. 5.

recognizes and cleaves a sorting signal different from LPXTG (121, 124). Staphylococcal SrtB anchors its only known substrate, IsdC, by cleaving a C-terminal NPQTN motif, between threonine and asparagine, and linking threonine to the pentaglycine cross bridges. The genes encoding SrtB and IsdC are both part of the *isd* locus, which is involved in bacterial heme iron uptake (121, 124). *L. monocytogenes* has also an alternative sortase B, whose coding sequence maps to an operon containing two genes encoding putative substrates, Lmo2185 (formerly SvpA) and Lmo2186 (16). Both proteins share homology to *S. aureus* IsdC and bear as putative sorting motifs NAKTN (Lmo2185) or NKVTN or NPKSS (Lmo2186), respectively. Inactivation of *srtB* impairs sorting of Lmo2185 (16). Lmo2185 and Lmo2186 are the only two SrtB substrates present in the *L. monocytogenes* cell wall proteome, consistent with the fact that there is no other protein with an NXXTX sorting signal encoded in the *L. monocytogenes* genome (146).

Work with *S. aureus* and analysis of other complete gram-positive genomes suggest that SrtB enzymes may have evolved to specifically target some proteins involved in iron uptake (49, 121). IsdC and Lmo2186 each contain one NEAT (*near transporter*) domain, and Lmo2185 contains three NEAT domains that are expected to play a role in iron transport (Fig. 2) (5). Interestingly, the *L. monocytogenes* operon containing the lmo2185, lmo2186, and *srtB* genes is regulated by the iron-responsive transcriptional repressor Fur and is induced under iron-deficient conditions (131). However, neither Lmo2185 nor Lmo2186 allows heme, hemoglobin, or ferrichrome utilization (131). The exact functions of these two surface proteins, which are conserved in all *Listeria* species, remain to be characterized more precisely.

Proteins with Noncovalent Association to the Cell Wall

A second group of cell surface proteins in gram-positive bacteria comprises those that are thought to bind to the cell wall by noncovalent interactions, most often via repeated domains (Table 1) (for extensive recent reviews, see references 42, 89, and 165).

GW modules. The first characterized protein of this type in *L. monocytogenes* was InlB, a protein of the internalin family required for *L. monocytogenes* entry into many eukaryotic cell types (15, 47, 73). The InlB C-terminal domain comprises three highly conserved tandem repeats of ~80 amino acids, which are termed GW modules as they begin with the dipeptide Gly-Trp (25). GW modules are necessary and sufficient to anchor InlB to the bacterial surface by binding LTAs (86). These modules also interact with eukaryotic molecules, namely, glycosaminoglycans (GAGs) and gC1q-R (87, 119).

Eight additional proteins carrying a variable number of GW modules are encoded in the *L. monocytogenes* EGDe genome; seven are putative autolysins containing cell wall hydrolase domains, while Lmo2713 is of unknown function (Table 1; Fig. 3A). Among the autolysins are Ami and Auto, which also are implicated in interactions with eukaryotic cells (32, 127). Auto is the only autolysin of this subfamily that is absent in *L. innocua*. Interestingly, the *aut* gene, encoding Auto, is in a locus comprising several genes presumably involved in TA synthesis, some of which are absent in *L. innocua*. Whether these genes products are required for association of Auto with the cell wall is at present unknown.

Association of the GW domain with LTA occurs whether the protein is produced within the bacterium or added externally (25,

A

B

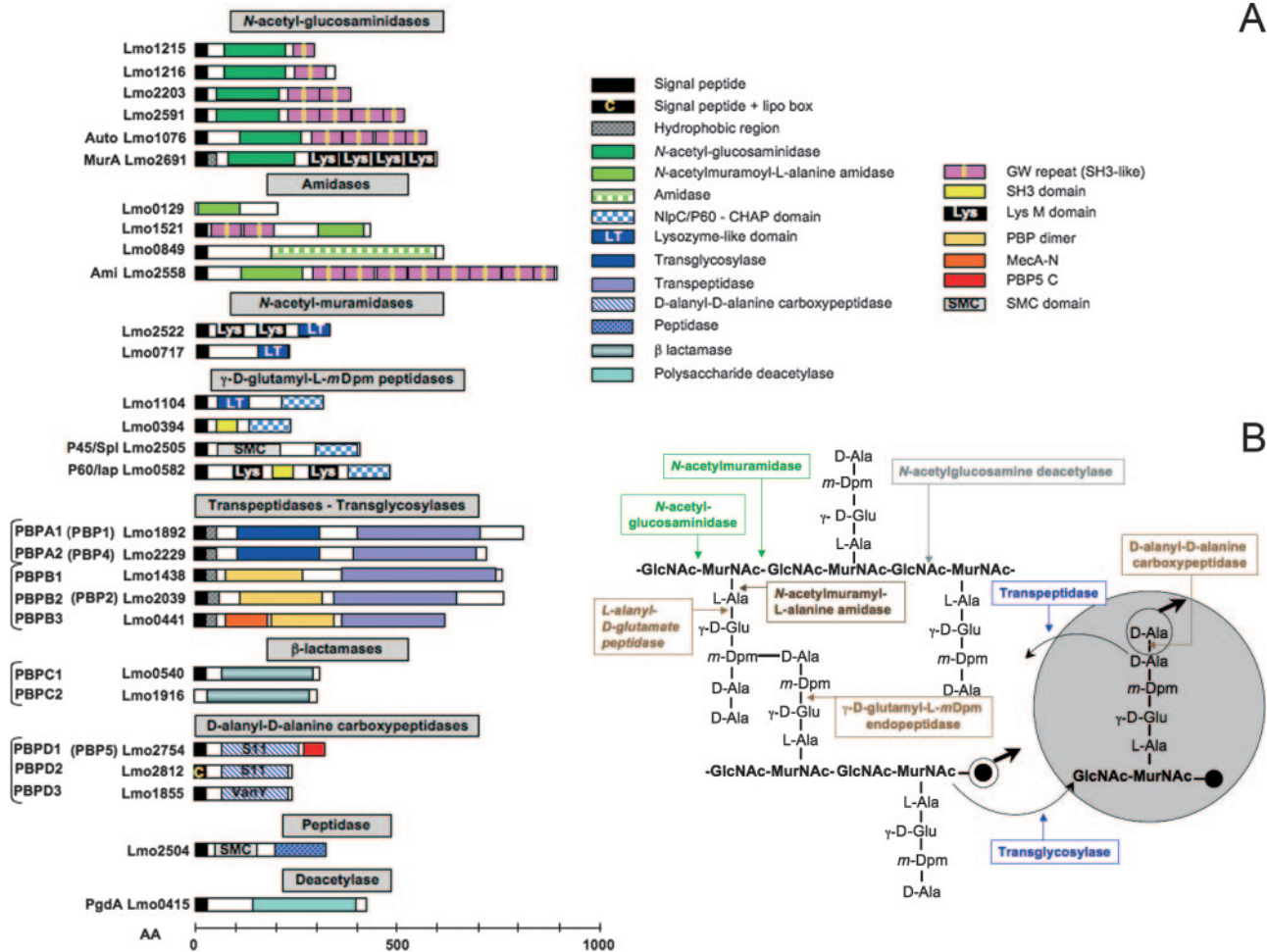


FIG. 3. Surface proteins predicted to be involved in cell wall metabolism. (A) Schematic representation of *L. monocytogenes* surface proteins potentially involved in cell wall synthesis, hydrolysis, or modification. AA, amino acids. Most of the enzymatic activities are deduced from domain sequence similarities and require biochemical experimental validation. (B) Cell wall synthetase and hydrolase activities on *Listeria* peptidoglycan. Glycan chains of GlcNAc and MurNAc are interlinked by direct peptide linkage between the *meso*-diaminopimelic acid (*m*-Dpm) residue of one wall peptide and the D-alanyl residue at position 4 of the adjacent wall peptide. A peptidoglycan precursor linked to the undecaprenyl pyrophosphate group (black circle) is represented by a spotted circle. The precursor is incorporated to the preexisting peptidoglycan by the formation of two bonds: (i) a transglycosylase splits the pyrophosphate bond between the undecaprenyl group and the MurNAc of a nascent glycan strand and forms a glycosidic bond between MurNAc and the hydroxyl group of the GlcNAc of the precursor molecule, and (ii) a transpeptidase forms a DD-peptide bond between the carboxyl group of the D-Ala of the precursor and the amino group of an *m*-Dpm residue present in a peptide moiety of the cell wall. An example of each type of bond attacked by different specific murein hydrolases is also shown. (Adapted from reference 82 with permission.)

