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**Inactivation of the gene encoding the cationic antimicrobial peptide resistance factor MprF increases
biofilm formation but reduces invasiveness of *Listeria monocytogenes***

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Running title: Biofilm formation in *Listeria monocytogenes*

Keywords: *Listeria monocytogenes*, mutagenesis library, biofilm, *mprF*

21 **Abstract**

22 Aims

23 To understand the genetics involved in surface attachment and biofilm formation of *Listeria*
24 *monocytogenes*.

25 Methods and Results

26 An *in vitro* screen of a Himar1 transposon library of *L. monocytogenes* strain 15G01 identified three
27 transposants that produced significantly different biofilm levels when compared to the wild-type strain;
28 two mutants exhibited enhanced biofilm formation and one produced less biofilm biomass than the wild-
29 type. The mutant 15G01 *mprF*::Himar1, which had a transposon insertion in the *mprF* gene, was selected
30 for further analysis. The mutant produced a more densely populated biofilm on solid surfaces such as
31 stainless steel and polystyrene, as determined using scanning electron and light microscopy. The 15G01
32 *mprF*::Himar1 mutant remained viable in biofilms, but showed an increase in sensitivity to the cationic
33 antimicrobial gallidermin. The mutant also displayed reduced invasiveness in CaCo-2 intestinal cells,
34 suggesting virulence properties are compromised by the inactivation of *mprF*.

35 Conclusions

36 Biofilm formation and gallidermin resistance of *L. monocytogenes* is influenced by *mprF*, but this trait is
37 associated with a compromise in invasiveness.

38 Significance

39 The presence of pathogenic microorganisms in the food processing environment can cause a significant
40 problem, especially when these microorganisms are established as biofilms. This study shows that the
41 inactivation of the *mprF* gene results in enhanced biofilm formation and abiotic surface attachment of
42 *Listeria monocytogenes*.

Introduction

The presence of pathogenic microorganisms in the food processing environment makes their management challenging. *Listeria monocytogenes* is the causative agent of listeriosis, a severe infection in humans that has a particularly high mortality rate amongst the vulnerable population (Vázquez-Boland et al. 2001; Dussurget et al. 2004). Infection with *L. monocytogenes* is thought to primarily occur through consumption of contaminated food such as dairy products, ready-to-eat meat and seafood as well as vegetables and fruit. Contamination of foods with *L. monocytogenes* primarily occurs during processing (Autio et al. 1999; Fonnesbech Vogel et al. 2001) and the ability to form biofilms after attachment to biotic and abiotic surfaces is thought to confer protection from environmental stress. The formation of *L. monocytogenes* biofilms can result in greater resistance to detergents, biocides, antimicrobial and sanitizing agents (Borucki et al. 2003; Møretrø and Langsrud 2004; Moltz and Martin 2005). Consequently, surface attachment and biofilm formation may be important for the environmental persistence of *L. monocytogenes* (Carpentier and Cerf, 2011), with biofilms acting as sources for secondary contamination of food. The role of genetic factors involved in biofilm formation were studied (Taylor et al. (2002), and this led to the discovery that the alarmone guanosine pentaphosphate is important for biofilm formation. However, the complex process of *L. monocytogenes* biofilm formation remains relatively poorly understood.

The seafood industry is of high economic value to New Zealand, with approximately 600,000 tonnes of seafood harvested each year. In 2017 alone, 127,760 tonnes of seafood valued at US\$1.15 billion (total export earning) was exported, particularly to European and Asian markets (<http://www.seafoodnewzealand.org.nz/our-industry/key-facts>). Contamination of seafood products remains a concern to the industry, highlighted by the voluntary recall by a mussel-processing company of 280 tonnes of Greenshell™ mussel meat that was possibly contaminated with *L. monocytogenes* in 2007 (<http://www.stuff.co.nz/dominion-post/archive/national-news/140211/Listeria-found-in-tonnes-of-export-Kiwi-mussel-meat>). Contamination of seafood products in New Zealand is believed to be the result

of persistence of the pathogen either on the food or on equipment in the processing plant (Carpentier and Cerf 2011).

A survey of *L. monocytogenes* in seafood processing plants in New Zealand identified a number of pulsotypes that predominate (Cruz and Fletcher 2011). Of these, pulsotype 5132 (MLST ST-321) was identified as the most prevalent and persistent, which produced greater biofilm mass than sporadic variants (Nowak et al. 2017).

The ST-321 *L. monocytogenes* isolates displayed low invasiveness in mammalian cell culture cells, linked to truncation of the primary virulence factor internalin A (InlA) (Cruz et al. 2014).

In this study, we have used the *L. monocytogenes* ST-321 (pulsotype 5132) isolate 15G01 to construct a mariner-based transposon mutant library to investigate the mechanisms associated with biofilm formation of *L. monocytogenes*. Screening of this *L. monocytogenes* 15G01 transposon mutant library revealed three transposants that had altered biofilm formation when compared to the wild-type, and one of these mutants has been further characterized.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains used in this study are listed in Table 1. Strains of *L. monocytogenes* were routinely cultured on Brain Heart Infusion (BHI) broth and agar (Oxoid Limited, UK) and Tryptic Soy Broth enriched with 0.6% yeast extract (TSBYE) (Difco, USA). For biofilm assays, TSBYE and modified Welshimer's broth (MWB, Himedia, India) were used. Lysogenic broth (LB, Difco, USA) was used for culturing *Escherichia coli* strains. Antibiotics used (final concentration) in this study were: erythromycin (ery) (5 µg ml⁻¹) (Duchefa, Biochemie, The Netherlands); kanamycin (kan) (50 µg ml⁻¹) (MP Biomedicals, Illkirch, France) and nalidixic acid (nal) (20 µg ml⁻¹) (Fort Richards, NZ). *L. monocytogenes* was grown at 37°C, with the exception of

transposants and transconjugants, which were grown at 30°C. *E. coli* strains were also cultured at 37°C, except during conjugation experiments, when they were grown at 30°C. All liquid cultures were grown shaking at 200 rpm unless indicated otherwise.

Transposon mutagenesis of *L. monocytogenes* 15G01

A transposant library of *L. monocytogenes* 15G01 was constructed using the Himar1 mariner-based transposition system following the protocol of Cao *et al.* (2007). The number of transposon mutants required for the library was calculated following method described in Russell and Sambrook (2001). Transposon mutants were deposited in the 96 wells of a microtiter plate (Costar, Corning, NY) containing 250 µl BHI/well supplemented with erythromycin. The 96-well plates were then incubated overnight at 37°C and stored as glycerol-stocks at -80°C prior to use.

