INTRODUCTION

Listeria (L.) monocytogenes is an opportunistic bacterial pathogen, which is well adapted to both extracellular dwelling in outdoor and food factory environments (Carpentier & Cerf, 2011; Vivant, Garmyn, & Piveteau, 2013) and intracellular survival when ingested by a host (Vazquez-Boland et al., 2001). In the host, L. monocytogenes may pass the intestinal tract without causing disease or may cause self-limiting gastroenteritis (Gahan & Hill, 2014). However, when host barriers are crossed by invasion of cells, L. monocytogenes causes listeriosis, a potentially life-threatening infection associated with septicemia, abortions, and neurological disease (Disson & Lecuit, 2012; Oevermann, Zurbriggen, & Vandevelde, 2010; Siegman-Igra et al., 2002). Despite its low incidence rate, listeriosis is considered...
as a major public health threat due to the high fatality rate (Maertens de Noordhout et al., 2014; Swaminathan & Gerner-Smidt, 2007).

Its remarkable niche adaptability is due to the large set of genes that allows L. monocytogenes to resist stressful environmental conditions and to invade and survive within phagocytic and non-phagocytic host cells (Chaturongakul, Raengpradub, Wiedmann, & Boor, 2008; Kazmierczak, Mithoe, Boor, & Wiedmann, 2003). Many essential genes for intracellular survival of L. monocytogenes have been identified in strain EGD-e, a widely used reference strain of the species, belonging to lineage II (Chakraborty, Hain, & Domann, 2000; Portnoy, Chakraborty, Goebel, & Cossart, 1992; Schnupf & Portnoy, 2007; Vazquez-Boland et al., 2001). Six of them are located on the Listeria pathogenicity island number 1 (LIPI-1), one of which is the transcriptional activator prfA (Scortti, Monzo, Lacharme-Lora, Lewis, & Vazquez-Boland, 2007; Vazquez-Boland et al., 2001). PrfA regulates the transcription of the other 5 essential virulence genes on LIPI-1 including hly, plcA, plcB, mpl (vacuolar escape), and actA (invasion, intracellular movement, intercellular spread, and avoidance of autophagy)

and additionally other important genes outside of LIPI-1 including inIA and inIB (Alvarez & Agaisse, 2016; Kanki, Naruse, & Kawatsu, 2018; Kocks et al., 1992; Phelps et al., 2018; Suarez, Gonzalez-Zorn, Vega, Chico-Calero, & Vazquez-Boland, 2001). The proteins encoded by the latter two genes initialize bacterial internalization into non-phagocytic cells by interacting with the two host membrane proteins E-cadherin and c-Met, respectively (Bierne & Cossart, 2007).

Studies indicate that strain diversity in L. monocytogenes is relevant in the context of infection and environmental survival. Recent epidemiological studies have shown that the relative prevalence of L. monocytogenes strains differs between clinical infection and environment (Dreyer et al., 2016; Maury et al., 2016; Orsi, Bakker, & Wiedmann, 2011). Of the four phylogenetical lineages known, lineage I strains are significantly more prevalent in clinical infection of both humans and ruminants than in the environment. This is particularly true for strains from specific sequence types (ST, as determined by multilocus sequence typing (MLST)), namely ST1 and 4. These two ST additionally behave hypervirulent and hyperinvasive
in experimental in vivo and in vitro infections (Dreyer et al., 2016; Guldimann et al., 2015; Maury et al., 2016).

Previous studies from our group have shown that a ST1 strain from lineage I isolated from bovine rhombencephalitis (JF5203) behaves hyperinvasive compared to lineage II strains including EGD-e (Dreyer et al., 2016; Rupp, Bartschi, Frey, & Oevermann, 2017) suggesting that, besides the well-known virulence genes, yet uncharacterized virulence genes may contribute to cellular invasion and virulence of certain strains. Therefore, the aim of this study was to assess the impact of lineage I-specific virulence candidate genes on the cellular infection process. Emphasis was put on four genes of lineage I that encode surface proteins with internalin-like structure. To investigate their effect on cellular infection, CNS-derived cell lines (fetal bovine brain cells, human microglia cells) and non-CNS-derived cell lines (bovine macrophage cells, human adenocarcinoma cells) that represent the various target cells of *L. monocytogenes* were infected with a ST1 parental strain and deletion mutants derived thereof.