86). It displays specificities, as GW modules of InlB do not bind to the surface of *L. innocua* or to that of *Streptococcus pneumoniae* (86). In addition, the strength of the association seems to increase with the number of GW modules. Thus, an InlB variant bearing the eight GW modules of the autolysin Ami binds more efficiently to the cell surface (25). The InlB GW modules are structurally related to Src homology 3 (SH3) domains found in many eukaryotic adaptor proteins, but the binding site for proline-rich ligands of bona fide SH3 domains is obstructed (119, 133). Interestingly, two non-GW surface proteins, the autolysin P60 and Lmo0394 (Fig. 3), also contain an SH3-related domain (Pfam PF08239). Whether this domain interacts with cell wall polymers is unknown. A related bacterial SH3 domain (Pfam PF08460) mediates binding to the cell wall of lysostaphin, a bacteriocin secreted by *Staphylococcus simulans* (9).

GW modules like those in *Listeria* promote surface localiza-

tion of several staphylococcal surface autolysins, such as AtlC from *Staphylococcus caprae* (2), AtlE from *Staphylococcus epidermidis* (77), and Aas from *Staphylococcus saprophyticus* (78). Domains containing 20-amino-acid repeat units beginning with GW but weakly related to *Listeria* GW modules are present in *S. pneumoniae* surface proteins, such as LytA (55, 56, 183) and PspA (197), or in clostridial proteins, such as CspA of *Clostridium acetobutylicum* (158) or the toxin ToxB of *Clostridium difficile* (56). These modules are responsible for binding of the protein to choline residues that decorate the TAs and LTAs in these species (29, 63).

WxL domain. A novel type of cell wall association domain of 160 to 190 amino acids, termed the WxL domain since it contains two conserved sequence motifs with the Trp-x-Leu signature, was recently identified in surface proteins of *Lactobacillus plantarum* and *Enterococcus faecalis* (28, 168). WxL

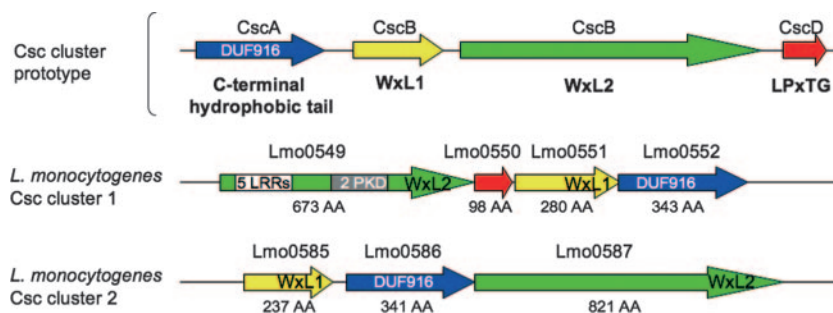


FIG. 4. The two Csc Clusters in *L. monocytogenes*. The first line shows a characteristic four-component Csc cluster, with the predicted surface-anchoring domain of Csc proteins indicated below. The two *L. monocytogenes* Csc clusters are schematically represented, with genes corresponding to Csc components indicated in blue (CscA like), yellow (CscB like), green (CscC like), and red (CscD like), respectively. The number of amino acids (AA) in each gene product is indicated. Cluster 2 does not contain any *cscD*-like gene. PKD, PKD repeats; DUF 919 (Pfam PF06030), domain of unknown function. (Adapted from reference 168 with permission.)

proteins are present in many low-GC gram-positive bacteria and belong to the so-called Csc family of surface proteins. Csc gene clusters typically encode CscA, a protein with a conserved DUF916 domain of unknown function and a C-terminal transmembrane anchor; CscB and CscC, which display a C-terminal WxL domain; and CscD, a small LPXTG protein (Fig. 4). Csc proteins are proposed to form a multicomponent complex at the bacterial surface, although there is still no evidence for such a hypothesis (168). The EGDe *L. monocytogenes* genome contains two Csc-like gene clusters, among which four genes encode WxL domain-containing proteins (Lmo0549, Lmo0551, Lmo0585, and Lmo0587). Lmo0549 contains an N-terminal LRR region and hence is a member of the internalin family. Two *E. faecalis* WxL proteins, EF2686 and EF2250, are also internalin-like proteins. The WxL domain of EF2686 promotes the association of the protein at the bacterial surface by interacting with peptidoglycan (28). It is tempting to speculate that listerial WxL proteins are attached at the bacterial surface by a similar mechanism, although this hypothesis remains to be experimentally addressed.

LysM domain. Six listerial proteins, including P60 and MurA, carry one to four copies of a domain of ~40 amino acids called the lysine motif (LysM, Pfam PF01476) domain (Table 1). The LysM domain is found in a variety of enzymes involved in bacterial cell wall degradation and is also present in many other bacterial proteins with various enzymatic or binding activities (10). Several LysM-containing proteins, such as staphylococcal immunoglobulin G (IgG) binding proteins and *Escherichia coli* intimin, are involved in bacterial pathogenesis. LysM domains are also present in some eukaryotic proteins, possibly as a result of horizontal gene transfer from bacteria. The LysM domain is thought to be a general peptidoglycan binding module. Indeed, the C-terminal domain of the *Enterococcus faecalis* N-acetylglucosaminidase AtlA, which contains six LysM modules, bind to highly purified peptidoglycan (50). The LysM domain can adopt a beta-alpha-alpha-beta conformation, with the beta strands forming an antiparallel beta sheet and the two alpha helices packing on one side of this sheet (10). These structures show no similarity to other bacterial cell surface domains. The peptidoglycan binding activity of LysM domains has not been demonstrated for any listerial proteins. However, proteomic analyses of *L. monocytogenes* extracts suggest that LysM domain-containing proteins are sur-

face exposed. The autolysins P60 and MurA, which contain two and four LysM domains, respectively, are present in purified cell wall fractions (34). Lmo1303 and Lmo2522, which contain one and two LysM domains, respectively, are detected in supernatant fractions, indicating that these proteins can translocate across the plasma membrane (179). One LysM domain is also found in Lmo1941, a protein with a transmembrane hydrophobic domain that is detected in the *L. monocytogenes* membrane fraction (193). Finally, a LysM domain is present in Lmo0880, a cell wall LPXTG protein (Fig. 2) (146). In this case, the LysM domain could play a role in the topological distribution within the cell wall of a protein attached by a sortase A-dependent mechanism.

Peptidase peptidoglycan binding domain. One protein, Lmo1851, is predicted to possess an N-terminal signal peptide (179), a transmembrane domain, and a C-terminal domain that is also found at the N or C termini of a variety of peptidases (Table 1) (Fig. 5). This domain of ~70 amino acids is composed of three alpha helices and may have a general peptidoglycan binding function (see INTERPRO entry IPR002477 and Pfam PF01471). Many of the proteins having this domain are uncharacterized. Lmo1851 is detected in the *L. monocytogenes* membrane fraction (193), suggesting that it may be associated with the membrane via its N-terminal hydrophobic region and stabilized inside the cell wall via its C-terminal peptidoglycan binding domain. However, this hypothesis requires experimental validation.

Membrane-Bound Proteins

Proteins with hydrophobic tails. Proteins carrying a signal peptide can be retained in the membrane bilayer by hydrophobic segments generally present at the N- or C-terminal part of the protein. Proteins associated with the membrane by their C terminus contain a carboxyl-terminal stretch of hydrophobic residues followed by few charged residues thought to serve as a stop-transfer signal. This class includes the surface protein ActA, which promotes *Listeria* intracellular motility (94); two CscA-like proteins as mentioned above (Fig. 4); and four proteins of unknown function (Table 2). It also includes Lm0058, an ortholog of EssA, a component of the Wss(WGX100) specific secretion pathway (43). Finally, it includes two proteins, Lmo0528 and Lmo0529, which are similar to a UDP-glucose

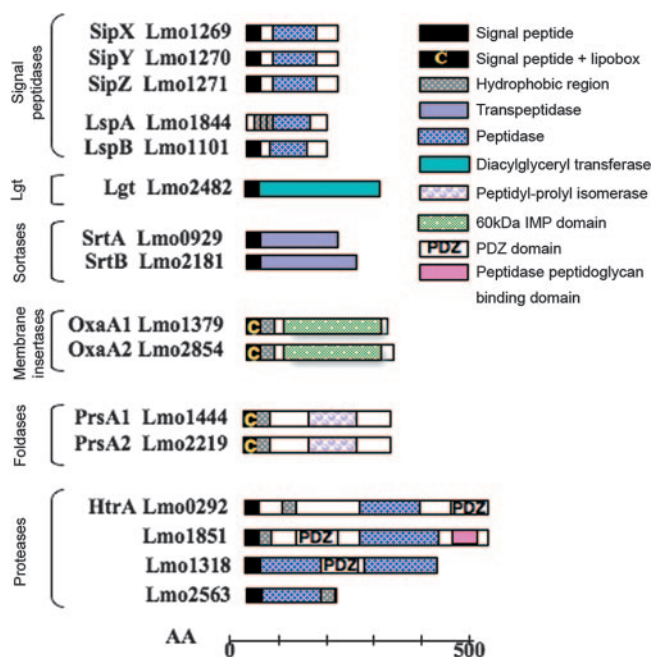


FIG. 5. Surface proteins predicted to be involved in protein processing, folding, and anchoring at the cell surface. A schematic representation of *L. monocytogenes* surface proteins potentially involved in modifications or degradation of proteins at the surface is shown. AA, amino acids.

dehydrogenase and UDP-glucose glycosyltransferase, respectively. Whether these proteins play a role in exopolysaccharide production at the bacterial surface is an interesting possibility that has to be tested.

