



31 **Abstract**

32

33 *Listeria monocytogenes* is a Gram-positive pathogen responsible for the manifestation  
34 of human listeriosis, an opportunistic foodborne disease with an associated high  
35 mortality rate. The key to the pathogenesis of listeriosis is the capacity of this  
36 bacterium to trigger its internalization by non-phagocytic cells and to survive and  
37 even replicate within phagocytes. The arsenal of virulence proteins deployed by  
38 *L. monocytogenes* to successfully promote the invasion and infection of host cells has  
39 been progressively unveiled over the past decades. A large majority of them are  
40 located at the cell envelope, which provides an interface for the establishment of close  
41 interactions between these bacterial factors and their host targets. Along the multistep  
42 pathways carrying these virulence proteins from the inner side of the cytoplasmic  
43 membrane to their cell envelope destination, a multiplicity of auxiliary proteins must  
44 act on the immature polypeptides to ensure that they not only mature into fully  
45 functional effectors but also are placed or guided to their correct position in the  
46 bacterial surface. As the major scaffold for surface proteins, the cell wall and its  
47 metabolism are critical elements in listerial virulence. Conversely, the crucial physical  
48 support and protection provided by this structure make it an ideal target for the host  
49 immune system. Therefore, mechanisms involving fine modifications of cell envelope  
50 components are activated by *L. monocytogenes* to render it less recognizable by the  
51 innate immunity sensors or more resistant to the activity of antimicrobial effectors.  
52 This review provides a state-of-the-art compilation of the mechanisms used by  
53 *L. monocytogenes* to organize its surface for virulence, with special focus on those  
54 proteins that work “behind the frontline”, either supporting virulence effectors or  
55 ensuring the survival of the bacterium within its host.

## 1. Introduction

*Listeria monocytogenes* is a ubiquitous Gram-positive bacillus and the causative agent of human listeriosis, a rare foodborne infectious disease with a high and particularly severe incidence in immunocompromised individuals and other risk groups, such as pregnant women and neonates. In these hosts, the invasive form of the illness can be symptomatically manifested as septicemia and meningoenzephalitis, or abortions and neonatal infections, which contribute to an estimated mortality rate of 20-30% of clinical cases (Swaminathan and Gerner-Smidt, 2007; Allerberger and Wagner, 2010). The success of this facultative intracellular pathogen results from the ability to promote its own internalization by non-phagocytic cells, which enables the bacterium to overcome important pathophysiological barriers, such as the intestinal epithelium, the blood-brain barrier and the placenta (Lecuit, 2007), and to survive and proliferate inside the host immune phagocytic cells. Decades of studies have contributed to the characterization and comprehension of the *L. monocytogenes* intracellular life cycle (Pizarro-Cerdá et al., 2012). Once internalized, *L. monocytogenes* quickly induces the lysis of its containing vacuole to reach the nutrient-rich cytoplasmic compartment where it can multiply (Gaillard et al., 1987). An actin-based motility machinery allows the bacterium to move in the cytosol and spread to neighboring cells (Ireton, 2013), thus disseminating the infection without re-exposure to the host extracellular immune surveillance.

To efficiently infect cells, *L. monocytogenes* makes use of a large array of virulence effectors that act in one or more steps of the cellular infection cycle (Camejo et al., 2011). The majority of these factors comprise proteins located at the surface of the bacterial cell, in association with the cell envelope or secreted to the extracellular milieu. Their extracytoplasmic localization allows these proteins to interact directly with host cell targets and induce the effects necessary for the establishment of infection. Annotation of the first sequenced genome of *L. monocytogenes* (EGD-e, serotype 1/2a) (Glaser et al., 2001) revealed the presence of 133 genes coding for surface proteins, corresponding to nearly 5% of the complete genome. Interestingly, a comparison with the genome of the phylogenetically close but non-pathogenic *L. innocua* signaled surface proteins as the major difference between both species, highlighting their potential role in *Listeria* pathogenesis (Cabanés et al., 2002). The characterization of this important subset of proteins has allowed us to better understand the role of immediate key virulence effectors of *L. monocytogenes* and to acknowledge the paramount importance of numerous other individual and multicomponent systems of proteins in the promotion and support of their activity.

This review focuses on the various backstage surface players that have been shown to enable *L. monocytogenes* to be fully equipped and proficient as a human pathogen. These include major intervenients in the mechanisms of surface protein processing and localization, the latter of which also relies on the presence of diverse surface-binding protein motifs or domains. Cell envelope modifications that optimize the surface display of virulence proteins and protect the bacterium from external aggression will be covered, as well as substrate uptake and metabolite/drug efflux systems necessary for bacterial survival. Finally, relevant content regarding the genetic and post-translational regulation of these surface events will be addressed.

## 2. Secretion systems

As bacterial surface proteins are being synthesized by cytoplasmic ribosomes, their surface export signal directs them to the plasma membrane, where a specialized secretion system will assist in their transposition to the other side of the membrane. Once outside, the protein can then be associated with a cell envelope component, depending on signals and features encoded in its sequence. Apart from the canonical Sec-dependent pathway, which mediates the secretion of most typical surface proteins in Gram-positive bacteria (Schneewind and Missiakas, 2013), other non-classical secretion systems identified in *L. monocytogenes* include the Tat system, the fimbriin protein exporter (FPE) system, the flagellar export apparatus (FEA), the Esx-1/Wss system, and prophage holins (Desvaux and Hébraud, 2006). So far, only components of the Sec and FEA systems were shown to be required for *Listeria* virulence, although it is believable that further characterization of the other systems will also reveal some degree of contribution to the infectious process.

### 2.1. Sec system

The Sec system is the classical and most important protein translocation system in prokaryotes, enabling the transport of N-terminal signal peptide-containing polypeptides (preproteins) across the cytoplasmic membrane, to be either associated with the cell surface or further released into the extracellular environment. This multimeric system has been thoroughly characterized in *E. coli* and *B. subtilis*, where it comprises a translocon complex of the integral membrane proteins SecYEG, which forms a protein-specific transport channel in the plasma membrane; the peripheral ATP-dependent motor protein SecA, which primes and drives the passage of unfolded substrates through the translocon; and a number of accessory components whose functions include recognition, folding and membrane integration of translocated proteins (Papanikou et al., 2007; du Plessis et al., 2011; Chatzi et al., 2013) (Figure 1).

The biological significance of this system is reflected by its striking conservation degree among bacterial species, including *Listeria* spp. (Desvaux and Hébraud, 2006). The requirement of Sec-mediated translocation in the composition of the cell wall and secretory proteomes of *L. monocytogenes* was mostly inferred from bioinformatic predictions coupled with proteomic analyses (Glaser et al., 2001; Calvo et al., 2005; Trost et al., 2005; Bierne and Cossart, 2007). Although informative, these studies did not directly address the operability of the system and the specific contribution of each component to the secretion process. Recently, Burg-Golani *et al.* tackled this issue by investigating the particular role of the listerial SecDF component, which in *E. coli* mediates the later steps of translocation by assisting the unfolded polypeptide to exit from the translocon channel (Tsukazaki et al., 2011). They identified this membrane protein as a chaperone essential for the secretion and optimal activity of LLO, PlcA, PlcB and ActA. Accordingly, deletion of SecDF induced defects in phagosomal evasion and cytosolic growth in macrophages, as well as reduced virulence in mice (Burg-Golani et al., 2013).

Unlike Gram-negative species, several Gram-positive bacteria express an additional copy of the SecA ATPase, called SecA2. This paralogous protein fulfills the same role as SecA but, in contrast, is not essential for bacterial viability and possesses a

156 much more limited set of substrates (Rigel and Braunstein, 2008; Bensing et al.,  
157 2013). Comparative secretomics implicated *L. monocytogenes* SecA2 in the export of  
158 a number of surface and secretory proteins that include known virulence factors, such  
159 as the autolysins p60 (or CwhA, previously Iap) and MurA (or NamA) (Lenz and  
160 Portnoy, 2002; Lenz et al., 2003) (see “Peptidoglycan turnover”), and the fibronectin-  
161 binding protein FbpA (Dramsı et al., 2004) (see “Unknown mechanism of  
162 association”). Stressing the importance of SecA2-driven secretion in *Listeria*  
163 pathogenesis, a  $\Delta secA2$  strain revealed impairment in intercellular spread and reduced  
164 virulence in the mouse model. This phenotype partly overlaps with that of a p60  
165 mutant, suggesting that abnormal secretion of this autolysin is a contributing factor to  
166 the  $\Delta secA2$  virulence impairment (Lenz et al., 2003). Secretion of OppA, an  
167 oligopeptide-binding lipoprotein necessary for optimal replication inside macrophages  
168 and in mice organs (Borezee et al., 2000) (see “Transport systems”), was shown to be  
169 also reduced in the absence of SecA2 (Lenz et al., 2003), although contradicted by  
170 more recent data (Renier et al., 2013). Interestingly, proteins normally found in the  
171 cytoplasm (lacking an N-terminal signal peptide) were also identified as SecA2  
172 substrates, namely, the manganese-dependent superoxide dismutase Sod, which  
173 provides bacterial resistance against host-generated toxic oxygen species  
174 (Archambaud et al., 2006); and LAP, an alcohol acetaldehyde dehydrogenase required  
175 for adhesion to enterocytes under anaerobic conditions (Burkholder et al., 2009).

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## 177 **2.2. Flagellar export apparatus**

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179 *L. monocytogenes* actively moves in its environment by expressing flagella uniformly  
180 around its surface. This motility is temperature-dependent, exhibiting a peak between  
181 20–25 °C that decreases steadily to a near complete absence of flagella-driven  
182 movement at 37 °C (Peel et al., 1988). The bacterial flagellum is a highly complex  
183 and conserved structure, well characterized in Gram-negative enteric species. It  
184 comprises five main components: (i) the basal body, (ii) the rotor/switch, (iii) the  
185 hook and hook/filament junctions, (iv) the filament with its cap, and (v) the flagellar  
186 export apparatus (FEA) (Macnab, 2003, 2004) (Figure 1). The FEA is homologous to  
187 a type III secretion system, and once assembled into the membrane core structure, it  
188 mediates the translocation of all the external components of the flagellum. The  
189 mechanisms through which substrates are recognized by and recruited to the FEA  
190 have not yet been elucidated (Macnab, 2003, 2004). The listerial FEA is predicted to  
191 transport twelve proteins that make up the hook, rod and filament structures. The  
192 majority of the FEA components characterized in Gram-negative species are encoded  
193 in a large flagella/chemotaxis-dedicated gene cluster in *L. monocytogenes* (Desvaux  
194 and Hébraud, 2006). Evidence of the contribution of the FEA system towards  
195 *L. monocytogenes* virulence was provided by a study on the role of FliI in flagellar  
196 biogenesis (Bigot et al., 2005). FliI is a cytosolic ATPase that, in a complex with  
197 FliH, binds and carries substrates to the entrance of the export channel, releasing them  
198 after ATP hydrolysis (Fan and Macnab, 1996; Minamino et al., 2011). Depletion of  
199 FliI in *L. monocytogenes* was shown to abolish flagellar assembly with concomitant  
200 loss of bacterial motility. The absence of flagella translated into a dramatic decrease  
201 of the levels of adhesion and internalization by epithelial and macrophage cells, albeit  
202 with no significant impact on bacterial proliferation inside cells or mouse organs  
203 (Bigot et al., 2005).

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### 3. Surface protein maturation: processing of precursor polypeptides

Surface proteins exported *via* the Sec system are synthesized as immature polypeptide precursors that undergo post-translational modifications necessary to reach their final location in the cell envelope, where they can fully exert their activity. Preprotein processing generally involves the post-translocational cleavage of signal sequences by specialized surface proteases: N-terminal secretion signal peptides are removed by signal peptidases, while sortases further cleave the C-terminal sorting domain of proteins targeted for covalent binding to the cell wall (Schneewind and Missiakas, 2013). Another type of preprotein modification occurs specifically in lipoproteins and consists in the addition of a lipid anchor to the N-terminal end of the lipoprotein precursor to enable its membrane anchoring (Figure 1). In *L. monocytogenes*, interference with these events results in drastic changes in the surface proteome with negative consequences for bacterial physiology and virulence.

#### 3.1. Signal peptidases

Before completing translocation *via* the Sec system, bacterial proteins are committed to one of two fates: (i) remain integrated within the plasma membrane or (ii) be released to either interact with cell envelope structures or diffuse to the extracellular milieu. The latter situation requires the enzymatic cleavage of the N-terminal signal peptide by a specific family of membrane-bound serine proteases, called signal peptidases (SPases) (Paetzel et al., 2000; Auclair et al., 2012). Depending on features present in their substrates, SPases are categorized into three classes: type I enzymes process canonical unmodified preproteins, while type II members act upon lipid-modified polypeptides (lipoproteins); finally, prepilins are processed by type III SPases (Paetzel et al., 2000).