## METHODS

### 2.1 Selection of candidate genes

For the identification of genes specific to lineage I, comparative whole genome analysis of 121 lineage I and 104 lineage II genomes was performed (Aguilar-Bultet et al., 2018). One hundred and sixty-seven genes were found to be present in lineage I genomes but absent from lineage II genomes (Aguilar-Bultet et al., 2018). Four genes (LMJF5203_00388, LMJF5203_01291, LMJF5203_02767 and LMJF5203_02537) of 167 genes were chosen for analysis based on their internalin-like protein structure (Appendix 1). All candidate genes specify for potential proteins with a leucine-rich repeat (LRR) binding motif involved in host receptor recognition and interaction, which is found in internalin proteins (Bierne & Cossart, 2007). Furthermore, LMJF5203_00388, LMJF5203_01291, LMJF5203_02767 possess a LPXTG sortase recognition motif. Additionally, LMJF5203_00388 and LMJF5203_02537 are found in most strains of clonal complexes (CCs) belonging to lineage I and III, whereas LMJF5203_02767 and LMJF5203_01291 are present in most lineage I CCs but not in lineage III. None of the four genes was found in lineage II strains.

### 2.2 Expression of *Listeria monocytogenes* candidate genes by reverse-transcription PCR

Virulence gene mRNA expression was assessed in stationary phase bacteria, which were used for the gentamicin protection assay. Liquid broth cultures were grown overnight, and bacteria were collected by centrifugation at 3,220 g for 5 min. Total RNA was extracted using RiboPure™ Bacteria kit (Ambion, Life technologies). Reverse transcription was performed using GoScript Reverse-Transcription System (Promega) based on manufacturer’s instruction. sigB and gyrA were used as control genes for RNA expression. DNA contamination was excluded by PCR using RNA that was not reverse-transcribed. mRNA expression was assessed using following primers d0388_inside_fw, d0388_inside_rv, d1291_inside_fw, d1291_inside_rv, d2537_inside_fw, d2537_inside_rv, d2767_inside_fw, d2767_inside_rv, sigB_fw, sigB_rv, gyrA_fw, and gyrA_rv (Table 1), with amplification parameters as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 2 min, annealing at 55°C for 30 s, and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min.

### 2.3 Bacterial strains

*Listeria monocytogenes* strain JF5203 (NCBI Reference Sequence: NZ_LT985474.1; https://www.ncbi.nlm.nih.gov/nuccore/NZ_LT985474.1)
belonging to phylogenetic lineage I, clonal complex 1, sequence type 1, isolated from a rhombencephalitis case in cattle was used as parental strain for cell invasion experiments and generation of the deletion mutants. Listeria monocytogenes strain EGD-e, belonging to lineage II, clonal complex 9, sequence type 35 was used as a reference strain in order to confirm hyperinvasiveness of our parental strain. The deletion mutants LMJF5203_Δ00388 and LMJF5203_Δ02767 were generated using the pHoss1 plasmid, and LMJF5203_Δ01291 and LMJF5203_Δ02537 using the pMAD plasmid as previously described (Abdelhamed, Lawrence, & Karsi, 2015; Arnaud, Chastanet, & Debarbouille, 2004; Rupp et al., 2017). The upstream and downstream flanking regions of the genes of interest were amplified with the Expand High Fidelity Plus PCR system (Roche Diagnostics, Rotkreuz, Switzerland) using the amplification primer pairs d00388_1_fw_SalI/d00388_2_rv; d00388_3_fw/d00388_4_rv_XmaI; d01291_1_fw_SalI/d01291_2_rv; d01291_3/fw/d01291_4_rv_XmaI; d2416_1-fw_SalI/d02537_2_rv; d2416_3-fw/d02537_4_rv_XmaI; d02767_1_fw_SalI/d02767_2_rv; and d02767_3-fw/d02767_4_rv_XmaI (Table 1). Subsequently, amplicons of the flanking regions were joined via overlap extension PCR with the primer pairs d00388_1 fw_SalI/d00388_4_rv_XmaI; d01291_1_fw_SalI/d01291_4_rv_XmaI; d02537_1 fw_SalI/d02537_4_rv_XmaI; and d02767_1 fw_SalI/d02767_4_rv_XmaI. The fused DNA fragments were inserted into the SalI- and XmaI-digested pMAD and pHoss1 plasmids, respectively, by ligation with T4 ligase to create pMad_Δ01291, pMad_Δ02537, pHOSS_Δ00388, and pHOSS_Δ02767. The parental strain JF5203 was transformed with these deletion plasmids as described (Abdelhamed et al., 2015; Arnaud et al., 2004). Deletion of the genes of interest was confirmed by colony PCR using the flanking region primer pairs as described above and additional primers binding to the ORF regions of the deleted genes (Table 1).

