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Adaptations of intracellular bacteria to vacuolar or cytosolic niches

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Abstract

Invasive bacteria colonise their host tissues by establishing niches inside eukaryotic cells, where they grow either in the cytosol or inside a specialised vacuole. These two distinct intracellular lifestyles both present benefits but also impose various constraints on pathogenic microorganisms, in terms of nutrient acquisition, space requirements, exposure to immune responses, and ability to disseminate. Here we review the major characteristics of cytosolic and vacuolar lifestyles and the strategies used by bacteria to overcome challenges specific to each compartment. Recent research providing evidence that these scenarios are not mutually exclusive is presented, with the dual lifestyles of two foodborne pathogens, *Listeria monocytogenes* and *Salmonella* Typhimurium, discussed in detail. Finally, we elaborate on the conceptual implications of polyvalence from the perspective of host-pathogen interactions.

Keywords

Pathogenic bacteria, invasive bacteria, cytosol, vacuole, intracellular lifestyle.

Highlights

The classical dichotomy between intracellular bacteria living either inside vacuoles or in the cytosol is defied by evidence that some of them can accommodate dual lifestyles.

Heterogeneity among populations of intracellular bacteria that inhabit different niches allows alternative strategies for growth, immune evasion, dissemination or persistence.

Adaptation of *Salmonella* Typhimurium to growth in the cytosol of intestinal epithelial cells leads to a population of invasion-primed bacteria that are released in the gut lumen by inflammatory cell death, and thereby constitutes a reservoir for chronic faecal shedding.

Listeria monocytogenes, originally featured as the archetype of cytosolic bacteria, is also found to inhabit durably several vacuolar niches. Strikingly, vacuolar residence requires the pore-forming toxin listeriolysin O (LLO), initially known as a facilitator of vacuole escape.

Living within or beyond endomembranes

Whilst pathogenic bacteria are conventionally classified as extracellular or intracellular infectious agents according to their preferential replication niche, the lifestyles of intracellular pathogens themselves differ considerably depending on the intracellular compartments they colonise during infection. Bacteria replicating in compartments delimited by cell endomembranes (often referred to simply as “vacuoles”), or in the cytosol, face drastically distinct constraints regarding physical confinement, control of biochemical parameters, access to nutrients, and exposure to host immune sensing, signalling, and responses (**Table 1**).

Table 1. Benefits and constraints of intracellular lifestyles

Considered criteria	Cytosolic		Vacuolar	
	Benefits	Constraints	Benefits	Constraints
Endolysosomal maturation	Avoidance of endolysosome fusion	Requirement to disrupt membranes; DAMPs	Reduced volume, the parameters of which can be better controlled	Requirement to subvert/adapt the endolysosomal pathway
Nutrient acquisition	Direct access to host cytosol nutrients	—	—	Nutrients must be imported across the membrane
Growth and population behaviour	Broad cytosolic space	—	Confinement promoting bacterial communication	Limited PCV size
Motility and dissemination	Actin-based motility	—	Possibility of bulk release in membrane-enclosed compartments	Dependence on host trafficking (microtubules, SNAREs etc.)
Antimicrobial defences	Cell-to-cell spread (avoidance of humoral immunity)	Detection by cytosolic sensors and autophagy	Sheltered from cytosolic sensors and autophagy ^(a)	Target of humoral defences after cell exit ^(b)

^a Vacuolar pathogens must however maintain vacuole integrity to avoid autophagy.

^b While this holds true for exocytosis or complete host cell lysis (**Figure 1**), in other instances such as *Chlamydia* extrusions, release in membrane-bound vesicles protects bacteria from host extracellular defences.

Accordingly, a wealth of strategies enables invasive bacteria to thrive in a given compartment. In recent years, our understanding of the molecular role of bacterial effectors in niche adaptation, as well as the corresponding contribution of host cell processes, has been considerably refined. Here we illustrate some of these advances with examples representative of different intracellular lifestyles. We then discuss how recent work on pathogens such as *Salmonella* and *Listeria*, which can endure both vacuolar and cytosolic lifestyles, is changing our views on classical infection schemes and opening novel tracks of investigation.

Endomembranes as confinement barriers

Evading or co-opting the endolysosomal pathway after entry

Upon adhesion to a cell surface, invasive bacteria rely on a variety of mechanisms to promote their uptake into their host cell, before initiating an intracellular lifestyle [1]. **Figure 1** provides an overview of typical invasion-multiplication-dissemination cycles in host cells.

Some bacteria gain rapid access to the host cytosol after internalisation due to disintegration of the membranes of the entry vacuole. By escaping the endolysosomal pathway, cytosolic bacteria

avoid lysosomal acidification and degradative enzymes. For instance, the foodborne bacterium *Listeria monocytogenes* relies on three virulence factors that mediate vacuolar escape by destabilising vacuole membranes: the pore forming toxin listeriolysin O (LLO, [see Glossary](#)) and two phospholipases, PlcA and PlcB [2-4] ([Figure 1](#)). For *Shigella flexneri*, the translocation of effectors such as IpgD through the type III secretion system (T3SS) leads to a cascade of host phosphoinositide signalling and to exocyst-assisted clustering of infection-associated macropinosomes around internalisation vacuoles, causing their disintegration [5-7]. In addition, an actin cocoon that assembles around the *Shigella*-containing vacuole has been recently proposed to promote the clearance of vacuole membrane debris and subsequent bacterial cytosolic motility [8].

