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Review

Biotechnological applications of *Listeria*'s sophisticated infection strategies

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Summary

Listeria monocytogenes **is a Gram-positive bacterium that is able to survive both in the environment and to invade and multiply within eukaryotic cells. Currently** ***L. monocytogenes*** **represents one of the most well-studied and characterized microorganisms in bacterial pathogenesis. A hallmark of** ***L. monocytogenes*** **virulence is its ability to breach bodily barriers such as the intestinal epithelium, the blood–brain barrier as well as the placental barrier to cause severe systemic disease. Curiously, this theme is repeated at the level of the interaction between the individual cell and the bacterium where its virulence factors contribute to the ability of the bacteria to breach cellular barriers.** ***L. monocytogenes*** **is a model to study metabolic requirements of bacteria growing in an intracellular environment, modulation of signalling pathways in the infected cell and interactions with cellular defences involving innate and adaptive immunity. Technical advances such as the creation of** ***LISTERIA*****-susceptible mouse strains, had added interest in the study of the natural pathogenesis of the disease via oral infection. The use of attenuated strains of** ***L. monocytogenes*** **as vaccines has gained considerable interest because they can be used to express heterologous antigens as well as to somatically deliver recombinant DNA to eukaryotic cells. A novel vaccine concept, the use of non-viable but metabolically active bacteria to induced immunoprotective responses, has been developed with** ***L. monocytogenes*****. In this mini-review, we review the strategies used by** ***L. monocytogenes*** **to subvert the cellular functions at different stages of the infection cycle in the host and examine how these properties are being exploited in biotechnological and clinical applications.**

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Introduction

Listeria monocytogenes is a ubiquitous, rapidly growing, Gram-positive intracellular bacterium with an unusually broad ecological niche and host range. It thrives in diverse environments such as soil, water, various food products, humans and animals. Infection of humans and animals has been traced to contaminated foods and can lead to serious, often fatal disease. In humans, disease is most common among pregnant women, newborns and immunocompromised individuals (Schlech, 2000). In humans, it causes a range of clinical manifestations from asymptomatic intestinal carriage and gastroenteritis to invasive and disseminated disease. Septicemia, meningoencephalitis and infection of the fetus in pregnant women are the most serious manifestations of listeriosis. The gastrointestinal tract is the primary site of entry for pathogenic *Listeria* species. After invasion of the small intestine, *L. monocytogenes* can spread to and infect the liver, spleen, central nervous system and, in pregnant women, the placenta (Vazquez-Boland *et al.*, 2001).

Listeria monocytogenes enters cells by adopting the endocytosis pathway and is able to propagate in most cell types, including macrophages, endothelial cells, fibroblasts, hepatocytes and other epithelial cells. Entry of *L. monocytogenes* into mammalian cells is a dynamic process that requires actin polymerization and membrane remodelling, and is an excellent example of how a bacterium can manipulate host–cell signalling and harness endocytic pathways to its advantage. The life cycle of *L. monocytogenes* reflects its remarkable adaptation to intracellular survival and multiplication in many cell types and has been used as a model for evaluation of the host–cellular interactions. Recent reviews (Dussurget *et al.*, 2004; Pamer, 2004; Hain *et al.*, 2006; Hamon *et al.*, 2006) have highlighted cell biological and immunological aspects of listerial biology. Here, we review the strategies used by *L. monocytogenes* to subvert the cellular functions at different stages of the infection cycle in the host

and examine them in the context of current and future clinical and biotechnological applications.

Host and bacterial factors in infection

Listeria monocytogenes is able to breach bodily barriers such as the intestinal epithelium, the blood–brain barrier as well as the placental barrier to cause severe systemic disease (Fig. 1). At the cellular level, *L. monocytogenes* is able to promote its own uptake into host cells through pathogen-induced phagocytosis. Following internalization in a vacuole, the bacterium lyses its membrane-bound compartment and multiplies in the cellular cytoplasm. In this particular environment, *L. monocytogenes* polymerizes the host actin to induce its own random movement, eventually reaching the host plasma membrane and invading adjacent cells through the formation of membrane protrusions and cell-to-cell spread (Pizarro-Cerda and Cossart, 2006). Mechanisms of *Listeria*-mediated entry into eukaryotic cells and its interaction with cell organelles are depicted in Fig. 2.

Invasion of non-phagocytic cells by *L. monocytogenes* is mediated by at least two bacterial surface proteins,

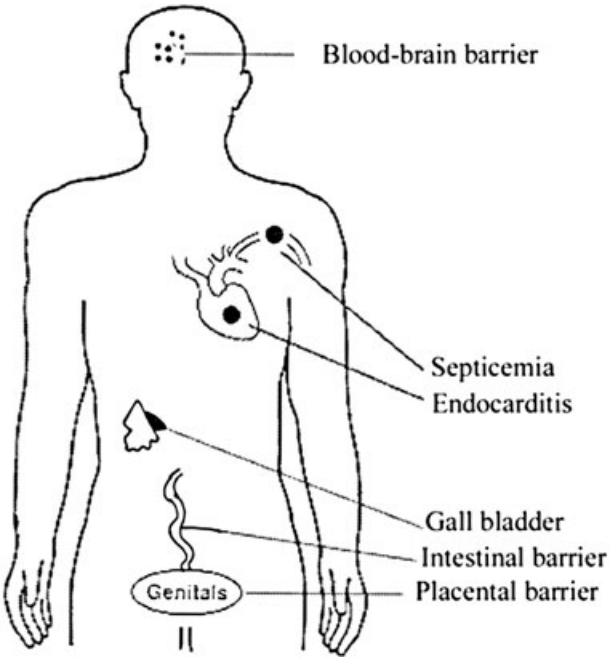

Fig. 1. *Listeria monocytogenes* crosses all important barriers in human body.

