Physiological studies on pH and salt tolerance and the utilization of diverse carbon sources by *Listeria monocytogenes*

Lisa Maria Schulz[1], Alicia Konrath[2], Jeanine Rismondo[3]*

Department of General Microbiology, Institute of Microbiology and Genetics, GZMB, Georg-August University Göttingen, Grisebachstr. 8, 37077 Göttingen, Germany

[1] lisamaria.schulz@uni-goettingen.de

[2] alicia.konrath@t-online.de

[3] jrismon@gwdg.de

* Corresponding author

Running title: Growth of *L. monocytogenes* in *Listeria* synthetic medium

Funding

This work was funded by the German research foundation (DFG) grant RI 2920/3-1 to JR.

Conflict of interest

The authors declare no conflict of interest. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Author Contributions

LMS: Investigation, formal analysis, conceptualization, methodology, supervision, validation, writing – review & editing. AK: Investigation, formal analysis, writing – review & editing. JR: Investigation, conceptualization, supervision, funding acquisition, writing – original draft.

Abstract

The foodborne pathogen *Listeria monocytogenes* can grow in a wide range of environmental conditions. For the study of the physiology of this organism, several chemically defined media have been developed over the past decades. Here, we examined the ability of *L. monocytogenes* wildtype strains EGD-e and 10403S to grow under salt and pH stress in *Listeria* synthetic medium (LSM). Furthermore, we determined that a wide range of carbon sources could support growth of both wildtype strains in LSM. However, for hexose phosphate sugars such as glucose-1-phosphate, *L. monocytogenes* strains need to be pre-grown under conditions, where the major virulence regulator PrfA is active. In addition, growth of both *L. monocytogenes* wildtype strains was observed when LSM was supplemented with the amino acid sugar *N*-acetylmannosamine (Man*N*Ac). We were able to show that some of the proteins encoded in the operon *lmo2795-nanE*, such as the Man*N*Ac-6-phosphate epimerase NanE, are required for growth in presence of Man*N*Ac. The first gene of the operon, *lmo2795,* encodes a transcriptional regulator of the RpiR family. Using electrophoretic mobility shift assays and quantitative real time PCR analysis, we were able to show that Lmo2795 binds to the promoter region of the operon *lmo2795-nanE* and activates its expression.

Introduction

The foodborne pathogen *Listeria monocytogenes* is a Gram-positive, facultative anaerobic, rod-shaped bacterium, which is ubiquitously found in nature. It can survive as a saprophyte on decaying plant material, in soil or in wastewater. *L. monocytogenes* and other *Listeria* species can also enter food processing facilities and food chains, for instance due to contaminated water or raw materials (Lourenco *et al.*, 2022). Several recent outbreaks of *L. monocytogenes* were associated with contaminated fruits and vegetables, such as melons, apples or mushrooms, or contaminated meat products (Matle *et al.*, 2020; Lachmann *et al.*, 2021; Townsend *et al.*, 2021). The intracellular pathogen *L. monocytogenes* is the causative agent of the disease listeriosis. In healthy individuals, the intake of *L. monocytogenes* via the consumption of contaminated food products mostly leads to mild symptoms, such as fever and

56 a self-limiting gastroenteritis. However, for immunocompromised individuals, the elderly,
57 newborns and pregnant women, the infection with *L. monocytogenes* can cause more severe
58 symptoms, such as muscle aches, vomiting, diarrhea, encephalitis or meningitis. For pregnant
59 women, it can also lead to premature or still birth (Radoshevich and Cossart, 2018). This is
60 achieved by its ability to cross all human barriers, namely the intestinal, placental and blood-
61 brain barriers (Lecuit, 2005).

62 The survival of *L. monocytogenes* in food processing facilities and within the host is
63 enabled due to its ability to grow under diverse stress conditions. Previous studies reported
64 that *L. monocytogenes* can grow at temperatures between 1 and 45°C, in a pH range from 4.0
65 to 9.5 or in the presence of up to 10% salt (Patchett *et al.*, 1992; Arizcun *et al.*, 1998; Vasseur
66 *et al.*, 2001). *L. monocytogenes* is also able to use a variety of carbon sources ranging from
67 simple monosaccharides (glucose, fructose) to more complex sugars (maltodextrin) and
68 polyols (glycerol, D-arabitol, D-xylitol) (Gopal *et al.*, 2010; Deutscher *et al.*, 2014; Kentache *et*
69 *al.*, 2016). The ability to take up such a wide range of carbon sources is facilitated by a diverse
70 set of transporters, which are encoded in the genome of *L. monocytogenes*. Most of the carbon
71 sources are imported into the cell via the phosphoenolpyruvate:carbohydrate
72 phosphotransferase system (PTS). The genome of *L. monocytogenes* wildtype strain EGD-e
73 contains 86 PTS genes, which encode for 29 complete and 12 incomplete PTS complexes
74 (Glaser *et al.*, 2001; Stoll and Goebel, 2010). These PTS transporters are involved in the
75 transport of diverse carbon sources such as glucose, fructose, D-arabitol and D-xylitol
76 (Deutscher *et al.*, 2014; Kentache *et al.*, 2016). Some of these PTS transporters have also
77 overlapping substrate specificities. For instance, the two PTS systems ManLMN (MptACD)
78 and MpoABCD, encoded by *lmo0096-0098* and *lmo0784-0781*, respectively, are both involved
79 in the transport of glucose and mannose (Stoll and Goebel, 2010; Aké *et al.*, 2011). Inactivation
80 of all PTS systems, e.g. by deleting the phosphotransferase system enzyme I encoding gene
81 *ptsI*, did not abolish fermentation of some sugars such as glucose, fructose or mannose,
82 suggesting that *L. monocytogenes* possesses also other sugar transporters (Deutscher *et al.*,
83 2014). Indeed, *L. monocytogenes* encodes three GlcU-like non-PTS permeases (Lmo0169,

84 Lmo0176, Lmo0424), which could be involved in the import of these sugars (Aké *et al.*, 2011;
85 Deutscher *et al.*, 2014). The import of L-rhamnose is accomplished by the major facilitator
86 superfamily protein Lmo2850, which uses a proton symport mechanism (Fieseler *et al.*, 2012;
87 Deutscher *et al.*, 2014; Zeng *et al.*, 2021). Within the host, *L. monocytogenes* uses mainly
88 glucose-1-phosphate, glucose-6-phosphate and glycerol as carbon sources (Ripio *et al.*, 1997;
89 Grubmüller *et al.*, 2014). The transporter Hpt, also named UhpT, takes up hexose phosphates
90 from the host cell cytosol (Chico-Calero *et al.*, 2002), while the glycerol uptake facilitator GlpF
91 facilitates the import of glycerol (Joseph *et al.*, 2008). The expression of *hpt* thereby depends
92 on PrfA, the main virulence regulator of *L. monocytogenes* (Ripio *et al.*, 1997; Chico-Calero *et*
93 *al.*, 2002). *L. monocytogenes* possesses eight predicted carbohydrate ATP-binding cassette
94 (ABC) transporters. One of these transporters, which is encoded by *lmo2123-2125*, is required
95 for the import of maltose and maltodextrin (Gopal *et al.*, 2010). The remaining seven ABC
96 transporters have not yet been studied.

