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Spontaneous virulence loss in natural populations of *Listeria monocytogenes*

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31 **Abstract**

32 *Listeria monocytogenes* (*Lm*) pathogenesis depends on its ability to escape from the
33 phagosome of the host cells via the action of the pore-forming toxin listeriolysin O (LLO).
34 Expression of the LLO-encoding gene (*hly*) requires the transcriptional activator PrfA, and
35 both *hly* and *prfA* genes are essential for *Lm* virulence. Here we used the hemolytic activity of
36 LLO as a phenotypic marker to screen for spontaneous virulence-attenuating mutations in *Lm*.
37 Sixty (0.1%) non-hemolytic isolates were identified among a collection of 57,820 confirmed
38 *Lm* strains isolated from a variety of sources. In most cases (56/60), the non-hemolytic
39 phenotype resulted from nonsense, missense or frameshift mutations in *prfA*. Five strains
40 carried *hly* mutations leading to a single amino acid substitution (G299V) or a premature stop
41 codon causing strong virulence attenuation in mice. In one strain, both *hly* and *gshF*
42 (encoding a glutathione synthase required for full PrfA activity) were missing due to genomic
43 rearrangements likely caused by a transposable element. The PrfA/LLO loss-of-function
44 mutants belonged to phylogenetically diverse clades of *Lm* and most were identified among
45 non-clinical strains (57/60). In line with the extremely low frequency of loss of virulence
46 mutations, we show that *prfA* and *hly* are under purifying selection. Although occurring at a
47 low frequency, PrfA^{-}/LLO^{-} mutational events in *Lm* lead to niche restriction and open an
48 evolutionary path for obligate saprophytism in this facultative intracellular pathogen.

49

50 **Importance**

51 The hemolytic phenotype of *Lm* is a key identification criterion in food and clinical
52 microbiology. Here we characterized 60 non-hemolytic *Lm* strains, identified by screening a
53 vast collection of natural *Lm* isolates collected in the context of epidemiological surveillance
54 of listeriosis. Phenotypic and genomic analyses demonstrated that the absence of hemolysis
55 was due to loss-of-function mutations in *prfA* or *hly*, leading to strong virulence attenuation in

56 mice. We also identified the first natural *Lm* strain which spontaneously lost the *gshF* gene,
57 required for the PrfA-dependent transcriptional activation of *hly* and other virulence genes.
58 Previous phylogenomic studies have indicated that some non-pathogenic *Listeria* species
59 derive from pathogenic ones, and the virulence-attenuating mutations characterized in this
60 study illustrate the possible early events that could have determined their emergence and
61 evolution.

62

63 **Introduction**

64 *Listeria monocytogenes* (*Lm*) is a foodborne pathogen that can cause a severe invasive disease
65 in people and animals, called listeriosis. As a facultative intracellular bacterium, *Lm* has
66 evolved a range of virulence determinants allowing intracellular survival (1, 2). One key
67 virulence factor is listeriolysin O (LLO), a pore-forming toxin responsible for the
68 characteristic ß-hemolytic phenotype of *Lm* that allows the bacterium to escape from the
69 phagosome of host cells and replicate intracellularly (3, 4). LLO is encoded by *hly*, located in
70 the *Listeria* Pathogenicity Island 1 (LIPI-1) (5). Expression of the genes within this central
71 pathogenicity locus, including *hly*, is under the control of the transcriptional activator PrfA,
72 the master regulator of *Lm* virulence genes (6, 7).

73 The hemolytic activity conferred by LLO is considered a cardinal marker for *Lm* detection
74 and/or identification in clinical and food microbiology. *Lm* is divided into four phylogenetic
75 lineages (8-10), 13 serotypes (11) that can be approximated by PCR serogrouping (12), and
76 more than 100 clonal complexes (CCs, as defined by multilocus sequence typing (MLST))
77 (13), which are unevenly virulent (14). Weakly or non-hemolytic *Lm* strains have been
78 reported (15-19), but the frequency and phylogenetic diversity of the strains displaying an
79 altered hemolysis phenotype is unknown, as well as their underlying genetic and
80 microbiological features.

81 This study aimed at (*i*) estimating the frequency of naturally-occurring non-hemolytic *Lm* isolates and their distribution among *Lm* lineages and MLST clonal complexes, (*ii*) understanding the molecular bases of the non-hemolytic phenotype and (*iii*) assessing its impact on virulence. By using phenotypic and genomic approaches, mutagenesis and *in vivo* assays, we show that mutations leading to loss of hemolytic activity in *Lm*, although rare, affect a wide range of clonal complexes of the major lineages I and II and lead to a decreased virulence.

Results

Identification and characterization of non-hemolytic *Lm* strains

We examined the prevalence of non-hemolytic *Lm* strains among the 57,820 *Lm* isolates collected between 1987 and 2008 at the French National Reference Centre for *Listeria* (NRCL) and the WHO Collaborating Centre for *Listeria* (WHOCCL). Sixty *Lm* isolates (0.1%) were identified as non-hemolytic on horse blood agar plates. These were isolated from food (n = 33), food production environments (n = 2), non-human unknown sources (n = 22) and human clinical cases (n = 3). Phenotypic characterization using the API *Listeria* system confirmed all 60 non-hemolytic isolates as *Lm*. These belonged to lineages I (n = 23, 38.3%) and II (n = 37, 61.7%) and were grouped within serogroups IIa (n = 36), IVb (n = 13), IIb (n = 10) and IIc (n = 1) (**Table S1**). MLST showed that the 60 non-hemolytic isolates belonged to 15 different clonal complexes, including the “hypovirulent” CC9 (n = 1), CC121 (n = 3), CC31 (n = 20) and ST13 (n = 3) (14, 20), but also the “hypervirulent” CC1 (n = 3), CC2 (n = 7), CC4 (n = 1) and CC6 (n = 1) (14) (**Table S1** and **Fig. 1**). Core genome MLST (cgMLST) typing identified 39 different cgMLST types (CTs) (21). Nine CTs comprised more than one strain, suggesting a possible epidemiological link between them (21) (**Table S1**). In particular, among the twenty non-hemolytic CC31 strains, ten belonged to CT878 and two