Southern hybridization was used to assess the copy number of the mariner transposon in randomly selected individual mutants from the transposon library and whether the transposon insertions were random. Genomic DNA was extracted from the wild-type and the mutants using the DNeasy Blood and Tissue kit (Qiagen). Two to three micrograms of DNA from each extract was digested with *Pst*I (New England BioLabs) and separated by electrophoresis in a 0.7% agarose gel. The DNA was transferred to a N+ nylon membrane (Amersham Hybond-N+; GE Healthcare Life Sciences), the DNA was cross-linked by exposure of the membrane to UV light for 2 min, and Southern hybridisation performed using a transposon-specific probe (*ermC*). The 400-bp *ermC* probe was amplified from pMC38 using the primers Marq206 and Marq254 as indicated by Cao *et al.* (2007). The probe was then labelled using DIG-High Prime (Roche, Germany) for the non-radioactive labelling of DNA with DIG-11-dUTP alkali-labile, using random oligonucleotides as primers (Roche, Germany), following the manufacturer's instructions. DIG-labelled DNA was detected by an anti-DIG- alkaline phosphatase (AP, Fab fragments (Roche, Germany)) and visualisation of hybridised probe was performed using the NBT/BCIP ready-to-use tablets (Roche, Germany) for the sensitive detection of AP following the manufacturer's instructions.

High throughput screening for changes in biofilm formation

A set of 2,000 mutants from the transposant library were screened for their capacity to form biofilms using a modified crystal violet assay (Djordjevic et al. 2002; Cruz and Fletcher 2011; Nowak et al. 2015). To perform the screen, an aliquot of 200 µl TSBYE was added to each well of a 96-well plate and the media in each well was inoculated with a single transposant. After 24 h incubation at 37°C, a new 96 well-plate containing 200 µl TSBYE or MWB was inoculated with those cultures using a pin replicator. After a further 48 h incubation at 30°C each well was subsequently washed with distilled H₂O three times using a microtiter plate washer (ELx50, Biotek). The remaining biofilm was stained with a 150 µl aqueous 1% solution of crystal violet (CV). After addition of the CV solution and sealing with parafilm, the 96-well plate was incubated for 45 min at 30°C. Each well was then washed six times with distilled H₂O and the remaining CV was dissolved using 96% ethanol. Finally, after 1 h incubation at room temperature, the optical density (OD) of the dissolved crystal violet produced by each mutant was measured at a wavelength of 595 nm. Biofilm formation as expressed by CV staining by each mutant was measured in three independent experiments and the average CV staining was calculated. Mutants with means at least 2 standard deviations above or below that of the wild-type strain were selected. Biofilm formation of selected mutants was then confirmed in a subsequent independent experiment with eight wells used per mutant.

Localising transposon insertion sites in mutants of *L. monocytogenes* 15G01

To identify the sites of transposition in selected mutant, arbitrary PCR was used to amplify DNA directly flanking either end of the transposon. Arbitrary PCR was carried out as described previously with the following modifications: the DNA flanking the left and right ends of the transposon was amplified by Invitrogen *Taq* polymerase using primers Marq 207/ Marq 255 and Marq 207/ Marq 269, respectively (Cao et al. 2007). The program for the first round of the arbitrary PCR consisted of an initial denaturation cycle at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, an extension step at 72°C for 1 min, and a final extension cycle at 72°C for 7 min. For the second round of

the arbitrary PCR, High Fidelity AccuPrime *Taq* polymerase (Invitrogen) was used to amplify 1 µl of the PCR product from the first round used as a template, using the same PCR programme but with primers Marq 208 / Marq 256 and Marq 208 / Marq 270 for the amplification of the left and right ends of the transposon. The PCR products were then gel purified and sequenced using primers Marq 257 and Marq 271, respectively. DNA sequencing of the arbitrary PCR amplicons was performed using an Automatic Sequencer 3730x by Macrogen Inc. (Korea). The insertion sites of the transposons were determined by alignment of the transposon flanking sequences from each mutant to the genome sequence of *L. monocytogenes* 15G01 (accession number: GCA_900162555) using Geneious v6.1 (Drummond et al. 2013). A representative genome of 15G01 with the locations of the insertion sites was generated using DNAPlotter (version 1.11) (Carver et al. 2009).

Construction of strains for genetic complementation

Genetic complementation of the 15G01 *mprF*::Himar1 mutant was performed using the site-specific integrative vector pIMK (Monk et al. 2008). Initially, the *mprF* gene (and its predicted promoter region) was amplified from DNA of *L. monocytogenes* 15G01 using the primers MPRFFWD (5'-AAGCTGCAGGTCGCTTTGGTAATGGCA-3') and MPRFREV2 (5'-AACGGATCCTGCGGCATCGAACTGCTTCA-3') containing the *Pst*I and *Bam*HI restriction sites, respectively (underlined). The resulting PCR amplicon was then digested with *Pst*I and *Bam*HI and the digested product was cloned into pIMK by ligation using the LigaFast™ Rapid DNA Ligation System (Promega). *E. coli* S17 was subsequently transformed with the recombinant vector as well as with pIMK, and transformants were selected on LB agar plates supplemented with kan. Conjugation was performed to mobilise the pIMK plasmids into the 15G01 *mprF*::Himar1 mutant according to the method of Azizoglu et al. (2014), with some modifications. Briefly, cultures of the donor *E. coli* strains were grown overnight in LB broth containing kan at 30°C under shaking. At the same time, the recipient *Listeria* transposon mutant was incubated overnight at 37°C with shaking in BHI containing ery. Three ml of the donor culture and 1.5 ml of a pre-warmed (45°C; 10 min) recipient culture were then mixed together and centrifuged at 2050 g for 8 min. The resulting bacterial pellet was

washed with 10 ml of BHI broth and centrifugation was repeated. The bacterial pellet was re-suspended in 500 µl of BHI broth and deposited in the centre of a BHI agar plate, the plate was incubated overnight, and the resulting bacterial growth was scraped from the plate and re-suspended in 2 ml BHI broth. Finally, a 100 µl aliquot of each mix was spread-plated onto BHI agar plates containing kan and nal, and the plates were incubated at 30°C for 2-3 days. The authenticity of transconjugants was confirmed by PCR with the gene-specific primers used for cloning (Table 1). The empty vector, pIMK, was conjugated into the transposon mutant for comparative purposes.

Measuring growth and viability of *L. monocytogenes* 15G01 and the 15G01 *mprF*::Himar1 mutant

The growth of 15G01 *mprF*::Himar1 (6B4), with a gene disruption in *mprF*, was compared to that of the wild-type to eliminate the possibility that biofilm formation was altered as a result of impaired planktonic growth of mutants. To determine growth, *L. monocytogenes* 15G01 and the 15G01 *mprF*::Himar1 mutant strain were grown overnight in a 96-well plate in TSBYE at 37°C. One well per strain was in two independent experiments used to measure the OD_{595nm} with an automated plate reader (SPECTROstar Omega, BMG Labtech) at 30°C for 50 h. The results of two independent experiments were analysed to identify differences in the four parameters of a logistic growth curve $OD = A + \frac{C}{(1 + \exp(B * (Time - M)))}$ (A=left hand plateau, B=measure of steepness, M=mid-point of the rise, C=amount of climb) using the FITCURVE command in Genstat (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK). To see if there were any consistent differences between strains, allowing for the variability of the replicates, the parameter estimates for the strain-replicate combinations were tested using t-tests.

