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David Ribet, Pascale Cossart

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2 **How bacterial pathogens colonize their hosts**
3 **and invade deeper tissues**
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8 **David Ribet^{1,2,3,*} and Pascale Cossart^{1,2,3,*}**
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19 ¹Institut Pasteur, Unité des Interactions Bactéries-Cellules, Département de Biologie
20 Cellulaire et Infection, F-75015 Paris, France.

21
22 ²INSERM, U604, F-75015 Paris, France.

23
24 ³INRA, USC2020, F-75015 Paris, France.
25
26
27

28 * Correspondence should be addressed to D.R. (david.ribet@pasteur.fr) and P.C.
29 (pascale.cossart@pasteur.fr)
30

31 **Abstract**

32

33 Bacterial pathogens have evolved a wide range of strategies to colonize and invade
34 human organs, despite the presence of multiple host defense mechanisms. In this review, we
35 will describe how pathogenic bacteria can adhere and multiply at the surface of host cells,
36 how some bacteria can enter and proliferate inside these cells, and finally how pathogens may
37 cross epithelial or endothelial host barriers and get access to internal tissues, leading to severe
38 diseases in humans.

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48 **Keywords**

49 bacterial invasion; bacterial adhesion; microbiota; host barrier; host-pathogen interactions;

50 *Listeria*

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52 **1. Introduction**

53

54 The human body harbours a large number of bacteria but their localisation in healthy
55 individuals is normally restricted to certain body areas such as the skin, the mucosae of buccal
56 and nasal cavities, vagina and, most importantly, the gastrointestinal tract [1],[2],[3],[4],[5],[6].
57 The internal tissues are normally sterile. In some circumstances, however, some opportunistic
58 pathogens are able to enter the host by taking advantage of injuries or breaches in one of the
59 different host barriers. In addition, *bona fide* pathogens have evolved mechanisms to cross
60 host barriers and reach deeper organs where they proliferate and lead to severe disease for
61 their host.

62 In this review, we will describe the diversity of mechanisms used by bacterial
63 pathogens to colonize and invade human organs. We will first focus on the capacity of these
64 bacteria to adhere and to proliferate at the surface of host cells and tissues, despite a wide-
65 range of defense mechanisms used by the host. We will then present how some bacteria are
66 able to enter and to proliferate inside host cells. Finally we will discuss how some pathogens
67 can cross host barriers and get access to deeper tissues thereby promoting their dissemination
68 inside their host.

69

70 **2. Colonization of host surfaces**

71

72 The respiratory, digestive and urogenital mucosa represent a surface area of
73 approximately 300 to 400 square meters (*i.e.* 200-fold larger than that of the skin) and thus
74 constitute major sites of contact with bacteria. These mucosa are composed of three layers: an
75 epithelium, a layer of loose connective tissue called lamina propria, and a thin layer of smooth
76 muscles. These surfaces constitute frontline barriers limiting the invasion by both commensal
77 and pathogenic bacteria. Despite the different defense mechanisms occurring at the level of

78 these barriers, pathogenic bacteria have evolved various molecular strategies to adhere to
79 these epithelia and to proliferate at their surface.

80

81 **2.1 Host epithelia and associated defense mechanisms**

82

83 Epithelia of diverse organs in contact with the extracellular milieu, and thus with
84 environmental bacteria, are covered by a mucus layer that allows a protection against
85 intruders. The intestinal mucus layer, for example, plays a key role in limiting invasion by
86 commensal bacteria of the microflora or by foodborne pathogenic bacteria [7] (Fig. 1 and 2).
87 This mucus is mainly composed of glycoproteins called mucins, digestive enzymes,
88 antimicrobial peptides and immunoglobulins. Bacteria are often found at the top of this
89 intestinal mucus layer, where they interact with mucins, whereas the inner layer of mucus,
90 where the concentration of antimicrobial compounds is high, is normally devoid of bacteria
91 [8]. Mucins are produced and secreted in the intestine by goblet cells, a specialized cell-type
92 of the intestinal epithelium. Their production can be modulated in response to microbial
93 products or inflammation [7]. The level of antimicrobial peptides, predominantly secreted by
94 Paneth cells from intestinal crypts, can also be regulated by the presence of microorganisms.
95 Indeed, whereas α -defensins are constitutively expressed, other antimicrobial peptides such as
96 REG3 γ (Regenerating islet-derived protein 3 γ) or cryptdins are produced in response to the
97 detection of pathogen-associated molecular patterns (PAMPs) that activates TLR (Toll-like
98 receptors) or NOD (nucleotide-binding oligomerization domain-containing protein) signalling
99 pathways [9],[10],[11],[12]. IgA, produced by B cells in the lamina propria and secreted into
100 the mucus via epithelial cells, are also involved in limiting bacterial association with the
101 intestinal epithelium and intestinal barrier crossing [13],[14]. Finally, in addition to these
102 molecules with antimicrobial activities, shedding of mucus can be another mechanism to

103 prevent bacterial adhesion to epithelial surface, as reported in the case of gastric mucus
104 colonized by the pathogenic bacterium *Helicobacter pylori* [15]. Interestingly, some bacterial
105 pathogens have evolved mechanisms to go through this mucus layer in order to reach
106 epithelial cells. They either produce proteases and directly target host mucins, locomote via
107 flagella-based motility or resist to antimicrobial products [16],[17],[18],[19],[20],[21] (Fig. 2).
108 Interactions between pathogenic bacteria and host mucus thus constitute a challenging issue
109 during host infection.