97 For many studies on the physiology and stress tolerance of *L. monocytogenes*, bacteria
98 were cultured in complex medium such as brain heart infusion (BHI) or tryptic soy broth (TSB).
99 In their natural habitat or within their host, *L. monocytogenes* likely encounters more limiting
100 growth conditions. Within the last decades, several minimal media have been developed which
101 allow for the growth of *L. monocytogenes* (Welshimer, 1963; Premaratne *et al.*, 1991; Phan-
102 Thanh and Gormon, 1997; Tsai and Hodgson, 2003; Jarvis *et al.*, 2016; Whiteley *et al.*, 2017).
103 In this study, we aimed to characterize the growth of *L. monocytogenes* wildtype strains EGD-
104 e and 10403S under stress conditions (low pH or high salt) in the recently developed *Listeria*
105 synthetic medium (LSM) (Whiteley *et al.*, 2017). To our knowledge, growth of *L.*
106 *monocytogenes* in LSM was so far only assessed with glucose as a sole carbon source. We
107 therefore determined, which other carbon sources can facilitate growth of *L. monocytogenes*
108 EGD-e and 10403S in LSM. This analysis revealed a range of carbon sources that can support
109 growth of both *L. monocytogenes* wildtype strains such as glucose, mannose and cellobiose.
110 We could further show that glucose-1-phosphate and glucose-6-phosphate could only facilitate
111 growth of both wildtype strains when they have been pre-grown under PrfA-activating

112 conditions. Interestingly, *L. monocytogenes* could also grow in LSM medium containing *N*-
113 acetylmannosamine (Man*N*Ac) as a sole carbon source, which has not yet been shown so far.
114 In *Escherichia coli*, Man*N*Ac is imported and phosphorylated via the PTS transporter ManXYZ.
115 Man*N*Ac-6-phosphate is then converted to *N*-acetylglucosamine(Glc*N*Ac)-6-phosphate by the
116 Man*N*Ac-6-phosphate epimerase NanE and subsequently to fructose-6-phosphate by the
117 Glc*N*Ac-6-phosphate deacetylase NagA and the glucosamine-6-phosphate deaminase NagB
118 (Plumbridge and Vimr, 1999). *L. monocytogenes* possesses one NanE homolog encoded by
119 *lmo2801*. The *nanE* gene is part of the *lmo2795-nanE* operon, which codes for an RpiR-type
120 transcriptional regulator, a member of the repressor, ORF, kinase (ROK) family, the EIIA and
121 EIIBC components of a PTS system, a haloacid dehydrogenase-like (HAD) hydrolase and a
122 putative oxidoreductase. Deletion of *lmo2797* and *lmo2799* encoding the PTS components did
123 not affect the growth of *L. monocytogenes* in the presence of Man*N*Ac. In contrast, a strain
124 lacking NanE was unable to grow suggesting that NanE is essential for the catabolism of
125 Man*N*Ac in *L. monocytogenes*.
126

127 **Experimental procedures**

128 **Bacterial Strains and growth conditions.** All strains and plasmids used in this study are
129 listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium and *Listeria*
130 *monocytogenes* strains in brain heart infusion (BHI) medium or *Listeria* synthetic medium
131 (LSM) at 37°C unless otherwise stated. Where required, antibiotics and supplements were
132 added to the medium at the following concentrations: for *E. coli* cultures, ampicillin (Amp) at
133 100 µg ml^{-1}, kanamycin (Kan) at 50 µg ml^{-1}, and for *L. monocytogenes* cultures,
134 chloramphenicol (Cam) at 7.5 µg ml^{-1} and Kan at 50 µg ml^{-1}.

135 LSM was prepared in accordance with the recipe from Whiteley et al. with minor
136 modifications (Whiteley *et al.*, 2017). L-leucine, L-methionine and L-valine were used instead
137 of the corresponding DL-enantiomers. L-arginine·HCl, riboflavin, $Na_2HPO_4 \cdot 7\ H_2O$, $CoCl_2 \cdot 7\ H_2O$
138 and L-cysteine·2·HCl were substituted for L-arginine, riboflavin 5' monophosphate sodium salt
139 hydrate, $Na_2HPO_4 \cdot 12\ H_2O$, $CoCl_2 \cdot 6\ H_2O$ and L-cysteine·HCl·H_2O, respectively. The pH of LSM

140 was adjusted with HCl, where indicated. Sodium chloride was added to LSM for
141 characterization of the growth of *L. monocytogenes* strains under salt stress. To test the ability
142 of *L. monocytogenes* to grow on different carbon sources, 1% glucose, which is contained in
143 LSM, was replaced by 1% of the following carbon sources: glycerol, glucosamine (Glc*N*), *N*-
144 acetylglucosamine (Glc*N*Ac), *N*-acetylmannosamine (Man*N*Ac), glucose-1-phosphate (Glc-1-
145 P), glucose-6-phosphate (Glc-6-P), mannose, maltose, mannitol, sucrose, rhamnose,
146 trehalose, succinate, cellobiose, galactose and *N*-acetylneuraminic acid (Neu5Ac).
147 For growth under PrfA-activating conditions, LSM was freshly prepared with 10-fold
148 less of the branched chain amino acids (80 µM L-isoleucine, 80 µM L-leucine and 90 µM L-
149 valine).
150
151 **Growth assays.** Overnight cultures of the indicated *L. monocytogenes* strains were diluted in
152 fresh BHI medium to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.3 was reached.
153 Cells of 2 ml culture were collected per condition, washed twice with 1 ml of the corresponding
154 LSM and re-suspended in 1 ml LSM. The cultures were adjusted to an OD_{600} of 0.2 and 100 µl
155 of the cell suspension were transferred into wells of a 96-well plate containing 100 µl of the
156 corresponding LSM. The plate was incubated at 37°C with orbital shaking in the BioTek Epoch
157 2 microplate reader and the OD_{600} was measured every 15 min for at least 36 h. Averages and
158 standard deviations of at least three independent growth assays were plotted.
159 To test whether growth of *L. monocytogenes* in LSM Glc-1-P and Glc-6-P was possible
160 after pre-growth of the indicated strains under PrfA-activating conditions, overnight cultures of
161 the indicated *L. monocytogenes* strains were diluted in LSM glucose with low concentrations
162 of BCAA to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.3 was reached. Cells
163 were collected, washed and used to inoculate a 96-well plate containing the corresponding
164 LSM with low concentrations of BCAA as described above.
165
166 **Strain and plasmid construction.** All primers used in this study are listed in Table 2. For
167 markerless deletion of *lmo2795*, 1-kb fragments up- and downstream of *lmo2795* were

