Listeria monocytogenes
Membrane Trafficking and Lifestyle: The Exception or the Rule?

Javier Pizarro-Cerda1-3 and Pascale Cossart1-3

1Unité des Interactions Bactéries-Cellules, Institut Pasteur, Paris F75015, France
2INSERM, U604, Paris F75015, France
3INRA, USC2020, Paris F75015, France; email: javier.pizarro-cerda@pasteur.fr, pascale.cossart@pasteur.fr

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Abstract
Listeria monocytogenes is an intracellular bacterial pathogen that promotes its internalization within nonprofessional phagocytes by interacting with specific host cell receptors. L. monocytogenes resides transiently in a membrane-bound compartment before escaping into the host cell cytosol where bacterial proliferation takes place. Actin-based motility then promotes cell-to-cell pathogen spread. Extensive studies on cytoskeleton rearrangements, membrane trafficking, and other events have established this microorganism as an archetype of cellular function subversion for intracellular parasitism. Here we discuss the most significant membrane trafficking pathways hijacked by L. monocytogenes during the host cell infection process and compare them to those of other intracellular pathogens, in particular Shigella flexneri, Salmonella enterica, and Mycobacterium tuberculosis.
INTRODUCTION

Invasin of *Yersinia pseudotuberculosis* was the first reported bacterial gene product involved in the invasion of host eukaryotic target cells (Isberg et al. 1987), which opened the door to a new field that emerged at the boundaries of classical cell biology and microbiology: cellular microbiology. During the same period, the hemolysin gene of *Listeria monocytogenes* was characterized and became not only the first *L. monocytogenes* virulence factor to have its gene characterized but also the first bacterial gene product for which a function critical to bacterial survival within host cells was attributed (Mengaud et al. 1996). The prototype internalin (also known as InlA) is a cell wall covalently anchored protein, which binds the cellular adhesion molecule E-cadherin and induces bacterial entry into polarized human epithelial cells (Gaillard et al. 1991, Mengaud et al. 1996) (Figure 2). E-cadherin is normally involved in homophilic interactions for the establishment and maintenance of cell-to-cell contacts in epithelial tissues, and *L. monocytogenes* exploits the E-cadherin localization at the intestinal barrier to invade enterocytes during the initial stages of host colonization (Lecuit et al. 2001). E-cadherin is normally involved in homophilic interactions for the establishment and maintenance of cell-to-cell contacts in epithelial tissues, and *L. monocytogenes* exploits the E-cadherin localization at the intestinal barrier to invade enterocytes during the initial stages of host colonization (Lecuit et al. 2001). E-cadherin is also involved in the bacterial invasion of the syncitio-trophoblast layer at later placental infection stages (Disson et al. 2008). Through studies in human epithelial cell lines, it has been shown that, during host cell invasion, *L. monocytogenes* hijacks the molecular machinery associated with the cytoplasmic tail of E-cadherin. β- and α-catenins, which are have evolved fascinating adaptations to interact with and hijack host cell functions, and in the following sections we compare their molecular strategies with those of *L. monocytogenes* with the goal of drawing common themes in membrane trafficking subversion and intracellular lifestyle.

ENTRY INTO TARGET CELLS

Subversion of Host Cell Receptors for Pathogen Internalization

With the analysis of the *L. monocytogenes* genome, it was highlighted that this pathogen possesses an important arsenal of surface proteins that modulate bacterial interactions with the environment, in particular with host eukaryotic cells (Bierne & Cossart 2007). Among these bacterial surface molecules, the internalin family is characterized by the presence of amino-terminal leucine-rich repeat modules implicated in protein-protein interactions. Two members of this family are critical for the internalization of *L. monocytogenes* within nonprofessional phagocytic cells through interaction with cell-specific receptors (Bierne et al. 2007).
Entry: InlA & InlB

Vacuole lysis: LLO

Double-membrane vacuole lysis: LLO, PlcA, PlcB

Intracellular movement and cell-to-cell spread: ActA

Figure 1

Model of the *Listeria monocytogenes* intracellular life cycle. Interaction of the bacterial surface proteins InlA and/or InlB with specific cellular receptors at the plasma membrane of host cells induces the internalization of *L. monocytogenes* in a vacuole that is subsequently lysed by the pore-forming activity of the cholesterol-dependent cytolysin lysteriolysin O (LLO). Once in the host cell cytoplasm, *L. monocytogenes* proliferates and moves via the polymerization of host actin (stippled regions) triggered by the bacterial surface protein ActA. Bacteria that reach the host plasma membrane induce the formation of membrane protrusions that invade neighboring cells, and *L. monocytogenes* will be located in a double-membrane vacuole that is lysed by the activity of LLO and two bacterial phospholipases, PlcA and PlcB. Redrawn with permission from the *The Journal of Cell Biology* (Tilney & Portnoy 1989).

required to establish a functional link between E-cadherin and the actin cytoskeleton in adherens junctions (Imamura et al. 1999), are recruited by *L. monocytogenes* to entry sites and are required for actin remodeling during bacterial invasion (Lecuit et al. 2000). The Arp2/3 complex and its activator cortactin, which direct actin assembly in nascent cellular adhesive contacts in a Rac-dependent manner (Helwani et al. 2004, Kovacs et al. 2002), are also implicated during *L. monocytogenes* internalization (Sousa et al. 2007). Study of the InlA/E-cadherin invasion pathway allowed the identification of a novel guanosine-activating protein for RhoA and Cdc42 named ARHGAP10 that is involved in the recruitment of α-catenin to the *L. monocytogenes* internalization site and necessary for α-catenin recruitment to cell-cell junctions (Sousa et al. 2005). The unconventional myosin VIIa and its ligand vezatin are also functionally involved in the formation of adherens junctions and in the entry of *L. monocytogenes* in epithelial cells (Sousa et al. 2004).