Measuring the hydrophobicity and surface charge of planktonic cells

The MATH (microbial adhesion to hydrocarbon) test was used to evaluate the bacterial cell surface hydrophobicity through separation of bacterial cells to an aqueous phase and a hydrocarbon phase. In this test, a fresh colony of each strain was picked from the TSBYE agar plates and used to inoculate 10 ml of TSBYE. The cultures of *L. monocytogenes* 15G01 and the 15G01 *mprF*::Himar1 mutant were incubated

overnight (18 h) at 35°C with shaking (200 rpm, Orbit 19 Labshaker, Labnet, NJ). Five millilitres of each overnight culture was washed three times with phosphate buffered saline (PBS, pH 7.2) (2050 g, 10 min, 20°C) and then re-suspended in the same volume of PBS. The OD_{600nm} was adjusted to 0.8. The culture (2.5 ml) was then mixed with 2.5 ml hexadecane (Fisher Scientific, UK), a non-polar solvent, and vortexed for 1 min. The OD₆₀₀ of the aqueous phase was measured after 15 min incubation at room temperature using a microtiter plate reader. The hydrophobicity was then calculated using the following equation:

$$\text{hydrophobicity}[\%] = \frac{(A_0 - A_1)}{A_0} * 100$$

with A₀ being the OD of the bacterial suspension before addition of hexadecane and A₁ the OD of the aqueous phase after mixing with hexadecane. The MATH was carried out three times with n=1.

Hydrophobic interactive chromatography (HIC) was used as a second method of evaluating hydrophobicity. To conduct HIC, cultures of each strain were grown as described above. The overnight cultures (100 µl) were then used to inoculate 9.9 ml MWB and the new cultures were grown at 35°C with shaking (200 rpm, Orbit 19 Labshaker, Labnet, NJ) until mid-log phase. Cultures were centrifuged at 1000 g for 20 min and re-suspended in 0.05 mol l⁻¹ phosphate buffer (pH 7.0) to an OD_{600nm} of 0.5. Chromatography columns were created from Pasteur pipettes. These were plugged with glass wool, filled with 1 ml of the resin (Phenyl-sepharose fast flow (Sigma-Aldrich, USA)) and washed ten times with 0.05 mol l⁻¹ phosphate buffer (pH 7.0). The re-suspended culture (300 µl) was then loaded into each column and washed with 900 µl 0.05 mol l⁻¹ phosphate buffer. The absorbance of the eluent and of a ¼ dilution of the original culture were determined at 600 nm and the percentage of bacteria retained in the hydrophobic column was calculated as follows: $\% \text{ retained} = \frac{(A_0 - A_1)}{A_0} * 100$ where A₀= OD_{600nm} of ¼ dilution of bacterial culture re-suspended in phosphate buffer and A₁= OD_{600nm} of the eluted bacterial suspension. HIC was carried out three times with n=2.

Cytochrome C is a cationic polymer that will bind to anionic structures on the bacterial cell allowing the surface charge to be measured. Thus, the cytochrome C assay was performed as described previously (Kang et al. 2015) with minor modifications. An aliquot of 100 µl of an overnight culture grown in TSBYE

216 at 37°C was used to inoculate 10 ml of MWB. The culture was grown overnight at 30°C and cells were
217 harvested by centrifugation at 2050 g for 10 min. The pelleted cells were washed twice with 0.02 mol l⁻¹
218 MOPS (Sigma-Aldrich, USA) buffer, pH 7.1 and re-suspended to an OD_{600nm} of 0.7. The culture (1.5 ml) was
219 transferred to a 2 ml Eppendorf tube and centrifuged (1485 g, 10 min). The cells were then re-suspended
220 in half the volume of 0.5 mg ml⁻¹ cytochrome C in MOPS buffer (0.02 mol l⁻¹, pH 7.1) and incubated for 10
221 min at room temperature. The cells were again centrifuged for 5 min (6000 g), the supernatant was
222 transferred to a 96-well plate and the OD was measured at 410 nm. The quantity of bound cytochrome C
223 was calculated using the following equation: *bound Cytochrome C* [%] = $\frac{(A_0 - A_1)}{A_0} * 100$, with A₀ being
224 the OD measurement of the supernatant of the blank at 410 nm and A₁ the OD measurement of the
225 supernatant of the sample.

226 Data from the hydrophobicity and cytochrome C assays were analysed using analysis of variance (ANOVA)
227 using with Genstat version 17 (2016) and Microsoft Excel software.

228 **Measuring the viability and metabolic activity of biofilm cells in *L. monocytogenes* 15G01 and an 15G01**
229 ***mprF::Himar1* mutant**

230 For enumeration of the viable cells in a biofilm, the 6 x 6 drop plate method was used (Chen et al. 2003).
231 *L. monocytogenes* strains were grown in TSBYE overnight at 37°C and subcultured in 96-well plates
232 containing MWB to grow biofilms. After 48 h the biofilms were gently washed three times with double-
233 distilled H₂O) and then resuspended in 200 µl Dulbecco's phosphate buffered saline (DPBS (without Mg²⁺
234 and Ca²⁺)) (Thermo Fisher, New Zealand) by rigorous pipetting. The suspension was transferred to a new
235 96-well plate. Ten-fold serial dilutions were prepared in DPBS and 10 µl drops of each dilution were plated
236 on BHI agar plates. Plates were incubated at 37°C for 24 h and colonies were enumerated, counts averaged
237 and the data plotted against the OD_{595nm} values from CV staining. The experiment was performed in
238 duplicate with five replicates for each strain. The results for the wild-type and the mutant were compared
239 using the Student's t-test, with p=0.05 as the significance level.

Biofilms were grown for 48 h in MWB at 30°C as described above. The metabolic activity of the cells in the biofilm was examined by adding the tetrazolium salt (XTT, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) (Sigma-Aldrich, USA) and phenazine methosulfate (PMS) (Sigma-Aldrich, USA), as described previously with minor modifications (Koban et al. 2012; Celis 2014). The XTT solution was prepared at a concentration of 1 mg ml⁻¹ in PBS (pH 7.2) and a stock solution of PMS at a concentration of 5 mmol l⁻¹. PMS solution (25 µl) was added to 5 ml XTT solution and 200 µl were added into each well and incubated in the dark at 37°C for 3h. The OD_{450nm} was measured and the values obtained were blank corrected. Analysis of variance was applied to each measure (OD, CV and XTT) to test the difference between the two strains, and results compared to see if estimates were similar.