168 amplified by PCR using primer pairs LMS346/LMS347 and LMS348/LMS349, respectively.
169 The resulting PCR fragments were fused by PCR using primers LMS346/LMS349. The
170 deletion fragment was subsequently digested with *Xba*I and *Sac*I and ligated into plasmid
171 pKSV7 that had been cut with the same enzymes. Plasmid pKSV7-Δ*lmo2795* was recovered
172 in *E. coli* XL1-Blue yielding strain EJR88. For markerless deletion of *lmo2796*, *lmo2799*,
173 *lmo2800* and *nanE*, 1-kb fragments up- and downstream of the corresponding gene were
174 amplified by PCR using primer pairs LMS395/LMS396 and LMS397/LMS398 (*lmo2796*),
175 LMS411/LMS412 and LMS413/LMS414 (*lmo2799*), JR211/LMS408 and LMS409/LMS410
176 (*lmo2800*), LMS401/LMS402 and LMS403/LMS404 (*nanE*). The resulting PCR fragments
177 were fused by PCR using primers LMS395/LMS398 (*lmo2796*), LMS411/414 (*lmo2799*),
178 JR211/LMS410 (*lmo2800*) and LMS401/LMS404 (*nanE*). The Δ*lmo2799*, Δ*lmo2800* and
179 Δ*nanE* deletion fragments were digested with *Bam*HI and *Pst*I and ligated into *BamH*I/*Pst*I cut
180 pKSV7. Plasmids pKSV7-Δ*lmo2796,* pKSV7-Δ*lmo2799* and pKSV7-Δ*nanE* were recovered in
181 *E. coli* XL10-Gold yielding strains EJR238, EJR122 and EJR210, respectively. pKSV7-
182 Δ*lmo2800* was recovered in *E. coli* XL1-Blue yielding strain EJR121. For the construction of
183 pKSV7-Δ*lmo2798*, 1-kb fragments up- and downstream of *lmo2798* were amplified by PCR
184 using primer pairs LMS417/LMS418 and LMS419/LMS420. The resulting PCR fragments were
185 fused by PCR using primers LMS417/LMS420, cut with *BamH*I and *EcoR*I and ligated into
186 *BamH*I/*EcoR*I cut pKSV7. pKSV7-Δ*lmo2798* was transformed into *E. coli* XL10-Gold yielding
187 strain EJR123. For markerless deletion of *lmo2797*, 1-kb fragments up- and downstream of
188 *lmo2797* were amplified by PCR using primer pairs JR142/JR143 and JR144/JR145. The
189 resulting PCR fragments were fused by PCR using primers JR142/JR145. The Δ*lmo2797*
190 deletion fragment was cut with *BamH*I/*EcoR*I and ligated with plasmid pKSV7. Plasmid pKSV7-
191 Δ*lmo2797* was recovered in *E. coli* XL1-Blue yielding strain EJR164. Plasmids pKSV7-
192 Δ*lmo2795*, pKSV7-Δ*lmo2796*, pKSV7-Δ*lmo2797*, pKSV7-Δ*lmo2798*, pKSV7-Δ*lmo2799*,
193 pKSV7-Δ*lmo2800* and pKSV7-Δ*nanE* were transformed into *L. monocytogenes* strain EGD-e
194 and genes *lmo2795*, *lmo2797*, *lmo2798*, *lmo2799*, *lmo2800* and *nanE* deleted by allelic

195 exchange as previously described (Camilli *et al.*, 1993) yielding strains EGD-e Δ*lmo2795*
196 (LJR123), Δ*lmo2796* (LJR264), EGD-e Δ*lmo2797* (LJR204), EGD-e Δ*lmo2798* (LJR132),
197 EGD-e Δ*lmo2799* (LJR135), EGD-e Δ*lmo2800* (LJR206) and EGD-e Δ*nanE* (LJR207). To
198 construct strain EGD-e Δ*lmo2799* Δ*lmo2797* (LJR205), pKSV7-Δ*lmo2797* was transformed
199 into *L. monocytogenes* strain LJR204 and *lmo2797* deleted by allelic exchange.
200 For the purification of Lmo2795 with an N-terminal His-tag, the *lmo2795* was amplified
201 using primer pair AK004/AK005. The resulting PCR product was digested with *BamH*I and *Sal*I
202 and ligated into pWH844. Plasmid pWH844-*lmo2795* was recovered in *E. coli* DH5α yielding
203 strain EJR131.
204

205 **RNA extraction.** RNA of the indicated *L. monocytogenes* strains was isolated following a
206 previously published method with minor modifications (Hauf *et al.*, 2019). Briefly, a single
207 colony was used to inoculate 10 ml LSM medium with 1% glucose as sole carbon source and
208 cultures were grown overnight at 37°C. Next day, cultures were used to inoculate 30 ml of
209 fresh LSM medium with 1% glucose to an OD_{600} of 0.1. When the cultures reached an OD_{600}
210 of 0.5 ± 0.05, 25 ml of the cell suspensions were harvested by centrifugation for 15 min at
211 4,000 rpm and 4°C and the pellet was snap frozen in liquid nitrogen and stored at -80°C. To
212 isolate the RNA, pellets were re-suspended in 1 ml killing buffer (20 mM Tris, pH 7.5, 5 mM
213 $MgCl_2$, 20 mM NaN_3), transferred to 1.5 ml microtubes and centrifuged at 13,000 rpm for 60
214 sec. Cells were then re-suspended in 1 ml lysis buffer I (25 % sucrose; 20 mM Tris-HCl pH 8,
215 0.25 mM EDTA) and 2 µl lysozyme (100 mg ml^{-1}) and incubated for 5 min on ice, followed by
216 centrifugation at 5,000 rpm and 4°C for 5 min. Pellets were then re-suspended in 300 µl lysis
217 buffer II (3 mM EDTA; 200 mM NaCl) and added to pre-heated (95°C) lysis Buffer III (3 mM
218 EDTA; 200 mM NaCl; 1 % SDS). Samples were incubated for exactly 5 min at 95°C and 600
219 µl phenol/chloroform/isoamylalcohol (25:24:1) (PCI) was added. After shaking the samples at
220 700 rpm for 5 min, the two phases were separated by centrifugation at 13,000 rpm for 5 min
221 and the upper aqueous phase was transferred to a new 1.5 ml microtube containing 600 µl

222 PCI. The extraction was repeated with 600 µl chloroform/isoamylalcohol (25:1). Finally, the
223 upper phase was transferred into an RNAse free tube and RNA was precipitated by addition
224 of 0.1 x volume 3 M sodium acetate (pH 5.2) and 1.5 volumes 96 % ethanol and incubated at
225 20°C overnight. To precipitate the RNA, samples were centrifuged at 13 000 rpm for 15 min,
226 washed in 70 % ethanol and dried under the fume hood. Finally, samples were re-suspended
227 in 25 µl RNAse free H_2O.

228 To avoid DNA contamination, 5 µg isolated RNA were digested with 5 µl DNase I (1 U
229 μl^{-1}, Thermo Scientific) for 40 min at 37°C. The reaction was stopped by addition of 2.5 µl 25
230 mM EDTA and incubation at 65°C for 10 min. To verify that the isolated RNA is free of DNA, a
231 check PCR was performed using primers LMS169 and LMS170. Genomic DNA from *L.*
232 *monocytogenes* EGD-e was used as a control.

