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# Accepted Manuscript

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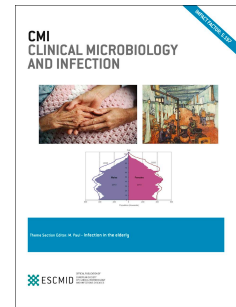
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3 **Title:** Reassessing the role of Internalin B in *Listeria monocytogenes* virulence using the  
4 epidemic strain F2365

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32

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41 **Abstract**

42 **Objectives:** We investigated the contribution to virulence of the surface protein  
43 Internalin B (InIB) in the *L. monocytogenes* lineage I strain F2365, which caused in  
44 California in 1985 one of the deadliest listeriosis outbreaks.

45 **Methods:** The F2365 strain displays a point mutation that hampers expression of InIB.  
46 We rescued the expression of InIB in the *L. monocytogenes* lineage I strain F2365  
47 introducing a point mutation in the codon 34 (TAA to CAA) and investigated its  
48 importance for bacterial virulence using *in vitro* cell infection systems and a murine  
49 intravenous infection model.

50 **Results:** We show in HeLa and JEG-3 cells that the F2365 InIB+ strain expressing InIB  
51 was  $\approx$ 9-fold and  $\approx$ 1.5-fold more invasive than F2365, respectively. In livers and spleens  
52 of infected mice at 72 h post-infection bacterial counts for F2365 InIB+ were significantly  
53 higher compared to the F2365 strain ( $\approx$ 1 log more) and histopathological assessment  
54 showed that the F2365 strain displayed a reduced number of necrotic foci compared to  
55 the F2365 InIB+ strain (Mann-Whitney test).

56 **Conclusions:** We describe a critical role for InIB during infection of non-pregnant  
57 animals by a *L. monocytogenes* strain from lineage I. Our study suggests that a  
58 spontaneous mutation in InIB could have prevented more severe human morbidity and  
59 mortality during the 1985 California listeriosis outbreak.

60

61 **Keywords:** *Listeria monocytogenes*, Internalin B, epidemic, infection, invasion

62

## 63 Introduction

64 *Listeria monocytogenes* is a facultative intracellular bacterium that causes listeriosis (1).  
65 After ingestion of contaminated food, *L. monocytogenes* disseminates to the liver,  
66 spleen, brain and/or the placenta (1). *L. monocytogenes* infections can be fatal, as  
67 exemplified by the 2017-2018 outbreak of listeriosis in South-Africa which affected 1060  
68 patients, of who 216 died ([http://www.nicd.ac.za/wp-](http://www.nicd.ac.za/wp-content/uploads/2018/07/Listeriosis-outbreak-situation-report-_26July2018_fordistribution.pdf)  
69 [content/uploads/2018/07/Listeriosis-outbreak-situation-report-](http://www.nicd.ac.za/wp-content/uploads/2018/07/Listeriosis-outbreak-situation-report-_26July2018_fordistribution.pdf)  
70 [\\_26July2018\\_fordistribution.pdf](http://www.nicd.ac.za/wp-content/uploads/2018/07/Listeriosis-outbreak-situation-report-_26July2018_fordistribution.pdf)). Strains of *L. monocytogenes* are grouped into lineage  
71 I, lineage II and lineage III (1). Major listeriosis epidemics have been associated to  
72 lineage I strains (2). However, the majority of reports investigating listeriosis  
73 pathophysiology have studied essentially strains from lineage II: e.g. EGD, EGD-e and  
74 10403S (3, 4).

75 The most important virulence factors of *L. monocytogenes* strains are encoded in the  
76 *inlA-inlB* locus and in the pathogenicity islands LIPI-1, LIPI-3 and LIPI-4 (2, 3). The *inlA-*  
77 *inlB* locus encodes for internalin A (InlA) and InlB, two bacterial surface proteins that  
78 bind the host cell receptors E-cadherin and Met, respectively, to induce bacterial uptake  
79 into non-phagocytic eukaryotic cells (1). Expression of the *inlA-inlB* locus and LIPI-1 is  
80 regulated by the transcriptional regulator PrfA (1, 5). Importantly, the strain EGD  
81 displays a PrfA mutation leading to constitutive production of InlA and InlB (5, 6).  
82 However, only one isolate carrying a PrfA mutation that leads to the constitutive  
83 production of InlA, InlB and LIPI-1 virulence factors has been found in *L.*  
84 *monocytogenes* clinical strains (2, 6).