2.4 | Whole genome sequencing of deletion mutants

Listeria monocytogenes mutants were grown overnight at 37°C in Bacto Brain Heart Infusion (BHI, Chemie Brunschwig, 237500), and genomic DNA was extracted using the DNA extraction kit (Invitrogen, PureLink™ Microbiome, DNA purification Kit, A29789). The whole genomes of the mutant strains were sequenced in GATC Biotech on an Illumina® HiSeq 4000 (150 bp paired-end reads) platform according to the manufacturer’s protocols. Genome coverage was between 200x and 300x. The Illumina reads of the different mutants were mapped to the whole genome of the parental strain in the Geneious software (Geneious 8.1.9, Biomatters Limited) to check the targeted deletion and to exclude spontaneous off-target mutations (NCBI Access number: PRJNAS04399; https://www.ncbi.nlm.nih.gov/bioproject/PRJNAS04399).

2.5 | Mammalian cell lines

The bovine macrophage cell line (BoMac), the human microglia cell line (HMC-3), and the human epithelial colorectal adenocarcinoma cell line (Caco-2) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamax (Life Technologies, Zug, Switzerland) supplemented with 10% fetal calf serum (FCS) (Bioswisstec, Schaffhausen, Switzerland), 100U/ml penicillin and 10 µg/ml streptomycin (Life Technologies). Fetal bovine brain cells (FBBC-1) were grown in a DMEM/F12 mix (1:1, Life Technologies) supplemented with 10% FCS, 50 ng/ml epithelial growth factor, 50 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Sigma-Aldrich, Buchs, Switzerland), 100 U/ml penicillin, 10 µg/ml streptomycin, and 1x N2 supplement (Life Technologies).

2.6 | Axenic growth in broth

Single colonies of mutant strains and the parental strain were inoculated into BHI-broth and grown overnight. The following day, fresh broth was inoculated with overnight culture at an OD600 of 0.05, and the OD600 was measured every 30 min for 7 hr. Bacterial growth was quantified in three independent experiments. Growth curves were fitted using a logarithmic scale in base 10, and generation time was calculated.

2.7 | Gentamicin protection assay

Cells were grown to confluency in 24-well plates with DMEM medium supplemented with 10% FCS and without penicillin/streptomycin. FBBC-1 cells were differentiated by incubation with 100 µM forskolin (Merck-Millipore, Schaffhausen, Switzerland) during 18 hr prior to infection (Takenouchi, Iwamaru, Sato, Yokoyama, & Kitani, 2009). Cells were starved in DMEM medium without FCS during 1 hr before inoculation. Overnight cultures of bacteria were added...
at $10^6$ CFUs per well corresponding to a multiplicity of infection (m.o.i) of 5:1. One hour following inoculation, cells were washed twice with phosphate-buffered saline (PBS) and DMEM medium supplemented with 10% FCS and 50 µg/ml gentamicin (Sigma-Aldrich) was added. FBBC-1 cells were further supplemented with 100 µM forskolin. At different time points (2, 4, 8, and 24 hr p.i.), cells were washed twice with PBS and then lysed with 0.5% ice-cold Triton-X100 (Sigma-Aldrich) and finally plated on BHI-plates in several dilutions (1:1, 1:10, 1:100, 1:1,000, 1:10,000) for CFU quantification. Resulting CFU numbers were normalized to the inoculum. At least three independent experiments using triplicates were performed. The 2-hr time point was used as an indicator for cellular invasion (Gaillard, Berche, Moulier, Richard, & Sansonetti, 1987; Sabet, Lecuit, Cabanes, Cossart, & Bierne, 2005). To estimate the intracellular fitness of strains, the number of intracellular duplications and duplication time between time intervals was calculated according to $d = t/3.3 \log (n_2/n_1)$, with $d =$ duplication time, $t =$ time interval, $n_1 =$ intracellular cfu number at the beginning of the time interval, and $n_2 =$ intracellular cfu number at the end of the time interval.

### 2.8 | Immunofluorescence

For microscopical assessment of the gentamicin protection assay, cells were grown on glass coverslips, which were coated with poly-D-lysine hydrobromide for Caco-2 cells. Coverslips were removed from the 24-wells plates at the time points indicated above and fixed in 4% paraformaldehyde (PFA Sigma-Aldrich) for 30 min at room temperature (RT). The coverslips were then washed three times in PBS supplemented with 0.5% Tween (PBS-T), and cells were permeabilized with 0.5% Triton X-100 for 30 min at RT. In order to block nonspecific labeling, cells were incubated with PBS-T containing 10% normal goat serum (Dako, Baar, Switzerland) for 30 min and then incubated with rabbit Listeria O antiserum (BD, Allschwil, Switzerland, 1:200) in PBS-T with 10% of goat serum for 1 hr at RT. Coverslips were then washed three times in PBS-T and then incubated with Alexa Fluor 488- conjugated goat anti-rabbit IgG secondary antibody (Life technologies, 1:500) and with DAPI (Invitrogen, Carlsbad, CA, USA, T3604, 1:10,000) for one hour in the dark. Coverslips were washed three times with PBS-T, rinsed with distilled water, dried and mounted on Superfrost Plus glass.
slides (Menzel-Gläser) with Glycergel Mounting Medium (Dako, Glostrup, Denmark). Cell cultures were imaged using an Olympus Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan), equipped with 405-nm and 488-nm laser channels. The number of infection foci as a measure of invasion was quantified at 24 hr p.i. Additionally, size of 5 foci per strain was measured as an indicator of spread in the BoMac cell line. Three independent experiments were performed.