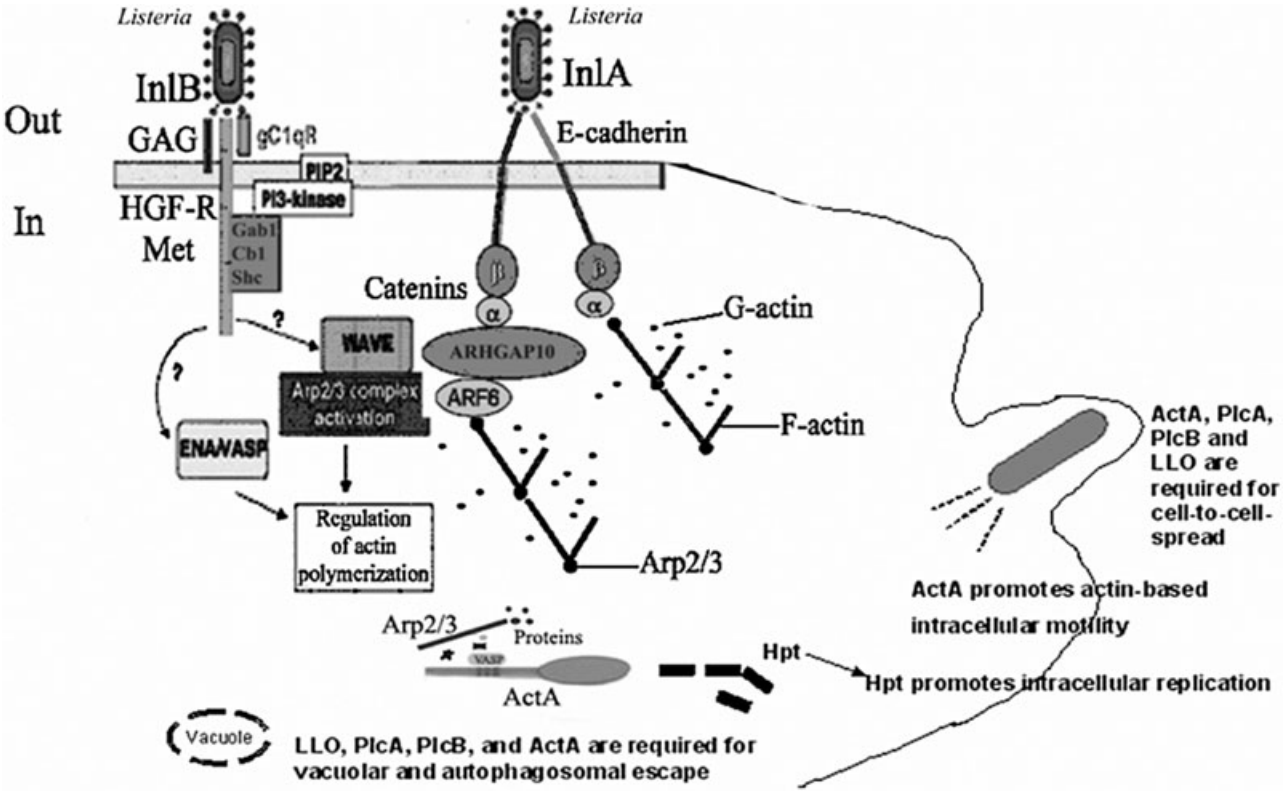

Fig. 2. Mechanisms of *Listeria*-mediated entry into eukaryotic cells. Zipper mechanism: bacterial uptake is promoted from the 'outside' by the interaction between bacterial adhesins and host cell surface molecules. Interaction activates host cell signalling pathways that result in moderate actin cytoskeleton rearrangements. The signalling component acronyms and their translations are given in Table 2.

Table 1. Examples of eukaryotic targets hijacked by *Listeria monocytogenes*.