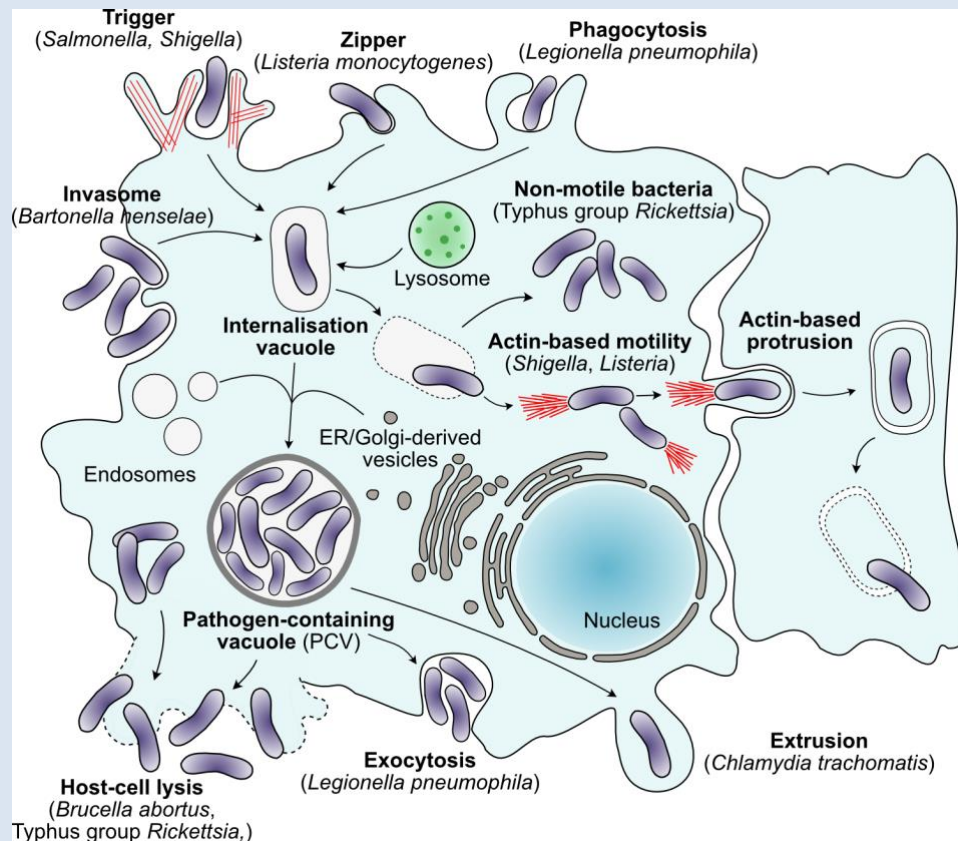


Figure 1. Classical cytosolic versus vacuolar lifestyles of intracellular bacteria

Intracellular bacteria enter their host cells by inducing cytoskeletal remodelling (trigger, zipper) by entering as large aggregates (invasome), or by phagocytosis. Once inside the internalisation vacuole, cytosolic bacteria rapidly break down the vacuole membrane and replicate in the cytosol. Actin-based motility enables a variety of cytosolic bacteria, although phylogenetically unrelated, to move inside the infected cell and disseminate to other cells via cell-to-cell spread. Most vacuolar bacteria hamper phagolysosomal maturation by interfering with the endocytic pathway, and then create a replication-permissive vacuole (PCV) by modulating host-cell trafficking pathways, before exiting the host cell through various mechanisms, such as inducing host-cell lysis, exocytosis or extrusion. Abbreviations: ER, endoplasmic reticulum; PCV, pathogen-containing vacuole.

By contrast, vacuolar bacteria co-opt the cell endomembrane system, living and replicating therein ([Table 1](#)). Often they interfere with the endolysosomal pathway via manipulation of host cell trafficking, thus preventing or surviving the fusion of lysosomes with internalisation vacuoles, which in turn are transformed into pathogen-containing vacuoles (PCVs) permissive for replication ([Figure 1](#)) [9]. For instance, following their uptake by phagocytes, some of the effectors translocated by *Legionella pneumophila* across phagosome membranes lead to the recruitment of COPII proteins, SNAREs, and endoplasmic reticulum (ER)-derived vesicles through the subversion of small GTPases

or through membrane phosphoinositide conversion [10]. In addition, *L. pneumophila*-secreted kinase LegK2 inhibits actin polymerisation on the surface of phagosomes, which impedes their fusion with late endosomes and lysosomes [11]. Thus, by preventing endocytic trafficking towards degradative compartments, *L. pneumophila* remodels phagosomes into inhabitable *Legionella*-containing vacuoles (LCVs).

Instead of preventing endolysosomal trafficking, other bacteria adapt and respond to vacuole maturation. *Brucella*- or *Coxiella*-containing vacuoles (BCVs or CCVs, respectively) are decorated with lysosomal markers such as LAMP1, indicative of phagolysosomal targeting [12,13]. For *Brucella* spp., phagolysosomal acidification induces the expression of the type IV secretion system (T4SS), which in turn is necessary for the fusion of the BCV with the ER, providing a replication-permissive niche to bacteria that survive this process [14,15]. *Coxiella burnetii* is one of the few vacuolar bacteria that is well adapted to the phagolysosomal environment itself, as lysosomal acidity and contents activate its virulence and promote metabolic adaptation to its host [12,16]. However, it was recently demonstrated that, even for this species, the T4SS was necessary to modulate the terminal maturation and acidification of the CCV, thereby maintaining appropriate conditions for bacterial growth [17].

Nutrient uptake and space requirements for growth

Intracellular bacteria can find various supplies of nutrients within their host cells, such as glucose and its derivatives, glycerol and amino acids [18]. Iron uptake is also critical as its tight sequestration by host ferritin, which prevents toxicity to host cells, limits its availability for bacterial growth [19].

Cytosolic bacteria benefit from a broad volume to replicate as well as direct access to cytosolic nutrients (Table 1). By contrast, vacuolar residence restricts access to nutrients that do not freely diffuse across phospholipid bilayers [20]. This barrier is overcome by mechanisms allowing the import of nutrients across the vacuolar membrane. The *Chlamydia trachomatis* vacuole can directly engulf resources from the cytoplasm, fuse with nutrient-containing vacuoles, or exchange lipids with adjacent organelles [21], while the *Salmonella*-containing vacuole (SCV) of *Salmonella enterica* sv Typhimurium (*S. Typhimurium*) is connected to a continuous network of tubular vesicles, which provides access to endosomal nutrient supplies [22]. Uptake of essential micronutrients can otherwise be sustained via specific bacterial effectors, such as the recently characterised transition metal transporter MavN from *L. pneumophila* which allows the transport of iron and other transition metals to the LCV [23].