Quantifying eDNA and carbohydrates in biofilms produced by *L. monocytogenes* 15G01 and an 15G01 *mprF*::Himar1 mutant

Biofilms were grown in 96-well plates as described in the previous paragraph. To disrupt the biofilm and measure the eDNA content, a method described by Wu and Xi (Wu and Xi 2009) was used, with modifications. To determine the amount of eDNA in the biofilm, N-Glycosidase solution (200 µl of a 1U ml⁻¹ 0.9% NaCl- solution) was added to each washed well and then incubated for 30 min at 37°C. Proteinase K was then added at a final concentration of 5 µg ml⁻¹ and incubated for another 30 min at 37°C. Disruption of the biofilm was supported by rigorous pipetting. Eighteen wells per strain were pooled together and filtered through a 0.2 µm membrane (cellulose acetate). Five hundred µl of the obtained solution was used to determine extracellular carbohydrates present in a biofilm using the phenol-sulphuric assay (DuBois et al. 1956). An aqueous 5% phenol solution (500 µl) was added to a 500 µl sample. Concentrated H₂SO₄ (2.5 ml) was quickly added and the solution incubated at room temperature for 30 min. Glucose content was determined with a spectrophotometer at 490 nm using a glucose standard curve.

To determine the eDNA content in the biofilm, the supernatant was precipitated using the CTAB (cetyltrimethylammonium bromide) method as described previously (Corinaldesi et al. 2005). To determine the amount of eDNA in the supernatant, the supernatants of the biofilms from 12 wells were pooled together and centrifuged at 5225 g for 10 min at room temperature. The eDNA of the supernatant (200 µl) was measured with the NanoDrop 2000 (Thermo Fisher, New Zealand) after using a commercially available kit (DNeasy blood and tissue kit, Qiagen) for extraction. The amount of eDNA present in the biofilm and supernatant and the carbohydrate amount were standardized against the OD_{595nm} values obtained after CV staining and compared using the Student's t-test.

Visualising biofilms on stainless steel and polystyrene surfaces

Scanning electron and fluorescence microscopy were used to compare biofilm production on stainless steel coupons (5 mm x 5 mm) coated with cooked mussel juice by *L. monocytogenes* 15G01 and the 15G01 *mprF::Himar1* mutant. Stainless steel coupons coated with mussel juice were used as a negative control. The stainless steel coupons were prepared as described previously (Borucki et al. 2003). SEM was performed as described previously (Borucki et al. 2003), with some modifications. Briefly, cooked mussel juice was prepared from Greenshell™ mussels, which were stored for 24 h at 10°C and then steamed without addition of water. Once all mussels had opened and had released their intervalvular juice, the liquid was collected and autoclaved at 121°C for 15 min. The coupons were pre-treated with alkali detergent for 2 h at 45°C and then rinsed with dd H₂O and autoclaved at 121°C for 15 min. The coupons were coated by immersion in cooked mussel juice for 4 h at 60°C, and then placed into MWB. Where appropriate, coupons were inoculated with bacteria by adding 30 µl of an overnight culture grown in TSBYE in 2.97 ml MWB. After incubation of the coupons for 7 days at 30°C, loosely attached cells on the coupons were removed by washing the coupons in PBS. After finally rinsing the coupons with 0.1 mol l⁻¹ cacodylate buffer (pH 7.2) (Acros Organics, NJ, USA), the coupons were fixed in 2% glutaraldehyde (Acros Organics, NJ, USA) and 0.1% ruthenium red (Acros Organics, NJ, USA) solution in 0.1 mol l⁻¹ cacodylate buffer overnight at 4°C. Coupons were then rinsed to remove unbound dye and dehydrated in a series of

dilutions of ethanol for 10 min (30, 50, 60, 70, 90% ethanol). The final three 10 min rinses were performed in 100% ethanol. The coupons were then critical point dried (BalTec CPD030 (BalTec AG, Balzers, Lichtenstein)) and sputter coated with gold (Leica EM ACE200, (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland)) before being visualised by SEM (FEI Quanta 250 SEM (Fei Company, Hillsboro, OR)).

Fluorescence microscopy was used to visualise biofilm formation of bacterial strains on polystyrene. For visualisation under fluorescence, the LIVE/DEAD *BacLight* bacterial viability kit (Life Technologies, Thermo Fisher, NZ) was used to label living cells green (SYTO9, membrane permeable stain) and dead cells red (propidium iodide, a non-membrane permeable stain). Six-well plates (tissue treated, Greiner, Germany) were filled with 2.97 ml MWB and inoculated with 30 µl of an overnight bacterial culture grown in TSBYE at 37°C before being incubated at 30°C for 48 h. The biofilms were then washed twice with 0.8% NaCl to remove loosely attached cells and stained with 1 ml fluorescent stain prepared according to the manufacturer's manual. A fluorescence microscope (Olympus, BX51 fitted with the XC30 digital camera) was used at 100× magnification to capture images.

Measuring growth of *L. monocytogenes* 15G01 and the 15G01 *mprF*::Himar1 mutant in the presence of antimicrobial peptide

Resistance of bacterial strains to the cationic gallidermin (ChemCruz – Santa Cruz Biotechnology Inc.,TX) was assessed as described previously by Thedieck et al. (2006) , with some modifications. Briefly, cultures of bacteria were grown overnight in BHI broth at 37°C and then washed and re-suspended in M9 minimal medium (<http://cshprotocols.cshlp.org/content/2010/8/pdb.rec12295>) to a final concentration of 10³ colony forming units (CFU) ml⁻¹. Thirty microliters of each resulting bacterial suspension was plated in triplicate on BHI agar plates containing increasing concentrations of gallidermin (0 – 3.5 µg ml⁻¹). The number of colonies for each strain growing on the inoculated BHI plates was enumerated after incubation at 37°C for 48 h. Colony counts on agar plates were analysed with a Poisson generalized linear model (GLM) with a logarithmic link (McCullagh and Nelder 1989) and the predicted mean CFU and associated 95% confidence limits were obtained on the log. scale within analyses. The analysis was carried out with

Genstat (Payne et al. 2017). A similar experiment in liquid culture, was also run as indicated by Thedieck et al. (2006).

Invasion assays

The invasiveness of *L. monocytogenes* 15G01 and the 15G01 *mprF*::Himar1 mutant were compared using a Caco-2 intestinal cell line (Gaillard et al. 1987). Experiments were conducted as previously described by Cruz et al., (2014). The invasion efficiency was calculated as the percentage of the initial inoculum recovered from a Caco-2 monolayer. The relative invasion efficiency was subsequently calculated by comparison of the invasion efficiency for each strain to that of the wild-type 15G01 (Nowak et al. 2015). Three independent assays were performed using each mutant, with each assay carried out in duplicate. For statistical analysis of the invasion data, the logarithms of % invasion were analysed to stabilise the variance. An ANOVA model was fitted with fixed effects for experiment and strain, and a random effect/block term for experiment x strain. The invasion efficiency was compared to that of *L. monocytogenes* Scott A (American Type Culture Collection 49594™), which has been widely used in virulence studies (Lammerding et al. 1992).