The other major internalin-like molecule involved in entry is InlB, a bacterial effector loosely bound to the *L. monocytogenes* lipoteichoic acid, which can be released...
Entry of *Listeria monocytogenes* in host polarized cells via the InlA invasion pathway. In polarized epithelial cells, the bacterial protein InlA interacts with its receptor E-cadherin located in lipid rafts to promote cellular invasion. Caveolin-1, associated with lipid rafts, is involved in the Src-dependent phosphorylation of E-cadherin. This phosphorylation triggers the ubiquitination of E-cadherin by the ubiquitin ligase Hakai and the recruitment of a clathrin coat. β-catenin interacts directly with the cytoplasmic tail of E-cadherin to promote recruitment of α-catenin, which in turn interacts with actin and with other effectors including the Rho/Cdc42 guanosine activating protein ARHGAP10 and the small GTPase Arf6. Myosin VIIA provides the tracking force along actin filaments to promote bacterial internalization while its ligand vezatin mediates attachment to the membrane. Src is also involved in the phosphorylation of cortactin that promotes Arp2/3 complex activation and actin polymerization. Rac is implicated in this signaling cascade at a still-unknown position.

from the cell wall and interacts with three host cell molecules to produce successful bacterial entry: Its C-terminal domain binds extracellular matrix glycosaminoglycans (Jonquieres et al. 2001) and the receptor for the globular part of the complement molecule C1q (gC1q-R) (Braun et al. 2000), whereas its N-terminal leucine-rich repeats activate the hepatocyte growth factor receptor Met, a member of the receptor tyrosine kinase family (Shen et al. 2000) (Figure 3). Stimulation of Met with InlB mimics the
Figure 3

Entry of *Listeria monocytogenes* in host epithelial cells via the InlB invasion pathway. In a wide variety of epithelial cells, the interaction between InlB and its main signaling receptor Met leads to bacterial internalization. InlB interacts not only via its N-terminal domain with Met, but also through its C-terminal domain with extracellular glycosaminoglycans (GAGs) and with the gC1q-R. Activation of the tyrosine receptor kinase Met leads to its autophosphorylation and recruitment/phosphorylation of several protein adaptors including CrkII, Gab1, Shc, and Cbl, which in turn are involved in the recruitment of the type I PI 3-kinase (PI3K). Cbl is also a ubiquitin ligase required for Met ubiquitination and promoting clathrin recruitment and receptor internalization via endocytosis. The type I PI 3-kinase produces PIP3, and redistribution of this phosphoinositide within lipid rafts is involved in the activation of Rac (through recruitment of an as-yet-identified effector), which activates Wave and Arp2/3 for actin polymerization. Sept2, a new cellular effector required for InlB-mediated entry downstream of Met, is depicted (purple oval next to the plasma membrane) as well as the type II PI4Kα (red oval associated to the plasma membrane) involved in the production of PIP in a PI 3-kinase-independent signaling pathway.

physiological stimulation of the receptor with its natural ligand, the hepatocyte growth factor: Met dimerizes, autophosphorylates, and triggers the recruitment/phosphorylation of several protein adaptors including Gab-1, Cbl, Shc, and CrkII (Dokainish et al. 2007, Ireton et al. 1999). These adaptors are involved in the recruitment to the bacterial entry site of the phosphatidylinositol 3-kinase type I (PI 3-kinase) (Ireton et al. 1996), which is implicated in the activation of Rac-1 and the orchestration of a complex signaling cascade that leads not only to actin polymerization via the Arp2/3 complex, WASP-related proteins, and Ena/VASP (Bierne et al. 2005) but also actin depolymerization via LIM kinase and cofilin (Bierne et al. 2001). Recent research examining the InlB/Met signaling pathway has revealed the role of novel molecular partners including two type II phosphatidylinositol 4-kinases
implicated in a cascade independent of the PI 3-kinase pathway (Pizarro-Cerdá et al. 2007). Novel cytoskeletal proteins of the septin family also modulate \textit{L. monocytogenes} entry via the InlB-dependent pathway (Mostowy et al. 2009) (Figure 4).

Subversion of surface cellular adhesion/attachment receptors for invasion has been reported for other bacterial pathogens. Invasin, an invasion protein of \textit{Yersinia enterocolitica} and \textit{Y. pseudotuberculosis} (Isberg et al. 1987), promotes bacterial entry by binding members of the integrin family of extracellular matrix binding receptors (Isberg & Leong 1990). Similar to the interaction between \textit{L. monocytogenes} and E-cadherin, \textit{Y. pseudotuberculosis} takes advantage of the molecular machinery associated to the cytoplasmic tail of \(\beta_1\) integrins to orchestrate actin rearrangements required for bacterial entry: The kinases FAK and Src as well as the small GTPases Rac1 and Arf6 are subverted from their normal integrin-mediated signaling to favor actin polymerization via the Arp2/3 complex that induces pathogen internalization (Alrutz et al. 2001, Wong & Isberg 2003). It is important to note that \textit{Y. pseudotuberculosis} proliferates in the host as an extracellular pathogen and that cellular invasion is relevant during the early phases of host infection only (Bliska & Casadevall 2009). Probably as a strategy for persistence in host tissues, several other extracellular pathogens that bind extracellular matrix proteins access the intracellular space by stimulating the integrin-signaling pathway. For example, the \textit{Staphylococcus aureus} fibronectin-binding adhesin A binds fibronectin to engage \(\alpha_5\beta_1\) integrin and induces endothelial cell invasion in a FAK-, tensin-, cortactin-, and Arp2/3-dependent manner (Agerer et al. 2005, Massey et al. 2001). In a similar way, \textit{Streptococcus pneumoniae} binds vitronectin to promote entry in endothelial and epithelial cells via \(\alpha V\beta 3\) integrin engagement, integrin-linked kinase activation, and PI 3-kinase recruitment (Bergmann et al. 2009).