106 belonged to CT2659, suggesting that the overrepresentation of CC31 could be in part due to
107 multiple sampling of the same source in the context of an epidemiological investigation.
108 These results show that non-hemolytic strains are phylogenetically very diverse and that the
109 loss of hemolytic activity is caused by independent events across the *Lm* population.
110 To investigate the impact of the loss of hemolytic activity in *Lm* fitness, we analyzed the
111 growth of all non-hemolytic strains in BHI at 22°C and 37°C, using EGDe as control (**Fig.**
112 **S1**). At 22°C, in a large majority of cases, the growth of non-hemolytic strains was within the
113 same range as EGDe, as revealed by the areas under their growth curves (AUCs). On the
114 contrary, at 37°C, temperature in which *prfA* is known to be maximally expressed (22), most
115 of the non-hemolytic strains showed lower growth (lower AUCs) than EGDe. Some of the
116 non-hemolytic strains showed particularly decreased fitness in one or both temperature: CLIP
117 2000/86467 ($PrfA_{T170*}$, at 22°C), CLIP 1998/75799 ($PrfA_{I51*}$-LLO_{N261*}, at 37°C) and, at both
118 temperatures, strains CLIP 1998/76801 (Δ*hly*-Δ*gshF*), CLIP 1996/70991 ($PrfA_{Q21*}$), CLIP
119 1994/58618 ($PrfA_{A129P}$) and CLIP 1996/71614 ($PrfA_{Y207*}$) (**Fig. S1**).

120

121 **Molecular basis of non-hemolytic phenotype – PrfA variants and activity**

122 The central regulator of *Listeria* virulence, PrfA, is required for the expression of a set of key
123 virulence determinants, known as the PrfA regulon, including the *hly* gene (6, 7, 23).
124 Consequently, mutations altering the function of either PrfA or LLO could lead to a non-
125 hemolytic phenotype. Sequence analyzes identified frameshifts and missense and nonsense
126 mutations in *prfA* in 56 non-hemolytic strains, leading to amino-acid substitutions or protein
127 truncations in PrfA (**Fig. 1**; **Table S1**). Phenotypic analysis in PrfA-activating and non-
128 activating conditions using the PrfA-dependent virulence factors PlcB (phospholipase C) and
129 Hpt as reporters (see Materials and Methods) (24) confirmed the complete loss of function of
130 the central virulence gene regulator in all of these strains (**Fig. 1; Fig. S2**).

131 Forty-three out of the 56 PrfA^{-} strains, distributed in lineages I and II, expressed a truncated
132 PrfA at 14 distinct positions distributed along the entire PrfA protein (**Table S1)**. All analyzed
133 strains of CC59 and CC31 exhibited a truncation at positions 59 and 185, respectively,
134 suggesting a common ancestor for each of these groups of strains. Seven PrfA^{-} strains
135 presented a single amino-acid substitution in PrfA as compared to the reference strain EGDe
136 (accession number: NC_003210). Among them, one occurred in the β-roll region of PrfA
137 (G72D, strain CLIP 1997/75561, CC9). Mutations located in this region are known to affect
138 PrfA activation or the ability of PrfA to form a stable complex with the RNA polymerase and
139 initiate transcription of the target virulence genes (25-27). One PrfA^{-} mutation occurred in the
140 DNA-binding helix-turn-helix (HTH) domain of PrfA (G175C, strain CLIP 2006/01642,
141 CC6) and two others in its C-terminal part (K220T, strains CLIP 1994/60344, CLIP
142 2000/80770 and CLIP 2001/87255, all ST13; and L221F, strain CLIP 1994/56373, CC1).
143 These regions are known to be important for the binding of PrfA to PrfA-binding sites of
144 target DNAs (25, 26). In addition, the A129P substitution, located between the β-roll and the
145 hinge αD regions, occurred in a CC224 strain (CLIP 1994/58618). Finally, six of the PrfA^{-}
146 strains, all belonging to CC155, showed a reversion of the *prfA* stop codon due to the
147 insertion of 5 nucleotides at position 712 in the *prfA* sequence, leading to a longer PrfA
148 protein (238 amino acids in EGDe *vs* 293 amino acids in the CC155 strains of this study).
149 One of the four non-hemolytic mutants (CC1 strain CLIP 1998/76801) exhibited a wild-type
150 (WT) PrfA sequence as compared to EGDe, but showed a PrfA^{-} phenotype. This observation
151 suggested that a mechanism interfering upstream of PrfA function was affected. Glutathione,
152 synthetized by *Lm* through the glutathione synthase encoded by *gshF* (*lmo2770*), is critical for
153 PrfA activation (28). Interestingly, although it is part of the *Lm* core genome (14, 21), *gshF*
154 was absent in the genome of the CLIP 1998/76801 strain (**Fig. 1**) (see below), which could
155 explain the absence of PrfA activity in this strain.

156

157 **Analysis of spontaneous LLO mutants**

158 Analysis of *hly* sequences in the 60 non-hemolytic strains identified multiple mutations
159 leading to amino acid substitutions in LLO (**Table S1**). Several substitutions (N31H, S35L,
160 V438I and K523S) were identified in at least 48 hemolytic *Lm* strains of our database
161 (~ 4,100 genomes), suggesting that they do not cause LLO loss of function. However, a
162 S250N substitution was only found in three non-hemolytic strains of this study (CLIP
163 2008/01432, 2008/01433 and 2008/01435, all CC77) and could therefore result in LLO loss
164 of function. Since these strains also expressed a truncated and non-active PrfA, which is
165 sufficient to explain the non-hemolytic phenotype of these strains, we did not pursue this
166 further.

167 Two out of the three non-hemolytic strains showing a WT PrfA sequence and a $PrfA^+$
168 phenotype (CC121 strains CLIP 2007/01406 and CLIP 2007/01014) exhibited a single amino
169 acid substitution in LLO (hly_{G299V} or LLO_{G299V}), which was not present in any of the other
170 strains. The third strain (CC2, CLIP 1989/13656) harbored a premature stop codon at position
171 484 in LLO (hly_{C484*} or LLO_{C484*}). The absence of any other specific feature in these three
172 strains that could be linked to the loss of hemolytic activity suggested that the G299V
173 mutation and the truncation at position 484 in LLO could be the cause of the loss of hemolytic
174 activity in these strains. In addition, two CC7 strains expressing a truncated PrfA (CLIP
175 1998/75799 and CLIP 1989/14490) also showed a premature stop codon in LLO at position
176 261 (hly_{N261*}) due to the insertion of one nucleotide.

