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Title

How the study of *Listeria monocytogenes* has led to new concepts in biology

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Abstract

The opportunistic intracellular bacterial pathogen *Listeria monocytogenes* has in 30 years emerged as an exceptional bacterial model system in infection biology. Research on this bacterium has provided considerable insight into how pathogenic bacteria adapt to mammalian hosts, invade eukaryotic cells, move intracellularly, interfere with host cell functions and disseminate within tissues. It also contributed to unveil features of normal host cells pathways and unsuspected functions of previously known cellular proteins. This review provides an updated overview of our knowledge on this pathogen. In many examples, findings on *Listeria monocytogenes* provided the basis for new concepts in bacterial regulation, cell biology and infection processes.

Key words

Listeria monocytogenes, cellular microbiology, virulence, microbiota, RNA-based regulation, PrfA, actin, post-translational modifications

Introduction

Research on infection by pathogenic micro-organisms aims at understanding the molecular mechanisms used by pathogens to establish an infection. It often provides insights into fundamental aspects of cell biology and immunology. For example, the mechanism used by enveloped virus to enter host cells helped explaining receptor-mediated endocytosis and intracellular transport. *Listeria monocytogenes*, the causative agent of listeriosis, has also led to a number of discoveries in various fields that we will discuss in this review.

L. monocytogenes was identified by E.G.D. Murray in 1926 as a Gram-positive bacillus responsible for epidemic cases of mononucleosis in rabbits and guinea pigs in animal care houses in Cambridge (UK) and named as *Bacillus monocytogenes* [1]. A few years later, it was shown to cause sporadic cases of meningitis in humans [2] and was subsequently recognized as an opportunistic food-borne pathogen of human, cattle and wild animals [3]. This bacterium is responsible for gastroenteritis in healthy individuals, and meningitis and septicemia in immunocompromised individuals. Pregnant women, particularly those in their third trimester, can develop *L. monocytogenes* induced chorioamnionitis (an infection of placental tissues) and infection of the foetus leading to abortion in 20-50% of cases [4]. Cases of listeriosis are generally sporadic but epidemics occur, such as that of 2011 in USA that resulted from contaminated cantaloupes and for which 147 cases and 33 deaths have been reported [5]. The center for disease control (CDC) estimates that *L. monocytogenes* causes approximately 1600 human cases and 260 deaths annually in the USA (mean annual incidence 0.26 cases per 100,000 individuals, [6]). In Europe, in 2011, 1470 cases of listeriosis were reported with a mortality rate of 12.7% (mean annual incidence 0.39 cases per 100,000 residents, [7]). Early diagnosis of listeriosis and antibiotic treatment (involving both amoxicillin and gentamicin) are critical to prevent the devastating outcome and the neurological consequences of the disease.

L. monocytogenes is widespread in the environment and is commonly found in water, soil and decaying vegetation. It is particularly well adapted to growth in high salt concentrations and/or at low temperatures, conditions normally used for food conservation and making it difficult for the food industry to rely on these conditions to control listerial contamination. Following ingestion of contaminated food (such as cheese, deli meat, vegetables, dairy products...), *L. monocytogenes* survives acidic conditions of the stomach and bile acids encountered within the gastrointestinal [8]. It crosses the intestinal barrier by invading the intestinal epithelium. *Via* the lymph and the blood, it reaches the liver and the spleen. Bacteria can then disseminate to the brain and the placenta, after crossing the blood-brain barrier and the materno-fetal barrier, respectively. The bacterium has the remarkable ability to invade, survive and actively multiply within professional phagocytes and a number of non-phagocytic cells, to spread directly from cell-to-cell and to evade innate immunity (**Figure 1**). During infection, *L. monocytogenes* uses a series of virulence factors whose production is spatiotemporally regulated by both protein-mediated and RNA-mediated regulatory mechanisms (for reviews,[9, 10]). These secreted or surface-exposed virulence factors allow *L. monocytogenes* to exploit and/or compromise host cell functions and also promote its survival.

This review highlights some of the strategies used by *L. monocytogenes* to subvert its host functions focusing on those in which this bacterium has opened new perspectives in bacterial gene expression regulation, cell biology, innate immunity and *in vivo* infection. We report how the study of virulence gene expression regulation in *L. monocytogenes* has led to never described mechanisms of regulation. We then discuss how bacterial infection has enabled us to explore or even reconsider cell biology and immunology processes. We highlight progress in infection biology, focusing on the effect of *L. monocytogenes* on gut

microbiota. At the end of this review, we discuss future perspectives offered by the study of host-pathogen interactions.

New concepts in microbial gene expression regulation

PrfA, a tightly controlled regulator

Environmental pathogens, such as *L. monocytogenes*, are microorganisms that normally spend a large part of their lifecycle in the environment, and when present into human hosts can cause disease. This suggests that these pathogens must maintain a broad array of survival strategies to maintain life under these various conditions. In addition, to colonize their hosts successfully, they must regulate virulence gene expression appropriately. Coordinated virulence gene expression in *L. monocytogenes* is orchestrated by PrfA, a transcription factor of the cAMP receptor protein (CRP) family with a consensus binding site in the -35 region of the promoter [11, 12]. PrfA directly regulates transcription of the major virulence factors, including the surface protein internalins InlA and InlB, the secreted protein InlC, the pore-forming toxin listeriolysin O (LLO), the phospholipases PlcA and PlcB, the metalloprotease Mpl, the actin-polymerizing protein ActA, the hexose phosphate transporter Hpt, and is therefore referred to as the master virulence regulator in *L. monocytogenes* [12, 13]. Strains lacking *prfA* are totally avirulent [11, 13].