Vacuolar bacteria additionally face significant space constraints as they remain confined in vacuoles throughout their infectious cycle (Table 1). To accommodate growth, the fusion of PCVs with other PCVs or endomembrane compartments provides the main source of additional membrane supplies and space [20]. The maintenance of enlarging PCVs involves host cell trafficking mechanisms, as well as bacterial effectors that mimic host cell fusion proteins or regulate the organelle fusion machinery. Meanwhile, whereas confinement inside vacuoles represents an obstacle to bacterial proliferation, it should be noted that it can favour interbacterial communication, which may facilitate collective behaviour and impact the coordinated regulation of bacterial metabolic adaptation and virulence (Box 1).

Box 1. Enclosed spaces as bacterial meeting points

The enclosed volume of a vacuole poses a spatial constraint that can in turn promote exchange of information and/or genetic material between bacteria. Quorum sensing has received consistent attention as a virulence-promoting mechanism for vacuolar bacteria such as *L. pneumophila* and *Brucella* spp. [20] and could facilitate collective behaviour among the population of bacteria forming microcolonies inside endomembrane compartments. The *Legionella* quorum-sensing system Lqs is an important regulator of *L. pneumophila* virulence, and was recently shown to be responsible for the formation of highly virulent and antibiotic-resistant persisters in amoebas and macrophages [76]. Strikingly, Lqs

drives phenotypic heterogeneity within LCVs, including the emergence of a flagellated sub-population of bacteria that is primed for dissemination upon host cell bursting [77]. In *Staphylococcus aureus*, the quorum-sensing regulator Agr responsible for bacterial virulence is also induced in acidifying phagosomes [78]. By driving the expression of toxins such as haemolysins, diffusion-sensing by the Agr regulon is required for phagosomal escape of staphylococci [79]. Quorum-sensing genes were also found to contribute to the establishment of protective, biofilm-like structures in phagosomes by the Crohn's disease-associated, adherent invasive *E. coli* strain LF82 [80].

As an alternative mechanism, gene regulatory signalling induced in the phagosome environment can be transferred through genetic mobile elements such as prophages. In *L. monocytogenes*, it was shown that the phagosome environment promoted the excision of a prophage harboured in the bacterial *comK* gene, in a process dubbed active lysogeny [81,82]. Prophage excision and reactivation of *comK* support phagosome escape and *Listeria* intracellular growth. Given that a subset of *Listeria* can also survive or replicate durably inside endomembrane compartments such as SLAPs, eSLAPs and LisCVs [71,74,75], it would be informative to explore the status of prophage activation, metabolic and virulence gene expression for this sub-population of bacteria, to investigate how this status impacts bacterial metabolism and fitness, and to question whether it might prime, or on the contrary, tone down, bacterial virulence before later steps of infection.

Beyond regulatory functions, inter-strain horizontal gene transfer has been reported to occur between co-cultured isolates of *Chlamydia* spp. in tissue cultures [83,84]. Vacuolar bacteria could thereby acquire new genes through genetic recombination during their vacuolar lives, thus opening, in an evolutionary perspective, a window of potential diversification in bacteria that are genetically insulated in other regards, especially when considering obligate intracellular bacteria.

Facing host cell defence mechanisms

As intracellular bacteria colonise their vacuolar or cytosolic niches they face recognition by host cell immune sensors, the nature of which differ depending on the compartment ([Table 1](#) and [Figure 2](#)). In each niche, bacteria have evolved strategies to hide away from immune detection and evade host cell defence mechanisms [24].

Detection by immune sensors

Upon bacterial encounter with host cells, pathogen-associated molecular patterns (**PAMPs**) and damage-associated molecular patterns (**DAMPs**) are sensed by pattern-recognition receptors (**PRRs**), leading to the activation of cell autonomous immunity [25,26]. Part of this sensing is common to all intracellular bacteria, such as detection of PAMPs by plasma membrane Toll-like receptors (**TLRs**) during adhesion and entry ([Figure 2](#)) [27]. Inflammasomes also constitute central signalling hubs that integrate sensing of various cues. Their activation drives caspase-mediated immune responses, including the secretion of proinflammatory cytokines and cell death by pyroptosis that assist in counteracting bacterial replication [28]. However, besides these common defence lines, an array of sensors and clearance mechanisms are specific to the vacuolar or cytosolic compartments.

In the cytosol, bacteria are exposed to cytosolic immune sensors and to entrapment in autophagosomes ([Figure 2](#)) [29,30]. Among cytosolic sensors, the stimulator of interferon genes **STING** senses cyclic dinucleotides and subsequently induces the expression of interferon-stimulated genes ([Figure 2](#)). The bacterial metabolite cyclic-di-AMP, which is secreted by cytosolic *L. monocytogenes* (but also eventually produced by vacuolar bacteria like *C. trachomatis*), is a direct activator of STING [31,32]. Alternatively, cyclic GMP-AMP (**cGAMP**) can be produced by the host cGAMP synthase (**cGAS**) upon sensing of cytosolic DNA [33]. Bacterial RNA released by cytosolic bacteria such as *L. monocytogenes* is additionally sensed by retinoic acid-inducible gene I (**RIG-I**)-like receptors (**RLRs**), leading to the activation of type-I interferon responses ([Figure 2](#)) [34-36].

Entrapment of bacteria that gain access to the cytosol inside double-membraned autophagosomes is followed by targeting for lysosomal degradation, a process known as xenophagy ([Figure 2](#)) [37]. Upon vacuole rupture, either bacterial surface components or glycans exposed on remnants of

damaged membranes act as “eat-me” signals that are recognised by host E3 ubiquitin ligases and galectins, respectively [38]. Some of the recognition mechanisms involved in xenophagy have recently been described in detail. For instance, the host E3 ubiquitin ligase RNF213 was shown to sense lipid A of Gram-negative lipopolysaccharide, and thereafter initiate ubiquitin coating at the surface of cytosol-exposed *Salmonella* [39]. Ubiquitin-dependent signalling cascades then drive the recruitment of the autophagic machinery [40].