233

234 **Quantitative real-time PCR (qRT-PCR).** qRT-PCR was carried out using the One-Step
235 reverse transcription PCR kit, the Bio-Rad iCycler and the Bio-Rad iQ5 software (Bio-Rad,
236 Munich, Germany). Three biological replicates were performed. Primer pairs LMS375/LMS376
237 and LMS169/170 were used to determine the transcript amounts of *rplD* and *gyrB*,
238 respectively, which were used as internal controls. Transcript amounts for *lmo2796* and *nanE*
239 were monitored using the primer pairs LMS384/LMS385 and LMS369/LMS377, respectively.
240 The average of the cycle threshold (C_T) values of *rplD* and *gyrB* were used to normalize the
241 C_T-values obtained for *lmo2796* and *nanE*. For each strain, the fold changes of *lmo2796* and
242 *nanE* expression were calculated using the $\Delta\Delta C_T$-method.

243

244 **Purification of His-Lmo2795.** For the overexpression of His-Lmo2795, plasmid pWH844-
245 *lmo2795* was transformed into *E. coli* BL21 and grown in 2x LB. When cultures reached an
246 OD_{600} of 0.6-0.8, expression was induced by the addition of IPTG at a final concentration of 1
247 mM and the cells were grown for 2 h at 37°C. Cells were collected by centrifugation, washed
248 once with 1x ZAP buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl) and the cell pellet stored at
249 -20°C. The next day, cells were re-suspended in 1x ZAP (50 mM Tris-HCl, pH 7.5, 200 mM

250 NaCl) and lysed by three passages (18,000 lb/in2) through an HTU DIGI-F press (G.
251 Heinemann, Germany). The resulting crude extract was centrifuged at 46,400x g for 60 min
252 followed by protein purification using a Ni^{2+} nitrilotriacetic acid column (IBA, Göttingen,
253 Germany). His-Lmo2795 was eluted using imidazole and elution fractions analyzed using
254 SDS-PAGE. Selected elution fractions were subsequently subjected to dialysis against 1x ZAP
255 buffer at 4°C overnight. The protein concentration was determined according to the method of
256 Bradford (Bradford, 1976) using the Bio-Rad protein assay dye reagent concentrate. Bovine
257 serum albumin was used as standard. The protein samples were stored at -80°C until further
258 use.
259

260 **Electrophoretic mobility shift assay (EMSA).** EMSA was performed as previously described
261 with some modifications (Dhiman *et al.*, 2014). Briefly, the upstream region of the *lmo2795-*
262 *nanE* operon containing a putative promoter site was amplified using primer pair
263 LMS430/LMS431. A 204 bp region within the operon, which served as an unspecific control,
264 was amplified using primers LMS428/LMS429. Binding reactions were performed at 25°C for
265 20 min in 20 µl binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% glycerol,
266 5 mM EDTA, 20 mM DTT and varying amounts of His-Lmo2795 (3.5 – 14 µg), as well as 250
267 pmol of DNA. To ease loading of the samples into the wells, 1 µl DNA loading dye was added
268 to each sample. A 60-min pre-run was carried out at 70 V prior to sample loading. The samples
269 were separated on an 8% polyacrylamide native gel in 0.5x TBE buffer (50 mM Tris-HCl, pH
270 10, 50 mM boric acid, 1 mM Na_2EDTA) at 4°C for 2.5 h at 50 V. The gel was stained in 50 ml
271 0.5x TBE buffer containing 5 µl HDGreen™ Plus DNA dye (INTAS, Göttingen, Germany) for 3
272 min, followed by a 5 min washing step in 0.5x TBE buffer, three 20 sec washing steps in H_2O
273 and an additional 30 min washing step in H_2O. DNA bands were visualized using a GelDoc™
274 XR+ (BioRad, Munich, Germany).
275

276 **Results**

277 **Growth of *L. monocytogenes* wildtype strains under salt and pH stress**

278 For most physiological studies, which aimed at the characterization of salt and pH resistance
279 of *L. monocytogenes*, bacteria were grown in complex media such as BHI or TSB. In nature
280 as well as within the host, *L. monocytogenes* likely encounters limited access to nutrients, such
281 as carbon and nitrogen sources or vitamins. We therefore assessed the ability of the *L.*
282 *monocytogenes* wildtype strains EGD-e and 10403S to grow under high salt and low pH in the
283 chemically defined medium LSM. Growth of EGD-e was barely affected in the presence of 1%
284 NaCl. In the presence of 2, 3 and 4 % NaCl, growth of EGD-e was delayed as compared to
285 LSM. At a concentration of 5% salt, EGD-e was still able to grow, but only reached an optical
286 density of around 0.3 after 36 h. The *L. monocytogenes* wildtype strain 10403S showed a lower
287 salt resistance as compared to EGD-e. Growth of 10403S was already impaired in LSM
288 containing 3% salt. In the presence of 4% and 5% salt, 10403S could only reach an optical
289 density of around 0.2 after 36 h (Fig. 1A). To assess the pH resistance, we compared the
290 growth of *L. monocytogenes* strains grown in standard LSM, which has a pH of 8.7, and LSM
291 adjusted to a pH of 8, 7, 6 and 5.5. Growth of the *L. monocytogenes* wildtype strain EGD-e
292 was similar in LSM with a pH of 7 as compared to standard LSM, while growth of 10403S was
293 slightly reduced. Both strains had a severe growth defect in LSM with a pH of 6 and were
294 unable to grow in LSM with a pH of 5.5 (Fig. 1B). Overall, we observed that the *L.*
295 *monocytogenes* wildtype strain 10403S was less tolerant to salt and pH stress than EGD-e
296 (Fig. 1).

297

298 **Growth of *L. monocytogenes* in LSM with different carbon sources**

299 Over the last decades, several chemically defined media have been developed that support
300 growth of *L. monocytogenes* (Pine *et al.*, 1989; Premaratne *et al.*, 1991; Phan-Thanh and
301 Gormon, 1997; Tsai and Hodgson, 2003; Jarvis *et al.*, 2016; Whiteley *et al.*, 2017). Most of
302 these media contain glucose as the sole carbon source. Other carbon sources such as fructose
303 and mannose were shown to support growth of *L. monocytogenes* in Hsiang-Ning Tsai medium
304 (HTM) or modified Welshimer's Broth (MWB) (Premaratne *et al.*, 1991; Tsai and Hodgson,

