The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle

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*Listeria monocytogenes* is an intracellular Gram-positive pathogen and the etiological agent of listeriosis, a human foodborne disease potentially fatal for certain risk groups. The virulence of *L. monocytogenes* is supported by a highly complex and coordinated intracellular life cycle that comprises several crucial steps: host cell adhesion and invasion, intracellular multiplication and motility, and intercellular spread. The completion of each stage is dependent on the orchestrated activity of specialized bacterial factors, in turn tightly controlled by a specific set of regulators. Some virulence factors and modulators also assume an important role in bacterial resistance and evasion to host defense mechanisms. In the last years, the advent of genomics promoted an increasingly prolific identification and functional characterization of new Listeria virulence factors. In this review, we summarize the current knowledge on nearly 50 molecules deployed by *L. monocytogenes* to promote its cell infection cycle.

**Introduction**

*Listeria monocytogenes* was isolated for the first time by E.G.D. Murray and colleagues in 1926, during an epidemic in animal care houses that affected specially guinea pigs and rabbits. They named it originally *Bacterium monocytogenes*, because of the large number of monocytes that were found in the blood of infected animals. The following year, Pirie isolated an identical bacterium from the liver of several gerbils (*Iatera lobenquiae*). He named it *Listerella hepatolytica*, in honor of Sir Joseph Lister, a pioneer in the field of antisepsis. In 1940, to harmonize the nomenclature, the genus *Listeria* belongs to the Firmicutes division, characterized by low GC DNA content (38%). *Listeria* spp are flagellated, non-spore-forming and facultative anaerobic Gram-positive bacilli. This genus contains eight species: *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayi*, and the newly identified *L. rocouriaae* and *L. marthii*. These ubiquitous bacteria are commonly isolated from plants, soil and water and *L. monocytogenes* is capable to contaminate agriculture environments, animal feed and foods. The pathogenic species *L. monocytogenes* causes disease in humans and animals, whereas *L. ivanovii* only affects animals, mainly sheep and cattle. The remaining species are not pathogenic.

*Listeria monocytogenes* is the etiological agent of listeriosis, an infectious disease with a mortality rate of 20–30% in certain risk groups. Two forms of listeriosis are caused by *L. monocytogenes*: a non-invasive form that in immunocompetent individuals develops as a febrile gastroenteritis and an invasive form that in immunocompromised hosts, such as the elderly and patients undergoing immunosuppressive therapy, can manifest as septicemia or meningoencephalitis. Moreover, the fetus can also acquire invasive listeriosis from its infected mother through the placenta. Perinatal listeriosis increases the probability of abortion, stillbirth or birth of a baby with generalized infection, sepsis or meningitis. *L. monocytogenes* is also able to induce a broad variety of uncommon focal infections; cases of endocarditis, cutaneous infection, joint infection, myocarditis and necrotizing fasciitis have been described. Among the *L. monocytogenes* strains, those of serovars 1/2a, 1/2b and 4b are responsible for 95% of human infection cases.

This potentially fatal disease was considered a zoonose until the early 80s, when it was identified as an important human foodborne infection in industrialized countries. In 1981, the foodborne transmission of listeriosis was documented for the first time, following an outbreak of invasive disease with high case-fatality rate in Canada. In spite of the high rates of contamination with *L. monocytogenes* in certain food products, listeriosis is rare (incidence in Europe in 2009 = 0.4/100,000) in comparison to other foodborne illnesses (e.g., salmonellosis incidence in Europe in 2009 = 23.7/100,000). Nevertheless, listeriosis was the most frequent cause of death due to the consumption of contaminated food in Europe in 2009, with a case fatality of 16.6% (270 deaths) as compared with the 0.08% (90 deaths) for salmonellosis.

In general, *L. monocytogenes* infects the human host via the oral route through uptake of contaminated food products and is...
able to cross three tight physiological barriers: the intestinal, the blood-brain, and the feto-placental barriers. By crossing the intestinal barrier, the bacterium is absorbed from the intestinal lumen, traversing the epithelial cell layer, and if the immune system does not control the infection, the pathogen disseminates to the bloodstream and mesenteric lymph nodes. The interaction of this alcohol acetaldehyde dehydrogenase with Hsp60, its host cell receptor, promotes bacterial adhesion to intestinal cells. Anaerobic growth induces significant increases in the level of lap transcript and Lap secretion via the accessory secretion system SecA2 (see regulation section). Oral administration of lap-deficient strains to mice confirmed that Lap is essential for full virulence.

Ami is a 99 kDa protein exhibiting N-acetylmuramoyl-L-alanine amidase activity. Its N-terminal domain is responsible for cleavage of the amide bond between N-acetylmuramic acid and L-alanine residues in the peptidoglycan. Its C-terminal cell wall-anchoring (CWA) domain contains eight glycine-tryptophan (GW) modules, through which Ami associates to the bacterial surface, putatively by interaction with lipoteichoic acids (LTAs). The CWA domain of Ami has been shown to mediate bacterial adhesion to human epithelial cells. In addition, an ami mutant is attenuated in the liver of intravenously infected mice, suggesting a role in L. monocytogenes virulence.

Cell Infection and Virulence Determinants

L. monocytogenes is a facultative intracellular pathogen able to invade, survive and multiply inside epithelial cells, macrophages and dendritic cells. Following adhesion and invasion of the host cell, the bacterium quickly disrupts the internalization vacuole and escapes into the cytoplasm, where it can replicate. In this compartment, L. monocytogenes employs an actin-based process of motility to impel itself within the host cell. To get inside a neighboring cell, it generates a double membrane protrusion which can result in the formation of a secondary vacuole. This structure is also lysed, allowing the bacterium to initiate a new cycle of infection in contiguous cells. This intracellular life cycle occurs not only in vertebrate cells but was also documented in invertebrate cells. Below, we will present L. monocytogenes virulence factors involved in the various steps of the cell infection cycle.

Adhesion. Adhesion of L. monocytogenes to the surface of mammalian cells is the initial step of the cell infection cycle, and a critical aspect of the host-pathogen interaction, as the bacterium is in direct contact with host cells. L. monocytogenes adherence facilitates cell invasion by connecting bacteria to host cells and activating host cell signaling pathways through the engagement of host cell receptors. This stage involves a number of bacterial surface adhesion factors.