2.9 Statistical analyses

For comparison of intracellular CFU dynamics and axenic growth between deletion mutants and JF5203, nonparametric Kruskal-Wallis analyses followed by Dunn’s multiple comparison and nonparametric Mann-Whitney tests were performed for each time point using GraphPad Prism (GraphPad Software, La Jolla California USA, www.graphpad.com).

3 RESULTS

This study aimed to investigate the putative involvement of lineage I-specific genes encoding for internalin-like proteins in the hyperinvasive behavior of lineage I strains. To this end, whole genome analysis of 121 lineage I and 104 lineage II genomes was realized and among 167 genes that were associated with lineage I, 4 internalin-like genes were identified. A clinical ST1 (CC1, lineage I) strain, which is hyperinvasive compared to EGD-e from CC9, lineage II (Appendices 2 and 3 and Rupp et al., 2017) expressed all four internalin-like genes in vitro (Figure 1)
and therefore was used to generate the respective deletion mutants (LMJF5203_Δ00388, LMJF5203_Δ01291, LMJF5203_Δ02537, and LMJF5203_Δ02767). Whole genome sequencing of the four deletion mutants and comparison to the parental strain confirmed the deletion and excluded the presence of spontaneous genomic off-target mutations (results not shown). Reverse-transcription PCR confirmed absence of expression in the deletion mutants (Figure 1). Mutants were tested for axenic growth in broth and in different cell lines representing different targets of *L. monocytogenes* (FBBC-1 and HMC-3 as model for CNS infection, BoMac and Caco-2 as model for non-CNS target cells).

### 3.1 | Deletions do not affect fitness of *L. monocytogenes* mutants

When grown in BHI medium, the four deletion mutants generated (LMJF5203_Δ00388, LMJF5203_Δ01291, LMJF5203_Δ02537, and LMJF5203_Δ02767) showed growth curves similar to the parental strain JF5203 indicating that the deletions did not exhibit any defect in extracellular growth and fitness (Figure 2).

### 3.2 | Hyperinvasive behavior of ST1 is independent of LMJF5203_Δ00388, LMJF5203_Δ01291, LMJF5203_Δ02537, and LMJF5203_Δ02767

Confirming previous studies, the parental strain JF5203 (ST1, CC1) was hyperinvasive compared to EGD-e (CC9) as indicated by higher CFU counts from 2 hr p.i. on and by the higher number of infection foci in the analyzed cover slips (Appendices 2 and 3). The intercellular spread was similar between EGD-e and JF5203 (Appendix 3). Deletion of LMJF5203_00388, LMJF5203_02537, and LMJF5203_01291 in the hyperinvasive strain JF5203 did not result in any significant reduction of invasion of various CNS and non-CNS cell lines (Figures 3–6) as indicated by similar CFU numbers at 2 hr p.i. Supporting these results, the number of infection foci as determined by immunofluorescence at 24 hr p.i. was similar between deletion mutants and parental strain (Figure 7a–e). Also, kinetics of intracellular duplication were similar to the parental strain as indicated by similar increase in CFU numbers at later timepoints (4, 8, and 24 hr, Appendix 4). None of the four deletions had an effect on the size and shape of infection foci in the BoMac cell line indicating that the genes are not involved in intercellular spread (Figure 7a–d,f).

![Figure 5](image_url)
**Listeria monocytogenes** has a clonal population structure that is organized in four phylogenetic lineages with lineages I and II being the major lineages (Maury et al., 2016; Nightingale, Windham, & Wiedmann, 2005). Within these two lineages, hyper- and hypovirulent clones have been identified (Orsi et al., 2011). Hypovirulent clones of *L. monocytogenes* generally belong to lineage II (Jacquet et al., 2004; Maury et al., 2016; McLauchlin, 1990; Orsi et al., 2011), while hypervirulent clones have been predominantly found in lineage I, which is also the most prevalent lineage in animal and human infection (Chenal-Francisque et al., 2011; Jacquet et al., 2004; Kim et al., 2018; Maury et al., 2016; Orsi et al., 2011). Of those, strains belonging to sequence type (ST) 1 are particularly prevalent in CNS infection of ruminants and humans (Dreyer et al., 2016; Maury et al., 2016) and behave hyperinvasive in vitro compared to the reference strain EGD-e and other strains from lineage II (Dreyer et al., 2016; Guldimann et al., 2015; Rupp et al., 2017). Differences in genomic gene content suggest that lineage I strains harbor specific genes that may confer hyperinvasion and hypervirulence resulting in the higher prevalence of these strains in clinical infection (Dreyer et al., 2016; Maury et al., 2016). Therefore, we investigated the impact of four genes encoding for surface proteins with internalin-like structure (LMJF5203Δ00388, LMJF5203Δ02767, LMJF5203Δ02537, and LMJF5203Δ01291) on cellular invasion, intracellular survival, and intercellular spread.