Proteins may also be tethered to the cell membrane by an amino-terminal hydrophobic stretch, which may be the signal peptide itself if it remains uncleaved (176). Sortases SrtA and SrtB, as well as several proteins involved in protein folding or in cell wall synthesis, such as penicillin binding proteins (PBPs), belong to this class of proteins (Fig. 3 and 5). Whether they remain attached to the bacterial surface needs to be experimentally verified. Nevertheless, some of them have been detected in the *L. monocytogenes* membrane fraction by proteomics (193).

Lipoproteins. Lipoproteins are anchored to the membrane by covalent N-terminal lipidation. This process is directed by a specific signal peptide sequence characterized by a lipobox with a conserved cysteine residue (174). Two steps are necessary and sufficient for maturation of lipoproteins. Following signal peptide-directed export of the prolipoprotein, the prolipoprotein diacylglycerol transferase (Lgt) catalyzes the transfer of a diacylglycerol moiety from phosphatidylglycerol present in the membrane to the thiol of the conserved cysteine in the prolipoprotein. Subsequently, the signal peptide is removed by a specific lipoprotein signal peptidase (SPase) II (Lsp) enzyme, which cleaves within the lipobox to release the mature lipoprotein. Based on identification of a lipobox, it was possible to predict 68 lipoproteins in the *L. monocytogenes* EGDe genome sequence, the largest family of surface proteins (67). Recently, Baumgartner and colleagues were able to experimentally verify the genome predictions for 26 lipoproteins,

which were specifically released in supernatants of a Δ *lgt* strain (13). Listerial lipoproteins include 28 putative substrate binding proteins (SBPs) of ABC transporter systems, 19 proteins predicted to be involved in different enzymatic activities or other function, and 21 proteins of unknown function, the genes for six of which (Lmo0255, Lmo0460, Lmo0617, Lmo1340, Lmo2594, and Lmo2595) are absent from the *L. innocua* genome (Table 3).

Nonconventional Secreted Surface Proteins

Proteomic studies identified proteins that have no predicted signal sequence, and are assumed to have cytoplasmic functions, in the *L. monocytogenes* cell wall or supernatant fractions (160, 179). Among them are glycolytic enzymes, chaperones, and heat shock proteins, as well as proteins involved in detoxification and adaptation to atypical conditions, nucleic acid metabolism, transcription, and translation. Although these cytosolic proteins may be recovered in these fractions due to cell lysis, increasing evidence suggests that some of them may be specifically transported to the surface by alternative pathways. In *L. monocytogenes* the auxiliary secretory protein SecA2 is involved in export of a subset of listerial proteins, some of which lack recognizable Sec sequences (107). Recent reports identified superoxide dismutase as a protein dependent on SecA2 for secretion and surface association in *L. monocytogenes* (7). Superoxide dismutase is also secreted via the SecA2 pathway in *Mycobacterium tuberculosis* (27). Another study reports the unambiguous presence at the bacterial surface of FbpA (Lmo1829), a protein that does not possess any characteristic of a surface-exposed protein. FbpA is also exported via the SecA2-dependent secretion pathway (48). Orthologs of FbpA, including PavA of *S. pneumoniae* and Fbp54 of *S. pyogenes*, are also cell surface proteins (40, 81). Together these data suggest that SecA2-dependent export is a new type of secretion pathway that is partly responsible for *L. monocytogenes* virulence (7, 48, 107).

The autolysin-like protein Lmo0129 (Fig. 3) does not contain any obvious signal peptide, although it is detected in *Listeria* supernatant fractions (179). Interestingly, the gene encoding Lmo0129 is adjacent to a gene encoding a putative holin of the TcdE family, Lmo0128 (43), raising the possibility that Lmo0129 could be exported by a holin-like pathway (reviewed in references 70 and 191).

TABLE 2. *L. monocytogenes* EGDe proteins with a carboxyl-terminal hydrophobic tail

Protein ^a	Name	Predicted function
Lmo0058		Similar to EssA
Lmo0082		Unknown
Lmo0204	ActA	Actin polymerization, intracellular motility
Lmo0528		Similar to UDP-glucose 6-dehydrogenase
Lmo0529		Similar to UDP-glycosyltransferase
Lmo0552		Unknown, CscA like ^b
Lmo0586		Unknown, CscA like ^b
Lmo0701		Unknown
Lmo0821		Unknown
Lmo2061		Unknown

^a Proteins absent from *L. innocua* are indicated in boldface.

^b See Fig. 4.

TABLE 3. Lipoproteins of *L. monocytogenes* strain EGDe^a

Functional group	Protein ^b	Name	Predicted function
Substrate binding proteins of ABC TS ^c	Lmo0135		Similar to oligopeptide binding lipoproteins, ABC TS
	Lmo0152		Similar to oligopeptide binding lipoproteins, ABC TS
	Lmo0153		Similar to Zn(II) binding lipoproteins, ABC TS
	Lmo0181		Similar to sugar binding proteins, ABC TS
	Lmo0285		Similar to substrate binding lipoproteins, ABC TS
	Lmo0541		Similar to iron compound binding lipoproteins, ABC TS
	Lmo0768		Similar to sugar binding proteins, ABC TS
	Lmo0859		Similar to sugar binding proteins, ABC TS
	Lmo1016	GbuC	Similar to glycine betaine binding protein, ABC TS
	Lmo1041		Similar to molybdate binding proteins, ABC TS
	Lmo1073		Similar to metal ion binding proteins, ABC TS
	Lmo1388	TcsA	Similar to substrate binding lipoproteins, ABC TS; CD4 ⁺ T-cell-stimulating antigen
	Lmo1426	OpuC	Glycine betaine/carnitine/choline binding proteins, ABC TS
	Lmo1671		Similar to adhesion proteins and Mn/Zn binding proteins, ABC TS
	Lmo1730		Similar to sugar binding proteins, ABC TS
	Lmo1738		Similar to amino acid binding proteins, ABC TS
	Lmo1847	LpeA	Similar to manganese binding lipoprotein, ABC TS
	Lmo1959		Similar to ferrichrome binding proteins, ABC TS
	Lmo2007		Similar to sugar binding proteins, ABC TS
	Lmo2125		Similar to maltose/maltodextrin binding lipoproteins, ABC TS
	Lmo2184		Similar to ferrichrome binding lipoproteins, ABC TS
	Lmo2196	OppA	Similar to oligopeptide binding lipoprotein, ABC TS
	Lmo2349		Similar to amino acid binding lipoproteins, ABC TS
	Lmo2417		Similar to substrate binding lipoproteins, ABC TS, and to pheromone cOB1
	Lmo2431		Similar to ferrichrome binding lipoproteins, ABC TS
	Lmo2499		Similar to phosphate binding lipoproteins, ABC TS
	Lmo2569		Similar to dipeptide binding proteins, ABC TS
	Lmo2839		Similar to sugar binding proteins, ABC TS
Enzymatic activities	Lmo0013	QoxA	Similar to AA3-600 quinol oxidase subunit II
	Lmo0355		Similar to flavocytochrome <i>c</i> fumarate reductase chain A
	Lmo0517		Similar to phosphoglycerate mutase
	Lmo0945		Similar to metallo-beta-lactamase, DNA binding and competence protein (ComEC and ComEA of <i>B. subtilis</i>)
	Lmo1379	OxaA	Similar to membrane insertase OxaA ^d
	Lmo1444	PrsA	Similar to foldase PrsA ^d
	Lmo1800		Similar to protein tyrosine-phosphatase
	Lmo1903		Similar to thioredoxin
	Lmo2219	PrsB	Similar to foldase PrsA ^d
	Lmo2446		Similar to glycosidase
	Lmo2578		Similar to hydrolase
	Lmo2636		Similar to thiamine biosynthesis lipoprotein ApbE
	Lmo2642		Similar to serine/threonine protein phosphatase
	Lmo2812		Similar to D-alanyl-D-alanine carboxypeptidase ^e
	Lmo2854	OxaB	Similar to membrane insertase OxaA ^d
	Other	Lmo0366	
Lmo1757			Similar to sex pheromone staph-cAM373
Lmo2331			Similar to Gp32 bacteriophage A118 protein
Lmo2637			Similar to sex pheromone cAD1
Lmo0047			Unknown
Lmo0207			Unknown
Lmo0255			Unknown
Lmo0303			Unknown
Lmo0324			Unknown
Lmo0460			Unknown
Lmo0510			Unknown
Lmo0617			Unknown
Lmo0791			Unknown
Lmo0821			Unknown
Lmo0953			Unknown
Lmo1068			Unknown
Lmo1265			Unknown
Lmo1340			Unknown
Lmo1649			Unknown
Lmo1653			Unknown
Lmo2079			Unknown
Lmo2080			Unknown
Lmo2416			Unknown
Lmo2594			Unknown
Lmo2595			Unknown

^a Adapted from reference 13 with permission. Of note, Lmo1340 was identified as a new lipoprotein, and Lmo0810 was removed from the first lipoprotein annotation (67), as it was incorrectly annotated as a lipoprotein.