177 In the CLIP 1998/76801 strain mentioned above, *hly* could not be detected by PCR and the
178 *hly* region could not be assembled from Illumina reads. In order to resolve this region, we
179 sequenced this strain using the single molecule, real-time (SMRT) sequencing technology
180 (Pacific Biosciences, California, USA). The CLIP 1998/76801 complete genome (CC1,

181 2.84 Mb) was compared to the closely related F2365 complete genome (CC1, NCBI
182 accession number NC_002973) as reference. This showed that the LIPI-1 region had
183 undergone an inversion of more than 40 kb (**Fig. 2A**). This large rearrangement splitted LIPI-
184 1 into two parts with concomitant loss of *hly* and partial truncation of the 5' region of the
185 adjacent *mpl* gene. Six ORFs were inserted upstream of *mpl* in CLIP 1998/76801 as compared
186 to F2365, comprising genes encoding a transposition protein (*tnsB*) and a DNA invertase
187 (*hin*), which are likely the cause of the rearrangement, as well as cadmium resistance genes
188 (*cadA* and *cadC*) (**Fig. 2A**).
189 We confirmed that *gshF* is absent in CLIP 1998/76801, together with 12 other upstream and
190 downstream genes related to sugar metabolism (**Fig. 2B**). These genes were replaced by 11
191 ORFs encoding a transposition protein (*tnsB*), a DNA invertase (*hin*) and cadmium resistance
192 genes (*cadA* and *cadC*) similar to those inserted in the LIPI-1 region. In total, eight similar
193 copies of this transposable element were found in the CLIP 1998/76801 genome, as well as
194 many other large rearrangements and deletions (**Fig. 2C**). Similar transposable elements were
195 detected in one *L. ivanovii* strain in NCBI database (accession number KR780025.1; 99%
196 nucleotide identity, full length) and in 128 *Lm* strains (> 99.87% nucleotide similarity, full
197 length) out of the 4,091 genome sequences available at the NRCL at the time of the study.
198 These strains comprised 14.1% of all the CC1 strains (90/638, representing two distinct
199 monophyletic groups within the phylogeny of CC1, data not shown) and all the CC59 strains
200 (n = 38). No significant link of this element with food or clinical origins was found within
201 CC1.
202

203 **Assessment of *hly* and *prfA* transcription**

204 In order to test the effect of the identified mutations on *hly* and *prfA* transcription, qRT-PCRs
205 were performed for a representative set of non-hemolytic strains (one strain per type of loss-

206 of-hemolysis mutation, **Table S1**). All non-hemolytic strains showed *prfA* transcription levels
207 equivalent to or higher than EGDe, except for strains CLIP 1998/75799 ($PrfA_{I51*}$-LLO_{N261*}
208 mutations) and CLIP 1998/77604 ($PrfA_{T76*}$ mutation), which showed no amplification, likely
209 due to poor primer annealing (8 mismatches with the *prfA*-R primer) (**Fig. S3**). As expected,
210 strains with altered PrfA (aa substitution or truncation) showed no or extremely reduced *hly*
211 transcription levels. These results show that for these strains the loss of hemolytic activity is
212 due to *prfA* post-transcriptional events leading to the absence of PrfA activity. In the strain
213 CLIP 2007/01406 (LLO_{G299V}), *hly* was transcribed at a similar level than in EGDe, whereas in
214 CLIP 1989/13656 (LLO_{C484*}), *hly* transcription was weaker.
215

216 ***In vitro* characterization of the hly_{G299V} and hly_{C484*} mutations**
217 In order to characterize the functional impact of the G299V substitution (CLIP 2007/01406
218 and CLIP 2007/01014) and of the truncation at position 484 in LLO (CLIP 1989/13656), we
219 introduced a plasmid containing either a wild type *hly* gene (hly_{WT}) or a mutated version of
220 this gene (hly_{G299V} or hly_{C484*}, encoding LLO_{G299V} and LLO_{C484*}, respectively) in a EGDΔ*hly*
221 strain. While EGDΔ*hly*:pPL2-hly_{WT} was hemolytic, EGDΔ*hly*:pPL2-hly_{G299V} or
222 EGDΔ*hly*:pPL2-hly_{C484*} remained non-hemolytic, as assessed on Columbia horse blood agar-
223 plates. These results demonstrate that the hly_{G299V} and hly_{C484*} mutations are responsible for
224 the absence of hemolytic activity in the strains CLIP 2007/01406, CLIP 2007/01014 and
225 CLIP 1989/13656.
226 Western blot analyses of culture supernatants detected lower amounts of LLO produced by
227 EGDΔ*hly*:pPL2-hly_{G299V} and EGDΔ*hly*:pPL2-hly_{C484*} bacteria as compared to the WT EGD
228 and EGDΔ*hly*:pPL2-hly_{WT} strains (**Fig. 3A**). qRT-PCR analyses showed that *hly* transcription
229 level in both EGDΔ*hly*:pPL2-hly_{G299V} and EGDΔ*hly*:pPL2-hly_{C484*} strains is comparable to
230 that observed in EGDΔ*hly*:pPL2-hly_{WT}, although slightly weaker for EGDΔ*hly*:pPL2-hly_{C484*}

231 (**Fig. 3B**). Furthermore, the EGDΔ*hly*:pPL2-*hly*$_{C484*}$ mutant produced a shorter LLO protein
232 as compared to strains harboring the *hly*$_{WT}$, confirming that the premature stop codon
233 identified in *hly* in the CLIP 1989/13656 strain leads to the production of a truncated LLO.
234 The *hly*$_{N261*}$ mutation (**Fig. 1**; **Table S1**) was not tested *in vitro* as this premature stop codon
235 is upstream of the *hly*$_{C484*}$ mutation, leading to an even shorter LLO.

236

237 **Virulence of *hly*$_{G299V}$ and *hly*$_{C484*}$ mutants**

238 We finally assessed the virulence of the EGDΔ*hly*:pPL2-*hly*$_{G299V}$ and EGDΔ*hly*:pPL2-*hly*$_{C484*}$
239 complemented strains relative to that of the EGDΔ*hly*:pPL2-*hly*$_{WT}$ and EGDΔ*hly*:pPL2 strains
240 upon intravenous injection in mice. EGDΔ*hly*:pPL2-*hly*$_{G299V}$ and EGDΔ*hly*:pPL2-*hly*$_{C484*}$
241 strains were four order of magnitude less abundant than the EGDΔ*hly*:pPL2-*hly*$_{WT}$ strain in
242 the liver and the spleen (**Fig. 3C**). This demonstrates that the virulence of *Lm* expressing
243 either LLO$_{G299V}$ or LLO$_{C484*}$ is strongly attenuated *in vivo*.