In this study, we confirmed that compared to strain EGD-e our ST1 strain JF5203 is hyperinvasive in the different cell systems studied (Rupp et al., 2017) and that invasion is dependent on the infected cell line suggesting cell type-specific interactions between *L. monocytogenes* and the host cell. Our results show that
despite their association with lineage I, none of the four investigated genes is involved in the hyperinvasiveness of the ST1 strain, independently of the type and host origin of cell line infected (macrophages, microglia, fetal brain cells, colon adenocarcinoma epithelium). Additionally, these genes do not contribute to the intracellular survival and intercellular spread of *L. monocytogenes* ST1. Our results show that despite the LRR binding and LPXTG sortase recognition motifs, LMJF5203_00388, LMJF5203_02537, LMJF5203_02767, and LMJF5203_01291 do not act as invasins and may have other functions, either during the infection process or outside the host. Similar has been shown for the internalins InlC, InlH, and InlJ (Bierne & Cossart, 2007). Hence, other factors likely contribute to the hyperinvasiveness of ST1/CC1 and its high prevalence in clinical infections. Alternatively, the function of the investigated genes may be redundant with other genes of *L. monocytogenes* and therefore the phenotype not be picked up in our systems. Certainly, in vitro infection assays are limited tools for the study of virulence factors as they do not reflect all aspects of the infectious process in vivo. Indeed, other virulence factors (*llsB*, *inlJ*) were shown to be involved in in vivo infection, while no particular phenotype could be attributed to these virulence factors in in vitro infections of cell lines (Quereda, Andersson, Cossart, Johansson, & Pizarro-Cerda, 2018; Quereda et al., 2017; Rupp et al., 2017; Sabet et al., 2008). Therefore, we cannot fully rule out an impact of these four genes on the infectious process either in other cell types, which were not represented in our study, or in more complex physiological systems including the immune system and host barriers where such membrane proteins may exhibit moonlighting functions on the bacterial cell surface (Copley, 2012). The cause for the association of these four internalin-like genes with lineage I remains to be determined.

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**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

**AUTHORS CONTRIBUTION**
AO designed the study. BG, SR, and AO conceived and designed the experiments. LAB performed the whole genome comparisons. BG, SR, and CM performed the bacterial cloning and infection experiments. BG and AO wrote the manuscript. CM, SR, LAB, and JF critically revised the manuscript.
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DATA ACCESSIBILITY
All data are included in the main manuscript. Raw data are available on request.

ETHICS STATEMENT
None required.

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APPENDIX 1

Amino acid sequences of LMJF5203_00388 (a), LMJF5203_01291 (b), LMJF5203_02537 (c), and LMJF5203_2767 (d). Domains are annotated in different colors (LPXTG Motif Signal peptide LRR (leucine rich repeat)). Signal peptide and LPXTG were determined using https://www.expasy.org, LRR motifs using https://www.expasy.org for LMJF5203_00388 and LMJF5203_01291 and www.lrrfinder.com for LMJF5203_02537 and LMJF5203_2767.
(a) MKSKGLFLYVVLALSIVIGTNVFIKIDAAAAAAPPAISQIFPDDALATEIQTLGKSTAEVTQTDLDTINS5LTTSKGIS
SLEGMTNLTGLTLGTLTNQVDSPKLGLTNMLQLQSGNPSIDSLALSINLKLQLADLINDAQVTDPISGLTNKLGLGL
YNQNLQNSGNYSLQHRSLVSNKLTLNDEQALSNSLVSALNENQNNINLGLSINLNLFLDLDSANQIVDTTPLAGL
TKVQTLYVSNQQISDVTVGLSSINLWLIDISQKNISINRPPLNTKLTIIQMTNLVNEPISFESTVTPNILKNIAEQITIDDP
SISDNGVYANEAVTWNLPYTPKSYTFIERTDIGNATGNGSGTVEQPLVQYFKATFNIDGQETTENVETGTLLEQPPTTPV
KEGYTFNGWYDAETGKTWKDYTADTMPANDITLYAQFSINSYTAIFDVGDIVSTQAVEYQLLLEPPAPTDGDYTEFKG
WYDAKTGGTWDFTNNQMMPANDITLYAQFSKDASSGGDGGGTDEGGGNSENSTEGAPNTSIDTINTHIVLPATCD
HVLPIFFIGTFLSALTLLRKK