85 All studies performed to understand the role of InIB in deep organ infection have used  
86 the EGD strain (7-10). While a clear contribution for InIB has been demonstrated for  
87 placental invasion (10), in spleen and liver infections it has been observed either a  
88 contribution for InIB in conventional mice (7) or no contribution for InIB in a transgenic  
89 humanized E-cadherin mouse model (10).

90 The genome of the lineage I strain F2365 responsible for the 1985 California outbreak,  
91 one of the deadliest bacterial food-borne outbreaks ever reported in the US (11), shows  
92 that the F2365 isolate carries a nonsense mutation in *inIB* (codon number 34 is TAA)  
93 (<http://genolist.pasteur.fr/>) (12). We thus decided to restore the expression of InIB in the  
94 F2365 strain and to examine the consequences of InIB expression during *in vitro* and *in*  
95 *vivo* infections.

96

## 97 **Materials and methods**

98 An isogenic mutant strain (F2365 InIB+, BUG3824) containing a functional InIB (a point  
99 mutation was introduced in the codon 34 (TAA to CAA)) was used. The InIB amino acid  
100 sequence of *L. monocytogenes* EGDe (lineage II) and F2365 (lineage I) strains has  
101 94% amino acid sequence identity (Supplementary figure 1). Cell infection was  
102 performed as described (13), using an MOI of 2 (phagocytic RAW 264.7), 5 (epithelial  
103 JEG-3 with InIA and InIB-dependent entry) or 25 (epithelial HeLa with only InIB-  
104 dependent entry). Luciferase reporter system experiments were performed creating a  
105 transcriptional fusion by cloning 308 nucleotides upstream from the *inIB* initiation codon  
106 into Swal–Sall-digested pPL2<sup>lux</sup> as described (3). For *in vivo* bioluminescence

107 experiments, mice were infected orally with  $5 \times 10^9$  F2365 InIB+<sup>*inIB::lux*</sup> (BUG4155) as  
108 described (3). Mouse infections were performed intravenously with  $10^4$  CFU of the  
109 indicated strain as reported (13). Half of the organs was used to assess bacterial load  
110 and the other half was used for histopathological analysis at 72 and 96 h after infection.  
111 This study was carried out in accordance with the French and European laws  
112 (86/609/EEC) and approved by the Animal Experiment Committee of the Institut Pasteur  
113 (approval no. 03-49).

114

## 115 Results

116 A point mutation in the *inIB* codon 34 (TAA to CAA) was performed to generate a F2365  
117 strain carrying a functional *inIB*, named F2365 InIB+ (Figure 1A) (13). Both F2365 and  
118 F2365 InIB+ strains were tested for entry into epithelial cells which express only the InIB  
119 receptor Met (HeLa), epithelial cells expressing both the InIB and the InIA receptors Met  
120 and E-cadherin respectively (JEG-3) or RAW 264.7 macrophages. Quantification of the  
121 number of viable intracellular *L. monocytogenes* showed that in HeLa and JEG-3 cells,  
122 the F2365 InIB+ strain was  $\approx 9$ -fold and  $\approx 1.5$ -fold more invasive than F2365, respectively  
123 reporting for the first time that chromosomal restoration of InIB promotes a gain of entry  
124 associated to the presence of the InIB receptor Met (Figure 1B). In macrophages,  
125 F2365 InIB+ and F2365 strain invaded similarly, showing that InIB does not play a role  
126 in entry into phagocytic cells.

127 In *L. monocytogenes* EGD, *inIA* and *inIB* are transcribed *in vitro* both individually and in  
128 an operon by PrfA-dependent and independent mechanisms (4, 14). Here, we



129 investigated whether *inlB* is transcribed *in vivo* from its own promoter in the epidemic  
130 lineage I *L. monocytogenes* F2365. For this purpose, we fused the 308 nucleotides  
131 located upstream from the *inlB* initiation codon to a Lux reporter plasmid and integrated  
132 it into the chromosome of the F2365 strain (F2365InlB<sup>+inlB:lux</sup>). Upon oral infection of  
133 twelve conventional BALB/c mice with  $5 \times 10^9$  *L. monocytogenes* F2365InlB<sup>+inlB:lux</sup>, no  
134 bioluminescent signal was detected in organs of infected animals from 24 h post-  
135 infection (p.i.) to 72 h p.i. (Supplementary Figure 2A). To discard the possibility that the  
136 absence of bioluminescence in the liver and spleen could be due to a low number of  
137 CFU in these organs, the two organs were dissected, homogenized, serially diluted, and  
138 plated onto BHI plates. Mice orally infected yielded  $\approx 1 \times 10^{7.5}$  CFU in the liver or spleen  
139 at 48 h p.i. (Supplementary Figure 2B).