In contrast with Gram-negative bacteria, which commonly express a single type I SPase, Gram-positive species often encode multiple members of this class (van Roosmalen et al., 2004). Bioinformatic analysis of the *L. monocytogenes* genome led to the identification of three neighboring genes encoding paralogous type I SPases (SipX, SipY, SipZ) (Bonnemain et al., 2004). Up-regulation of these genes was detected in bacteria isolated from cells infected for 30 min, suggesting a role for these SPases in the early stages of cellular infection (invasion and/or phagosomal escape) (Raynaud and Charbit, 2005). Characterization of strains lacking one or more of these SPases demonstrated that SipZ was essential and sufficient to promote normal levels of protein secretion, bacterial multiplication (in broth and inside cultured cells) and virulence. Particularly,  $\Delta sipZ$  mutants exhibited a significantly reduced secretion of LLO and PC-PLC and a consequent lower hemolytic activity. SipX was also shown to be important for proliferation in mice organs (Bonnemain et al., 2004).

Following membrane translocation, lipoproteins are specifically cleaved by type II SPases that remove the N-terminal signal peptide upstream of a conserved lipid-modified cysteine residue (Nakayama et al., 2012). In *L. monocytogenes*, Lsp, a lipoprotein-specific SPase, was identified and deletion of its encoding gene gave rise to bacteria deficient in lipoprotein processing, which ultimately impaired their capacity to escape the phagosomal compartment and significantly attenuated their virulence. Immature and alternatively processed forms of the lipoprotein LpeA were detected in  $\Delta lsp$  bacteria (Réglier-Poupet et al., 2003a). Considering that LpeA was

256 shown to promote *L. monocytogenes* entry into non-phagocytic cells, these  
257 presumably non-functional LpeA variants might partly contribute for the reduction in  
258 host cell invasion associated with this mutant strain (Réglier-Poupet et al., 2003b)  
259 (see “Lipobox motif”).

260

### 261 **3.2. Lipoprotein lipidation**

262

263 The Lsp-mediated processing of an N-terminal signal peptide-containing lipoprotein  
264 typically occurs after the newly synthesized polypeptide has been modified with a  
265 membrane-anchoring lipid. This step is catalyzed by an enzyme called Lgt (for  
266 lipoprotein diacylglycerol transferase), which catalyzes the covalent linkage of a  
267 phospholipid-derived diacylglycerol moiety to the sulfhydryl group of a cysteine  
268 residue located in a lipobox motif, at the end of the signal peptide (Kovacs-Simon et  
269 al., 2011). Whereas Lgt activity is crucial for the growth of Gram-negative bacteria,  
270 the same does not necessarily occur in certain Gram-positive genera, such as  
271 Firmicutes (Hutchings et al., 2009). Indeed, *L. monocytogenes* lipoproteins could still  
272 have their signal peptide removed by Lsp without previous Lgt-mediated lipidation,  
273 thus suggesting a less strict pathway for lipoprotein maturation (Baumgärtner et al.,  
274 2007). Regarding its role in *Listeria* virulence, Lgt was described as being specifically  
275 required for replication inside eukaryotic cells, probably due to its role in sensing  
276 and/or collecting nutrients from the host cell cytosol. To support this argument,  
277 comparative secretomics revealed over 20 different lipoproteins that were solely or  
278 increasingly secreted in the absence of Lgt, the large majority of which comprised  
279 putative ABC transporter components associated with intake of nutrients (see  
280 “Nutrient uptake”) and sensing systems (Baumgärtner et al., 2007).

281

### 282 **3.3. Sortases**

283

284 To become stably associated with the Gram-positive cell wall, surface protein  
285 precursors have to be processed by an enzyme called sortase A. This trans-  
286 cytoplasmic, membrane-bound transpeptidase recognizes polypeptides with a C-  
287 terminal sorting signal sequence containing a signature LPXTG motif (Schneewind et  
288 al., 1992). Sortase A then promotes cell wall anchoring of these proteins via a two-  
289 step mechanism: (i) the catalytic site cysteine breaks the peptide bond between the  
290 threonine and glycine residues of the LPXTG motif, forming an intermediate protein-  
291 sortase complex linked by a thioester bond; (ii) the amine group of a muropeptide  
292 (peptidoglycan precursor) attacks the thioester bond, releasing the cleaved protein  
293 from the sortase A active site, and forms a new amide bond with the carboxyl group  
294 of the new C-terminal threonine (Ton-That et al., 1999) (Figure 1). The ubiquity of  
295 LPXTG proteins and the conservation of the sorting motif were investigated and  
296 validated across a wide range of Gram-positive species (Navarre and Schneewind,  
297 1999; Pallen et al., 2001; Comfort and Clubb, 2004), highlighting the importance of  
298 this sortase A-mediated anchoring mechanism in Gram-positive physiology. The  
299 virulence of Gram-positive pathogens is also dependent of sortase activity, as many  
300 virulence factors are expressed as cell wall-anchored surface proteins (Navarre and  
301 Schneewind, 1999). Bioinformatics analysis of the sequenced *L. monocytogenes*  
302 genome predicted the existence of a large number of putative LPXTG protein-  
303 encoding genes (Cabanes et al., 2002). Soon after, the listerial homologue of  
304 sortase A was identified and shown to be of chief importance for the surface  
305 anchoring of internalin A (InlA), one of the two major invasion-promoting proteins,

306 and consequently for bacterial entry into eukaryotic cells and full virulence in mice  
307 (Bierne et al., 2002; Garandeau et al., 2002). In the following years, the role of  
308 sortase A in *L. monocytogenes* infection became more important as several novel  
309 virulence effectors were found to be associated with the bacterial surface through the  
310 activity of this enzyme (see “LPXTG and NXXTX sorting signals”).

311

312 A second sortase-encoding gene, *srtB*, was identified in *L. monocytogenes* in a locus  
313 far apart from *srtA*. It encodes a protein 23% identical with sortase A and with  
314 sortase-like motifs (Bierne et al., 2002, 2004). The existence of two or more sortase  
315 paralogues is not uncommon in Gram-positive bacteria (Pallen et al., 2001), and, in  
316 particular, sortase B orthologs are expressed in other species, such as *S. aureus* and  
317 *B. anthracis* (Zhang et al., 2004). Similarly to *S. aureus* (Mazmanian et al., 2001), the  
318 two listerial sortases do not display overlapping or redundant activities, indicating that  
319 they act upon different classes of substrates. Moreover, the substrate spectrum of  
320 sortase B is more limited than that of sortase A, with only two proteins identified as  
321 sortase B substrates (Bierne et al., 2004; Pucciarelli et al., 2005) (see “LPXTG and  
322 NXXTX sorting signals”). Sequence alignment of several known and putative  
323 sortase B substrates indicated that the enzyme recognizes an NXXTX consensus motif  
324 sequence. Whereas *L. monocytogenes*  $\Delta$ *srtA* mutants are significantly less virulent,  
325 the inactivation of *srtB* yields no effect, indicating that sortase B-processed proteins  
326 have no role in listerial virulence (Bierne et al., 2004).

327

#### 328 **4. Surface protein localization: anchoring domains**

329

330 Once surface proteins have been translocated, they are able to associate with  
331 components of the cell envelope via specific binding domains encoded in their  
332 sequence. For instance, sortase substrates contain a characteristic C-terminal sorting  
333 domain that allows their covalent attachment to the cell wall. On the other hand,  
334 proteins containing domains with tandem repeated sequences often display a more  
335 labile interaction with secondary cell wall components. In addition, proteins may also  
336 span the plasma membrane, provided that they contain adequately long stretches of  
337 hydrophobic residues to act as transmembrane regions (Figure 1). Notwithstanding,  
338 proteins lacking recognizable surface-binding sequences have been found associated  
339 with the bacterial surface through mechanisms that still require elucidation.

340

#### 341 **4.1. Cell wall association**

342

##### 343 **4.1.1. LPXTG and NXXTX sorting signals**

344

345 The precursors of proteins covalently anchored to the Gram-positive cell wall by a  
346 sortase A-dependent mechanism feature a C-terminal sorting signal of about 30–40  
347 residues comprising (i) an LPXTG pentapeptide motif, (ii) a hydrophobic domain and  
348 (iii) a short positively charged tail (Schneewind et al., 1992). Whereas the  
349 hydrophobic and charged domains of the sorting signal can display variability in their  
350 sequence and/or length, the LPXTG motif is very conserved (Fischetti et al., 1990;  
351 Schneewind et al., 1992). Studies with C-terminal truncates of staphylococcal  
352 protein A revealed that proper cell wall anchoring requires a complete sorting signal,  
353 and hinted that the hydrophobic and charged residues downstream of the LPXTG  
354 motif are responsible for retaining the polypeptide in the bacterial membrane until its  
355 recognition by sortase A (Schneewind et al., 1992, 1993). The LPXTG motif is

356 accommodated in the sortase A active site, where a catalytic cysteine initiates  
357 cleavage of the peptide bond between the threonine and the glycine residues. The  
358 cleaved protein becomes temporarily bound to the sortase (Ton-That et al., 1999),  
359 which seems to prevent its diffusion to the extracellular medium. The protein is then  
360 transferred to its final acceptor, lipid II (peptidoglycan precursor), which establishes a  
361 new bond between the amine group of a cross-bridge residue (*meso*-diaminopimelic  
362 acid in *L. monocytogenes*) and the C-terminal threonine carboxyl group of the surface  
363 protein (Ton-That et al., 1997). Proteins with LPXTG motifs are found in a  
364 multiplicity of Gram-positive organisms (Navarre and Schneewind, 1999; Mazmanian  
365 et al., 2001; Hendrickx et al., 2009; Pérez-Dorado et al., 2012). *L. monocytogenes*  
366 stands out as the species with the largest number, encoding 41 proteins (over 1% of its  
367 genome) (Glaser et al., 2001; Cabanes et al., 2002), seven of which are currently  
368 described as virulence factors. InlA, important for entry into epithelial cells and  
369 virulence in mice (Gaillard et al., 1991; Lingnau et al., 1995), was the first to be  
370 identified, long before the *L. monocytogenes* genome was sequenced. The list  
371 comprises four other internalin family members (Bierne and Cossart, 2007) – InlF  
372 (Kirchner and Higgins, 2008), InlH (Pucciarelli et al., 2005; Personnic et al., 2010),  
373 InlJ (Sabet et al., 2005, 2008) and InlK (Dortet et al., 2011) – with roles in host cell  
374 adhesion and immune evasion, and two non-internalins, Vip (Cabanes et al., 2005)  
375 and LapB (Reis et al., 2010), important for entry into cells.

376

377 A subset of covalently attached cell wall proteins feature a different sorting motif,  
378 characterized by an NXXTX consensus sequence that targets surface protein  
379 precursors for sortase B processing (Comfort and Clubb, 2004; Mariscotti et al.,  
380 2009). Sortase B enzymes have few substrates, which are usually encoded by genes  
381 arranged in an operon together with *srtB* (Marraffini et al., 2006). Interestingly, they  
382 are involved in heme-iron scavenging and uptake (Mazmanian et al., 2002; Maresso  
383 and Schneewind, 2006; Xiao et al., 2011; Klebba et al., 2012), indicating that the  
384 sortase B anchoring mechanism may have evolved differently from sortase A to  
385 become more specialized in the anchoring of proteins required for iron homeostasis.  
386 *L. monocytogenes* encodes only two proteins with NXXTX motifs (Bierne et al.,  
387 2004), both of which require sortase B for cell wall anchoring (Pucciarelli et al.,  
388 2005). One of them, SvpA, is a surface-associated protein required for iron uptake  
389 and bacterial persistence in mouse organs (Newton et al., 2005). The other listerial  
390 sortase B substrate, Lmo2186, possesses two putative sorting motifs, NKVTN and  
391 NPKSS (underlined residue is common to both), but only the latter is necessary for  
392 surface anchoring (Mariscotti et al., 2009). SvpA was first characterized as a  
393 virulence factor, as its depletion resulted in deficient escape from macrophage  
394 phagosomes (Borezée et al., 2001). However, more recent data indicate that neither  
395 SvpA nor Lmo2186 are used by *L. monocytogenes* to promote infection (Newton et  
396 al., 2005), agreeing with results demonstrating that sortase B is dispensable for  
397 virulence (Bierne et al., 2004).