(b) MKRKISIIVVGMFFQSLTTPFITEAKEENKEEINPKSKITKGLTNSLYKTKLITGETDYGDSVFPSALAKVAAKATGS
ENTQQLVTQADLLNKISLNGYNKGISVTGDLLNNVTSISLNNQQVTIDPSIDQLPNLVSLSVKNNQISSSLILNAQNQLPK
LTDDDIENNDLDNTIDQDPQLVDVKTSGYTLKRLKKTVIAKKNPEVLNLQGYTIRNVYFQSVASTLKVSLNPVRK
NLRENSINELKVTLDAIEDPLGENELTDTVDFNQLNPLNLKTLDSKQNEEVLVDKTDVENSENLQLQLNLKLQLSIDQJPL
VQDQPQLVDVKTSDYKELSALTTVIAKNKPEVLNLGYPQIMQNYFQSVASTLKVSLNPVRKVLNLRENSINELVTDL
AIEDPLGENELTDVDNQLNPLNLKTLDSKQNEEVLVDKTDKENLQLMTLNNQNNLAQINLQVDQPQLVDVKS
DYKELSALTTVIAKKNPEVLNLGYPQIMQNYFQSVASTLKVSLNPVRKVLNLRENSINELVTDL
AIEDPLGENELTDVDNQLNPLNLKTLDSKQNEEVLVDKTDKENLQLMTLNNQNNLAQINLQVDQPQ

(c) MKNLFRLFLVSIVIIGVVSFKAADANETDVPYPLPARIIDVPFDENLAEDMIVENFGKDKVDTVQIQQDQAVDSATLGLGY
FTNYLTDLDQLMGLNAYFTVNNNIMYQTPTQMTFTGFPLDLPKLDDLDRAEGNLSELPENITVPDYQNYPLELYLSDL
NRIVGGLPNFNSIPKLETLMMSGLSASEVPDFNKLNNKQNFTQNFRETMDTFHLDLVSMDLSYNYLNLVPPT
IVDKVILGQJGTLPDQNVVFEDTNLTPYVTQLDDGLRISGFEQEVWIRDNESNEIYNAKVDYDEVTKIQIIPNLDK
GEYTIDFNGIEPYIEEGEVMMYSVKITIN

(d) MLQRREWGFICAFIFLFLFPITGSAETSDYETINGNEATITDYGQSTDITPTTLGNENYTVTAINGNAFSGKRTLNTVT
IPNTVITIGDAFTINSLEQVLPSNVQTIGRNSFSNVKNLEKITYSTALKNPIQALANLKTTPATVESIDAFENNFI
TNITIQPNPLQMAYQAFAATQLSTLIVPNSHLPNQDFQDASAHLLDTNFDLTLANGITYNQAELNFSAEPLST
FSLSFTGHNRSYDISEYGPSCSKPFYFYKTKVPLYSKDAKSNLNLSTRLDSGIGENYTTPKIDGYTLEKTPGNAQTQ
FSETLQNVITIEYKATAVQNNTGTVYDESGKLKTDVLTGEVNVTYQTSHKDIAYKJLQKVEGNESGFTSPATVTYI
YEKIANSDNNTNEMTDNSTTLSTNDTENSEATKTVKLNTSNILPTTGDSDKDALFGLSSTLLTSTSFFFFKRS
APPENDIX 2
Infection of BoMac (a), Caco-2 (b), FBBC-1 (c) and HMC-3 (d) with LMJF5203 (red) and EGD-e (blue) in the gentamicin exclusion assay. At the indicated time points, cells were lysed for CFU counting. Three independent experiments were performed in triplicates. Single CFU data are presented as dots, bars indicate the mean, and error bars indicate the standard error of the mean (SEM). Statistical analysis (nonparametric Mann–Whitney test) did reveal significant difference between EGD-e and LMJF5203.
APPENDIX 3
Immunofluorescence of BoMac after 24 hr of infection with (a) LMJF5203 and (b) EGD-e. *Listeria monocytogenes* are shown in green and nuclei in blue. EGD-e shows decreased foci numbers compared to the parental strain LMJF5203 (c) but similar foci size (d).