110

111 Besides mucus, an important actor in the control of pathogen invasion is the
112 microbiota, which is mainly constituted of commensal bacteria living on human mucosal
113 surfaces. In the human intestine, this microbiota plays fundamental roles in digestion, as well
114 as in intestinal epithelial metabolism and proliferation. In addition, it plays a key role in the
115 resistance to foodborne infections by directly competing with enteric pathogens [22]. Indeed,
116 inhibitory metabolites, such as acetate or butyrate, can be released by different species of
117 commensal bacteria. These bacteria can furthermore utilize various nutrients of the intestinal
118 lumen, which would then be no longer available for incoming pathogens. Finally, the
119 microbiota is involved in the regulation of the host immune system [23]. It has been shown in
120 particular that germ-free mice, *i.e.* animals devoid of normal microbiota, display poorly
121 developed intestinal mucosal lymphoid follicles, called Peyer's patches. They also have an
122 altered composition of CD4⁺ T cells and IgA-producing B cells in the lamina propria [24],[25].
123 Despite the protection provided by the microbiota, some enteric pathogens are able to
124 efficiently colonize the gut and even cross the intestinal barrier. Triggering of inflammation
125 can be considered as a mechanism used by pathogens to alter the microbiota composition,
126 thereby allowing them to outcompete luminal commensals [26]. Inflammation of the gut is
127 characterized by an increase in the quantity of mucosal antimicrobial peptides to which

128 pathogens may exhibit higher resistance compared to commensals [20],[21],[22]. Mucosal
129 inflammation also leads to the production of specific compounds that can be used by
130 pathogens such as particular glycosylated proteins or tetrathionate [27],[28]. This last molecule
131 is indeed used by the murine enteric bacterial pathogen *Salmonella* Typhimurium, which uses
132 it as a terminal electron acceptor during anaerobic respiration, giving to this pathogen a
133 growth advantage over fermenting commensal bacteria in this inflamed environment [28] (Fig.
134 2). Together, the overall crosstalks and interactions between commensal bacteria, enteric
135 pathogens and host are crucial in the establishment and progression of intestinal diseases [26].

136

137 Finally, in addition to mucus and to the microbiota, epithelial cell renewal plays an
138 important role in the control of bacterial colonization [29]. In the gut, cells of the intestinal
139 epithelium have a very high turnover rate. Indeed, new epithelial cells are constantly
140 generated at the level of intestinal crypts. These cells are then migrating along the villi to be
141 finally extruded at the tip of these villi after about one week. There is a tight balance between
142 the self-renewal of cells and their elimination that is crucial to homeostasis and epithelium
143 integrity. Induction of epithelial cell death has been characterized as a defensive mechanism
144 used by the host to limit infection by enteric pathogens [29]. Cell death indeed allows the
145 elimination of damaged cells and limits persistent bacterial colonization. The global
146 upregulation of the epithelial turnover furthermore facilitates the repair of epithelial injuries
147 and decreases the intestinal permeability induced by some pathogens. Consistently, the
148 intestinal epithelium turnover rate constitutes a target for pathogens. Some bacteria can
149 indeed block epithelial cell death to preserve their replication niches, whereas others trigger
150 cell death to facilitate their egress or induce breaches in the epithelial barrier in order to
151 access the underlying tissues [29] (Fig. 3).

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2.2 Mechanisms of bacterial adhesion to host cells

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Adhesion of bacteria to host surfaces is a crucial aspect of host colonization as it prevents the mechanical clearing of pathogens and confers a selective advantage towards bacteria of the endogenous flora. Accordingly, bacteria have evolved a very large arsenal of molecular strategies allowing them to target and adhere to host cells.

Pili, which are polymeric hair-like organelles protruding from the surface of bacteria, represent a first class of structures involved in the binding of bacteria to host cells [30],[31]. The base of these structures, initially discovered in gram-negative bacteria, is anchored to the bacterial outer membrane, whereas the tip is usually an adherence factor conferring the binding specificity of these structures. For example, UPEC, which are uropathogenic strains of *E. coli* colonizing the urinary tract and involved in kidney infections, display pyelonephritis-associated (P) pili at their surface. The tip of these pili is constituted by an adhesion factor called PapG, that binds to glycosphingolipids of the kidney epithelium [32]. Some UPEC strains also possess Type I pili at their surface, which have binding specificity to D-mannosylated receptors, such as the uroplakins of the bladder [33]. Type IV pili constitute another class of polymeric adhesive surface structures expressed by different gram-negative bacteria [34]. These pili are composed of thousands of copies of the major pilin protein that are first synthesized in the bacterial cytoplasm and then translocated across the inner membrane and proteolytically processed. Only the processed forms of pilin are competent for polymerization. The assembled pili then pass through the outer membrane via a channel formed by the secretin protein [34]. Once formed, these pili can aggregate laterally to form bundles. Type IV pili have the ability to retract through the bacterial cell wall, while the pilus tip remains attached to its target surface, allowing the so-called “twitching motility”, a flagella-independent mode of motility important for efficient colonization of host surfaces

178 [35]. In the case of *Neisseria meningitidis*, a bacterium found in the human nasopharynx but
179 which may occasionally get access to the host blood stream leading to sepsis and meningitis,
180 type IV pili play a role in the formation of microcolonies attached to vascular endothelial cells
181 [36]. Adhesion of bacteria to vascular endothelium is normally impaired by the shear stress,
182 which represents the hydrodynamic forces generated by the blood components circulating at
183 high speed through the vessels. Local and temporal drops in this shear stress, such as those
184 observed in some brain capillaries, may however be compatible with an attachment of
185 “seeding” *N. meningitidis* to the vascular endothelium [37]. Once attached, these bacteria are
186 much more resistant to shear stress and can start to proliferate leading to the formation of
187 microcolonies. This resistance to shear stress is in part due to the induction of filopodia-like
188 cell projections, that can be observed inside or surrounding *Neisseria* microcolonies, and to
189 the recruitment of several host proteins underneath bacteria [38]. Furthermore, upon divisions,
190 bacteria remain aggregated via their type IV pili. It is interesting to note that, in some
191 instance, some bacteria may modify their pili (via a post-translational modification of specific
192 pili subunits) in order to destabilize pilus fiber interactions, leading to the detachment of these
193 bacteria from the original microcolony and facilitating their dissemination to distant sites of
194 infection in other vessels or in the cerebrospinal fluid in the cases of blood-brain barrier
195 crossing (see below) [39]. In addition, antigenic variation has been observed for *Neisseria* type
196 IV pili, allowing expression of new variants during infection and escape of this pathogen from
197 the host immune system [34].

198 In the last decade, pili structures have also been observed in gram-positive bacteria.
199 Two types of pili have been described so far in these species. The first class consists in
200 “sortase-assembled pili”, in which successive pilin subunits are linked by isopeptide bonds
201 after translocation across the bacterial membrane. This linkage is catalysed by bacterial
202 transpeptidases called sortases allowing the formation of completely covalent polymers that

203 are eventually linked to the cell wall peptidoglycan [40]. The second class consists in “type
204 IV-like pili”, which are similar to type IV pili of Gram-negative bacteria, even though the
205 lack of outer membranes and the thick peptidoglycan structures of Gram-positive bacteria
206 imply differences in the assembly mechanisms of these filaments [34]. Many studies are now
207 deciphering the role of these pili in the adhesion of gram-positive pathogens to host cells and
208 in pathogenesis.