244

245 **Discussion**

246 Virulence gene polymorphisms leading to *Lm* attenuation have been previously described and
247 have been associated with strains of lower pathogenic potential. The best characterized are
248 those affecting the invasion-associated *inlA* gene, found in a large proportion (>25-30%) of
249 lineage II food isolates but extremely rare among lineage I strains, more frequently associated
250 with clinical cases (13, 21, 29, 30). Mutations leading to more radical *Lm* virulence
251 attenuation have also been characterized, particularly those affecting the *prfA* gene (31-33),
252 but their frequency and distribution across the *Lm* population remained undetermined. Here
253 we examined the occurrence of "loss-of-virulence" mutations in *Lm* by screening a wide and
254 diverse panel of strains for hemolytic activity. Our data shows that non-hemolytic *Lm* mutants
255 occur at low frequency (0.1%) and are phylogenetically diverse, including strains belonging

256 to hypovirulent and hypervirulent clonal complexes (14). This indicates that the underlying
257 mutational events are not linked to the genetic background of the strains.
258 *Lm* hemolytic phenotype depends on two essential virulence determinants, the central
259 virulence regulator PrfA and LLO, encoded by *prfA* and *hly*, respectively. Indeed, all non-
260 hemolytic strains identified in this study carried mutations in either of these genes. The large
261 majority of non-hemolytic strains (56/57,820; 95%) carried *prfA* mutations (frameshifts,
262 missense or nonsense nucleotide changes, or reversion of the stop codon into a glutamine
263 codon). Although no PrfA activity could be detected and *hly* was not transcribed in these
264 strains, *prfA* was transcribed at similar levels to EGDe. This suggests that the loss of PrfA
265 activity in these strains likely results from PrfA misfolding, instability and/or inactivating
266 amino-acid substitution. Some inactivating amino acid substitutions in PrfA occurred in the β-
267 roll, HTH motif or C-terminal domain, in line with the critical role of these regions in PrfA
268 activity (25-27, 31). As PrfA is the major transcriptional regulator of *Lm* virulence genes and
269 is essential for its pathogenicity (23, 34), the virulence of $PrfA^-$ strains are expected to be
270 highly attenuated as previously described (31-33). The first *Lm* strain naturally producing a C-
271 terminally-extended PrfA polypeptide (55 residues longer) was identified in this study and
272 showed no PrfA activity and no *hly* transcription.
273 Comparatively to PrfA, non-hemolytic *hly* mutants with affected LLO activity were less
274 frequent (5/57,820; 0.01%) in our study. Our analysis identified for the first time a
275 spontaneous amino-acid substitution in LLO (hly_{G299V}) and premature stop codons in *hly*
276 (hly_{N261*} and hly_{C484*}) leading to the loss of LLO activity. Lower quantities of LLO were
277 detected in the culture supernatants of the EGDΔ*hly*:pPL2-hly_{G299V} and
278 EGDΔ*hly*:pPL2-hly_{C484*} constructs than for the EGD and EGDΔ*hly*:pPL2-hly_{WT} strains. The
279 quantity of *hly* transcripts was similar in the EGDΔ*hly*:pPL2-hly_{G299V} and in the
280 EGDΔ*hly*:pPL2-hly_{WT} control, indicating that LLO_{G299V} is likely less stable than WT LLO. In

281 contrast, EGDΔ*hly*:pPL2-*hly*$_{C484*}$ showed a lower *hly* transcription level, suggesting an
282 impaired stability of *hly*$_{C484*}$ transcript, relative to that of WT *hly*. *In vivo* experiments
283 confirmed that the non-hemolytic strains harboring the *hly*$_{G299V}$ or *hly*$_{C484*}$ mutations have
284 strongly attenuated virulence in mice. In line with these results, only three non-hemolytic
285 strains were isolated from human samples. Although we did not have access to the detailed
286 clinical data of these patients (dating back from the 1980s and 90s), one possibility would be
287 that they were heavily immunocompromised, mirroring previous reports on isolation of the
288 non-pathogenic *Lm* relative *Listeria innocua* from immunosuppressed individuals (35).
289 One of the LLO$^-$ strains (CLIP 1998/76801) underwent huge genomic rearrangements that
290 likely caused the loss of *hly* and *gshF,* encoding a glutathione synthase reported as being
291 required for PrfA activity (28). CLIP 1998/76801 is the only strain in our entire genome
292 database (~ 4,100 entries) that lacks *gshF*. Interestingly, each copy of the transposable
293 element that likely caused the genomic rearrangements observed in this strain carried putative
294 cadmium resistance determinants that could be advantageous in environments in which
295 virulence determinants are not needed. Similar transposable elements were detected in
296 monophyletic groups of CC1 and CC59 strains, suggesting that it has been horizontally
297 transmitted in the *Lm* population.
298 The predominance of PrfA$^-$ mutants among the non-hemolytic strains could reflect the fact
299 that *prfA* is a pleiotropic regulatory gene that controls the expression of a number of virulence
300 determinants, the expression of which is known to entail a significant fitness cost in non-host
301 conditions (24). Our results show that, at 22°C, the majority of PrfA$^-$ strains have similar
302 fitness than EGDe, suggesting that the absence of PrfA activity does not impact *Lm* fitness in
303 non-pathogenic conditions. Nevertheless, a reduced fitness was observed at 37°C (mammalian
304 host temperature), comparatively to EGDeΔ*prfA.* This result suggests that non-hemolytic
305 strains are more adapted to a non-pathogenic lifestyle, independently of PrfA. Consistent with

this, most of the non-hemolytic *Lm* isolates were from non-clinical origins. The ratio of non-synonymous and synonymous substitutions (dN/dS) estimated for *prfA* (dN/dS=0.08892) and *hly* (dN/dS=0.03674) using a dataset of 100 genomes representative of *Lm* phylogenetic diversity (14) confirmed that, similarly to *Lm* core genes (dN/dS=0.05353 in average, (21)), these genes are under purifying selection. Thus, any deleterious mutations affecting these genes tend to be eliminated from *Lm* population. The exceptionally low frequency of deleterious mutations in *prfA* and *hly* indicates that there is a strong necessity for *Lm* to retain its virulence capacity (36). Our results also suggest that, although exceptionally, once strains loss their virulence capacity (*e.g.* due to a *prfA* mutation), other virulence genes may become unneeded and prone to accumulate mutations, as observed in our PrfA$^-$/LLO$^-$ and PrfA$^-$/GshF$^-$ strains. Previous studies have already identified strains with multiple mutations occurring in several major virulence genes (20). Strains with virulence attenuating mutations are therefore prone to enter into an evolutionary path towards obligate saprophytism. The *Lm* phylogenomic clade comprises another pathogenic species, *Listeria ivanovii*, with a full complement of PrfA-regulated genes, as well as non-pathogenic species, some of which contain remnants thereof (e.g. *Listeria seeligeri* or *L. innocua*) (37, 38). While infrequent, spontaneous virulence-disabling mutations, as those described here, could have been key initial events in the emergence and evolution of the *Lm*-related non-pathogenic *Listeria* species.