APPENDIX 4
Fold change, number of duplications and duplication time of *Listeria monocytogenes* JF5203 and isogenic deletion mutants in BoMac, HMC-3, FBBC-1, and CaCo-2.

| Time interval | 2–4 hr | 4–8 hr | 8–24 hr | 2–24 hr |
|---------------|--------|--------|---------|---------|
| BoMac         |        |        |         |         |
| Fold change   |        |        |         |         |
| LMJF5203      | 4,583582522 | 15,5596285 | 22,50513127 | 1605,039885 |
| LMJF5203_Δ00388 | 2,651962832 | 16,66801311 | 24,31400553 | 1074,750801 |
| LMJF5203_Δ02767 | 2,463174925 | 29,05865569 | 19,31753369 | 1382,682455 |
| Number of duplications |        |        |         |         |
| LMJF5203      | 2,196475648 | 3,95973571 | 4,492182075 | 10,64839343 |
| LMJF5203_Δ00388 | 1,407060556 | 4,059010234 | 4,60371568 | 10,06978647 |
| LMJF5203_Δ02767 | 1,300519086 | 4,860896057 | 4,271839009 | 10,43325415 |
| Duplication time in minutes |        |        |         |         |
| LMJF5203      | 54,63297538 | 60,61010571 | 213,7046059 | 123,9623619 |
| LMJF5203_Δ00388 | 85,28417593 | 59,12771492 | 208,527213 | 131,08493026 |
| LMJF5203_Δ02767 | 92,27084884 | 49,37361284 | 224,72757 | 126,5185321 |
| Strain           | 2–4 hr | 4–8 hr | 8–24 hr | 2–24 hr |
|------------------|--------|--------|---------|---------|
| **BoMac**        |        |        |         |         |
| **Fold change**  |        |        |         |         |
| LMJ5203          | 1,767764298 | 38,05098039 | 20,02135293 | 1346,739601 |
| LMJ5203_Δ01291   | 5,585830102  | 5,842304537  | 32,27726472 | 1053,340148 |
| LMJ5203_Δ02537   | 2,860383944  | 9,45748831  | 59,48329712 | 1608,678883 |
| **Number of duplications** |        |        |         |         |
| LMJ5203          | 0,821925928  | 5,24986172  | 4,323467562 | 10,39525521 |
| LMJ5203_Δ01291   | 2,481771693  | 2,54637562  | 5,01246462 | 10,04075568 |
| LMJ5203_Δ02537   | 1,51620881  | 3,241039133  | 5,89442712 | 10,65166066 |
| **Duplication time in minutes** |        |        |         |         |
| LMJ5203          | 145,9985576  | 45,71548981 | 222,0439928 | 126,9810094 |
| LMJ5203_Δ01291   | 48,35255407  | 94,24561552 | 191,5234424 | 131,4642087 |
| LMJ5203_Δ02537   | 79,1447716  | 74,05032465 | 162,8660983 | 123,9243384 |
| **HMC-3**        |        |        |         |         |
| **Fold change**  |        |        |         |         |
| LMJ5203          | 6,435560863  | 6,813925927 | 208,8039615 | 9156,352501 |
| LMJ5203_Δ00388   | 8,348928598  | 6,519884194 | 141,51048  | 7702,988202 |
| LMJ5203_Δ02767   | 6,58260637  | 7,44976417  | 183,3785193 | 8992,674766 |
| **Number of duplications** |        |        |         |         |
| LMJ5203          | 2,686065886  | 2,76846131  | 7,706005273 | 13,16055279 |
| LMJ5203_Δ00388   | 3,061591071  | 2,70484634  | 7,1476509  | 12,9112025 |
| LMJ5203_Δ02767   | 2,718658929  | 2,897194804 | 7,518680844 | 13,13453458 |
| **Duplication time in minutes** |        |        |         |         |
| LMJ5203          | 44,6750244  | 86,68997736 | 124,5781655 | 100,299704 |
| LMJ5203_Δ00388   | 39,19530637  | 88,72962448 | 134,361097 | 102,2367978 |
| LMJ5203_Δ02767   | 44,13940958  | 82,83875136 | 127,6819724 | 100,4984221 |
| **HMC-3**        |        |        |         |         |
| **Fold change**  |        |        |         |         |
| LMJ5203          | 1,514319923  | 1,943310072 | 67,11636437 | 197,5095779 |
| LMJ5203_Δ01291   | 0,97695924  | 5,25072548  | 51,50628647 | 245,4750107 |
| LMJ5203_Δ02537   | 0,883355971  | 5,881794187 | 57,42170845 | 298,3470049 |
| **Number of duplications** |        |        |         |         |
| LMJ5203          | 0,598670028  | 0,958516114  | 6,068592663 | 7,625778806 |
| LMJ5203_Δ01291   | 0,139719016  | 2,39247475  | 5,686676222 | 7,939432356 |