305 2003). Glucose could also be replaced by the amino sugars Glc*N*Ac and *N*-acetylmuramic acid
306 in MWB (Premaratne *et al.*, 1991). Interestingly, lactose and rhamnose could support growth
307 of *L. monocytogenes* in ACES-buffered chemically defined (ABCD) medium, but not in HTM
308 and/or MWB (Table 3) (Pine *et al.*, 1989; Premaratne *et al.*, 1991; Tsai and Hodgson, 2003).
309 Similarly, glycerol was shown to support growth of *L. monocytogenes* in HTM medium, while
310 only weak growth could be observed for MWB with glycerol as sole carbon source (Table 3)
311 (Premaratne *et al.*, 1991; Tsai and Hodgson, 2003). To our knowledge, no information is
312 available on the carbon sources that support growth of *L. monocytogenes* in LSM. We
313 therefore performed growth experiments with *L. monocytogenes* wildtype strains EGD-e and
314 10403S in LSM containing 1% of selected carbon sources. This analysis revealed that growth
315 of both strains was similar in LSM containing Glc*N*Ac, Glc*N*, mannose, cellobiose or trehalose
316 as sole carbon source compared to glucose (Fig. 2, Table 3). Both strains were also able to
317 use glycerol as carbon sources, while only weak growth was obtained for maltose, rhamnose
318 and succinate (Fig. 2, Table 3). No growth was observed for both strains, when glucose was
319 replaced with galactose, sucrose, glucose-1-phosphate (Glc-1-P), glucose-6-phosphate (Glc-
320 6-P) or mannitol (Fig. 3A, Table 3). The hexose phosphate transporter Hpt, which is required
321 for the import of Glc-1-P and Glc-6-P, is expressed in a PrfA-dependent manner. The
322 expression and activity of PrfA is controlled on transcriptional, translational and post-
323 translational level (reviewed in (Xayarath and Freitag, 2012; Gaballa *et al.*, 2019)). One factor
324 involved in the control of *prfA* expression is the global regulator CodY, which senses the
325 presence of branched chain amino acids (BCAAs). In the presence of high levels of BCAAs,
326 CodY acts as a *prfA* repressor, while expression of *prfA* is induced when BCAA levels drop,
327 which occurs upon invasion of host cells (Lobel *et al.*, 2015). We therefore tested whether
328 reduction of the BCAA concentration in LSM could lead to growth of *L. monocytogenes*
329 wildtype strains EGD-e and 10403S on Glc-1-P and Glc-6-P as sole carbon source. While both
330 strains grew fine in LSM with low concentrations of BCAA and glucose as sole carbon source,
331 no growth was observed in LSM with Glc-1-P or Glc-6-P (Fig. 3A). For growth analysis, *L.*
332 *monocytogenes* strains were pre-grown in BHI complex medium, in which PrfA is inactive

333 (Renzoni *et al.*, 1997). We thus wondered whether the strains have simply not sufficient time
334 to activate PrfA and by this also do not induce the expression of *hpt*, encoding the Glc-1-P and
335 Glc-6-P importer, when they are transferred into LSM containing the hexose phosphate sugars.
336 To test this, we pre-grew *L. monocytogenes* strains EGD-e and 10403S in LSM glucose with
337 low levels of BCAA and subsequently transferred them either into the same medium as a
338 control, or into LSM with low levels of BCAA and containing Glc-1-P or Glc-6-P as sole carbon
339 source. A strain lacking the virulence regulator PrfA was used as a control. Indeed, EGD-e and
340 10403S were now able to metabolize Glc-1-P and Glc-6-P, while the *prfA* mutant was unable
341 to grow (Fig. 3B). In contrast, absence of PrfA results in a growth advantage in LSM with
342 glucose as sole carbon source, suggesting that the expression and/or activity of PrfA is a
343 burden for *L. monocytogenes*, which has previously been shown (Vasanthakrishnan *et al.*,
344 2015; Friedman *et al.*, 2017).

345 The amino sugar Man*N*Ac, which has not been tested for any of the other chemically
346 defined media, was able to support growth of *L. monocytogenes* strains EGD-e and 10403S
347 (Fig. 2A, Table 3). Man*N*Ac is the precursor of the sialic acid *N*-acetylneuraminic acid
348 (Neu5Ac), which can serve as a carbon source for *Staphylococcus aureus* inside the host
349 (Angata and Varki, 2002; Vimr *et al.*, 2004; Olson *et al.*, 2013). However, Neu5Ac could not
350 support growth of *L. monocytogenes*, suggesting that this organism does not contain the
351 necessary catabolic pathway (Table 3).

352

353 **The *lmo2795-nanE* operon is required for Man*N*Ac catabolism**

354 In *E. coli*, Man*N*Ac is imported into the cell by the PTS transporter ManXYZ yielding Man*N*Ac-
355 6-phosphate. Man*N*Ac-6-phosphate is subsequently converted into Glc*N*Ac-6-phosphate by
356 the Man*N*Ac-6-phosphate epimerase NanE. The genome of *L. monocytogenes* strain EGD-e
357 contains one homolog of NanE encoded by *lmo2801* (37% identity, 58% similarity). Based on
358 the presence of the NanE homolog, it was already previously proposed that the *lmo2795-nanE*
359 operon might be involved in the transport of Man*N*Ac (Deutscher *et al.*, 2014). In addition to
360 NanE, the *lmo2795-nanE* operon encodes an RpiR-type regulator, a member of the repressor,

361 ORF, kinase (ROK) family, the EIIA and EIIBC components of a PTS system, a putative HAD
362 hydrolase and a putative oxidoreductase (Fig. 4A). To test whether these proteins are required
363 for the catabolism of Man*N*Ac, single deletion mutants and a mutant lacking both PTS
364 components, Lmo2797 and Lmo2799, were constructed in the EGD-e wildtype background
365 and their growth analyzed. All deletion mutants were able to grow in LSM containing glucose,
366 however, growth was enhanced for *L. monocytogenes* strains Δ*lmo2799* and Δ*lmo2797*
367 Δ*lmo2799*, lacking one or two of the PTS components, and the *nanE* deletion mutant (Fig. 4B-
368 C). In contrast, growth using Man*N*Ac as sole carbon source was reduced for the *lmo2795* and
369 *lmo2798* deletion strains lacking the RpiR transcriptional regulator and the putative HAD
370 hydrolase, respectively, and was nearly abolished for the strain lacking NanE (Fig. 4D-E).
371 Surprisingly, deletion strains Δ*lmo2799* and Δ*lmo2797* Δ*lmo2799* showed a better growth in
372 LSM with Man*N*Ac, similar to what was observed for the glucose containing LSM (Fig. 4C-E),
373 indicating that this PTS system is either not involved in the import of Man*N*Ac or that Man*N*Ac
374 can also be imported by other PTS systems of *L. monocytogenes*.
375
376 **Expression of the *lmo2795-nanE* operon is controlled by Lmo2795**
377 RpiR-type transcriptional regulators are often involved in the control of sugar metabolic
378 pathways in Gram-positive and Gram-negative bacteria (Sørensen and Hove-Jensen, 1996;
379 Yamamoto *et al.*, 2001; Jaeger and Mayer, 2008; Li *et al.*, 2017; Aleksandrzak-Piekarczyk *et*
380 *al.*, 2019). We thus wondered whether Lmo2795 regulates the expression of the *lmo2795-*
381 *nanE* operon. First, we conducted EMSA experiments to assess whether Lmo2795 binds to
382 the promoter region of the operon. For this purpose, increasing concentrations of purified His-
383 Lmo2795 were incubated with the DNA fragment containing the putative promoter of the
384 *lmo2795-nanE* operon. The migration of the DNA fragment was retarded in the presence of
385 His-Lmo2795 as compared to the DNA sample that did not contain the protein, suggesting that
386 a protein-DNA complex was formed. In addition, a so-called supershift could also be observed,
387 whose intensity increases with increasing protein concentrations. In contrast, no retardation of
388 the unspecific DNA control was observed in the presence of His-Lmo2795 (Fig. 5A). To test