Lap, originally named surface protein p104, is a 104 kDa adhesion protein, present in every Listeria. The interaction between lap and its host cell receptor, promotes bacterial adhesion to intestinal cells. Anaerobic growth induces significant increases in the level of lap transcript and Lap secretion via the accessory secretion system SecA2 (see regulation section). Oral administration of lap-deficient strains to mice confirmed that Lap is essential for full virulence.

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FbpA displays a strong homology to atypical fibronectin-binding proteins, such as Streptococcus pneumoniae PavA, S. pyogenes Fbp54, and S. gordonii FbpA. FbpA is exposed at the surface of the bacteria despite lacking a canonical signal peptide, as its streptococcal homologs. FbpA binds to immobilized human fibronectin in a dose-dependent and saturable manner, and increases adherence of L. monocytogenes to epithelial cells when exogenous fibronectin is present. The expression of FbpA affects the protein levels of two major Listeria virulence factors described below: LLO and InIB. Thus, FbpA seems to work not only as a fibronectin-binding protein but also as a chaperone that stabilizes and/or ensures the proper secretion of LLO and InIB.

InlJ belongs to the internalin family, which is characterized by the presence of leucine-rich repeats (LRRs). The structure of the internalin domain of InlJ was solved and shows that the LRRs of InlJ differ from those of other internalins, in particular by having one LRR-defining hydrophobic residue replaced with a conserved cysteine. The C-terminus displays an LPXTG motif recognized by the sortase A (SrtA) enzyme, which covalently
anchors LPXTG-containing proteins to the peptidoglycan (see regulation section). The \textit{inlJ} gene is only found in the genomes of \textit{L. monocytogenes} strains. An \textit{inlJ} deletion mutant is significantly less virulent following intragastric and intravenous infection of mice. Interestingly, InlJ is only efficiently expressed at the surface of bacteria recovered from the liver and blood of infected animals. Moreover, adhesion to human epithelial cells was promoted in \textit{L. monocytogenes} or \textit{L. innocua} strains expressing \textit{inlJ} via an heterologous promoter. InlJ has also been shown to bind MUC2 (the major component of intestinal mucus) by its internalin domain. InlJ seems thus to act as a \textit{L. monocytogenes}-specific sortase-anchored adhesin whose expression is specifically induced in vivo.

The cysteine transport-associated protein, CtaP, was identified in a study aiming to detect novel proteins secreted in response to
activation of PrfA, the main transcriptional activator of *Listeria* spp virulence genes (see regulation section). CtaP is a protein functionally associated with cysteine transport, acid resistance, bacterial membrane integrity and host cell adhesion. Indeed, absence of CtaP increased the permeability and acid sensitivity of the membrane, and reduced the adherence of bacteria to epithelial cells. CtaP deletion mutants also display significant attenuation after intragastric and intravenous inoculation of mice.

LapB is a SrtA-anchored LPXTG protein that was identified using comparative genomics. *lapB* is absent from non-pathogenic *Listeria* species, and its expression was shown to be positively regulated by PrfA and highly increased in infected mouse spleens. LapB is necessary for adhesion to and entry into mammalian epithelial cell lines and for virulence in intravenous or orally infected mice. The adhesion function of LapB is localized in its N-terminal domain, which probably interacts with a receptor in the host cell.

ActA, the only bacterial determinant necessary for actin-based motility of *L. monocytogenes* (see intracellular motility section), was shown to be also involved in cell attachment and entry by recognition of heparan sulfate.

RecA participates in DNA repair and activates the SOS response. Recently, it was shown to contribute to acid and bile resistance and to the ability of *L. monocytogenes* to bind to and enter into human intestinal epithelial cells. In addition, increased *recA* transcription was detected following exposure to low pH and increased bile concentrations, as well as during adhesion and invasion of intestinal epithelial cells. However, the role of RecA in the adhesion process appears to be accessory. RecA seems therefore to contribute for bacterial colonization of the human gastrointestinal tract and translocation of the intestinal epithelium.

InlF, another member of the internalin family, was found to be a *L. monocytogenes*-specific protein that promotes increased host cell binding and entry, under particular conditions. Indeed, inhibition of the activity of the host cell kinase ROCK in murine fibroblasts and hepatocytes, as well as human fibroblasts and epithelial cells, was shown to enhance bacterial adhesion and invasion in an InlF-dependent manner. The identification of the host cell receptor of InlF is the subject of ongoing studies. This internalin is an example of a virulence factor whose function may be only significant under specific host cell conditions or during infection of specific host species.

**Invasion.** Entry of *L. monocytogenes* in macrophage cells is mostly driven by the macrophage itself. In contrast, entry into non-professional phagocytes is induced by several *Listeria* factors, and in particular, by the interaction of bacterial surface proteins with specific host receptors.

Internalins A (InlA) and B (InlB) were the first proteins identified as mediators of *Listeria* entry into different non-phagocytic cell types, and are two major *L. monocytogenes* invasion proteins. Both proteins belong to the internalin family (25 genes in *L. monocytogenes* EGD-e) whose members contain a signal peptide sequence, followed by the LRR region that allows protein-protein interactions, and a conserved inter-repeat (IR) domain followed by several other repeats and different C-terminal motifs. Nineteen internalins, including InlA, contain an LPXTG motif predicted to bind covalently the protein to the cell wall. The C-terminal domain of InlB contains GW repeats that are involved in non-covalent cell wall interactions.