209 In addition to pili, a wide range of bacterial surface factors with adhesive properties
210 have been described. These adhesins recognize various classes of host molecules including
211 transmembrane proteins such as integrins or cadherins, or components of the extracellular
212 matrix such as collagen, fibronectin, laminin or elastin [30],[31],[41],[42]. Some of these
213 adhesins, after allowing the binding of bacteria to host cell surfaces, are also triggering the
214 internalization of bacteria inside host cells (see below).

215 As already mentioned in the case of *Neisseria*, shear stress can decrease adhesion of
216 bacteria bound to host surfaces under fluid flow. For many bacterial adhesins, the probability
217 of bond breaking increases with the tensile force derived from shear stress. However, in some
218 instances, shear stress does not inhibit but rather promotes adhesion. This counter-intuitive
219 phenomenon can be explained by the existence of specific force-strengthened bonds, called
220 ‘catch bonds’ [43]. These bonds can be observed for example with the *E. coli* FimH adhesin,
221 which exhibits a shear-enhanced binding to mannose [43].

222 In parallel to these canonical mechanisms of bacterial adhesion, the EPEC (Entero-
223 Pathogenic *E. coli*) and EHEC (Entero-Hemorrhagic *E. coli*) pathogens, which are responsible
224 respectively for diarrheal disease in children, and severe foodborne infections, use a very
225 particular mechanism to create an intimate contact with host cells: they inject an effector,
226 called Tir, that inserts into the host cell plasma membrane and serves as an “exogenous”
227 receptor for the bacterial surface protein intimin [44]. Tir is delivered into the host cell

228 cytoplasm via EPEC or EHEC type III secretion system (T3SS), a complex of proteins
229 forming a needle-like structure that traverses the bacterial cell wall and the host-cell plasma
230 membrane [45]. Binding of bacterial intimin to the extracellular domain of Tir is followed by
231 the recruitment of host cell cytoskeleton regulators such as the Wiskott-Aldrich syndrome
232 protein (N-WASP) and the actin-related protein 2/3 (Arp2/3) complex that locally remodels
233 the actin cytoskeleton [44]. This remodelling leads to the retraction of the host cell absorptive
234 microvilli and to the creation of a pedestal under the attached bacterium, thereby creating the
235 characteristic “attaching and effacing” lesions induced by this pathogen. Tir thereby tethers
236 the bacteria to the host epithelial cell surface and provides a direct connection between the
237 bacteria and the host’s cytoskeleton. These bacterial factors are essential for pathogenesis as
238 mutants of intimin/Tir interaction do not colonize the intestine and are avirulent in animal
239 models of infection [46].

240 Adhesion of bacteria to host surfaces is finally a key element in the formation of
241 biofilms, *i.e.* matrix-enclosed microbial assemblies that can adhere to biological or non-
242 biological surfaces. Biofilm formation constitutes a protected mode of growth that allows
243 bacteria to survive in hostile environment. In the context of infectious diseases, biofilms may
244 be critical as matrix-embedded bacterial aggregates are more resistant to host defences or
245 antibiotic treatments. The exact *in vivo* role of these biofilms during bacterial infections now
246 constitutes an active field of research [47],[48].

247 In conclusion, adhesion represents a crucial step for extracellular bacteria that
248 facilitates their persistence in the host. For intracellular bacteria, it is a first essential step that
249 precedes their internalization within host cells.

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253 **3. Establishment and maintenance of an intracellular lifestyle**

254

255 An intracellular lifestyle provides diverse advantages for bacterial pathogens: they
256 become inaccessible to humoral and complement-mediated attack; they avoid shear stress-
257 induced clearance and get access to a wide range of nutrients, provided they display the
258 metabolic pathways to use them. However, host cells also possess different mechanisms
259 specifically targeting these intracellular bacteria. Intracellular pathogens have therefore
260 developed different strategies to successfully establish and maintain an intracellular infection.

261

262 **3.1 Getting inside host cells**

263

264 Professional phagocytes, such as macrophages or M cells of the intestinal Peyer's
265 patches, represent a frontline defense against pathogens. These cells also constitute a niche for
266 bacteria with an intracellular lifestyle, as they naturally internalize foreign particles. After
267 being phagocytosed by macrophages, bacteria such as *Mycobacterium tuberculosis*, the agent
268 of tuberculosis, or *Legionella pneumophila*, the bacterium responsible for Legionnaire's
269 disease, block the acidification of the phagosome and its fusion to lysosomes, thereby
270 avoiding killing and allowing sustained survival in these cells. The ability of some of these
271 phagocytes to migrate through tissues furthermore provides an interesting way for pathogens
272 to disseminate inside their host.

273 Many bacteria can also induce their internalization into non-professional phagocytes.
274 Two main mechanisms of entry are involved in this case, namely the zipper and the trigger
275 mechanisms. Both of them rely on the activation of signalling cascades leading to the
276 reorganization of the actin cytoskeleton at the level of the host plasma membrane [41],[49]
277 (Fig. 4).

278 In the case of the zipper mechanism, engagement of bacterial proteins with host
279 membrane proteins normally involved in cellular adhesion such as cadherins or integrins,
280 leads to the recruitment of various host factors involved in the strengthening of cell-cell or
281 cell-matrix contacts. Due to the small size of bacteria, induction of a response normally
282 strengthening cell attachment to extracellular matrix or neighbouring cells results in this case
283 to bacterial engulfment. *Listeria monocytogenes* (hereafter referred to as *Listeria*), a gram-
284 positive food-borne pathogen responsible for human listeriosis, induces its internalization into
285 non-phagocytic cells via a zipper mechanism [49],[50],[51]. Internalization of *Listeria* is
286 mediated by two surface proteins, InlA and InlB, which respectively target E-cadherin and the
287 hepatocyte growth factor receptor Met, which are both host plasma membrane proteins
288 [52],[53]. Met is ubiquitously expressed in human cells, whereas E-cadherin is expressed only
289 in specific cell types, such as epithelial cells. Interaction of InlA with E-cadherin triggers the
290 same signalling cascade as the one normally observed for E-cadherin/E-cadherin interactions
291 [50],[51]. This leads in particular to the recruitment of different host factors at the site of
292 bacterial entry such as α - and β -catenin, myosin VIIa and vezatin [54]. InlB interaction with
293 its receptor Met results in the recruitment of Gab1, Cbl and Shc, the activation of PI3 kinase
294 and in actin remodelling at the site of entry [51]. Clathrin-mediated endocytosis machinery
295 was shown to be involved in the early steps of *Listeria* internalization after the initial contact
296 between InlA and InlB with their receptors and before cytoskeleton rearrangements [55].
297 Ultrastructural analysis by electron microscopy revealed the presence of isolated clathrin-
298 coated pits assembled at *Listeria* entry foci [56]. Because these structures cannot internalize
299 large particles such as bacteria, it was proposed that the observed clathrin coated pits serve as
300 a platform for cytoskeletal rearrangements at bacteria-host adhesion sites [50],[51],[56].
301 Septins, a class of host proteins forming non polar filaments and participating to the cell