Uropathogenic \textit{Escherichia coli} (UPEC), a bacterium responsible for human urinary infections, is another extracellular pathogen that can invade host cells facultatively to establish a bacterial reservoir involved in recurrent urinary infections (Anderson et al. 2003). The UPEC adhesin FimH binds extracellular matrix
components such as laminin (Kukkonen et al. 1993); however, functional interaction between FimH and the monomannose moiety of the tetraspanin molecule uroplakin 1a leads to invasion of human bladder epithelial cells (Zhou et al. 2001). Bacterial entry requires the activation of small GTPases Cdc42 and Rac1, phosphorylation of FAK, PI 3-kinase recruitment, and actin binding by α-actinin/vinculin (Martinez & Hultgren 2002, Martinez et al. 2000). The obligate intracellular pathogen Rickettsia conorii, responsible for Mediterranean spotted fever, also invades host cells in a receptor-dependent manner: R. conorii takes advantage of its outer membrane protein B (rOmpB) to interact with the Ku70 subunit of the DNA-dependent protein kinase to invade host epithelial cells (Chan et al. 2009, Martinez et al. 2005). Among the intracellular molecules engaged by the rOmpB/Ku70 interaction are the protein adaptor Cbl, the kinase Src, Cdc42, PI 3-kinase, cortactin, and the Arp2/3 complex (Martinez & Cossart 2004).

Molecular mimicry and subversion of normal signaling cascades involved in the physiological function of surface receptors such as tyrosine receptor kinases, cadherins, or integrins are thus a common feature of intracellular bacterial pathogens, which exploit these signaling pathways to reorganize the cortical actin cytoskeleton and to produce membrane rearrangements required for bacterial engulfment. It is important to mention that the dynamics of actin filaments can be modulated by other cytoskeletal proteins including microtubules (Rodriguez et al. 2003), and disruption of microtubules inhibits the entry of several pathogens including L. monocytogenes and Campylobacter jejuni (Biswas et al. 2003, Kuhn 1998). In these cases, however, the specific molecular pathways involved in the potential actin-microtubules cross talks have not been explored. Very recent work on UPEC shows that histone deacetylase 6 (HDAC6), which deacetylates α-tubulin, can modify microtubule stability and affect kinesin-1 recruitment while inhibiting UPEC entry into bladder cells. This finding led Dhakal & Mulvey (2009) to propose that HDAC6 can modulate directional trafficking of kinesin-1 and associated cargos such as WAVE2, highlighting a molecular pathway that could link actin and microtubules during bacterial entry. As mentioned above, septins, a family of small GTPases that have the property to form nonpolarized filaments, which associate to actin and microtubules and are increasingly recognized as new elements of the cytoskeleton (Toolely et al. 2009), modulate the entry of L. monocytogenes in host cells (Mostowy et al. 2009) (Figure 4). These results should open new avenues through which to study the interaction of different cytoskeletal elements during bacterial invasion.

Lipid Rafts and Membrane Organization at Pathogen Entry Sites

Cellular receptors are located in membranes, and the physical environment of specific membrane domains affects the behavior of the receptors subverted by pathogens for entry into cells. Lipid rafts are specialized membrane microdomains enriched in cholesterol and sphingolipids (Simons & Ikonen 1997). Protein-lipid and protein-protein interactions participate in the formation of these dynamic structures, which serve as platforms to cluster signaling molecules that participate in a wide variety of processes including phagocytosis, cell migration, and immune responses (Lasserre et al. 2008). In many cases, their presence is required for bacterial entry into host cells. The invasion of target cells by L. monocytogenes requires the integrity of membrane microdomains (Seveau et al. 2004). Lipid raft markers such as glycosylphosphatidylinositol (GPI)-linked proteins, myristoylated and palmitoylated peptides, and the ganglioside GM1 are detected by immunofluorescence at bacterial entry sites (Seveau et al. 2004). Cholesterol depletion using the water-soluble cyclic oligosaccharide methyl-β-cyclo-dextrin (MβCD) reversibly inhibits L. monocytogenes entry in mouse fibroblasts expressing the human E-cadherin or in green monkey Vero kidney epithelial cells expressing a functional
Met receptor (Seveau et al. 2004). The presence of E-cadherin in lipid rafts is necessary for its initial clustering and interaction with InlA to promote bacterial entry (Seveau et al. 2004). In contrast, the initial interaction of InlB with Met does not require membrane cholesterol, but downstream signaling that leads to actin polymerization is cholesterol and lipid microdomain dependent (Seveau et al. 2004). PI 3-kinase activation downstream of Met is not affected by cholesterol depletion using MβCD, but activation of Rac1 downstream of PI 3-kinase is inhibited in cholesterol-depleted cells, which suggests that the spatial distribution of 3′-phosphoinositides produced by the PI 3-kinase within membrane microdomains is critical for Rac1 activation and consequently for actin polymerization at L. monocytogenes entry sites (Seveau et al. 2007).