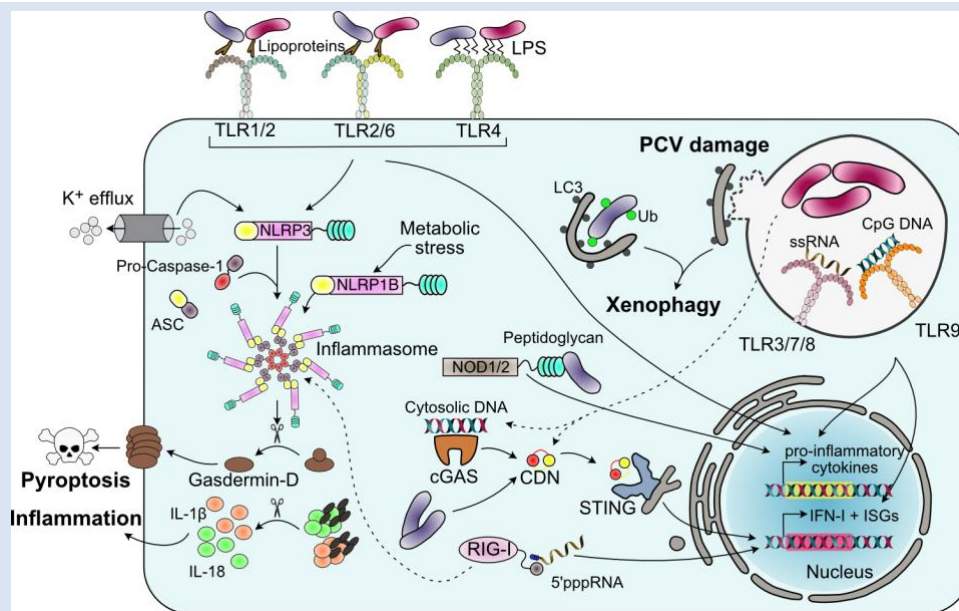


Figure 2. Detection of intracellular bacteria by host immune sensors

Bacterial pathogen-associated molecular patterns (PAMPs) are detected by several sensors upon bacterial infection. Activation of plasma membrane Toll-like receptors (TLRs) and NOD-like receptors (NLRs) induces the NF- κ B dependent transcription of pro-inflammatory cytokines and contribute to inflammasome activation. Other cellular stresses such as potassium efflux and metabolic stresses induce inflammasome formation, leading to the activation of caspase 1 that cleaves pro-nterleukin (IL)-1 β and pro-IL-18 into the pro-inflammatory cytokines IL-1 β and IL-18, and activate Gasdermin-D, thereby leading to pyroptosis. Detection of intracellular DNA by cyclic GMP-AMP synthase (cGAS), of cyclic dinucleotides (CDNs) by stimulator of interferon genes (STING), of triphosphorylated RNAs by retinoic acid-inducible gene I (RIG-I) and activation of vacuolar TLRs lead to the transcription of type-I interferons (IFN-I) and interferon-stimulated genes (ISGs). Damaged pathogen-containing vacuoles (PCVs) or ubiquitinated bacteria serve as “eat-me” signals that induce autophagy. Whilst cytosolic bacterial PAMPs can activate plasma membrane TLRs, RIG-I, inflammasome, and xenophagy, vacuolar bacteria activate mainly plasma membrane and vacuolar TLRs, as well as autophagy when the PCV membrane is disrupted. Abbreviations: LPS, lipopolysaccharide; ssRNA, single stranded RNA.

From the perspective of cytosolic surveillance, PCVs may thus be regarded as shelters against detection, unless their membrane integrity is compromised (**Table 1**). Bacterial effectors such as *L. pneumophila* SdhA contribute to this protection by eliciting the maintenance of LCV membrane integrity; indeed, *sdhA* deletion enhances bacterial release in the cytosol, impairs bacterial growth and induces caspase-11-dependent inflammation and pyroptosis [41,42]. However, inside vacuoles, bacteria face sensing by vacuolar TLRs (**Figure 2**). For instance, vacuolar TLR9 was shown to launch an efficient innate immune response against *L. pneumophila* in murine lung macrophages [43], and TLR3 plays a protective role against *Chlamydia* infections of human oviduct epithelial cells [44].

Evasion from antimicrobial mechanisms

As a countermeasure to detection by sensors and induction of cell-autonomous antimicrobial defences, many bacteria have evolved strategies to impede recognition, actively suppress downstream signalling, or remain shielded from host responses, depending on the threats they face in each

compartment. A number of intravacuolar pathogens can attenuate TLR signalling. For instance, the T4SS effector BtpB of *Brucella abortus* interacts, via its Toll/interleukin-1 receptor (TIR) domain, with TIR domains in downstream targets of TLRs, resulting in inhibition of NF- κ B translocation and reduced inflammatory responses in dendritic cells [45]. Although their identity has not been elucidated yet, effectors secreted by *L. pneumophila* through its type II secretion system (T2SS) also inhibit TLR2 signalling in human macrophages [46].

With regard to cytosolic bacteria, most of them have evolved mechanisms for avoidance of cytosolic surveillance and autophagy [24,38]. Actin-polymerising *S. flexneri* can be trapped inside cages of septin cytoskeletal filaments that inhibit their proliferation and target them to autophagy [47]. This process is supported by mitochondria, which in turn *S. flexneri* can oppose by stimulating the fragmentation of the mitochondrial network [48]. In *L. monocytogenes*, the surface-exposed internalin-family protein InlK recruits the Major Vault Proteins (MVPs), likely creating a disguise of vault particles that prevents recognition by sensors and thus autophagosome assembly [49]. In addition, actin polymerisation by the actin nucleation promoting factor ActA thwarts autophagic detection [50], and actin-based motility drives *L. monocytogenes* away from nascent autophagosomes [51]. Lastly, in *Rickettsia parkeri*, two methyltransferases were recently found to directly methylate lysin residues of the bacterial outer-membrane proteins, thereby preventing their ubiquitination and targeting to autophagy [52].

