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1           **Making sense of the biodiversity and virulence of *Listeria monocytogenes***

2

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21

22 **Abstract**

23 *Listeria monocytogenes* is a foodborne pathogen responsible for listeriosis, an infection which  
24 can manifest in humans as bacteremia, meningoenzephalitis in immunocompromised patients  
25 and the elderly, and fetal-placental infection in pregnant women. Reference strains from this  
26 facultative intracellular bacterium have been instrumental to investigate basic mechanisms in  
27 microbiology, immunology and cell biology. The integration of bacterial population genomics  
28 with environmental, epidemiological and clinical data allowed the uncovering of new factors  
29 involved in the virulence of *L. monocytogenes* and its adaptation to different environments.  
30 This review illustrates how these investigations have led to a better understanding of the  
31 bacterium's virulence and the driving forces that shaped it.

32

33 **Highlights**

- 34 • *Listeria monocytogenes* is a foodborne opportunistic bacterial pathogen
- 35 • Classical genetic approaches in reference strains allowed identification of core  
36 virulence genes
- 37 • Investigation of the species diversity of *L. monocytogenes* allowed uncovering hypo-  
38 and hypervirulent strains and new virulence genes
- 39 • Hypervirulent strains are host-associated, whereas hypovirulent strains are associated  
40 with food-processing environments
- 41 • Accumulation of loss-of-function mutations in virulence pathways leads to niche  
42 restriction and obligate saprophytism

43

## 44 **Introduction**

45 *Listeria monocytogenes* is the causal agent of listeriosis, a foodborne infection associated with  
46 high morbidity and mortality. The virulence of *L. monocytogenes* depends on its ability to  
47 colonize the gut and disseminate in the host. This Gram-positive bacterium was first isolated  
48 by G. Hülphers in Sweden, from rabbit liver necrosis [1] and from a human meningitis case in  
49 1921 by J. Dumont [2]. It was then isolated and characterized by E.G.D. Murray in rabbits  
50 and guinea pigs from a breeding facility in the UK in 1926, and J.H. Pirie in wild gerbils in  
51 South Africa in 1927 [3, 4]. Human listeriosis is characterized by septicemia and central  
52 nervous system (CNS) infections, which mostly occur in immunocompromised and/or elderly  
53 individuals, and maternal-fetal infections, as well as rare anatomically localized infections [5-  
54 12]. *L. monocytogenes* can also induce gastroenteritis in otherwise healthy individuals when  
55 the ingested inoculum is large [13]. The foodborne origin of human listeriosis was formally  
56 proven in the 1980s [14]. Although listeriosis is relatively rare compared to other foodborne  
57 infections [15, 16], its high fatality rate (approximately 20% to 30%, despite effective  
58 antibiotic treatment [5]) makes it a significant public health concern [17]. Recently, the  
59 largest recorded outbreak occurred in South Africa, where 937 individuals were affected, with  
60 a 27% mortality rate [18]. *L. monocytogenes* has also been detected in the feces of healthy  
61 people, but the prevalence of *L. monocytogenes* fecal carriage in the general population is  
62 unknown [19, 20]. *L. monocytogenes* is therefore mostly considered as an opportunistic  
63 pathogen.

64 *L. monocytogenes* is not only an economical and public health issue, but is also a powerful  
65 model to study basic microbiology, host biology including innate and adaptive immunity and  
66 cell biology [21-24]. The identification of *L. monocytogenes* virulence factors in reference  
67 strains in cell culture models allowed deciphering the underlying mechanisms of its  
68 interactions with the host, and also addressing basic cell biology questions [22]. Further  
69 studies combining epidemiologic data with comparative genomics analyses of food- and  
70 clinically-associated strains allowed the uncovering of new genes involved in *L.*  
71 *monocytogenes* virulence [25]. Current investigations indicate that virulence factors are key  
72 for the adaptation of *L. monocytogenes* to its host and its optimal spread in the environment,  
73 and may modify our understanding of how this bacterium has acquired and selected these  
74 genes.

75

76 **Uncovering virulence genes of *L. monocytogenes* by mutagenesis of reference strains**

77 It was shown early on, using *in vitro* and *in vivo* approaches, that *L. monocytogenes* can enter  
78 macrophages, survive and be transferred to adjacent cells [26]. *In vivo* experiments in guinea  
79 pigs indicated that *L. monocytogenes* can be released in the cytoplasm of intestinal epithelial  
80 cells, surrounded by electron dense material [27], which would later come to be identified as  
81 polymerized actin. Entry of *L. monocytogenes* in non-phagocytic epithelial cells was reported  
82 *in vitro* in the human enterocyte-like cell line Caco-2 [28]. Most of the factors involved in key  
83 phenotypes of the cellular microbiology of *L. monocytogenes*, namely entry into host cells,  
84 escape from the internalization vacuole and spread from cell to cell, were characterized in the  
85 1980s and 1990s and shown to be critical for virulence *in vivo*. They were mostly discovered  
86 by a combination of molecular and cell biology approaches, using transposon bacterial  
87 mutagenesis, gene knock-out and complementation studies coupled with infection of cultured  
88 macrophages and epithelial cell lines. These factors were characterized in the laboratory  
89 reference strains EGD, 10403S or EGDe, which all belong to the serovar 1/2a but to two  
90 distinct lineage II clonal complexes (CCs; see below). EGD (CC7) derives from a strain  
91 isolated in guinea pig by E. G. D. Murray, hence its name [3]. 10403S (CC7) is a  
92 streptomycin-resistant strain derived from 10403, a strain isolated from a human listeriosis  
93 case [29]. The origin of EGDe (CC9) is more difficult to trace. It was supposed to be an EGD  
94 isolate, but genome analyses have revealed that both strains are actually unrelated [30, 31].