Lipid rafts are required for the entry of many other pathogens within target cells. As mentioned above, FimH mediates UPEC internalization in human bladder cells by interacting with uroplakin 1a (Zhou et al. 2001). This receptor is associated with lipid rafts, and cholesterol depletion inhibits bacterial entry in host cells (Duncan et al. 2004). FimH is also involved in the entry of UPEC in macrophages and mast cells, and the fate of UPEC in these cells depends on whether bacteria interact with the GPI-anchored protein CD44 present in lipid rafts via FimH or whether bacteria are opsonized: In the first case, bacteria replicate in compartments that do not fuse with lysosomes; in the second case, opsonized bacteria are efficiently internalized but are degraded in lysosomes (Baorto et al. 1997, Shin et al. 2000).

Shigella flexneri and Salmonella enterica, the agents of bacterial dysentery and systemic typhoid fever, respectively, are enteropathogens that invade target cells by injecting bacterial effectors into their host cell cytosol through a macromolecular syringe-like apparatus known as the type 3 secretion system (T3SS) (Blocker et al. 2001, Kubori et al. 1998). At the S. flexneri entry site, GPI-anchored/lipid raft-associated proteins distinctly accumulate around the bacteria during epithelial cell invasion (Lafont et al. 2002). Initial binding of the S. flexneri T3SS effector IpaB to the cellular transmembrane protein CD44 takes place in detergent-resistant microdomains and is cholesterol dependent (Lafont et al. 2002). It has been proposed that rafts activate the S. flexneri T3SS to functionally translocate effectors into target cells (van der Goot et al. 2004). For S. enterica, its T3SS effector SipB, which is homologous to S. flexneri IpaB, binds cholesterol with high affinity prior to effector delivery and cellular invasion (Hayward et al. 2005).

Other bacterial species exploit lipid rafts during infection: Mycobacterium tuberculosis, the agent of human tuberculosis, survives within macrophages if entry takes place in cholesterol microdomains that allow the recruitment of coronin-1, a coat protein that may participate in the inhibition of lysosomal fusion with M. tuberculosis-containing compartments (Ferrari et al. 1999, Gatfield & Pieters 2000). The disruption of lipid rafts also inhibits cellular invasion by R. conorii (Martinez et al. 2005), Brucella abortus (Watarai et al. 2002), Campylobacter jejuni (Wooldridge et al. 1996), and some strains of Chlamydia (Stuart et al. 2003), among others. Also interesting is the fact that the internalization of many bacterial toxins from extracellular pathogens, including VacA from Helicobacter pylori, cholera toxin from Vibrio cholerae, and anthrax toxin from Bacillus anthracis, takes place via lipid raft-mediated endocytosis (Gupta et al. 2008, Saslowsky & Lencer 2008, Abrami et al. 2003).

The studies discussed above indicate overall that the organization of the plasma membrane in specialized microdomains is critical for the efficient orchestration of signaling during bacterial invasion processes. However, the specific structure of these specialized microdomains is far from completely understood, and emphasis on the role of cholesterol in the organization of these microdomains may have underscored the importance of other mechanisms of lateral segregation for membrane constituents—for example, the underlying skeleton, as proposed in the fence-picket model (Kusumi & Suzuki 2005), or the tetraspan web, which depends
on the ability of tetraspanins to interact with various other surface proteins to form a network of molecular interactions clearly distinct from lipid raft microdomains (Espenel et al. 2008). The next challenge is to identify precisely the specificities of these different membrane microdomains in order to understand their unique functional contributions to the signaling cascades subverted by intracellular bacterial pathogens.

**Clathrin and Caveolin: New Players and New Links with the Cytoskeleton**

Endocytosis is the internalization of macromolecules in cells, and several endocytic routes have been described; the main and best-characterized ones are clathrin- and caveolin-dependent mechanisms (Conner & Schmid 2003). Caveolin-1 is a protein that binds cholesterol, inserts as a loop into the inner leaflet of the plasma membrane, and self-associates to form coats on the surface of membrane invaginations, i.e., caveolae (Glenney & Soppet 1992). Because caveolin-1 binds cholesterol, the functions of caveolae and lipid rafts are often associated (Head et al. 2006, Pelkmans et al. 2005). Clathrin-mediated endocytosis requires the recruitment to endocytic sites of specific assembly proteins that recognize the cargo to be internalized in some cases and direct the targeting and assembly of clathrin coats to these internalization sites (Semerdjieva et al. 2008). The GTPase dynamin is involved in the fission of vesicles from the plasma membrane both in clathrin- and caveolin-dependent processes (Yao et al. 2005, Rappoport et al. 2008).

Analysis of the signaling cascade triggered by the InlB/Met interaction led to the demonstration that clathrin is required for cellular invasion by *L. monocytogenes* (Veiga & Cossart 2005, Veiga et al. 2007) (Figure 3). Indeed, the adaptor protein Cbl recruited by Met to the bacterial entry site is a ubiquitin ligase that is involved in the monoubiquitination of Met to induce its endocytosis (Petrelli et al. 2002). Met is also monoubiquitinated by Cbl upon cellular stimulation by InlB, and short interfering RNA (siRNA) depletion of several components of the endocytic machinery, including dynamin, clathrin, and the clathrin-interacting protein eps15, blocks the entry of *L. monocytogenes* in target cells (Veiga & Cossart 2005). Clathrin, dynamin, and auxillin (this latter protein is required for clathrin-coated vesicles disassembly) are detected by confocal microscopy at the *L. monocytogenes* invasion foci, and real-time imaging analysis of the infection process indicates that the dynamics of clathrin recruitment at bacterial invasion sites differ from the behavior observed during the formation of classical clathrin-coated pits and vesicles during endocytosis of smaller cargo (Veiga et al. 2007). Interestingly, depletion of clathrin or dynamin by siRNA inhibits actin polymerization, suggesting that the recruitment of the clathrin-endocytic machinery precedes the actin rearrangements required for InlB-mediated entry (Veiga et al. 2007).