302 cytoskeleton, constitute another player involved in the late step of *Listeria* entry process
303 [57],[58] (Fig. 4).

304 In the case of the trigger mechanism, bacteria activate signalling pathways leading to
305 large-scale cytoskeletal rearrangements characterized by the formation of membrane
306 protrusions called ruffles [30],[41]. The extending ruffles then fold over and fuse back to the
307 cell surface, thereby entrapping nearby bacteria (Fig. 4). This cellular process can normally be
308 observed in response to soluble growth factors. *Salmonella* induces its internalization into
309 non-phagocytic cells via a trigger mechanism. To do so, it injects directly in the host cell
310 cytoplasm, via one of its two T3SS, a set of sophisticated bacterial effectors that trigger
311 cellular responses [59]. Some of these effectors activate host cell Rho GTPases such as Cdc42
312 and Rac that spatiotemporally stimulate actin cytoskeleton rearrangements and allow
313 membrane ruffling. Other effectors control these events and permit recovery of the
314 cytoskeleton's normal architecture after infection, by deactivating Rho GTPases [59].

315 Interestingly, *Salmonella* exhibit target site preferences for internalization in tissue
316 culture. Selection of these preferred loci involve a “near surface swimming” mode, in which
317 flagella-driven motility allows bacteria to land onto and scan the host cell layer for
318 “promising” entry sites [60]. For non-motile bacteria such as *Shigella flexneri*, the bacterial
319 pathogen responsible for bacillary dysentery, it was reported that bacteria can get in contact
320 with the epithelial layer via filopodial-like extensions emanating from the host cells. Upon
321 bacterial contact, filopodia retract and bring *Shigella* in contact with the cell body where
322 invasion occurs [61].

323 Finally, it should be noted that in the course of infection, one given pathogen can be
324 internalized in different cell types and express different set of virulence genes. In such cases,
325 each infected cell type may have a particular role for disease progression.

326

327 **3.2 Diversity of intracellular compartments used for bacterial replication**

328

329 After internalization, intracellular bacteria can replicate in three main classes of
330 compartments. The first class is constituted by lysosomes-like vacuoles, which have an acidic
331 pH and contain hydrolytic enzymes. The second corresponds to intracellular non acidic
332 vacuoles that do not fuse to lysosomes and are usually remodeled by the pathogen. The third
333 compartment is the cytosol in which some pathogens can reside after escape from their
334 internalization vacuole.

335 *Coxiella burnetti*, the agent of Q fever, is a well-known example of an intracellular
336 bacterium able to survive in a lysosomal-like compartment [41],[62]. Following
337 internalization, the *Coxiella*-containing phagosome develops into a parasitophorous vacuole
338 harbouring lysosomal properties such as acidic pH, the presence of hydrolases and cationic
339 peptides. Despite these harsh environmental conditions, *Coxiella* is able to efficiently
340 replicate in this compartment although the mechanisms used by this pathogen to survive in
341 this type of vacuole are poorly understood [62].

342 In addition to lysosomal-like vacuoles, there is a great diversity of non-acidic
343 intracellular vacuoles in which pathogenic bacteria may reside [41]. In particular, pathogens
344 are able to remodel the properties of these vacuoles by altering their proteic and lipidic
345 composition or their trafficking and interaction with other vacuolar compartment of the host.
346 *Salmonella*, for example, resides after internalization in vacuoles that undergo acidification
347 but do not behave as lysosomes. Several effectors secreted by the second T3SS of *Salmonella*
348 play important roles in the remodelling of these *Salmonella*-containing vacuoles [63]. Some
349 effectors secreted across the vacuolar membrane remodel locally the actin cytoskeleton,
350 allowing the polymerization of an actin basket surrounding these vacuoles and regulating
351 bacterial virulence [64], [65]. Other effectors block the recruitment of NADPH oxidase

352 responsible for the production of bactericidal compounds that normally kill intracellular
353 bacteria [66].

354 Finally, some pathogens such as *Listeria* are able to escape from their internalization
355 vacuoles and get access to the host cell cytosol [41],[67]. In the case of *Listeria*, this escape is
356 mediated by LLO, a pore-forming toxin secreted by the bacteria and the two bacterial
357 phospholipases PC- and PI-PLC (phosphatidylcholine and phosphatidylinositol-specific
358 phospholipases C) [68]. Once in the cytosol, *Listeria* is able to replicate and to move inside
359 cells using actin-based motility [67]. This intracellular motility leads to the formation of
360 bacteria-containing protrusions and cell-to-cell spread. In some cases, protrusion formation is
361 associated with plasma membrane damage due to LLO's pore forming activity. LLO also
362 promotes the release of bacteria-containing vesicles from the host cell, covered with exofacial
363 phosphatidylserine (PS), that can be recognized by PS-binding receptor expressed by
364 macrophages, and phagocytosed. This mechanism, known as efferocytosis and normally used
365 by the host to phagocytose dying or dead cells, is here exploited by *Listeria* to promote cell-
366 to-cell spread and facilitate bacterial access to macrophages, which are key targets of *Listeria*
367 during systemic infection [69].