140 To analyze the potential contribution of InlB to the F2365 InlB<sup>+</sup> virulence, we performed  
141 intravenous inoculations of BALB/c mice with the F2365 and the F2365 InlB<sup>+</sup> strains. In  
142 all the organs tested at 72 h, bacterial counts for F2365 InlB<sup>+</sup> were significantly higher  
143 compared to the F2365 strain ( $\approx 1$  log more) (Figure 1C) (Mann-Whitney test was used  
144 for statistical significance).

145 Furthermore, histopathologic assessment showed that the F2365 strain displayed a  
146 reduced number of necrotic foci in the spleen and liver 72 h p.i. compared with the  
147 F2365 InlB<sup>+</sup> strain (Figure 1D and Supplementary Figure 3). The ratio necrosis / total  
148 area was significantly higher in the spleen of mice infected with the F2365 InlB<sup>+</sup> strain  
149 at 72 h (Figure 1D).

## 150 Discussion

151 In our study chromosomal restoration of InIB promoted a gain of entry into eukaryotic  
152 cells associated to the presence of the InIB receptor Met.

153 Previous studies performed in our laboratory demonstrated that less than  $\approx 10^7$  *L.*  
154 *monocytogenes* CFUs distributed across the entire intestine were sufficient to produce a  
155 bioluminescent signal in this organ (3). The present results suggest therefore that *in vivo*,  
156 *inIA* and *inIB* are transcribed in an operon from a promoter located upstream of *inIA*.

157 The present *in vitro* and *in vivo* results demonstrate that InIB expression increases the  
158 virulence of the F2365 InIB+ strain, and show that InIB plays an essential role in spleen  
159 and liver infection by lineage I *L. monocytogenes*. InIB is highly conserved in the  
160 genome of *L. monocytogenes*, suggesting a critical role for this molecule during  
161 infections (2). In conclusion, it could be speculated that a spontaneous mutation in InIB  
162 could have prevented a more severe *L. monocytogenes* disease during the 1985  
163 California outbreak.

#### 164 **Transparency declaration**

165 The authors have no conflicts of interest to declare.

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177

### 178 **Author contributions**

179 J.J.Q., P.C., and J.P.C. designed research; J.J.Q., O.D., M.A.N., J.M.T., I.M.R.G.,  
180 J.G.L., L.C., and J.P.C. performed research; J.J.Q., O.D., J.G.L., P.C., and J.P.C.  
181 analyzed data; and J.J.Q., P.C., and J.P.C. wrote the paper.

182

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221

## 222 **Figure legends**

223 **Figure 1.** (A) *InlB* protein levels detected by Western blot. Levels of a loading control  
224 protein, EF-Tu, are shown for comparison. (B) Role of *InlB* in an epidemic *L.*  
225 *monocytogenes* entry. Eukaryotic cells were incubated with the bacteria for 30 min  
226 (JEG-3 and RAW 264.7) or 1 h (HeLa) at 37°C. Following this incubation, extracellular  
227 bacteria were neutralized by adding complete fresh medium containing 40 µg/ml of  
228 gentamicin. At 2h post infection (p.i.), cells were lysed to determine the number of viable  
229 intracellular *L. monocytogenes*. Bars show numbers of viable intracellular bacteria. The  
230 mean and standard deviation are shown. Three independent experiments with 6  
231 replicates in each experiment were performed. One representative experiment is shown  
232 (\*\*\*\*P<0.0001, \*\*P<0.01, ns: no statistical significance, by Student's t test. Error bars  
233 represent standard deviation). (C) BALB/c mice were intravenously inoculated with 10<sup>4</sup>

234 *L. monocytogenes* F2365 and F2365 InIB+. Half of the organ was used to assess  
235 bacterial load and the other half was used for histopathological analysis. CFU in the  
236 spleen and liver were assessed at 72 h and 96 h p.i.. Each dot represents the value for  
237 one mouse. Two mice infected with the F2365 InIB+ strain died in before 96 h.  
238 Statistically significant differences were evaluated by the Mann–Whitney test. \*P < 0.05.  
239 (D) Histopathological analysis of the number of necrotic foci and the ratio necrotic  
240 area/total area were recorded in spleens and livers from the same infected mice (\*P <  
241 0.05 by the Mann–Whitney test).

