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Genomic differences between *Listeria monocytogenes* EGDe isolates reveals crucial roles for SigB and wall rhamnosylation in biofilm formation.

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Summary

Listeria monocytogenes is a Gram-positive Firmicute that causes food-borne infections, in part due to its ability to use multiple strategies, including biofilm formation, to survive adverse growth conditions. As a potential way to screen for genes required for biofilm formation we harnessed the ability of bacteria to accumulate mutations in the genome over time, diverging the properties of seemingly identical strains. By sequencing the genomes of four laboratory reference strains of the commonly used *L. monocytogenes* EGDe, we showed that each isolate contains SNPs compared with the reference genome. We discovered that two SNPs, contained in two independent genes within one of the isolates, impacted biofilm formation. Using bacterial genetics and phenotypic assays, we confirmed that *rsbU* and *rmlA* influence biofilm formation. RsbU is the upstream regulator of the alternative sigma factor, SigB and mutation of either *rsbU* or *sigB* increased biofilm formation. In contrast, deletion of *rmlA*, which encodes the first enzyme for TDP-L-rhamnose biosynthesis, resulted in a reduction in the amount of biofilm formed. Further analysis of biofilm formation in a strain that still produces TDP-L-rhamnose, but which cannot decorate the wall teichoic acid with rhamnose (*rmlT* mutant), showed that it is the decorated wall teichoic acid that is required for adhesion of the cells to surfaces. Together these data uncover novel routes by which biofilm formation by *L. monocytogenes* can be impacted.

Importance

Biofilms are an important mode of growth in many settings. Here we have looked at small differences in the genomes of the bacteria *Listeria monocytogenes* isolate EGDe and used them to find out how biofilms form. This is important fundamental information may help new treatments to be developed and also highlights the fact that isolates of the same identity often diverge.

Introduction

Biofilms are complex communities of microbial cells that are encased within a self-produced extracellular matrix. The biofilm matrix provides protection from environmental insults, increasing the tolerance of cells to antimicrobial agents and biocides (1). *Listeria monocytogenes* is a Gram-positive bacterium that causes the food-borne infection listeriosis. In susceptible individuals (e.g. people who are immunocompromised) the mortality rate of Listeria infections has been estimated to be up to 30%. Biofilms of *L. monocytogenes* can form on machinery in food processing plants, contributing to food contamination (2) and potentially leading to the closure of manufacturing facilities for deep clean processes (3). Thus, routes to inhibit or disrupt biofilm formation by *L. monocytogenes* could present one means of reducing Listeria infections. It is currently known that biofilm formation by *L. monocytogenes* is dependent on an active flagellum (4). Moreover two major transcription factors, SigB and PrfA, and the virulence factor, ActA have been shown to contribute to biofilms formation (5-7). However there are still many unanswered questions regarding the molecular processes underpinning *L. monocytogenes* biofilm formation.

Reference strains of bacteria are widely used in laboratories as research models for the study of bacterial behaviour and physiology (8). However, mutations can be inadvertently introduced into the genome during routine culture, modifying the strains derived from the designated laboratory reference strain (9). Diverging mutations within laboratory reference strains can contribute to differences in observed phenotypic behaviour between different research groups. For example, a *B. subtilis* laboratory reference strain 168 was identified as a non-rugose biofilm forming strain (10); however, it has been shown that some variants can form biofilms (11). By sequencing a collection of 12 sublines of 168, it was revealed that the *epsC* gene, which is essential for biofilm, carried point mutations in the non-rugose biofilm isolates. *L. monocytogenes* EGDe, serovar 1/2a, is widely used for molecular and cellular studies as the model organism (12) and we chose to use this isolate in our studies. We predicted that if we were able to identity genomic variations between *L. monocytogenes* isolates used by different laboratories this could potentially shed light on the underlying genetics of biofilm formation. Using a comparative sequencing approach we identified and connected genomic variations in *L. monocytogenes* EGDe isolates with differences in biofilm formation. More specifically, our bioinformatic analysis and experimental approaches revealed two genes, *rsbU* and *rmlA*, involved in biofilm formation. This work contributes to our understanding of biofilm formation by an important human pathogen.

Materials and Methods

Growth media and additives

Brain-Heart Infusion medium (BHI, 237500, BD Biosciences) was used for propagating *L. monocytogenes* strains. Strains were routinely grown either in liquid BHI, on BHI solidified with 1.5% (w/v) select agar, or in liquid Modified Welshimer's Broth (MWB) (6.56 g/L KH_2PO_4, 16.39 g/L Na_2HPO_4, 0.41 g/L $MgSO_4 \cdot 7H_2O$, 10 g/L glucose, 0.088 g/L ferric citrate, 0.1 g/L leucine, 0.1 g/L isoleucine, 0.1 g/L valine, 0.1 g/L methionine, 0.1 g/L arginine, 0.1 g/L cysteine, 0.6 g/L glutamine, 0.5 mg/L riboflavin, 1.0 mg/L thiamine, 0.5 mg/L biotin, and 0.005 mg/L lipoic acid). Starter cultures were prepared by inoculating a single colony of *L. monocytogenes* grown on BHI-agar into 5 ml of BHI, which was grown with shaking. The growth medium was supplemented with selective antibiotics (100 µg/ml ampicillin (Amp), 5 µg/ml erythromycin (Ery), or 50 µg/ml X-gal (5-Bromo-4-Chloro-3-Indolyl-D-Galactopyranoside)) during cloning and constructing mutant strains as required.

Strains, plasmids and primers

Complete details of the strains, plasmids and primers used in this study are provided in Table S1-3.

Growth Measurement

To follow the growth of *L. monocytogenes* strains starter cultures were grown at 37°C for ~20 h and inoculated into 100 ml of BHI at a starting OD_{600} of 0.05. The cultures were incubated in a water bath with shaking at 200 rpm and the OD_{600} measured every hour. Alternatively, growth over time was monitored using a plate reader (Synergy 2, BioTek Instruments). The starting cultures were subcultured into MWB at an initial OD_{600} of 0.01 in 200 µl per well in a round bottom polystyrene 96-well plate.The OD_{600} was measured every hour during incubation at 30°C for 48 h without shaking.

Motility

Semi-solid (0.3% w/v) agar was prepared in BHI or MWB media. Starter cultures for each strain were grown at 30ºC for up to 48 hours. To seed the strains, the OD_{600} of starting cultures was normalised to 1.0, and 1 µl of the normalised culture was stabbed into the centre of a semi-solid agar plate. A negative control, the non-motile strain EGDe Δ*flaA* (13), was included. The seeded semi-solid agar plates were incubated at 30°C and after 24 and 48 h of incubation images were captured using a DSLR camera (Nikon D3200 with Nikkor 18-55mm

lens). Quantification of motility was performed by measuring the diameter of the zone occupied by the cells. For each sample, the diameters of the swarm were measured at two positions. The average of the two values was used for further statistical analysis.