Expression and activity of PrfA are tightly regulated at the transcriptional and post-transcriptional levels (**Figure 2** and as discussed in the next section). Until recently, there were several lines of evidence suggesting that PrfA is also regulated at the post-translational level. Firstly, proteins of the CRP family generally require the binding of small-molecule cofactors or other forms of post-translational modification for full activity. Secondly,

mutations within an identified PrfA cofactor-binding pocket impair PrfA activation in the cytosol and reduce bacterial virulence [14, 15]. Finally, it was clear that PrfA is activated in the cytosol of host cells, but, even after decades of study, the biochemical mechanism by which PrfA detects the intracellular environment was not understood. Last year, by analyzing how *L. monocytogenes* recognizes and responds to its intracellular niche of the mammalian cell cytosol, Reniere and colleagues demonstrated that glutathione, found abundantly in the host cytoplasm, represents a critical signaling molecule activating virulence of this intracellular pathogen [16]. They showed that bacterial or host glutathione induces an allosteric change in the structure of PrfA and consequently activates expression of *L. monocytogenes* virulence factors (**Figure 2**). This study also suggested that *L. monocytogenes* uses the glutathione concentration to differentiate extracellular and intracellular environments and to regulate the switch from saprophytism to virulence. Since then, *Burkholderia pseudomallei*, an intracellular Gram-negative bacterium, was reported to sense the presence of reduced glutathione in the host cytosol, through the membrane-bound histidine sensor kinase VirA, leading to activation of its type VI secretion system, critical for infection in mammalian host [17]. Taken together, these two studies highlight that the same ubiquitous molecule can regulate virulence in both Gram-positive and Gram-negative pathogenic bacteria, through different sets of regulators and mechanisms. It seems that the unifying theme for *L. monocytogenes* and *B. pseudomallei* is the need to recognize the intracellular environment and to turn on their appropriate virulence genes necessary for pathogenesis in the cytosol. Given the central role glutathione plays in virulence regulation, it could represent a potential novel target for new anti-microbial drug strategies. In addition, one may speculate that other low-molecular-weight thiols (coenzyme A, mycothiol and bacillithiol) also play roles in activation of virulence gene expression in other pathogens.

Unconventional mechanisms regulating bacterial gene expression

Successful host infection by pathogenic micro-organisms relies on the coordinated expression of many virulence factors and genes involved in the infection process. Over the last decade, much has been learned about the complexity of bacterial gene expression and the different levels of regulation. Regulatory RNAs are increasingly being recognized as important players in many physiological and adaptive responses in pathogenic bacteria [18-20]. The first noncoding RNAs (ncRNAs) in *L. monocytogenes* were identified by co-immunoprecipitation with Hfq, a small RNA-binding protein required for the activity of small regulatory RNAs in prokaryotes [21] and by an *in silico*-based approaches [22]. Since then, the use of high-density tiling arrays and RNA deep sequencing provided a picture of the whole *L. monocytogenes* transcriptome in multiple conditions, allowing the discovery of many regulatory RNAs [23-28] and the annotation of hundreds of regulatory RNAs in *L. monocytogenes*, among which some play regulatory roles in virulence [29]. Public access and navigation through the results of these different studies are facilitated by *Listeria* browsers ([30] and [31] (unpublished)). 305 *L. monocytogenes* ncRNAs have now been reported, with 155 trans-acting sRNAs, 46 cis-regulatory RNAs and 104 antisense RNAs (asRNAs) [32]. They may play important roles in virulence gene expression. The study of some of these transcripts has revealed unconventional mechanisms of bacterial gene expression regulation and highlighted an intricate interlink between RNA-based regulatory mechanisms. These mechanisms have been recently reviewed and two reviews are highly recommended [29, 33]. Here, we will briefly illustrate that studying *L. monocytogenes* led to the discovery of (i) the first RNA thermosensor regulating virulence (5'-UTR of *prfA*), (ii) the first riboswitch-derived RNA able to act *in trans* (*sreA*), (iii) a riboswitch-regulated antisense RNA (*aspocR*), (iv) the sequestration of an antiterminator by a riboswitch-regulated ncRNA (*rli55*), (v) the ribo-regulation of antibiotic resistance genes, (vi) the excludon concept and (vii) an atypical CRISPR.

RNA thermosensor. Soon after the characterization of PrfA as an activator of the expression of *hly*, the gene encoding LLO, the expression of PrfA-dependent virulence genes was found to be thermoregulated in *L. monocytogenes* [34]. In addition, at low temperature, PrfA is undetectable, although *prfA* transcripts are present [35]. The mechanism of this regulation relies on the 5'-UTR of the *prfA* transcript, which is a thermosensor adopting alternative structures at different temperatures which therefore inhibit or activate PrfA translation [36] (**Figure 2**). Indeed, at low temperatures (30°C), the 5'-UTR of *prfA* adopts a stable stem-loop structure, occluding the Shine-Dalgarno (SD) sequence and thereby preventing the binding of the 30S ribosomal subunit, whereas at higher temperatures (37°C), such as those encountered in warm-blood organisms, the stem-loop structure opens, exposing the SD site and leading to PrfA translation. The PrfA RNA thermosensor was the first RNA thermosensor regulating virulence discovered. RNA-based thermosensors, while rare, have also been identified in other bacteria, such as *Escherichia coli* [37]. They control the expression of different genes, including genes involved in virulence [38]. For example, one RNA thermosensor located upstream of the virulence regulator gene *lcrF* of *Yersinia pseudotuberculosis* has been described to regulate expression of the *ycs* type III secretion genes and the *yop* effectors and to promote tissue colonization [39].