^b Proteins absent from *L. innocua* are indicated in boldface.

^c TS, transporter systems.

^d See Fig. 5.

^e See Fig. 3.

Another possible mechanism is deduced from electron microscopy studies performed 40 years ago that revealed the presence of small vesicles attached to the listerial membrane. These vesicles could be derived from small membrane extrusions (65). This observation is reminiscent of a well-described phenomenon in gram-negative bacteria. Many of them release vesicles from their outer membranes as secretory vehicles for proteins, lipids, and immunomodulatory compounds (189; for a review, see reference 99). These vesicles mediate bacterial binding and invasion, cause cytotoxicity, and modulate the host immune response. It is tempting to speculate that such phenomenon occurs in gram-positive bacteria. *L. monocytogenes* would be a nice model with which to test this hypothesis.

Finally, some proteins may also reach the cell surface through cosecretion and interaction with other surface proteins. Together, these results highlight the possibility of Sec-independent mechanisms of secretion and surface association that clearly deserve future attention.

FUNCTIONS OF SURFACE PROTEINS AND THEIR RELEVANCE IN PATHOGENESIS

Interfering with the surface-anchoring pathways represents a powerful tool for analyzing the diverse classes of surface proteins and their role during the *Listeria* infectious process. This approach has proven to be successful for LPXTG proteins and lipoproteins. An *srtA* mutant, in which LPXTG proteins are missorted, displays a severe attenuation phenotype following oral infection, with a more pronounced effect than an internalin mutant, pointing to the role of several LPXTG proteins in pathogenesis (16, 17). Similarly, interfering with the processing of lipoproteins affects intracellular survival of *L. monocytogenes* (13, 148). However, from these global approaches it is not known whether the alteration of a predominant single protein or the cumulative effect of alterations of several proteins is responsible for the attenuated phenotype. In order to answer this question, it is necessary to examine each protein individually or in groups of functional categories. An in-depth examination of domains present in predicted surface proteins may help in this classification prior to the essential experimental validation.

Cell Wall Metabolism

The cell wall is a highly dynamic structure that expands during cell growth and must be cleaved during cell division and lysis. Cell wall remodeling is also critical in numerous cellular processes, including formation of flagella, sporulation, biofilm formation, chemotaxis, genetic competence, and protein secretion. Growth and breakage of the peptidoglycan involve several enzymes, synthetases, and hydrolases, whose activities must be coordinated in time and space (82). Autolysins break the glycosidic bonds between the peptidoglycan monomers and also break the peptide cross-bridges that link the rows of sugars together (169). Transglycosylases (also referred to as glycosyltransferases) insert and link new peptidoglycan monomers into the breaks in the peptidoglycan. Finally, transpeptidases reform the peptide cross-links between the rows and layers of peptidoglycan (Fig. 3B).

Knowledge of the enzymatic activities involved in cell wall

assembly and turnover is important, as peptidoglycan synthetases are targets for many antibiotics and cell wall components are potent determinants of inflammation. *L. monocytogenes* cell wall derivatives are recognized by membrane-bound immune sensors, such as the macrophage scavenger receptor class A and Toll-like receptors (TLRs), or by intracytosolic sensors, such as Nod proteins, thereby stimulating host immunity. Macrophage scavenger receptor class A- and TLR2-deficient mice are highly susceptible to *L. monocytogenes* infection (85, 178). Nod2-deficient mice are susceptible to oral bacterial infections (92). Evidence for sensing of *L. monocytogenes* by Nod1 in vitro has also been recently reported (134). Together, these observations suggest that enzymes promoting the release of bacterial components are likely to be important modulators of the host innate (20) as well as adaptive (122) immune responses.

Autolysins. By digesting the cell wall peptidoglycan, autolysins are involved in numerous cellular processes, including cell growth and division, cell wall turnover, motility, chemotaxis, protein secretion, differentiation, and pathogenicity (140, 169). These hydrolytic enzymes are also responsible for the active release of immunogenic cell wall components. There are several types of autolysins, categorized by the nature of the bonds hydrolyzed in the peptidoglycan (Fig. 3B). Based on domain homology search data and a few studies using zymogram assays, we can expect the existence of several autolysins in *L. monocytogenes*. However, a direct demonstration of cleavage activities of these enzymes at specific bonds will require further biochemical studies.

(i) N-Acetylglucosaminidases and N-acetylmuramidases. Two types of enzymes hydrolyze the sugar backbone of peptidoglycan between the alternating MurNAc and GlcNAc residues of the glycan chains. *N*-Acetylmuramidases, which are related to lysozyme, cleave the β -1,4 glycosidic bond between MurNAc and GlcNAc (Fig. 3B), whereas *N*-acetylglucosaminidases hydrolyze the bond between GlcNAc and MurNAc by liberating free reducing groups of GlcNAc. Three listerial proteins, Lmo0717, Lmo1104, and Lmo2522, possess a putative N-terminal *N*-acetylmuramidase domain. Six proteins, among which are the GW protein Auto and the LysM protein MurA (Fig. 3A), possess a domain with similarities to the catalytic domain of *N*-acetylglucosaminidases. Both Auto and MurA display cell wall hydrolase properties (31, 37). However, an *aut* deletion mutant has a morphology similar to that of the wild-type strain and has no defect in septation and cell division, while a *murA* mutant grows as long chains during exponential growth. Interestingly, Auto is required for entry of *L. monocytogenes* into different nonphagocytic eukaryotic cell lines, and an *aut* mutant displays a reduced virulence (31). How Auto specifically contributes to pathogenicity is unknown, though it may be linked to the maintenance of the cell surface architecture and/or to the release of immunologically active cell wall components.

(ii) Amidases. The *Listeria* genome encodes four proteins with a putative amidase domain known to hydrolyze the bond between the glycan chain and the peptide side chain of peptidoglycan. Two are GW proteins, Ami and Lmo1521, and two are proteins without any obvious modules for targeting at the bacterial surface, Lmo0129 and Lmo0849. Ami is the only amidase for which autolysin activity was reported (25). Like in

InIB, the GW modules appear to promote Ami adhesion to host cells (127). Since the InIB GW modules bind cellular matrix proteoglycans (87), it is possible that Ami GW modules exert a similar function. Thus, Ami may act as a complementary adhesion during infection.

(iii) γ -D-Glutamyl-(L)-meso-diaminopimelate peptidases. Another important group of *L. monocytogenes* autolysins is the P60 subfamily. P60, encoded by the *iap* (invasion-associated protein) gene, is a major 60-kDa autolysin first described as being involved in the *L. monocytogenes* invasion process (100). P60-defective mutants display an abnormal morphology, characterized by the presence of filamented cells containing fully formed septa (72, 196). The four members of the P60 family, Spl (P45), Lmo0394, Lmo1104, and P60 itself, share in their C-terminal region an NlpC/P60 domain (Pfam PF00877) related to the CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain, which is present in many proteins predicted to function in peptidoglycan hydrolysis (12, 151). For instance, this domain is present in *Bacillus subtilis* LytE and LytF and in *S. aureus* LytA and LytN autolysins (4). All CHAP superfamily enzymes characterized to date hydrolyze diverse substrates that contain a γ -D-glutamyl moiety. Although not yet biochemically validated, it is proposed that the P60 family fulfills the same function as *Bacillus sphaericus* endopeptidase II, which hydrolyzes the γ -D-glutamyl-meso-diaminopimelate linkage in the cell wall peptides (Fig. 3B) (169).