Chitinase Activity

Chitinase activity was tested as described previously (14). Starter cultures were grown at 37°C for ~20 h. The cultures of the strains were normalised to an OD_{600} of 1.0 and 10 µl was spotted onto an LB agar plate supplemented with colloidal chitin at a final concentration of 2% (w/v). The plates were then incubated at 30°C for 24, and 48 h prior to imaging using a DSLR camera (Nikon D3200 with Nikkor 18-55mm lens).

Cell Wall Teichoic Acid Analysis

Extraction of the cell wall teichoic acids from *L. monocytogenes* was performed as described (15). Starter cultures were grown at 37°C for ~8 h and inoculated into 50 ml of MWB at an initial OD_{600} of 0.01, which was incubated at 30°C for ~17 h with shaking at 200 rpm. The cells were harvested by centrifugation at 3800×g for 10 min. The cell pellet was washed with 20 ml of MES buffer (50 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5) and centrifuged at 3800×g for 10 min. The cell pellet was resuspended in 1 ml of MES buffer supplemented with 4% (w/v) SDS and boiled at 99°C for 1 h. The SDS-treated cells were harvested by centrifugation at 17000×g for 10 min. The cell pellets were washed with MES buffer containing 2% (w/v) NaCl twice, rinsed with MES buffer and resuspended in 1 ml of MES buffer with 0.4 g acid-washed glass beads (≤106 µm, cat# G4649-500G, Sigma-Aldrich) per sample. The cells were lysed by vortexing at the highest speed for 10 min with the tube lying horizontally. The glass beads were discarded after centrifugation at 1000×g for 5 min, and the cell lysate was harvested for the following steps. The proteins in the samples were digested with 20 µg/ml proteinase K (03508811103, Roche) in 20 mM Tris-HCl pH8.0 at 50°C for 2 h. After centrifuging at 17000×g for 10 min, the pellet was treated with 1 ml of 0.1 M NaOH for 17 h with shaking at 1200 rpm, 25°C on Thermomixer R (Eppendorf). The supernatant was harvested by centrifugation at 14000×g for 15 min, and 0.1 ml of 1 M HCl was added to each sample. The liquid was dialysed into milli-Q water using a 1 kDa dialysis membrane (132105, Spectrum). The dialysed samples were dried by SpeedVac (RVC2-25 with CT02-05, Christ). Each sample was resuspended with 100 µl of WTA loading buffer (20 mM tris-HCl, 20 mM tricine, 10% (v/v) glycerol) for further analysis by native polyacrylamide gel electrophoresis. The gel was rinsed with milli-Q water and stained with Alcian blue staining

145 solution (5% (v/v) acetic acid, 30% (v/v) ethanol, and 1 mg/mL alcian blue 8GX) for 1 h. An
146 image of the stained gel was taken after incubation in destaining solution (5% (v/v) acetic
147 acid, 30% (v/v) ethanol) for 20 min.

148 *Biofilm Formation*

149 Starter cultures were grown at 37°C for ~20 h and the OD_{600} normalised to 0.01 in MWB.
150 150 µl of the diluted cultures were subcultured into the Calgary biofilm device (cat# 445497
151 for the lid, and cat# 262162 for the plate, Nunc, Thermo Scientific) and incubated at 30°C for
152 12 to 48 hours. The biomass of the biofilm formed was determined by crystal violet staining.
153 The cultures were discarded by aspiration, each well rinsed three times with 1.2 volume of 1×
154 PBS (8 g/L NaCl, 0.2 g/L KCl, 2.56 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.2 g/L KH_2PO_4, pH 7.4), and cells
155 incubated with 1.3 volume of 0.1% (w/v) crystal violet (diluted from 2.3% solution in milli-Q
156 water, HT901-8FOZ, Sigma-Aldrich) for 1 hr at room temperature. The staining solution was
157 aspirated, and the peg washed with 1.5 volume of 1× PBS three times. The biofilm was
158 destained by incubation with 30% (v/v) acetic acid for 30 min at room temperature. The
159 absorbance of the stained 30% (v/v) acetic acid was measured at a wavelength of 595 nm. For
160 each replicate the A_{595} of a medium only control was used as the background reading.

161 *Scanning Electron Microscopy*

162 Biofilms formed on the pegs of the Calgary biofilm device were fixed for scanning electron
163 microscopy (SEM) largely as described previously (16). The protocol involved two different
164 stages of fixation, critical point drying and sputter coating with platinum prior to final
165 imaging. The biofilm-coated pegs were first rinsed with 1× PBS three times and fixed with
166 200 µl per well of primary fixative for 2 hr at room temperature. The primary fixative
167 comprised 2.5% (v/v) glutaraldehyde, 4% (w/v) paraformaldehyde, 75 mM L-lysine, 0.075%
168 (w/v) alcian blue in 1× PBS. Next, the pegs were removed from the Calgary biofilm device
169 using diagonal pliers. A secondary fixation step was included after a brief wash with 1× PBS.
170 The secondary fixative was composed of 1% (w/v) osmium tetroxide (diluted from 4% stock,
171 75632, Sigma-Aldrich). After 1 h of secondary fixation, the biofilms were treated with a
172 gradient ethanol series (50%, 70%, 90%, and 99.9% (v/v)). The biofilm-coated pegs were
173 transferred into a chamber to be critical point dried. Biofilm-coated pegs were stuck onto a 25
174 mm sample stub (AGG3023, Agar Scientific) with carbon stickers (AGG3303, Agar
175 Scientific) and conductive carbon double-sided tape (AGG3939, Agar Scientific). The sample
176 stub carrying the biofilm-coated pegs was sputter coated with 25 nm thick platinum to create

177 a conductive surface. The biofilms were imaged with field emission SEM (JSM-7400f, Jeol).
178 All images were taken with 5k V detected by lower secondary electron (LEI) detector. The
179 number of cells in each image was counted manually with a cell counter plug-in in ImageJ.

180 *Electrocompetent cells*

181 To insert plasmids into *L. monocytogenes* strains electrocompetent cells were prepared as
182 described previously (17). Plasmid DNA (1 μg) was gently mixed with 50 μl of
183 electrocompetent cells prior to incubation on ice for 10 min. The cells were transferred into a
184 chilled electroporation cuvette (1652089, Bio-Rad) and electroporated at 10k V/cm, 400 Ω,
185 and 25 μF. A recovery medium, 1 ml of 0.5 M sucrose supplemented BHI medium, was
186 gently added to each electroporation reaction. Following incubation at 30°C for 90 min
187 without shaking, 150 μl of the cell suspension was plated onto a BHI agar plate supplemented
188 with antibiotics as required.