Riboswitch-derived regulatory transcript. PrfA expression is also regulated by trans-acting riboswitch-derived transcripts. Typical riboswitches are present in the 5' UTRs of the mRNA they control and, upon binding of a ligand (metabolite, metal ions, cyclic-di-GMP...), change their conformation usually resulting in translation arrest, premature transcription arrest or both [40]. Transcripts regulated by riboswitches usually encode genes that are involved in the biosynthesis of the molecule that regulates the riboswitch. The S-adenosyl-methionine (SAM) riboswitch SreA regulates *in cis* expression of genes involved in methionine and cysteine metabolism, but interacts also with the 5'UTR of *prfA* mRNA [41]

(**Figure 2**). This interaction decreases *prfA* translation. This is the first and so far unique example of a riboswitch derived RNA able to act *in trans*. In addition, this nutrient-mediated trans-regulation of *prfA* expression coupled to its temperature-mediated cis-regulation described above allows *L. monocytogenes* to sense its environment and regulate *prfA* expression accordingly. The large number of mechanisms that regulate PrfA is indicative of its importance during infection.

Riboswitch-regulated non coding RNA. Another atypical riboswitch is one dependent on vitamin B₁₂, located at the 3' end of a gene and in antisense orientation to the downstream gene *pocR* and controlling the expression of an RNA antisense to *pocR*, *aspocR* [42] (**Figure 3A**). PocR is a transcriptional factor activating expression of genes involved in propanediol catabolism and vitamin B₁₂ biosynthesis, both pathways being intimately linked as propanediol utilization requires vitamin B₁₂ as a cofactor. In absence of vitamin B₁₂, the riboswitch allows transcription of *aspocR* and thus blocks that of *pocR*. In presence of vitamin B₁₂, the riboswitch arrests prematurely the transcription of *aspocR*, enabling that of *pocR*. Therefore, this mechanism ensures the transcription of *pocR* and thereby that of the PocR-regulon only when both propanediol and vitamin B₁₂ are present in the growth medium.

Another *L. monocytogenes* vitamin B₁₂ riboswitch is involved in regulation of the ethanolamine utilization operon (*eut*) [43] (**Figure 3B**). In the presence of ethanolamine, the *eut* operon is activated by the EutVW two-component system in which the sensor histidine kinase EutW phosphorylates the antiterminator EutV. However, in absence of vitamin B₁₂, the activated EutV protein is sequestered by a ncRNA called *rli55*, whose expression is controlled by a vitamin B₁₂ riboswitch. In presence of vitamin B₁₂, the riboswitch terminates prematurely the transcription of *rli55*, EutV is no longer titrated and can therefore mediate antitermination, allowing expression of *eut* genes. Thus, upregulation of the *eut* operon only occurs when the two nutrients are present - the substrate ethanolamine and the co-factor

vitamin B12. The expressions of *pocR* and *eut* operon are both regulated by vitamin B12 riboswitches and antisense RNA or an anti-terminator, respectively. This multiple layer of regulation indicates a complex and tightly regulated interlink between RNA-based regulators in *L. monocytogenes*. Such complex and layered RNA-based regulation of utilization of ethanolamine has also been reported in the pathogen *Enterococcus faecalis* [44].

Riboswitches regulating antibiotic resistance genes. The vast majority of antibiotics affecting translation have RNA binding properties and it has been speculated that RNA-based regulation could be involved in antibiotic resistance genes expression. Recently, several antibiotics resistance genes regulated *via* termination-based ribo-regulators were identified in *L. monocytogenes* [24]. Indeed, the discovery of ribo-regulators that specifically respond to antibiotics occurred by applying term-seq, a method enabling quantitative mapping of all exposed RNA 3' ends, to *L. monocytogenes* bacteria grown in the presence or absence of antibiotic, such as lincomycin. This method was also performed on *Bacillus subtilis*, *E. faecalis* and human oral microbiota and allowed the identification of many genes ribo-regulated by antibiotics, illustrating that conditional transcription represents a general feature for antibiotic resistance. The discovery of such new regulatory mechanisms controlling antibiotic resistance genes has provided a new window on antibiotic resistance expression, suggesting that these ribo-regulators might be excellent drug targets.

Excludon. High-resolution transcriptome of *L. monocytogenes* highlighted a class of very long asRNAs (lasRNAs) which display a dual function as both asRNA and mRNA and encoded by an “excludon” [27, 28]. An excludon typically consists of two divergent transcriptional units overlapped by a long asRNA that inhibits the expression of one of the two divergent operons, but whose distal part harbors information as an mRNA, activating therefore the expression of the other operon (**Figure 3C**). The first excludon discovered was the *mogR* locus in *L. monocytogenes* and excludons are conserved in other *Listeria* species

and also in a more distantly related bacterium *Clostridium difficile* [28]. It is highly possible that excludons exist not only in bacteria, but also in other organisms.

CRISPR. A class of ncRNAs, named CRISPRs (clustered regularly interspaced short palindromic repeats) mediates prokaryotic adaptive immunity against invading bacteriophages and conjugative plasmids [45]. CRISPR RNAs processing and function rely on nucleases or helicases encoded by CRISPR-associated (*cas*) genes. Unlike other CRISPRs, *L. monocytogenes rliB* is an atypical member of the CRISPR family, devoided of *cas* genes [46]. Biochemistry experiments showed that it is a substrate for polynucleotide phosphorylase (PNPase), an enzyme which acts as an exoribonuclease of single stranded target RNAs and that PNPase is required for the *rliB*-CRISPR mediated DNA interference. Therefore, characterization of the *rliB*-CRISPR shed light on a new and unexpected function for PNPase in CRISPR system.