95

#### 96 LIPI-1 genes

97 A phenotypic characteristic of *L. monocytogenes* is its ability to completely lyse red blood  
98 cells (beta hemolysis), due to the expression of a hemolysin encoded by *hly* and named  
99 listeriolysin O (LLO) [32, 33]. Screening and characterization of nonhemolytic mutants  
100 obtained by transposon mutagenesis indicated that hemolysin expression is critically needed  
101 for *L. monocytogenes* virulence [34]. Further characterization of  $\Delta hly$  mutant in intestinal  
102 Caco-2 cells showed that LLO's pore-forming toxin is involved in escape of *L.*  
103 *monocytogenes* of its internalization vacuole [28]. Later analysis involved the characterization  
104 of two phospholipases, PlcA and PlcB (activated by the metalloprotease Mpl), which are also  
105 involved in vacuolar escape [35, 36]. Once in the cytosol, *L. monocytogenes* can polymerize  
106 actin and form so-called "actin comet tails" via its surface protein ActA, which propel *L.*  
107 *monocytogenes* in the cytoplasm, and allow its spread from cell to cell [37, 38]. The study of  
108 *actA* and *plcA* transposon and deletion mutants indicated that they are both critical for *L.*  
109 *monocytogenes* virulence [39, 40]. Analysis of an avirulent non-hemolytic variant of the  
110 NCTC 7973 guinea pig strain (isolated by E.G.D. Murray) allowed the characterization of a

111 transcription factor involved in *hly* expression which was named PrfA for "Positive regulatory  
112 factor of listeriolysin (*lisA*, the other designation for *hly*)" [41, 42]. PrfA was further shown to  
113 be the major regulator of *L. monocytogenes* virulence genes *in vivo*, including *prfA*, *hly*, *actA*,  
114 *plcA*, *mpl*, *plcB* and the nucleomoduline *orfX* [43], which are all encoded by the Listeria  
115 pathogenicity island 1 (LIPI-1) [44] (Figure 1A). PrfA is also transcriptionally regulated by  
116  $\sigma^B$ , that controls the general stress response in *L. monocytogenes*, is regulated post-  
117 transcriptionally and requires a glutathione co-factor for full activation [45, 46].

118

### 119 *inlAB*

120 Transposon mutagenesis was also used to screen for mutants that are unable to enter Caco-2  
121 cells. This approach allowed for the identification and characterization of internalin (InlA), a  
122 leucine-rich repeats (LRR)-containing protein anchored to the bacterial peptidoglycan by a  
123 Leu-Pro-X-Thr-Gly (LPXTG) motif [47]. The receptor of InlA, namely E-cadherin (Ecad),  
124 which is expressed on epithelial cells and forms *adherens* junctions, was identified by affinity  
125 chromatography [48]. Further analyses indicated that the InlA-Ecad interaction is species-  
126 specific, as mouse and rat Ecad are not a receptor for InlA, in contrast to human Ecad. Indeed,  
127 InlA interaction with Ecad requires a proline in the 16<sup>th</sup> amino acid position of Ecad, which is  
128 present in permissive species (human, rabbit, guinea pig, gerbil), and is replaced by a  
129 glutamic acid in non-permissive murine species (mouse, rat), preventing InlA interaction with  
130 Ecad in the latter [49]. In guinea pigs and transgenic mice expressing human Ecad, InlA-Ecad  
131 interaction is critical for *L. monocytogenes* to cross the intestinal barrier, at the villus level, by  
132 transcytosis across goblet cells [50, 51]. Live imaging in intestinal organoids has now shown  
133 that *L. monocytogenes* hijacks E-cadherin recycling for its: (i) entry into; (ii) transepithelial  
134 transfer across; and (iii) exocytosis from goblet cells, in a dynamin-, microtubule- and Rab11-  
135 dependent manner, respectively [52]. *inlA* is located in an operon comprising a downstream  
136 gene called *inlB* [47]. InlB is also an LRR-containing protein; however, it is not covalently  
137 linked to the bacterial surface by an LPXTG motif, but loosely attached to it by its Gly-Trp  
138 (GW) motif. InlB mediates the entry of *L. monocytogenes* in cultured cells by interacting with  
139 c-Met/HGFR, which is ubiquitously expressed [53, 54]. InlB-c-Met interaction is also  
140 species-specific: it occurs in humans, mice and gerbils but not in rabbits nor guinea pigs [55].  
141 InlB is not involved in *L. monocytogenes* crossing of the intestinal barrier [55]. In order to  
142 study the role of the InlA-Ecad and InlB-c-Met interactions in organs other than the intestine,  
143 knock-in mice KIE16P, in which the glutamic acid in 16<sup>th</sup> position of the Ecad is replaced by  
144 a proline, were generated [56]. In contrast to the intestinal barrier where only InlA is

145 involved, InlA and InlB are both required and act in a conjugated manner in *L.*  
146 *monocytogenes* crossing of the placental barrier in gerbils and in KIE16P mice [56, 57].

147

### 148 **Uncovering virulence genes of *L. monocytogenes* by comparative *Listeria* genomics**

149 While classical genetics has identified major virulence factors in *L. monocytogenes*, whole  
150 genome sequencing of pathogenic bacterial species, which was initiated with *Mycoplasma*  
151 *genitalium* [58], opened a new avenue for identifying genes involved in virulence, by  
152 comparison of the genomic content of pathogenic and phylogenetically closely related non-  
153 pathogenic bacterial species. Indeed, comparison of the genome sequences of *L.*  
154 *monocytogenes* EGDe and the nonpathogenic *Listeria innocua* CLIP 11262 reference strains  
155 allowed identification of putative virulence factors present in *L. monocytogenes* but absent in  
156 *L. innocua* [59] (Figure 1B).