189 *Construction of deletion strains*

190 In-frame deletions of protein coding regions on the chromosome were introduced by the
191 pMAD based approach (18). First, the pMAD based plasmid was modified such that it could
192 be used for allelic exchange. Both upstream and downstream regions of the gene to be deleted
193 were either amplified and fused with a *Kpn*I restriction enzyme site using PCR or were
194 synthesized commercially. The modified DNA sequences were first inserted into intermediate
195 cloning vectors, pUC19 or pUC57, prior to ligation into pMAD. The pMAD vector
196 containing the required insert was introduced into the desired parental strain. The recovered
197 cells were spread onto BHI agar plates supplied with 5 μg/ml Ery and 50 μg/ml X-gal and
198 incubated at 30°C for 72 h. The resultant colonies were collected and inoculated into BHI
199 medium containing 5 μg/ml Ery and incubated at 39°C with shaking at 200 rpm for 17 h. The
200 cultures were serially diluted to a factor of 10^{-6} and isolated on 5 μg/ml Ery and 50 μg/ml X-
201 gal supplemented BHI agar plates that were incubated at 39°C for 48 h. Blue coloured
202 colonies were used to inoculate liquid BHI, and the cells incubated at 30°C for 17 h without
203 shaking and then for 4h with shaking at 200 rpm. The cultures were serially diluted to a factor
204 of 10^{-6} and isolated on 50 μg/ml X-gal supplemented Tryptic Soy Agar plates. The plates
205 were incubated at 37°C for 72 h to allow the formation of white colonies. Each white colony
206 was inoculated in 5 ml of BHI medium and incubated at 37°C with shaking at 200 rpm for
207 ~17 h. Deletions were confirmed using PCR and DNA sequencing.

208 *Statistical Analysis*

209 GraphPad Prism 7 was used to generate plots and analyse data. Statistical analysis of the data
210 were performed by one-way ANOVA with Dunnett's multiple comparison tests. * represents
211 $p \leq 0.05$, and ** represents $p \leq 0.01$.

212 *Genome sequencing*

213 Genomic DNA was extracted from starter cultures incubated at 37°C for ~17-20 h. The cells
214 were harvested by centrifugation at 3500×g for 10 min and the cell pellet suspended in 180 µl
215 of enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, pH 8.0, 1.2% (v/v) triton X-100
216 containing 20 mg/ml lysozyme). The cells were lysed at 37°C for 30 min after which the cell
217 lysate was applied to the DNeasy Blood & Tissue Kit (69504, QIAGEN). The final product
218 was eluted in water and stored at -20°C.

219 Illumina next generation sequencing was performed by Genome Sequencing Unit at the
220 Tayside Centre for Genome Analysis. The DNA was quantified using the QuBit 2.0 DNA kit,
221 and 1 µg of DNA was sheared into 300 bp fragments using a Covaris M220 Focused
222 Ultrasonicator. Paired end libraries were generated using the Illumina TruSeq DNA sample
223 preparation guide, and sequenced using the Illumina MiSeq Reagent kit v2 on the Illumina
224 MiSeq platform.

225 *Sequence Analysis*

226 The list of the single nucleotide polymorphisms (SNPs) was acquired by aligning the reads to
227 the published genome (NC_003210). The sequence data were analysed using MiSeq
228 Reporter, and alignment to the reference genome was done using Burrows-Wheeler Aligner
229 (19) and variant calling to identify SNPs was performed using the Genome Analysis Toolkit
230 UnifiedGenotyper (20). Sequence data has been deposited in the European Nucleotide
231 Archive under study accession PRJEB35200 and ERZ1188925.

232 To determine if the A118 prophage was integrated, genome assemblies of strains WT1030
233 and WT1032 were carried out using the BugBuilder (21) pipeline, using SPAdes (22) for
234 contig assembly (version 3.13.1, coverage cutoff=5, kmer size=auto, and 'careful' mode
235 enabled). Scaffolding was carried out with the Mauve Contig Mover (23) (version 2.4.0) with
236 NC_003210.1 as a reference sequence, followed by automated gap closure using Pilon 1.23
237 (24). Annotation of the assembled sequences was carried out using Prokka 1.13.4 (25).
238 Assembled genomes were aligned against NC_003210.1 using pairwise comparisons with

NCBI Blast (26) (blastn version 2.7.1, e-value cut-off=0.01), and alignments visualized using the Artemis Comparison Tool (27).

Additional bioinformatics analysis performed in this study used CLC Main Workbench 8 to organise the DNA sequences. Basic Local Alignment Search Tool (BLAST) was used to align sequences of nucleic acids (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ExPASy translation tool was used to assess the impact of the mutations on the protein sequences (https://web.expasy.org/translate/). The alignment of the protein sequences was generated by ClustalOmega (28).

248 **Results**

249 ***Assessing growth and flagellar based motility***

250 Four different *L. monocytogenes* EGDe isolates were obtained for this study and hereafter
251 referred to as WT_{1030}, WT_{1031}, WT_{1032}, and WT_{1033} (Table 1). The designation of the bacteria
252 used in the study as EDGe was initially based on information obtained from the source
253 supplying them and was later confirmed by whole genome sequencing. We first compared the
254 growth of each strain and assayed motility. Growth was monitored in shaking culture
255 conditions using BHI and in static culture conditions using MWB. We did not identify any
256 statistically significant differences in the growth rate or final yield of the four isolates for
257 either condition (Figure 1 A and B). Next, we assessed flagellar-based motility by
258 quantifying the ability of the cells to spread on semi-solid agar plates using EGDe Δ*flaA* as a
259 negative control (13). The four EGDe isolates and EGDe Δ*flaA* were spotted onto BHI and
260 MWB based semi-solid agar plates that were incubated at 30°C, the permissive temperature
261 for motility by *L. monocytogenes* (13, 29). As expected, the EGDe Δ*flaA* strain did not spread
262 away from the inoculation point (Fig. 1 C and D). In contrast, the four EGDe stocks spread
263 out from the seeding point over time, although WT_{1030} showed reduced motility on BHI by
264 comparison with the other three isolates (Fig. 1 C and D, Fig. S1). These data indicate that
265 any differences in biofilm formation observed are not due to impaired growth or mutation of
266 the flagellar genes.

267 ***Differences in chitinase activity***

268 Certain regions of the *L. monocytogenes* genome are prone to incorporating mutations during
269 growth (30), including *rsbS*, *rsbU*, and *rsbV* (31). The products of these genes comprise part
270 of the complex regulatory system that activate the alternative sigma factor, Sigma B (SigB)
271 (32). In turn, SigB controls a large regulon in *L. monocytogenes* that includes the genes *chiA*
272 and *chiB*, which encode extracellular chitinases (14). Thus, to test if SigB regulation was
273 disrupted due to mutations in the *rsb* genes we examined chitinase activity (31). After
274 spotting the four EGDe isolates onto chitin-rich agar, we noted that two of the isolates
275 displayed clear evidence of chitinolytic activity: WT_{1031} and WT_{1032}. In contrast, colonies
276 formed by WT_{1030} and WT_{1033} had less distinct clearance zones, suggesting altered expression
277 of members of the SigB regulon (Fig. 2). These gross phenotypic differences are indicative of
278 genomic variations existing between the four EGDe isolates.