398

#### 399 **4.1.2. GW module**

400

401 Many surface proteins interact non-covalently with the cell wall through a domain  
402 containing a variable number of tandemly arranged sequences, called GW modules,  
403 whose name derives from the presence of a conserved glycine (G)-tryptophan (W)  
404 dipeptide. This cell wall association motif was first discovered in *L. monocytogenes*  
405 InlB (Braun et al., 1997), an internalin-like protein that promotes entry into

406 hepatocytes, epithelial and endothelial cells (Braun et al., 1998; Parida et al., 1998).  
407 The C-terminal cell wall association domain (CWA) of InlB contains three GW  
408 modules, which are required and sufficient to confer cell wall-binding properties  
409 (Braun et al., 1997). InlB variants lacking the CWA are unable to associate to the  
410 surface of non-invasive *Listeria* and promote their entry into eukaryotic cells (Braun  
411 et al., 1998). Structural analysis of the GW module revealed an interesting  
412 resemblance with SH3 domains, known to be involved in protein-protein interaction  
413 in signal transduction pathways, but steric hindrance discarded a functional SH3-like  
414 activity for GW modules (Marino et al., 2002). Lipoteichoic acids (LTAs) were  
415 identified as the “surface anchor” of InlB, binding to its CWA. The interaction with  
416 these cell envelope glycopolymers is highly specific, as LTAs from *L. innocua* or *S.*  
417 *pneumoniae* are not able to capture InlB (Jonquières et al., 1999). The CWA of InlB  
418 also enables its association with glycosaminoglycans present at the surface of host  
419 cells and with the receptor of the complement C1q globular part (gC1q-R),  
420 significantly potentiating InlB-mediated invasion (Braun et al., 2000; Jonquières et  
421 al., 2001; Banerjee et al., 2004; Asano et al., 2012). The binding strength of proteins  
422 containing GW modules is proportional to the number of modules. This is illustrated  
423 by comparing the surface association levels of InlB and Ami, another GW protein  
424 with autolytic activity and an important role in bacterial adhesion to host cells  
425 (Milohanic et al., 2000, 2001; Asano et al., 2012). Containing eight GW modules,  
426 Ami is found exclusively in association with the bacterial surface, whereas InlB (only  
427 three modules) is detected in the cell envelope and secreted fractions (Braun et al.,  
428 1997). *L. monocytogenes* encodes seven other GW proteins, all of which have a  
429 predicted amidase domain in common with Ami (Cabanes et al., 2002), hinting that  
430 they also may possess autolytic functions. Indeed, one of them, Auto, was described  
431 to behave also as an autolysin (Cabanes et al., 2004) (see “Peptidoglycan turnover”).  
432 Staphylococcal autolysins are also associated to the bacterial surface via structural  
433 motifs resembling the listerial GW modules (Oshida et al., 1995; Heilmann et al.,  
434 1997; Hell et al., 1998; Allignet et al., 2001), strongly suggesting that this cell wall  
435 association protein motif has evolved with the specific purpose of mediating the  
436 reversible surface binding of proteins with autolytic activity (Milohanic et al., 2001).

437

### 438 **4.1.3. LysM domain**

439

440 Lysin motif (LysM) domains are encountered in proteins from a broad variety of  
441 organisms, such as plants, fungi, bacteria and viruses (Buist et al., 2008). Initially  
442 found in bacterial and phage lysins, from which the motif took its name (Birkeland,  
443 1994), the LysM domain is characterized by a variable number of roughly 40–80-  
444 residue repeats, spaced by stretches rich in serine, threonine and asparagine (Buist et  
445 al., 1995). The consistent presence of this domain in proteins expressing cell wall-  
446 degrading activity suggested that LysM repeats are important for retention of these  
447 enzymes within the peptidoglycan (Joris et al., 1992; Birkeland, 1994). This  
448 hypothesis was validated through binding studies using the LysM domains of  
449 *Lactococcus lactis* and *Enterococcus faecalis* autolysins (Steen et al., 2003; Eckert et  
450 al., 2006). Further studies singled out *N*-acetylglucosamine (GlcNAc) as the  
451 peptidoglycan moiety bound by LysM (Buist et al., 2008). However, instead of an  
452 expected uniform surface distribution, many LysM-containing proteins appear  
453 localized to specific sites by the excluding action of cell wall components, such as  
454 LTAs (Steen et al., 2003), or modifications, such as *O*-acetylation (Veiga et al., 2007)  
455 (see “Modification of cell envelope components”). LysM domains are found in six

456 *L. monocytogenes* proteins (Bierne and Cossart, 2007), two of which, p60 and MurA,  
457 have been characterized as autolysins with a relevant role in infection (Lenz et al.,  
458 2003) (see “Peptidoglycan turnover”). The p60 sequence contains a C-terminal  
459 NlpC/p60 domain putatively responsible for the peptidoglycan peptidase activity  
460 (Anantharaman and Aravind, 2003; Layec et al., 2008), and an N-terminal region with  
461 two LysMs separated by an SH3-like domain (Bierne and Cossart, 2007), which  
462 presumably mediate protein binding to peptidoglycan. Unlike p60, MurA contains  
463 four C-terminal LysM repeats (Carroll et al., 2003), which may be important to  
464 position the catalytic site of this autolysin in a manner distinct of p60, so as to  
465 optimize its activity. A third LysM protein of *L. monocytogenes* (Lmo2522) was  
466 recently characterized as one of two novel listerial resuscitation-promoting factors,  
467 i.e. muralytic enzymes important for jump-starting the growth in dormant bacteria  
468 (Pinto et al., 2013).

469

## 470 **4.2. Membrane association**

471

### 472 **4.2.1. Lipobox motif**

473

474 Bacterial lipoproteins contribute to important physiological roles, such as substrate  
475 binding and transport, antibiotic resistance, signaling and folding of secreted proteins  
476 (Sutcliffe and Russell, 1995; Hutchings et al., 2009), and were also shown to take an  
477 active part in virulence-associated processes, such as adhesion, invasion, and  
478 immunomodulation (Kovacs-Simon et al., 2011; Nakayama et al., 2012). As  
479 described above, lipoproteins are expressed as immature polypeptides, which are  
480 converted to prolipoproteins by the addition of a lipid moiety at a specific motif in the  
481 distal portion of the N-terminal signal peptide. This motif, called lipobox, is  
482 characterized by a four-residue sequence containing a conserved cysteine (Sutcliffe  
483 and Harrington, 2002; Babu et al., 2006). The sulfhydryl group of the cysteine  
484 establishes a thioester bond with phospholipid-derived diacylglycerol in a reaction  
485 catalyzed by Lgt (Kovacs-Simon et al., 2011). The N-terminal lipid group inserts into  
486 the outer leaflet of the lipophilic plasma membrane, thus enabling the retention of the  
487 protein at the cell surface once the signal peptide is cleaved. In *L. monocytogenes*, the  
488 biological importance of lipoproteins is emphasized by their preponderance in the  
489 surface proteome: 68 of 133 surface proteins were predicted to be lipoproteins, based  
490 on the presence of an N-terminal lipobox (Glaser et al., 2001), and 26 were later  
491 confirmed experimentally (Baumgärtner et al., 2007). Interestingly, nearly half of the  
492 listerial lipoproteins are presumed to act as substrate-binding components of ABC  
493 transporter systems (Bierne and Cossart, 2007), performing the equivalent functions  
494 of periplasmic solute-binding proteins in Gram-negative bacteria (Tam and Saier,  
495 1993). Such is the case of the above-mentioned lipoproteins OppA, which participates  
496 in the oligopeptide uptake, and LpeA, which belongs to the LraI family of  
497 manganese-importing ABC transporter components (Novak et al., 1998), although  
498 supporting evidence for this function in *L. monocytogenes* have yet to be obtained.  
499 Another substrate-carrying lipoprotein, OpuC, operates in the transport of L-carnitine,  
500 important for bacterial osmotolerance and, without which, *L. monocytogenes* is unable  
501 to efficiently persist in mice organs (Sleator et al., 2001) (see “Osmolarity and bile  
502 tolerance”). Fifteen other lipid-anchored proteins were predicted to have enzymatic  
503 activities (Bierne and Cossart, 2007). Among them, the best studied and with a  
504 significant contribution to infection is the surface chaperone PrsA2 (see  
505 “Chaperones”).

506

#### 507 **4.2.2. Hydrophobic tail**

508

509 Surface proteins can also be associated to the bacterial membrane through an N- or C-  
510 terminal tail comprised of hydrophobic residues that spans and stably inserts the  
511 protein in the lipid bilayer, during translocation. The orientation of the proteins in the  
512 membrane is pre-determined by the presence and localization of positively charged  
513 residues relative to the membrane-spanning domain (stop-transfer signals) (Dalbey et  
514 al., 2011). From the ten predicted *L. monocytogenes* surface proteins with a putative  
515 C-terminal hydrophobic tail (Bierne and Cossart, 2007), only ActA has been  
516 biochemical and functionally characterized (Kocks et al., 1992; Domann et al., 1992).  
517 This key virulence factor promotes intracellular motility and intercellular spread by  
518 triggering the polymerization of host cell actin into a comet-like tail of actin filaments  
519 that propels the bacterium through the cytoplasm, towards neighboring cells (Kocks et  
520 al., 1995; Monack and Theriot, 2001). ActA was also shown to enable  
521 *L. monocytogenes* to escape autophagy (Yoshikawa et al., 2009) and to play a key role  
522 in persistence within the host and transmission from the host back to the environment  
523 (Travier et al., 2013). A large number of listerial enzymes linked with cell wall  
524 metabolism and surface protein processing – such as sortases (Mazmanian et al.,  
525 2000), signal peptidases (Paetzel et al., 2000) and penicillin-binding proteins (PBPs,  
526 see “Peptidoglycan assembly”) – are anchored to the bacterial membrane by an N-  
527 terminal hydrophobic tail (Bierne and Cossart, 2007), which in many cases  
528 corresponds to a signal peptide sequence lacking a type I cleavage site.

529

#### 530 **4.3. Unknown mechanism of association**

531

532 Several proteins secreted by *L. monocytogenes* lack recognizable surface-targeting  
533 sequences and a number of them are associated with the cell envelope, despite having  
534 no predicted surface-binding domains (Schaumburg et al., 2004; Trost et al., 2005).  
535 Consistent and, in some cases, significant secretion of the same proteins in different  
536 studies seems to discard, or at least minimize, the contribution of bacterial cell lysis to  
537 their extracytoplasmic localization. In turn, it suggests that they use a non-classical  
538 type of secretion mechanism (Schaumburg et al., 2004). So far, the only example of  
539 an unconventionally secreted and surface-associated protein with a described  
540 virulence-promoting function in *L. monocytogenes* is FbpA. Like many streptococcal  
541 fibronectin-binding proteins, FbpA lacks all the classical cell surface sorting and  
542 anchoring sequences, yet it was detected in the bacterial plasma membrane after  
543 subcellular fractionation. It was shown to facilitate *in vitro* adhesion to hepatocytes  
544 and to support liver infection in mice (Dramsı et al., 2004). As mentioned before (see  
545 “Sec system”), FbpA secretion is driven by SecA2, which promotes the export of  
546 other signal peptide-lacking substrates, many normally resident in the cytoplasm  
547 (Lenz et al., 2003).

548

#### 549 **5. Surface protein quality control**

550

551 The accumulation of non-natively configured or damaged proteins composes a threat  
552 to cell viability as it may lead to the formation of toxic aggregates. To prevent this,  
553 bacteria have evolved mechanisms of protein quality control, which rely on the  
554 coordinated activity of molecular chaperones and proteases to repair misfolded

555 substrates, degrade irreparably damaged molecules and activate stress response  
556 pathways (Wickner et al., 1999).