Materials and methods

Bacterial strains and growth media

The 60 non-hemolytic *Lm* isolates included in this study were identified among a collection of 57,820 *Lm* strains collected between 1987 and 2008 by the French National Reference Centre for *Listeria* (NRCL) and World Health Organization Collaborating Centre for *Listeria*

331 (WHOCCL) in the context of the epidemiological surveillance of listeriosis. This global
332 collection included isolates of food (n = 36,630), clinical (n = 5,980), environmental
333 (n = 3,647), veterinary (n = 1,713) and unknown (n = 9,850) origins. Isolates were revived by
334 plating them onto Columbia Agar and single colonies were grown on Columbia Agar slants.
335 *Lm* strains were routinely grown in BHI at 37°C and *Escherichia coli* strains were grown at
336 37°C in LB broth or agar plates.

337

338 **Phenotypic characterization of *Listeria* isolates**

339 Miniaturized enzymatic and sugar fermentation tests (API-*Listeria* identification
340 microgallery, BioMérieux, France), in combination with the hemolytic activity assessment of
341 strains, were used for phenotypic identification of *Listeria* species (39). Hemolytic activity
342 was tested on Columbia horse blood agar-plates (BioMérieux, France). *Lm* CLIP 74910 and
343 *Listeria innocua* CLIP 74915 were used as positive and negative controls of hemolysis,
344 respectively.

345

346 **Genome sequencing and analyses**

347 Genomic DNA was extracted using the DNeasy Blood and Tissue extraction kit (Qiagen,
348 Denmark) and used for whole genome sequencing on Illumina NextSeq 500 (2 x 150 bp)
349 platform (Illumina, California, USA). Reads were trimmed with AlienTrimmer (Criscuolo &
350 Brisse 2013) to eliminate adapter sequences and discard reads with Phred scores of ≤ 20. *De*
351 *novo* assembly of Illumina reads was performed using SPAdes Genome Assembler 3.1 (40).
352 The complete genome of the CLIP 1998/76801 strain was obtained by PacBio RS II
353 sequencing (Pacific Biosciences, California, USA) using DNA purified with the Wizard
354 genomic DNA purification kit (Promega, Wisconsin, USA). Genome annotation was
355 performed using Prokka 1.11 (41).

356 PCR-serogroups (12, 42), MLST profiles (13) and cgMLST profiles (21) were deduced from
357 genome assemblies using the BIGSdb-*Lm* platform (http://bigsdb.pasteur.fr/listeria; (21)).
358 Genome assemblies were made publicly available in the BIGSdb-*Lm* platform (**Table S1**).
359

360 **Assessment of *prfA* and *hly* evolutionary trends**

361 *prfA* and *hly* sequences were extracted from 100 genomes that were selected to represent the
362 species diversity based on MLST and PFGE typing (14) and aligned using Muscle 3.8 (43).
363 This dataset included genomes from 13 food isolates, 45 human clinical isolates, 19 animal
364 isolates, 1 environmental isolate and 22 isolates of unknown origin. They comprised 41
365 genomes of lineage I, 53 of lineage II, 5 of lineage III, and 1 of lineage IV and represented 5
366 singletons and 34 clonal complexes based on MLST. No non-hemolytic strain was included in
367 this analysis. Alignments were used to estimate the non-synonymous and synonymous ratios
368 (dN/dS) of *prfA* and *hly* using the *codeml* program, included in the PAML 4.4 package (44).
369

370 **Assessment of PrfA activity**

371 PrfA activity was assessed by measuring the activity of PrfA-regulated *plcB* and *hpt* gene
372 products as previously described (45, 46). For PlcB, lecithinase tests were performed in egg-
373 yolk BHI, for Hpt, glucose-1-phosphate acidification tests were carried out in phenol red
374 broth, in both cases with and without 0.5% w/v activated charcoal (Merck, New Jersey,
375 USA). Medium supplementation with charcoal leads to the partial activation of PrfA,
376 presumably due to sequestration of repressor substances from the culture medium. Three *Lm*
377 genotypes from strain P14 were used as controls: (i) $prfA_{\mathrm{WT}}$ characterized by an activable
378 PrfA phenotype (lack of PlcB and Hpt activity in normal medium and strong activity in
379 charcoal-supplemented medium), (ii) Δ*prfA* which remains negative for PlcB and Hpt activity

380 in the presence of charcoal; and (iii) constitutively activated *prfA** with strong PlcB and Hpt
381 activity independently of charcoal supplementation (24, 45, 47).
382
383 **RNA extractions**
384 Non-hemolytic strains and EGDΔ*hly*:pPL2-hly_{WT}, EGDΔ*hly*:pPL2, EGDΔ*hly*:pPL2-hly_{G299V}
385 and EGDΔ*hly*:pPL2-hly_{C484*} constructs were cultured overnight on BHI agar at 37°C. One
386 colony was used to inoculate 5 ml of BHI broth. After overnight growth at 37°C, 500µl of
387 culture was added to 10ml of BHI broth and the whole exponential phase culture (at 37°C)
388 was centrifuged at 5,000 g for 5 min. The pellet was suspended with 400 µl of resuspension
389 buffer (10% glucose, 12.5 mM TRIS, 10 mM EDTA in nuclease-free water) and transferred
390 to a lysing tube (containing 0.1 mm of ceramic breads, 500 µl of acid phenol and 60 µl of
391 EDTA 0.5 M). The Precellys24 homogenizer (Bertin Instruments, France) was used at
392 6,500 rpm, for 2 x 23 s (10 s break), and the resulting mixture was centrifuged at 14,000 g at
393 4°C for 10 min. The upper aqueous phase was transferred into a tube containing 1 ml of
394 Trizol and 100 µl of chloroform, mixed by inversions and centrifuged. The upper aqueous
395 phase was transferred into a tube containing 200 µl of chloroform, mixed by inversions and
396 centrifuged. The upper aqueous phase was transferred into a storage tube (containing 650 µl
397 of isopropanol and 65 µl sodium acetate 3 M), mixed by inversions, precipitated 20 min
398 at -20°C and centrifuged during 20 min. The supernatant was rinsed twice with ethanol 75%.
399 The air-dried pellet was dissolved in 300 µl of nuclease-free water. RNA concentrations were
400 measured with the DeNovix DS-11 Spectrophotometer (DeNovix, Delaware, USA) and
401 diluted to obtain 500 ng of RNA in 12.5 µl of nuclease-free water.
402
403 **Quantification of *hly* and *prfA* transcripts by qRT-PCR**