157 Sequencing of EGDe revealed the presence of 25 genes that code for proteins of the internalin  
158 family, characterized by the presence of an LRR domain. Seventeen of them had not been  
159 described before, including InlJ, an LPXTG anchored protein involved in virulence, whose  
160 exact function remains to be determined [60, 61] and InlP (*lmo2470*), a secreted protein  
161 involved in the infection of the placenta, among other organs [62, 63].

162 Comparative genomics also revealed the presence of GW motif-containing proteins other than  
163 InlB in EGDe (and which were absent in *L. innocua* CLIP 11262) that are involved in  
164 virulence. As an example, the GW-containing protein Auto is an autolysin involved in cell  
165 entry and virulence [64].

166 Another gene that is absent in *L. innocua*, named *bsh*, has been identified by this strategy.  
167 This PrfA-regulated virulence gene encodes for a bile salt hydrolase, which increases  
168 resistance of *L. monocytogenes* to bile, and enhances liver infection and bacterial faecal  
169 carriage [65], an essential property for a foodborne pathogen.

170

### 171 **Intraspecies genomic studies and identification of novel virulence genes**

#### 172 *The biodiversity of L. monocytogenes*

173 The biodiversity of *L. monocytogenes* was first investigated via serotyping, based on *Listeria*  
174 somatic and flagellar antigens, which allowed identifying the four main serotypes (1/2a, 1/2b,  
175 1/2c and 4b) associated to listeriosis [66]. However, this method does not allow establishing  
176 reliable phylogenetic links between strains. Multilocus enzyme electrophoresis differentiated  
177 two main lineages, I and II [67], while gene variation studies and multi-locus genotyping  
178 assays have led to the delineation of two minor lineages III and IV [68, 69]. In order to

179 determine the population structure of *L. monocytogenes*, multi-locus sequence typing  
180 (MLST), allowing to translate sequence diversity into numerical profiles, called sequence  
181 types (STs), was set up with seven housekeeping genes [70]. Using this technique, isolates  
182 having at least 6 alleles in common out of these seven genes are grouped into the same clonal  
183 complex (CC). With advances in whole genome sequencing, this method has been extended  
184 to larger sets of loci (typically thousands) to allow strain differentiation at the level of the  
185 whole- or core genome (wgMLST or cgMLST, respectively) [71-75]. A standard  
186 nomenclature system has been proposed based on the cgMLST scheme of 1,748 core loci  
187 [75]. Isolates sharing less than 150 and 7 allelic mismatches are grouped into sublineages  
188 (SL) and cgMLST types (CT), respectively, which allows for efficient inter-laboratory  
189 communication, in particular for emerging strain types [76] (Figure 2A). In addition, the  
190 compilation of genotypic data in dedicated databases, such as BIGSdb-*Lm*  
191 (<http://bigsdb.pasteur.fr/listeria>) or GenomeTrakr (hosted at  
192 <https://www.ncbi.nlm.nih.gov/pathogens>) allow for in-depth studies of the global spread and  
193 biodiversity of *L. monocytogenes*.

194

#### 195 *Hypervirulent strains belong to distinct CCs compared to hypovirulent strains*

196 *L. monocytogenes* can be divided into four evolutionary lineages [77], 13 serotypes [78]  
197 grouped into 6 genoserogroups [79] and more than 170 clonal complexes and 300 sublineages  
198 (<http://bigsdb.pasteur.fr/listeria>). Of these, lineage I (serotypes 4b and 1/2b) and II (serotypes  
199 1/2a and 1/2c) isolates represent the majority of clinical isolates. The most prevalent CCs and  
200 SLs are geographically and temporally widespread [70, 80, 81]. However, an uneven  
201 prevalence is observed among food vs. clinical cases [25, 75, 80], as previously noted at the  
202 lineage and serotype levels [77]. While lineage II, in particularly sublineages SL9 (CC9) and  
203 SL121 (CC121) are mostly associated with food, lineage I isolates, and in particular  
204 sublineages SL1 (CC1), SL2 (CC2), SL4 (CC4) and SL6 (CC6) are strongly associated with  
205 clinical cases in the Western world [25, 75, 82-87] (Figure 2B). In Asia, specifically in China  
206 and Taiwan, the most prevalent sublineage in both food and clinical cases is SL87 (CC87,  
207 lineage I, serotype 1/2b) [84, 88, 89], suggesting that food habits and/or local specificities are  
208 involved in geographic diversity of *L. monocytogenes*.

209 A large study of 6,663 *L. monocytogenes* isolates of clinical and food origins prospectively  
210 collected in France between 2005 and 2013 reported a higher prevalence of CC9 and CC121  
211 (both of lineage II) isolates in food samples, while CC1, CC6, CC2 and CC4 (all of lineage I)  
212 were the most prevalent in clinical cases [25]. In addition, CC1 and CC4 were found to be



213 statistically associated with more invasive forms of listeriosis (maternal-neonatal and CNS  
214 infections). Similar results have been observed within farm environments, with CC1 being  
215 also associated with CNS infection in ruminants in Switzerland [90]. This could reflect a  
216 difference in virulence and/or in exposure of at-risk patients to these strains. Association of  
217 CCs to clinical cases is inversely correlated to patients' immunosuppressive comorbidities,  
218 suggesting that these are more virulent than CCs most associated with food [25]. *In vivo*  
219 studies, which compared CC1, CC4, CC6, CC9 and CC121 isolates and the laboratory  
220 reference strains EGDe and 10403S in a mouse model permissive for oral *L. monocytogenes*  
221 infection, unambiguously showed that strains from CCs most associated with clinical cases  
222 (CC1, CC4 and CC6) are hypervirulent when compared to laboratory reference strains EGDe  
223 and 10403S, as they induced a more pronounced body weight decrease and are more  
224 neuroinvasive [25]. In contrast, CC9 and CC121 are hypovirulent when compared to these  
225 laboratory reference strains. Of note, CC9 and CC121 are not fully avirulent, since they can  
226 still infect laboratory animals, although to a lower degree, as well as immunodeficient patients  
227 [25]. This heterogeneity in virulence among lineages and CCs led to an investigation of the  
228 putative molecular mechanisms involved in both hypo- and hyper-virulence.