Besides P60, P45 (Spl) is the only protein of this family in *L. monocytogenes* known to exhibit peptidoglycan-lytic activity (163). Interestingly, P45 combines the C-terminal CHAP domain with an N-terminal domain related to the SMC (structural maintenance of chromosomes) family of proteins. The SMC proteins are essential for successful chromosome transmission during replication and segregation of the genome in all organisms (80). They form heterodimers and are core components of large multiprotein complexes. Lmo2504 is another putative surface protein with a C-terminal peptidase domain and an SMC-related N-terminal domain (Fig. 3). It is possible that P45 and Lmo2504 somehow couple the peptidoglycan remodeling to the segregation of the nucleoid, although further studies are required to test this hypothesis. Lmo1104 also combines the C-terminal CHAP domain with another N-terminal module. In this case, the N-terminal region shows similarity to a lytic transglycosylase domain (Pfam PF01464). From this, Lmo1104 may thus have a dual function in hydrolyzing the cell wall. Lmo1104 is the only putative autolysin of the group that is absent in *L. innocua*.

A link between this class of surface proteins and *Listeria* pathogenicity has been established in the case of P60. Rough mutants expressing lower levels of P60 enter less efficiently into certain eukaryotic cells, suggesting a role for P60 in invasion (100). A P60-deficient mutant is attenuated in virulence after intravenous infection of mice and is impaired in its intracellular motility due to mislocalization of the actin-polymerizing factor ActA (107, 138). In addition, 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 (95). P60 is also a major protective antigen that induces both T-CD8 and Th1 protective immune responses (64, 75). Finally, it was recently reported that P60 contributes to sub-

version of NK cell activation and production of gamma interferon (83).

(iv) Phage endolysins. The genome of *L. monocytogenes* EGDe contains one prophage inserted in the *comK* locus (67, 111). This cryptic phage is highly homologous to phage A118 (111, 112, 114), a temperate bacteriophage attacking *L. monocytogenes* serovar 1/2. Like A118, the Φ EGDe element possesses a gene encoding a putative endolysin (Lmo2278, homologous to Ply118 [115]) followed by a gene encoding a holin (homologous to Hol118 [115]) thought to trigger secretion of the endolysin (188). The Φ EGDe endolysin is not characterized but could function like Ply118 endolysin, an L-alanyl-D-glutamate peptidase that cuts the amide bonds between L-Ala and D-Gln within the peptidoglycan peptide bridges (Fig. 3A) (115). Interestingly, endolysin Ply500 from phage A500 has the same enzymatic activity as Ply118, although it targets the cell wall of *Listeria* serovars 4, 5, and 6. The specificities of the endolysins are conferred by their different cell wall binding domains, which are thought to recognize *Listeria*-specific surface carbohydrates (113). Supporting this idea, phage PSA endolysin has a cell wall binding domain closely related to that of Ply500 and targets the same serovars, while having different enzymatic activity than Ply500 (96).

PBPs: transglycosylases, transpeptidases, β -lactamases, and D-alanyl-D-alanine carboxypeptidases. Peptidoglycan assembly is a multistep process carried out by different enzymatic activities. Transglycosylase enzymes catalyze the insertion of peptidoglycan precursors into the growing end of the bacterial cell wall. Subsequently, transpeptidase enzymes join the peptide of a precursor with that of the preexisting peptidoglycan in order to cross-link the sugar chains (Fig. 3B). Penicillins and cephalosporins block the formation of the peptide cross-links and induce osmotic lysis of the bacterium by binding to the transpeptidase enzyme. Bacteria may resist the action of these β -lactam antibiotics by mutating transpeptidases or by expressing β -lactamase, an enzyme hydrolyzing β -lactam rings. All these enzymes belong to the family of PBPs.

A first study reported that *L. monocytogenes* had five PBPs, as detected by their ability to bind benzylpenicillin (186). By searching the genome, Guinane and colleagues recently found seven surface proteins with similarities to PBPs (71). Our own in silico research identified 10 PBP-like proteins, which we classified into four families as represented in Fig. 3A. The first family, including PBPA1 and PBPA2 (formerly PBP1 and PBP4 [186]), corresponds to class A PBPs, which are bifunctional proteins with an N-terminal transglycosylase domain and a C-terminal transpeptidase domain. The second family, including PBPB1, PBPB2, (formerly PBP2 [186]), and PBPB3, corresponds to class B PBPs, which have a C-terminal transpeptidase domain and an N-terminal PBP dimerization domain (Pfam PF03717) involved in interaction with other proteins during cell morphogenesis. The third family encompasses two putative PBPs, PBPC1 and PBPC2, which contain a β -lactamase class C domain (Pfam PF00144). PBPC1 is expected to be at the bacterial surface, while PBPC2 lacks any known cell surface association domain. Finally, the fourth family includes three proteins, PBPD1, PBPD2, and PBPD3, that contain a D-alanyl-D-alanine carboxypeptidase domain. This domain may catalyze the removal of the C-terminal D-alanine residue from peptidoglycan pentapeptides (Fig. 3B). PBPD1 (formerly

PBP5 [186]) and PBPD2 are related to the peptidase S11 family (Pfam PF00768), represented by *E. coli* PBP5, while PBPD3 is related to the VanY DD-carboxypeptidase family (Pfam PF02557).

L. monocytogenes clinical isolates are resistant to cephalosporins but are, fortunately, susceptible to penicillin and ampicillin, which constitute the major treatment for listeriosis in combination with the aminoglycoside gentamicin. However, several recent findings provide evidence of the emergence of multidrug-resistant *L. monocytogenes* strains, including strains with resistance to penicillin and ampicillin (38, 143, 157, 170). Dissemination of these resistant traits in clinical strains of *L. monocytogenes* could have important medical consequences. It is therefore important to characterize listerial PBPs.

Recent studies have confirmed the enzymatic activity of PBPD1 (Lmo2754) (97) and of PBPA2 (Lmo2229) (198). Moreover, analysis of mutant strains suggests that PBPB3 (Lmo0441) and, to a lesser extent, PBPA2 (Lmo2229) are central to β -lactam resistance in *Listeria* (71). Of interest is that PBPB3 shares similarities with MecA, the key determinant of β -lactam resistance in methicillin-resistant *S. aureus* strains (109). Furthermore, disruption of some *pbp* genes attenuates *L. monocytogenes* virulence, probably due to alteration of the cell wall metabolism (71).

Deacetylase. A last important peptidoglycan-modifying enzyme is PgdA (Lmo0415), an enzyme recently characterized in *L. monocytogenes* as a GlcNAc deacetylase (21). *pgdA* mutants of *L. monocytogenes*, like those of *S. pneumoniae* (187), are hypersensitive to lysozyme, one of the first-line defense mechanisms in the human host against invading bacteria. Inactivation of *pgdA* also dramatically attenuates virulence and induces a massive beta interferon response in a TLR2- and Nod1-dependent manner. Peptidoglycan deacetylation is therefore essential for resistance against the innate immune response (21).

Enzymes involved in assembly and modification of TAs and LTAs. In addition to the assembly of cross-linked peptidoglycan, several enzymes are required for the synthesis of polyanionic TAs and LTAs, which make up an important polyanionic network with ion-exchange properties at the bacterial surface. However, polymerization of TAs and LTAs occurs intracellularly (14). Therefore, enzymes involved in cell wall polymer synthesis are expected to be cytosolic and not surface exposed, although a signal peptide is proposed to be present in two of them in *L. monocytogenes*, TagO (Lmo2519) and TagB (Lmo1088) (179). Enzymes involved in D-alanine esterification, a common modification found in TAs and LTAs of many gram-positive bacteria, are cytosolic or membrane-bound proteins (130). The synthesis of D-alanyl-LTA requires four proteins that are encoded by the *dlt* operon (1): *dltA* (Lmo0974) encodes a cytoplasmic D-alanine-D-alanyl carrier protein ligase (designated DltA or Dcl); *dltB* (Lmo0972) encodes the D-alanyl carrier protein Dcp, which is secreted; and *dltC* (Lmo0973) and *dltD* (Lmo0971) encode membrane-associated proteins, each having a signal peptide according to the Pfam annotation. However, the current model is that DltD would function intracellularly, providing binding sites for DltA and Dcp on the cytoplasmic leaflet, while DltB would form a channel for the secretion of D-alanyl-Dcp towards the cell wall, where D-alanylation occurs (130). This pathway still needs clarification.

Protein Processing, Folding, and Anchoring

Following translocation across the Sec channel, most proteins have their signal peptide cleaved by an SPase. The *L. monocytogenes* genome encodes five membrane-bound SPases (Fig. 5). Three of them, SipX, SipY, and SipZ, are type I SPases, whose predicted function is to remove the signal peptides of preproteins exported by the general secretory pathway. SipZ appears to be the major SPase I for listerial secreted virulence factors (22). Two other SPases, LspA and the as yet undescribed LspB, belongs to class II SPases involved in maturation of lipoproteins. LspA processes at least lipoprotein LpeA (148), while LspB remains to be characterized.