Event	Host target	Effector protein/ phospholipid	Mechanism hijacked
Bacterial invasion	Human E-cadherin	InlA	Cytoskeleton dynamics linked to intercellular adherence and junction formation
	Glycoprotein gC1qR; Met/hepatocyte growth factor receptor (HGF); glycosaminoglycans (GAGs)	InlB	Activation of HFG receptor and PI-3-kinase-mediated signalling
Lifestyle in cytosol	Phagolysosome	LLO	Lyses the vacuole, pore formation
	Protein kinase C (PKC) Cascade	PI-PLC	Promotes escape of the bacterium from a macrophage-like cell phagosome
	Microsomal-6-phosphate transporter; translocase	Hpt	Mimicry of hexose-P intracellular growth
	Arp2/3 complex; actin; VASP	ActA	Promotes actin-based motility

Internalin A (InlA) and Internalin B (InlB) (Dramsi *et al.*, 1995; Cabanes *et al.*, 2002). In mice, *L. monocytogenes* invasion and replication within the gastrointestinal tract is independent of InlA and is restricted to the Peyer's patches, suggesting a predominant role for specialized phagocytic M-cells in bacterial uptake (Jensen *et al.*, 1998; Lecuit *et al.*, 1999). Similarly in rats, intestinal translocation rates are low and independent of the *InlAB* locus, suggesting a passive process that does not involve InlA or InlB (Pron *et al.*, 1998). In contrast, *L. monocytogenes* can directly invade enterocytes in guinea pigs, which are naturally susceptible to listeriosis (Lecuit *et al.*, 2001).

Utilizing two wild-type strains, differentially marked by their susceptibility to erythromycin, it was found that only a single bacterium was necessary to cause placental infection, and that *L. monocytogenes* trafficked from maternal organs to the placenta in small numbers. Surprisingly, bacteria trafficked in large numbers from the placenta to maternal organs. Once colonized, the placenta becomes a nidus of infection resulting in massive reseeding of maternal organs, where *L. monocytogenes* cannot be cleared until trafficking is interrupted by expulsion of the infected placental tissues (Bakardjiev *et al.*, 2006).

Listeria monocytogenes can replicate in the murine gall bladder where its replication is extracellular and intraluminal (Hardy *et al.*, 2004). The resistance of *L. monocytogenes* to acidic conditions (Cotter *et al.*, 2000) and to bile salts (Begley *et al.*, 2005; Sleator *et al.*, 2005) makes this pathogen particularly adept at infecting the gastrointestinal tract.

Invasion of target cells by *Listeria monocytogenes*

The surface proteins internalins InlA and InlB have been identified as the main bacterial factors involved in the invasion of polarized and non-polarized cells respectively (Gaillard *et al.*, 1991). Their presence is essential for effective invasion of these cell types. Transfer of the internalins to recombinant non-pathogenic *L. innocua* strains retains the specificity imparted by InlA and InlB respectively; however, levels of invasion remain low (~10%) when compared with the level achieved by parental *L. monocytogenes* strains implying a requirement for additional factors for effective invasion. Indeed, other molecules, unrelated to the internalin family, have been recognized as necessary to complete the host cell invasion programme (Cabanes *et al.*, 2004; Dramsi *et al.*, 2004; Cabanes *et al.*, 2005; Chatterjee *et al.*, 2006).

InlA

Invasion of *L. monocytogenes* of epithelial cells depends on the interaction between the bacterial surface protein InlA and the host protein E-cadherin. *L. monocytogenes* exploits the dynamic nature of epithelial renewal and junctional remodelling for entry (Pentecost *et al.*, 2006). InlA induces local cytoskeletal rearrangements in the host cell to stimulate uptake of *L. monocytogenes* by epithelial cells (Table 1). The usefulness of studying the expression of internalin as a marker of virulence in humans has been suggested (Jacquet *et al.*, 2004).

InlA is anchored covalently to the bacterial cell wall by an LPTTG motif in its C-terminal domain. It is characterized by the presence of a leucine-rich repeat domain in its N-terminal domain, followed by an inter-repeat domain. These two domains are sufficient to promote bacterial entry into target cells (Lecuit *et al.*, 1997). The distal intracellular domain anchors the adhesive complexes to the cortical actin cytoskeleton through interaction with catenins (Perez-Moreno *et al.*, 2003). β-Catenin binds in particular to the distal domain of the intracytoplasmic tail of E-cadherin and recruits the actin-binding protein α-catenin. A novel cellular component, ARHGAP10 (Rho GTPase-activating protein 10), has been identified that is involved in the recruitment of α-catenin to cell–cell junctions and is essential for listerial entry (Sousa *et al.*, 2005a). Furthermore, ARHGAP10 was shown to control the activity of RhoA and CDC42, two proteins that regulate cell–cell junction formation.

E-cadherin is present in several human barriers, such as the intestinal or the fetoplacental barrier (Lecuit *et al.*,

2001). The blood–brain barrier also harbours E-cadherin-expressing cells, suggesting that *L. monocytogenes* could also target the central nervous system by subverting E-cadherin function (Lecuit, 2005). It has been suggested that *L. monocytogenes* exploits the same molecular scaffold involved in the formation of adherens junctions for inducing its entry into target cells (Sousa *et al*., 2005b).