557

## 558 **5.1. Chaperones**

559

560 Sec-mediated export requires that proteins are kept in an unfolded configuration to  
561 pass through the translocase channel (Desvaux and Hébraud, 2006). Immediately after  
562 transposing the membrane, proteins must properly fold into their native conformation  
563 to acquire their activity and become less susceptible to proteolytic attacks, which  
564 occur at a high frequency in the extracytoplasmic environment (Sarvas et al., 2004). A  
565 specific group of ATP-dependent chaperones, called foldases, assist in the correct  
566 post-translocational folding of secreted proteins. The number and variety of these  
567 enzymes is another distinguishing element between Gram-negative and Gram-positive  
568 bacteria. Whereas the former are known to express multiple foldases with different  
569 selectivities (Merdanovic et al., 2011), the latter species are not as well supplied  
570 (Sarvas et al., 2004). PrsA, a ubiquitous Gram-positive lipoprotein with peptidyl-  
571 prolyl *cis-trans* isomerase activity (Drouault et al., 2002), was originally identified in  
572 *B. subtilis*, where mutations in its encoding gene were responsible for deficient  
573 secretion of exoproteins [Kontinen, 1991]. Critical for *B. subtilis* viability, PrsA was  
574 shown to have also chaperone activity, promoting optimal secretion levels without  
575 influencing protein translocation, and prevented the degradation of exported proteins  
576 (Vitikainen et al., 2001). *L. monocytogenes* encodes two PrsA homologues, PrsA1  
577 and PrsA2, neither of which are essential for bacterial survival. Despite highly similar  
578 amino acid sequences, only PrsA2 displayed chaperone activity comparable to the  
579 PrsA of *B. subtilis* (Alonzo et al., 2009; Alonzo and Freitag, 2010) (Figure 1). Up-  
580 regulation of *prsA2* was detected in *L. monocytogenes* isolated from infected  
581 macrophages (Chatterjee et al., 2006), suggesting a role for PrsA2 in the adaptation to  
582 the host intracellular environment. This increased expression is not directly controlled  
583 by PrfA, the major virulence gene regulator (Zemansky et al., 2009) (see  
584 “Transcriptional regulation”), in spite of the presence of a putative PrfA-binding  
585 sequence upstream of *prsA2* (Glaser et al., 2001) and data showing increased PrsA2  
586 secretion in strains expressing hyperactive forms of PrfA (Port and Freitag, 2007).  
587 Further studies confirmed the involvement of PrsA2 in intracellular replication, cell-  
588 to-cell spread and virulence in a mouse model (Chatterjee et al., 2006; Alonzo et al.,  
589 2009). In particular, PrsA2 is determinant for the proper secretion and activity of  
590 major virulence factors, such as listeriolysin O (LLO), metalloprotease (Mpl) and  
591 phosphatidylcholine-specific phospholipase C (PC-PLC) (Alonzo et al., 2009;  
592 Zemansky et al., 2009; Forster et al., 2011).

593

## 594 **5.2. Proteases**

595

596 The high temperature requirement A (HtrA) family of proteases is one of the most  
597 conserved in all living organisms and the most predominant group of bacterial  
598 extracytoplasmic proteases (Figure 1). Some HtrA proteases, like the archetypal  
599 *E. coli* DegP (Krojer et al., 2008), also exhibit ATP-independent chaperone activity  
600 (Clausen et al., 2002), and this dual role is important for other biological processes,  
601 such as activation of stress responses and virulence (Clausen et al., 2011). Indeed,  
602 HtrA was shown to promote host cell invasion and survival inside macrophage  
603 phagosomes in a number of bacterial pathogens, such as *Helicobacter pylori*, *Yersinia*  
604 *enterocolitica* and *Salmonella enterica* serovar Typhimurium (Ingmer and Brøndsted,

605 2009; Hoy et al., 2010). A similar intracellular phenotype was also observed in HtrA-  
606 depleted *L. monocytogenes* mutants (Stack et al., 2005; Wilson et al., 2006),  
607 indicating that a functional HtrA is crucial for bacterial survival in the stress-inducing  
608 environment of the macrophage phagosome, possibly by promoting the degradation  
609 and preventing the accumulation of stress-damaged proteins. HtrA was also shown to  
610 be required for efficient colonization of mouse organs (Stack et al., 2005; Wilson et  
611 al., 2006). Interestingly, like PrsA2, up-regulation of *htrA* was detected in  
612 intracellular bacteria (Chatterjee et al., 2006) and higher amounts of the protein were  
613 secreted by a *L. monocytogenes* strain expressing a constitutively active variant of  
614 PrfA (Port and Freitag, 2007).

615

## 616 **6. Cell envelope metabolism**

617

618 The cell envelope is a fundamental and defining structure of prokaryotes. In Gram-  
619 positive bacteria, it comprises the plasma membrane and a cell wall, which provides  
620 physical and morphological support and protection against external aggression. Its  
621 mesh-like constituent, the peptidoglycan, also acts as a biological scaffold for the  
622 surface positioning of proteins and other glycopolymers with relevant physiological  
623 roles. The remodeling of the cell wall is vital for bacterial growth and division, and  
624 requires a dynamic balance between peptidoglycan biogenesis, assembly and  
625 turnover. Coordination between these processes is mandatory to prevent  
626 morphological malformations and concomitant functional defects, such as the  
627 mislocalization of surface molecules.

628

629 From an immunological perspective, the cell wall is a particularly relevant structure.  
630 Cell wall turnover events can generate fragments that are specifically recognized by  
631 and activate the host innate immune system. In turn, innate immunity effectors, such  
632 as lysozyme and cationic peptides, target the cell wall to promote bacterial death by  
633 lysis. The introduction of specific modifications in components of the cell envelope is  
634 a strategy developed by bacteria to render them undetectable to both immune  
635 recognition and to the bacteriolytic activity of host defense enzymes and peptides  
636 (Davis and Weiser, 2011).

637

### 638 **6.1. Peptidoglycan assembly**

639

640 Peptidoglycan is assembled outside of the bacterial cell through the polymerization  
641 and bridging of subunits generated on the cytoplasmic side of the membrane.  
642 Following translocation, these building blocks are transferred and integrated into  
643 existing peptidoglycan chains by the action of a multifunctional family of surface  
644 proteins called penicillin-binding proteins (PBPs) (Figure 2). These membrane-  
645 anchored proteins are categorized into high molecular weight (HMW) PBPs – the  
646 major intervenients in peptidoglycan assembly – and low molecular weight (LMW)  
647 PBPs, both of which are characterized by the presence of an archetypal DD-peptidase  
648 domain (Macheboeuf et al., 2006). In HMW PBPs, the peptidase domain is located at  
649 the C-terminus and catalyzes the crosslinking of adjacent glycan strands through their  
650 subunit stem peptides (transpeptidation). Additionally, they may contain an N-  
651 terminal domain that displays transglycosylase activity, necessary for the elongation  
652 of *N*-acetylglucosamine (GlcNAc)-*N*-acetylmuramic acid (MurNAc) glycan strands.  
653 LWM PBPs perform roles linked to peptidoglycan maturation and recycling  
654 (Macheboeuf et al., 2006; Sauvage et al., 2008). The peptidase domain cleaves the D-

655 Ala-D-Ala bond of a peptidoglycan subunit stem peptide, releasing the terminal  
656 alanine residue and forming a new bond between the remaining alanine and the  
657 diamino acid residue from a stem peptide in a different strand. Penicillin and other  $\beta$ -  
658 lactam compounds take advantage of their structural similarity with the D-Ala-D-Ala  
659 dipeptide to bind irreversibly to and inhibit most PBPs, thus promoting bacterial death  
660 by perturbing cell wall synthesis (Tipper and Strominger, 1965; Ghuysen, 1994). *In*  
661 *silico* studies have allowed the identification of ten PBP-like protein-encoding genes  
662 in *L. monocytogenes* (Guinane et al., 2006; Korsak et al., 2010) and  $\beta$ -lactam-binding  
663 assays confirmed that nine of them expressed functional PBPs (Korsak et al., 2010).  
664 They comprise five HMW proteins – class A members PBPA1 and PBPA2 (former  
665 PBP1 and PBP4) and class B members PBPB1, PBPB2 (former PBP3 and PBP2) and  
666 PBPB3 – and four LMW PBPs, including the carboxypeptidase PBPD1 (former  
667 PBP5) and two  $\beta$ -lactamases (Korsak et al., 2010). Studies on listerial PBPs have  
668 largely focused on their biochemical characterization, namely through the  
669 determination of their affinity to several  $\beta$ -lactam derivatives (Gutkind et al., 1990;  
670 Pierre et al., 1990; Vicente et al., 1990; Guinane et al., 2006; Zawadzka-Skomial et  
671 al., 2006). In some cases, mutational approaches allowed the elucidation of the roles  
672 of some PBPs towards *L. monocytogenes* virulence. For instance, PBPB1, PBPD1,  
673 but mostly PBPA2 and PBPC1, were found to be important for the colonization of the  
674 mouse spleen (Guinane et al., 2006). The depletion of these PBPs resulted in variable  
675 degrees of morphological defects (Guinane et al., 2006; Korsak et al., 2010), and the  
676 pleiotropic effects elicited by such modifications are likely to be responsible for the  
677 attenuated virulence.

678

## 679 **6.2. Peptidoglycan turnover**

680

681 The reshaping of the bacterial cell wall is vital for many physiological processes,  
682 particularly cell growth and division, and thus depends on a dynamic equilibrium  
683 between the degradation and recycling of cell wall components (Popowska, 2004;  
684 Vollmer et al., 2008). Peptidoglycan turnover relies on the activity of another family  
685 of surface-associated enzymes, called autolysins, which catalyze the hydrolysis of  
686 every existing covalent bond in the mature peptidoglycan matrix. The nature and  
687 location of the bond(s) cleaved by an autolysin is determined by its functional  
688 specificity within the broader family of peptidoglycan hydrolases (Vollmer et al.,  
689 2008). *N*-acetylglucosaminidases (NAGases) and *N*-acetylmuramidases (NAMases)  
690 cleave the glycosidic bond between glycan chain residues GlcNAc and MurNAc,  
691 respectively after GlcNAc and MurNAc; *N*-acetylmuramyl-L-alanine amidases (or  
692 simply amidases) separate the stem peptide from the sugar strand by breaking the  
693 bond between MurNAc and L-alanine; finally, endo- and carboxypeptidases hydrolyse  
694 the amide bonds within and between stem peptides (Vollmer et al., 2008) (Figure 2).  
695 The existence of multiple autolysins sharing the same activity and substrate attests for  
696 the functional redundancy associated with peptidoglycan hydrolases, which has  
697 complicated the characterization of the role of individual cell wall-degrading  
698 enzymes.

699

700 The genome of *L. monocytogenes* EGD-e is predicted to encode six NAGases, four  
701 NAMases, four amidases, and a multiplicity of peptidoglycan peptidases, but only a  
702 few have been experimentally validated (Popowska, 2004; Bierne and Cossart, 2007;  
703 Pinto et al., 2013). The only predicted NAGases with confirmed peptidoglycan  
704 hydrolase activity are MurA and Auto, although their specific NAGase activity

705 remains to be verified (Carroll et al., 2003; Cabanes et al., 2004). MurA is necessary  
706 for proper cell separation during growth and its absence or malfunction results in  
707 virulence defects, namely in adhesion to host cells (Lenz et al., 2003; Alonzo et al.,  
708 2011). Auto is important for entry into non-phagocytic cells and virulence in mice and  
709 guinea pigs (Cabanes et al., 2004). The contribution of both autolysins towards  
710 *Listeria* virulence occurs possibly through different mechanisms. This is suggested by  
711 their distinct CWA domains – MurA contains LysM repeats, Auto has GW modules –  
712 which hint at a differential cell wall localization, and their relative importance for cell  
713 wall remodeling, as *murA* mutants cannot separate well and grow in filaments, while  
714 *aut* mutants maintain a normal morphology (Carroll et al., 2003; Cabanes et al.,  
715 2004). Two putative *L. monocytogenes* amidases contain C-terminal GW module  
716 repeats, suggesting similar surface association requirements, and among them is the  
717 virulence-promoting adhesin Ami (see “GW module”). Although none of the  
718 NAMases have been deeply characterized in a virulence-oriented perspective, two  
719 were recently shown to possess lysozyme-like activity in the presence of cell wall  
720 substrate and to be required for stimulating the replication of quiescent bacteria,  
721 possibly through their impact in cell wall reshaping and thus in cell growth and  
722 division (Pinto et al., 2013). On the other hand, IspC, a putative NAMase-like protein  
723 with a highly significant contribution to *Listeria* infection, was identified in a serotype  
724 4b strain (Wang and Lin, 2007, 2008). Interestingly, IspC mutants were not affected  
725 in their growth *in vitro* and cell morphology, but showed cell type-dependent defects  
726 in nearly every step of the cellular infection cycle (Wang and Lin, 2008).

727  
728 Common to many peptidoglycan hydrolases is the presence of an NlpC/p60 domain,  
729 related to the CHAP (cysteine, histidine-dependent amidohydrolase/peptidase)  
730 superfamily. Interestingly, most NlpC/p60 proteins are found in the genus *Bacillus*  
731 and *Listeria*, but not in *Staphylococcaceae*, which express proteins with another  
732 CHAP-type domain (Bateman and Rawlings, 2003; Layec et al., 2008). This is most  
733 likely a reflection of the affinity of the NlpC/p60 domain for the  $\gamma$ -D-glutamyl-*meso*-  
734 diaminopimelic acid bond (Rigden et al., 2003), which is replaced by a  $\gamma$ -D-glutamyl-  
735 L-lysine linkage in staphylococci. Four *L. monocytogenes* proteins contain putative  
736 NlpC/p60 domains and were predicted to possess cell wall hydrolase activity (Bierne  
737 and Cossart, 2007). Two of them, p45 (or Spl) and p60, have been studied and their  
738 function validated. Spontaneous mutants that secreted lower amounts of this protein,  
739 also known as CwhA (cell wall hydrolase A), showed a filamentous morphology and  
740 reduced host cell invasion efficiency, suggesting that p60 was required for entry into  
741 non-phagocytic cells (hence its first name, Iap, for invasion-associated protein).  
742 Indeed, exogenously added p60 not only restored the invasiveness potential (Kuhn  
743 and Goebel, 1989), but also disrupted the bacterial chains into individual cells, due to  
744 its cell wall-degrading activity (Wuenscher et al., 1993). Lack of functional p60  
745 results in septum abnormalities that disrupt actin-based intracellular motility,  
746 impairing optimal cell-to-cell spread and, overall, virulence (Pilgrim et al., 2003; Hess  
747 et al., 1996; Faith et al., 2007). The immunomodulatory properties of the p60 have  
748 been previously addressed (Pamer, 1994; Geginat et al., 1999; Humann et al., 2007;  
749 Sashinami et al., 2010) and a recent study implicated specifically the N-terminal  
750 region in NK cell activation upon bacterial infection (Schmidt et al., 2011).