As described above, mature proteins exhibiting a specific C-terminal sorting signal are processed by sortases, while lipoproteins are processed by Lgt (Fig. 5) (13, 16, 17, 62). Proteins that will remain at the surface inserted in the membrane will be processed by components that catalyze efficient lateral transport into or across the plasma membranes. These include the conserved YidC/Oxa1/Alb3 proteins family that are believed to function as membrane insertases, possibly functioning also as membrane chaperones (reviewed in references 139 and 182). Two *L. monocytogenes* lipoproteins, Lmo1379 (OxA) and Lmo2854 (OxB), share homology with YidC/Oxa1 and may thus target membrane-bound surface proteins (43). Future work is needed to validate this hypothesis.

The majority of proteins that are translocated across the cytoplasmic membrane are delivered to the membrane-cell wall interface essentially unfolded. They must then be folded into their native configuration without blocking the translocation machinery in the membrane, by forming inadequate interactions with the cell wall or insoluble aggregates. Misfolded or aberrant proteins have to be cleared off the translocase and the cell wall. Bacteria encode membrane- and cell wall-associated foldases and proteases that act as folding catalysts and quality control machines (159). Several genes in the *L. monocytogenes* genome encode proteins likely to be involved in folding and degradation of polypeptides at the bacterial surface (Fig. 5). PrsA (Lmo1444) and PrsB (Lmo2219) are lipoproteins related to the PrsA protein of *B. subtilis*. PrsA is a parvulin-type peptidyl-prolyl *cis/trans* isomerase, which assists posttranslocational folding of exported proteins and their stabilization at the bacterial surface. Interestingly, lmo2219 is preceded by a PrfA box, in contrast to lmo1444 (67), and is regulated by PrfA, the major regulator of *Listeria* virulence gene expression (13, 126). In addition, lmo2219 is upregulated during the intracytosolic phase of the *Listeria* cellular infectious process (39). We therefore suggest that PrsB could play a specific role in bacterial adaptation to the host.

lmo0292 (*degP*) encodes an HtrA-like serine protease related to *B. subtilis* YycK (172). HtrA is a protease that can also function as a chaperone. The listerial HtrA-like protease is essential for optimal growth under stress conditions and is involved in listerial pathogenesis (172, 195). Interestingly, like the gene encoding PrsB, the *htrA* gene is upregulated intracellularly (39). In addition to its N-terminal protease domain (Pfam PF00089), HtrA possesses a C-terminal PDZ domain. PDZ domains consist of ~80 amino acids compactly arranged in a globular structure thought to function in protein or peptide binding (Pfam PF00595). They are found in diverse sig-

naling proteins, in both bacteria and eukaryotes. We found two other proteins with protease and PDZ domains in the *L. monocytogenes* genome, Lmo1851 and Lmo1318 (Fig. 5). Lmo1851 carries a peptidase domain of the S41 family (Pfam PF03572), to which the ClpP protease belongs. Lmo1318 is a hypothetical zinc metalloprotease of the S2P/M50 peptidase family (Pfam PF02163), such as the membrane-bound *E. coli* protease EcfE (90). Related to Lmo1318 is Lmo2563, which is also predicted to be a S2P/M50 peptidase albeit without a PDZ domain and sharing similarities with the *B. subtilis* sporulation factor SpoIVFB (35). Interestingly, S2P/M50 proteases, by catalyzing cleavage of membrane-bound proteins, seem to be involved in a variety signal transduction pathways, some of which play roles in host-pathogen interaction (117). Whether Lmo1851, Lmo1318, and Lmo2563 function as cell surface proteases will require further investigation.

Adhesion to Host Cells

As their name implies, adhesins are specialized surface proteins that mediate bacterial adhesion. They specifically recognize receptors on the surface of target host cells, determining tissue tropism of the pathogen. Among these host receptors are components of the extracellular matrix, a complex structural entity surrounding and supporting cells within mammalian tissues (24). The extracellular matrix is composed of three major classes of biomolecules: (i) structural proteins, including collagen and elastin; (ii) specialized proteins, such as fibrillin, fibronectin, and laminin; and (iii) proteoglycans, which are high-molecular-weight components composed of a protein core to which long chains of repeating disaccharide units termed GAGs are attached. Besides proteoglycans, other high-molecular-weight glycosylated proteins, such as mucins, are likely to play an important role in the bacterial adherence to epithelia. Mucins, either secreted or membrane bound, form a major part of a protective biofilm on the surface of epithelial cells (142, 154).

Sequence comparisons with previously characterized proteins suggest that some domains found in listerial proteins may play a role in adhesion to host cells.

Collagen binding domain. Five LPXTG proteins (Lmo0159, Lmo0160, Lmo0880, Lmo2158, and Lmo2576) are predicted to have a collagen binding domain (Pfam PF05737) (Fig. 2), which is found in the collagen binding adhesins of several other gram-positive pathogens (137, 199). The prototype member of this family is the collagen binding adhesin Cna of *S. aureus*. Cna participates in the infectious process of pathogenic *S. aureus* (52), suggesting that the ability to interact with collagen provides a general advantage to the bacteria in pathogenesis. Cna is composed of an N-terminal A region that carries the collagen binding activity and a C-terminal region of B repeats (CnaB-like repeats) (see below) (137). The Cna A region is composed of three subdomains, N1, N2, and N3 (199). Crystal structure determination of the N1 and N2 domains suggest a model in which both domains, each taking an Ig-like fold, cooperate to wrap around and hug the collagen triple helix (199). The collagen binding domain present in listerial proteins shows similarity to N2, which is the minimal collagen binding domain of Cna. Further work is required to explore the func-

tion of these listerial proteins, especially their possible binding to collagen.

Fibronectin binding domain. FbpA (Lmo1829) is a *Listeria* surface fibronectin binding protein required for intestinal and liver colonization of *L. monocytogenes* following oral infection (48). FbpA has strong homology to atypical fibronectin binding proteins, such as PavA of *S. pneumoniae* (81) and Fbp54 of *S. pyogenes* (40). FbpA binds to immobilized human fibronectin in a dose-dependent and saturable manner and increases adherence of wild-type *L. monocytogenes* to HEp-2 cells in the presence of exogenous fibronectin. Like FbpA, Lmo0721 is another reported listerial fibronectin binding protein that lacks conventional secretion/anchoring signals (66). Whether it is exposed at the bacterial surface has not been formally demonstrated.

Proteoglycan binding domain. As mentioned above, InlB GW repeats bind not only to LTAs present at the bacterial surface but also to GAGs. GW repeats binding to GAGs releases InlB from the bacterial surface (87). Furthermore, the presence of GAGs on the cell surface significantly increases InlB-dependent invasion, suggesting that binding of GW repeats to cellular GAGs could enhance interaction of InlB with its cellular receptor, Met. It is not known whether GW modules present in autolysins also bind GAGs. However, the purified GW domain of Ami binds eukaryotic cells, suggesting that a similar interaction may explain the role of Ami in listerial adhesion to host cells (127).

RGD motif. Three listerial surface proteins contain an arginine-glycine-aspartate (RGD) motif: ActA, the LPXTG protein Lmo1666, and the lipoprotein Lmo0460. The RGD motif is found in various proteins of the extracellular matrix and is known to interact with integrins. Although the RGD motif in ActA does not seem to contribute to *Listeria* adhesion to host cells (3), it may be a potential integrin binding site in Lmo1666 and Lmo0460.

Mucin binding domains. Twelve LPXTG proteins (Fig. 2) and one protein with a membrane anchor domain, Lmo0576, contain a MucBP (mucin binding protein) domain (Pfam PF06458). Identified in MUB, an extracellular mucus binding protein of *Lactobacillus reuteri* 1063, this domain is arranged as tandem repeats that were shown to trigger adhesion to mucus material (152). MucBP repeats are present in 1 to 14 copies in listerial proteins. According to the Pfam database, MucBP repeats are present only in *Lactobacillales* and *Listeria* bacterial species. Sequence analysis indicates that the MucBP repeats vary in size, for instance, ranging from ~60 amino acids in listerial proteins to almost 200 amino acids in MUB proteins of *L. reuteri* (19, 152). The difference in size between *Listeria* MucBP and *Lactobacillus* MUB repeats can be explained by the presence of a distinct N-terminal region in the MUB domain. MUB repeats and MucBP repeats may therefore be different functional units (19). At present, there is no experimental evidence for a role of listerial MucBP binding to mucins.