229

### 230 Virulence factors found in the core genome

231 While *inlA* is present in all *L. monocytogenes* strains, some strains, including the reference  
232 laboratory strain LO28 (CC9, lineage II), express a truncated and therefore nonfunctional  
233 InlA [91]. Epidemiological analysis indicated that clinical strains, and most significantly  
234 those from maternal-fetal infections, express full-length InlA more frequently than strains  
235 from food origins [70, 92]. InlA is truncated in the majority of hypovirulent CC9 and CC121  
236 isolates as well as in other lineage II clonal complexes (e. g. CC331, CC199 and CC321[75]).  
237 Hypovirulence can therefore, at least in part, be accounted for by InlA truncation, leading to  
238 decreased translocation across the intestinal barrier and placental infection [50, 56, 93].

239 Similarly to *inlAB*, LIPI-1 genes are present in all *L. monocytogenes* SLs and are therefore  
240 core virulence genes (Figure 1A). As indicated above, non-pathogenic *L. innocua* lacks LIPI-  
241 1 and *inlAB* genes, however rare *L. innocua* strains contain both LIPI-1 and *inlA* that are  
242 functional *in vitro* and *in vivo* [94-99]. This indicates that *L. monocytogenes* and *L. innocua*  
243 species likely evolved independently from a common pathogenic ancestor harboring both the  
244 LIPI-1 and *inlAB* virulence loci. Speciation likely occurred upon differential environmental  
245 selective pressures, with *L. innocua* evolving in an environment where virulence genes are not  
246 needed [100]. In contrast, 0.1% of *L. monocytogenes* strains from both lineage I and II are

247 non-hemolytic. 93.3% of these non-hemolytic strains harbor at least one loss-of-function  
248 mutation in *prfA*, and the remaining 6.7% harbor *hly* mutations leading to LLO inactivation.  
249 In both of these cases, this results in a strong decrease in virulence, suggesting that niche  
250 restriction leading to sub-speciation may still occur within *L. monocytogenes* species [101].

251

## 252 Virulence factors encoded by the accessory genome

### 253 LIPI-3

254 The LIPI-3 virulence cluster, which is absent in *L. monocytogenes* reference strains but  
255 present in lineage I F2365 (SL1/CC1) and H7858 (SL6/CC6) outbreak strains, has also been  
256 characterized (Figure 1A). It consists of 8 genes, including a hemolytic and cytotoxic factor,  
257 listeriolysin S (LLS), involved in virulence [102]. LLS, encoded by *llsA*, acts as a bacteriocin  
258 and alters microbiota composition, resulting in an increase in the luminal amount of *L.*  
259 *monocytogenes*, while *llsB*, another gene of LIPI-3, plays a role in the systemic phase of the  
260 infection [103, 104]. Functional LIPI-3 or its remnants can also be present in some *L. innocua*  
261 strains [98, 105], where its role is not defined, reinforcing the hypothesis that the two species  
262 share a common pathogenic ancestor. The presence of LIPI-3 in *Lm* sublineages SL1 (CC1),  
263 SL4 (CC4) and SL6 (CC6), which are the most associated with clinical cases [75, 98], and its  
264 absence in lineage II strains, suggests a possible role in their hypervirulence.

265

### 266 LIPI-4

267 Comparative genomic analyses derived from biodiversity studies allowed the characterization  
268 of LIPI-4, a putative phosphotransferase system (PTS) present in SL4, a sublineage of the  
269 lineage I, which strongly associated with a clinical origin (Figure 2B), particularly in CNS  
270 and placental infections [25]. LIPI-4 is involved in placental and CNS infection in an *in vivo*  
271 model of listeriosis, by a so far unknown mechanism (Figures 1A and 2A) [25]. Interestingly,  
272 LIPI-4, is also present in the *L. monocytogenes* sublineage that is the most prevalent in Asia,  
273 SL87 (CC87) [106], as well as in *L. innocua* [98]. Its actual role in such a non-virulent species  
274 is currently unknown.

275

### 276 LIPI-2

277 LIPI-2 is a genomic region discovered in *Listeria ivanovii*, a *Listeria* species which is  
278 pathogenic for mammals, mainly ovines and bovines, but also rarely humans [108, 109].  
279 LIPI-2 was first described as an *L. ivanovii*-specific genomic region that encodes a  
280 sphingomyelinase involved in phagosome disruption, as well as 10 genes of the internalin

281 family [110]. This region is involved in *L. ivanovii* virulence, together with the *L. ivanovii*  
282 LIPI-1 orthologous region, both *in vitro* and in an *in vivo* sheep model of infection [110]. *L.*  
283 *monocytogenes* isolates belonging to a new sublineage of the major lineage II (SL626/CC33,  
284 serovar 4h) recovered from an ovine listeriosis outbreak in China have been characterized as  
285 hypervirulent [107]. These isolates contain a partial LIPI-2 locus, comprising only the  
286 sphingomyelinase *smcL* and the internalins *i-inlF* and *i-inlE* genes (Figure 1A),  
287 likely by acquisition of exogenous DNA from *L. ivanovii*. The specific role of the partial  
288 LIPI-2 in hypervirulence of this newly described *L. monocytogenes* sublineage remains to be  
289 elucidated.