Results

An *in vitro* screen of a *L. monocytogenes* 15G01 transposant library identifies mutants with altered biofilm formation when compared to the wild-type.

A library of *L. monocytogenes* 15G01 mutants was constructed using the Himar-1 mariner transposon delivery system. The number of mutants needed was calculated for a 90% probability to hit a gene of interest (Russell and Sambrook 2001). The library had a plasmid retention rate of less than 1.5%, as calculated according to Cao et al. (2007). An initial screen of 2,000 mutants from this library using an *in vitro* biofilm plate assay, identified three mutants with significantly altered biofilm formation compared

with the wild-type when grown in both TSBYE and MWB (with at least 2 standard deviations above or below wild-type strain) (Table 2).

The transposon insertion site in the genome of each mutant was located by semi-arbitrary PCR of the chromosomal regions immediately flanking the transposon (Figure 1). In transposant 15G01 *mprF*::Himar1 (6B4), the transposon has inserted into the 3' end of a gene encoding a putative phosphatidylglycerol lysyltransferase known as MprF, which is a multi-peptide resistance factor produced by many Gram-positive bacteria that confers resistance to cationic antimicrobial peptides (Bao et al. 2012; Peschel et al. 2001). MprF is also involved in virulence of *L. monocytogenes* and other bacteria (Mandin et al. 2005; Bao et al. 2012). Mutant 6B4 was renamed 15G01 *mprF*::Himar1. The other two mutants (21B6 and 12F11) had the insertion in the stop codon of the *pta* gene, which encodes a phosphate acetyl transferase, and in the promoter region of a gene encoding the protein translocase subunit SecA2, respectively. Transposant 21B6 and 12F11 were renamed 15G01 *pta*::Himar1 and 15G01 *secA2*::Himar1, respectively. The three insertion sites were mapped onto the genome of *L. monocytogenes* 15G01 to show their relative positions (Figure 1B), and they were found to be located distantly from one another.

The mutant 15G01 *mprF*::Himar1 was selected for further analysis to assess its involvement in biofilm formation and to investigate a potential connection between CAMP (cationic antimicrobial peptide) resistance and biofilm formation.

The planktonic growth, the hydrophobicity and the surface charge of the 15G01 *mprF*::Himar1 mutant are not affected by the transposon insertion.

Hydrophobicity, surface charge and growth of planktonic cultures of the wild-type strain and the 15G01 *mprF*::Himar1 were assessed to identify a potential link with biofilm formation. Planktonic growth of the 15G01 *mprF*::Himar1 mutant was similar ($p > 0.05$) to that of the wild-type when grown for 50 h in MWB, for three of the four parameters analysed. The mutant and wild-type differed significantly in the mid-point of the rise (M) ($p = 0.005$), which means that statistically the growth for the 15G01 *mprF*::Himar1 mutant

was slower when compared with the wild-type, albeit only slightly. These data suggest that the greater biofilm biomass produced by the 15G01 *mprF*::Himar1 mutant in the initial CV screen was not a result of a large difference in the planktonic growth of the mutant (Supplementary Figure S1).

The MATH-test and HIC tests, which both determine hydrophobicity, as well as the cytochrome C assay that measures surface charge of the bacterial cells were used to determine whether there were differences in the surface characteristics of the 15G01 *mprF*::Himar1 mutant and the wild-type strain that might correlate with the differences in biofilm formation. There were no statistically significant differences in hydrophobicity or surface charge of planktonic cells when the mutant was compared to the wild-type (Supplementary Table S1).

The viability of the 15G01 *mprF*::Himar1 mutant in biofilms is not affected, but metabolic activity is greater and production of eDNA and carbohydrate is lower than the wild-type.

Cell enumeration, applied to calculate the quantity of viable cells in the biofilm, and metabolic activity, as measured using the XTT assay, showed a significant correlation with the greater biofilm levels of the 15G01 *mprF*::Himar1 mutant relative to the wild-type; cell counts were 6.99 (SD 0.16) log₁₀ CFU/well compared to 7.39 (SD 0.07) ($p < 0.001$), and metabolic activity was 0.08 (SD 0.02) OD_{450nm} compared to 0.22 (SD 0.08) ($p < 0.001$) in the wild-type and the 15G01 *mprF*::Himar1 mutant, respectively (Table 3). In contrast, eDNA content in the biofilm produced by the 15G01 *mprF*::Himar1 mutant (4.87 ng, SD1.29) was lower than that in the wild-type (6.55 ng, SD 0.29) ($p = 0.096$) when adjusted per biofilm unit, as was the eDNA content of the supernatant ($p < 0.001$). The carbohydrate amount in the biofilm produced by the 15G01 *mprF*::Himar1 mutant was also significantly lower ($p = 0.022$) than in the wild-type strain.

Inactivation of *mprF* alters biofilm formation on stainless steel surfaces covered with mussel juice.

Further characterisation of the biofilm produced by 15G01 *mprF*::Himar1 using SEM confirmed that inactivation of *mprF* results in greater production of biofilm mass than observed in the wild-type on stainless steel surfaces contaminated with mussel juice, i.e. the mutant produced densely packed cells on the organic matter contaminating the stainless steel coupons after 7 days at 30°C (Figure 2). A live/dead stain also confirmed that the majority of the cells in the biofilms were viable (Figure 3).

Inactivation of *mprF* results in increased sensitivity to cationic peptides such as gallidermin

The minimal inhibitory concentration (MIC) of cationic antimicrobial peptides was decreased upon inactivation of *mprF* in *L. monocytogenes* EGD-e (Thedieck et al. 2006). To confirm that *mprF* also plays a role in antimicrobial peptide sensitivity of the *L. monocytogenes* 15G01, growth of the 15G01 *mprF*::Himar1 mutant and the wild-type was compared in the presence of increasing concentrations of gallidermin. The effect of gallidermin on solid media showed the transposant being clearly more susceptible to gallidermin than the wild-type (Figure 4 and Supplementary Figure S2). The growth of 15G01 *mprF*::Himar1 was completely inhibited at 1 µg ml⁻¹ while the wild-type strain was able to grow at 3.5 µg ml⁻¹. Genetic complementation using the integrative vector pIMK carrying a wild-type copy of *mprF* resulted in partial restoration of the wild-type gallidermin sensitivity. A plot showing the behaviour of the three isogenic strains to the antimicrobial effect of gallidermin is presented in Figure 4 and photographs of the cultures in agar plates are shown in Supplemental Figure S2. Experiments in liquid media presented similar results with inhibition of the 15G01 *mprF*::Himar1 mutant growth at 1 µg ml⁻¹ gallidermin in four-fold BHI broth whereas the wild-type strain was able to grow at concentrations greater than 3.5 µg ml⁻¹ gallidermin (data not shown).