389 whether Lmo2795 activates or represses the expression of the *lmo2795-nanE*, we quantified
390 the expression of *lmo2796* and *nanE* via quantitative real-time PCR. To allow for a better
391 comparison of the gene expression, we decided to isolate RNA of the *L. monocytogenes*
392 wildtype strain EGD-e and the *lmo2795* mutant that were grown in LSM with glucose as sole
393 carbon source due to the observed growth deficit of the *lmo2795* mutant in Man*N*Ac-containing
394 LSM. This analysis revealed that the expression of both genes, *lmo2796* and *nanE*, was
395 reduced in the *lmo2795* mutant as compared to the wildtype strain (Fig. 5B), suggesting that
396 Lmo2795 acts as a transcriptional activator under the tested condition.
397

398 **Discussion**

399 The human pathogen *L. monocytogenes* is able to withstand diverse environmental stress
400 conditions and thrives in changing environments. However, most studies on physiology and
401 stress tolerance of this organism were performed in complex media without any nutrient
402 limitation, a condition that is rarely found in their natural habitat or during infection. Within the
403 last decades, several chemically defined media have been developed to study the physiology
404 of the human pathogen *L. monocytogenes* (Welshimer, 1963; Premaratne *et al.*, 1991; Phan-
405 Thanh and Gormon, 1997; Tsai and Hodgson, 2003; Jarvis *et al.*, 2016; Whiteley *et al.*, 2017).
406 In this study, we wanted to determine the growth range of *L. monocytogenes* strains EGD-e
407 and 10403S, two widely used wildtype strains, at low pH and high salt concentration in the
408 *Listeria* synthetic medium (LSM) (Whiteley *et al.*, 2017). This analysis showed that the growth
409 of both wildtype strains is already significantly reduced in the presence of 4% salt or when they
410 were grown at a pH of 5.5. In contrast, *L. monocytogenes* is able to grow in the presence of
411 up to 10% salt or in a pH range from pH 4 to 9.5, when a more complex medium is used
412 (Patchett *et al.*, 1992; Arizcun *et al.*, 1998; Vasseur *et al.*, 2001). We further observed that
413 strain 10403S was less salt and pH resistant as compared to EGD-e when grown in LSM.
414 Interestingly, 10403S grew better than EGD-e in BHI medium which was adjusted to a low pH
415 (Cheng *et al.*, 2015). Resistance of *L. monocytogenes* to pH stress is mainly conferred by the

416 activity of the glutamate decarboxylase activity (GAD) system, a process which depends on
417 the availability of extracellular glutamate (Cotter *et al.*, 2001; Feehily *et al.*, 2014). The two *L.*
418 *monocytogenes* wildtype strains EGD-e and 10403S seem to use two distinct GAD systems,
419 which confer a different degree of acid resistance. The GAD system used by 10403S was
420 shown to give a higher acid tolerance (Feehily *et al.*, 2014; Cheng *et al.*, 2015). However,
421 complex medium such as BHI likely contains higher glutamate levels than LSM, which can be
422 used by the GAD system. Thus, the GAD system of 10403S might be less efficient when the
423 cells are grown in LSM and are therefore less resistant to acid stress.

424 *L. monocytogenes* encodes a variety of import systems for carbon sources, such as
425 PTS systems or ABC transporters. Chemically defined media that were used to grow *L.*
426 *monocytogenes* typically include glucose as the sole carbon source (Welshimer, 1963; Pine *et*
427 *al.*, 1989; Premaratne *et al.*, 1991; Phan-Thanh and Gormon, 1997; Tsai and Hodgson, 2003;
428 Jarvis *et al.*, 2016; Whiteley *et al.*, 2017). However, glucose was also replaced in some of
429 these chemically defined media by other carbon sources to determine whether they can
430 support growth of *L. monocytogenes* (Table 3) (Pine *et al.*, 1989; Premaratne *et al.*, 1991; Tsai
431 and Hodgson, 2003). The capability of *L. monocytogenes* to utilize other carbon sources than
432 glucose in LSM was not yet investigated. We therefore determined the growth of *L.*
433 *monocytogenes* strains EGD-e and 10403S for 16 different carbon sources, ranging from
434 hexoses, hexose phosphates and amino sugars, as well as from mono- and disaccharides
435 (summarized in Table 3). Most of the tested carbon sources, such as mannose, cellobiose,
436 trehalose and glucosamine (Glc*N*) could be metabolized by *L. monocytogenes*. The hexose
437 phosphate sugars Glc-1-P and Glc-6-P, which are found in the cytoplasm of host cells, could
438 not support growth of *L. monocytogenes* Scott A in modified Welshimer's broth (MWB)
439 (Premaratne *et al.*, 1991), similar to the results obtained in our study. For the growth
440 experiments, *L. monocytogenes* strains were grown in BHI medium, in which the major
441 virulence regulator PrfA is inactive (Renzoni *et al.*, 1997), and directly transferred into MWB or
442 LSM. However, PrfA is required to activate the expression of the hexose phosphate transporter
443 Hpt. Therefore, characterization of growth and the general physiology of *L. monocytogenes* in

444 the presence of Glc-1-P and Glc-6-P as sole carbon source, requires a pre-growth under PrfA-
445 activating conditions, for instance in a chemically defined medium or in BHI medium containing
446 activated charcoal, Chelex or Amberlite XAD (Chico-Calero *et al.*, 2002; Portman *et al.*, 2017;
447 Gaballa *et al.*, 2021).

448 In this study, we could also show that *L. monocytogenes* is able to grow on different
449 amino sugars, such as Glc*N*, Glc*N*Ac and Man*N*Ac. The catabolism of Man*N*Ac usually
450 depends on the presence of the Man*N*Ac-6-phosphate epimerase NanE, which converts
451 Man*N*Ac-6-phosphate to Glc*N*Ac-6-phosphate. The genome of *L. monocytogenes* EGD-e
452 contains one gene encoding a NanE homolog, *lmo2801*. A strain lacking Lmo2801 is unable
453 to grow in LSM with Man*N*Ac as sole carbon source, suggesting that Lmo2801 is indeed the
454 only NanE homolog in *L. monocytogenes*. NanE is encoded in the *lmo2795-nanE* operon,
455 which also contains two components of a PTS system. Surprisingly, strains lacking one or both
456 components showed wildtype-like or even enhanced growth on Man*N*Ac, suggesting that they
457 are either not involved in Man*N*Ac import or that *L. monocytogenes* EGD-e encodes additional
458 Man*N*Ac importers. Absence of individual PTS systems can also lead to the overexpression of
459 another PTS system (Stoll and Goebel, 2010), which could explain why strains lacking the
460 EIIB/C component Lmo2799 grew better than the wildtype in LSM with glucose or Man*N*Ac.
461 The expression of the *lmo2795-nanE* operon is controlled by the RpiR-type transcriptional
462 regulator Lmo2795. Unfortunately, we were unable to determine and compare the expression
463 levels of the L. monocytogenes wildtype and the lmo2795 mutant, when they were grown in
464 Man*N*Ac-containing LSM due to the growth differences. However, qRT-PCR analysis revealed
465 that Lmo2795 acts as an activator of the *lmo2795-nanE* operon in glucose-containing LSM.
466 Based on the observation that a strain lacking NanE had a growth deficit in LSM with Man*N*Ac
467 as sole carbon source, we assume that Lmo2795 also activates the expression of the *lmo2795-*
468 *nanE* under this condition as we would expect at least wildtype-like growth of the *nanE* mutant,
469 if Lmo2795 acts as a repressor. Further analysis is required to support this hypothesis.