751

### 752 **6.3. Modification of cell envelope components**

753

#### 754 **6.3.1. Peptidoglycan: acetylation and deacetylation**

755  
756 Similar to autolysins, host-secreted hydrolases – such as lysozyme – bind to the  
757 bacterial cell wall and degrade the peptidoglycan. For this reason, they constitute one  
758 of the first and most important players of the host innate immune response against  
759 bacterial invaders. Because of their highly exposed peptidoglycan, Gram-positive  
760 bacteria are particularly susceptible, so they developed mechanisms to interfere with  
761 the activity of exogenous murolytic enzymes. In particular, the assembled  
762 peptidoglycan is modified by the addition of small molecules or large polymeric  
763 structures (Vollmer, 2008) (Figure 3). These changes prevent bacterial lysis and  
764 modulate the release of peptidoglycan fragments that can be recognized by specific  
765 host receptors and activate the innate immune response. For instance, the addition of  
766 *O*-linked (or removal of *N*-linked) acetyl groups to the peptidoglycan residues  
767 GlcNAc and MurNAc was shown to confer resistance to lysozyme and reduce the  
768 activation of the host immune response (Davis and Weiser, 2011). The deacetylation  
769 of GlcNAc and/or MurNAc is catalyzed by a deacetylase present in species  
770 containing *N*-deacetylated peptidoglycan (Vollmer, 2008). A significant proportion of  
771 the GlcNAc residues in the *L. monocytogenes* peptidoglycan was shown to be  
772 deacetylated, in a process exclusively dependent on the expression of PgdA (Boneca  
773 et al., 2007). In the absence of PgdA, *L. monocytogenes* is highly vulnerable to  
774 peptidoglycan hydrolases and cell wall-targeting antibiotics, dying rapidly inside  
775 macrophages and exhibiting attenuated virulence in the mouse model. Importantly,  
776 muropeptides derived from *N*-deacetylated peptidoglycan were less  
777 immunostimulatory than fully acetylated peptidoglycan fragments (Boneca et al.,  
778 2007; Popowska et al., 2009).

779  
780 Compared to *N*-deacetylation, the mechanism of peptidoglycan *O*-acetylation has a  
781 more stringent specificity (only affects MurNAc residues), but it is a more  
782 predominant event, having been detected in numerous Gram-negative and Gram-  
783 positive species (Vollmer, 2008). This modification is enzymatically mediated by an  
784 integral membrane protein called *O*-acetyltransferase, which exports acetyl-  
785 containing substrates from the cytoplasm and transfers the acetyl group to the  
786 MurNAc residues in the assembled peptidoglycan strands (Clarke et al., 2002). First  
787 discovered in *S. aureus* (Bera et al., 2005), the gene coding for such an enzyme, *oatA*,  
788 was also identified in *L. monocytogenes* (Aubry et al., 2011). Analysis of mutants  
789 revealed that the activity of the listerial OatA largely overlapped with that of PgdA, as  
790 it is required for resistance to lysozyme and other antimicrobial compounds, survival  
791 within macrophages and virulence in mice (Aubry et al., 2011; Rae et al., 2011).  
792 However, while OatA-deficient strains induced the secretion of pro-inflammatory  
793 cytokines in mouse livers, particularly IL-6, PgdA mutants failed to stimulate IL-6 *in*  
794 *vivo* (Aubry et al., 2011), although they did so in macrophage cell lines (Boneca et al.,  
795 2007). The non-overlapping immunomodulatory activities of OatA and PgdA provide  
796 *L. monocytogenes* with a higher versatility in the control of the host immune response.

### 797 798 **6.3.2. Secondary glycopolymers: LTA D-alanylation and WTA glycosylation**

799  
800 In addition to a myriad of surface proteins, the peptidoglycan of Gram-positive  
801 bacteria is densely decorated with a family of secondary glycopolymers called  
802 teichoic acids. These molecules generally consist of a polymeric backbone of  
803 phosphodiester-linked polyol repeats that is covalently bound either to the  
804 peptidoglycan matrix (wall teichoic acids, WTAs) or to the plasma membrane

805 (lipoteichoic acids, LTAs), via a linkage unit connected to MurNAc residues or  
806 phospholipids, respectively. The abundance of phosphate groups confers strong  
807 anionic properties to teichoic acids, which increase the net negative charge of the  
808 bacterial surface (Neuhaus and Baddiley, 2003). The size of the polyol subunit and  
809 the presence and nature of substituent groups vary between and even within species,  
810 to the point of being used as serotype markers. While LTAs have conserved  
811 polyglycerol-phosphate backbones (Reichmann and Gründling, 2011), WTA  
812 monomers are chemically more diverse, the most common including glycerol- or  
813 ribitol-phosphate (Brown et al., 2013).

814

815 WTA/LTA subunits can be typically substituted with sugars or esterified with D-  
816 alanine, as a result of the action of specific glycosyltransferases or of the products of  
817 the *dltACBD* operon (Neuhaus and Baddiley, 2003). This operon encodes a  
818 multicomponent complex of cytosolic and membrane-bound proteins that transport D-  
819 alanine residues from the cytoplasm and incorporate them into extracellularly located  
820 teichoic acid polymers (Reichmann et al., 2013) (Figure 3). Given that D-alanine is  
821 positively charged at physiological pH, the addition of this molecule to teichoic acids  
822 represents a mechanism used by bacteria to fine-tune their surface charge in response  
823 to adverse environmental conditions. This process is particularly important for the  
824 protection against the cationic antimicrobial peptides (CAMPs). Similarly to what was  
825 observed in *S. aureus* (Collins et al., 2002), failure to perform D-alanylation of WTAs  
826 due to genetic inactivation of the *dltACBD* operon results in a *L. monocytogenes*  
827 strain highly susceptible to several CAMPs and with significantly decreased virulence  
828 in the mouse model (Abachin et al., 2002). In addition, the mutant bacteria showed  
829 lower levels of adhesion, suggesting that the lack of D-alanylated WTAs, and likely  
830 the increased surface electronegativity, hinders bacterial attachment to host cells.

831

832 The similarities between WTAs and LTAs bring about a functional redundancy (Oku  
833 et al., 2009) that has complicated the understanding of the contribution of tailoring  
834 modifications to various aspects of bacterial physiology. The striking structural and  
835 biochemical diversity within WTAs, resulting from *O*-glycosylation of WTA  
836 monomers with a plethora of sugar molecules, provides additional complexity.  
837 Studies about the role of glycosidic substituents of WTAs have been mostly done in  
838 *S. aureus*, where the biogenesis and functions of teichoic acids have been better  
839 elucidated. They showed that sugar moieties confer immunogenic properties to WTAs  
840 (Juergens et al., 1963; Torii et al., 1964) and enable the binding of bacteriophages  
841 (Chatterjee et al., 1969). Interestingly, similar observations were made in  
842 *L. monocytogenes* (Wendlinger et al., 1996), which has only sugar-modified WTAs  
843 (Kamisango et al., 1983). This suggests an even more significant impact of WTA  
844 glycosylation in these processes. Evidences linking sugar modification of WTAs with  
845 *Listeria* virulence were obtained from studies using transposon-generated mutants.  
846 EGD (serotype 1/2a) mutants were screened in a mouse model for virulence  
847 attenuation (Autret et al., 2001) and multiple attenuated clones were found to contain  
848 an insertion in *gtcA*, a gene coding for a glycosyltransferase responsible for the  
849 tailoring of serotype 4b or 1/2a WTAs with galactose or GlcNAc, respectively  
850 (Promadej et al., 1999; Eugster et al., 2011) (Figure 3). In another study, the  
851 pathogenic potential of a serotype 4b *gtcA* mutant strain was shown to be strongly  
852 reduced in intragastrically infected mice. In addition, the absence of a functional  
853 GtcA protein decreased the ability to efficiently invade an enterocytic cell line,

854 suggesting that GtcA-mediated WTA glycosylation in *L. monocytogenes* 4b is  
855 important for the intestinal phase of listeriosis (Faith et al., 2009).

856

### 857 **6.3.3. Plasma membrane: phospholipid lysylation**

858

859 The mechanisms of resistance to CAMPs by Gram-positive species can also include  
860 modifications of the plasma membrane, namely from the extracellular side, to mask  
861 the negative charge of the bacterial cell surface that favors the interaction with  
862 cationic peptides (Weidenmaier et al., 2003). Thus, in parallel with the D-alanylation  
863 of LTAs, the anionic surface environment can be reduced, for instance, by the  
864 covalent binding of positively charged L-lysine residues to the negatively charged  
865 head groups of phospholipids in the outer leaflet of the plasma membrane (Staubitz et  
866 al., 2004) (Figure 3). L-lysylated phospholipids were shown to occur in Gram-positive  
867 species (Nahaie et al., 1984; Fischer and Leopold, 1999) through a process dependent  
868 on the expression and enzymatic activity of the membrane protein MprF (multiple  
869 peptide resistance factor) (Peschel et al., 2001; Thedieck et al., 2006). This protein  
870 was first identified in *S. aureus*, where mutants lacking *mprF* showed growth and  
871 survival defects in the presence of CAMPs from diverse origins. This phenotype  
872 resulted from a stronger binding of CAMPs to the bacterial surface and was correlated  
873 with the absence of lysylphosphatidylglycerol (L-PG) from the membrane (Peschel et  
874 al., 2001), indicating that phospholipid L-lysylation promotes the repulsion of  
875 CAMPs. A functional ortholog of MprF was identified in *L. monocytogenes* and, like  
876 its staphylococcal version, is responsible for the generation of L-PG and for  
877 conferring resistance to CAMPs. Importantly, in the absence of MprF, entry levels in  
878 epithelial cell lines and *in vivo* virulence were significantly reduced (Thedieck et al.,  
879 2006), confirming the role of this surface modification protein in *Listeria* infection  
880 and resistance to host defense peptides.

881

## 882 **7. Transport systems**

883

884 To survive and thrive in the host environment, *L. monocytogenes* developed crucial  
885 transport systems to acquire essential nutrients and to expel toxic compounds.

886

### 887 **7.1. Osmolyte uptake and bile acid extrusion**

888

889 For the majority of foodborne pathogens, the ability to sense and respond to the  
890 challenging environment of the gastrointestinal lumen is a key component of  
891 virulence. The osmolarity shift between the external environment and the small  
892 intestine triggers the synthesis of stress-related virulence factors, such as OpuC, an  
893 uptake system for carnitine, one of the most effective osmoprotectants in  
894 *L. monocytogenes* (Beumer et al., 1994). OpuC is essential for successful intestinal  
895 colonization and subsequent systemic infection, following ingestion of  
896 *L. monocytogenes* (Sleator et al., 2001). In contrast, the two other osmolyte  
897 transporters involved in the uptake of glycine betaine, BetL and Gbu, appear  
898 dispensable for *Listeria* virulence (Sleator et al., 2000; Wemekamp-Kamphuis et al.,  
899 2002).

900

901 Following ingestion and gastric digestion, bile represents the most significant  
902 challenge for bacteria. *L. monocytogenes* was shown to be relatively resistant to bile  
903 (Begley et al., 2002), by inducing different bile resistance/detoxification systems,

904 including a bile salt hydrolase (Bsh) important for colonization of the gastrointestinal  
905 tract (Dussurget et al., 2002). However, *L. monocytogenes* also expresses a bile  
906 exclusion system, BilE, which functions by actively extruding bile acids from the cell,  
907 inducing bile tolerance and the ability to infect mice via the oral route, a mechanism  
908 coordinately regulated by SigB ( $\sigma^B$ ) and PrfA (Sleator et al., 2005). Interestingly,  
909 osmolyte uptake systems appear to be required for the maintenance of  
910 *L. monocytogenes* bile tolerance, and the presence of carnitine seems to contribute to  
911 this process (Watson et al., 2009). In addition, genes involved in osmolyte uptake are  
912 responsive to bile salts, with *opuC* operon being highly expressed *in vitro* and *betL* in  
913 a mouse model of oral infection.