Survival of bacteria in each compartment thus requires that they have mechanisms for avoiding cell-autonomous immunity. In addition, cytosolic or vacuolar bacteria are not similarly exposed to circulating defences upon cell egress (**Box 2**), which impacts their probability of being targeted by the host immune system as well as their ability to disseminate.

Box 2. Avoidance of the humoral immune response by intracellular bacteria

After replication, vacuolar bacteria can leave the host cell by exocytosis, by inducing cell lysis, or by extrusion, before invading another target cell (**Figure 1**) [85]. Bacteria that are released into the extracellular space through exocytosis and lysis mechanisms can be detected by immune patrols and extracellular immune effectors such as neutralising antibodies and complement cascades. By contrast, *Chlamydia* spp. can escape the host cell inside a double-membraned compartment by extrusion [86]. Thus, as long as they stay enveloped, *Chlamydia* spp. remain sheltered from humoral immune responses. Then, upon their uptake by macrophages, these enveloped bacteria are protected from phagosomal bactericidal activities and may further spread infection [87].

As an alternative to release and exposure to the extracellular environment, a number of cytosolic pathogens can spread directly from cell to cell by forming membrane protrusions that end up inside a neighbouring cell (**Figure 1**). Bacterial actin-based motility, which drives the formation of protrusions, is mediated by surface effectors that recruit the host actin nucleation complex Arp2/3 [88]. To date, such effectors have been reported in *L. monocytogenes* (ActA), *S. flexneri* (IcsA), *Rickettsia* spp. (RickA) and *Burkholderia* spp. (BimA). Recruitment of Arp2/3 induces the polymerisation of F-actin at bacterial poles and the formation of actin comet tails that propel bacteria throughout the cytoplasm and generate sufficient forces to distort the plasma membrane [89]. Although belonging to distinct protein families, differing in structure and in their direct or indirect mechanism of Arp2/3 recruitment, the highly similar outcomes achieved by these four effectors is suggestive of convergent evolution, and thus of a strong selection pressure on intracellular bacteria for acquiring actin-based motility for cell-to-cell spread and immune evasion. However, not all intracellular bacteria employ this strategy. For instance, the causative agent of tularemia *Francisella tularensis* is devoid of actin-based motility; it was recently demonstrated that it could be transferred to uninfected macrophages by trophocytosis, a mechanism of plasma protein membrane exchange between cells [90].

For bacteria with dual lifestyles, the impact of their intracellular location on the probability that a subset of bacteria evades exposure to host defences and disseminates deserves closer investigation. For instance, one might speculate that while *Listeria* actin-based cell-to-cell spreading favours close-range tissue colonisation, a vacuolar lifestyle might support long-range dissemination of bacteria after bulk release in the intestinal lumen or in the circulation by plasma membrane lysis, or by an extrusion process similar to that of *Chlamydia*.