The 15G01 *mprF*::Himar1 mutant is less invasive than *L. monocytogenes* 15G01 in Caco-2 cell line

Invasion assays were performed using a Caco-2 cell line to compare the invasiveness of the 15G01 *mprF*::Himar1 mutant (and other mutants) to that of *L. monocytogenes* 15G01. These assays confirmed the 15G01 *mprF*::Himar1 mutant was significantly impaired in its capacity to invade this cell line. Indeed,

15G01 *mprF*::Himar1 had a mean relative invasion efficiency of 0.10 (SD 0.14) compared to that of the wild-type of 0.43 (SD 0.18). All 15G01 strains had a significantly lower invasion efficiency ($p < 0.001$) in Caco-2 cells when compared to the well characterised reference strain ScottA (5.8 (SD 2.6)).

Discussion

In this study we have identified *L. monocytogenes* genes to be involved in biofilm formation, and have shown for the *mprF* mutant that the biofilm phenotype is also associated with increased susceptibility to antimicrobial peptides and decreased levels of invasion in intestinal epithelial cells. The *mprF* mutant produced a biofilm in both MWB and TSBYE at 30°C, suggesting a nutrient-independent effect on biofilm formation. *mprF* encodes a lysylphosphatidylglycerol synthetase that uses phosphatidylglycerol as a substrate and forms lysyl-phosphatidylglycerol and diphosphatidylglycerol which are both major components of the cell wall (Thedieck et al. 2006). Studies have not only shown that reduced lysinylation decreases cationic antimicrobial peptide (CAMP)-resistance (Thedieck et al. 2006) but have also shown that gene disruption of *mprF* reduces the negative charge of the membrane (Ernst et al. 2009) due to a decreased amount of positively charged lysyl residues on the surface. It can therefore alter phenotypic behaviour and potentially the ability to produce a biofilm.

Biofilm formation of the wild-type and the 15G01 *mprF*::Himar1 mutant was assessed and compared by measuring total biofilm including live and dead cells as well as extrapolymeric substance (CV-assay), viable cell numbers (CFU) and viable but non-culturable (VBNC) cells (XTT-assay). Biofilm production as measured by the CV-assay, the XTT-assay and through cell enumeration, correlated significantly with each other, showing that increased biofilm biomass is accompanied by higher numbers of viable cells. Hence, disruption of *mprF* does not seem to influence cell viability.

The establishment of a biofilm is dependent on several environmental factors, which include, but are not limited to, the surface charge and hydrophobicity of the cell and the attachment site (Chmielewski

and Frank 2003). We compared hydrophobicity and surface charge of the wild-type and the 15G01 *mprF*::Himar1 mutant at 30°C and did not observe any correlation between surface properties and biofilm formation, which is in line with other findings (Chae et al. 2006; Poimenidou et al. 2016). Hydrophobicity increased with increasing temperature for 44 *L. monocytogenes* strains with a positive correlation between biofilm formation on glass and hydrophobicity at 12°C, but not for the other temperatures tested (Di Bonaventura et al. 2008). This highlights the importance of environmental conditions influencing biofilm formation.

Extracellular DNA (eDNA) is believed to serve as a key matrix component of microbial biofilms of *L. monocytogenes* (Harmsen et al. 2010; Bao et al. 2012). However, the mechanism of eDNA release remains elusive. It has been postulated that it is either released by active secretion (Liao et al. 2014) or by autolysis (Qin et al. 2007). In our study, eDNA measurements for the wild-type and 15G01 *mprF*::Himar1 were carried out for biofilms grown in a nutrient limited environment (MWB), which is known to induce the stress response of bacteria. DNase was able to disrupt *L. monocytogenes* biofilms grown in nutrient-limiting media, whereas no effect was observed in full strength media (Zetzmann et al. 2015). eDNA was found to be present in the matrix of biofilms grown in diluted media, but not in full strength media. Hence, nutrient limitation / stress might result in release of eDNA by cell death/lysis. The lower amounts of eDNA for the 15G01 *mprF*::Himar1 mutant may be linked to the changes in the membrane composition (due to altered production of lys-PG) and hindrance of eDNA release, but this will need further investigation.

The *mprF* transposant showed reduced resistance to gallidermin and other antimicrobials (Kang et al. 2015), despite no significant difference in surface charge as measured by the Cytochrome C assay. Furthermore, only partial antimicrobial resistance was achieved after successful complementation of the mutant 6B4 (15G01 *mprF*::Himar1) (Figure 4), which suggests its involvement in antimicrobial resistance, but also raises the question on whether proximity to other regulatory units is required for full restoration of the phenotype. However, another study was able to fully restore gallidermin resistance in *S. aureus* upon complementation with *mprF* (Ernst et al. 2009). Whether or not there are interspecies differences in function and regulation of *mprF* has yet to be determined.

The efficacy of certain antimicrobials is often reduced once the biofilm is formed, and sub-inhibitory concentrations of antimicrobials even increase biofilm formation (Ferrer et al. 2017). Our studies showed that the growth of the wild-type strain was not affected at sub-inhibitory gallidermin concentrations between 0–1.5 $\mu\text{g ml}^{-1}$. The 15G01 *mprF*::Himar1 mutant showed growth inhibition at a concentration of 1 $\mu\text{g ml}^{-1}$ gallidermin, while the wild-type was practically totally inhibited by 3.5 $\mu\text{g ml}^{-1}$ gallidermin in both solid and liquid media. Complementation partially restored resistance to gallidermin as growth was detected at gallidermin concentration of up to 2 $\mu\text{g ml}^{-1}$. Biofilm production is usually associated with *in vivo* virulence models. This phenomenon is directly linked to an increase in colonizing properties, antibiotic resistance and evasion of immune host system (Bao et al. 2012). In our previous study *L. monocytogenes* 15G01 was identified as the highest biofilm former of the tested isolates of New Zealand origin, and here we show that the *mprF* mutant shows further increased biofilm production (Nowak et al. 2017). The invasion assays confirmed the involvement of *mprF* in virulence (24.22% invasion by the mutant compared to the wild-type 15G01). The increased biofilm formation (i.e. increased cell numbers) by the mutant did not correspond to increased adhesion followed by invasion of eukaryotic Caco-2 cells. Further virulence assays using a mouse bacteraemia model are needed to confirm the pathogenicity effect of the isogenic strain 15G01 *mprF*::Himar1.

In conclusion, in this study we have identified three *L. monocytogenes* genes that are involved in biofilm formation, and for the *mprF* gene we have shown a link with antimicrobial peptide resistance and virulence properties, further supporting the view that phenotypes associated with environmental survival and virulence require tight control in *L. monocytogenes*. Finally, the insights gained in this study will be useful in developing future strategies to manage *L. monocytogenes* in food processing environments.