242

243 **Supplementary Figure 1.** CLUSTAL alignment showing inIB sequences in *L.*  
244 *monocytogenes* EGD-e and F2365. Note that *L. monocytogenes* F2365 carries a  
245 nonsense mutation in codon number 34

246

247 **Supplementary Figure 2. Bioluminescence imaging of *inIA*-independent *inIB***  
248 **transcription.** (A) Absence of induction of the *inIB* promoter in liver and spleen after  
249 intragastric inoculation of four mice with  $5 \times 10^9$  bacteria per BALB/c mouse. C- control  
250 non-infected mouse. Images were acquired at the indicated hours after infection with an  
251 IVIS Spectrum Imaging System. Images were taken before (top panel) and after (bottom  
252 panel) abdominal skin and peritoneum dissection showing liver and spleen removed  
253 from mice. (B) Bacterial counts in the spleen and liver of the same mice at 24, 48 and  
254 72 h p.i.

255

256 **Supplementary Figure 3. Spleen and liver histopathological lesions.** BALB/c mice  
257 were injected intravenously with  $10^4$  CFU of the indicated strains. Mice were killed at 72  
258 h p.i., and spleens and livers were removed. Half of the organ was used to assess  
259 bacterial load, and the other half was used for histopathological analysis. Arrowheads  
260 show necrotic foci.

261

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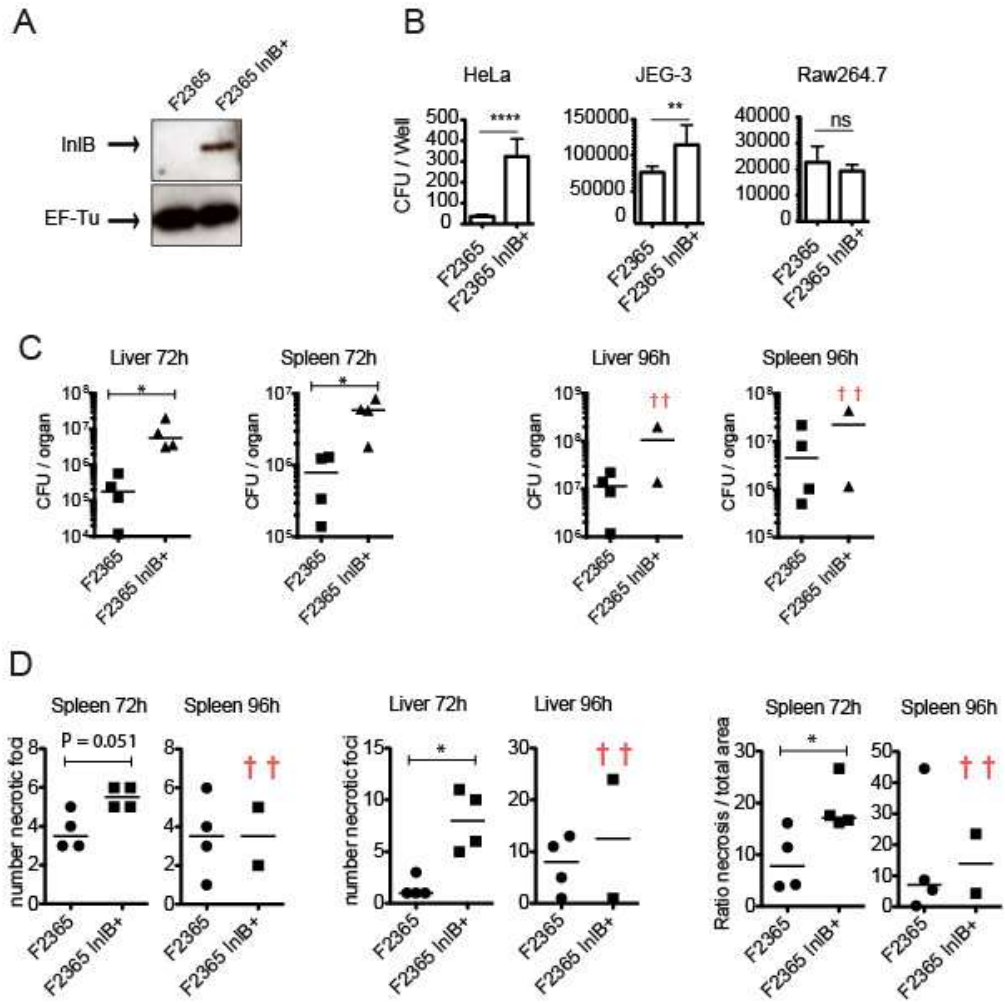


Figure 1