InlB

InlB causes subversion of the hepatocyte growth factor (HGF) receptor signalling to promote non-polarized cell invasion and exhibits a broader range of target cells than InlA (Gaillard *et al*., 1991; Dramsi *et al*., 1995). In contrast to InlA, InlB is loosely attached to the lipoteichoic acids through a series of modules called the GW domains (Braun *et al*., 1997). As with InlA, InlB harbours a series of leucine-rich repeats followed by an inter-repeat region in its N-terminal domain that are sufficient for inducing bacterial entry (Braun *et al*., 1999).

Binding of InlB to its cellular receptor Met, a protein tyrosine kinase, results in the entry of *L. monocytogenes* into different cell types. *In vivo*, Met is expressed mainly by cells of epithelial origin, whereas its ligand, HGF is produced mainly by fibroblasts and stromal cells. Binding of InlB activates the protein-tyrosine-kinase activity of Met, as well as the phosphatidylinositol 3-kinase (PI3K) and the Ras–mitogen-activated protein kinase (MAPK) pathways, all of which are required for the uptake process (Tang *et al*., 1996; Shen *et al*., 2000). Like HGF, InlB is able to induce the tyrosine autophosphorylation of Met and the recruitment of several adaptor molecules including Shc, Cbl and Gab1 (Basar *et al*., 2005; Sun *et al*., 2005). It leads to activation of the type IA phosphoinositide 3-kinase p85/p110 (Ireton *et al*., 1996) and of the small GTP binding protein Rac1 (Bierne *et al*., 2001), which are key events required for bacterial uptake. Both HGF and InlB also induce the ubiquitination and internalization of Met by a clathrin-mediated endocytosis mechanism (Li *et al*., 2005; Veiga and Cossart, 2005), leading to degradation of the receptor, and also to internalization of the bacteria. Recent evidence suggests that HGF-induced membrane ruffling and *Listeria* invasion mediated by the bacterial c-Met ligand InlB requires both Cdc42 and PI3-kinase signalling (Bosse *et al*., 2007).

The signalling pathways that are activated by InlB lead to cytoskeletal rearrangements and entry of *L. monocytogenes* (Table 2). Proteins of the Ena/VASP family (enabled homologue/vasodilator-stimulated phosphoprotein family), which promote actin-filament elongation, are also central to the process. In addition, cofilin, which is essential for depolymerization of actin filaments, functions successively as a stimulator and a downregulator of actin rearrangements that occur during the internalization process (Bierne *et al*., 2001; Bierne *et al*., 2005). Local actin remodelling at the site of InlB attachment is mediated by the recruitment and activation of the actin-nucleation complex, Arp2/3, which promotes actin nucleation and polymerization. Plasma-membrane microdomains known as lipid rafts have also been shown to be important for the entry of *L. monocytogenes* (Seveau *et al*., 2004).

Table 2. The signalling component acronyms and their translations involved in the mechanisms of *Listeria*-mediated entry into eukaryotic cells.

Acronym	Translation
ARF6	ADP-ribosylation factor 6
ARHGAP10	Rho GTPase-activating protein 10
Arp	Actin-related protein
Ena/VASP family	Enabled homologue/vasodilator-stimulated phosphoprotein
F-actin	Filamentous actin
G-actin	Globular actin
GAG	Glucosaminoglycans
ABI	Abl interactor 1
F-actin	Filamentous actin
GAB1	GRB2-associated binding protein 1
SHC	SRC-homology-2- domain-containing transforming protein C
WAVE	Wiskott–Aldrich syndrome protein (WASP)-family verprolin homologous protein.

Escape from the vacuole

The strong correlation between haemolytic activity and virulence in *L. monocytogenes* has been documented. Thus *hly* mutants were unable to escape from the internalization vacuole while wild-type bacteria were free in the cytoplasm (Gaillard *et al*., 1987). During infection, LLO has been shown to be required at a multitude of steps in the intracellular life cycle of *L. monocytogenes*, such as in modulating internalization, escape from the vacuole, proliferation in the cytoplasm and cell-to-cell spread (Cossart and Lecuit, 1998; Chakraborty, 1999; Vazquez-Boland *et al*., 2001). LLO enhances uptake of the bacterium, delays vacuole fusion with lysosomes (Henry *et al*., 2006; Shaughnessy *et al*., 2006), promotes its egress from the endocytic vesicle to the host cytoplasm (Hakansson *et al*., 2005; Alberti-Segui *et al*., 2007) and fosters the transfer of bacterial DNA (Grillot-Courvalin *et al*., 2002).

The *hly* gene is located within the virulence regulon that contains the most important genes involved in the pathogenesis of *L. monocytogenes*. LLO has narrow pH range of optimal activity, between pH 4.5 and 6.5, the optimal activity being at pH 5.5. The optimal pH range of LLO coincides with that observed in the bacterial internalization vacuole, suggesting that LLO is adapted to function only within the phagosomal compartment, a feature that would protect infected cells from the damaging activity of

LLO once the *L. monocytogenes* phagosome is lysed and the protein is free to interact with the cellular plasma membrane (Glomski *et al*., 2003). Recently, cleavage of LLO within the vacuolar compartment by the lysosomal aspartyl-protease cathepsin-D (Ctsd) was demonstrated. Enhanced susceptibility to *L. monocytogenes* infection of fibroblasts and bone-marrow macrophages had been shown (Schnupf *et al*., 2006). Increased intraphagosomal viability of bacteria in fibroblasts isolated from Ctsd-deficient mice compared with wild-type was observed. In addition, intracellular LLO was found to degrade in a proteasome-dependent manner, and prior to degradation, LLO was ubiquitinated and phosphorylated within the PEST-like sequence (Schnupf *et al*., 2006).