279 ***Whole genome sequencing***

We next sequenced the genomes of the EGDe strains using Illumina next-generation technologies. The reads were mapped to the published wild-type EGDe reference genome (NC_003210) and SNPs were identified in each of the four strains using variant detection (Table 2). Some of the SNPs initially identified (not shown in Table 2) in the WT_{1032} genome were close to the prophage A118 integration site; further bioinformatic analysis revealed that these were caused by excision of the prophage from the chromosome restoring a functional copy of *comK* (33, 34). Isolates WT_{1030} and WT_{1033} both contained a nonsense SNP in *rsbU*; this is consistent with the chitinase analyses which showed that these isolates generated a less distinct clearance zone on chitin containing growth medium. WT_{1031} contained the fewest SNPs, all of which were identified in the other EGDe isolates, and so was designated as the parental "wild-type" strain. These findings support the conclusion that variations in the genome have emerged between the EGDe isolates obtained from different sources.

Biofilm formation by the L. monocytogenes *EGDe stocks*

Having identified that the genomes of the four EGDe isolates were non-identical we assessed biofilm formation. The four EGDe stocks were inoculated into a 96-well microtiter plate platform where polystyrene pegs protruded from the lid into the well (this is also known as a Calgary biofilm device (35)). The EGDe Δ*flaA* strain, which has previously been shown to be impeded in biofilm formation (4), was included as a negative control. The cultures were incubated statically at 30°C and the biomass of each biofilm was measured every 12 hours for a 48 hour period. As expected, the EGDe Δ*flaA* exhibited lower A_{595} readings then those of the four EGDe stocks (Fig 3A), indicative of biofilm formation being reduced. Using the data from WT_{1031} as a baseline, the profile of biofilm biomass measured for the other three EGDe isolates was found to differ (Fig. 3A). Overall the biomass of WT_{1030} was lower at all time points (Fig. 3A), whereas the biomass of WT_{1033} started at a lower point than WT_{1031} but ended with higher measurements at later time points (Fig. 3A). The statistical analysis revealed the measurements for WT_{1032} to be comparable to those of the reference WT_{1031} (Fig. 3A). The findings indicate that excision of prophage A118 does not impact biofilm formation as assessed here.

We next imaged the adherent cells using scanning electron microscopy (Fig. 3B). This analysis was conducted at 30°C after biofilms were grown for 48 hours. Five regions of interest (ROI) were chosen for each sample that covered the top (liquid surface) to near to the bottom of the peg (Fig. S2A). We first compared the overall cell morphology of the EGDe isolates and concluded that there were no discernible differences (Fig. 3B). We next counted

313 the number of individual cells per field of view (FOV), and in doing so we noticed that dense
314 aggregates of cells encased in extracellular material were only encountered infrequently for
315 all of the strains. The biomass produced by WT_{1031} contained on average ~810±320
316 (mean±SD) cells per FOV (Fig S2B). Moreover, consistent with the measurements derived
317 from crystal violet staining, the number of cells per FOV calculated for WT_{1032} did not
318 significantly vary from those measured for WT_{1031}. In contrast, fewer cells were counted per
319 FOV for WT_{1030} while considerably more cells were detected in the WT_{1033} samples
320 (1255±539). It is worth noting that in some cases the cell density per FOV seemed to change
321 with the location on the peg (Fig. S2C); the region of the peg that was closer to the bottom of
322 the well had a higher number of cells that an equivalent region nearer the liquid-air interface.
323 This gradient of cell attachment was most apparent for the biofilms formed by WT_{1033} (Fig.
324 S2C). In summary the biomass measured using crystal violet and by counting the number of
325 adherent cells per FOV correlate well.

326 ***Linking genotype and biofilm formation***

327 Our data suggest that WT_{1030} is impeded in biofilm formation by comparison with WT_{1031}, a
328 phenotype that is a consequence of fewer cells attaching to the substratum. As detailed in
329 Table 2, the WT_{1030} genome contains 6 missense SNPs and three nonsense SNPs. To identify
330 which of these mutations was responsible for reducing cell attachment we constructed single
331 gene deletions in the coding regions that contained nonsense SNPs, *lmo0184*, *rmlA* (*lmo1081*)
332 and *rsbU*, using WT_{1031} as the parent. We reasoned that the nonsense SNPs were more likely
333 to have a significant impact on protein function than the missense SNPs and additionally
334 links to biofilm formation can be made for both *rsbU* and *rmlA*(36).

335 We checked if planktonic growth of the deletion strains was different from that of the
336 parental strain WT_{1031} (Fig S3). No significant differences were detected. Next, we measured
337 the biomass adhered to the pegs of the Calgary biofilm device for the deletion strains using
338 crystal violet staining. We discovered that deletion of *lmo0184* did not impact biofilm
339 formation compared with WT_{1031} (Fig. 4A) In contrast, deletion of either *rsbU* or *rmlA*
340 produced differences in the level of crystal violet staining measured over time. For the *rsbU*
341 deletion strain, the biomass was higher than that of WT_{1031} at 36 and 48 hours (Fig. 4A). For
342 the *rmlA* mutant strain, biofilm formation was reduced at 36 and 48 hours (Fig. 4A). These
343 findings were in agreement with the average number of cells adherent per FOV that were
344 visualised (Fig. 4B-G) and quantified following scanning electron microscopy (Fig. S4A and
345 S4B). Therefore we concluded that two genes that impact biofilm formation are mutated in

346 WT_{1030}: *rsbU* and *rmlA*. By constructing a double *rsbU rmlA* deletion strain in the WT_{1031}
347 background, we established that the impact of the *rmlA* mutation dominated the moderate
348 increase in biofilm observed when *rsbU* was deleted alone (see Fig. 4A, 4E and 4G).

349

350 ***When Sigma B is inactive cell adherence increases***

351 Deletion of *rsbU* enhances biofilm formation, whereas deletion of *rmlA* decreases biofilm
352 formation. RsbU is an upstream positive regulator of SigB (37), therefore one possible
353 interpretation of our data is that deletion of *rsbU* decreases transcription of the SigB regulon,
354 leading to an increase in *rmlA* transcription. While an effect of SigB on transcription of *rmlA*
355 has not been reported, this hypothesis would explain the enhanced biofilm capability of the
356 *rsbU* mutant and decreased biofilm level in the double *rsbU rmlA* strain, and in the *rmlA*
357 single mutant. Therefore, we first tested if the impact of mutating *rsbU* on biofilm formation
358 manifests as a consequence of SigB inactivation. If our hypothesis was correct then deletion
359 of *sigB* should phenocopy the *rsbU* mutation.