These results challenge the prevailing dogma establishing that the upper-limit size of clathrin-coated vesicles is 150 nm (Cheng et al. 2007), implying that larger particles cannot be internalized through a clathrin-dependent mechanism. It is interesting to note, however, that large clathrin assemblies have been detected surrounding the base of nascent phagosomes containing opsonized beads (Aggeler & Werb 1982). Clathrin had also been previously observed in human epithelial cells by transmission electron microscopy at the entry sites of *Chlamydia trachomatis* (Wyrick et al. 1989) and *E. coli* expressing the adhesins AfaE/AfaD (Jouve et al. 1997), and use of the drug monodansylecadaverin, which acts as an inhibitor of the transglutaminase that participates in clathrin-dependent endocytosis, suggested that *S. aureus* also requires clathrin for the invasion of cultured osteoblasts (Ellington et al. 1999).

The involvement of the endocytic machinery in bacterial entry is, in fact, a widespread mechanism, and the recruitment of clathrin and dynamin also occurs for the *Listeria innocua* InlA/E-cadherin-dependent invasion pathway, for other zippering bacteria that include...
expressing the invasin protein of *Y. pseudotuberculosis*, and for latex beads of 1 or 5 μm coated with epidermal growth factor or fibronectin, which suggests a specificity of the endocytic-machinery requirement for receptor-dependent entry (Veiga et al. 2007). In agreement with this hypothesis, the entry of pathogens that do not use a classical receptor-mediated entry mechanism, but use a T3SS-mediated entry as does *S. flexneri* or *S. enterica*, does not require clathrin (Green & Brown 2006, Veiga et al. 2007). In addition, the FimH-dependent internalization of UPEC in bladder cells requires clathrin (Eto et al. 2008) and, interestingly, a noninvading pathogen such as enteropathogenic *E. coli* (EPEC), which attaches to the extracellular surface and induces membrane reorganization and pedestal formation through the infection of T3SS effectors, recruits clathrin at its attachment site (Veiga et al. 2007). However, how clathrin is assembled at the EPEC attachment site or during the entry of the endocytic-machinery requirement for transcytosis of meningitis-inducing *E. coli* K1 in brain microvascular endothelial cells (Rohde et al. 2003), and they are required for the transcytosis of meningitis-inducing *E. coli* K1 in brain microvascular endothelial cells (Sukumaran et al. 2002). In summary, the traditional machinery associated with endocytosis appears critical for the internalization of larger particles such as invading bacteria. The molecular pathways that link endocytosis and actin polymerization have been difficult to establish, but recent work from several laboratories has identified connections between these different pathways (Galletta & Cooper 2009, Kaksonen et al. 2006). The study

These results demonstrate that caveolin-1 recruitment precedes clathrin recruitment.

Dependence on Cbl, clathrin, and caveolin-2 (but not caveolin-1) has been demonstrated recently for the entry of *E. coli* expressing the rOmpB protein of *R. conorii* in HeLa cells (Chan et al. 2009). However, the hierarchy of recruitment of clathrin and caveolin-2 following cellular stimulation with rOmpB as well as the structural differences that caveolin-2 confers to the bacterial invasion site in comparison with caveolin-1 are not known. It is important also to note that *Pseudomonas aeruginosa* colocalizes with caveolin-1 and caveolin-2 during the invasion of type I pneumocytes. In addition, siRNA-mediated depletion of both caveolins leads to a diminution in bacterial entry. However, the selective depletion of caveolin-2 has an identical susceptibility to *P. aeruginosa* infection as the combined caveolin-1 and caveolin-2 knock-down, and only tyrosine phosphorylation of caveolin-2 is critical for *P. aeruginosa* invasion, which suggests a more important role for caveolin-2 in this process (Zaas et al. 2005).

As mentioned above, caveolin-1 binds cholesterol and hence associates with lipid rafts. During UPEC entry in the bladder epithelium, caveolin-1 is detected at bacterial entry lipidic microdomains, and the reduction of caveolin-1 expression by siRNA inhibits bacterial invasion (Duncan et al. 2004). Lipid raft-associated caveolae and caveolin-1 act as entry ports for *Porphyromonas gingivalis* in oral epithelial cells (Sukumaran et al. 2002) for Group A *Streptococcus* into epithelial and endothelial cells (Sukumaran et al. 2002). In summary, the traditional machinery associated with endocytosis appears critical for the internalization of larger particles such as invading bacteria. The molecular pathways that link endocytosis and actin polymerization have been difficult to establish, but recent work from several laboratories has identified connections between these different pathways (Galletta & Cooper 2009, Kaksonen et al. 2006). The study
of bacterial internalization will probably help us further understand how these connections take place (Veiga et al. 2007). Clathrin clearly assembles in a nonclassical way at sites of bacterial entry, and solving the structure of these assemblies, as well as defining the cross talk between caveolin and clathrin, will be an exciting task for the near future.

LIFE IN AND ESCAPE FROM THE VACUOLE

Maturation of the Vacuole

After the invasion of target cells, *L. monocytogenes* is transiently trapped in a membrane-bound compartment before it destroys the vacuolar membrane to escape in the host cell cytoplasm. *L. monocytogenes* was proposed to modify the vacuole maturation through modulation of the activity of the small GTPase Rab5a (Alvarez-Dominguez et al. 2008) (Figure 5). Rab5a (but not Rab5c) overexpression increases the degradation of a *hly* mutant *L. monocytogenes*, which cannot escape from the phagocytic vacuole (Alvarez-Dominguez & Stahl 1999). In macrophages infected with wild-type *L. monocytogenes*, Rab5a facilitates the translocation of the small Rho GTPase Rac to the bacterial-containing compartment, which in turn activates the NADPH oxidase at the vacuolar membrane for bacterial killing (Prada-Delgado et al. 2001). However, *L. monocytogenes* inhibits the exchange activity of Rab5a from an inactive GDP- to an active GTP-bound form by expressing a 40-kDa protein (Lmo 2459) that ADP-ribosylates Rab5a in its inactive form (Alvarez-Dominguez et al. 2008, Prada-Delgado et al. 2005). How this enzyme is translocated across the vacuolar membrane to interact with Rab5a is currently unknown.