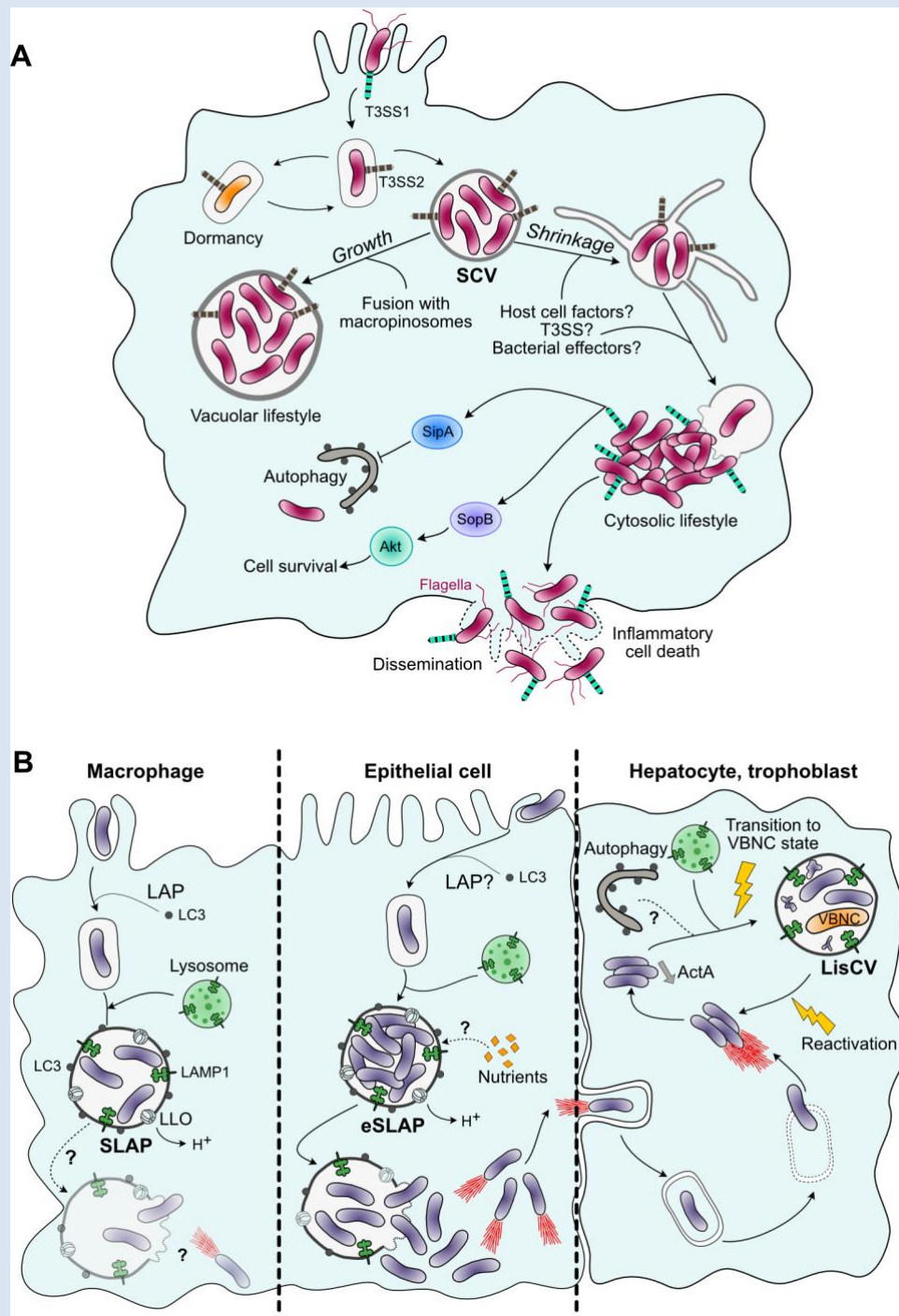


Figure 3. Dual intracellular lifestyles of *Listeria monocytogenes* and *Salmonella Typhimurium*

(A) Transition of *S. Typhimurium* from *Salmonella*-containing vacuoles (SCVs) to a cytosolic lifestyle in epithelial cells. Fusion of SCVs with macropinosomes favours the maintenance of a vacuolar lifestyle, whereas shrunk SCVs eventually rupture, after which bacteria initiate a cytosolic lifestyle where they hyper-replicate before inducing host cell death. Whereas the internalisation vacuole is turned into an SCV in a type III secretion system (T3SS2)-dependent manner, the ability of *Salmonella* to colonise the cytosol is T3SS1-dependent. The T3SS1-translocated effector SopB promotes cell survival by activating Akt-dependent signalling, while SipA inhibits autophagy, resulting in increased survival of cytosolic bacteria. *Salmonella* can also enter dormancy, either as viable but nonculturable (VBNC) persisters inside macrophage SCVs, or under a low metabolic state in a novel vesicular niche in epithelial cells. (B) Three vacuolar residence niches for *L. monocytogenes*. In macrophages and epithelial cells, bacteria reside in LC3⁺ and LAMP1⁺ vacuoles called respectively spacious *Listeria*-containing phagosomes (SLAPs) and SLAP-like compartments found in epithelial cells (eSLAPs). Replication in eSLAPs is more efficient than in SLAPs, allowing bacteria to eventually resume a cytosolic lifestyle. After

long-term infection in hepatocytes and trophoblasts, a subset of cytosolic bacteria stops expressing *actA* and becomes entrapped in secondary vacuoles called *Listeria*-containing vacuoles (LisCVs), where some bacteria enter a VBNC state. Through unknown mechanisms, VBNC bacteria can reactivate and resume a conventional lifestyle. Abbreviations: LAP, LC3-associated phagocytosis; LLO, listeriolysin O.

When intracellular bacteria play both sides

While bacterial adaptations to the vacuolar or cytosolic environments are usually opposed, as in the two previous sections, by contrasting the benefits and constraints of each lifestyle between distinct bacterial species, a growing body of evidence indicates that intracellular bacteria thought to be either exclusively cytosolic or exclusively vacuolar can in fact reside, for short or long parts of their intracellular lives, in either compartment. Some of them can successively occupy distinct intracellular niches, as is the case for *Mycobacterium tuberculosis*, which initiates its infectious cycle inside a vacuole before colonising the cytosol [53]. In other instances, dual lifestyles represent alternative routes after cell invasion, sometimes depending on the host cell type, and imply that bacteria can adapt to each environment. In this section, we illustrate this paradigm with an updated review of the intracellular niches occupied by two intracellular foodborne pathogens, *Salmonella* Typhimurium and *L. monocytogenes* (Figure 3) [previously reviewed respectively in 54,55].

Cytosolic replication of *S. Typhimurium*

S. Typhimurium displays a variety of intracellular behaviours and strategies of adaptations to specific niches, ranging from active replication to dormancy, depending on the host cell type and on the sensing of environmental cues (Figure 3A). In macrophages, while bacteria can replicate in SCVs, the stressful environment of this compartment can also lead to a dormant, viable-but-nonculturable (VBNC) state which favours *Salmonella* long-term persistence, including survival following antibiotic treatment [56]. In epithelial cells, induction of the stress response process known as the “stringent response” was recently shown to lead to a dormant, although culturable, state in a novel vesicular niche distinct from SCVs [57].

Despite being originally considered to inhabit vacuoles, *S. Typhimurium* can also replicate in the cytosol of epithelial cells (Figure 3A), but not of fibroblasts or macrophages [58]. Initial observations reported that a subpopulation of *S. Typhimurium* replicated in the cytosol of infected human cervical or colonic epithelial cell lines at a much higher rate than in the SCV [59,60]. *Salmonella* release into the cytosol proceeds mainly from events of growth and shrinkage of maturing SCVs [61]; growth results from fusion with infection-associated macropinosomes, whereas shrinkage is caused by tubule formation (Figure 3A). Perturbation of the growth/shrinkage cycle leads to vacuole rupture, bacterial escape and initiation of a cytosolic lifestyle. While the exact roles of host-cell factors and/or bacterial effectors in this SCV expansion/shrinkage cycle are not known [62-64], the role of some bacterial effectors involved in cytosolic survival and replication has been recently identified (Figure 3A). For effector secretion, the T3SS1 (the T3SS that mediates *Salmonella* invasion in epithelial cells) plays a critical role in *Salmonella* adaptation to the cytosolic environment [65,66]. Among the T3SS1-dependent effectors translocated by cytosolic *S. Typhimurium*, SopB was shown to activate Akt signalling, thereby promoting cell survival and extending the lifespan of cytosolic bacteria [67], while SipA promotes cytosolic bacteria survival and is implicated in autophagy evasion [68].