360 We constructed a *sigB* deletion in WT_{1031}, examined the level of chitinase activity and
361 assessed the impact on biofilm formation. As expected the *sigB* deletion strain did not display
362 chitinolytic activity (Fig. 2)(31). During biofilm formation, the *sigB* deletion strain was
363 initially observed to have a lower level of biomass adherent to the pegs compared with the
364 parental WT_{1031} strain. However, the value surpassed that of the parental strain at later time
365 points (Fig. 5A). As suggested by the crystal violet staining in Fig 5A, the *sigB* and *rsbU*
366 strains were shown to have similar numbers of cells attached per FOV when SEM imaging
367 was applied to the samples (Fig. 5B and S4A and S4B). Together these findings are
368 consistent with the conclusion that the impact of the SNP in *rsbU* on biofilm formation was
369 due to a reduction in *sigB* activity.

370 We next reasoned that if the reduction of SigB activity in the *rsbU* mutant impacted *rmlA*
371 transcription, this would manifest as an alteration in L-rhamnose decoration of the wall
372 teichoic acid (WTA). This is because RmlA is an enzyme in the TDP-L-rhamnose pathway.
373 TDP-L-rhamnose is used for the synthesis of cell wall carbohydrates (38-40) and for the
374 decoration of WTA in *L. monocytogenes* (41, 42). Therefore we extracted WTA from the
375 *sigB* and *rsbU* mutants and compared the apparent molecular mass with the WTA extracted
376 from the *rmlA* mutant. These analyses showed there was no gross difference in the apparent
377 molecular mass of WTA produced by the *sigB* and *rsbU* strains compared with the parental

strain, not at either a lower or higher position as would be expected for material with fewer or greater rhamnose moieties respectively. In contrast, for the *rmlA* mutant the molecular mass of WTA extracted was lower than that observed for WT_{1031}.The mobility of the WTA extracted from the *rmlA* mutant was comparable to that of the WTA extracted from EGDe isolate WT_{1030} (Fig. S5). Therefore, taking these data together, it is unlikely that *rsbU, or sigB,* are mediating their impact on biofilm formation via *rmlA* and its impact on WTA decoration.

***Sugar decoration of wall teichoic acids alters adhesion properties of* L. monocytogenes**

RmlA is the first enzyme in the pathway that catalyses the conversion of D-glucose-1-phosphate into TDP-L-rhamnose (41). We wanted to confirm if deletion of *rmlA* had an impact on biofilm formation due to the lack of the L-rhamnose moiety on WTA or if TDP-L-rhamnose was used in the synthesis of a different polymer. To do this, we constructed a derivative of WT_{1031} that still produced TDP-L-rhamnose but lacked the glycosyltransferase, RmlT, which is responsible for the transfer of TDP-L-rhamnose onto ribitol phosphate (41). Biofilm formation was measured for the 1031 Δ*rmlT* strain every 12 hours and found to be more comparable to that of the 1031 Δ*rmlA* strain than the WT_{1031} strain (Fig. 6A). Using SEM imaging to visualise the attached biomass, the 1031 Δ*rmlA* and 1031 Δ*rmlT* strains were shown to have similar numbers of cells attached per FOV (Fig. 6B and S4A and S4B). Therefore, as presence of the TDP-L-rhamnose pool in the *rmlT* mutant strain was not sufficient to allow biofilm formation, these findings suggest that decoration of WTA with L-rhamnose is needed for cell adhesion to the substratum. It is also possible that decoration of WTA with L-rhamnose is needed to promote the formation of clusters of the bacteria but further analysis would be needed to determine this conclusively.

Discussion

To study biofilm formation by the Gram-positive pathogen *Listeria monocytogenes* we chose an approach that was based on the hypothesis that diverged stocks of the EGDe wild-type may contain mutations that could impact biofilm formation. We proposed that identifying the mutations would allow us to link genotype with phenotype and thereby gain insights into the mechanisms underpinning biofilm formation in this pathogen. We sourced four EGDe isolates, checked planktonic growth and identified differences in chitinase activity. Using next-generation sequencing technologies we sequenced the genomes of the four isolates and identified genomic variations. Some features of the genomic sequencing data could be readily connected to phenotypic differences displayed by the four EGDe isolates. For example, RsbU is an upstream regulator of SigB activity (32, 43, 44) and in WT_{1030} and WT_{1033} a frameshift mutation that leads to the premature termination of translation is contained within *rsbU* (*lmo0892*) (Table 2). A consequence of the *rsbU* mutation may be that SigB is not activated and transcription of the genes in its regulon will not be triggered (44), although there is evidence showing that SigB retains partial activity in an RsbV mutant background (45). The presence of the SNP in *rsbU* correlated with the reduction of chitinolytic activity observed for WT_{1030} and WT_{1033} (Fig. 2). Additionally, WT_{1030} contains a nonsense SNP within the *rmlA* (*lmo1081*) coding region. RmlA is the first enzyme in a four-step reaction resulting in the synthesis of TDP-L-rhamnose (41), which is a substrate to transfer L-rhamnose onto the ribitol phosphate backbone of wall teichoic acid. The nonsense SNP in *rmlA* is predicted to disrupt TDP-L-rhamnose production resulting in a strain that carries WTA without the L-rhamnose decoration. The presence of this mutation correlates with the lower molecular weight of the WTA extracted from WT_{1030}.

We adapted and implemented a robust method of assessing biofilm formation by the four EGDe isolates. The biofilm formed under these conditions did not typically appear to generate an obvious extracellular matrix, when viewed by microscopy the biomass appeared to be isolated cells or small clusters that were adherent to the surface. This is different from the honeycomb arrangement of *L. monocytogenes* cells seen in some biofilms (46), but comparable to other studies where cells have been observed as an attached monolayer (47). Through our analysis we identified one strain (named here WT_{1030}) that displayed a defect in biofilm formation. Having ruled out that differences in growth or motility caused the differences in biofilm formation we observed, we used the details from the next-generation sequencing analysis to link *rmlA* to surface adhesion and biofilm formation. As detailed

434 above, RmlA is needed for TDP-L-rhamnose production and through assessing biofilm
435 formation lacking RmlT we were able to determine that the lack of L-rhamnose decoration of
436 wall teichoic acid was the factor influencing biofilm formation, rather than the loss of TDP-L-
437 rhamnose production *per se*. The defect in biofilm formation appeared to be due to reduced
438 cell surface adhesion. Our findings are consistent with data derived from a global transposon
439 screen of *L. monocytogenes* isolate 568 which identified *lmo1080* (*rmlT*) as needed for
440 biofilm formation at low temperature (48). In addition they are in line with experiments
441 which uncovered wall teichoic acids as a major polysaccharide present in the *L.*
442 *monocytogenes* biofilm matrix (49). However, exactly how the L-rhamnose decorated wall
443 teichoic acid aids cell surface interaction remains unknown.

444 We also strengthened the already identified connection between *sigB* and biofilm formation
445 and in doing reinforced the need to obtain dynamic data when analysing biofilm formation
446 using a microtitre plate based assay (7, 50). SigB has previously been found to promote
447 biofilm formation (51, 52). However, here for the *sigB* deletion strain a defect in biofilm
448 formation at early time points culminated in an enhanced level of biofilm produced at later
449 time points. We therefore conclude that SigB appears to suppress transcription of genes
450 involved in biofilm formation, perhaps those directly linked with matrix synthesis as deletion
451 resulted in greater adhesion and more extracellular material being deposited and visible by
452 SEM analysis.