290

### 291 Heterogeneity in virulence as a reflection of differential host adaptation?

292 The distribution of *L. monocytogenes* is wide. It has been isolated in humans and animals in  
293 all continents except Antarctica [80]. Linked to its ability to tolerate extreme temperatures,  
294 acidity and osmolarity, *L. monocytogenes* can persist and grow in soil, plants, water, food and  
295 silage [111-113]. However, the distribution of *L. monocytogenes* lineages and CCs is not  
296 homogeneous in these ecosystems, indicating that CCs emergence may be driven by their  
297 selective adaptation to the environment in which they are the most abundant [25, 77].

298 When investigating food samples, hypervirulent CC1, CC4 and CC6 were found to be  
299 statistically associated with dairy products, whereas hypovirulent *L. monocytogenes* clones,  
300 CC9 and CC121 were found associated with meat products [114], suggesting a differential  
301 adaptation of the CCs to their environment. Interestingly, CC9 and CC121 harbor genes  
302 involved in tolerance to benzalkonium chloride, a surface disinfectant commonly used in food  
303 production environments, and are more efficient than their hypervirulent counterparts to form  
304 biofilms in the presence of this disinfectant [114, 115]. Other stress resistance genes such as  
305 the SSI-1 and SSI-2 stress survival islets [116, 117] are also typically present in lineage II,  
306 and contribute to *L. monocytogenes* survival and growth in the stress conditions encountered  
307 in food and food processing environments (e.g. high salt concentrations, oxidative stress).  
308 Lineage I isolates typically display higher tolerance to low pH [118], which can be  
309 advantageous for the survival of *L. monocytogenes* in the host's gastric acidic environment.  
310 Consistent with these observations, strains of hypervirulent CCs colonize the gut more  
311 efficiently than those of hypovirulent CCs [114], which may account for their strong  
312 association with ruminant feces [119] and dairy products, which may result from milk fecal  
313 contamination [114] (Figure 3).

314

315 **Concluding remarks**

316 *L. monocytogenes* is a major model pathogen which has allowed scientific breakthroughs in  
317 microbiology, immunology, cell biology and host-pathogen interactions. It has also emerged  
318 as a powerful model to study how microbes evolve from their natural habitat to anthropic  
319 environments. While advances have been made in the discovery of genetic factors involved in  
320 virulence and adaptation to host vs. food processing environments, the mechanisms by which  
321 *L. monocytogenes* adapts to various environments remains to be fully deciphered. Seminal  
322 studies have indicated how the transcriptome in the EGDe reference strain responds to the  
323 switch from saprophytism to virulence [120]. Future large-scale transcriptomics and  
324 metabolomics studies with representative strains from different clonal complexes will enrich  
325 our understanding of how *L. monocytogenes* can adapt to the environments in which it thrives.  
326 Further studies will also be needed to decipher the forces driving hypervirulence and its  
327 relationship with adaptation to the host, and how this relates to capacity of *L. monocytogenes*  
328 to be shed back in the environment and transmitted to new hosts, as suggested by data from  
329 ruminants and animal models [114, 119]. The continuous analysis of biodiversity of *L.*  
330 *monocytogenes*, together with environmental, epidemiological, clinical and experimental data  
331 obtained from genome wide association studies will shed light into the mechanisms and  
332 selective pressures that drive *Listeria* adaptation to diverse environments.

333

334 **Figure Legends**

335 **Figure 1. Virulence genes of *L. monocytogenes***

336 A) Typical genetic organization of *Listeria* pathogenicity islands (LIPI; colored arrows).  
337 Flanking genes (gray arrows) are labeled based on their orthologs in *L. monocytogenes* EGDe,  
338 when existing. *Lm*, *L. monocytogenes*; *Lin*, *L. innocua*; *Liv*, *L. ivanovii*; \*, LIPI-2 genes  
339 present in *Liv* atypical *L. monocytogenes* CC33 isolates are shown in darker yellow.

340 B) Genome maps of *L. monocytogenes* EGDe and *L. innocua* CLIP11262 [59]. Inner circles  
341 represent the GC skew  $((G - C)/(G + C))$  and GC content  $((G+C)/(G+C+A+T))$  of *L.*  
342 *monocytogenes* EGDe. Gaps in *L. innocua* chromosome (blue) represent genomic regions  
343 absent in *L. monocytogenes* genome (red). The genomic positions of known *L.*  
344 *monocytogenes* virulence genes are shown and their common and/or locus names indicated.  
345 Internalin genes are highlighted in red. Triangles denote the genomic positions of LIPI  
346 regions in either EGDe (black triangle) or other *Listeria* species (open triangles), as detailed  
347 in panel A.

348

349 **Figure 2. Population structure of *L. monocytogenes***

350 A) Population structure of *L. monocytogenes* based on cgMLST profiles (adapted from [75];  
351  $N=1,696$  isolates collected from food and clinical samples, mostly in North America and  
352 Europe). Branches are colored by phylogenetic lineage (I-IV, shown in red, blue, green and  
353 gray, respectively) and collapsed by sublineage (SL). The vertical dashed bar indicates the  
354 established cgMLST cut-off to define SLs (i.e. 150 allelic differences out of 1748 loci). Main  
355 SLs are labeled on the branches and corresponding MLST clonal complexes (CCs) are shown  
356 in the first column, PCR serogroups, source types and continents of isolation are colored  
357 according the key panels (upper left). Dark blue boxes denote the presence of selected genetic  
358 traits: pathogenicity islands (LIPI-1 to -4), genes involved in teichoic acid biosynthesis  
359 (*gltAB*, *tagB* and *gtaA*), genes coding for internalins (*inlABCEFGHJK*), autolysins (*aut* and  
360 *autIVb*) and bile resistance (*bsh*), genes involved in benzalkonium resistance (*bcrABC*  
361 operon, *ermC*, *Tn6188::ermC* and *ermE*), stress resistance islets (SSI-1 and SSI-2) and  
362 biofilm formation and virulence (*comK*).