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Authors declare that there are no conflicts of interest, and that the research does not involve human participants and / or animals.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. Gene and genome context for the transposon insertion sites associated with three transposants (6B4, 12F11 and 21B6) of *Listeria monocytogenes* 15G01, which were generated using the Himar1 mariner-based transposition system and showed altered biofilm formation in a crystal violet assay. A) Coordinates of the transposon insertions and locus tag of genes hit by the transposon in *L. monocytogenes* 15G01. B) Representation of the genome of *Listeria monocytogenes* 15G01 showing the three transposon insertion sites (red bars). The outer ring represents the scale in bp, protein coding sequences are shown in turquoise and the inner ring (purple/green) shows the G+C% content plot with the GC skew. The image was generated using DNAPlotter available from www.sanger.ac.uk.

Figure 2. Scanning electron micrographs showing the biofilms produced by *L. monocytogenes* 15G01 (a and b) and a 15G01 *mprF*::Himar1 mutant (6B4) (c and d) on stainless steel coupons covered with mussel juice after 7 d incubation at 30°C as well as non-inoculated coupons covered with mussel juice (e and f). Micrographs were taken using a magnification of 5,000× (a and c), 10,000× (b and d) and 3,000× and 6,000× (e and f), respectively.

Figure 3. Fluorescence micrographs showing the viability of cells in the biofilms produced by *L. monocytogenes* 15G01 (a and b) and the 15G01 *mprF*::Himar1 mutant 6B4 (c and d) grown on polystyrene surfaces in MWB for 48 h at 30°C. The biofilms were stained with the LIVE/DEAD BacLight bacterial viability kit according to the manufacturer's instructions (Life Technologies, Thermo Fisher, New Zealand). Living cells were labelled with SYTO9 (green) and dead cells with propidium iodide (red). Scale bars represent 20 µm.

Figure 4. The effect of inactivation of *mprF* on the sensitivity of *L. monocytogenes* 15G01 to the cationic antimicrobial peptide gallidermin. The graph shows the percentage survival of *L. monocytogenes* 15G01, the 15G01 *mprF*::Himar1 mutant 6B4 and the 15G01 *mprF*::Himar1 mutant complemented using pIMK*mprF* after growth for 48 h in BHI agar containing increasing concentrations of gallidermin (0-3.5 µg ml⁻¹) at 37°C. Mean values of colony forming unit (CFU) counts were calculated from three experimental replicates and are expressed as a percentage of the titre in cultures without gallidermin. Error bars are 95% confidence limits. Note that the upper error for a mean of zero is difficult to obtain, and so is not shown.

Supplementary Figure legends (Online publishing)

Supplementary Figure S1. A. Growth curves for *L. monocytogenes* 15G01 (●) and the 15G01 *mprF*::Himar1 mutant (6B4, ●) cultured in MWB at 30C° for 50 h, as measured by optical density at a wavelength of 600 nm (OD₆₀₀). The growth curves displayed are the means of two experiments with n=1. **B.** Results of the statistical analysis of the growth curves of 15G01 and the 15G01 *mprF*::Himar1 mutant (6B4) obtained in two independent experiments analysed using the FITCURVE command in Genstat (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) to estimate the four parameters of a logistic growth curve (A=left hand plateau, B=measure of steepness, M=mid-point of the rise, C=amount of climb). Both the strains and the strain-replicate combinations differed significantly ($p < 0.001$ on 4 and 136 df for strain differences, $p < 0.001$ on 8 and 136 df for rep differences within strains). By comparing the sums of squares between the analyses, it appeared the replicates within each strain differed significantly. To see if there were any consistent differences between strains, allowing for the variability of the replicates, the parameters for the four strain-replicate combinations were tested using a t-test to see if one strain tended to have higher values than the other. The only significant difference ($p=0.005$) was for the M parameter; the growth curves had similar shape, but 15G01 *mprF*::Himar1 (6B4) was delayed by about 2 hours compared to 15G01.

Supplementary Figure S2. Photographs showing the effect of inactivating *mprF* on the sensitivity of *L. monocytogenes* 15G01 to the cationic antimicrobial peptide gallidermin. The photographs show the growth of colonies of *L. monocytogenes* 15G01, the 15G01 *mprF*::Himar1 mutant (6B4) and the 15G01 *mprF*::Himar1 mutant complemented using pIMK*mprF* after growth for 48 h on Brain Heart Infusion agar containing increasing concentrations of gallidermin (0-3.5 µg ml⁻¹). No growth of *mprF*::Himar1 mutant (6B4) was observed at 1 µg ml⁻¹ while the wild-type produced a few colonies even at 3.5 µg ml⁻¹. Complementation was partial, with the 15G01 *mprF*::Himar1 mutant complemented using pIMK*mprF* showing inhibition at 3.5 µg ml⁻¹.

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Genotype or phenotype	Source
<i>Listeria monocytogenes</i>		
15G01	Wild Type, pulsotype 5132	(Cruz and Fletcher 2011)
15G01 <i>mprF</i> ::Himar1 (6B4)	15G01 mutant 6B4 with Himar1 mariner-based transposon inserted in the <i>mprF</i> gene; Ery ^R , Kan ^S	This study
15G01 <i>mprF</i> ::pIMK	15G01 <i>mprF</i> ::Himar1 with pIMK integration; Ery ^R , Kan ^R	This study
15G01 <i>mprF</i> ::pIMK <i>mprF</i>	15G01 <i>mprF</i> ::Himar1 complemented with the <i>mprF</i> gene using plasmid pIMK <i>mprF</i> ; Em ^R , Kan ^R	This study
15G01 <i>secA2</i> ::Himar1 (12F11)	15G01 mutant 12F11 with Himar1 mariner-based transposon inserted in the intergenic region of <i>secA2</i> ; Ery ^R , Kan ^S	This study
15G01 <i>pta</i> ::Himar1 (21B6)	15G01 mutant 21B6 with Himar1 mariner-based transposon inserted in the <i>pta</i> gene; Ery ^R , Kan ^S	This study
<i>Escherichia coli</i>		
S17-1λpir	Cloning host strain	(de Lorenzo et al. 1990)
S17-1λpir::pIMK	S17-1λpir harbouring pIMK	This study
S17-1λpir::pIMK <i>mprF</i>	S17-1λpir harbouring pIMK <i>mprF</i>	This study
Plasmids		
pMC38	Mariner-based transposon delivery plasmid; Ery ^R	(Cao et al. 2007)
pIMK	Site-specific integrative vector used in <i>Listeria</i> , 5.1 kb, Kan ^R	[9]
pIMK <i>mprF</i>	pIMK carrying the wild-type <i>mprF</i> gene from 15G01	(Bahey-El-Din et al. 2008)

Table 2. Biofilm formation by *Listeria monocytogenes* 15G01 (wild-type) and three transposon mutants in TSBYE and MWB media after 48 h incubation at 30°C.