453 **Concluding Comments**

454 The use of laboratory reference strains was initially focussed on allowing the cooperation of
455 research groups around the world (8). It provides a baseline of commonality to compare
456 observations and accelerate the progression of research. Although this goal has been
457 accomplished, the approach also allows seemingly identical isolates of bacteria to
458 independently evolve in different laboratories (9, 11). Using a comparative sequencing
459 approach we have uncovered variations in the genomes of EGDe isolates used in laboratories
460 across the world. Moreover, we have reinforced the importance and necessity of obtaining
461 whole genome sequencing data to ensure that strains do not contain inadvertent mutations
462 when a new isolate is used in research settings.

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475

476 **References**

477 1. **Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S.** 2016. Biofilms:
478 an emergent form of bacterial life. Nature reviews. Microbiology **14:**563-575.
479 2. **Colagiorgi A, Bruini I, Di Ciccio PA, Zanardi E, Ghidini S, Ianieri A.** 2017. Listeria
480 monocytogenes Biofilms in the Wonderland of Food Industry. Pathogens **6**.
481 3. **Fagerlund A, Moretro T, Heir E, Briandet R, Langsrud S.** 2017. Cleaning and Disinfection of
482 Biofilms Composed of Listeria monocytogenes and Background Microbiota from Meat
483 Processing Surfaces. Applied and environmental microbiology **83**.
484 4. **Lemon KP, Higgins DE, Kolter R.** 2007. Flagellar motility is critical for Listeria monocytogenes
485 biofilm formation. Journal of bacteriology **189:**4418-4424.
486 5. **Travier L, Guadagnini S, Gouin E, Dufour A, Chenal-Francisque V, Cossart P, Olivo-Marin JC,**
487 **Ghigo JM, Disson O, Lecuit M.** 2013. ActA promotes Listeria monocytogenes aggregation,
488 intestinal colonization and carriage. PLoS pathogens **9:**e1003131.
489 6. **Price R, Jayeola V, Niedermeyer J, Parsons C, Kathariou S.** 2018. The Listeria
490 monocytogenes Key Virulence Determinants hly and prfA are involved in Biofilm Formation
491 and Aggregation but not Colonization of Fresh Produce. Pathogens **7**.
492 7. **van der Veen S, Abee T.** 2010. Importance of SigB for Listeria monocytogenes static and
493 continuous-flow biofilm formation and disinfectant resistance. Applied and environmental
494 microbiology **76:**7854-7860.
495 8. **Fux CA, Shirtliff M, Stoodley P, Costerton JW.** 2005. Can laboratory reference strains mirror
496 "real-world" pathogenesis? Trends in microbiology **13:**58-63.
497 9. **Read TD, Massey RC.** 2014. Characterizing the genetic basis of bacterial phenotypes using
498 genome-wide association studies: a new direction for bacteriology. Genome medicine **6:**109.
499 10. **McLoon AL, Guttenplan SB, Kearns DB, Kolter R, Losick R.** 2011. Tracing the domestication
500 of a biofilm-forming bacterium. Journal of bacteriology **193:**2027-2034.
501 11. **Gallegos-Monterrosa R, Mhatre E, Kovacs AT.** 2016. Specific Bacillus subtilis 168 variants
502 form biofilms on nutrient-rich medium. Microbiology **162:**1922-1932.
503 12. **Becavin C, Bouchier C, Lechat P, Archambaud C, Creno S, Gouin E, Wu Z, Kuhbacher A,**
504 **Brisse S, Pucciarelli MG, Garcia-del Portillo F, Hain T, Portnoy DA, Chakraborty T, Lecuit M,**
505 **Pizarro-Cerda J, Moszer I, Bierne H, Cossart P.** 2014. Comparison of widely used Listeria
506 monocytogenes strains EGD, 10403S, and EGD-e highlights genomic variations underlying
507 differences in pathogenicity. mBio **5:**e00969-00914.
508 13. **Grundling A, Burrack LS, Bouwer HG, Higgins DE.** 2004. Listeria monocytogenes regulates
509 flagellar motility gene expression through MogR, a transcriptional repressor required for
510 virulence. Proceedings of the National Academy of Sciences of the United States of America
511 **101:**12318-12323.
512 14. **Larsen MH, Leisner JJ, Ingmer H.** 2010. The chitinolytic activity of Listeria monocytogenes
513 EGD is regulated by carbohydrates but also by the virulence regulator PrfA. Applied and
514 environmental microbiology **76:**6470-6476.
515 15. **Carvalho F, Pucciarelli MG, Garcia-del Portillo F, Cabanes D, Cossart P.** 2013. Extraction of
516 cell wall-bound teichoic acids and surface proteins from Listeria monocytogenes. Methods in
517 molecular biology **966:**289-308.
518 16. **Fischer ER, Hansen BT, Nair V, Hoyt FH, Dorward DW.** 2012. Scanning electron microscopy.
519 Current protocols in microbiology **Chapter 2:**Unit 2B 2.
520 17. **Monk IR, Gahan CG, Hill C.** 2008. Tools for functional postgenomic analysis of listeria
521 monocytogenes. Applied and environmental microbiology **74:**3921-3934.
522 18. **Arnaud M, Chastanet A, Debarbouille M.** 2004. New vector for efficient allelic replacement
523 in naturally nontransformable, low-GC-content, gram-positive bacteria. Applied and
524 environmental microbiology **70:**6887-6891.