The LRR and IR regions of InlA were shown to be required and sufficient to promote entry of *Listeria* into human epithelial cells. The host cell receptor for InlA is E-cadherin, a transmembrane glycoprotein involved in cell-cell adhesion. The InlA/E-cadherin interaction is species-specific and, in humans, it is enabled by the presence of a proline residue at position 16 of the E-cadherin molecule. In contrast, mouse E-cadherin cannot interact with InlA because the proline is replaced by a glutamic acid residue. The crystal structure of the LRR region of InlA in association with the extracellular domain of human E-cadherin corroborated this observation and revealed that a hydrophobic pocket on the LRR accommodates the E-cadherin proline 16 residue. Oral inoculation of a transgenic mouse model that synthesizes the human E-cadherin in the intestine showed the crucial role of InlA in the crossing of the intestinal epithelium. The role of this interaction in the process of placental infection is still not clear and different studies have provided contradicting results. InlA was shown to interact with E-cadherin present at the basal and apical membranes of syncytiotrophoblasts and villous cytotrophoblasts, mediating the crossing of the feto-placental barrier. On the other hand, syncytiotrophoblasts were shown to be highly resistant to *L. monocytogenes* infection. Epidemiological studies showed that InlA plays a key role in human listeriosis: 96% of clinical strains express a full-length functional form of InlA, while only 65% of food-isolated strains contained the full-length form.

InlA/E-cadherin interaction is critical for epithelial cell invasion, as it activates complex signaling pathways leading to cytoskeletal reorganization. The E-cadherin extracellular domain is sufficient to promote InlA-dependent adherence, while the intracellular domain binding to catenins is required for the internalization process. ARHGAP10, an α-catenin binding partner, contributes to the recruitment of α-catenin to the adherens junctions and efficient bacterial invasion. Upon InlA/E-cadherin interaction, host Src kinase is activated leading to clathrin recruitment, actin polymerization at the entry site and E-cadherin endocytosis.

InlB is another surface-associated protein belonging to the internalin family. Similar to InlA, the LRR region of InlB is necessary and sufficient to promote invasiveness. The GW modules of InlB mediate the binding of this protein to the bacterial surface by non-covalent interactions with LTAs. The crystal structures of the LRR and LRR/IR domains and full-length protein indicate that InlB has a highly elongated structure, suggesting an interaction with multiple cell receptors, that cooperate in bacterial uptake. Indeed, various host receptors have been identified for InlB: gC1qR, c-Met and glycosaminoglycans (GAGs). A direct interaction between InlB and gC1qR has been shown. In addition, the InlB-mediated entry was impaired by anti-gC1qR antibodies. The receptor tyrosine kinase Met, the physiological receptor for the hepatocyte growth factor (HGF), was also identified as a host cell receptor for InlB. Through its GW modules, soluble InlB binds directly to cellular GAGs.
GAG receptors facilitate the detachment of InlB from the bacterial surface and InlB clustering at the host cell surface, thus promoting strong Met activation and bacterial entry.\(^{81,82}\) Although InlB has several receptors, it is well accepted that Met is the major InlB signaling receptor. InlB has the ability to induce Met autophosphorylation and the recruitment of adaptor proteins, such as Cbl, Shc and Gab1,\(^{83-85}\) that lead to the activation of PI3-kinase\(^{86}\) and small GTPase Rac1.\(^{87}\) Upon interaction with Met, InlB induces Met ubiquitination and bacterial internalization via a clathrin-mediated endocytosis mechanism.\(^{88}\) InlB-mediated entry is highly dependent on actin rearrangements taking place downstream the activation of Met, which involve host molecules (e.g., coflin, LIM kinase, WASP and VASP).\(^{87,89}\) Interestingly, the activation of Met by InlB is species-specific. In mice, InlB is not involved in intestinal barrier crossing,\(^{90}\) but is important for bacterial colonization of liver and spleen. In contrast, in guinea pigs and rabbits, there is no virulence attenuation when these animals are infected with a ΔinlB deletion mutant.\(^{91}\) Recent data showed the key role of InlB in the crossing of placental barrier,\(^{90}\) the cooperation of InlA and InlB appearing critical for the efficient placental invasion.

Vip is another LPXTG protein anchored to the peptidoglycan by SrtA.\(^{92}\) The vip gene is present in all L. monocytogenes lineages, including serovars commonly associated with human disease (1/2a, 1/2b and 4b), and is absent from non-pathogenic species.\(^{56}\) Vip is necessary for efficient Listeria entry into several epithelial cell lines. A vip deletion mutant shows virulence attenuation in different mouse organs (liver, spleen and brain) after oral or intravenous infection. The endoplasmic reticulum-resident chaperone Gp96 was identified as the host cell receptor for Vip, and the Vip-Gp96 interaction was shown to be crucial for Listeria invasion of eukaryotic cells.\(^{92}\) Gp96 binds to and activates monocytes and neutrophils, and is essential for the subcellular localization of Toll-like receptors (TLR).\(^{93}\) Therefore, in addition to promote cell invasion, the interaction of Vip with Gp96 could possibly interfere with TLR trafficking, leading to the control of the innate immune response by L. monocytogenes.\(^{92}\)

The aut gene was identified by comparative genomics and encodes Auto, a surface-associated GW repeat-containing protein that is absent from non-pathogenic L. innocua.\(^{25,94}\) Auto is an autolytic protein containing a signal peptide and a N-terminal N-acetylglucosaminidase domain that is activated by proteolytic cleavage and works optimally at an acidic pH.\(^{95}\) Like Ami and InlB, Auto is involved in the cooperation of InlA and InlB appearing critical for the efficient placental invasion.

The iap gene encodes p60, a surface protein with a murine hydrolase activity without which cells show irregular septum formation and become prone to form short bacterial cell filaments and hooked cell forms during exponential growth phase. In the late-stationary-growth phase, these abnormal bacterial cell filaments break into nearly normal-sized single bacteria.\(^{96,97}\) The analysis of spontaneous mutants that have lost the ability to invade mouse fibroblasts demonstrated that p60 is necessary for a successful invasion of host cells.\(^{96,98}\) An iap mutant was shown to have a decreased invasiveness of fibroblasts and epithelial cells.\(^{97}\) Purified p60 has the capacity to restore the cell internalization capacity of iap mutants\(^{99}\) and to bind to human enterocyte-like cells.\(^{100}\) p60 was also shown to confer increased cellular uptake when expressed in Salmonella typhimurium.\(^{99}\) The iap mutant is highly attenuated in infected mice after intravenous injection, stressing the importance of p60 during infection.\(^{97}\)