914

915 During replication in the cytosol of infected cells, *L. monocytogenes* uses two  
916 multidrug efflux pumps, MdrM and MdrT, to secrete the small second messenger  
917 cyclic-di-AMP (c-di-AMP) (Crimmins et al., 2008). Host recognition of c-di-AMP  
918 triggers the production of type I interferons, including IFN- $\beta$ , which, surprisingly,  
919 further promote *L. monocytogenes* virulence (Woodward et al., 2010). However,  
920 unregulated expression of MdrT was shown to significantly restrict virulence *in vivo*,  
921 by an yet unknown mechanism (Schwartz et al., 2012). Curiously, *L. monocytogenes*  
922 MdrM and MdrT are strongly induced by bile through a mechanism mediated by the  
923 BrtA transcriptional regulator, which has been previously shown to be important for  
924 *L. monocytogenes* virulence in mice (Crimmins et al., 2008). BrtA is a bile sensor that  
925 binds to the *mdrT* promoter de-repressing *mdrT* transcription (Quillin et al., 2011). In  
926 addition to c-di-AMP, MdrT was also shown to work as an efflux pump for bile,  
927 acting in synergy with MdrM to induce bile resistance and promote colonization of  
928 the mouse liver and gallbladder.

929

## 930 **7.2. Nutrient uptake**

931

932 *L. monocytogenes* is auxotrophic for selected vitamins and amino acids and thus must  
933 acquire them directly from the host (Welshimer, 1963; Premaratne et al., 1991;  
934 Marquis et al., 1993; Tsai and Hodgson, 2003). A total of 331 genes (11.6% of the  
935 genome) encoding transport proteins were identified in *L. monocytogenes* EGD-e  
936 (Glaser et al., 2001), reflecting the ability of *Listeria* to adapt to and colonize a broad  
937 range of ecosystems, including the human host. Approximately one third of these  
938 systems are devoted to the transport of carbon sources. Contrarily to non-pathogenic  
939 *Listeria* spp., *L. monocytogenes* is able to metabolize glucose-1-phosphate, which is  
940 readily available in the intracellular compartment of the host, and its utilization is  
941 strictly dependent on PrfA (Ripio et al., 1997), which is fully activated in the host cell  
942 cytosol (Moors et al., 1999; Freitag and Jacobs, 1999; Renzoni et al., 1999).  
943 *L. monocytogenes* was shown to exploit hexose phosphates from the host cell cytosol  
944 as a source of carbon and energy for intracellular growth. Hexose phosphate uptake is  
945 mediated by the PrfA-regulated Hpt translocase, which is required for *Listeria*  
946 intracytosolic proliferation and virulence in the mouse model (Chico-Calero et al.,  
947 2002).

948

949 In addition to using phosphorylated sugars, *L. monocytogenes* may use host cytosolic  
950 peptides as a source of amino acids during intracellular growth (Marquis et al., 1993).  
951 Three distinct oligopeptide transport systems have been described as required for  
952 virulence in *L. monocytogenes*. OppA is an oligopeptide-binding protein encoded by  
953 the first gene of an oligopeptide permease (Opp) operon (*oppA*, *oppB*, *oppC*, *oppD*,

954 and *oppF*) (Borezee et al., 2000). This ATP-dependent oligopeptide carrier is capable  
955 of transporting peptides with as many as eight residues (Verheul et al., 1998). OppA  
956 was shown to mediate the transport of oligopeptides and to be involved in  
957 intracellular growth of *L. monocytogenes* in bone marrow-derived macrophages, but  
958 an *oppA* deletion mutant was only slightly less virulent than the wild type in the  
959 mouse model (Borezee et al., 2000). DtpT is a di- and tripeptide transporter that was  
960 shown to be required for growth when the essential amino acids leucine and valine  
961 were supplied as peptides. This transporter appears to be also involved in salt stress  
962 protection and to contribute to mouse model pathogenesis (Wouters et al., 2005). CtaP  
963 (for cysteine transport-associated protein) is the substrate-binding component of  
964 another oligopeptide transport system shown to be required for *L. monocytogenes*  
965 virulence (Port and Freitag, 2007). Other than cysteine transport, this multifunctional  
966 protein is associated with acid resistance, bacterial membrane integrity and adherence  
967 to host cells. In addition, a *ctaP* deletion mutant is severely attenuated following  
968 intragastric and intravenous inoculation of mice (Xayarath et al., 2009).

969

970 Thiamine pyrophosphate is an essential cofactor thiamine (vitamin B1)-derived  
971 cofactor that is involved in central metabolism and amino acid biosynthesis. Because  
972 *L. monocytogenes* lacks the gene encoding the ThiC protein, responsible for the  
973 synthesis of the thiamine precursor hydroxymethylpyrimidine, it is unable to  
974 synthesize thiamine in absence of this precursor (Schauer et al., 2009). However, the  
975 thiamine transporter ThiT was shown to be required for the uptake of this nutrient  
976 and, more broadly, for the intracellular growth of *L. monocytogenes*, indicating that  
977 thiamine acquisition is a critical step for bacteria that proliferate in the cytoplasm of  
978 host cells.

979

980 Successful pathogens obtain iron from the host environment. However, free iron is  
981 toxic at excessive concentrations and bacteria must regulate its accumulation (Stauff  
982 and Skaar, 2009). Whereas the host developed mechanisms for iron sequestration,  
983 pathogens engineered membrane transport systems for iron utilization during  
984 infection (McLaughlin et al., 2011). Iron acquisition is mediated by a number of  
985 distinct systems that have been characterized in *L. monocytogenes*. In particular, it  
986 requires the activity of the putative ABC-transporter encoded by the *hup*  
987 chromosomal locus, since a mutant for *hupC* is impaired in haem uptake and shows  
988 decreased virulence (Jin et al., 2006). Interestingly, the SrtB-anchored protein SvpA  
989 seems to play a role in the capture of the iron porphyrin (Xiao et al., 2011). In most  
990 bacteria, including *L. monocytogenes*, iron homeostasis is controlled by the ferric  
991 uptake regulator Fur (Andrews et al., 2003). FrvA is a Fur-regulated virulence factor  
992 absolutely required for the growth of *L. monocytogenes* under iron-restricted  
993 conditions and for systemic infection. FrvA is required for the uptake of haem but is  
994 also essential for resistance to heme toxicity as well as maintenance of iron  
995 homeostasis. Sensitivity to heme toxicity may account for the significant attenuation  
996 of virulence during the systemic phase of infection in the murine infection model  
997 (McLaughlin et al., 2012).

998

## 999 **8. Regulation of cell surface-associated mechanisms**

1000

1001 The spatial and temporal expression of bacterial cell wall components is crucial for  
1002 their optimal function, in particular regarding virulence. This process must be tightly  
1003 regulated in response to the variable stimuli of the host environment to allow *Listeria*

1004 to adapt to the changing host conditions, subvert host cellular mechanisms and  
1005 neutralize host defenses.

1006

### 1007 **8.1. Transcriptional regulation**

1008

1009 PrfA is the major *L. monocytogenes* transcriptional regulator of virulence  
1010 determinants (Chakraborty et al., 1992; de las Heras et al., 2011). Mutants lacking a  
1011 functional PrfA are unable to grow in infected cells and are almost avirulent in mice  
1012 (Freitag et al., 1993). A recent study showed that PrfA activation is dispensable for  
1013 vacuole escape but required for efficient bacterial dissemination and survival *in vivo*  
1014 (Deshayes et al., 2012). The core PrfA regulon is composed of the ten virulence genes  
1015 first identified as being PrfA-dependent (Scortti et al., 2007), seven of them being  
1016 related with the bacterial surface (*actA*, *hly*, *inlAB*, *mpl* and *plcAB*). In addition, nearly  
1017 160 other *L. monocytogenes* genes were shown to have their expression directly or  
1018 indirectly dependent on PrfA. Among these genes, several encode virulence factors  
1019 involved in *Listeria* cell envelope architecture, composition and modification (*ntpT*,  
1020 *frvA*, *hpt*, *inlH*, *lapB*, *opuC* and *prsA2*) (Ripio et al., 1998; Dussurget et al., 2002;  
1021 Milohanic et al., 2003; Raynaud and Charbit, 2005; Marr et al., 2006; Reis et al.,  
1022 2010). PrfA activates transcription by binding to a PrfA box, a palindromic sequence  
1023 (tTAACanntGTtAa) in the promoter of the target gene (Scortti et al., 2007; Freitag et  
1024 al., 2009). PrfA integrates both environment- and bacteria-elicited signals to ensure  
1025 the proper spatio-temporal transcription of its regulon. The expression of PrfA itself is  
1026 simultaneously controlled by an RNA thermosensor mechanism that only enables  
1027 translation of the *prfA* mRNA at temperatures close to 37 °C, and by a trans-acting  
1028 riboswitch (Johansson et al., 2002; Loh et al., 2009). An unstructured 5'-coding  
1029 region of the *prfA* mRNA was recently identified as required for efficient translation  
1030 (Loh et al., 2012). Its activity is postulated to be regulated through an allosteric shift  
1031 mediated by a cofactor yet to be identified. Notwithstanding, the positive charge  
1032 within the PrfA binding pocket was shown to contribute to the intracellular activation  
1033 of PrfA, presumably by facilitating the binding of an anionic cofactor (Xayarath et al.,  
1034 2011). Links between carbon metabolism and PrfA-dependent transcription suggest  
1035 that host nutrient availability may work as an intracellular localization signal for  
1036 *L. monocytogenes*, ensuring the strongest induction levels in the host cell cytoplasm  
1037 and repression outside of the host environment (Freitag et al., 2009; Eisenreich et al.,  
1038 2010).

1039

1040  $\sigma^B$  is the major regulator of the class II stress response genes. Several transcriptomic  
1041 and proteomic analyses revealed that  $\sigma^B$  regulates a large and diverse set of genes  
1042 (nearly 200) predicted to function in stress tolerance, carbohydrate metabolism,  
1043 transport, and also in cell envelope processes and virulence (Hain et al., 2008;  
1044 Mujahid et al., 2013). In particular, a great number of genes related to bacterial  
1045 surface architecture and maintenance, and involved in virulence, are regulated by  $\sigma^B$   
1046 (*actA*, *bilE*, *chiA*, *ntpT*, *hly*, *iap*, *inlABH*, *lapB*, *lpeA*, *opuC*, *plcAB*, *prfA*, *prsA2*, *sigB*).  
1047 Interestingly, a significant subset of these genes is co-regulated by PrfA (*actA*, *ntpT*,  
1048 *hly*, *inlABH*, *lapB*, *opuC*, *plcAB*, *prfA* and *prsA2*). PrfA and  $\sigma^B$  were shown to jointly  
1049 contribute to processes such as intracellular growth and virulence (Nadon et al., 2002;  
1050 Kazmierczak et al., 2006; Chaturongakul et al., 2011). In addition, evidences suggest  
1051 that  $\sigma^B$  fine-tunes *prfA* expression inside host cells to avoid overexpression of  
1052 virulence genes that may compromise the host cell (Ollinger et al., 2009).

1053

1054 VirR is the response regulator element of the VirR/VirS two-component system. VirR  
1055 was shown to be required for efficient mouse liver colonization and to positively  
1056 control the transcription of 17 genes (Mandin et al., 2005), among them, the surface-  
1057 related *dlt* operon and *mprF* (see “Modification of cell envelope components”). The  
1058 fact that VirR regulates the expression of both *dlt* and *mprF* genes suggests that the  
1059 VirR/VirS system plays a role in the modulation of *L. monocytogenes* resistance  
1060 against host cationic peptides and constitutes another important virulence regulon  
1061 involved in *Listeria* surface adaptation and pathogenesis.

1062

1063 Flagellar motility is an essential mechanism by which bacteria can adapt to and  
1064 survive in diverse environmental niches. Although flagella confer an advantage to  
1065 *L. monocytogenes* for host colonization (Dons et al., 2004; Bigot et al., 2005; O’Neil  
1066 and Marquis, 2006), listerial flagellin also stimulates host innate immune responses  
1067 (Hayashi et al., 2001). Consequently, at the physiological temperature of the host  
1068 (37 °C), *L. monocytogenes* shuts down flagellar motility, repressing expression and  
1069 assembly of flagellar components. Also required for virulence, this down-regulation is  
1070 governed by the reciprocal activities of the MogR transcriptional repressor and the  
1071 bifunctional flagellar anti-repressor/glycosyltransferase GmaR, which is kept  
1072 activated by the orphan response regulator DegU, at temperatures under 30 °C  
1073 (Gründling et al., 2004; Shen and Higgins, 2006; Kamp and Higgins, 2009). Recently,  
1074 GmaR was shown to function as a thermosensitive anti-repressor that integrates  
1075 temperature signals into transcriptional control of flagellar motility (Kamp and  
1076 Higgins, 2011).