363 B) Frequency of the most prevalent *L. monocytogenes* MLST clonal complexes in clinical ( $y$ -  
364 axis) versus food ( $x$ -axis) isolates, based on a exhaustively collected isolates dataset obtained  
365 in the scope of listeriosis surveillance ( $N=6,633$  isolates, France 2005-2013). (reproduced  
366 with permission from [25]). Each circle represents a clonal complex, and is colored according  
367 to its lineage. The size of each circle is proportional to the number of isolates it represents.  
368 The clonal complexes of common reference lab strains (CC7: strains EGD and 10403S; CC9:  
369 EGDe and LO28), are also indicated.

370

371 **Figure 3. Trade-off between host-associated and environmental lifestyles of *L.***  
372 ***monocytogenes***

373 Properties of *L. monocytogenes* strains adapted to a host environment (in red) versus of those  
374 adapted to saprophytism (natural and food-processing environments; in blue). Listeriosis  
375 results from the interplay between host and microbial factors: the less immunocompromised is  
376 the host, the more virulent *L. monocytogenes* strains need to be to cause disease. Thus,  
377 immunocompromised hosts tend to be susceptible to even saprophytic (hypovirulent) *L.*  
378 *monocytogenes*, whereas healthy hosts are usually only susceptible to host-associated  
379 (hypervirulent) strains, as shown in [25].

380

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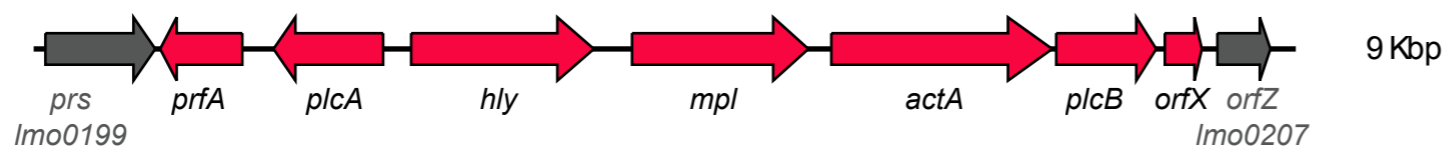
660

## Outstanding questions

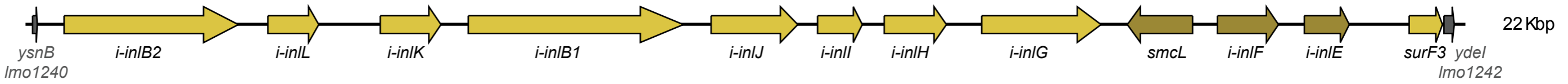
- What are the natural environments that have led to the current biodiversity of *L. monocytogenes*?
- What is the primary function of LIPI-1 genes, and other so-called “virulence genes”?
- What are the driving forces that have selected the virulence genes of *L. monocytogenes*?
- How does *L. monocytogenes* maintain its virulence since disease is not implicated in inter-host transmission?

(A)