Igt encodes a diacylglycerol transferase responsible for lipidation of prolipoproteins. Phenotypical studies of the Δigt mutant showed that it is defective in host cell invasion and intracellular survival. However, since intracellular multiplication seems to be delayed rather than fully abolished, this suggests either that lipoproteins are not directly implicated or that the non-lipidated variants are still active. Δigt exhibits increased susceptibility to cationic peptides, implying that the anchored lipoproteins contribute to the net charge of the bacterial surface.\(^{101}\)

GtcA was shown to be involved in glycosylation of the cell wall teichoic acids.\(^{102}\) gtcA mutation impairs L. monocytogenes infection in intragastrically inoculated mice. The gtcA mutant strain displayed reduced entry levels but normal multiplication inside Caco-2 cells, suggesting that the diminished ability of a gtcA mutant to induce its entry into intestinal epithelial cells may contribute in part for the decreased virulence in the gastro-intestinal tract.\(^{103}\)

LpeA is a 35 kDa homolog of S. pneumoniae PsA, a lipoprotein implicated in the streptococcal cell adherence. LpeA is required for L. monocytogenes entry into murine hepatocytes and human intestine epithelial cells. Intriguingly, an lpeA mutant survives better in macrophages and shows a weak virulence exacerbation, in comparison to the wild-type strain.\(^{104}\)

Mpf (multiple peptide resistance factor) was identified as a VirR-dependent virulence regulon gene.\(^{42}\) Mpf was shown to be implicated in the synthesis of lysophosphatidylglycerol (L-PG), a membrane phospholipid, and crucial for lysis of diphosphatidylglycerol (DPG).\(^{105}\) Inactivation of mpf results in a decreased invasivity in both epithelial cells and macrophages, and in attenuation of virulence in a mouse model of infection.

Inactivation of ActA (see intracellular motility section) impairs adhesion (see adhesion section) but also invasion of macrophages and epithelial cells, and expression of ActA in the non-invasive L. innocua is sufficient to confer at least partially the capacity to enter into epithelial cells.\(^{106,107}\)

Listeriolysin O (LLO) is the Listeria factor mainly responsible for bacterial vacuole evasion (see vacuole escape section), but was also shown to contribute for invasion of epithelial cells by inducing influx of calcium ions.\(^{108}\) This LLO-dependent calcium flux modulation was further demonstrated to cause a transient mitochondria network fragmentation which appears to briefly slow down the host cell bio-energetic state, promoting a more efficient entry of L. monocytogenes.\(^{109}\)
Flagella, which mainly work as motility effectors (see regulation section), and the SOS response activator RecA (see adhesion section) were also shown to exert a role in L. monocytogenes invasion of host cells. However, the specific functions of these proteins and their level of contribution to the entry process remain to be elucidated.

**Vacuole escape.** Subsequent to the invasion step, Listeria is found temporarily enclosed in a phagocytic vacuole, before it escapes to the host cell cytoplasm.

It was proposed that L. monocytogenes delays vacuole maturation through inhibition of the host cell GDPase Rab5a activity. The secreted form of Listeria GAPDH would be the responsible for Rab5a inactivation. However, how bacterial GAPDH is transported across the vacuolar membrane to interact with Rab5a is yet to be understood. An insertion mutagenesis strategy successfully created a viable GAPDH secretion mutant strain severely impaired in virulence, probably through its inability to inactivate phagosomal Rab5a. Listeriolysin O (LLO) is the main bacterial determinant responsible for the escape of L. monocytogenes from primary and secondary vacuoles and one of its major virulence factors. The gene that encodes LLO (hly) was the first virulence gene identified in Listeria, and is part of a locus that contains the main virulence genes (prfA, plcA, hly, mpl, actA and plcB). LLO is a secreted protein that belongs to the cholesterol-dependent cytolysin (CDC) toxin family. L. monocytogenes mutants deficient in LLO have difficulty to reach the host cell cytoplasm and are less virulent. The activity of LLO is optimal at the acidic pH of the phagocytic vacuole and decreases at the neutral pH of the cytoplasm, preventing excessive host cell damage. LLO binds to membranes as monomers that oligomerize into pore-forming complexes. Recently, it was shown that the cystic fibrosis transmembrane conductance regulator (CFTR) facilitates Listeria cytolicolic entry. It was proposed that CFTR transiently increases phagosomal chloride concentration after infection, potentiating LLO-mediated pore formation and vacuole lysis, which suggests that Listeria exploits mechanisms of cellular ion homeostasis to escape the phagosome. As other CDCs, LLO is a strong signaling protein that can induce several events in host cells, such as apoptosis, NFκB activation, upregulation of adhesion molecules and cytokines, activation of MAP kinase pathways and protein kinase C.

In addition, LLO influences the levels of intracellular calcium and stimulates phosphoinositide metabolism to favor bacterial entry. It is also a potent aggregator of lipid rafts and recently, LLO was shown to suppress the host immune response by decreasing protein SUMOylation (see evasion of host defense section).

Phagosomal membrane disruption is enhanced by the bacterial phosphatidylinositol-specific and broad-range phospholipases C, PI-PLC (plcA) and PC-PLC (plcb), respectively. The two enzymes cooperate with LLO in the lysis of primary and secondary vacuoles. PC-PLC is expressed as a proenzyme whose activation occurs in the acidified vacuolar environment and is mediated by the bacterial zinc metalloprotease Mpl. PrsA2 is a post-translocation chaperone reported to work at the bacterial membrane-cell wall interface to help in the folding of proteins translocated across the membrane. PrsA2 was shown to be important for L. monocytogenes pathogenesis by stimulating the activity and assuring the stability of LLO and PC-PLC. Absence of PrsA2 induces significant virulence attenuation in the mouse model and deficient bacterial cell-to-cell spread in host cell lines. PrsA2 is thus required for the stabilization and optimal activity of L. monocytogenes-secreted factors involved in vacuole escape. In addition, PrsA2 was shown to be also required for efficient Listeria intracellular replication (see below).