470

471 **Acknowledgements**

472 We thank Julia Busse for technical assistance, Fabienne Dreier and Tayfun Acar for their help
473 with constructing strains and plasmids and Pascale Cossart for sharing the *prfA* mutant. We
474 are grateful to Prof. Jörg Stülke for providing JR and LMS with laboratory space, equipment
475 and consumables and to the Göttingen Center for Molecular Biosciences (GZMB) for financial
476 support.

477

478 **Funding**

479 This work was funded by the German research foundation (DFG) grant RI 2920/3-1 to JR.

480

481 **Conflict of Interest**

482 The authors declare no conflict of interest. All co-authors have seen and agree with the
483 contents of the manuscript and there is no financial interest to report.

484

485 **Author Contributions**

486 LMS: Investigation, formal analysis, conceptualization, methodology, supervision, validation,
487 writing – review & editing. AK: Investigation, formal analysis, writing – review & editing. JR:
488 Investigation, conceptualization, supervision, funding acquisition, writing – original draft.

489

490 **References**

491 Aké, F.M.D., Joyet, P., Deutscher, J., and Milohanic, E. (2011) Mutational analysis of glucose
492 transport regulation and glucose-mediated virulence gene repression in *Listeria*
493 *monocytogenes*. *Mol Microbiol* **81**: 274–293.

494 Aleksandrzak-Piekarczyk, T., Szatraj, K., and Kosiorek, K. (2019) GlaR (YugA)-a novel RpiR-
495 family transcription activator of the Leloir pathway of galactose utilization in *Lactococcus*

496 *lactis* IL1403. *Microbiologyopen* **8**: e00714.

497 Angata, T. and Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto
498 acids: an evolutionary perspective. *Chem Rev* **102**: 439–469.

499 Arizcun, C., Vasseur, C., and Labadie, J.C. (1998) Effect of several decontamination
500 procedures on *Listeria monocytogenes* growing in biofilms. *J Food Prot* **61**: 731–734.

501 Bishop, D.K. and Hinrichs, D.J. (1987) Adoptive transfer of immunity to *Listeria*
502 *monocytogenes*. The influence of in vitro stimulation on lymphocyte subset
503 requirements. *J Immunol* **139**: 2005–2009.

504 Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities
505 of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

506 Camilli, A., Tilney, L.G., and Portnoy, D.A. (1993) Dual roles of *plcA* in *Listeria*
507 *monocytogenes* pathogenesis. *Mol Microbiol* **8**: 143–157.

508 Cheng, C., Yang, Y., Dong, Z., Wang, X., Fang, C., Yang, M., et al. (2015) *Listeria*
509 *monocytogenes* varies among strains to maintain intracellular pH homeostasis under
510 stresses by different acids as analyzed by a high-throughput microplate-based
511 fluorometry. *Front Microbiol* **6**: 15.

512 Chico-Calero, I., Suárez, M., González-Zorn, B., Scortti, M., Slaghuis, J., Goebel, W., and
513 Vázquez-Boland, J.A. (2002) Hpt, a bacterial homolog of the microsomal glucose- 6-
514 phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. *Proc Natl*
515 *Acad Sci U S A* **99**: 431–436.

516 Cotter, P.D., Gahan, C.G., and Hill, C. (2001) A glutamate decarboxylase system protects
517 *Listeria monocytogenes* in gastric fluid. *Mol Microbiol* **40**: 465–475.

518 Deutscher, J., Aké, F., Zebre, A., Cao, T., Kentache, T., Pham, Q., et al. (2014)
519 Carbohydrate utilization by *Listeria monocytogenes* and its influence on virulence gene
520 expression. In Listeria monocytogenes*: Food Sources, Prevalence and Management*

521 *Strategies*. pp. 49–76.

522 Dhiman, A., Bhatnagar, S., Kulshreshtha, P., and Bhatnagar, R. (2014) Functional
523 characterization of WalRK: A two-component signal transduction system from *Bacillus*
524 *anthracis*. *FEBS Open Bio* **4**: 65–76.

525 Feehily, C., Finnerty, A., Casey, P.G., Hill, C., Gahan, C.G.M., O'Byrne, C.P., and Karatzas,
526 K.-A.G. (2014) Divergent evolution of the activity and regulation of the glutamate
527 decarboxylase systems in *Listeria monocytogenes* EGD-e and 10403S: roles in
528 virulence and acid tolerance. *PLoS One* **9**: e112649.

529 Fieseler, L., Schmitter, S., Teiserskas, J., and Loessner, M.J. (2012) Rhamnose-inducible
530 gene expression in *Listeria monocytogenes*. *PLoS One* **7**: e43444.

531 Friedman, S., Linsky, M., Lobel, L., Rabinovich, L., Sigal, N., and Herskovits, A.A. (2017)
532 Metabolic genetic screens reveal multidimensional regulation of virulence gene
533 expression in *Listeria monocytogenes* and an aminopeptidase that is critical for PrfA
534 protein activation. *Infect Immun* **85**: e00027-17.

535 Gaballa, A., Guariglia-Oropeza, V., Wiedmann, M., and Boor, K.J. (2019) Cross talk between
536 SigB and PrfA in *Listeria monocytogenes* facilitates transitions between extra- and
537 intracellular environments. *Microbiol Mol Biol Rev* **83**: e00034-19.

538 Gaballa, A., Sunil, S., Doll, E., Murphy, S.I., Bechtel, T., Guariglia-Oropeza, V., and
539 Wiedmann, M. (2021) Characterization of the roles of activated charcoal and Chelex in
540 the induction of PrfA regulon expression in complex medium. *PLoS One* **16**: e0250989.

541 Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., et al. (2001)
542 Comparative genomics of *Listeria* species. *Science* **294**: 849–852.

543 Gopal, S., Berg, D., Hagen, N., Schriefer, E.-M., Stoll, R., Goebel, W., and Kreft, J. (2010)
544 Maltose and maltodextrin utilization by *Listeria monocytogenes* depend on an inducible
545 ABC transporter which is repressed by glucose. *PLoS One* **5**: e10349.