368 Although life in the cytosol provides access to a wide-range of different nutrients,
369 intracytosolic bacteria have also to face specific defense mechanisms such as autophagy
370 [70],[71]. Autophagy is a degradation process by which cytosolic components are delivered to
371 lysosomal compartments. This process involves the formation of a double-membrane
372 phagophore that closes to form an autophagosome, which then fuses to lysosomes leading to
373 the degradation of enclosed material. Selective autophagy permits the targeting of specific
374 components such as intracellular bacteria to autophagosomes via their detection by specific
375 receptors. Bacterial autophagy was first described as an important host response degrading
376 intracellular bacteria replicating in the cytosol. Of note, this process may also target

377 intravacuolar bacteria. Consistently, several pathogens have evolved mechanisms to avoid
378 autophagy-mediated degradation. This is the case for *Listeria*, which possesses at least two
379 virulence factors, ActA and InlK, that disguise the bacteria from recognition by the autophagy
380 machinery [72],[73]. In addition, autophagy was shown to favor bacterial replication in some
381 instances, revealing a much more complex interplay between autophagy and pathogens than
382 previously expected [70],[71].

383 Among the different advantages conferred by intracellular lifestyles, the
384 internalization of pathogens in specific cell types, such as cells migrating through host tissues,
385 facilitates the dissemination of bacteria in their host. In parallel, several pathogens have
386 evolved specific mechanisms to cross epithelial and endothelial barriers and to get access to a
387 wide range of host tissues.

388

389 **4. Crossing of host barriers**

390

391 Several types of sentinel cells, such as M cells, luminal macrophages or dendritic cells
392 (DCs) are continuously sensing the presence of pathogenic bacteria in the mucosal
393 environment. Although these cells are playing a key role in coordinating the innate and
394 adaptive immune response to limit the colonization of pathogens in the host, they also
395 constitute entry portals for pathogens.

396 M cells are specialized cells found in the intestinal epithelium and other epithelia in
397 humans. These cells have a function different from that of their neighbouring epithelial cells.
398 In the intestine, they continuously sample the lumen and transport luminal antigens across the
399 epithelial barrier to the underlying lymphoid tissue thereby contributing to intestinal
400 immunity [74] (Fig. 1). M cells are exploited by many different pathogens as a route of entry
401 to deeper tissues of the host. Intestinal ligated loop infection models have established that

402 *Salmonella* Typhimurium can cross the intestinal barrier via these M cells [75]. Indeed,
403 targeting of M cells by *S. Typhimurium* leads to M cells destruction, thereby introducing
404 breaches in the intestinal barrier. Bacteria are then able to spread rapidly to organs before the
405 establishment of an immune response [75]. In a similar fashion, *Shigella flexneri*, was shown
406 to target and enter into M cells allowing translocation of bacteria across the epithelial barrier,
407 without being toxic for these cells [76]. *S. flexneri* then reinvade epithelial cells basolaterally
408 and triggers an inflammatory response that disrupts the epithelium, thereby facilitating the
409 translocation of additional bacteria [76] (Fig. 1).

410 DCs constitute another cell type that sense antigens of the mucosal environment and
411 which play a central role in the adaptative immunity. These cells, present in mucosal tissues,
412 may migrate to mesenteric lymph nodes, where they interact with lymphocytes. The
413 phagocytic activity of these cells and their ability to migrate from periphery to circulation has
414 been exploited by different bacterial pathogens [77]. *S. Typhimurium*, for example, may be
415 taken up by DCs located in intestinal Peyer's patches, but also by inter-epithelial DCs that
416 send dendrites between absorptive cells without altering the epithelial permeability [78] (Fig.
417 1). This mechanism was proposed to also participate to the rapid crossing of the host
418 epithelium by *Salmonella* and to facilitate its dissemination in the host via reaching of the
419 bloodstream [78].

420 Translocation through non phagocytic cells of the intestinal epithelium is another key
421 mechanism used by pathogens to reach the lamina propria and to cause systemic infections. In
422 the case of *Listeria*, interaction between the bacterial InlA surface protein and the host
423 receptor E-cadherin is essential for crossing of the intestinal barrier [54],[79]. E-cadherin is a
424 key component of adherens junctions, and was first considered as being inaccessible for
425 bacteria located in the intestinal lumen. However, accessible E-cadherin has been reported to
426 be present at sites of cell extrusion at the tip of intestinal villi and at junctions between

427 mucus-secreting goblet cells and adjacent enterocytes. These two locations have been shown
428 to be sites of invasion for *Listeria* [80],[81] (Fig. 1). In addition to the well-established
429 intracellular lifestyle of *Listeria*, involving bacterial escape from the internalization vacuole,
430 entry of bacteria through accessible E-cadherin can also lead to its rapid translocation, via
431 transcytosis, across enterocytes, without bacterial escape from the internalization vacuole [81].
432 Of note, this mechanism of epithelium traversal has also been reported for *S. Typhimurium*
433 which can traffic to the basolateral side of epithelial cells after invasion and is then released in
434 the underlying lamina propria [82]. InlB, another *Listeria* internalin mediating its
435 internalization in host cells, is not required for crossing of the intestinal barrier. However, it
436 has been shown that this internalin is crucial, in addition to InlA, to cross the placental barrier
437 [83]. The proposed mechanism is that InlB, via the activation of PI3 kinase, potentiates InlA-
438 mediated downstream signalling, thereby increasing internalization efficiency at the level of
439 the placenta [84].

440 Increasing epithelial or endothelial permeability is another strategy widely used by
441 bacterial pathogens to cross host barriers [29],[36],[54]. Many pathogens are targeting cell-cell
442 junctions to increase barrier permeability, thereby enhancing bacterial dissemination in the
443 host (Fig. 3). For example, some T3SS effectors secreted by EPEC and EHEC destabilize
444 tight junctions and induce a loss of trans-epithelial resistance [29],[54]. *Vibrio cholerae*
445 secretes a metalloprotease, called HA/P (hemagglutinin/protease), which cleaves the
446 extracellular domain of host occludin, a key component of the tight junctions [85]. Another
447 pathogenic species of *Vibrio*, *V. parahaemolyticus*, delivers in the host cytoplasm a virulence
448 factor that AMPylates RhoGTPases (*i.e.* catalyses the covalent addition of AMP to
449 RhoGTPases), leading to the disruption of the actin cytoskeleton integrity and the rounding of
450 targeted cells [86]. *Neisseria meningitidis* is able to cross the host blood barrier by altering
451 endothelial permeability. This bacterium is thought to gain access to the cerebrospinal fluid

452 via the blood by crossing the vessel endothelium barrier. One proposed mechanism is that
453 upon binding to endothelial cells, *N. meningitidis* recruits host cell proteins involved in the
454 formation and the stabilization of adherens and tight junctions [87],[88]. Several junction
455 proteins are thereby depleted from cellular junctions and relocated beneath *Neisseria*
456 microcolonies. This process may lead to a destabilization of endothelial junctions and
457 increase the permeability of the vessels, facilitating bacterial escape from the blood vessel and
458 colonization of the cerebrospinal fluid.