Cellular infection by *L. monocytogenes* induces an autophagic response, which inhibits the growth of both the wild-type and a *act*A deletion mutant strain, impaired in cell-to-cell spreading. *Listeria monocytogenes* are targeted for degradation by autophagy during the primary infection and in the early phase of the intracellular cycle, following LLO-dependent vacuole perforation but preceding active multiplication in the cytosol. Expression of bacterial phospholipases is necessary for the evasion of autophagy (Py *et al*., 2007).

A role for LLO in promoting *L. monocytogenes* replication in vacuoles and a mechanism by which this pathogen can establish persistent infection in host macrophages has been suggested (Birmingham *et al*., 2008). LLO is required for the formation of spacious *Listeria*-containing phagosomes (SLAP) in which the replication rate of bacteria was found to be greatly reduced as compared with those replicating in the cytosol. This implies that *L. monocytogenes* may exist as slow-growing forms that can persist for long periods in host cells depending on the immune status of the host (Birmingham *et al*., 2008).

LLO has been identified as a tool used by *L. monocytogenes* to manipulate the intracellular Ca^{2+} level without direct contact of the bacterium with the target cell. As Ca^{2+} oscillations modulate cellular signalling and gene expression, LLO at sublytic concentrations induces a broad spectrum of Ca^{2+}-dependent cellular responses during *Listeria* infection (Repp *et al*., 2002). Non-lytic concentrations of listeriolysin were shown to induce Ca^{2+}-permeable non-selective cation channels in human embryonic kidney cells (Repp *et al*., 2002) and erythrocytes (Föller *et al*., 2007). At these concentrations listeriolysin triggers suicidal death of erythrocytes as evident by exposure of phosphatidyserine on the erythrocyte surface and depletion of intracellular K+ (Lang *et al*., 2003a; Lang *et al*., 2003b; Föller *et al*., 2007). In addition to effects observed at sublytic toxin concentrations, at high concentrations of the toxin, pore formation and erythrocyte lysis occurs (Jacobs *et al*., 1998).

The oligomerization of LLO reportedly forms lipid rafts which induce signalling through co-aggregation of raft's associated receptors/kinases (Gekara *et al*., 2005). Other effects of LLO in nucleated host cells include stimulation of phospholipase C with formation of inositoltrisphosphate and diacylglycerol, triggering of MAP kinase pathways (Tang *et al*., 1996), expression of cytokines (Kohda *et al*., 2002), MUC genes (Lievin-Le *et al*., 2005), cell adhesion molecules (Krull *et al*., 1997), exocytosis (Coconnier *et al*., 1998; Coconnier *et al*., 2000) and host cell apoptosis (Guzman *et al*., 1996).

Other bacterial factors implicated in vacuolar lysis include a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad range phospholipase C (PC-PLC). LLO is essential for bacterial escape from vacuoles of most cell lines, whereas the phospholipases are dispensable but enhance the efficacy of vacuolar escape. In the absence of PI-PLC, bacteria have a significant lowered ability to escape from primary vacuoles and a defect in the ability to spread from cell-to-cell (Camilli *et al*., 1993). The absence of PC-PLC does not affect escape from primary vacuoles but significantly reduces the bacterium's ability to escape secondary vacuoles (Smith *et al*., 1995). The two bacterial phospholipases appear to have overlapping functions, as a double phospholipase mutant is more deficient in escape from secondary vacuoles than the sum of the defects observed with individual mutants (Smith *et al*., 1995). PC-PLC activation is assisted by a metalloprotease (Mpl) and is dependent on cell-to-cell spread and vacuolar acidification, indicating that it occurs specifically in vacuoles (Poyart *et al*., 1993; Marquis *et al*., 1997). Both types of PI-PLC produce diacylglycerol as well as inositol-1-P that releases Ca^{2+} from the endoplasmic reticulum (Goldfine *et al*., 2000). Constitutive secretion of active PC-PLC during intracellular infection causes host membrane damage during cell-to-cell spread and results in virulence attenuation in the mouse (Yeung *et al*., 2007). During infection, PC-PLC is activated specifically in acidified vacuoles. PI-PLC activates a host protein kinase C (PKC) cascade which promotes escape of the bacterium from a macrophage-like cell phagosome (Wadsworth and Goldfine, 2002). Bacterial PI-PLC and host PKC beta are responsible for phagosome permeabilization, which precedes escape (Poussin and Goldfine, 2005).