1077

## 1078 **8.2. Spatiotemporal regulation of surface proteins**

1079

### 1080 **8.2.1. *In vivo* regulation**

1081

1082 Virulence is by definition expressed in a susceptible host and involves a dynamic  
1083 crosstalk with the pathogen. In response to the host environment, *L. monocytogenes*  
1084 tightly regulates and coordinates the expression of virulence factors to promote an  
1085 efficient infection (Chatterjee et al., 2006; Camejo et al., 2009; Toledo-Arana et al.,  
1086 2009; Joseph et al., 2006). This is particularly the case for virulence factors involved  
1087 in the modification of the bacterial surface (Figure 4). In the mouse intestinal lumen,  
1088 most of these genes are down-regulated as compared to exponential growth *in vitro*,  
1089 suggesting that they are either needed only for later stages of infection or their  
1090 premature expression hinders the progress of infection in the gastrointestinal phase.  
1091 Reversely, when infecting the mouse spleen, *L. monocytogenes* overexpresses most of  
1092 the virulence genes related with bacterial surface architecture and modification.  
1093 Globally, there is a good correlation between the expression status of these genes in  
1094 the different *in vivo* conditions analyzed and the role of the encoded proteins. For  
1095 example, the only surface factors up-regulated by *L. monocytogenes* in the mouse  
1096 intestinal lumen are InlA, InlH and SrtA, possibly to prepare bacteria for the invasion  
1097 of enterocytes. When inside host cells, *L. monocytogenes* expresses genes required for  
1098 vacuole escape, intracellular motility and multiplication (Figure 4). This reveals the  
1099 ability to fine-tune the expression of the factors involved in *Listeria* surface  
1100 architecture and modification in response to rapidly changing environmental  
1101 conditions, particularly in accordance with the infection phase. Interestingly, the  
1102 characterization of the cell wall proteome of bacteria proliferating within eukaryotic  
1103 cells revealed that that the adaptation of *L. monocytogenes* to the intracellular lifestyle

1104 involves changes in the relative abundance of certain surface proteins, such as InlA  
1105 and InlH (García-Del Portillo and Pucciarelli, 2012).

1106

### 1107 **8.2.2. Spatial regulation**

1108

1109 Besides the transcriptional control of virulence-associated factors, the regulation of  
1110 their localization is also essential to ensure a successful infection. As already  
1111 mentioned here, *L. monocytogenes* uses different mechanisms to target to its surface  
1112 and secrete proteins that contribute to the colonization of host tissues.

1113

1114 Many listerial virulence effectors (InlA, InlF, InlH, InlJ, InlK, Vip and SvpA) are  
1115 covalently attached to the peptidoglycan (see “Sortases”). Recently, the three-  
1116 dimensional localization of InlA, InlH, InlJ and SvpA in the cell envelope of *Listeria*  
1117 grown at different conditions was reported (Bruck et al., 2011). In this study,  
1118 peptidoglycan-anchored proteins were found positioned along the lateral cell wall in  
1119 non-overlapping helices, but could also be localized at the poles and distributed  
1120 asymmetrically when specific regulatory pathways were activated. For instance,  
1121 whereas InlA and InlJ (SrtA substrates) are enriched at the bacterial poles during  
1122 exponential growth, InlA and InlH relocate to the septum when entering stationary  
1123 phase or under oxidative stress. It was proposed that, in response to PrfA activation,  
1124 excess of InlA protein may saturate sidewall anchor points and thus reach the septum  
1125 (Bierne and Dramsi, 2012). The fact that *inlAH* are also under the control of  $\sigma^B$  could  
1126 suggest an interconnection between cell wall protein anchoring and  $\sigma^B$ -dependent  
1127 stress response. This is reinforced by a recent study showing that the activation of  
1128 InlA and InlH during the transit of *L. monocytogenes* towards stationary growth phase  
1129 is dependent on the functionality of SrtA (Mariscotti et al., 2012). In contrast, SvpA  
1130 (SrtB substrate) is present at the poles and excluded from the septum of under  
1131 conditions of low iron concentration. This suggests that *L. monocytogenes* can  
1132 reorganize the spatial localization of its surface virulence factors in response to  
1133 environmental changes, to best accommodate to the particular conditions of the each  
1134 stage of the infectious process. SrtA links LPXTG proteins to the peptidoglycan  
1135 precursor lipid II, whereas SrtB is proposed to anchor substrates directly to the mature  
1136 peptidoglycan (Marraffini and Schneewind, 2005), suggesting that *Listeria* uses  
1137 different sortases to anchor proteins to distinct sites of the bacterial surface.

1138

1139 The polar distribution of ActA on the *L. monocytogenes* surface is required for actin-  
1140 based motility and successful infection (see “Hydrophobic tail”) and appears to be a  
1141 consequence of different cell wall growth rates along the bacterium, of the relative  
1142 rates of protein secretion and degradation, and of bacterial growth (Rafelski and  
1143 Theriot, 2006). Interestingly, although anchored to the membrane, ActA was shown to  
1144 be specifically co-purified with the peptidoglycan fraction isolated from intracellular  
1145 bacteria (García-del Portillo et al., 2011).

1146

1147 DivIVA is a crucial topological factor required for completion of cell division in  
1148 *L. monocytogenes*. It not only affects *Listeria* cell separation, but also biofilm  
1149 formation, host cell invasion and virulence. DivIVA was shown to influence the  
1150 activity of the Sec system alternative ATPase SecA2, resulting in reduced  
1151 extracellular levels of the autolysins p60 and MurA and inducing a pronounced  
1152 chaining phenotype (Halbedel et al., 2012).

1153

1154 All these observations clearly highlight how *Listeria* developed a complex regulatory  
1155 network linking cell growth, cell wall dynamics, cell wall protein anchoring and  
1156 response to environmental conditions, and coordinating the spatiotemporal expression  
1157 and activity of surface virulence factors.

1158

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1994 *Staphylococcus aureus* and *Bacillus anthracis* reveal catalytic amino acid triad in  
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- 1996

1997 **Figure legends**

1998

1999 Figure 1. Mechanisms leading to the surface display of proteins involved in  
2000 *L. monocytogenes* virulence. Secretory proteins are exported through the bacterial  
2001 membrane by the action of specialized secretion systems. The classical Sec system  
2002 (yellow) recognizes and translocates protein precursors containing N-terminal export  
2003 signal sequences, which are cleaved afterwards by signal peptidases (Lsp or SipXYZ).  
2004 Depending on the presence and the nature of surface anchoring domains, the  
2005 processed protein can become membrane-associated through an anchor molecule  
2006 (lipoproteins) or by one (hydrophobic tail proteins) or more transmembrane domains  
2007 (integral membrane proteins). Alternatively, the protein can associate with cell wall  
2008 components through covalent or non-covalent interactions. Covalently cell wall-  
2009 anchored proteins require processing of a C-terminal sorting domain by sortases  
2010 (SrtAB), which cleave an internal signature sequence and append the C-terminus to  
2011 the stem peptide of peptidoglycan precursors. In contrast, non-covalently anchored  
2012 proteins associate less stringently with other cell wall components via cell wall-  
2013 binding repeat-rich domains (CBR). The flagellum (green) assembly machinery has  
2014 its own export system (dark green), which enables the delivery of rod and filament  
2015 components to the tip of the nascent flagellar structure. The coordinated activity of  
2016 surface chaperones and proteases (purple), such as PrsA2 and HtrA, ensures the  
2017 integrity of the *L. monocytogenes* surfaceome under stressful conditions.

2018

2019 Figure 2. *L. monocytogenes* peptidoglycan metabolism and the surface proteins  
2020 involved in its assembly and turnover. The peptidoglycan sacculus is polymerized  
2021 with cytoplasmic precursors with the help of penicillin-binding proteins (PBPs,  
2022 yellow). High-molecular-weight PBPs, such as PBPA2, contain transglycosylase  
2023 (TGD) and transpeptidase domains (TPD) that catalyze, respectively, glycan chain  
2024 elongation and stem peptide bridging between adjacent chains. Other PBPs include  
2025 the low-molecular-mass carboxypeptidases, which cleave the terminal D-alanyl-D-  
2026 alanine stem peptide bond (e.g. PBPD1), and beta-lactamases, which degrade PBP-  
2027 inhibiting antibiotics to promote bacterial survival (e.g. PBPC1). On the other hand,  
2028 the degradation of mature peptidoglycan, during bacterial elongation/division or  
2029 autolysis, is mediated by autolysins (green), a family of surface hydrolases that can  
2030 cleave the peptidoglycan at different sites: within the glycan chain (*N*-  
2031 acetylglucosaminidases or *N*-acetylmuramidases) or the stem peptide (endo- and  
2032 carboxypeptidases), or between both (*N*-acetylmuramoyl-L-alanine amidases).  
2033 Interestingly, autolysins commonly associate non-covalently with the bacterial surface  
2034 *via* cell wall-binding repeats, such as the GW modules in Ami, Auto and IspC, or the  
2035 LysM repeats in MurA and p60.

2036

2037 Figure 3. Modification of *L. monocytogenes* cell envelope components. To evade  
2038 recognition and targeting by the host immune system, *L. monocytogenes* expresses  
2039 surface proteins specialized in the introduction of fine modifications in cell envelope  
2040 components. For instance, the addition of positively charged molecules contributes to  
2041 the decrease of the overall negative charge of the bacterial surface, which in turn  
2042 reduces the cell wall-binding affinity of cationic antimicrobial peptides. The D-  
2043 alanylation of lipoteichoic acid (LTA) polymers by the DltABCD system (green) or  
2044 the lysylation of plasma membrane phospholipids by MprF (pink) are examples of  
2045 such approach. The glycosylation of wall teichoic acid (WTA) polymers was linked  
2046 with listerial virulence (e.g. GtcA-mediated addition of GlcNAc in serotype 1/2a or

2047 galactose in serotype 4b), although the mechanism itself and the specific role of each  
2048 sugar in this process are still unclear. Additionally, the peptidoglycan can be modified  
2049 to become less recognizable or more resistant to degradation by host hydrolases (e.g.  
2050 lysozyme) and prevent unwanted shedding of immunostimulatory muropeptides.  
2051 These changes include the addition or removal of acetyl groups from the glycan chain  
2052 amino sugars, catalyzed by two surface proteins, Oat and PgdA (purple), respectively.

2053

2054 Figure 4. *In vivo* and intracellular expression of virulence genes involved in  
2055 *L. monocytogenes* surface architecture and modification. In each condition, genes are  
2056 shown up-regulated (red) or down-regulated (green) in comparison with the  
2057 corresponding transcription levels during exponential growth in standard culture  
2058 conditions (BHI, 37 °C). lmo, gene number in the *L. monocytogenes* EGD-e genome;  
2059 lin, number of the orthologue gene in the *L. innocua* CLIP 11262.