SvpA is a 64 kDa protein that is covalently bound to the cell wall by sortase B (SrtB) but can also be detected in culture supernatants. The virulence of a svpA mutant was shown to be strongly attenuated in infected mice. This reduced virulence was the result of an impairment in the intracellular multiplication of the mutant bacteria, as most of these remained trapped inside phagosomes, in contrast with wild-type bacteria that quickly escaped to the cytoplasm. SvpA appears thus as a virulence factor of L. monocytogenes that promotes bacterial escape from macrophage phagosomes.

Vacuole lysis is also assisted by SipZ, a type I signal peptide implicated in the secretion of PC-PLC and LLO. Lsp is a signal peptidase responsible for the maturation of L. monocytogenes lipoproteins. Mutation of the lsp gene results in a decrease in the ability to escape from phagosomes during infection of eukaryotic cells, which leads to low levels of virulence. In addition, expression of lsp is highly upregulated by bacteria still enclosed in infected macrophage phagosomes, suggesting a role for Lsp and lipoprotein maturation in the phagosomal escape of L. monocytogenes.

Recently, ActA, the protein responsible for the actin polymerization events that supply the propulsive force for bacteria to move through the cytoplasm (described below), was implicated in maturation of the L. monocytogenes phagosome. The exact role of ActA in this phenomenon is still unknown but various studies suggest a role for ActA in vacuole lysis, including abnormal permeabilization of the vacuole to both small and large molecules and the presence of lower number of ActA mutants in the cytoplasm.

**Intracellular survival and multiplication.** L. monocytogenes is remarkably adapted to survival and multiplication inside macrophages and other cell types. Once it evades from the phagocytic vacuole, Listeria can start replicating in host cell cytosol with a doubling time similar to that of growing in pure culture.

Listeria makes use of glucose-1-phosphate present in the cytoplasm for its energetic requirements. This metabolic process depends on the synthesis of Hpt, a hexose phosphate transporter protein that enables bacterial intracellular multiplication and is necessary for proliferation in mouse organs.

The lipoate ligation enzyme LplA1 is among other bacterial proteins required for growth in host cells. This enzyme is essential to perform a critical lipoyl modification in the E2 subunit of pyruvate dehydrogenase, in the presence of limiting concentrations of available host lipoil substrates, which suggests that abortive growth is due to loss of pyruvate dehydrogenase function.

**pcyA** encodes a pyruvate carboxylase, an enzyme of the tricarboxylic acid cycle. A pcyA insertion mutant is unable to replicate in mammalian macrophages and epithelial cells.

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The *L. monocytogenes* genome encodes Fri, the unique ferroptin involved in iron storage. Vertebrates are devoid of free iron, ensuring that all bacterial pathogens encounter a period of iron starvation upon entering their hosts. Fri has the capacity to control the level of free iron and hydrogen peroxide, and is essential for virulence and optimal bacterial growth during the infection process.150,151

A *L. monocytogenes* mutant for relA, a gene encoding a (p)ppGpp synthetase, is unable to accumulate (p)ppGpp in response to amino acid starvation.152 In addition to be required for full virulence in mice, RelA was shown to play a critical role for bacterial intracellular growth in non-phagocytic cells and macrophages. These results suggest that, after phagosomal escape, the capacity to mount a stringent response is pivotal for maximal *Listeria* intracellular replication.

PrsA2 was also shown to be necessary for optimal viability of *L. monocytogenes* within the host cell cytosol.153 Proteomic analyses unveiled that PrsA2 appears to have been adapted to facilitate *L. monocytogenes* survival under conditions of increased protein secretion, such as occurs during PrfA activation within the host cell cytosol.154

The oligopeptide-binding protein OppA was shown to mediate the transport of oligopeptides, and to be involved in intracellular survival in macrophages and in bacterial growth in organs of infected mice.155 These results seem to indicate that the uptake of oligopeptides is important for the intracellular behavior.

Small non-coding RNAs (sRNAs) are widespread effectors of post-transcriptional gene regulation in bacteria. The discovery of 150 putative regulatory RNAs was recently reported in *Listeria*.156 A total of 29 regulatory RNAs, including small non-coding antisense RNAs, are differentially expressed intracellularly. Mutants for three of these intracellularly expressed sRNA candidates revealed that their expression is required for efficient growth of bacteria in macrophages. In addition, the three mutants were attenuated when assessed for growth in mouse and insect models of infection.156 These results reveal extensive sRNA expression as an important feature of bacterial regulation during intracellular growth.

Interestingly, *L. monocytogenes* was shown to be able to replicate in macrophage vacuoles. In livers of infected SCID mice, bacteria were observed in large LAMP-1-positive compartments that were called spacious *Listeria*-containing phagosomes (SLAPs). In vitro, SLAPs were also identified and described as non-acidic and non-degradative compartments generated via an autophagy-dependent pathway.157 LLO was shown to be necessary and sufficient for SLAP formation, revealing a role for LLO in the vacuolar replication of *L. monocytogenes*, and suggesting that this bacterium is able to induce chronic infection of host macrophages.157,158

**Intracellular motility and cell-to-cell spread.** The polarized bacterial surface protein ActA is one of the major virulence factors of *L. monocytogenes*. On its C-terminal domain, ActA has a transmembrane hydrophobic tail region that retains the protein at the bacterial cytoplasmic membrane.159,160 ActA is responsible for the polymerization of actin filaments at one pole of the bacteria, forming a structure resembling a comet tail that enables bacterial propulsion and movement in the host cell cytosol. This allows the invasion of neighboring cells by a process called cell-to-cell spreading.161 ActA is sufficient to promote bacterial intracellular motility in the absence of other *Listeria* factors. In addition, ActA-coated latex beads are able to polymerize actin in cellular extracts in vitro.162 To stimulate actin polymerization, ActA mimics the activity of the WASP family proteins, which are host cell actin-nucleating factors.165 The N-terminal region of ActA is sufficient for its activity as it contains consensus sequences present in proteins of the WASP family, including an actin monomer-binding domain and two acidic regions that interact with and activate the Arp2/3 complex, a host actin nucleator.164,165 However, ActA lacks regulatory sequences that are present in WASP proteins that bind to Rho family GTPases. The central domain of ActA, a proline-rich repeat region, is required to recruit proteins of the Ena/VASP protein family, which modulates bacterial speed and directionality.170,172 VASP plays a dual role in *Listeria* actin tail formation: on one hand, it recruits profilin, an actin monomer-binding protein, that promotes actin polymerization; and on the other hand, it reduces the frequency of actin filament branching, favoring the assembly of long parallel filaments.173,174 The interaction of ActA with VASP plays a key role in the infection because ActA mutants that do not interact with VASP show a defect in intracellular spread and are avirulent in the mouse model of infection.172