404 For the qRT-PCRs, cDNAs were generated prior to qPCRs. DNase treatment was performed
405 with the RNase-free DNase I (New England BioLabs, Massachusetts, USA) according to the
406 instructions of the manufacturer (by adding 0.5 µl of RNAseOUT, 0.5 µl of DNAseI and
407 1.5 µl of buffer 10x to the 12.5 µl of diluted RNAs; then 1.5 µl of EDTA 0.05 M). cDNAs
408 were generated by reverse transcription using the M-MLV Reverse Transcriptase (Invitrogen,
409 California, USA) and random hexamers for priming according to the instructions of the
410 manufacturer (by adding 2 µl of dNTPs 10 mM, 2 µl of random primers 2.5 µM and 3.5 µl of
411 nuclease-free water to the 16.5 µl of previously DNase treated sample; then 8 µl of First-
412 Strand buffer 5x, 4 µl of DTT 0.1 M and 2 µl of nuclease-free water; then 0.5 µl of M-MLV
413 RT).
414 All quantitative PCRs were prepared using SYBR® Green Real-Time PCR Master Mixes and
415 StepOnePlus™ Real-Time PCR System (Applied Biosystemss, California, USA). Each
416 primer pair was used in separated reactions using PCR mixtures containing 1 µl of each
417 primer 9 µM (**Table S2**), 5 µl of Sybr mix, 1 µl of cDNA diluted at 1:5 and 3 µl of
418 nuclease-free water. Real-time PCR reactions were carried out in MicroAmp™ Fast Optical
419 96-Well Reaction Plates (Applied Biosystems, California, USA) using the following protocol:
420 initial denaturation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 15 s and
421 primer annealing/elongation at 60°C for 1 min. Each strain was tested at least three times
422 using independent pre-cultures. *gyrB* was used as stable reference gene for normalization.
423 Results are shown as fold change of target gene expression relative to EGDe or EGD
424 (Relative Quantities, RQs), which were deduced from the cycle threshold values (CTs) using
425 the $2^{-\Delta\Delta CT}$ methodology.
426
427 **Fitness studies**

428 The microbial growth of non-hemolytic strains, EGDe, EGD and EGDeΔ*prfA* was monitored
429 over time in BHI at 22°C and 37°C using absorbance measurements (OD_{600nm}) through the
430 Bioscreen C® system (Oy Growth Curves Ab Ltd, Helsinki, Finland). Bacteria were first
431 cultured overnight on BHI agar at 22°C or 37°C and one colony was used to inoculate 5 ml of
432 BHI broth. After overnight growth, the stationary phase cultures were diluted to reach an
433 OD_{600nm} of 0.1 and transferred into Bioscreen C® 96-well plates. OD_{600nm} of non-inoculated
434 wells (blanks) were subtracted from inoculated ones to delete the background noise. Each
435 strain was tested three times. Mean OD_{600nm} per strain were used to calculate area under the
436 curves over time. For this, data were fitted to parametric models (Gompertz, modified
437 Gompertz, Logistic and Richards laws) using the "gcFit" function of the "grofit" R package
438 v.1.1.1-1 (48). The model that best fitted the data was selected by means of an AIC criterion
439 (49) and used to derive areas under the growth curves.
440

441 **DNA manipulations and cloning**

442 We used a two-step cloning strategy to introduce the wild type *hly* (hly_{WT}), hly_{G299V} or hly_{C484*}
443 in the *Lm* strain EGDΔ*hly*. First, we cloned separately the hly_{WT}, hly_{G299V} and hly_{C484*} gene
444 sequences into the *Listeria* integrative vector pPL2 (50). Primers used are listed in the **Table**
445 **S2**. To deliver plasmids into *Lm*, *Escherichia coli* S17.1 (colistin and nalidixic acid sensitive)
446 were transformed with the plasmids followed by conjugation with *Lm* EGDΔ*hly* (colistin and
447 nalidixic acid resistant). *Lm* EGDΔ*hly* were selected on 7µg/ml chloramphenicol (bacteria
448 containing the pPL2 derivatives), 10µg/ml colicin and 50µg/ml nalidixic acid (selection of
449 resistant *Lm vs* sensitive *E. coli*). Since all our constructs were made on a similar EGD
450 background, no impact of the PrfA* phenotype of EGD is expected on our results and
451 conclusions.
452

453 **Western blotting**

454 Protein extracts were obtained from EGD, EGDΔ*hly*, EGDΔ*hly*:pPL2, EGDΔ*hly*:pPL2-*hly*$_{WT}$,
455 EGDΔ*hly*:pPL2-*hly*$_{G299V}$ and EGDΔ*hly*:pPL2-*hly*$_{C484*}$ as follows. Bacteria were grown
456 overnight in BHI broth at 37°C. After centrifugation of bacterial cultures (30 min; 2,151 g),
457 all proteins of the supernatant were precipitated by using trichloroacetic acid (20%) and
458 washed using acetone. Proteins were then separated by SDS/PAGE (8% acrylamide gel and
459 3.9% stacking gel) and transferred to a polyvinylidene difluoride transfer membrane (Bio-
460 Rad, California, USA). The membrane was incubated overnight at 4°C with a blocking buffer
461 containing dried milk (5%), phosphate buffered saline (PBS, 1%) and Tween (0.1%) and
462 washed with PBS (1%) and Tween (0.1%). It was then incubated first with a polyclonal anti-
463 LLO (51, 52) or anti-InlC antibody (53) (1/20,000; 1h; room temperature) and second with
464 the anti-rabbit antibody (1/3,000; 1h; room temperature). The membrane was washed with
465 PBS (1%) and Tween (0.1%) between each incubation step with antibodies. Antibody-antigen
466 interactions were revealed using a SuperSignal West Pico Chemiluminescent substrate
467 (Thermo Fischer Scientific, Massachussetts, USA).