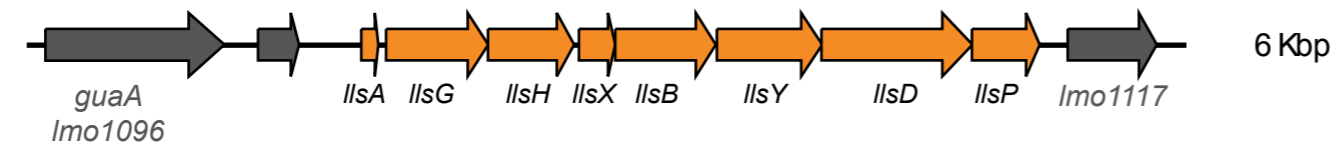
## LIPI-1

*Lm*, *Liv* and atypical *Lin*

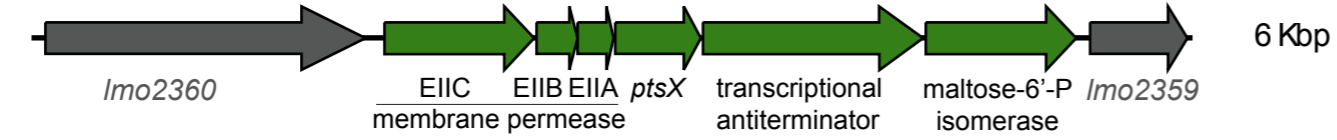
## LIPI-2

*Liv* and atypical *Lm* CC33\*

## LIPI-3

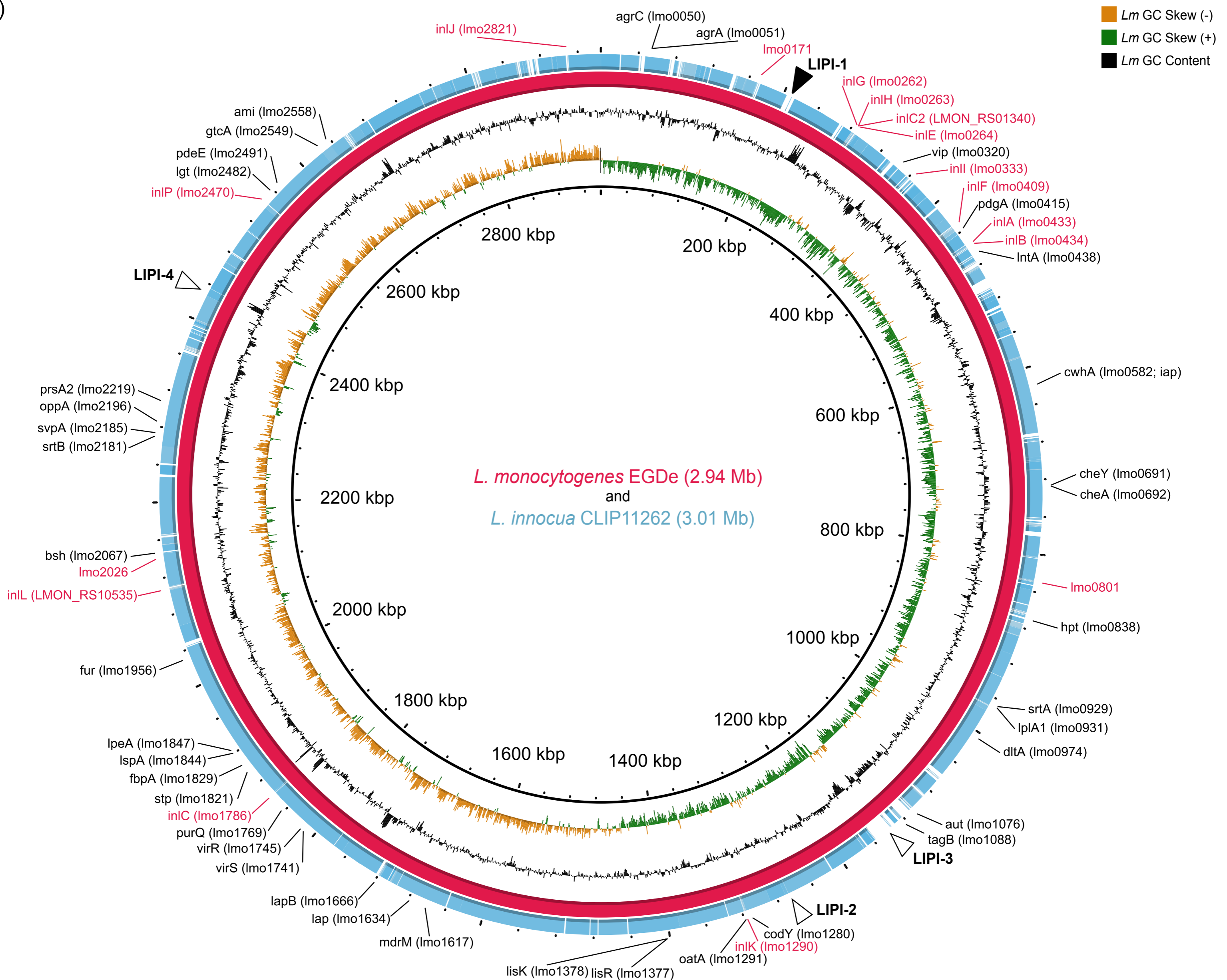
mainly *Lm* CC1, CC4, CC6 and some *Lin*

## LIPI-4

mainly *Lm* CC4 and *Lin*

Scale: 1 Kbp

(B)