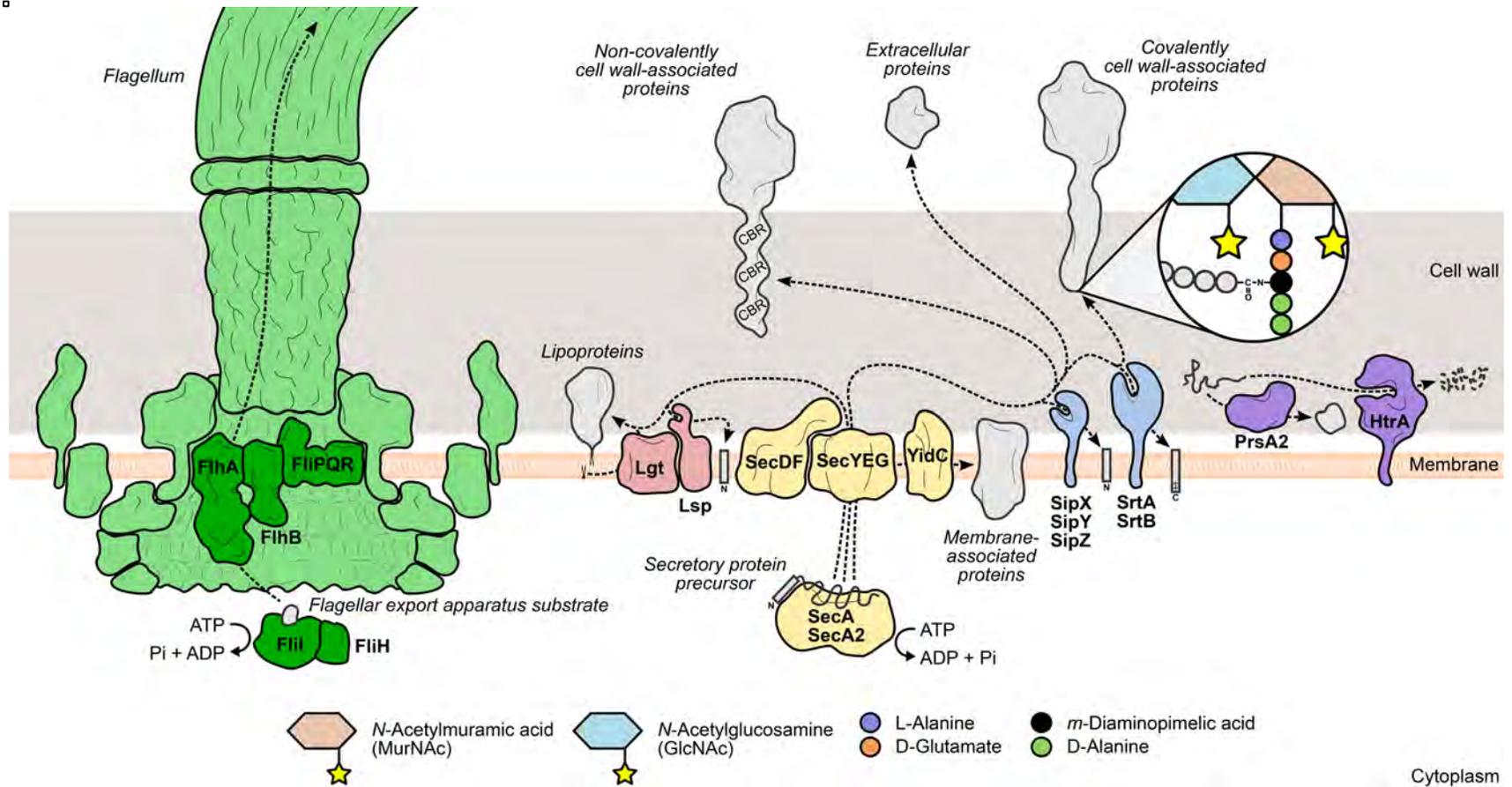


Figure 1

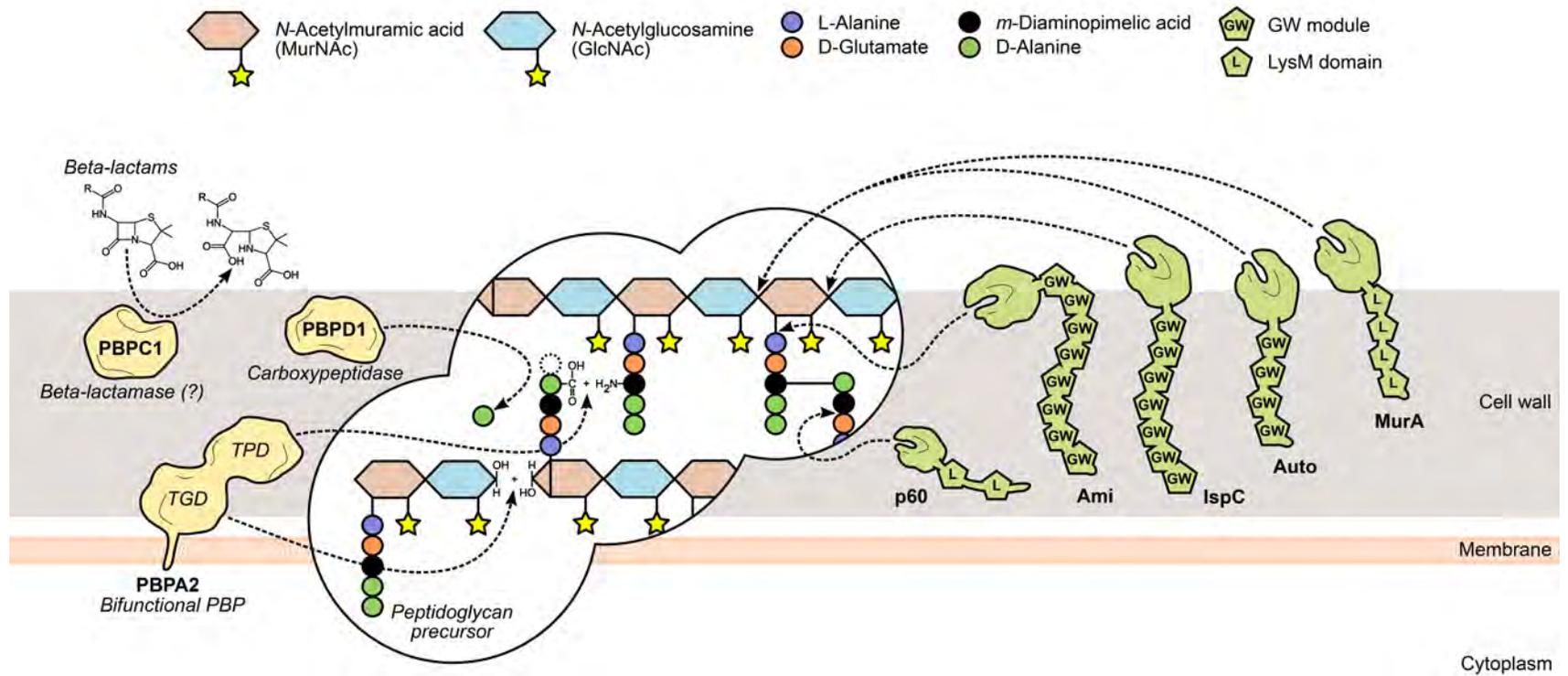


Figure 2

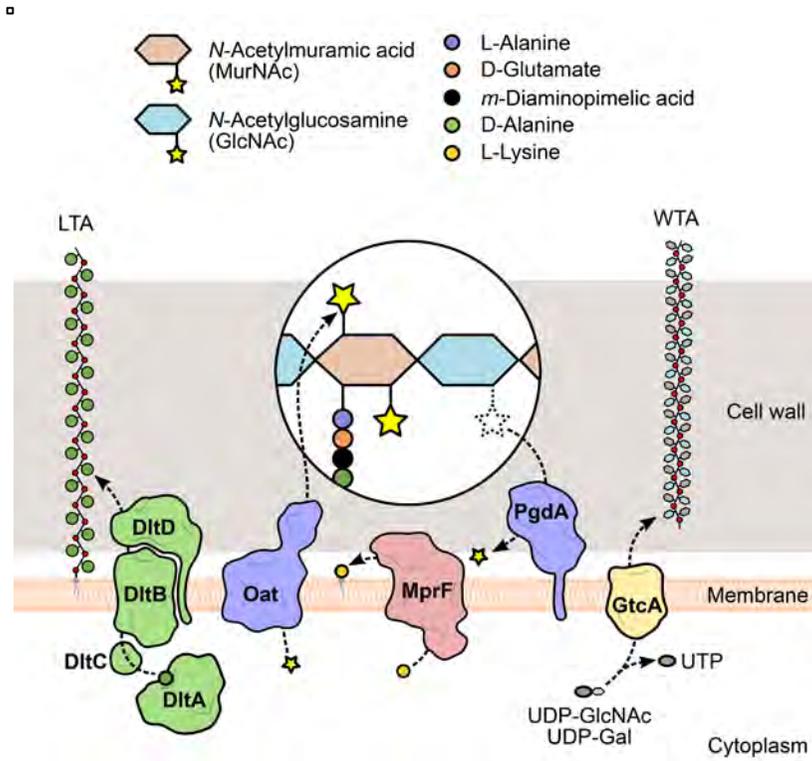


Figure 3

Gene	lmo	lin	Mouse intestine lumen	Human blood	Mouse spleen	Macrophages	Caco-2
<i>actA</i>	lmo0204						
<i>ami</i>	lmo2558						
<i>aut</i>	lmo1076						
<i>bilEA</i>	lmo1421						
<i>bilEB</i>	lmo1422	lin1461					
<i>ctaP</i>	lmo0135	lin0182					
<i>degU</i>	lmo2515	lin2659					
<i>divIVA</i>	lmo2020	lin2128					
<i>dltA</i>	lmo0974	lin0973					
<i>dltB</i>	lmo0973	lin0972					
<i>dltC</i>	lmo0972	lin0971					
<i>dltD</i>	lmo0971	lin0970					
<i>dtpT</i>	lmo0555	lin0564					
<i>fbpA</i>	lmo1829	lin1943					
<i>flaA</i>	lmo0690	lin0698					
<i>fliF</i>	lmo0713	lin0721					
<i>fliI</i>	lmo0716	lin0724					
<i>fri</i>	lmo0943	lin0942					
<i>frvA</i>	lmo0641	lin0644					
<i>fur</i>	lmo1956	lin2070					
<i>gmaR</i>	lmo0688	lin0696					
<i>gtcA</i>	lmo2549	lin2694					
<i>hly</i>	lmo0202						
<i>hpt</i>	lmo0838						
<i>htrA</i>	lmo0292						
<i>hupC</i>	lmo2429	lin2523					
<i>iap</i>	lmo0582	lin0591					
<i>inlA</i>	lmo0433						
<i>inlB</i>	lmo0434						
<i>inlF</i>	lmo0409						
<i>inlH</i>	lmo0263						
<i>inlJ</i>	lmo2821						
<i>inlK</i>	lmo1289						
<i>lap</i>	lmo1634	lin1675					

Gene	lmo	lin	Mouse intestine lumen	Human blood	Mouse spleen	Macrophages	Caco-2
<i>lapB</i>	lmo1666						
<i>lgt</i>	lmo2482	lin2625					
<i>lntA</i>	lmo0438						
<i>lpeA</i>	lmo1847	lin1961					
<i>lsp</i>	lmo1844	lin1958					
<i>mdrT</i>	lmo2588	lin2733					
<i>mogR</i>	lmo0674	lin0682					
<i>mpl</i>	lmo0203						
<i>mprF</i>	lmo1695	lin1803					
<i>murA</i>	lmo2691	lin2838					
<i>oat</i>	lmo1290						
<i>oppA</i>	lmo2196	lin2300					
<i>opuCA</i>	lmo1428	lin1467					
<i>opuCB</i>	lmo1427	lin1466					
<i>opuCC</i>	lmo1426	lin1465					
<i>opuCD</i>	lmo1425	lin1464					
<i>pgdA</i>	lmo0415	lin0436					
<i>plcA</i>	lmo0201						
<i>plcB</i>	lmo0205						
<i>prfA</i>	lmo0200						
<i>prsA2</i>	lmo2219	lin2322					
<i>secA2</i>	lmo0583	lin0592					
<i>secDF</i>	lmo1527	lin1562					
<i>sigB</i>	lmo0895	lin0894					
<i>sipX</i>	lmo1269	lin1308					
<i>sipY</i>	lmo1270						
<i>sipZ</i>	lmo1271	lin1310					
<i>sod</i>	lmo1439	lin1478					
<i>srtA</i>	lmo0929	lin0929					
<i>srtB</i>	lmo2181	lin2285					
<i>svpA</i>	lmo2185	lin2289					
<i>thiT</i>	lmo1429	lin1468					
<i>vip</i>	lmo0320						
<i>virR</i>	lmo1745	lin1856					

■ Up-regulated genes   ■ Down-regulated genes

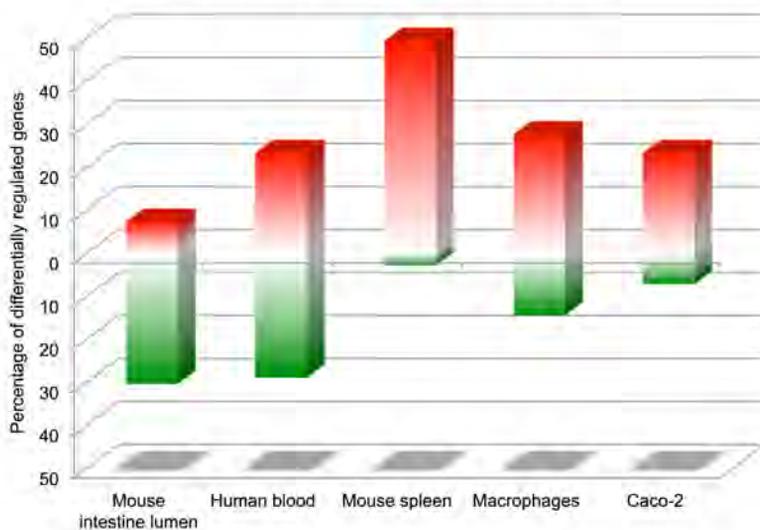


Figure 4