468

469 **Animal studies**

470 The virulence of *Lm* strains EGDΔ*hly*:pPL2-*hly*$_{WT}$, EGDΔ*hly*:pPL2-*hly*$_{G299V}$,
471 EGDΔ*hly*:pPL2-*hly*$_{C484*}$ and EGDΔ*hly*:pPL2 was assessed *in vivo*. Balb/c mice were infected
472 via intravenous route with 1.10^4 colony-forming units (CFUs) per animal. At 72 h post
473 infection, mice were sacrificed for spleen and liver dissection. CFUs were enumerated by
474 plating dilutions of the whole homogenized organs onto BHI plates. Statistical analyses were
475 done using the Mann–Whitney *U* test as compared with EGDΔ*hly*:pPL2-*hly*$_{WT}$.

476

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490

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660

660

661 **Figure legends**

662 **Figure 1: Phylogenetic tree summarizing all the genetic features causing the loss of**

663 **hemolytic activity among the 60 non-hemolytic *Lm* strains.**

664 Single-linkage based clustering was obtained based on the cgMLST allelic profiles, as

665 described previously (21). Scale bar indicates % of cgMLST similarity. Strain names have

666 been simplified to avoid redundancy and should be preceded by "CLIP". PrfA activities and

667 mutations (first and second columns, respectively), *gshF* presence/absence profile (third

668 column) and LLO mutations and presence/absence profile (fourth column) are mapped on the

669 phylogeny. Position and nature of amino acid substitutions are indicated in grey zones.

670 Positions of premature stop codons are indicated next to black asterisks in light pink zones.

671 Absence of *gshF* and *hly* in the CLIP 1998/76801 strain is indicated in black. MLST clonal

672 complexes are shown on the right. The black star highlights the CLIP 1998/76801 strain that

673 contains multiple copies of a transposable element that induced huge genomic

674 rearrangements. ND: unknown and non-human origin.

675

676 **Figure 2: Comparison of the CLIP 1998/76801 and F2365 genomes**

677 A. Gene content of the LIPI-1 region in F2365 (accession number: NC_002973) (top) in

678 comparison to the corresponding region in the non-hemolytic CLIP 1998/76801 strain

679 (bottom). LIPI-1 genes are highlighted in red. *mpl* is composed of 1532 bp in F2365 against

680 1133 bp in CLIP 1998/76801. B. Gene content of the *gshF* region in F2365 (top) in

681 comparison to the corresponding region in CLIP 1998/76801 (bottom). In A. and B., genes

682 that are present in CLIP 1998/76801 but absent in F2365 are indicated in orange. Genes

683 encoding the transposition protein (*tnsB*), the DNA-invertase (*hin*) and the cadmium

684 resistance genes (*cadA* and *cadC*) are indicated. C. Global comparison of the F2365 (top) and

685 the CLIP 1998/76801 (bottom) genomes. Positions of the eight copies of the transposable
686 element are indicated in dark blue. Identity percentages (indicated by grey zones of variable
687 intensities) between sequences were determined by nucleotide BLAST (54). Genome
688 comparisons were performed using Easyfig 2.1 (55).
689
690 **Figure 3: Characterization of the G299V substitution in LLO and the truncated LLO at**
691 **positon 484.**
692 A. Western blotting of the culture supernatants of EGD and EGDΔ*hly* complemented or not
693 with the pPL2 plasmid alone or containing hly_{WT}, hly_{G299V} or hly_{C484*}. LLO detection was
694 performed by using LLO-specific antibodies (above) and InlC-specific antibodies were used
695 as loading controls (below). B. qRT-PCR quantification of *hly* transcripts produced in BHI at
696 37°C by the EGDΔ*hly* strain complemented with the pPL2 plasmid alone or containing the
697 hly_{WT}, hly_{G299V} or hly_{C484*} genes. Each strain was tested at least three times using independent
698 pre-cultures. *gyrB* was used as stable reference gene for normalization. Results are shown as
699 fold change of *hly* expression relative to EGD (Relative Quantities, RQs). Each central bar
700 represents the mean of at least three replications. Error bars indicate standard deviations from
701 the means. C. *In vivo* characterization of the hly_{G299V} and hly_{C484*} mutations as compared to
702 the hly_{WT}. Each Balb/C mice were infected intravenously with 1.10^4 CFUs. Animals were
703 sacrificed 72 h after infection. Numbers of CFUs per organ are shown for all the tested
704 strains. No bacteria could be recovered from the liver of mice infected by
705 EGDΔ*hly*:pPL2-hly_{G299V} and EGDΔ*hly*:pPL2 strains; and from the spleen of mice infected by
706 EGDΔ*hly*:pPL2. Statistical analyses were done by a Mann-Whitney *U* test as compared with
707 EGDΔ*hly*:pPL2-hly_{WT}.