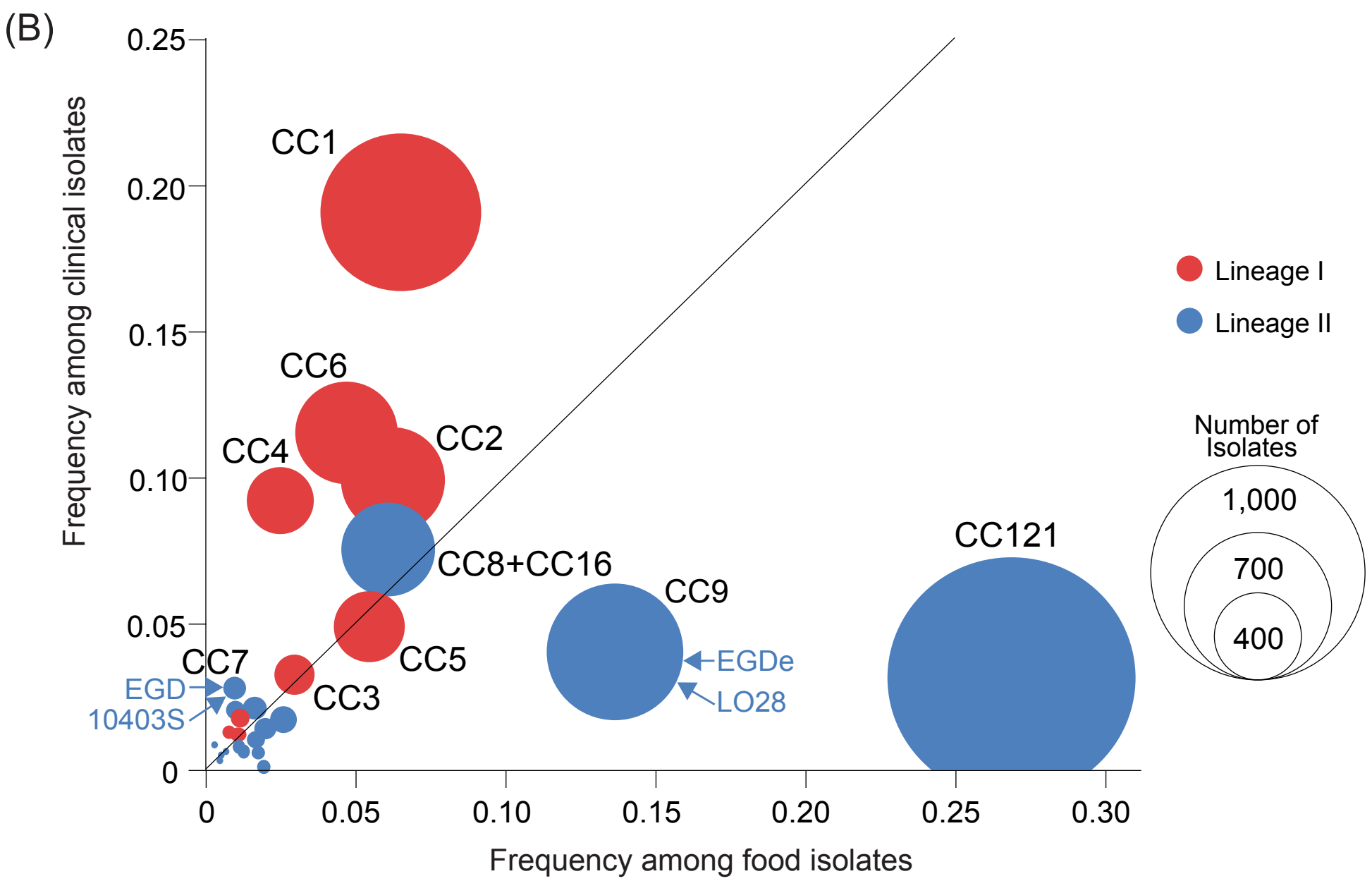
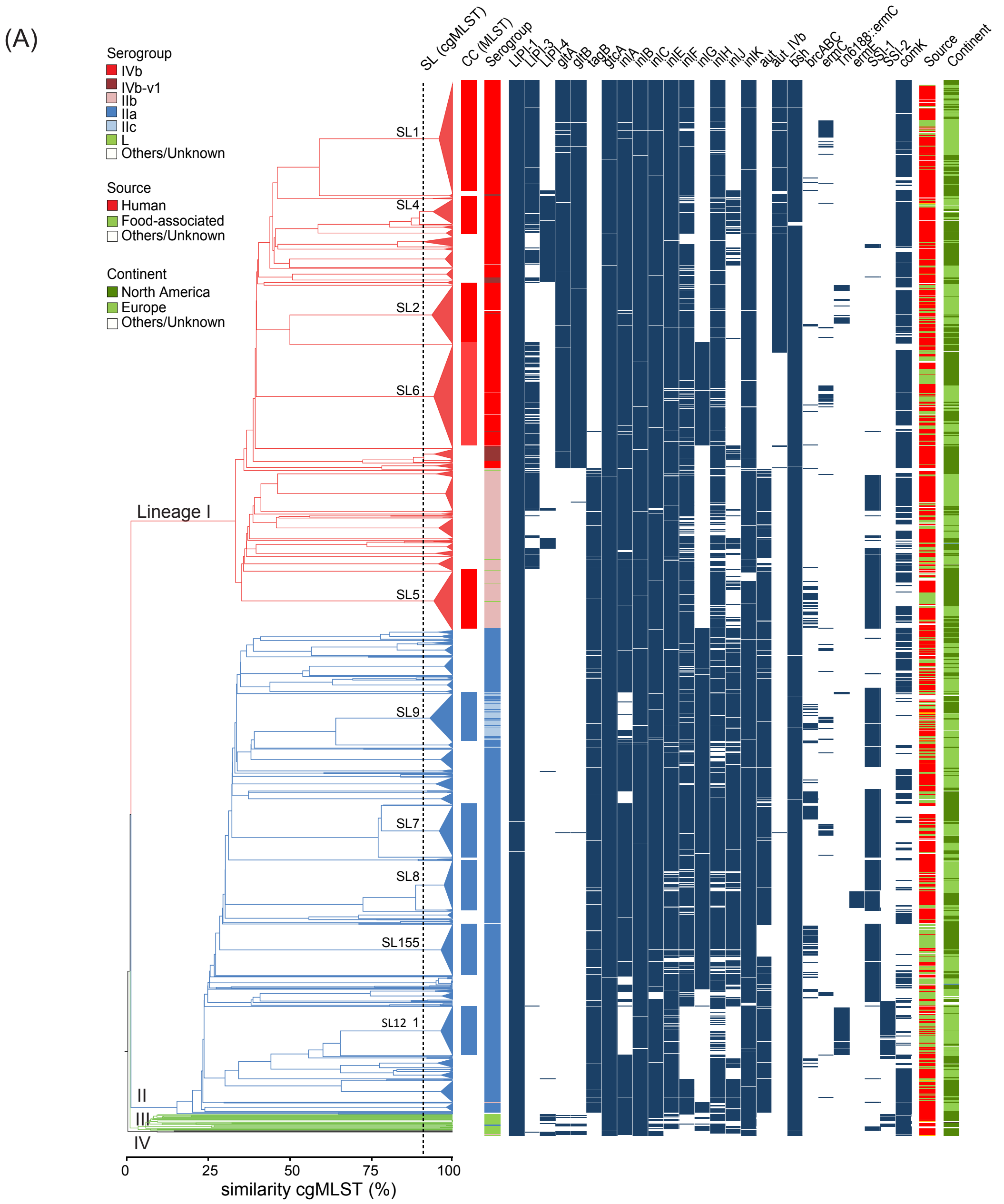


Figure 3



## Host-association

Virulence  
Active PrfA  
Active LIPIs  
Functional InIA  
Fecal shedding  
Transmission

Virulence

Immunodeficiency



Listeriosis depends on  
microbial and host factors



## Saprophytism

Loss of virulence  
Inactive PrfA  
Loss of LIPI-1 regulation  
Truncation of InIA  
Biofilm formation  
Resistance to antiseptics