A p60 mutant (see invasion section) was first shown to have intracellular motility and cell-to-cell spread levels drastically reduced in fibroblast and epithelial cell lines, since it failed to promote the formation of actin tails.97 The inability to fully complete cell division after septum formation, as a consequence of absence of p60, was shown to lead to ActA buildup at the presumed division sites, rendering the protein unavailable to induce actin polymerization into proper polar actin filaments.

Tuba and N-WASP are mammalian proteins known to regulate the structure of apical junctions in epithelial cells. Internalin C (InlC) contributes to formation of protrusions through inhibition of Tuba and N-WASP activity, probably by hindering the interaction between N-WASP and Tuba, which makes tense apical junctions become slack. Hence, it has been suggested that InlC favors bacterial dissemination by weakening cortical tension and therefore strengthening the capacity of motile bacteria to deform the plasma membrane into protrusions.175

**Regulation of Virulence Determinants**

Adaptability of pathogens is central to the infectious process and is determined not only by the bacterial factors allowing survival and multiplication within tissues, but also by the mechanisms required for the tight and coordinate regulation of their expression.

PrfA controls the expression of the key virulence determinants of *L. monocytogenes*.176 Products of the PrfA regulon mediate each step of the *Listeria* cellular infection cycle. The core PrfA regulon is composed of the ten virulence genes first identified as being PrfA-dependent.177 Independent studies have then identified nearly 160 *L. monocytogenes* EGD-e genes whose expression is directly or indirectly dependent on PrfA.177-182 *L. monocytogenes*
homodimeric PrfA activates transcription by binding to a palindromic promoter region (PrfA box) of canonical sequence rTAACAnntGTtAa, which contains seven conserved nucleotides (in capitals) and a tolerance of two mismatches. PrfA integrates both environment- and bacteria-elicited signals to ensure the proper spatio-temporal transcription of the regulon: strongest induction levels in the host cell cytoplasm and repression in the environmental habitat. Multiple mechanisms regulate PrfA expression and activity. The expression is simultaneously controlled by a RNA thermosensor mechanism, that allows translation at 37°C, and by a trans-acting riboswitch. This putative PrfA cofactor is yet to be identified, but links between carbon metabolism and PrfA-dependent transcription suggest that host nutrient availability may work as an intracellular localization signal for L. monocytogenes. Interestingly, a large proportion of the genes controlled by PrfA, and in particular the major virulence factors, were shown to be highly upregulated during infection of macrophages and mouse organs.

A recent temporal transcriptome analysis revealed that σB regulates a large and diverse set of genes (nearly 200) that are predicted to function in stress tolerance, carbohydrate metabolism, transport and cell envelope processes, and virulence. It is interesting to note that a significant subset of PrfA-regulated genes have potential σB promoter sequences. In addition, recent evidence has arisen suggesting that σB fine-tunes prfA expression inside host cells to avoid overexpression of virulence genes, which could result in excessive host cell damage and thus impair the success of bacteria in establishing infection. This finding highlights the overlap that exists between the control of the general stress response and the control of virulence-related functions.

VirR is a response regulator that belongs to one of the 17 Listeria two-component systems, VirR/VirS. The VirR/VirS system was identified by signature-tagged mutagenesis in a mutant showing reduced levels of liver colonization. VirR was shown to positively control the transcription of 17 genes. Among them, the dlt operon, involved in D-alanylation of cell wall LTAs and L. monocytogenes virulence (see adhesion section). VirR is also responsible for the activation of the mprF gene, which is implicated in phospholipid lysinylation, a modification that provides resistance to defensins (see invasion section). The fact that VirR regulates both dltA and mprF suggests that VirR/VirS system is important for the modulation of L. monocytogenes resistance against cationic peptides and constitutes another important virulence regulon involved in Listeria pathogenesis. The VirR regulon appeared highly regulated during infection. Indeed, most VirR-regulated genes (13 of 17), including virR, the dlt operon and mprF, were shown upregulated when Listeria infect mouse spleen, with 10 of these genes being also upregulated in infected macrophages. Reversely, 12 VirR-regulated genes are downregulated in bacteria present in the mouse intestine lumen.

MogR, DegU and GmaR are three regulators involved in the control of flagella production. Flagellar motility is an essential mechanism by which bacteria adapt to and survive in diverse environments. Although flagella confer an advantage to L. monocytogenes for colonization of the host (see invasion section), Listeria flagellins also stimulate host innate immune responses. Consequently, following initial infection, Listeria downregulates flagella production. At physiological temperatures (37°C and above) L. monocytogenes represses transcription of flagellar motility genes through MogR, a DNA-binding transcriptional repressor. At low temperatures (30°C and below), DegU, an orphan response regulator, activates expression of the anti-repressor GmaR that specifically inhibits MogR repression.

The RNA-binding protein Hfq plays important roles in bacterial physiology and is required for the activity of many small regulatory RNAs in prokaryotes. In L. monocytogenes, Hfq regulates multiple processes, such as stress tolerance and virulence and its responsiveness to stress is regulated by σB. From the 150 putative regulatory RNAs reported thus far, three Hfq-interacting small RNAs (sRNAs) were identified, and one functionally characterized. Three other appear to be required for efficient bacterial growth in macrophages (see intracellular multiplication section) and at least two are involved in virulence in mice.

Several expression analyses showed an overlap among regulons of key regulatory proteins of L. monocytogenes. In particular, PrfA and σB were shown to jointly contribute to processes like intracellular growth and virulence. Indeed, several virulence factors appeared under the control of one or even both of these transcriptional regulators (Fig. 2). Interestingly, most of the virulence genes described here are also upregulated in infected mouse organs. This supports the existence of complex regulatory networks that provide the cell with regulatory redundancies, along with the ability to fine-tune gene expression in response to rapidly changing environmental conditions, in particular when infecting the host.