Figure 1

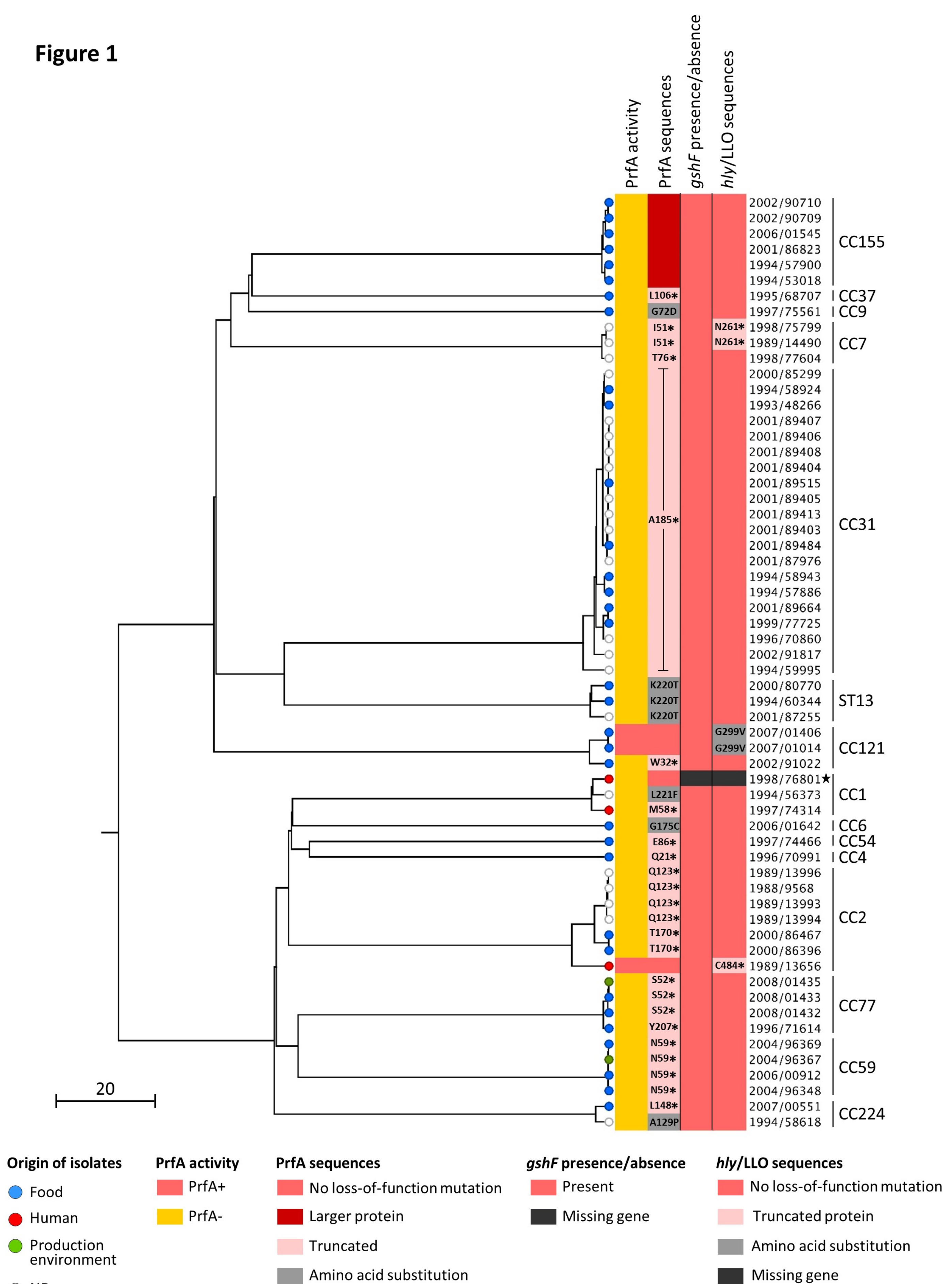

Figure 2

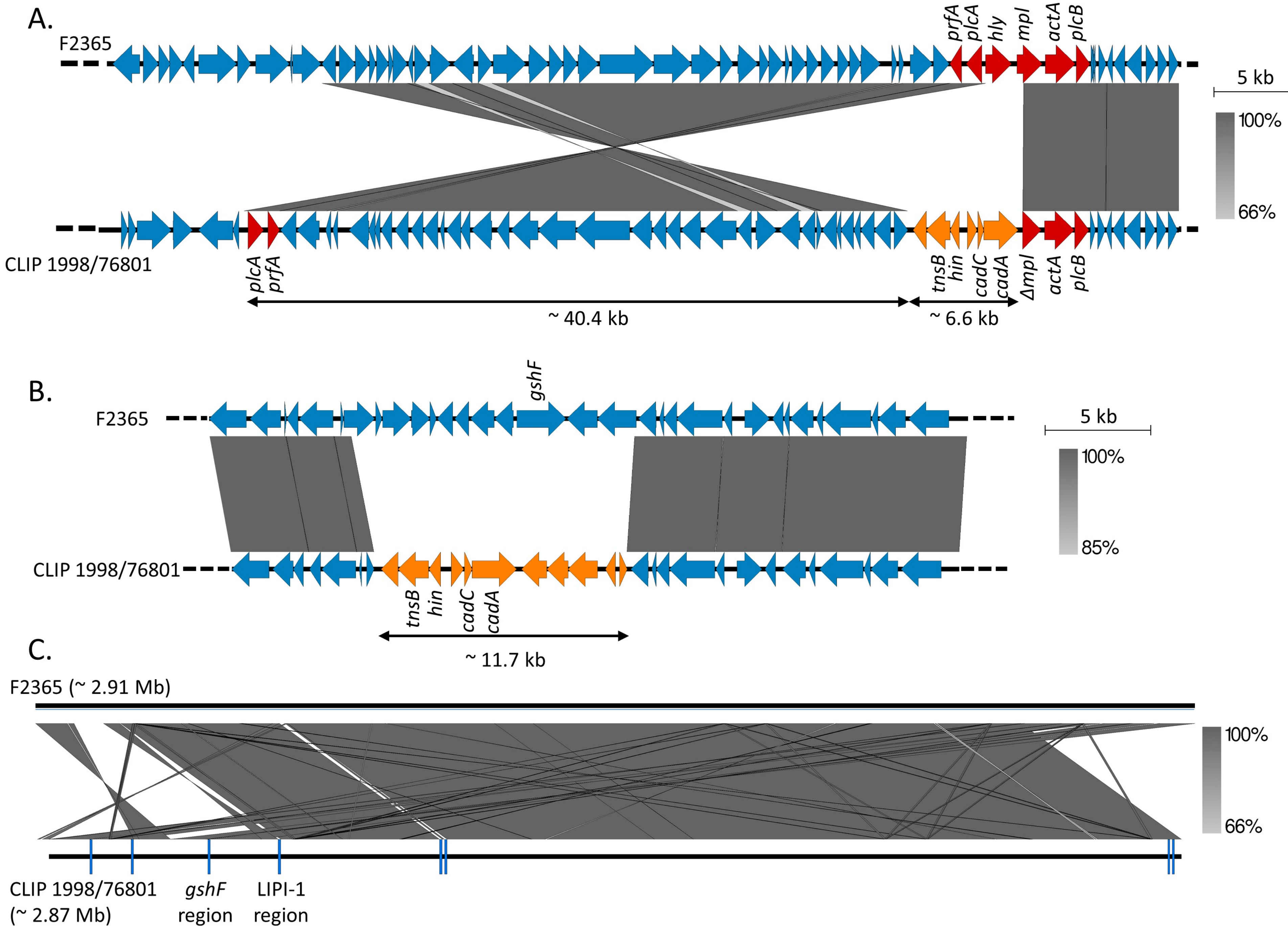

Figure 3