459 Finally, triggering of inflammation, in addition to its role on the microbiota discussed
460 above, was also proposed as a tool used by pathogens for host barriers disruption [89]. Some
461 inflammatory cytokines, such as TNF- α , indeed disrupt tight junctions and impair gut barrier
462 integrity, and thereby may facilitate access to deeper tissues for bacterial intruders [90].
463 Interestingly, it was shown that during the course of infection by *S. Typhimurium*, a major
464 portion of bacteria that invade epithelial cells is actually killed, but this fraction triggers the
465 inflammation response of the host that benefits to the surviving bacteria [91]. This example
466 illustrates the concept of phenotypic heterogeneity and cooperation for pathogenic bacteria,
467 where some bacteria from a given population may fulfil specialized functions for the benefits
468 of the overall community.

469

470 **5. Conclusion**

471

472 The diversity of niches that may be colonized by pathogenic bacteria in the human
473 body is huge. Bacteria have evolved various mechanisms to adhere to the surface of organs in
474 contact with the external milieu, such as the intestine. In addition, some bacteria can adopt an
475 intracellular lifestyle and get internalized inside various host cells types to replicate away
476 from the humoral host immune defenses. In this case, there is again a wide-range of strategies

477 adopted by pathogenic bacteria, which can be illustrated by the different cellular locations
478 they are able to use for replication. Finally, pathogenic bacteria can get access to deeper
479 tissues using various mechanisms to cross mucosal barriers, and access the bloodstream,
480 which is an entry portal for potentially all host organs, and is often associated to severe
481 clinical symptoms.

482 In addition to mucosal surfaces, the skin also corresponds to a preferential site of
483 contact with pathogens. As for mucosal barriers, the production of antimicrobial molecules
484 and the presence of specific immune cells play important roles in cutaneous defenses [3].
485 Whereas most pathogens are unable to cross the skin barrier, they can however access the
486 underlying tissues via ruptures in the skin, such as cuts, microlesions or bites (in particular for
487 pathogens transmitted via arthropod vectors).

488 With regard to the diversity of niches used by pathogenic bacteria for replication,
489 scientists have classified bacteria as extra- or intra-cellular or, for intracellular bacteria, as
490 intravacuolar or intracytosolic. However, increasing evidence now shows that bacteria
491 initially thought to remain strictly extracellular can indeed be found inside host cells as
492 exemplified by the case of *Staphylococcus aureus* [92]. In addition, some intracellular bacteria
493 can be observed both in vacuoles or free in the cytoplasm. This situation is well accepted for
494 some pathogens such as *Salmonella* but is more controversial for others such as
495 *Mycobacterium* or *Legionella*. Further work is therefore needed to clearly define the different
496 compartments where a given bacterium can be found, and more particularly during infection
497 *in vivo*. Deciphering the respective role of these compartments in the establishment of the
498 associated disease is also critical as some of them may only represent dead-ends during the
499 course of infection.

500 The frontier between commensals and pathogens is also not as straightforward as
501 expected. Indeed, some bacteria normally considered as commensals, can become pathogenic

502 when they escape their original niche and start to colonize deeper tissues. Bacteria belonging
503 to the microbiota, and therefore considered as commensals, can also become pathogenic if
504 their growth rate raises and if they outcompete other members of the intestinal flora. For *bona*
505 *fide* pathogens, variability in the expression of virulence factors has also been observed.
506 Indeed, virulence factors are not constitutively expressed and their production tightly depends
507 on the environmental conditions faced by the bacterium. As a given bacterium can be found,
508 depending on the stage of infection, in the intestinal lumen, inside epithelial cells or
509 professional phagocytes, or in the bloodstream, the set of virulence factors expressed in these
510 different conditions has to vary accordingly in order to face the different host defense
511 mechanism encountered. *Shigella*, for example, has the capacity to sense the gradient of
512 oxygen that is present between the anaerobic intestinal lumen and the oxygenized intestinal
513 tissues. Activation of its T3SS is effective only at its precise site of action, in relatively
514 oxygenized area, nearby intestinal epithelial cells, thereby allowing enhanced invasion and
515 virulence [93]. Similarly, intracellular *Salmonella* Typhimurium is sensing environmental pH
516 to coordinate the secretion of T3SS effectors. Assembly of one of its T3SS is indeed done
517 after internalization into host cells, in response to the acidification of the *Salmonella*-
518 containing vacuoles (SCVs) [94]. The corresponding T3SS effectors are further secreted only
519 after the tip of T3SS needle gets in contact with the neutral pH lying outside of the SCVs,
520 allowing a tightly regulated secretion of these bacterial molecules in the cytoplasm of the
521 infected cells [95].

522 It is also important to mention that the result of a bacterial infection is tightly
523 dependent on host susceptibility. Genetic polymorphism in the host population accounts for a
524 great variability in the type or intensity of responses triggered against the encountered
525 pathogen. The same bacterium can thus cause a large spectrum of clinical manifestations from
526 asymptomatic infection to fatal disease, depending on host genetic variability.

527 Finally, in addition to genetic-driven host susceptibility, it is now well-established that
528 the microbiota is playing a critical role to limit colonization and invasion by enteric
529 pathogens. Many studies are now highlighting that the composition of this microbiota may be
530 altered by various external parameters including overuse of antibiotics, changes in diet and
531 elimination of constitutive partners such as nematodes [96],[97]. For example, after antibiotic
532 treatment, the composition of the microbiota was demonstrated to be different from the
533 original one, and this modified microbiota may be more prone to colonization by specific
534 bacterial pathogens [98]. Characterization of the composition of microbiota from patients by
535 high throughput sequencing techniques will open new avenues for the development of
536 personalized diagnosis, the potential manipulation and modification of this microbiota, and
537 the development of new treatments to prevent and limit infections by enteric bacterial
538 pathogens.

