1 **Supplementary figure 1: Fitness analyzes of the non-hemolytic strains.**

2 Growth analyzes of all non-hemolytic strains were performed in BHI at 22°C (A) and 37°C
3 (B). Areas under the growth curves (AUC) were calculated for each strain using the "gcFit"
4 function of the "grofit" R package v.1.1.1-1. AUC values are shown for each strain (being
5 represented by one black dot) per type of loss-of-hemolysis mutation. Each central bar
6 represents the mean of at least three replications. Error bars indicate standard deviations from
7 the means. Horizontal black lines indicate the AUC values obtained for EGDe. Black circles
8 surround strains that show the most impaired growth.

9

10 **Supplementary figure 2: Assessment of PrfA activity.**

11 PrfA activity was assessed using, as reporter, the lecithinase activity of PlcB on egg yolk BHI
12 agar plates both in PrfA-non-activating (without charcoal, left) and -activating conditions
13 (with 0.5% w/v activated charcoal, right). Two time points are shown: 24 h (above) and 48 h
14 (below). *prfA*$_{WT}$: P14$_{WT}$; Δ*prfA*: P14Δ*prfA*; *prfA**: P14*prfA**; 1: CLIP 2008/01432; 2: CLIP
15 1996/70860; 3: CLIP 2001/89406; 4: CLIP 2008/01435; 5: CLIP 2001/89407. Positions of the
16 strains are identical in all pictures. All non-hemolytic strains showed similar activity profiles
17 as strains 1 to 5.

18

19 **Supplementary figure 3: Quantification of *prfA* and *hly* transcripts for a representative**
20 **set of non-hemolytic strains.**

21 Fold change of *prfA* (above) and *hly* (below) transcription relative to EGDe (Relative
22 quantities, RQs) are shown for a representative set of non-hemolytic strains (**Table S1**, one
23 strain per type of loss-of-hemolysis mutation). *prfA* and *hly* expression levels were
24 normalized according to *gyrB* expression. Each central bar represents the mean of at least

25 three replications. Error bars indicate standard deviations from the means. Horizontal black
26 lines indicate the RQs of 1 on the y-axes.
27

28 **Supplementary Table 1: Characteristics and amino-acid modifications identified in PrfA**
29 **and LLO for the 60 non-hemolytic strains.**

30 Lineages, PCR serogroups, sequence types and clonal complexes (MLST) as well as cgMLST
31 types were deduced from genome sequences using the BIGSdb-*Lm* platform
32 (http://bigsdb.pasteur.fr/listeria). All genome assemblies are publicly available in the BIGSdb-
33 *Lm* platform using the identifiers listed in the first column. Assemblies of both Illumina and
34 PacBio reads are made available for the CLIP 1998/76801 strain. Positions of amino-acid
35 substitutions and stop codons in PrfA and LLO are indicated. Stop codons are indicated with
36 black stars and highlighted in blue; amino acid substitutions are highlighted in orange. No
37 *bglA* allele could be deduced based on the Illumina reads of the CLIP 2004/96369 strain, and
38 no ST could therefore be assigned, but a clonal complex could be deduced based on the 6
39 other MLST alleles. Strains used for the quantification of *hly* and *prfA* transcripts are notified
40 in the "Used in *hly* and *prfA* qRT-PCR assays" column.
41

42 **Supplementary table 2: Primers used in this study.**

43 All primers used for *hly* complementation in a EGDΔ*hly* background (EGDΔ*hly*:pPL2-hly_{WT},
44 EGDΔ*hly*:pPL2-hly_{G299V} and EGDΔ*hly*:pPL2-hly_{C484*} constructs) as well as the primers used
45 for qPCR assays are listed. Restriction enzymes recognition sites are underlined.
46