Despite the protective action of these effectors, hyper-replicating cytosolic bacteria induce more epithelial cell extrusion and inflammatory cell death than their vacuolar counterparts [59]. Enhanced cell extrusion is assumed to favour the fast dissemination of a population of bacteria that are primed for invasion, owing to their expression of genes involved at invasive stages such as T3SS1 and flagellin. Compartment-specific transcriptional profiling of intracellular *Salmonella* has recently confirmed that the cytosolic population expressed an array of virulence genes involved in host cell

adherence and entry [66]. Efficient release of this invasion-primed population of *Salmonella* in the intestinal lumen could transiently outcompete the microbiota and favour fresh colonisation of the mucosa as cells are renewed, while exacerbating diarrhoea. Recent evidence obtained in a mouse model for *Salmonella* infection strengthens this model, in which repeated cycles of cytosolic replication in intestinal epithelial cells, extrusion into the gut lumen, and subsequent reinfection sustain chronic faecal shedding [69].

Residence of *L. monocytogenes* inside vacuoles

L. monocytogenes was regarded for a long time as the archetype of cytosolic bacteria. Upon internalisation, it was classically described to escape rapidly from entry vacuoles, replicate in the cytosol, and then colonise other cells via actin-based motility [70]. However, closer investigation revealed that it could also lastingly reside inside vacuoles (**Figure 3B**).

The residence and slow replication of *L. monocytogenes* inside primary vacuoles called spacious *Listeria*-containing phagosomes (SLAPs) was observed both in macrophages of immunodeficient mice and in cultured macrophage cell lines (**Figure 3B, left**) [71]. SLAPs are initiated by LC3-associated phagocytosis (LAP), a mechanism that combines molecular pathways of phagocytosis and autophagy [72,73]. Their biogenesis is LLO-dependent but occurs only when bacteria express moderate amounts of the pore-forming toxin. Weakened LLO activity has thus been hypothesised to modulate the pH of SLAPs, albeit not enabling efficient vacuolar escape. SLAPs are also decorated with the lysosomal protein LAMP1, indicative of phagolysosomal fusion despite their neutral pH. It has therefore been proposed that SLAPs result from a “stalemate” between the inability of macrophages to clear bacteria and the failure of *L. monocytogenes* to exit the phagosome and replicate efficiently [71]. Whether bacteria can eventually reach the cytosol after long residence and replication within SLAPs, thereby resuming cytosolic growth and cell-to-cell spread, remains an open question.

Similar structures called epithelial SLAPs (eSLAPs) have recently been observed in colon adenocarcinoma cell lines (**Figure 3B, centre**) [74]. Like SLAPs, eSLAPs derive from primary vacuoles and are LAMP1- and LC3-positive. *L. monocytogenes* can replicate inside eSLAPs in an LLO-dependent manner, but replication rates are strikingly higher than in SLAPs, approaching the efficiency of cytosolic growth. Interestingly, LLO exerts two opposite functions in eSLAPs. On the one hand, it is required for eSLAP biogenesis, likely allowing membrane permeation and nutrient uptake, and perhaps also stimulating vacuole membrane repair mechanisms that drive its expansion. On the other hand, LLO-mediated vacuole membrane permeation facilitates cytosolic release of bacteria after several hours of residence within eSLAPs, followed by a conventional cytosolic lifestyle.

Although a process analogous to LAP is likely involved in eSLAP biogenesis, the precise mechanisms underlying their formation, growth and maintenance requires further studies, including the identification of host and/or bacterial factors that allow efficient replication of *L. monocytogenes* without inducing membrane rupture. Replication within eSLAPs might provide alternative properties to a subset of *Listeria*, which may include: modulation of virulence gene expression; priming of bacteria for a specific step of barrier crossing or colonisation of deeper organs; enhancing the ability to disseminate at long range rather than by cell-to-cell spreading (**Box 2**); delaying of host sensing, cytosolic immune responses, and cell death. Moreover, although eSLAPs are unlikely to increase overall bacterial loads, they may facilitate collective behaviour (**Box 1**).

Finally, *L. monocytogenes* was recently shown to persist in late endomembrane compartments displaying lysosome-like features in hepatocytes and trophoblasts, called *Listeria*-containing vacuoles (LisCVs) (**Figure 3B, right**) [75]. LisCVs are observed during prolonged *L. monocytogenes* infections (72 h) and are derived from the recapture of bacteria into secondary vacuoles after a prior cytosolic stage. They are decorated with LAMP1 but, unlike SLAPs and eSLAPs, are acidic and not

labelled by LC3. Interestingly, bacteria inside LisCVs enter a VBNC state. Eventually, VBNC bacteria can switch back to a replicative state upon passage of host cells. Given their properties, LisCVs are proposed to be responsible for asymptomatic carriage of *L. monocytogenes* and for tolerance to antibiotic treatments, prompting their further in-depth investigation.

Given the broad variety of intracellular niches that *Listeria* can take advantage of, careful evaluation of the impact of this wealth of alternative strategies on bacterial population dynamics, virulence, and pathogenesis is now critical. For instance, it will be instructive to characterise the diversity of cell types allowing the persistence or replication of *Listeria* inside vacuoles *in vivo* and to evaluate to which aspects of listeriosis they contribute, as well as to infer mechanisms of niche adaptation from transcriptional profiling of bacteria contained in each compartment.

Concluding remarks

We have illustrated earlier how invasive bacteria can take advantage of their host cell by adapting to the constraints and benefits imposed by their intracellular niche, especially with regard to replication, nutrient acquisition, and evasion of antimicrobial defences (**Table 1**). Despite the differences between vacuolar and cytosolic lifestyles, a growing number of intracellular bacteria are found to make the best of living in either compartment. Whereas we have described in this review dual lifestyles as exceptions, they may prove more widespread than initially suspected, and, by providing complementary strategies, could constitute niches of persistence or alternative routes of dissemination within tissues.