Concepts in cell biology derived from the analysis of *L. monocytogenes* infections

L. monocytogenes is able to invade a number of non-phagocytic cells. This entry is induced by interactions between bacterial surface proteins, InlA and InlB, and their respective receptors E-cadherin (E-Cad) and Met expressed at the host cell surface. These interactions trigger cell-signalling cascades, leading to actin polymerisation and host cell membrane remodelling, which in turn allow listerial engulfment via a zipper mechanism and formation of an internalisation vacuole. After escape from the vacuole, bacteria multiply and move in the cytosol, via recruitment of the actin-nucleating complex Arp2/3 by the surface-exposed bacterial protein ActA and polymerisation of an actin filaments network at one pole of the bacterium. These networks, called actin comet tails, propel the bacterium across the cytoplasm and eventually into neighbouring cells. The efficient spreading between epithelial

cells requires the interaction of the secreted virulence protein InlC and the mammalian adaptor protein Tuba [47]. In the infected cell, *L. monocytogenes* impairs organelles function and target post-translational modifications at various stages of the infection process to manipulate the cell host response. We illustrate below how the research on *L. monocytogenes* has greatly contributed to our understanding of the structure and dynamics of the host cell cytoskeleton and to deepen our knowledge of several other central cellular processes (**Figure 4**).

Discovery of the Arp2/3 complex, the first actin nucleator identified in eukaryotic cells

The study of actin-based motility is one of the best examples highlighting how the study of a bacterial-induced process can yield insight into basic cellular processes. Indeed, ActA and *L. monocytogenes* have been exceptionally instrumental in the discovery of Arp2/3. It was first shown that *L. monocytogenes* polymerises actin at one side of the bacterium, producing an actin tail allowing bacterial motility [48, 49]. Then, it was discovered that this polymerisation of host actin is mediated by the bacterial surface protein ActA [50, 51], that it recruits the host Arp2/3 complex and that it mimics the host cell WASP proteins [52]. The Arp2/3 complex consists of seven subunits and has been classically considered as a single molecular entity since its discovery 20 years ago. However, recent studies suggest that some subunits of the complex are dispensable in some specific cellular contexts ([53-55], for review, [56]). In the case of *L. monocytogenes* infection, it was for example shown that the ARPC5 subunit is neither required for *L. monocytogenes* entry into host cells nor for actin tail formation and that the ARPC4 subunit is required for actin tail formation, probably at initial stages, but not for cell invasion [55]. These findings therefore highlight a previously unsuspected versatility in Arp2/3 complex composition and function [56].

L. monocytogenes is not the only microorganism that exploits actin for intracellular movement. (for review, [57]). *Shigella flexneri* recruits on its surface, *via* the protein IscA, N-WASP which in turn activates Arp2/3 to mediate actin-based motility [58]. *Rickettsia conorii* and *Rickettsia parkerii* possess two actin-polymerizing proteins: RickA and Sca2. RickA activates the Arp2/3 complex and is involved in early stages of bacterial intracellular motility, while Sca2 is responsible for the late actin-based motility *via* a mechanism independent of the Arp2/3 complex [59, 60]. *Mycobacterium marinum*, virulent *Burkholderia* and vaccinia virus also move *via* an actin-based motility requiring Arp2/3 functions [61-65].

A role for clathrin and two myosins in bacterial entry

Investigations on InlA and InlB-mediated entries have clearly shown that these two bacterial proteins are essential for *L. monocytogenes* entry into non-phagocytic mammalian cells (for review, [66]). An important finding concerning the mechanisms underlying *L. monocytogenes* entry into host cells has been the discovery that the clathrin-dependent endocytic machinery is crucial in actin polymerization and therefore bacterial entry [67]. Indeed, RNA interference of major components of the endocytic machinery (e.g. clathrin, dynamin and cortactin) inhibited *L. monocytogenes* entry. It had before been thought that clathrin was only involved in endocytosis of small molecules (nutrients and signalling molecules) or relatively small objects (<150nm) [68]. Therefore, the fact that the endocytic machinery is involved in bacterial internalization indicated that clathrin can internalize objects much larger than previously appreciated [67, 69]. Clathrin also mediates entry of large viruses and other bacteria using zipper mechanism [70-72] and also pedestal formation by the extracellular enteropathogenic *E. coli* (EPEC) [72]. Interestingly, by analysing the molecular events during early stages of *L. monocytogenes* and EPEC infection, it was

observed that Myosin VI, a myosin that moves on actin filaments towards the minus ends, accumulates at the *L. monocytogenes* entry sites but not at the EPEC-induced actin-based pedestals [73]. Knockdown of Myosin IV inhibits *L. monocytogenes* entry but does not reduce the recruitment of the clathrin-actin machinery at bacterial adhesion sites, suggesting the Myosin VI is the last component of this machinery and provides the pulling force for bacterial internalisation. In addition, by investigating whether the clathrin/actin interactions may also be involved in E-Cadherin-mediated cell-cell adhesion, it was discovered that clathrin is involved in the formation of adherens junctions between epithelial cells by reorganisation of actin [74]. Altogether these findings revealed a novel role for clathrin as an essential promoter of actin polymerisation and rearrangement, and reinforced the view that unrevealing pathogen infection strategies can reveal unappreciated features of normal host cells pathways.

Another unconventional myosin, the myosin VIIa, together with its ligand vezatin, are recruited at adherens junctions and at the entry site of *L. monocytogenes*, localize with actin and are required for *L. monocytogenes* entry [75]. To our knowledge, *L. monocytogenes* is the only bacterium described so far to require two unconventional myosins for its internalization.