Listeriolysin O and Vacuolar Escape

Lysis of the *L. monocytogenes* internalization compartment is mediated via the expression and activity of listeriolysin O (LLO), which is one of the major *L. monocytogenes* virulence factors (Cossart et al. 1989, Kathariou et al. 1989). LLO is one of the major *L. monocytogenes* virulence factors (Cossart et al. 1989, Kathariou et al. 1989).

**Figure 5**

*Listeria monocytogenes* residency in its internalization vacuole and escape to the host cytoplasm. After internalization in target cells, *L. monocytogenes* resides in a membrane-bound compartment before lysing the vacuole membrane to escape to the cytosol. (a) Activation of the small GTPase Rab5a is important for the Rac-dependent recruitment of the NADPH oxidase to the phagosomal membrane to favor bacterial killing. *L. monocytogenes* counterbalances this signaling cascade by producing a 40-kDa protein Lmo 2459, which is transported through the vacuole membrane (by an unidentified translocation step) and ADP-ribosylates/blocks Rab5a in an inactive GDP-bound form. (b) The host γ-interferon-inducible lysosomal thiol reductase (GILT) reduces the bacterial-produced listeriolysin O (LLO) and favors its oligomerization into an LLO pore, which is implicated in the reduction of vacuolar Ca²⁺ levels (inhibiting the recruitment of the lysosomal protein LAMP1). PI- and PC-PLC cooperate with LLO in the disruption of the vacuolar membrane. Acidification (H⁺) of the vacuole is important for LLO activity and PC-PLC maturation by a bacterial metalloprotease (not shown). (c) Disruption of the vacuolar membrane leads to bacterial translocation to the host cytoplasm and escape from phagosomal killing.
1987). LLO belongs to the large family of cholesterol-dependent cytolysins including perfringolysin O from *Clostridium perfringens* and streptolysin O (SLO) from *Streptococcus pyogenes*, and monomers of these toxins bind to cholesterol-containing membranes in which they oligomerize to form 20–30-nm-diameter pores (Bhakdi et al. 1985, Sekiya et al. 1993). Despite several decades of research, LLO’s precise mechanism of action on membranes and the molecular events that lead to membrane perforation are still elusive. The initial hypothesis that cholesterol is the cellular receptor for LLO, and therefore primarily required for toxin binding to target membranes, was refuted by the observation that LLO monomers that are preincubated with cholesterol can still efficiently bind membranes and oligomerize but are unable to form pores (Jacobs et al. 1998). Work performed with perfringolysin O and SLO suggests that cholesterol is critical for the prepore-to-pore transition (Giddings et al. 2003). Acidification of the *L. monocytogenes*-containing vacuole is required for membrane permeabilization (Beauregard et al. 1997), in agreement with the observation that LLO presents a higher hemolytic potential at acidic pH (Geoffroy et al. 1987) (Figure 5). However, acidification may be required for synthesis, release, or activation of other bacterial or host factors involved in phagosomal escape. Rupture of the phagosomal membrane is enhanced by a bacterial phosphatidylinositol-specific and a broad-range phospholipase C (PI- and PC-PLC, respectively) (Smith et al. 1995), and activation/release of the proPC-PLC to mature PC-PLC by a *L. monocytogenes*-encoded metalloprotease is precisely dependent on acidic pH (Marquis & Hager 2000). LLO also requires maturation through reduction of disulfide bonds, and full induction of the LLO lytic activity in vivo requires its reduction by the γ-interferon-inducible lysosomal thiol reductase (GILT), an enzyme that is delivered from lysosomes to maturing compartments of the endocytic/phagocytic pathway in antigen-presenting cells; *L. monocytogenes* replication in GILT-negative macrophages is impaired owing to delayed escape from the vacuole (Singh et al. 2008) (Figure 5).

What are the effects of LLO insertion on the *L. monocytogenes*-containing vacuole? LLO induces small-membrane perforations, which allow Ca\(^{2+}\) leakage from vacuoles shortly after infection and lead to an increase in the vacuolar pH and inhibition of vacuolar maturation, as measured by the delayed acquisition of the lysosomal-associated membrane protein 1 (LAMP1) in wild-type *L. monocytogenes*-containing compartments compared with those of LLO-negative mutants (Henry et al. 2006, Shaughnessy et al. 2006). Ca\(^{2+}\) favors endosomal fusion with lysosomes (Pryor et al. 2000), and calcium release could account for the LLO-mediated alteration of vacuolar maturation (Shaughnessy et al. 2006). In addition, LLO is inefficient in lysis of late-stage vacuoles that are positive for LAMP1 (Shaughnessy et al. 2006). Overall, these observations indicate that LLO favors the inhibition of vacuolar maturation and fusion with lysosomes in order to protect *L. monocytogenes* from the destructive effects of mature lysosomal enzymes. LLO may also make pores to mediate the translocation of PI- and PC-PLC to the cytosol, where they could access their substrate phospholipids present in the cytosolic leaflet for the bacteria-containing vacuole, thereby favoring vacuolar escape (Sibelius et al. 1996, Wadsworth & Goldfine 2002). This potential mechanism of translocation is reminiscent of that reported for the SLO of *S. pyogenes* and may be similar to the T3SS in gram-positive bacteria (Madden et al. 2001). More recently, it has been proposed that bacterial vacuolar lysis that is dependent on LLO (and not on PI- and PC-PLC) leads to a host cell innate response that triggers the control of *L. monocytogenes* intracellular growth by autophagy (Py et al. 2007) (see next section).