Ig-like fold domains. The Ig fold is an important structure, playing essential roles in the vertebrate immune response, cell adhesion, and many other processes. Novel three-dimensional structures of domains from distantly related proteins have revealed that unrelated sequences may take a similar Ig fold, which is characterized by the presence of β sheets. Thus, nearly

all *Listeria* LPXTG proteins possess Ig-like domains that fall into one of five distinct sequence families: the Cna collagen binding domain described above, the Cna protein B-type domain, the LRR-adjacent domain, the PKD repeat domain, and the bacterial Ig-like domain (Big 3). Two main functions may be attributed to these domains. They may be directly involved in adhesion by interacting with carbohydrates on the bacterial or host cell surface. Alternatively, they may serve as a stalk that projects a ligand binding region from the bacterial surface, thus facilitating bacterial interaction with host cells. For instance, the receptor binding domain of intimin in enteropathogenic *Escherichia coli*, invasins in *Yersinia*, or internalin in *Listeria* is presented at the tip of an elongated structure, in which the repeat region is used as a spacer domain or a stabilizer for the whole structure (133).

(i) **CnaB-like repeat domain.** As mentioned above, staphylococcal Cna has a nonrepetitive collagen binding A region followed by a repetitive B region. The B region has one to four 23-kDa repeat units [B(1) to B(4)], depending on the strain of origin. Each B repeat has two domains, D1 and D2, each exhibiting an inverse Ig-like fold. Modeling studies suggest an accordion structure in which the B repeat units could project the A region from the cell surface and aid in binding to collagen (41). Six listerial LPXTG proteins contain repeats related to the D domain of CnaB repeats (Pfam PF05738) (Fig. 2), among which four possess a collagen binding domain (Lmo0159, Lmo0160, Lmo2178, and Lmo2576).

(ii) **LRR-adjacent Ig-like domain.** Eleven LPXTG proteins of the internalin family contain the LRR-adjacent Ig-like domain (Pfam PF08191) (Fig. 2). It is a small beta-strand domain fused to the C-terminal end of the LRR region (133, 164). This domain belongs to the family of Ig-like domains in that it consists of two sandwiched beta sheets. The beta strands in one of the sheets is, however, much smaller than those in most standard Ig-like domains. The function of this domain appears to be mainly structural, forming a common rigid entity with the LRR. It is thought to facilitate the presentation of the adjacent LRR domain for protein-protein-interactions.

(iii) **PKD repeat domain.** Eleven LPXTG proteins contain the PKD repeat domain (Pfam PF00801) (Fig. 2). The PKD repeat domain was first identified in the human polycystic kidney disease 1 (PKD1) protein as 16 Ig-like repeats, each of ~80 amino acid in length. PKD1 is involved in adhesive protein-protein and protein-carbohydrate interactions. PKD1 Ig-like repeats II to XVI are involved in strong calcium-independent homophilic interactions in vitro (84), suggesting that PKD1 may be involved in cell-cell adhesion through its cluster of Ig-like repeats. However, the function of PKD1 repeats in listerial proteins remains totally unknown. For instance, InII, the longest internalin-like protein (Fig. 2), possesses eight PKD repeats, but no detectable role could be attributed to InII in vitro or in virulence assays (156).

(iv) **Big 3 domain.** Four LPXTG proteins contain a domain with an Ig-like fold called the Big 3 domain (Pfam PF07523) (Fig. 2). This domain is present in one copy in the internalin-like Lmo2026, Lmo0732, and Lmo0171 proteins and is repeated seven times in the Lmo0842 protein. Interestingly, Lmo0842 repeats are related to those found in the Alp family in streptococci, which comprises alpha, Rib, R28, and Alp2 proteins, which are important surface proteins known to confer

protective immunity (110). Alp repeats are themselves related to repeats of ~80 amino acids found in biofilm-associated proteins (Bap) in staphylococci and in Esp in enterococci (102), as well as to HYR (hyalin repeat) domains identified in several eukaryotic proteins (33). By BLAST analysis, we found that PKD repeats present in *Listeria* LPXTG proteins are also weakly similar to the Bap family repeats. Together, these data suggest an evolutionary relationship between all these repeated domains.

Invasion

Two surface proteins, the LPXTG protein internalin A (InIA) and the GW protein InIB, are major *L. monocytogenes* invasins, promoting bacterial entry into mammalian cells (47, 61; for a review, see reference 73). InIA is sufficient for bacterial uptake into epithelial cells and is required for *Listeria* to cross the intestinal and placental barriers (104). InIA-dependent entry is mediated by human Ecad, a cell adhesion molecule that is required for the integrity of adherens junction of polarized epithelial cells and is connected to cytoskeletal proteins. InIA-Ecad interaction is species specific and occurs between InIA and human or guinea pig Ecad but not mouse or rat Ecad (73, 104). The Pro residue at position 16 in human Ecad is essential for InIA recognition. InIB mediates entry into a variety of cell types by interaction with the hepatocyte growth factor receptor Met (167). InIB also interacts via its GW modules with gC1qR, a ubiquitous molecule first identified as the receptor for C1q, the first component of the complement cascade, and with GAGs (26, 73, 119). In vivo, InIB is required for efficient mouse liver colonization (91) and plays a role in invading human placenta in conjunction with InIA (105). Recent studies reveal species specificity for InIB and show that InIB does not recognize or stimulate guinea pig or rabbit Met (91). The LPXTG protein Vip (Lmo0320) is another surface virulence factor required for *L. monocytogenes* entry into some mammalian cells (32). Vip interacts with the mammalian heat shock protein Gp96, a member of the Hsp protein family that plays significant roles in protein folding and in the modulation of both the innate and adaptive immune responses. Vip-Gp96 interaction is critical for bacterial entry into cells. Vip could thus appear as a new type of virulence factor, exploiting Gp96 as a receptor triggering a signaling pathway required for cell invasion and/or to subvert the host immune response during infection (32).

Motility

Flagellar movement. Bacterial movement is produced through the action of the flagella. The flagellum comprises a long filament that acts as a propeller, which is anchored to the cell envelope by a flexible hook and basal body (116). The growth of the bacterial flagellar filament occurs at its distal end by self-assembly of flagellin transported from the cytoplasm through the channel of the flagellum (116). Listerial flagellin is produced and assembled at the cell surface when *L. monocytogenes* is grown at between 20 and 25°C, whereas its production is markedly reduced at 37°C. However, it is important to note that flagellin expression is still high at 37°C in some laboratory-adapted strains and in approximately 20% of clini-

cal isolates (192). Flagellin is encoded by the *flaA* gene (lmo0690), which is located in a 38-kb locus that comprises 26 other genes encoding putative flagellar machinery proteins (18, 43). Listerial flagellin is posttranslationally modified, being glycosylated with β -O-linked GlcNAc (161). Whether glycosylation is essential for flagellar assembly or in some way facilitates interactions of *Listeria* with either other cells or environmental substrates is currently unknown.

Flagellin from *L. monocytogenes* stimulates TLR5 (76), which is expressed by intestinal epithelial cells, monocytes, and dendritic cells. TLR5 activation induces the production of tumor necrosis factor (TNF) alpha, an important cytokine in host resistance to enteric listeriosis. In addition, flagella seem to facilitate the adhesion to and invasion of eukaryotic host cells by *L. monocytogenes* (45). However, the relationship between flagellin and virulence is not clear. On one hand, no differences in virulence, bacterial clearance, or induction of *L. monocytogenes*-specific T-lymphocyte responses between *L. monocytogenes* strain 10403S and an isogenic *flaA* deletion strain were observed in the mouse infection model, suggesting that flagellin is not essential for pathogenesis (192). On the other hand, inactivation of the regulator of *flaA* expression, MogR, induces overexpression of FlaA and causes defects in bacterial division, intracellular spread, and virulence in mice (166). Finally, virulence of *flaA* mutants is diminished relative to that of the wild-type strain in tumor necrosis factor alpha receptor-p55^{-/-} mice, suggesting that flagella participate in the triggering of tumor necrosis factor secretion, leading to protection against infection (45).

Intracellular movement. The well-known listerial surface protein ActA mediates polymerization of host actin to propel the bacterium through the cytoplasm of its host (for recent reviews, see references 68 and 73). ActA is the first bacterial protein identified to promote actin nucleation, by mimicking its eukaryotic counterparts, the Wiskott-Aldrich syndrome (WASP) family of proteins. The N-terminal region of ActA has homology to the C-terminal region of WASP, and both ActA and the WASP proteins act as nucleation-promoting factors for the Arp2/3 complex. The other major ligand of ActA is the Ena/vasodilator-stimulated phosphoprotein, which binds the central proline-rich domain of ActA and accelerates *L. monocytogenes* actin-based motility. A growing number of intracellular pathogens, such as *Rickettsia conorii*, *Shigella flexneri*, *Mycobacterium marinum*, *Burkholderia pseudomallei*, and vaccinia virus, are being recognized as displaying surface proteins that trigger actin polymerization for intra- and intercellular movement (68, 173).