A role of septins in actin cytoskeleton rearrangements

Septins constitute a family of 13 different GTP-binding proteins in humans involved in the regulation of cytokinesis, neurogenesis, membrane remodelling and cytoskeleton dynamics. They assemble into hetero-oligomeric complexes, form nonpolar filaments, bundles or rings, and associate with cellular membranes, actin filaments and microtubules. They are now considered as the fourth component of the cytoskeleton together with actin, microtubules and intermediate filaments [76]. SEPT9 has been found to associate with the *L.*

monocytogenes phagosome [77]. SEPT9, and its interacting partners SEPT2 and SEPT11, are recruited at the site of entry of *L. monocytogenes* and of the pathogenic bacteria *S. flexneri* [78, 79]. When cells are treated with cytochalasin D, an actin polymerisation inhibitor, septin recruitment is impaired indicating that actin polymerisation happens before septin assembly [79, 80]. Surprisingly, SEPT2 contributes to bacterial invasion, while SEPT11 limits it, suggesting that different septins may play different roles in the tuning of actin-based molecular events [78, 79].

In addition to be recruited at the site of bacterial entry, septin rings assemble around both *L. monocytogenes* and *S. flexneri* actin comet tails, suggesting that septin recruitment is a general pattern wherever actin polymerisation is taking place [81]. Septins can also entrap *Shigella* into cage-like structures that restrict cell-to-cell spread and therefore dissemination of the bacteria [81]. In the case of *L. monocytogenes*, no efficient septin caging has been observed, but the above-reported studies indicate that septins play a role as scaffold for protein recruitment in several biological processes, including host-pathogens interactions.

Interaction with mitochondria

Bacterial pathogens have evolved mechanisms to target eukaryotic organelles such as the nucleus, the mitochondria or the ER-Golgi system (for review, [82]). Although *L. monocytogenes* does not invade these host cell organelles, it has developed several strategies to interfere with their functions, i.e. by inhibiting phagosomal killing and lysosomal function, by activating the ER stress response, by modulating mitochondrial dynamics, by modulating host gene expression *via* interference with chromatin-based regulation (for review, [83]).

Research on *L. monocytogenes* has contributed to the identification of a novel mechanism of mitochondrial fission. Mitochondria are dynamic organelles that constantly undergo fusion and fission to regulate their size and subcellular distribution, and their function is tightly related to their morphology. They act as central hubs for energy production, apoptosis regulation, calcium homeostasis, biosynthesis of amino acids, lipids and nucleotides and innate immune signaling. Infection with *L. monocytogenes* transiently causes mitochondrial network fragmentation *via* the secreted pore-forming toxin LLO [84]. However, LLO-induced mitochondrial fragmentation does not follow canonical pathways. It is independent of the key fission component Drp1, revealing a novel and unique fission mechanism Drp1-independent [85]. On the other hand, it was shown that ER and actin play an active role in regulating LLO-induced mitochondrial fission. In parallel to this latter study, a novel link between mitochondria and septins has been described. Indeed, SEPT2 directly interacts with Drp1 and is required for efficient localization of Drp1 at mitochondria, thus introducing septins as new players in mitochondrial dynamics [86]. In addition, mitochondria are required for an efficient assembly of septin cages to entrap *S. flexneri* and restrict their dissemination, identifying a new role for mitochondria in host defence [87].

Mitochondrial dynamics are also targeted by *Vibrio cholerae*, *via* a type III secretion system effector protein, VopE, which binds to mitochondrial Rho GTPases involved in mitochondria movement [88]. Other mitochondrial functions, such as apoptosis, protein trafficking, coordination of immune signaling, are also targeted by other pathogenic bacteria (for review, [89]).

Interaction with lysosomes

Lysosomes are acidic organelles containing hydrolytic enzymes such as cathepsins and other proteases that are specialized in the degradation and recycling of macromolecules. They are able to fuse and degrade the content of a wide range of vesicles, including phagosome containing bacteria. Different stress conditions may alter the integrity of lysosomal membranes (a process known as lysosomal membrane permeabilisation (LMP)), resulting in the leakage of lysosomal content to the cytosol. It was recently shown that the secreted pore-forming toxin LLO induces LMP and the release of cathepsins in the host cytosol *via* its pore-forming activity [90]. Other cholesterol-dependent cytolysins, such as perfringolysin O and pneumolysin, secreted by other extracellular pathogenic bacteria, also induce LMP [90]. These findings unveil a novel activity of bacterial cholesterol-dependent cytolysins that may interfere with important host cellular pathways linked to lysosomal functions. So far, only few pathogenic bacteria were reported to target lysosomes by mechanisms differing from the one observed with *L. monocytogenes* [91-95].

Interaction with nuclear components: epigenetics and Listeria monocytogenes infection

To persist and replicate in host cells, pathogenic bacteria, such as *L. monocytogenes*, can directly modulate cellular signaling pathways activated upon bacterial entry and/or recognition of bacterial PAMP (Pathogen-Associated Molecular Patterns) by a variety of host receptors. In addition to the interference with transcriptional signaling cascades, the concept emerged that pathogenic bacteria can manipulate host cell transcription activity by directly acting at the nuclear level by interfering with chromatin-based regulation [96, 97]. In eukaryotic cells, DNA is packaged with histones into chromatin, a dynamic structure that maintains the stability and accessibility of the DNA genome. In addition to uncover post-translational modifications of histones induced by *L. monocytogenes* infection, research

aiming at exploring the effect of *L. monocytogenes* on chromatin-based regulation helped to characterize better host cells components such as SIRT2 and BAHD1.