Several other pathogens escape from their internalization compartments to replicate in the host cytoplasm, but the molecular events leading to vacuolar escape are poorly understood. In the case of the *Rickettsia* species, phospholipase A\(_2\) (PLA\(_2\)) activity was reported for
Rickettsia prowazekii, and PLA2 was proposed to be involved in phagosomal escape (Ojcius et al. 1995). However, completion of the R. prowazekii and R. conorii genomes indicates that no such gene could be identified, but a gene that codes for a phospholipase D, which is expressed and detected in rickettsial lysates, could account for the previously described PLA2 activity (Renesto et al. 2003). Whether this enzyme plays a role in bacterial escape from the vacuole remains to be demonstrated. A similar situation has been reported for R. typhi because a hemolysin has been cloned and characterized in this pathogen (Radulovic et al. 1999), but its possible role in vivo has not been demonstrated.

Vacuolar escape of S. flexneri remains an elusive issue. In J774 murine macrophages, IpaB mutants remained trapped in a phagocytic vacuole, suggesting that IpaB plays a role in vacuolar lysis (High et al. 1992). Subsequent comparative studies of the S. flexneri IpaC protein and its S. enterica homolog SipC showed that S. enterica SipC mutants expressing IpaC were found in the cytoplasm of host cells, which suggests a role for IpaC in S. flexneri escape from the vacuole (Osiecki et al. 2001). Subsequently, mutation of the leucine-rich repeat protein IpaH was shown to slow down S. flexneri cytoplasmic localization, implicating this protein in the facilitation of vacuolar escape (Fernandez-Prada et al. 2000).

Recently, escape of M. tuberculosis and M. leprae from their phagosome to the host cytoplasm of myeloid cells has been described and proposed to depend on the type 7 secretion system ESX-1 (van der Wel et al. 2007); however, these observations remain controversial in the field. M. marinum, a pathogen responsible for tuberculosis in fish and also in humans, uses the ESX-1 system to secrete the protein ESAT-6, which has been directly implicated in the formation of vacuolar pore and bacterial escape from phagocytic vacuoles (Smith et al. 2008). Additionally, invasive S. pyogenes may be able to escape phagosomes through SLO-induced membrane perforation and cytoplasmic bacteria may be destroyed by innate defense mechanisms (Nakagawa et al. 2004) (see next section), but the functional relevance of vacuolar escape for Group A Streptococcus has not been discussed.

Work concerning bacterial escape from vacuoles has focused on the assumption that maintenance of the vacuolar membrane is dependent only on the activity of bacterial proteins or toxins that damage the vacuole physical integrity. It is important to mention that in the case of S. enterica, an intracellular pathogen that normally proliferates in a membrane-bound compartment, deletion of the protein SifA leads to bacterial localization in the host cell cytoplasm (Beuzon et al. 2000), which indicates that bacteria can also actively participate in the maintenance of the vacuolar membrane. A similar situation is probably true in the case of Legionella pneumophila (Isberg 2009), highlighting that bacterial interactions with host cell molecular pathways are also implicated in a complex balance between vacuolar membrane maintenance and disruption.

**INTRACYTOSOLIC SURVIVAL AND AUTOPHAGY**

Autophagy is an important cellular process involved in the degradation of cellular components in the cytoplasm, which allows protein turnover in starving cells or the removal of malformed or superfluous subcellular components by sequestration in a double-membrane compartment termed the autophagosome, which may be derived from the rough endoplasmic reticulum (rER) (Klionsky 2007). Analysis of rER markers coupled with morphological analyses permitted the initial characterization of this pathway, and several bacterial species including Brucella abortus and Legionella pneumophila have been shown to interact with autophagosomes to establish a successful intracellular replication niche within host cells (Pizarro-Cerdà et al. 1998). Only recently has the molecular machinery involved in autophagosome biogenesis been partially unraveled, and it has been demonstrated that this process can be used by the cell as an innate defense mechanism.
Figure 6
Autophagy as a host innate defense mechanism against *Listeria monocytogenes*. Bacterial localization in the host cytosol triggers the formation of autophagosomes to control bacterial proliferation. (a) The bacterial surface protein ActA brings vasodilator-stimulated phosphoprotein (VASP) and, indirectly, profilin to the bacterial tail, which induces the recruitment of actin monomers that are polymerized by the Arp2/3 complex into actin comet tails. This actin-based motility system allows *L. monocytogenes* to move in the host cytoplasm and to escape capture by autophagic vacuoles. (b) A ΔActA *L. monocytogenes* mutant actively producing LLO will be trapped by LC3/LAMP1-positive autophagosomes originally derived from the endoplasmic reticulum, thus inducing bacterial killing. Besides ActA expression, the secretion of phospholipases PI-PLC and PC-PLC seems important for wild-type bacteria to escape from autophagy.