Intracellular motility and cell-to-cell spread

Cell-to-cell spread is a fundamental step in the infection cycle of *L. monocytogenes* that strictly depends on the formation of bacteria-induced protrusions. Bacterial movement inside the cytoplasm is a major virulence factor in that it is necessary for efficient colonization of the infected tissues. The bacterial surface protein ActA was identified as the molecular determinant required for the

induction of this actin-based motility system (Domann *et al*., 1992). ActA is a polarized surface protein anchored non-covalently to the *L. monocytogenes* cell wall by a transmembrane hydrophobic tail motif on its carboxyl terminal domain that traverses the cytoplasmic bacterial membrane (Domann *et al*., 1992; Kocks *et al*., 1992). Encapsulation of cytoplasmic bacteria by actin filaments was observed and it was used by the parasites to move inside infected cells and to spread to neighbouring cells (Tilney and Portnoy, 1989).

ActA is able to polymerize actin-enriched structures named 'actin comet tails' by mimicking the activity of a eukaryotic family of actin nucleating factors, the Wiskott–Aldrich syndrome protein (WASP) family of proteins. WASP proteins present a C-terminal region called VCA (for verprolin homology, cofilin homology and acidic regions). VCA favours actin polymerization by binding monomeric globular actin and a seven-polypeptide complex comprising the actin related proteins Arp2 and Arp3 (Stradal *et al*., 2004). Ena/VASP proteins enhance actin filament elongation via the recruitment of profilin:actin complexes to sites of active actin remodelling such as the tips of spreading lamellipodia and the surface of intracellular *Listeria* (Sechi and Wehland, 2004). Moreover, Ena/VASP proteins not only enhance actin filament elongation but also influence the activity of the Arp2/3 complex and counteract the inhibition of actin polymerization by capping proteins. Thus, Ena/VASP proteins act as multifunctional organizers of the actin cytoskeleton. ActA has also been reported to participate in the attachment and entry of *L. monocytogenes* in certain cell lines (Suarez *et al*., 2001) by interacting with glycosaminoglycans, but the precise mechanisms underlying this phenomenon are still unknown.

The ability to spread from cell to cell without coming in contact with the extracellular milieu allows the bacterium to propagate through tissues and avoid contact with circulating antibodies or other extracellular bactericidal compounds. *Listeria monocytogenes* is driven through the infected host cytoplasm by a comet tail of actin filaments that serves to project the bacterium out of the cell surface, in pseudopodia, to invade neighboring cells. The characteristics of pseudopodia differ according to the infected cell type (Sechi *et al*., 1997). The exit of a comet tail from bulk cytoplasm into a pseudopodium was associated with a reduction in total F-actin, the shedding of α-actinin, and the accumulation of ezrin, a member of the ezrin, radixin and moesin (ERM) protein family (Sechi *et al*., 1997). Ezrin, that functions as a key membrane-cytoskeleton linker, accumulates at *Listeria* protrusions (Pust *et al*., 2005). The ability of *Listeria* to induce protrusions and effectively spread between adjacent cells depends on the interaction of ERM proteins with both a membrane component such as CD44 as well as actin filaments. Interfering with either of these interactions or with ERM proteins phosphorylation reduces the number of protrusions and alters their morphology, resulting in the formation of short and collapsed protrusions. As a consequence, *Listeria* cell-to-cell spread is severely impaired. Thus, ERM proteins are exploited by *Listeria* to escape the host immune response and to succeed in the development of the infection (Pust *et al*., 2005).

Cell extrusion from the villus tips is a rapid and continuous process (Babyatsky and Podolsky, 2003). Therefore, in addition to providing a means for *L. monocytogenes* to evade the immune system (Portnoy *et al*., 2002), actin-based cell-to-cell spread within the villus tip may be beneficial to maintain an intracellular replicative niche in the face of a dynamically renewing system. The ability of *L. monocytogenes* to thrive and spread intracellularly is clearly responsible for its capacity to cause severe invasive disease and to disseminate to distant organs.

Intracellular lifecycle: virulence factor versus adaptive factors

The observation that non-pathogenic listeriae such as *L. innocua* are unable to grow efficiently in the host cytoplasm (Goetz *et al*., 2001; Slaghuis *et al*., 2004) has led to the suggestion that adaptation is required to enable growth of *L. monocytogenes* in the intracellular milieu. *Listeria innocua* equipped with the *prfA*, *hly* and *hpt* genes of *L. monocytogenes* did not show significantly increased cytosolic replication, which indicated that expression of this sugar phosphate uptake system was not sufficient for extensive listerial replication in the cytosol of host cells (Slaghuis *et al*., 2004). Recently, we have demonstrated that introduction of virulence gene cluster into *L. innocua* recapitulates many aspects of cellular infection by *L. monocytogenes* (Hain *et al*., 2008). However, the efficacy of growth of recombinant *L. innocua* strain is much lower than that of *L. monocytogenes* EGD-e, indicating additional factors are required for efficient growth in the host cytoplasm. Adaptive gene expression permits intracellular pathogens to successfully persist and disseminate during encounters with the host defences in the diverse intracellular microenvironments within the cell.