539

540 **CONFLICT OF INTEREST**

541

542 The authors declare no conflict of interest

543

544

545

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547

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557

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784

785 **FIGURE LEGENDS**

786

787 **Figure 1 : Routes of invasion by enteric pathogens in the human small intestine**

788 The epithelium of the small intestine is composed of absorptive enterocytes, mucus-producing
789 goblet cells, M cells, as well as proliferating stem cells and Paneth cells located in intestinal
790 crypts. The intestinal epithelium is covered by a mucus layer containing secreted IgA,
791 antimicrobial peptides and other types of antimicrobial compounds that limit the colonization
792 by commensal bacteria or foodborne pathogens. Peyer's patches and the overlaying follicle-
793 associated epithelium, M cells and dendritic cells constitute specialized regions of the
794 intestine that continuously sample the intestinal luminal content (adapted from [99]). *Listeria*
795 *monocytogenes* can cross the host intestinal barrier at sites of cell extrusion at the tip of the
796 villi or at junctions between goblet and absorptive epithelial cells. *Salmonella* Typhimurium
797 can cross the intestinal epithelium by targeting absorptive cells, M cells of Peyer's patches or
798 dendritic cells sampling the intestinal lumen. *Shigella flexneri* also target M cells for crossing
799 the intestinal barrier and then re-infect epithelial cells basolaterally.

800

801 **Figure 2 : Schematic representation of events leading to *Salmonella* overgrowth in the** 802 **intestine**

803 Invasion of intestinal epithelial cells by *Salmonella* triggers an inflammatory response leading
804 to the release of antimicrobial peptides and the production of ROS (Reactive Oxygen Species)
805 by neutrophils. H₂S, a fermentation end product generated by commensal bacteria, is oxidized
806 into thiosulfate by the colonic epithelium and then into tetrathionate by ROS. In contrast to
807 fermenting bacteria of the microbiota, *Salmonella* can use this tetrathionate as a terminal
808 electron acceptor to support growth in anaerobic conditions. The use of tetrathionate, in

809 addition to *Salmonella* resistance to antimicrobial molecules, allow this pathogen to out-
810 compete commensal bacteria in this inflamed context.

811

812 **Figure 3 : Bacterial virulence factors manipulating epithelial cell functions**

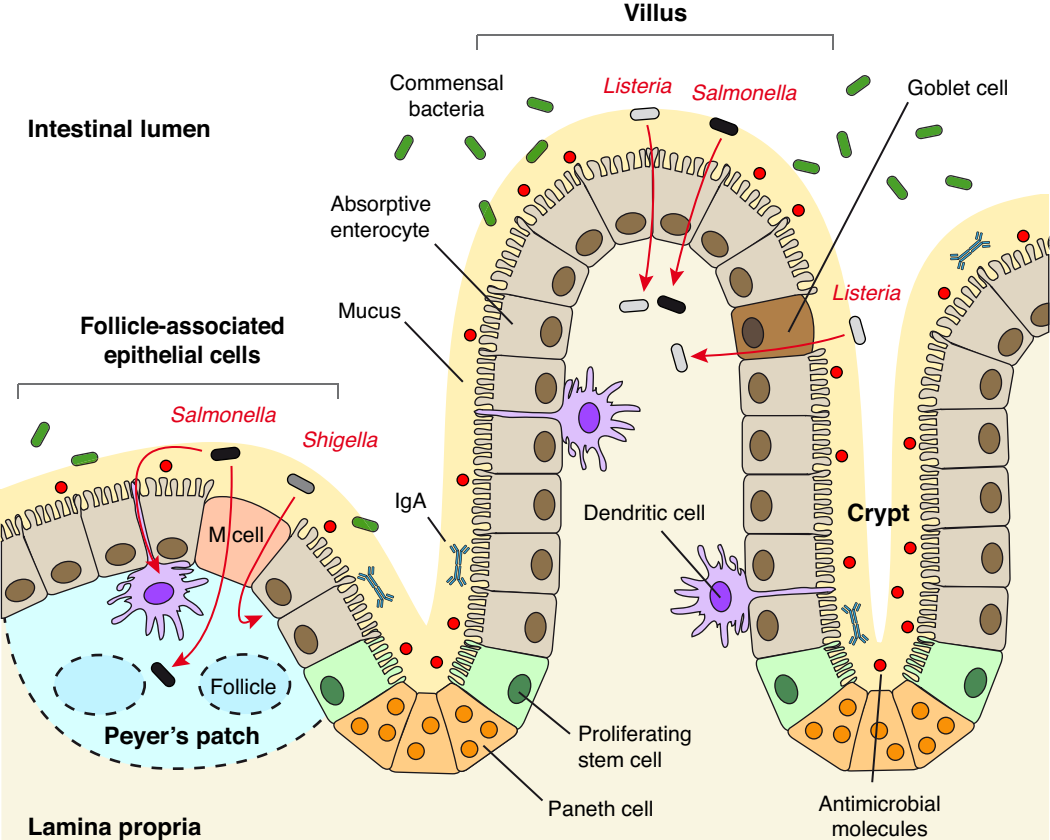
813 Examples of bacterial virulence factors targeting host proteins involved either in epithelial
814 cell-cell junctions or in cell death and turnover. CagA, an effector of *Helicobacter pylori*,
815 interacts with ZO-1, a component of tight junctions, and Par1, a regulator of cell polarity, and
816 triggers disruption of epithelial tight junctions. This virulence factor also disrupts adherens
817 junctions by targeting E-cadherin (Ecad) and promoting the release of β -catenin (β cat) from
818 the adherence complex. *Vibrio cholerae* secretes the metalloprotease HA/P which degrades
819 the extracellular domain of occluding (Ocl), another component of tight junctions. EPEC
820 injects different effectors in the host cytoplasm, such as MAP, EspF or EspG that also target
821 and disrupt tight junctions. AvrA, a factor delivered by *Salmonella* Typhimurium possesses
822 acetyltransferase activity towards MAP kinase kinase (MAPKK) and plays a major role in the
823 downregulation of both inflammatory and c-Jun N-terminal kinase (JNK)-mediated epithelial
824 cell death responses to infection. CagA, in addition to its alteration of cell-cell junctions,
825 promotes cell proliferation by upregulating ERK, a pro-survival factor, and Mcl1, an anti-
826 apoptotic factor. In contrast to AvrA or CagA, some pathogenic *E. coli* strains express Cif, an
827 effector that blocks the cell cycle by inactivating Nedd8-conjugated Cullin-RING E3
828 Ubiquitin ligases (CRLs).