After vacuolar escape, the *L. monocytogenes* surface protein ActA promotes cellular actin polymerization and bacterial movement in the host cell cytoplasm (Kocks et al. 1992) (Figure 6). In macrophages infected with a *L. monocytogenes* ΔActA mutant, chloramphenicol treatment was found to lead to bacterial sequestration of previously cytoplasmic bacteria into double-membrane organelles that resembled autophagosomes, which were decorated initially with the eER marker protein disulfide isomerase and subsequently with the lysosomal LAMP1 marker. This prompted Rich et al. (2003) to propose that the autophagic pathway could be used as a host defense mechanism against invading pathogens. Yuan and collaborators (Py et al. 2007) observed that autophagy limits the *L. monocytogenes* intracellular growth during the early time points after cellular invasion and vacuolar escape: Permeabilization of the vacuole by LLO (but not by phospholipases) triggers the bacterial sequestering in autophagosomes labeled by the microtubule-associated protein 1 light chain 3 (LC3) autophagic degradation compartments (Py et al. 2007). ActA was proposed as the main bacterial factor required for autophagosome escape during the first 3 hours after infection, but other virulence factors seem to be important to evade autophagy at later time points (Birmingham et al. 2007). In *Drosophila*, recognition of the *L. monocytogenes* peptidoglycan by the peptidoglycan-recognition protein (PGRP)-LE is crucial for the induction of autophagy and the prevention of bacterial intracellular growth (Yano et al. 2008). In SCID mice, LLO function has been highlighted again as required for the formation of spacious *L. monocytogenes*-containing phagosomes of autophagic origin (Birmingham et al. 2008).

After the establishment of a functional link between autophagy and control of *L. monocytogenes* intracellular proliferation (Rich et al. 2003), other reports documented the role of autophagy as an innate host defense mechanism against bacterial pathogens such as *M. tuberculosis* (Gutierrez et al. 2004). After internalization in macrophages, *M. tuberculosis*...
remains associated with a membrane-bound compartment and blocks the maturation of this compartment at an early phagosomal stage (Sturgill-Koszycki et al. 1994, Via et al. 1997). Autophagy stimulation by starvation or rapamycin treatment leads to M. tuberculosis phagosome colocalization with the LC3 autophagosomal marker and inhibition of mycobacterial intracellular survival (Gutierrez et al. 2004). The murine immunity-related p47 guanosine triphosphatase Irgm1 and its human ortholog IRGM trigger autophagy to eliminate intracellular mycobacteria (Singh et al. 2006). And ubiquitin-derived peptides with bactericidal activity against mycobacteria accumulate in autophagolysosomes and enhance bacterial killing (Alonso et al. 2007).

Autophagy has been demonstrated as a defense mechanism against S. pyogenes: In mouse embryonic autophagy-deficient Atg5−/− fibroblasts, bacteria that escape from vacuoles via an SLO-dependent mechanism are able to multiply and be released from infected cells (Nakagawa et al. 2004). The intercellular spread Λ (IcsA) protein of S. flexneri, required for the actin-based motility of this bacterium in the cytosol of host cells, is targeted by Atg5, thereby triggering autophagocytosis and bacterial killing. However, the T3SS effector IcsB blocks the IcsA/Atg5 interaction by directly interacting with Atg5 and, in this way, S. flexneri is able to escape autophagy (Ogawa et al. 2005). In the case of S. enterica, some bacteria-containing vacuoles that have been damaged by the activity of the T3SS, SPI-1 (normally required only for cellular invasion and not for intracellular survival), are targeted by autophagosomes and control potential bacterial replication in the host cell cytoplasm (Birmingham et al. 2006).

Cytoplasmic and membrane-bound intracellular pathogens are thus targeted by autophagy for destruction. The deciphering of the molecular events that trigger this new innate immune response will reveal whether a single pathway is always activated or whether diversity also exists in the formation of autophagosomes and different signaling cascades are activated in each case.

CELL-TO-CELL SPREAD

As mentioned above, L. monocytogenes moves in the host cell cytosol using an actin-based motility process. In nonconfluent cells, bacteria that reach the cellular plasma membrane induce the formation of long membrane protrusions that extend with bacteria at their tips but, surprisingly, never lyse or break. Actin-based motility is sufficient to promote protrusion formation, as revealed by an E. coli strain expressing invasin from Y. pseudotuberculosis for cellular invasion, LLO from L. monocytogenes to facilitate vacuolar escape, and IcsA of S. flexneri to produce actin polymerization (Monack & Theriot 2001). In confluent monolayers, bacterial protrusions penetrate in neighboring cells and lead to the formation of a double-membrane compartment from which L. monocytogenes escapes to initiate a new infection cycle. The term paracytophagy has been proposed to describe a normal cellular mechanism that allows epithelial cell engulfment of membrane fragments from neighboring cells, which is exploited in part by L. monocytogenes to form productive protrusions (Robbins et al. 1999). The inhibition of interaction between actin and members of the ezrin/radixin/moesin family results in the formation of short and collapsed protrusions, suggesting that ezrin, in particular, is critical for cell-to-cell spread (Pust et al. 2005). A recent study suggested that PI- and PC-PLC are required for the lysis of the inner membrane of the double-membrane vacuole formed after protrusion formation in secondary cells and that LLO lyses the outer membrane of this compartment (Alberti-Segui et al. 2007).

Few studies have been performed to address the cell-to-cell spread issue in other bacterial pathogens. In the case of S. flexneri, E-cadherin expression seems to be required for a productive protrusion formation (Sansonetti et al. 1994). Clearly, cell-to-cell spread is a complex phenomenon that requires inventive cell manipulation techniques to be fully addressed, but it is worth investigating given its key role during infection.
CONCLUSIONS
Survival and replication of bacterial pathogens within mammalian cells rely on a variety of molecular strategies. In this review, we describe in detail the membrane trafficking pathways hijacked by _Listeria_ and highlight that other bacteria, although using different effectors, can target the same pathways to subvert cellular functions and to hamper deleterious host responses. Knowledge of the specific cells or tissues in which these events occur in vivo will be critical for a full understanding of the infection process and poses a real challenge for future research.

DISCLOSURE STATEMENT
The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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