L. monocytogenes was one of the first reported examples of an intracellular bacterium having an effect on histone modifications in host cells [98]. The *L. monocytogenes* pore-forming toxin LLO triggers dephosphorylation of histone H3 on serine 10 and deacetylation of histone H4 [98]. In addition, upon binding to its receptor Met, internalin InlB induces the translocation of the host histone deacetylase sirtuin 2 (SIRT2) from the cytoplasm to the chromatin, where it deacetylates histone H3 on lysine 18, leading to modifications of the host cell transcriptome [99]. So far, SIRT2 was considered to only regulate in the cytoplasm microtubule dynamics through deacetylation of alpha-tubulin and NF- κ B gene expression through deacetylation of p65 and also controls adipocyte differentiation and autophagy through deacetylation FOXO1 (for review, [100]). SIRT2 is also present in the nucleus in the G2/M cell cycle transition and targets nuclear proteins such as p53. In addition, *Sirt2*^{-/-} mice can control listeriosis more efficiently than wild-type mice [99]. Therefore, the study of *L. monocytogenes* unveiled an additional nuclear function for SIRT2 in deacetylating histone H3 specifically on lysine 18, a process which is actively exploited by the bacteria to promote its survival. *Mycobacterium tuberculosis*, *Helicobacter pylori* and other bacteria also modulate immune responses by epigenetic mechanisms, such as DNA methylation or histone modifications [96].

L. monocytogenes secretes into the cell cytosol LntA, a small protein belonging to the emerging class of nucleomodulins [101, 102]. It was shown that LntA accumulates in the host cell nucleus and interacts directly with the previously unknown chromatin-associated repressor BAHD1, releasing it from its specifically targeted promoters and therefore allowing transcription of targeted genes [102, 103]. In parallel to the study of the listerial effector LntA, the role of BAHD1 has been characterized in non-infected cells. This protein acts as a

silencer by recruiting at specific promoters a set of proteins that coordinate heterochromatin assembly [104]. Therefore, *L. monocytogenes* has helped identifying and characterizing BAHD1.

Other nucleomodulins targeting chromatin such as the *Chlamydia trachomatis* histone-methyltransferase NUE, the *S. flexneri* phosphothreonine lyase OspF and the *Shigella flexneri* ubiquitin ligase IpaH9.8 have also been reported to deregulate host cell functions (for reviews [96, 101]).

Role of SUMO in bacterial infections

L. monocytogenes was the first example of a bacterium able to dampen the host response by interfering with the host SUMOylation machinery [105]. It was shown that the LLO toxin triggers the degradation of Ubc9, an essential enzyme of the SUMOylation machinery, leading to a global decrease in the level of host SUMOylated proteins. As hyper-SUMOylation impaired infection efficiency, it was suggested that deSUMOylation induced by LLO is critical to promote *L. monocytogenes* infection. Recently there have been a growing number of examples involving SUMOylation in the host-pathogen cross talk [106-109].

A method combining SILAC-based quantitative proteomics and immunocapture of SUMO-modified peptides was recently developed to identify SUMOylation sites and to compare the SUMOylome of two different cell populations [110]. This method was used to characterize proteins deSUMOylated upon LLO treatment and identified DNA-binding proteins and transcription factors as candidate host factors for which SUMO-regulation may play an important role during *L. monocytogenes* infection. This study may also lead in a near

future to the characterization of the role of SUMO in the function of these proteins in non-infected conditions.

Role of ISG15 in bacterial infection

Recently, the role for ISG15, an interferon-stimulated di-ubiquitin-like protein, known as an antiviral protein, was studied during *L. monocytogenes* infection [111]. ISG15 expression is induced by *L. monocytogenes in vitro* in epithelial cells as well as *in vivo* in mouse liver three days post-intravenous infection. ISG15^{-/-} MEFs (Mouse Embryo Fibroblasts) and ISG15-deficient mice are more susceptible to *L. monocytogenes*. In addition, infection leads to ISGylation of ER and Golgi proteins and increases secretion of interleukines Il-6 and Il-8. These findings indicate that ISG15 and ISGylation counteract *L. monocytogenes* infection and reveal a new role of ISG15 as a modulator of canonical secretion.

Listeria monocytogenes and immunology research

As mentioned above *L. monocytogenes* has led to the discovery of new concepts in innate immunity. In addition, *L. monocytogenes* is used as a tool in all major immunology papers since decades. Indeed, in 1962, Mackaness showed that *L. monocytogenes* can resist killing by macrophages in a mouse model and that a primary infection by *L. monocytogenes* induces a protective cellular immune response and protection against a secondary infection [112]. Further studies then showed that the clearance of the bacteria is T-cell mediated [113]. Since then, *L. monocytogenes* has become a useful model for evaluation of the cellular

interactions that are crucial for the induction and establishment of the host T-cell response. This topic has been deeply reviewed [114-116].

Animal models of listeriosis

Mouse models have been widely used for many decades and most of the currently available findings on pathophysiology and immunology of *L. monocytogenes* infections has been generated in mice. However, in mouse, E-cad presents a single amino-acid difference compared to human E-cad, the receptor for InlA [117]. This mutation on the 16th amino acid of mouse E-cad prevents it from binding InlA, thereby explaining the inadequacy of the mouse model for studying the pathophysiology of listeriosis after oral inoculation, the normal route of infection. To confer the mouse permissiveness to the InlA-E-cad interaction, mouse lines expressing human E-cad have been engineered and have been instrumental to show the essential role of InlA-E-cad interaction for crossing of the intestinal barrier [118, 119]. On the other hand, a murinized InlA, able to recognize the murine receptor E-Cad, was created by substituting two amino acids (S192N and Y369N) [120]. However, this murinized InlA mediates not only E-Cad dependent internalization but also N-Cadherin dependent internalization leading to enhanced innate immune responses and intestinal barrier damage [121].

A variety of other species such as gerbils, guinea pigs, zebrafishes, wax moths, chicken embryos, non-human primates have also occasionally been used (for reviews, [9, 122, 123]). However, it is extremely difficult to have an optimal animal model that mimics perfectly the infection process in humans, especially knowing that listeriosis appears to be an infection of immuno-compromised individuals.