The host and bacterial mechanisms leading to these alternative lifestyles, the evolutive cost of their maintenance, and the disparity of scenarios that may take place depending on host cell types, location within tissues, and responses, are only now starting to be unravelled (see **Outstanding Questions**). Among promising tracks to be explored in-depth, permissiveness to alternative lifestyles is in all likelihood strongly conditioned by host cell properties. For instance, only the cytosol of epithelial cells can sustain the growth of *S. Typhimurium*, contrary to that of macrophages [58,66]. Likewise, it is expected that specific intracellular environments determine bacterial gene expression, survival, and growth in a cell-type-dependent manner for other pathogens. Another, nonmutually exclusive hypothesis is that stochastic variations in bacterial gene expression result in heterogeneous bacterial populations replicating in different intracellular compartments.

The end-up influence of dual lifestyles when considering bacterial fitness and infection outcomes also remains to be fully understood. The ability to replicate in distinct intracellular compartments could represent bet-hedging strategies that would benefit the overall bacterial population and maximise its infectious potential by allowing a multiplicity of options. In addition, bacteria residing in vacuoles in a latent or low replication state are potential candidates for causing asymptomatic carriage or clinically challenging antibiotic resistance. In coming years, we expect that integrated studies on animal models or on organoids will help to draw a more complete and dynamic view the heterogeneity of cycles and variety of host niches colonised by bacterial pathogens *in vivo* and of their contribution to pathogenesis, from the stage of cell and organ colonisation to that of pathogen dissemination, or to the onset of disease chronicity.

Outstanding questions

What are the evolutive benefits and costs of bacterial dual lifestyles? Although the genetic and metabolic investments required for maintaining versatility are likely to be costly at the level of the individual bacterium, this flexibility could pay off at the population level by allowing bacterial replication even when one of the two lifestyles is hampered.

Does bistability in bacterial gene expression condition individual intracellular fate? In each niche, exposure to differing environmental cues also likely drives changes in metabolic and virulence gene expression that should impact bacterial adaptation.

To which extent does the host cell status (cell type, metabolism, autonomous immunity, turnover rate) influence the ability of bacteria to inhabit each niche?

Can we assess the qualitative and quantitative contributions of dual lifestyles to bacterial fitness and infection outcomes? Beyond local bacterial loads, to which extent do these alternative strategies contribute to long-term silent infections, to tuning penetrance within tissues, to dissemination throughout the body, or to shedding?

What is the relevance of the emerging diversity of *L. monocytogenes* vacuolar residence types to the host-bacteria crosstalk, and how do these niches contribute to infection dynamics? All vacuolar niches can in theory promote inter-bacterial communication, protect from cytosolic PAMP sensors and autophagy, and/or provide signalling platforms from the vacuole membrane. LisCVs, and to a lesser extent SLAPs, could offer a long-term protective niche against antimicrobial defences and favour persistent infections. In eSLAPs, which occur in epithelial cells with a high turnover rate, *Listeria* might find a temporary replicative niche where cytosolic sensing is delayed, and perhaps be later released in bulk for short- or long-distance dissemination.

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Declaration of interests

No interests are declared.

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Glossary

cGAMP: cyclic GMP-AMP, a cyclic dinucleotide produced by the cGAMP synthase (**cGAS**).

LAP: LC3-associated phagocytosis. A specific phagocytosis pathway that integrates some features of non-canonical autophagy.

LLO: listeriolysin O, a pore-forming toxin secreted by *Listeria monocytogenes*. Its ability to permeate membranes triggers pleiotropic effects on target cells and accounts for a large part of virulence.

NLR: NOD-like receptor, a family of cytosolic receptors that regulate inflammation and apoptosis. The NOD (for nucleotide-binding oligomerisation) is characteristic of this family.

PAMP: pathogen-associated molecular pattern. PAMPs refer to a broad variety of microbial molecular cues which can be sensed by host receptors named **PRRs** (for pattern-recognition receptors), such as NLRs, RLRs, and TLRs. By extension, **DAMPs** (for damage-associated molecular patterns) refer to molecular cues that act as danger signals, by reporting on impairments to cellular integrity.

PCV: pathogen-containing vacuole. Depending on the pathogen, BCV, CCV, LCV, LisCV or SCV refer, respectively, to *Brucella*-, *Coxiella*-, *Legionella*-, *Listeria*- or *Salmonella*-containing vacuoles. The *Listeria*-specific PCVs encountered in macrophages were named SLAPs, for spacious *Listeria*-containing phagosome; by analogy, eSLAPs represent SLAP-like compartments found in epithelial cells.

RLR: RIG-I-like receptor. A family of cytosolic PRRs that sense RNA of viral or bacterial origin. The family is named after named after the **RIG-I** (for retinoic acid-inducible gene I) receptor that senses uncapped 5'-triphosphorylated RNA.

STING: stimulator of interferon genes. This PRR anchored in endoplasmic reticulum membranes acts as a cytoplasmic DNA sensor and a signalling hub which activates type-I interferon production.

TLR: Toll-like receptor. A family of membrane-associated PRRs that sense a wide array of PAMPs. TLRs harbour a conserved TIR (for Toll/interleukin-1 Receptor) domain mediating protein-protein interaction and involved in signal transduction. TIR domains are encountered in other protein families specialised in immune signalling, such as the interleukin-1 receptor, and have also been acquired by secreted effectors of intracellular bacteria.

T2SS, T3SS, T4SS: Respectively, types II, III, and IV secretion systems, specialised in the export of proteins by bacteria. The T2SS is formed by the assembly of general secretory proteins allowing the ATP-dependent translocation of protein cargoes through the inner and outer membranes of Gram-negative bacteria. The T3SS adopts a needle-like structure, the base of which bulges through the inner and outer membranes of Gram-negative bacteria, and the tip of which contacts the membrane of target cells. The T4SS creates an ATP-dependent, gated translocation channel through prokaryotic membranes, usually connected to a secretion pilus. T4SSs allow the secretion of effector proteins, and mediate the export of nucleic acids during conjugation.

VBNC: viable but nonculturable. A state in which bacteria cannot be cultured despite keeping a low metabolic activity.