Apart from the role of the dedicated virulence factors in overcoming cellular barriers it is apparent that adaptive changes in bacterial cellular physiology and metabolism during infection have been instrumental in making *L. monocytogenes* a successful facultative intracellular pathogen. Studies on the gene expression profile of *L. monocytogenes* inside the vacuolar and cytosolic environments of the host cell by using whole-genome microarray and mutant analyses revealed mobilization of approximately 17% of the total genome to enable adaptation for intracellular growth (Chatterjee *et al*., 2006).

Intracellularly expressed genes showed responses typical of glucose limitation within bacteria, with a decrease in the amount of mRNA encoding enzymes in the central metabolism and a temporal induction of genes involved in alternative-carbon-source utilization pathways and their regulation. Adaptive intracellular gene expression involved genes that are associated with virulence, the general stress response, cell division and changes in cell wall structure and included many genes with unknown functions (Chatterjee *et al*., 2006). Strain and species-specific differences in expression suggest heterogeneity in the gene pool required for intracellular survival of *L. monocytogenes* in host cells.

Listeria monocytogenes exploits hexose phosphates (HP) from the host cell as a source of carbon and energy to fuel intracellular growth (Chico-Calero *et al*., 2002). Transcription of *hpt*, a bacterial homolog of the mammalian translocase that transports glucose 6-phosphate from the cytosol into the endoplasmic reticulum in the final step of gluconeogenesis and glycogenolysis, is regulated by PrfA and is activated upon entry into the cytosol of the host cell. The regulatory effect on PrfA caused by glucose may be exploited by *L. monocytogenes* to fine-tune the levels of intracellular virulence gene expression. Glucose is clearly a potential growth substrate for intracytosolic bacteria. However, in *L. monocytogenes* the uptake of that sugar triggers downregulation of the PrfA regulon. HP uptake, in contrast, does not provoke the repressor effect triggered by glucose (Ripio *et al*., 1997; Agaisse *et al*., 2005), making the use of a carbon source compatible with the induction of *prfA* and the PrfA-dependent virulence genes necessary for the completion of the intracellular life cycle. This mechanism may be important for the maintenance of the intracellular replication niche of *L. monocytogenes*, as it could help preventing cytotoxicity caused by an excessive number of the cytosolically growing bacteria (Chico-Calero *et al*., 2002). Other bacterial pathogens such as *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri* and *Chlamydia trachomatis* have a homologue of *hpt*, indicating that this transporter system could have a general role in intracellular survival (Hamon *et al*., 2006). Hpt appears to be important adaptive factor for intracellular survival and is a clear example of how adaptation to intracellular parasitism by microbial pathogens involves mimicry of physiological mechanisms of their eukaryotic host cells.

Additional genes expressed during intracellular growth have been detected to be under the regulation of the PrfA transcriptional activator protein. These include lmo0206 (*orfX*) and lmo0207 (*orfZ*), encoding gene products of unknown function as well as *prsA* which encodes a product homologous to the post-translocation molecular chaperone protein PrsA of *Bacillus subtilis* (Chatterjee *et al*., 2006).

Table 3. The different areas of biotechnology where the knowledge on *Listeria* is being harnessed toward application.

Biotechnological application	
Intracellular parasitism	Pathogenesis
	Pathophysiology
Patho-Biotechnology	Diagnostics
	DNA based vaccines
Model for cell biologist	T-cell responses
	Host pathogen interactions
	Cellular processes
Tumor Biology	Cytosolic drug delivery
New drug discovery	Inhibitors of intracellular replication
Drug delivery to target cells	hpt
	LLO

Listeria monocytogenes lacking the lipoate protein ligase LplA1 has been found defective for growth specifically in the host cytosol and less virulent in animals by a factor of 300 (O'Riordan *et al*., 2003), suggesting the use of host-derived lipoic acid may be a critical process for *in vivo* replication of bacterial pathogens.

Implications for Biotechnology

Listeria monocytogenes has become a paradigm for the study of host–pathogen interactions and bacterial adaptation to mammalian hosts (Hamon *et al*., 2006). Analysis of *L. monocytogenes* infection has provided considerable insight into how bacteria invade cells, move intracellularly and disseminate in tissues, as well as to generate tools to address fundamental processes in cell biology. The vast amount of knowledge that has been gathered through in-depth comparative genomic analyses and *in vivo* studies makes *L. monocytogenes* one of the best-studied bacterial pathogens. The different areas of biotechnology where the knowledge on *Listeria* is being harnessed toward application are given in Table 3.

Listeria monocytogenes infection has been a useful model for evaluation of the cellular interactions that are crucial for the initiation of the host T-cell response. Indeed, the ability of *L. monocytogenes* to invade and replicate in different cell types has been extensively studied and has revealed the sophisticated relationship between the bacterium and its host. As a facultative intracellular bacterium, *L. monocytogenes* has adapted to live within the cytosol of the host cell. Cytosolic delivery of an antigen facilitates the generation of a productive CD8+ T-cell response to the particular antigen. A new concept for vaccines was recently propagated using *L. monocytogenes* whereby, recombinant psoralen-inactivated *L. monocytogenes* lacking *uvr*AB genes vaccines induced potent CD4(+) and CD8(+) T-cell responses and protected mice against virus challenge in an infectious disease model as well as provided therapeutic benefit in a mouse cancer model (Brockstedt *et al*., 2005). Microbially inert but meta-

bolically active vaccines used either as a recombinant vaccine platform or as a modified form of the pathogen itself may have broad use for the treatment of infectious disease and cancer.