Besides the transcriptional control of virulence-associated genes, the regulation of the secretion and surface anchoring of virulence factors is also essential to promote infection. Indeed, pathogenic bacteria target to their surface and secrete proteins that contribute to the colonization of host tissues and resistance to host immune responses. Listeria uses different mechanisms for the surface targeting and secretion of its virulence factors.

In L. monocytogenes, several LPXTG proteins play a crucial role in the infectious process (InlA, InlF, InlH, InlJ and Vip). This type of proteins are covalently attached to the bacterial cell wall following a transpeptidation reaction catalyzed by sortase A (SrtA). In accordance with the role of the virulence factors anchored by SrtA to the bacterial cell wall, a srtA-deficient mutant is defective in entry of eukaryotic cells and is impaired in the colonization of the liver and spleen after oral inoculation in mice.

The auxiliary protein secretion system (SecA2) of L. monocytogenes was shown to be involved in the secretion of several virulence factors involved in different steps of the infectious process (LAP, FbpA, p60, OppA, MurA and Sod). In accordance, SecA2 expression promotes bacterial adhesion and invasion, intracellular multiplication and cell-cell spread and is required for virulence in mice. In addition, SecA2-secreted
autolysins can promote the release of specific surface components that interfere with host pattern recognition (see evasion of host defense section). Interestingly, the secA2 gene, as well as genes encoding SecA2-secreted proteins, are coordinately regulated in infected cells and host organs. In particular, it was observed an upregulation of most genes encoding SecA2 substrates, including all the SecA2 components and SecA2-secreted virulence factors, in bacteria infecting mouse spleens, which strongly suggests their involvement in pathogenesis.

**Evasion and Modulation of Host Defenses**

*L. monocytogenes*, as other intracellular pathogens, developed strategies to hijack host cell functions to escape cellular defenses and immune responses.

PgdA, a peptidoglycan N-deacetylase, was identified in *L. monocytogenes*. The molecular characterization of the *L. monocytogenes* peptidoglycan further evidenced the deacetylation of N-acetylmuramyl residues. Peptidoglycan is the main structural component of the bacterial cell wall and is easily exposed to the host, constituting an important target for the innate immune system. Inactivation of the *pgdA* gene revealed the critical role of peptidoglycan deacetylation in bacterial virulence. A deletion mutant showed extreme susceptibility to the bacteriolytic activity of lysozyme, and growth was drastically affected following oral and intravenous inoculations of mice. In macrophages, the Δ*pgdA* mutant was rapidly killed and induced a very strong IFNβ response in a TLR2- and Nod1-dependent manner. Listeria peptidoglycan N-deacetylation appears thus critical for evasion of the host innate defenses.

p60 has been shown to indirectly enhance natural killer (NK) cell activation and increase innate IFNγ production, induce proinflammatory cytokines and modulate host immune response through an unknown mechanism. Coordinated peptidoglycan hydrolysis by p60 and MurA, a surface-associated N-acetylmuramidase, presumably generates a muramyl glycopeptide that is known to alter host inflammatory responses. Therefore, it has been suggested that the SecA2-dependent secretion of p60/MurA may promote release of muramyl peptides that overturn host pattern recognition. Nonetheless, the *L. monocytogenes* EGD-e genome encodes other peptidoglycan hydrolases that may participate in this process.

Flagellin is a potent proinflammatory protein that activates TLR5. However, flagella-dependent motility was shown to promote *L. monocytogenes* cell invasion (see invasion section). In addition, flagellin was also shown to be required for intestinal and liver colonization in the early phase of murine listeriosis. It was thus proposed that, through the control of a complex regulatory system (see regulation section), *L. monocytogenes* regulates flagella synthesis in time and space in order to promote gastro-intestinal colonization and cell invasion, and to evade host defenses at later stages of the infectious process.

Pgl, a 6-phosphogluconolactonase, is the second enzyme in the pentose-phosphate pathway, and was identified in a genetic screen aimed to find *L. monocytogenes* mutants that stimulated changes in host IFNβ expression levels. Infection with *L. monocytogenes* elicits a host cytotoxic surveillance response characterized by the expression of IFNβ. The *pgl* mutant induced high levels of IFNβ and, whereas growth in macrophages was not different from that of wild-type bacteria, the mutant strain showed a growth defect in vivo.

Interferons are released by mammalian cells upon attack by microbial pathogens, alerting neighboring cells to prepare a defense that includes the activation of so-called IFN-stimulated genes. In some cases, the IFN response accelerates clearance of bacteria, but in other instances, it results in a more severe disease. LntA was recently identified as a protein secreted by...
L. monocytogenes that controls the expression of IFN-stimulated genes. The mechanism allows the bacterium to govern both the induction and repression of the host cell immune response, perhaps to optimize conditions for specific stages of infection or colonization of specific tissues.\(^{214}\)

Besides the LLO functions above described (see vacuole escape section), LLO is also required for upregulation of adhesion molecules and chemokines in endothelial cells infected by L. monocytogenes.\(^{123}\) LLO was also shown to be critical for dephosphorylation of histone H3 and deacetylation of histone H4, during the early phase of infection,\(^{219}\) through a mechanism that involves LLO-induced pore-dependent efflux of potassium ions.\(^{216}\) Indeed, reduced levels of histone modifications were associated to modulation of specific host cell gene expression, suggesting that LLO-mediated genetic reprogramming of the host cell could be an additional mechanism whereby L. monocytogenes manipulates the host immune response. Since mitochondria constitute important innate immune response signaling integrators, the short-term fragmentation of these organelles, as elicited by extracellularly secreted LLO, may be another way for Listeria to interfere with host immune response-activating events to its own advantage.\(^{109}\) Post-translational modifications allow cells to quickly, locally and specifically modify the activity or interactions between key proteins. Some of these modifications, including phosphorylation and ubiquitinylation, can be induced by pathogens. It was recently shown that infection with L. monocytogenes results in a decrease in the amount of cellular SUMO-conjugated proteins. LLO triggers this event by inducing a proteasome-independent degradation of Ubc9, a critical enzyme of the SUMOylation machinery, and a proteasome-dependent degradation of some SUMOylated proteins. In addition, SUMO overexpression impairs bacterial infection. Listeria thus dampens the host response by reducing the SUMOylation level of proteins required for infection.\(^{130,217}\) Proliferation of CD4\(^+\) T cells upon T cell receptor (TCR) activation is highly decreased in the presence of LLO or wild-type L. monocytogenes but not of an LLO mutant. Expression data indicate that T cell unresponsiveness induced by LLO is caused by the induction of a calcium-nuclear factor of activated T cells (NFATC) cell-dependent transcriptional program supporting the transcription of negative regulators of TCR signaling. These findings contribute to a better understanding of how Listeria silences adaptive immune responses, enabling the persistence of the pathogen in the host.\(^{218}\)