19. **Li H, Durbin R.** 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics **25:**1754-1760.
20. **McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA.** 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research **20:**1297-1303.
21. **Abbott JC.** 2017. BugBuilder - An Automated Microbial Genome Assembly and Analysis Pipeline. bioRxiv**:**148783.
22. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA.** 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Journal of Computational Biology **19:**455-477.
23. **Darling AE, Mau B, Perna NT.** 2010. progressiveMauve: Multiple Genome Alignment with Gene Gain, Loss and Rearrangement. PloS one **5:**e11147.
24. **Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK, Earl AM.** 2014. Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement. PloS one **9:**e112963.
25. **Seemann T.** 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics **30:**2068-2069.
26. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research **25:**3389-3402.
27. **Carver TJ, Rutherford KM, Berriman M, Rajandream M-A, Barrell BG, Parkhill J.** 2005. ACT: the Artemis comparison tool. Bioinformatics **21:**3422-3423.
28. **Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG.** 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology **7:**539.
29. **Peel M, Donachie W, Shaw A.** 1988. Temperature-dependent expression of flagella of Listeria monocytogenes studied by electron microscopy, SDS-PAGE and western blotting. Journal of general microbiology **134:**2171-2178.
30. **Orsi RH, Borowsky ML, Lauer P, Young SK, Nusbaum C, Galagan JE, Birren BW, Ivy RA, Sun Q, Graves LM, Swaminathan B, Wiedmann M.** 2008. Short-term genome evolution of Listeria monocytogenes in a non-controlled environment. BMC Genomics **9:**539.
31. **Quereda JJ, Pucciarelli MG, Botello-Morte L, Calvo E, Carvalho F, Bouchier C, Vieira A, Mariscotti JF, Chakraborty T, Cossart P, Hain T, Cabanes D, Garcia-Del Portillo F.** 2013. Occurrence of mutations impairing sigma factor B (SigB) function upon inactivation of Listeria monocytogenes genes encoding surface proteins. Microbiology **159:**1328-1339.
32. **Tiensuu T, Guerreiro DN, Oliveira AH, O'Byrne C, Johansson J.** 2019. Flick of a switch: regulatory mechanisms allowing Listeria monocytogenes to transition from a saprophyte to a killer. Microbiology.
33. **Rabinovich L, Sigal N, Borovok I, Nir-Paz R, Herskovits AA.** 2012. Prophage excision activates Listeria competence genes that promote phagosomal escape and virulence. Cell **150:**792-802.
34. **Loessner MJ, Inman RB, Lauer P, Calendar R.** 2000. Complete nucleotide sequence, molecular analysis and genome structure of bacteriophage A118 of Listeria monocytogenes : implications for phage evolution. Molecular microbiology **35:**324-340.
35. **Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A.** 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. Journal of clinical microbiology **37:**1771-1776.
36. **Griffiths A, Miller J, Suzuki D.** 2000. An Introduction to Genetic Analysis. 7th edition.

. New York: W. H. Freeman.

37. **Delumeau O, Dutta S, Brigulla M, Kuhnke G, Hardwick SW, Volker U, Yudkin MD, Lewis RJ.** 2004. Functional and structural characterization of RsbU, a stress signaling protein phosphatase 2C. The Journal of biological chemistry **279:**40927-40937.

38. **Ma Y, Pan F, McNeil M.** 2002. Formation of dTDP-rhamnose is essential for growth of mycobacteria. Journal of bacteriology **184:**3392-3395.

39. **Tsukioka Y, Yamashita Y, Oho T, Nakano Y, Koga T.** 1997. Biological function of the dTDP-rhamnose synthesis pathway in Streptococcus mutans. Journal of bacteriology **179:**1126-1134.

40. **van der Beek SL, Zorzoli A, Canak E, Chapman RN, Lucas K, Meyer BH, Evangelopoulos D, de Carvalho LPS, Boons GJ, Dorfmueller HC, van Sorge NM.** 2019. Streptococcal dTDP-L-rhamnose biosynthesis enzymes: functional characterization and lead compound identification. Molecular microbiology **111:**951-964.

41. **Carvalho F, Atilano ML, Pombinho R, Covas G, Gallo RL, Filipe SR, Sousa S, Cabanes D.** 2015. L-Rhamnosylation of Listeria monocytogenes Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane. PLoS pathogens **11:**e1004919.

42. **Kamisango K, Fujii H, Okumura H, Saiki I, Araki Y, Yamamura Y, Azuma I.** 1983. Structural and immunochemical studies of teichoic acid of Listeria monocytogenes. Journal of biochemistry **93:**1401-1409.

43. **Wiedmann M, Arvik TJ, Hurley RJ, Boor KJ.** 1998. General stress transcription factor sigmaB and its role in acid tolerance and virulence of Listeria monocytogenes. Journal of bacteriology **180:**3650-3656.

44. **Shin JH, Brody MS, Price CW.** 2010. Physical and antibiotic stresses require activation of the RsbU phosphatase to induce the general stress response in Listeria monocytogenes. Microbiology **156:**2660-2669.

45. **Utratna M, Cosgrave E, Baustian C, Ceredig RH, Byrne CP.** 2014. Effects of Growth Phase and Temperature on Activity within a Listeria monocytogenes Population: Evidence for RsbV-Independent Activation of at Refrigeration Temperatures. BioMed Research International **2014:**11.

46. **Guilbaud M, Piveteau P, Desvaux M, Brisse S, Briandet R.** 2015. Exploring the diversity of Listeria monocytogenes biofilm architecture by high-throughput confocal laser scanning microscopy and the predominance of the honeycomb-like morphotype. Applied and environmental microbiology **81:**1813-1819.

47. **Renier S, Hebraud M, Desvaux M.** 2011. Molecular biology of surface colonization by Listeria monocytogenes: an additional facet of an opportunistic Gram-positive foodborne pathogen. Environmental microbiology **13:**835-850.

48. **Piercey MJ, Hingston PA, Hansen LT.** 2016. Genes involved in Listeria monocytogenes biofilm formation at a simulated food processing plant temperature of 15 degrees C. International journal of food microbiology **223:**63-74.

49. **Brauge T, Sadovskaya I, Faille C, Benezech T, Maes E, Guerardel Y, Midelet-Bourdin G.** 2016. Teichoic acid is the major polysaccharide present in the Listeria monocytogenes biofilm matrix. FEMS microbiology letters **363:**fnv229.

50. **Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, Desvaux M, Di Bonaventura G, Hebraud M, Jaglic Z, Kacaniova M, Knochel S, Lourenco A, Mergulhao F, Meyer RL, Nychas G, Simoes M, Tresse O, Sternberg C.** 2017. Critical review on biofilm methods. Critical reviews in microbiology **43:**313-351.

51. **Lee JJ, Lee G, Shin JH.** 2014. sigma(B) affects biofilm formation under the dual stress conditions imposed by adding salt and low temperature in Listeria monocytogenes. Journal of microbiology **52:**849-855.

52. **Lee T, Jun SH, Choi CW, Kim SI, Lee JC, Shin JH.** 2018. Salt stress affects global protein expression profiles of extracellular membrane-derived vesicles of Listeria monocytogenes. Microbial pathogenesis **115:**272-279.

632 Table 1 The *Listeria monocytogenes* EGDe isolates used in this study

Strain	**Reference***	**Origin**
WT_{1030}	ANG882	Carmen Buchrieser via Angelika Gründling
WT_{1031}	ANG873	Martin Loessner via Angelika Gründling
WT_{1032}	EGDe	University College Cork
WT_{1033}	BAA-679	Carmen Buchrieser via ATCC

* The strain name used in the originating lab.