Microbiota and *L. monocytogenes* infection

With the recent appreciation of the important roles played by the intestinal microbiota in health and diseases, a number of studies have highlighted its specific role in protection against pathogen infection. However, many enteric pathogens have developed strategies to outcompete the intestinal community, leading to successful infection [124, 125]. In the case of *L. monocytogenes* infection, it was shown that the intestinal microbiota provides protection against oral infection, as germfree mice are more susceptible to *L. monocytogenes* infection than conventional mice [126]. Thorough transcriptional analysis of the host intestine of uninfected conventional and germfree mice and of *L. monocytogenes*-infected conventional and germfree mice indicated for the first time that host microRNAs expression triggered by *L. monocytogenes* infection is downregulated by intestinal microbiota, highlighting interplays between the pathogenic bacteria, the intestinal microbiota and the host. MicroRNAs are short ncRNAs that regulate eukaryotic gene expression by base pairing to target mRNAs and then inhibiting their translation. Modulation of host miRNAs is one of the various strategies used by bacterial pathogens to counteract host cell defenses (for review, [127]). The intestinal miRNA signature upon *L. monocytogenes* infection led to the establishment a list of predicted mRNA targets whose expression was affected by *L. monocytogenes* and the intestine microbiota [126]. Further investigations of the function of these miRNAs modulated by microbiota and/or by *L. monocytogenes* infection and of their putative mRNA targets will most likely provide new insights into gut homeostasis and also the impact of the host intestinal microbiota upon bacterial infection.

Some *L. monocytogenes* strains were recently shown to secrete listeriolysin S (LLS), a virulence factor only present in a subset of epidemic strains and specifically expressed in the intestine of orally infected mice [128, 129]. LLS is a bacteriocin altering the host intestinal microbiota and favoring an increase in *L. monocytogenes* persistence. Indeed, a strain

lacking LLS encoding gene displayed reduced bacterial loads in the intestine. In addition, analyzing the microbial community composition in the intestine indicated that LLS expression led to a decrease in *Allobaculum* and *Alloprevotella* genera, known to produce butyrate and acetic acid, respectively which inhibit either *L. monocytogenes* virulence gene expression or its growth. So far, bacteriocins were reported to be produced *in vivo* by commensal bacteria or by probiotics and to selectively inhibit the growth of competing bacteria, such as pathogens [125, 130, 131]. As an example, the commensal bacterium *Bacteroides thuringiensis* secretes a bacteriocin (thuricin CD) that targets *Clostridia*, including the pathogen *C. difficile* [132]. Therefore, LLS was the first bacteriocin described in *L. monocytogenes* to outcompete the mouse intestinal microbiota, leading to a successful infection. It is very likely that other pathogenic bacteria produce also bacteriocins and their study might reveal new mechanisms of pathogenicity and will help to decipher the complex relationship between the microbiota and pathogens.

Conclusions and future perspectives

Since the original paper of its discovery published ninety years ago [1], *L. monocytogenes* has become one of the most documented bacteria and listeriosis one of the most studied infectious diseases. Recent achievements were presented recently at the XIXth ISOPOL meeting (International Symposium on Problems of *Listeria* and Listeriosis) and clearly illustrate the complexity of *L. monocytogenes* physiology and the infectious process [133]. The present review has highlighted the many facets opened following studies of *L. monocytogenes* and listeriosis. Important progress to come should arise from studies of *L. monocytogenes* interacting with the gut microbiota, from studies examining the differences

among the various strains circulating in the clinic and in the environment and from *in vivo* imaging of the various cells, tissues, organs affected by the infection.

Executive summary

- *L. monocytogenes* is an intracellular pathogenic bacterium whose virulence factors expression is controlled by multiple RNA-mediated regulatory mechanisms (thermosensor, atypical riboswitches, excludon, CRISPR)
- Glutathione is used by *L. monocytogenes* as an specific indicator of intracellular environment and activates the activity of PrfA, the master virulence regulator, leading to expression of virulence factors
- *L. monocytogenes* manipulates several actin-related proteins (Arp2/3 complex, clathrin, unconventional myosins, septins) to promote its entry and/or its propagation
- *L. monocytogenes* alters mitochondria dynamics and lysosome function by novel mechanisms
- Research on *L. monocytogenes* broadly contributed to the emerging field named patho-epigenetics
- *L. monocytogenes* disturbs SUMOylation and ISGylation of infected cells
- *L. monocytogenes* infection occurs in the context of a complex microbiota, and understanding the interplays between the pathogenic bacteria, the intestinal microbiota and the host will be a major challenge.

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Reference annotation (* of interest, ** of considerable interest)

* Cossart PNAS 2011 Comprehensive review of *Listeria* interactions with the host

** Cossart FEBS 2014 Review describing most recent achievements in the understanding of the host-bacterial crosstalk

** Reniere Nature 2015 Highlight of the role of glutathione in activating PrfA activity in host cytoplasm and therefore virulence gene expression in *L. monocytogenes*

** Dar Science 2016 Discovery of a broad role for conditional termination in regulating antibiotic resistance in bacteria using a high-throughput method

** Toledo Arana nature 2009 Extensive transcriptomic analysis of *L. monocytogenes* grown in several different conditions

** Lebreton RNA biology 2016 Comprehensive review of regulatory RNAs in *L. monocytogenes*

** Johansson cell 2002 Discovery of the RNA thermosensor regulating the expression of the master virulence regulator, PrfA

* Loh cell 2009 Discovery of the first trans-acting riboswitch in *L. monocytogenes*

** Mellin Science 2014 and ** DebRoy Science 2014 both demonstrate that riboswitches regulate RNA

* Pizarro-Cerda trends in cell biology 2016 Opinion on the diversity of Arp2/3 complexes

* Escoll nature reviews microbiology 2016 Comprehensive review of the mechanisms used by bacterial pathogens to target host organelles

* Hamon PNAS 2007 Demonstrates that *L. monocytogenes* induces dephosphorylation as well as deacetylation of histones

** Lebreton Science 2011 Identifies a secreted listerial virulence factor, LntA, targeting the chromatin repressor BAHD1 and therefore activating expression of interferon response genes.