Other Functions

Noninvasin internalin-like proteins. Data resulting from a *Listeria* DNA macroarray analysis identified six genes encoding surface proteins of the internalin family always found in all *L. monocytogenes* strains associated with listeriosis: *inlA*, *inlB*, *inlH*, *inlE*, *inlI*, and *inlJ* (46). Besides the invasion factors InlA and InlB, InlH (Lmo0263) and InlJ (Lmo2821) were associated with *Listeria* pathogenesis (156, 164). Screening of a bank of transposon mutants by signature-tagged mutagenesis in the mouse model revealed another internalin-like protein, Lmo2026 (formerly ORF626), as a factor possibly involved in

listerial multiplication in the brain (8). The functions of InlH, InlJ, and Lmo2026 are unknown and do not seem to be related to the internalization process. LRR-containing proteins are widely represented in eukaryotes and participate in many biologically important processes, such as hormone-receptor interactions, enzyme inhibition, cell adhesion, cellular trafficking, regulation of gene expression, apoptosis signaling, and pathogen recognition. LRR domains appear to function primarily in providing a versatile structural framework for the formation of protein-protein interactions (93). Many bacteria, either pathogenic or commensal, also possess LRR domains (see Pfam PF00560 and PF07723). Some representatives play a role in host-pathogen interactions, such as YopM in *Yersinia pestis*, the IpaH family in *Shigella flexneri*, SspH in *Salmonella enterica* serovar Typhimurium, Slr in group A streptococci, and IIsA in *Bacillus cereus* (53, 54, 74, 150, 184).

Listerial internalins are likely to play a wide range of functions, not only those dedicated to host-pathogen interactions. For instance, Lmo0327 is an internalin-like protein associated with an autolytic activity. However, this conclusion is based on phenotypes of an lmo0327 mutant (141), and further work is required to confirm whether Lmo0327, which has no domain related to peptidoglycan hydrolysis, is a bona fide autolysin.

Transporters. Among the 28 lipoproteins that are putative SBPs of ABC transporter systems (Table 3), two have been associated with *Listeria* pathogenesis, OppA (Lmo2196) and LpeA (Lmo1847). OppA, which mediates the transport of oligopeptides, is required for bacterial growth at low temperature and intracellular survival in cultured macrophages as well as in organs of mice during the early phase of infection in vivo (23). Recent data suggest that OppA is subject to a PrfA-dependent posttranslational modification (13). Four other listerial proteins, Lmo0152, Lmo0135, Lmo2044, and Lmo2569, are structurally related to OppA, having an SBP bac-5 domain (Pfam PF00496) shared by proteins involved in active transport of solutes across the cytoplasmic membrane.

LpeA contains an SBP domain of the bac-9 family (Pfam PF01297). It is related to several streptococcal adhesins, including PsaA of *S. pneumoniae*, FimA of *Streptococcus parasanguis*, ScaA of *Streptococcus gordonii*, SsaB of *Streptococcus sanguis*, and EfmA of *Enterococcus faecium* (149). These proteins belong to a family of surface-associated proteins designated lipoprotein receptor-associated antigen I (LraI). The LraI family constitutes a new family of extracellular SBPs specific for metal (Zn or Mn) ions. LpeA displays a metal binding site formed by His-67, His-139, Glu-205, and Asp-280, as described for PsaA. Interestingly, LpeA-defective bacteria efficiently adhere to eukaryotic cells but fail to penetrate in vitro into epithelial and hepatocyte cells, while they survive better in macrophages than do wild-type bacteria and display a weak exacerbation of virulence (149). LpeA might act directly through a cell receptor or indirectly through its hypothetical function as an ABC transporter, potentially influencing the cell sensing or an intracellular cascade associated with cell entry through the uptake of polypeptides, oligopeptides, and multiple sugars. We found two other listerial proteins related to LpeA, Lmo0153 and Lmo1671, but nothing is yet known about their function.

TABLE 4. Surface proteins involved in *L. monocytogenes* virulence

Protein	Name	Bacterial surface association type	Function(s)	Reference(s) for experimental validation
Lmo0433	InIA	LPXTG	Adhesion and invasion	Reviewed in references 73 and 104
Lmo0263	InIH	LPXTG	Unknown	164
Lmo2821	InIJ	LPXTG	Unknown	156
Lmo2026	Lmo2026	LPXTG	Unknown	8
Lmo0320	Vip	LPXTG	Invasion	32
Lmo0434	InIB	GW	Invasion and signaling	Reviewed in references 15 and 73
Lmo1076	Auto	GW	Cell wall turnover and invasion	31
Lmo2558	Ami	GW	Cell wall turnover and adhesion	127
Lmo0582	P60	LysM	Cell wall turnover and invasion	100
Lmo1847	LpeA	Lipoprotein	Metal transport and invasion	149
Lmo2196	OppA	Lipoprotein	Oligopeptide transport and intracellular survival	23
Lmo0204	ActA	Hydrophobic tail	Actin-based intracellular motility	Reviewed in reference 68
Lmo1829	FbpA	Unknown	Adhesion	48

CONCLUSIONS AND FUTURE DIRECTIONS

Listeria surface proteins appear mosaic, often combining unique and repeated segments. Each domain could fold independently, but all domains likely function in concert with each other. Several questions remain to be addressed, in three major directions.

Pathogenic Potential

Several surface proteins contribute to *L. monocytogenes* virulence (Table 4). However, relatively few have received extensive biochemical characterization. In addition, functional analysis is still required for many uncharacterized proteins that may bind eukaryotic molecules. It will be especially interesting to address whether these proteins play redundant functions, work together, and/or act in macromolecular complexes. It will be also of major importance to address aspects of host specificities. InIA and InIB entry pathways are not functional in all animal models due to species specificity of interaction with their cellular receptors (91, 104). Such host specificities could occur for other surface proteins.

Spatio-Temporal Expression

Relatively few genes encoding surface proteins are known to be modulated during the *Listeria* infectious process in cultured cells in vitro (39, 88), and only a subset of surface proteins have been detected by proteomic approaches (13, 34, 146). It is likely that surface proteins are produced at different time points of the growth phase, with their expression depending upon various environmental changes, such as temperature, nutrients, or host factors. Identification of growth conditions during which they are expressed is a prerequisite for recognizing their mechanisms of function. Moving from cellular to tissue microbiology should help us to discover when these bacterial effectors are expressed and subsequently play a role in *L. monocytogenes* pathogenesis.

Localization

The spatial and temporal localization of a given protein in the bacterial cell wall is probably critical for its optimal function. For instance, Auto and MurA autolysins both have a glucosaminidase domain, but mutant bacteria elongate in one case and not in the other, suggesting that their glucosaminidase activities operate on distinct sets of muropeptides or regions of the peptidoglycan via different cell wall binding domains. Another example is provided by the intracellular motility factor ActA, whose asymmetric localization at one pole of the bacterium triggers the unidirectional movement of *L. monocytogenes* (94). It was recently suggested that the polarization of ActA might be a direct consequence of the differential cell wall growth rates along the bacterium (147). InIA has a polarized distribution similar to that of ActA and may thus follow the same rule (103, 147).

The distribution of cell wall-associated proteins is likely to also depend on the localization of the secretion apparatus. For *S. pyogenes*, controversial studies indicate that secretion could occur at the single Exportal site (153) or at multiple sites (36, 175), with a key role of signal peptide sequences in addressing secretion to a subcellular localization (36). It will be very interesting to test whether such information is present on *Listeria* signal sequences.

Finally, some proteins may be buried in the cell wall, such as InIB, which is not accessible to antibodies at the surface unless the peptidoglycan is digested with a muramidase (86). However, InIB acts as an invasion protein that needs to be accessible to interact with its host receptor, suggesting that external factors regulate its accessibility, possibly by acting on GW repeats. This hypothesis is supported by the demonstration that GW repeats bind to cellular proteoglycans and that these interactions are required for efficient entry (87). Other internal-like proteins, such as *E. faecalis* EF2686 (ElrA), *S. pyogenes* Slr, and *S. agalactiae* BrID are also buried in the cell wall (28, 190). The accessibility of these proteins at the bacterial surface

may be modulated, affecting their function during the infectious process in vivo. It is thus of interest to understand what contributes to the topological distribution of proteins at the surface.

Strain and Species Variability

Although a number of surface proteins similar to those of strain EGDe has been reported to exist in the three other *L. monocytogenes* strains whose genomes are known (129), it is clear that each strain displays specific repertoires of surface proteins. It will be of interest to address to what extent surface proteins present in "pathovariants" are playing a role in successful infection. A large project of sequencing various different strains, from different environments and with different host tropisms, will soon provide important data to measure gene content variability (*Listeria monocytogenes* Sequencing Project. Broad Institute of Harvard and MIT, <http://www.broad.mit.edu>). It should reveal the core set of *Listeria* virulence factors, especially among surface proteins.

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