829

830 **Figure 4 : “Zipper” versus “trigger” mechanisms of bacterial entry inside host cells**

831 Schematic representation of internalization of *Listeria monocytogenes* via a “zipper”
832 mechanism (A), or *Salmonella* Typhimurium via a “trigger” mechanism (B). In the “zipper”
833 mechanism, engagement of bacterial surface proteins with host proteins induce cytoskeleton

834 and membrane rearrangements, leading to the internalization of the bacterium. In the “trigger”
835 mechanism, injection of effectors by the bacterium in the host cell cytoplasm triggers large-
836 scale cytoskeletal rearrangements and ruffles formation allowing the bacterium to be engulfed
837 and internalized. (C) Schematic representation of the multiple molecular pathways activated
838 by bacterial surface proteins (in this case, InlA and InlB from *Listeria monocytogenes*)
839 leading to the internalization of bacteria by a “zipper” mechanism (?: unknown interaction or
840 component) (adapted from [67]).



Intestinal lumen

Villus

Commensal bacteria

Listeria

Salmonella

Goblet cell

Absorptive enterocyte

Mucus

Listeria

Follicle-associated epithelial cells

Salmonella

Shigella

M cell

IgA

Dendritic cell

Crypt

Paneth cell

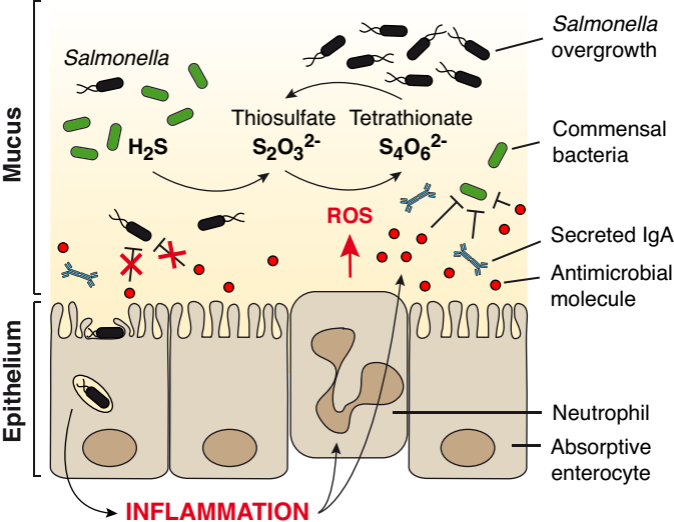
Proliferating stem cell

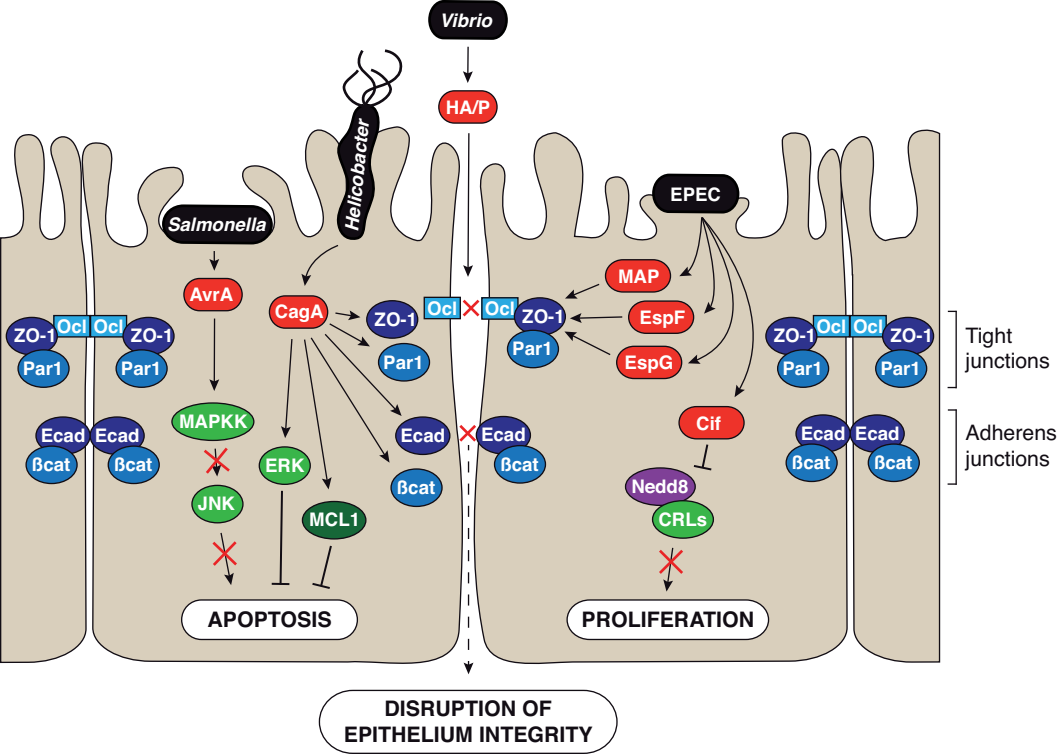
Antimicrobial molecules

Follicle

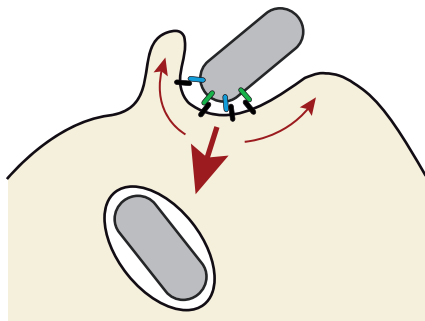
Peyer's patch

Lamina propria

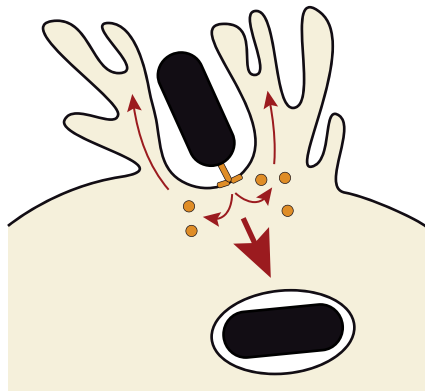




A Zipper mechanism
(*Listeria monocytogenes*)



B Trigger mechanism
(*Salmonella Typhimurium*)



C