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635 Table 2 Analysis of single nucleotide polymorphisms using whole genome sequencing data

Relative position in genome [a]	**Gene**	**Ref** [b]	**WT 1030** [e]	**WT 1031** [e]	**WT 1032** [e]	**WT 1033** [e]	**EGDe Δ*flaA*** [e]	**Alteration of amino acid** [c]	**Type of mutation** [d]
188308	*lmo0184*	G	T	-	-	T	-	148 E to stop codon	Nonsense
189757	*lmo0185*	C	A	-	-	-	-	-	Synonymous
264578	*lmo0247*	G	T	T	T	T	T	-	Synonymous
280225	*rpoC*	C	G	-	-	-	-	1166 I to M	Missense
435968	intergenic	C	A	A	A	A	A	Intergenic	intergenic
929469	*rsbU*	C	CTT	-	-	CTT	-	245L to F, frameshift	Nonsense
1116367	*lmo1081 (rmlA)*	G	T	-	-	-	-	241 E to stop codon	Nonsense
1442124	intergenic	C	A	A	A	A	A	Intergenic	Intergenic
1890030	*lmo1814*	C	A	-	-	A	-	82 G to W	Missense
2003900	*aroF*	C	A	-	-	-	-	138 V to F	Missense
2207164	*lmo2125*	T	G	-	-	-	-	400 Q to P	Missense
2734614	*lmo2660*	C	A	-	-	-	-	211 G to V	Missense
2836724	*lmo2757*	G	-	-	A	-	-	354 R to C	Missense
2849710	*lmo2769*	G	-	-	-	T	-	247 Y to stop codon	Nonsense
2943565	intergenic	G	T	T	T	T	T	Intergenic	Intergenic

a The relative locations of the SNPs present in the strains are compared with NC_003210.

b The nucleotide present at the corresponding relative position in NC_003210.

c The codon of the coding sequences with SNPs were analysed by ExPASy translation tool and followed by BLAST with the original amino acid sequences.

d The type of SNPs categorised into intergenic, synonymous, missense and nonsense.

e '-' indicates no difference from the reference genome

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642 **Figure 1: Growth and motility of the four *L. monocytogenes* EGDe isolates.** (**A**) Growth
643 in BHI medium in shaking conditions at 37°C; (**B**) Growth in MWB medium in static
644 conditions at 30°C. For both (A and B) the value presented is the mean of 2 independent
645 experiments and the error bars represent the standard deviation; (**C, D**) Motility of the four
646 isolates assessed after 24 and 48 hours at 30°C using (**C**) BHI or (**D**) MWB soft agar. The
647 EGDe Δ*flaA* strain was used as a negative control. Representative images are presented.

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649

650 **Figure 2: Chitinase activity of the four *L. monocytogenes* EGDe isolates.** Chitinolytic
651 activity assessed using LB agar containing 2% (w/v) chitin. Incubation was at 30°C for 120
652 hours. The genotypes of the strains tested are as follows: 1 and 3) 1031 Δ*sigB*; 2 and 4) 1031
653 Δ*rsbU*; 5) WT_{1031}; 6) WT_{1030}; 7) WT_{1032}; 8) WT_{1033}. The 1031 Δ*sigB* and 1031 Δ*rsbU* strains
654 were used as controls.

655

Figure 3: Biofilm formation of the four *L. monocytogenes* EGDe isolates. (**A**) The biomass of the four EGDe isolates adherent to the substratum was quantified over time when incubated at 30ºC. The EGDe Δ*flaA* strain was used as a negative control. The values presented are the mean of 29 independent experiments for the EGDe isolates and 4 experiments for the Δ*flaA* strain. The error bars are the standard error of the mean. The data were analysed by one-way ANOVA comparing with WT1031 with "*" representing a *p* value of ≤0.05 and "**" representing a *p* value of ≤0.01. (**B**) The biomass adherent to the substratum was imaged using scanning electron microscopy after 48 hours incubation. The representative image shown was taken at the midpoint of the peg. The scale bars represent 1 μm.

668

669 **Figure 4: RmlA and RsbU influence biofilm formation by *L. monocytogenes* EGDe.** (**A**)
670 The biomass of WT_{1031}, WT_{1030}, WT_{1031} Δ*lmo0184* (LSW1024), WT_{1031} Δ*rsbU* (LSW1028),
671 WT_{1031} Δ*rmlA* (LSW1040), and WT_{1031} Δ*rmlA* Δ*rsbU* (LSW1051) strains that was adherent to
672 the substratum was quantified. The samples were incubated at 30ºC for the time points
673 indicated. The values presented for WT_{1031} and WT_{1030} are reproduced from Figure 3. The
674 mean of ≥ 4 experiments are presented for the remaining strains. The error bars are the
675 standard error of the mean. The data were analysed by one-way ANOVA comparing with
676 WT1031 with "*" representing a *p* value of ≤0.05 and "**" representing a *p* value of ≤0.01;
677 (**B-G**) The biomass adherent to the substratum was imaged using scanning electron
678 microscopy for (**B**) WT_{1031}; (**C**) WT_{1030}; (**D**) WT_{1031} Δ*lmo0184*, (**E**) WT_{1031} Δ*rsbU*, (**F**)
679 WT_{1031} Δ*rmlA* and (**G**) WT_{1031} Δ*rmlA* Δ*rsbU*. The representative image shown was taken at
680 the midpoint of the peg after 48 hours incubation. The scale bars represent 1 μm.

681

682 **Figure 5: SigB influences biofilm formation by *L. monocytogenes* EGDe.** (**A**) The biomass
683 of WT_{1031}, WT_{1031} Δ*rsbU* (LSW1028), and WT_{1031} Δ*sigB* (LSW1026) strains that was
684 adherent to the substratum was quantified. The samples were incubated at 30ºC for the time
685 points indicated. The values presented for WT_{1031} and WT_{1031} Δ*rsbU* are reproduced from
686 Figure 3 and Figure 4. The mean of ≥ 4 experiments are presented for the WT_{1031} Δ*sigB*
687 strain. The error bars are the standard error of the mean. The data were analysed by one-way
688 ANOVA comparing with WT1031 with "*" representing a *p* value of ≤0.05 and "**"
689 representing a *p* value of ≤0.01; (**B**) The biomass adherent to the substratum was imaged
690 using scanning electron microscopy for WT_{1031} Δ*sigB*. The representative image shown was
691 taken at the midpoint of the peg after 48 hours incubation. The scale bar represents 1 μm.

692

693

694 **Figure 6: Decoration of the wall teichoic acid with L-rhamnose is needed for cell**
695 **adhesion by *L. monocytogenes* EGDe.** (**A**) The biomass of WT_{1031}, WT_{1030}, WT_{1031} Δ*rmlA*
696 (LSW1040), and WT_{1031} Δ*rmlT* (LSW1039) strains that was adherent to the substratum was
697 quantified. The samples were incubated at 30°C for the time points indicated. The values
698 presented for WT_{1031}, WT_{1030}, and WT_{1031} Δ*rmlA* are reproduced from Figure 3 and Figure 4.
699 The mean of ≥ 4 experiments are presented for the WT_{1031} Δ*rmlT* strain. The error bars are
700 the standard error of the mean. The data were analysed by one-way ANOVA comparing with
701 WT1031 with "*" representing a *p* value of ≤0.05 and "**" representing a *p* value of ≤0.01;
702 (**B**) The biomass adherent to the substratum was imaged using scanning electron microscopy
703 for WT_{1031} Δ*rmlT* after 48 hours incubation. The representative image shown was taken at the
704 midpoint of the peg. The scale bar represents 1 μm.

705