| LMJ5203_Δ02537   | 0,17893169  | 2,556256303  | 5,84352435 | 8,220847484 |
| **Duplication time in minutes** |        |        |         |         |
| LMJ5203          | 200,443088  | 250,3870269 | 158,1915369 | 173,0970742 |
| LMJ5203_Δ01291   | 858,8662255  | 100,3145383 | 168,8156482 | 166,2587375 |
| LMJ5203_Δ02537   | 670,6414487  | 93,88729907 | 164,2844185 | 160,5673871 |
| Time interval | Strain | 2–4 hr | 4–8 hr | 8–24 hr | 2–24 hr |
|---------------|--------|--------|--------|---------|---------|
| **FBBC-1**    |        |        |        |         |         |
| **Fold change** |       |        |        |         |         |
| LMJF5203      | 4.39689568 | 5.320430045 | 95.81982574 | 2241.5492 |
| LMJF5203_Δ00388 | 6.053174311 | 4.884853081 | 66.02136972 | 1952.177112 |
| LMJF5203_Δ02767 | 5.006113685 | 4.730859638 | 89.36151736 | 2116.36858 |
| **Number of duplications** |       |        |        |         |         |
| LMJF5203      | 2.136485304 | 2.411542862 | 6.582252285 | 11.13028045 |
| LMJF5203_Δ00388 | 2.597691896 | 2.288315172 | 6.044681165 | 10.9306823 |
| LMJF5203_Δ02767 | 2.323691054 | 2.242102357 | 6.481581778 | 11.0473519 |
| **Duplication time in minutes** |       |        |        |         |         |
| LMJF5203      | 56.16701402 |         |         |         |         |
| LMJF5203_Δ00388 |           |         |         |         |         |
| LMJF5203_Δ02767 |           |         |         |         |         |
| **CaCo-2**    |        |        |        |         |         |
| **Fold change** |       |        |        |         |         |
| LMJF5203      | 3.631826228 | 4.19075885 | 44.22098102 | 673.0829592 |
| LMJF5203_Δ01291 | 4.627432239 | 4.470073756 | 33.17831577 | 686.2922478 |
| LMJF5203_Δ02537 | 6.431270966 | 4.573448713 | 29.32636263 | 862.5788256 |
| **Number of duplications** |       |        |        |         |         |
| LMJF5203      | 1.860695175 | 2.06728622 | 5.46659126 | 9.39460521 |
| LMJF5203_Δ01291 | 2.210211864 | 2.160298636 | 5.052168748 | 9.422679248 |
| LMJF5203_Δ02537 | 2.685103875 | 2.193282473 | 4.874126239 | 9.75212587 |
| **Duplication time in minutes** |       |        |        |         |         |
| LMJF5203      | 64.49202512 | 116.0942291 | 175.6099983 | 140.5056422 |
| LMJF5203_Δ01291 | 54.29343763 | 111.0957513 | 190.017406 | 140.0875447 |
| LMJF5203_Δ02537 | 44.69100846 | 109.4250298 | 196.9583784 | 135.3497356 |
| **Time interval** |        |        |        |         |         |
| **Strain** |        |        |        |         |         |
| **2–4 hr** |        |        |        |         |         |
| **4–8 hr** |        |        |        |         |         |
| **8–24 hr** |        |        |        |         |         |
| **2–24 hr** |        |        |        |         |         |
| Strain          | 2–4 hr | 4–8 hr  | 8–24 hr | 2–24 hr |
|----------------|--------|---------|---------|---------|
| **CaCo-2**     |        |         |         |         |
| **Fold change**|        |         |         |         |
| LMJF5203       | 5,738236211 | 13,60616922 | 19,96733907 | 1558,958242 |
| LMJF5203_Δ01291| 7,261276156 | 13,73673343 | 32,34485431 | 3226,27679  |
| LMJF5203_Δ02537| 7,228074597 | 9,665179038 | 42,3800878  | 2960,699849 |
| **Number of duplications** |        |         |         |         |
| LMJF5203       | 2,520607357 | 3,766189032 | 4,319570181 | 10,6063657 |
| LMJF5203_Δ01291| 2,860223122 | 3,77996707  | 5,015464309 | 11,6556545 |
| LMJF5203_Δ02537| 2,853611395 | 3,272796457 | 5,405314672 | 11,53172252 |
| **Duplication time in minutes** |        |         |         |         |
| LMJF5203       | 47,6075735 | 63,72489484 | 222,243345  | 124,4535526 |
| LMJF5203_Δ01291| 41,95476888 | 63,49261662 | 191,4080015 | 113,2497536 |
| LMJF5203_Δ02537| 42,05197673 | 73,33178312 | 177,6029812 | 114,4668541 |