As *Listeria* survives within antigen-presenting cells after phagocytosis, it is an ideal vector for the delivery of antigens to be processed and presented through both the class I and II antigen-processing pathways (Singh and Paterson, 2006). Listeriolysin O-deficient *L. monocytogenes* impart protective immunity and that this highly attenuated strain may be a useful platform for vaccine delivery (Hamilton *et al*., 2006). The function of LLO as a pH-sensitive phagosomal lysin has been exploited for vaccine development to intracellular pathogens and cancer cells either for delivery of pH-sensitive liposomes containing nucleic acid-based compounds or protein antigens or expression of LLO by attenuated bacterial vaccine strains expressing the desired antigens or carrying DNA vaccine vectors (Provoda and Lee, 2000; Dietrich *et al*., 2003). Indeed, an LLO expressing tuberculosis vaccine strain *Mycobacterium bovis* bacille Calmettee-Guérin (BCG) is currently being tested in clinical trials for vaccine efficacy (Grode *et al*., 2005).

A major obstacle to achieving effective DNA-based therapeutics is efficient delivery of the DNA to its site of action in the cell. *Listeria* has been used for DNA delivery in two ways. Intracellular bacteria have been shown to transfer eukaryotic expression plasmids to mammalian host cells *in vitro* and *in vivo*. This can be used to induce immune responses towards protein antigens encoded by the plasmid, to complement genetic defects or even to direct the production of proteins in appropriate organs. The ease of generating such vehicles makes this a highly attractive area for further research (Weiss and Chakraborty, 2001). Functional transfer of GFP-CFTR (cystic fibrosis transmembrane conductance regulator) to eukaryotic cells using attenuated *L. monocytogenes* mediated gene transfer (bacteriofection) has been demonstrated (Krusch *et al*., 2002; Zelmer *et al*., 2005). Second, LLO, the haemolytic toxin of *L. monocytogenes,* is capable of facilitating transfection using an anionic liposome-entrapped polycation-condensed DNA delivery system (LPDII). This anionic delivery vehicle represents the successful combination of the LPDII system for condensation of the DNA with the unique endosomolytic properties of LLO for improved transfection using plasmid DNA (Lorenzi and Lee, 2005).

In causing disease, pathogens outmaneuver host defences through a dedicated arsenal of virulence determinants that specifically bind or modify individual host molecules. Newly emerging diseases mostly involve existing pathogens whose arsenal has been altered to allow them to infect previously inaccessible hosts. A single amino acid difference in the mouse cellular receptor for InlA, E-cadherin, prevented it from binding InlA, thereby showing the inadequacy of the mouse model for study of the invasive role of InlA (Lecuit *et al*., 1999). After analysing the recognition complex of the InlA protein and its human receptor E-cadherin, two single substitutions of amino acids in InlA has been found to increase binding affinity by four orders of magnitude and extend binding specificity to include formerly incompatible murine E-cadherin. A versatile murine model of human listeriosis has been created by substitution of amino acids (Wollert *et al*., 2007).

Drosophila melanogaster has been used as a model for many genetic and immunological studies and it has been successfully used to test *L. monocytogenes* virulence (Mansfield *et al*., 2003). Many new host factors important for entry into the host cell, escape from the vacuole and intracellular growth of *L. monocytogenes*, have been revealed using genome-wide RNA-interference screens in *D. melanogaster* S2 cells (which are macrophage-like cells) (Agaisse *et al*., 2005; Cheng *et al*., 2005).

An emerging area is the use of *L. monocytogenes* as a model to screen for efficacy of antibiotics during intracellular growth. Discrepancies between resistance *in vitro* and therapeutic efficacy *in vivo* can result from differential *in vitro-in vivo* expression of bacterial determinants of antibiotic susceptibility. Thus Hpt, an adaptive factor promoting listerial virulence is induced *in vivo*, and render cells *L. monocytogenes* very sensitive to fosfomycin treatment during intracytoplasmic growth but not during growth in broth cultures (Scortti *et al*., 2006).

Listeria monocytogenes have evolved sophisticated strategies to overcome host defences, to interact with the immune system and to interfere with essential host systems. Exploring the knowledge of these strategies can help in the design of more technologically robust and effective biotechnological and clinical applications. The term ‘patho-biotechnology’ has been coined to describe the exploitation of these valuable traits in biotechnology, medicine and food (Sleator and Hill, 2007). The approach shows promise for the development of novel vaccine and drug delivery systems. The genetic tractability of *L. monocytogenes*, the availability of the complete genome sequence of this intracellular pathogen, its ability to cope with stress and its ability to traverse the gastrointestinal tract and induce a strong cellular immune response make *L. monocytogenes* an ideal model organism for demonstrating the patho-biotechnology concept.

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