inlH is a virulence gene whose expression is dependent on \(\sigma^B\) and is specifically induced during stationary phase and in the mouse intestinal lumen.\(^{187,219}\) InlH is not involved in bacterial invasion of cultured cell lines or intestinal cells in vivo. However, the virulence decrease of L. monocytogenes strains lacking inlH is accompanied by an increase in IL-6 production in infected tissues, during the systemic phase of murine listeriosis. This suggests that InlH improves bacterial survival in tissues by interfering with the inflammatory response.\(^{219}\)

Superoxide dismutases (SODs) are enzymes that protect organisms against superoxides and reactive oxygen species (ROS) produced during active metabolism. ROS are major mediators of phagocyte microbicidal activity. Listeria encodes a single SOD that plays a critical role in intracellular survival in macrophages and is required for full virulence, demonstrating that the antioxidant potential is a critical factor for L. monocytogenes pathogenesis.\(^{207,220,221}\) Strikingly, SOD can be phosphorylated and inactivated inside infected cells, highlighting a possible cross-talk between the host cell and the pathogen.\(^{107}\)

ActA plays also a pivotal role in evading autophagy. Autophagy is employed by the host cell as an innate mechanism to restrain the growth and survival of intracellular pathogens. Some bacteria are targeted by autophagy during early stages of infection. L. monocytogenes exploits the biomimetic property of ActA to camouflage itself with host proteins, such as Ena/VASP and the Arp2/3 complex, thereby escaping recognition by the autophagic system.\(^{222,223}\)

Finally, in addition to the role of MprF and Lgt in cell invasion (see invasion section), these proteins were shown to be critical for resistance to cationic antimicrobial peptides (CAMPs) and could represent another mechanism developed by L. monocytogenes to evade the host immune response.\(^{101,105}\)

**Conclusions**

In order to cause an infection, Listeria has many obstacles to overcome. As a foodborne intracellular pathogen, Listeria must first resist into the intestinal track: recognize and target its preferred cells, adhere and enter into these cells, delay phagosome maturation, resist to lysozyme, escape into the cytoplasm, control the production of different factors as toxins in order to allow infection without alerting the host, scavenger nutrients to replicate, polymerase cellular actin to move, and identify pathways to infect other cells. The exploitation of this complex niche was only made possible due to the development of an sophisticated cellular infectious cycle. This process features the intervention of a myriad of bacterial factors to overcome each step of this cycle. Various virulence factors can be implicated in the same cycle stage, although some are preponderant for maintaining the ability of L. monocytogenes to cause infection. On the other hand, a number of virulence factors were shown to operate in multiple steps, whereas the action of others is more restricted. The controlled spatio-temporal expression of these virulence effectors, as exerted by a number of protein and non-protein modulators, is vital for the adaptability of this pathogen to the specificities of the host intracellular environment and for the promotion of an efficient infection. The multiplicity and apparent redundancy of virulence strategies deployed by Listeria probably reflects the diversity of situations that Listeria must face during the infectious process and the multiple levels of attack and counter defense that are needed to overcome host defenses. Developing this arsenal, Listeria is capable to infect an extraordinary large variety of cells, tissues, organs and hosts. The host-pathogen interaction must be view as a highly dynamic process, where the pathogen is always developing strategies to improve its infectious capacity and circumvent defenses implemented by the host. In this context, some of the Listeria factors described here emerge as major virulent determinants (LLO, InIA, InIB, ActA, PrfA). Genes encoding these factors have the particularity to be absent from non-pathogenic Listeria species, controlled by
the major virulence regulator and expressed during in vivo infection. However, number of other virulence-related genes are contributing to the complexity of the Listeria infectious process. This is particularly the case of the adhesion and invasion infection steps where some factors appear to directly promote the process and can be considered as adhesins and invasins. Other virulence-associated proteins seem to contribute indirectly to these processes, for example by modifying the structure of the bacterial surface, and may be present in both pathogenic and nonpathogenic strains.

However, loss or modification in any of one of these factors may be determinant for attenuation in virulence. Interestingly, when we analyzed the presence of this arsenal of virulence factors in the different Listeria strains sequenced, we observed a clear correlation between the number of genes present and the virulence of the strain (Fig. 2). Most of the described virulence determinants are present in virulent strains (L. monocytogenes serovars 1/2a and 4b). Some of these virulence genes appear absent from the non-pathogenic L. monocytogenes strain, and roughly half of these factors are not encoded by the genome of non-pathogenic Listeria species. This is in particular the case of all the major virulence determinants.

Targeting bacterial virulence factors could be a promising approach to develop new strategies against resisting organisms.

In this context, the lack of a single, fixed or universal set of virulence factors could be perceived as a disadvantage. However, this could also allow the development of more specific agents directed against pathogenesis. The advantage is that inhibitors against specific virulence targets would probably not affect host cells and endogenous flora, and would not induce resistance.

The extraordinary complexity and coordination of the Listeria infectious process is noticeable and it seems reasonable that more virulence factors and regulators remain to be identified so that a complete and in-depth understanding of the intricate mechanisms leading to L. monocytogenes pathogenicity can be achieved.

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