			Wild-type 15G01	15G01 <i>mprF</i> ::Himar1 (6B4)	15G01 <i>pta</i> ::Himar1 (21B6)	15G01 <i>secA2</i> ::Himar1 (12F11)
Biofilm formation* [OD _{595nm}]	TSBYE	mean	0.376	0.469	0.986	0.035
		SD	0.049	0.082	0.174	0.002
	MWB	mean	0.916	1.541	1.324	0.311
		SD	0.186	0.074	0.027	0.005

*Biofilm formation for each strain was measured in two independent experiments, each with 6 replicates, and the mean biofilm mass and the standard deviation (SD) were calculated.

Table 3. Viability, composition and metabolic activity of the biofilms produced by *Listeria monocytogenes* 15G01 and the 15G01 *mprF*::Himar1 mutant 6B4 after growth in MWB medium for 48 h at 30°C.

Biofilm trait measured	15G01	15G01 <i>mprF</i> ::Himar1
Cell enumeration [$\log_{10}$ CFU/well]	6.994 (0.160)	7.391 (0.067)
Metabolic activity [OD_{450nm}]*	0.079 (0.018)	0.217 (0.084)
eDNA in biofilm [ng/ OD_{595nm}]**	6.550 (0.289)	4.873 (1.294)
eDNA in supernatant [μ g/ OD_{595nm}]**	1.237 (0.085)	0.534 (0.062)
Biofilm carbohydrate content [μ g/ OD_{595nm}]**	7.835 (0.956)	5.186 (0.822)

*mean values for two experiments each with 5 to 6 technical replicates. **mean values for three experiments each with n=1. The standard deviation for each measurement is shown in brackets underneath the respective mean.

A.

B.

Figure 1. Gene and genome context for the transposon insertion sites associated with three transposants (6B4, 12F11 and 21B6) of *Listeria monocytogenes* 15G01, which were generated using the Himar1 mariner-based transposition system and showed altered biofilm formation in a crystal violet assay. A) Coordinates of the transposon insertions and locus tag of genes hit by the transposon in *L. monocytogenes* 15G01. B) Representation of the genome of *Listeria monocytogenes* 15G01 showing the three transposon insertion sites (red bars). The outer ring represents the scale in bp, protein coding sequences are shown

in turquoise and the inner ring (purple/green) shows the G+C% content plot with the GC skew. The image was generated using DNAPlotter available from www.sanger.ac.uk.

Figure 2

Figure 3

Figure 4. The effect of inactivation of *mprF* on the sensitivity of *L. monocytogenes* 15G01 to the cationic antimicrobial peptide gallidermin. The graph shows the percentage survival of *L. monocytogenes* 15G01 (★), the 15G01 *mprF*::Himar1 mutant 6B4 (●) and the 15G01 *mprF*::Himar1 mutant complemented using pIMK*mprF* (▲) after growth for 48 h in BHI agar containing increasing concentrations of gallidermin (0-3.5 $\mu\text{g}\cdot\text{mL}^{-1}$) at 37°C. Mean values of colony forming unit (CFU) counts were calculated from three experimental replicates and are expressed as a percentage of the titre in cultures without gallidermin. Error bars are 95% confidence limits. Note that the upper error for a mean of zero is difficult to obtain, and so is not shown.

A

B

	Experiment	A (left hand plateau)		B (measure of steepness)		M (mid-point of the rise)		C (amount of climb)	
		Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
15G01	1	0.123	0.012	0.360	0.014	12.91	0.12	1.158	0.015
	2	0.111	0.013	0.351	0.014	12.66	0.13	1.112	0.015
6B4	1	0.163	0.009	0.446	0.018	14.98	0.10	1.075	0.011
	2	0.146	0.010	0.367	0.013	14.84	0.11	1.192	0.013
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
15G01		0.117	0.008	0.356	0.006	12.79	0.18	1.135	0.033
6B4		0.155	0.012	0.407	0.056	14.91	0.10	1.134	0.083
P from t-test		0.069		0.328		0.005		0.983	

Supplementary Figure S1. A. Growth curves for *L. monocytogenes* 15G01 (●) and the 15G01 *mprF*::Himar1 mutant (6B4, ●) cultured in MWB at 30°C for 50 h, as measured by optical density at a wavelength of 600 nm (OD₆₀₀). The growth curves displayed are the means of two experiments with n=1. **B.** Results of the statistical analysis of the growth curves of 15G01 and the 15G01 *mprF*::Himar1 mutant (6B4) obtained in two independent experiments analysed using the FITCURVE command in Genstat (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) to estimate the four parameters of a logistic growth curve (A=left hand plateau, B=measure of steepness, M=mid-point of the rise, C=amount of climb). Both the strains and the strain-replicate combinations differed significantly ($p <$

0.001 on 4 and 136 df for strain differences, $p < 0.001$ on 8 and 136 df for rep differences within strains). By comparing the sums of squares between the analyses, it appeared the replicates within each strain differed significantly. To see if there were any consistent differences between strains, allowing for the variability of the replicates, the parameters for the four strain-replicate combinations were tested using a t-test to see if one strain tended to have higher values than the other. The only significant difference ($p=0.005$) was for the M parameter; the growth curves had similar shape, but 15G01 *mprF*::Himar1 (6B4) was delayed by about 2 hours compared to 15G01.

Wt 15G01: plates containing gallidermin from 0 to 3.5ug/ml

Mutant 6B4: plates containing gallidermin from 0 to 3.5 ug/ml

Complemented 6B4: plates containing gallidermin from 0 to 3.5ug/ml

Supplementary Figure S2. Photographs showing the effect of inactivating *mprF* on the sensitivity of *L. monocytogenes* 15G01 to the cationic antimicrobial peptide gallidermin. The photographs show the growth of colonies of *L. monocytogenes* 15G01, the 15G01 *mprF*::Himar1 mutant (6B4) and the 15G01 *mprF*::Himar1 mutant complemented using pMK*mprF* after growth for 48 h on Brain Heart Infusion agar containing increasing concentrations of gallidermin (0-3.5 $\mu\text{g ml}^{-1}$). No growth of *mprF*::Himar1 mutant (6B4) was observed at 1 $\mu\text{g ml}^{-1}$ while the wild-type produced a few colonies even at 3.5 $\mu\text{g ml}^{-1}$. Complementation was partial, with the 15G01 *mprF*::Himar1 mutant complemented using pMK*mprF* showing inhibition at 3.5 $\mu\text{g ml}^{-1}$.

Supplementary Table S1. Surface properties of planktonic cultures *Listeria monocytogenes* 15G01 and an 15G01 *mprF*::Himar1 (6B4).

Trait measured in planktonic cells	15G01	15G01 <i>mprF</i> ::Himar1
MATH test for hydrophobicity [%]	18.96 (6.49)	12.37 (5.02)
Hydrophobic interaction chromatography test [%]	43.79 (5.08)	47.96 (3.31)
Cytochrome C assay [bound cytochrome C [%]]	29.39 (3.21)	33.26 (1.62)

*The MATH and hydrophobicity interaction chromatography assays were performed three times (n=3), whereas the cytochrome C assay was performed twice (n=2). Standard deviation is shown in brackets adjacent to the respective mean.