* Baumler Nature 2016 and * Rolhion Philos Trans R Soc Lond B Biol Sci 2016 Two comprehensive and recent reviews on the interactions between the gut microbiota and pathogenic bacteria

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Figure legends

Figure 1. Intracellular life cycle of *L. monocytogenes*. Bacteria entry is mediated by the interactions of internalins InlA and InlB with their respective receptors E-cadherine and Met expressed at the surface of the host cell ①. Bacteria escape from the endocytic vacuole *via* the action of secreted factors, the pore forming toxin listeriolysin O (LLO), two phospholipases PlcA and PlcB and the metalloprotease Mpl ②. Bacteria then multiply in the cytosol and factors such as the internalin InlC, the sugar uptake system Hpt and the O-acetyltransferase OatA favor intracellular growth and immune escape ③. The surface-exposed bacterial protein ActA induces the polymerisation of actin through the recruitment of the Arp2/3 complex ④. Polymerised-actin filaments, called actin comet tails, and the interaction of InlC with the actin-binding protein Tuba allow cell-to-cell spread of the bacteria ⑤. LLO, PlcA and PlcB allow the rupture of the two-membrane vacuole ⑥. Figure adapted from [10] and reprinted from [9] with the permission of PNAS.

Figure 2. Multiple mechanisms of regulation of PrfA expression and activity. Post-transcriptional control of *prfA* expression, adapted from [10]. In the environment, at low temperatures, the 5'-UTR of *prfA* mRNA forms a closed stem-loop structure, masking the Shine-Dalgarno (SD) sequence and thereby preventing binding of ribosome. At 37°C, the stem-loop structure melts, liberating the SD and allowing translation initiation. The SreA small RNA, which is the product of a SAM riboswitch, can also bind to the 5'-UTR of *prfA* mRNA and block its translation. Upon entry into host cells, the temperature switches to 37°C, *prfA* mRNA is translated but the produced PrfA protein stays inactive. After vacuolar escape, the glutathione (GSH) found in the host cytoplasm, enters in bacteria, binds to PrfA, changes its structure and thereby activates transcription of PrfA-regulated genes (*prg*), leading to production of virulence factors.

Figure 3. RNA-mediated regulations of *L. monocytogenes* gene expression. **A.** The vitamin B₁₂ riboswitch (RS) regulates the expression of the antisens *aspocR*, inspired from [29]. In absence of vitamin B₁₂, the RS, *aspocR* is produced and inhibits the translation of *pocR*. In presence of vitamin B₁₂, the RS terminates prematurely the transcription of *aspocR*, allowing production of PocR transcription factor, provided that propanediol is also present in the medium. **B.** Sequestration of EutV by a B₁₂ riboswitch, inspired by [33]. In presence of ethanolamine (Ea), the antiterminator EutV is phosphorylated by EutW. In absence of vitamin B₁₂, phosphorylated EutV is sequestered by *rli55* and the *eut* genes are not transcribed. In presence of vitamin B₁₂, the transcription of *rli55* is terminated prematurely by the vitamin B₁₂ riboswitch (RS). Free phosphorylated EutV can induce antitermination and expression of the *eut* genes. **C.** Example of an excludon, where a long antisens *anti0677* overlaps with the *fli* operon, encoding the flagellum export apparatus, adapted from [29]. *mogR* is encompassed by 2 transcripts. The short one allows the production of MogR, a

transcriptional repressor of the flagellum genes. The long one also allows the production of the *anti0677*. Therefore, the excludon ensures that, *via* two mechanisms (inhibition of *fli* mRNAs mediated by the *anti0677* and repression mediated by MogR), flagellum production is switched off.

Figure 4. Subversion of host cell functions by *L. monocytogenes*. ① Actin, clathrin and septin (green fluorescence) are recruited at the site of entry of *L. monocytogenes* (red fluorescence). Reprints from [9] with the permission of PNAS. ② *L. monocytogenes* (green fluorescence) forming actin comet tails (red fluorescence) in infected Jeg3 cell and spreading to neighboring cell (courtesy of JJ. Quereda). Secretion of LLO induces: ③ degradation of E2-SUMO ligase and a global desumoylation of host cell proteins, ④ lysosomal membrane permeabilisation (LMP) leading to neutralisation of lysosomes (characterised by a decreased intensity of staining with LysoTracker, an acidophilic dye, courtesy of JK. Malet) and release of cathepsins in the host cytosol, and ⑤ calcium influx promoting the fragmentation of the mitochondrial network (courtesy of F. Stravru). The secreted internalin InlC interacts with IKK α , preventing NF- κ B activation and thus dampening the innate immune response ⑥. *L. monocytogenes* also controls host gene transcription, adapted from [10] : A) LLO induces an efflux of cellular potassium leading to dephosphorylation of histone H3 on serine 10 (H3deP) and deacetylation of H4 (H4deAc), which decreases the expression of host genes, B) interaction of InlB with its receptor Met targets SIRT2 to chromatin, where it deacetylates histone H3 on lysine 18 (H3K18deAc), which represses host genes transcription, and C) secreted LntA accumulates in the host nucleus, interacts with the chromatin-associated repressor BAHD1 and thereby promotes transcription of interferon-stimulated genes (ISG).