546 Grubmüller, S., Schauer, K., Goebel, W., Fuchs, T.M., and Eisenreich, W. (2014) Analysis of
547 carbon substrates used by *Listeria monocytogenes* during growth in J774A.1
548 macrophages suggests a bipartite intracellular metabolism. *Front Cell Infect Microbiol* **4**:
549 156.

550 Hauf, S., Herrmann, J., Miethke, M., Gibhardt, J., Commichau, F.M., Müller, R., et al. (2019)
551 Aurantimycin resistance genes contribute to survival of *Listeria monocytogenes* during
552 life in the environment. *Mol Microbiol* **111**: 1009–1024.

553 Jaeger, T. and Mayer, C. (2008) The transcriptional factors MurR and catabolite activator
554 protein regulate *N*-acetylmuramic acid catabolism in *Escherichia coli*. *J Bacteriol* **190**:
555 6598–6608.

556 Jarvis, N.A., O'Bryan, C.A., Ricke, S.C., Johnson, M.G., and Crandall, P.G. (2016) A review
557 of minimal and defined media for growth of *Listeria monocytogenes*. *Food Control* **66**:
558 256–269.

559 Joseph, B., Mertins, S., Stoll, R., Schär, J., Umesha, K.R., Luo, Q., et al. (2008) Glycerol
560 metabolism and PrfA activity in *Listeria monocytogenes*. *J Bacteriol* **190**: 5412–5430.

561 Kentache, T., Milohanic, E., Cao, T.N., Mokhtari, A., Aké, F.M., Ma Pham, Q.M., et al. (2016)
562 Transport and catabolism of pentitols by *Listeria monocytogenes*. *J Mol Microbiol*
563 *Biotechnol* **26**: 369–380.

564 Lachmann, R., Halbedel, S., Adler, M., Becker, N., Allerberger, F., Holzer, A., et al. (2021)
565 Nationwide outbreak of invasive listeriosis associated with consumption of meat
566 products in health care facilities, Germany, 2014-2019. *Clin Microbiol Infect Off Publ Eur*
567 *Soc Clin Microbiol Infect Dis* **27**: 1035.e1-1035.e5.

568 Lecuit, M. (2005) Understanding how *Listeria monocytogenes* targets and crosses host
569 barriers. *Clin Microbiol Infect* **11**: 430–436.

570 Li, J., Evans, D.R., Freedman, J.C., and McClane, B.A. (2017) NanR regulates *nanI*

571 Sialidase expression by *Clostridium perfringens* F4969, a human enteropathogenic
572 strain. *Infect Immun* **85**: e00241-17.

573 Lobel, L., Sigal, N., Borovok, I., Belitsky, B.R., Sonenshein, A.L., and Herskovits, A.A. (2015)
574 The metabolic regulator CodY links *Listeria monocytogenes* metabolism to virulence by
575 directly activating the virulence regulatory gene prfA. *Mol Microbiol* **95**: 624–644.

576 Lourenco, A., Linke, K., Wagner, M., and Stessl, B. (2022) The saprophytic lifestyle of
577 *Listeria monocytogenes* and entry into the food-processing environment. *Front Microbiol*
578 **13**: 789801.

579 Mandin, P., Repoila, F., Vergassola, M., Geissmann, T., and Cossart, P. (2007) Identification
580 of new noncoding RNAs in *Listeria monocytogenes* and prediction of mRNA targets.
581 *Nucleic Acids Res* **35**: 962–974.

582 Matle, I., Mbatha, K.R., and Madoroba, E. (2020) A review of *Listeria monocytogenes* from
583 meat and meat products: Epidemiology, virulence factors, antimicrobial resistance and
584 diagnosis. *Onderstepoort J Vet Res* **87**: e1–e20.

585 Olson, M.E., King, J.M., Yahr, T.L., and Horswill, A.R. (2013) Sialic acid catabolism in
586 *Staphylococcus aureus*. *J Bacteriol* **195**: 1779–1788.

587 Patchett, R.A., Kelly, A.F., and Kroll, R.G. (1992) Effect of sodium chloride on the
588 intracellular solute pools of *Listeria monocytogenes*. *Appl Environ Microbiol* **58**: 3959–
589 3963.

590 Phan-Thanh, L. and Gormon, T. (1997) A chemically defined minimal medium for the optimal
591 culture of *Listeria*. *Int J Food Microbiol* **35**: 91–95.

592 Pine, L., Malcolm, G.B., Brooks, J.B., and Daneshvar, M.I. (1989) Physiological studies on
593 the growth and utilization of sugars by *Listeria* species. *Can J Microbiol* **35**: 245–254.

594 Plumbridge, J. and Vimr, E. (1999) Convergent pathways for utilization of the amino sugars
595 *N*-acetylglucosamine, *N*-acetylmannosamine, and *N*-acetylneuraminic acid by

596 *Escherichia coli*. *J Bacteriol* **181**: 47–54.

597 Portman, J.L., Dubensky, S.B., Peterson, B.N., Whiteley, A.T., and Portnoy, D.A. (2017)
598 Activation of the *Listeria monocytogenes* virulence program by a reducing environment.
599 *MBio* **8**: e01595-17.

600 Premaratne, R.J., Lin, W.J., and Johnson, E.A. (1991) Development of an improved
601 chemically defined minimal medium for *Listeria monocytogenes*. *Appl Environ Microbiol*
602 **57**: 3046–3048.

603 Radoshevich, L. and Cossart, P. (2018) *Listeria monocytogenes*: towards a complete picture
604 of its physiology and pathogenesis. *Nat Rev Microbiol* **16**: 32–46.

605 Renzoni, A., Klarsfeld, A., Dramsi, S., and Cossart, P. (1997) Evidence that PrfA, the
606 pleiotropic activator of virulence genes in *Listeria monocytogenes*, can be present but
607 inactive. *Infect Immun* **65**: 1515–1518.

608 Ripio, M.T., Brehm, K., Lara, M., Suárez, M., and Vázquez-Boland, J.A. (1997) Glucose-1-
609 phosphate utilization by *Listeria monocytogenes* is PrfA dependent and coordinately
610 expressed with virulence factors. *J Bacteriol* **179**: 7174–7180.

611 Schirmer, F., Ehrt, S., and Hillen, W. (1997) Expression, inducer spectrum, domain structure,
612 and function of MopR, the regulator of phenol degradation in *Acinetobacter*
613 *calcoaceticus* NCIB8250. *J Bacteriol* **179**: 1329–1336.

614 Smith, K. and Youngman, P. (1992) Use of a new integrational vector to investigate
615 compartment-specific expression of the *Bacillus subtilis spoIIM* gene. *Biochimie* **74**:
616 705–711.

617 Sørensen, K.I. and Hove-Jensen, B. (1996) Ribose catabolism of *Escherichia coli*:
618 characterization of the *rpiB* gene encoding ribose phosphate isomerase B and of the
619 *rpiR* gene, which is involved in regulation of *rpiB* expression. *J Bacteriol* **178**: 1003–
620 1011.

621 Stoll, R. and Goebel, W. (2010) The major PEP-phosphotransferase systems (PTSs) for
622 glucose, mannose and cellobiose of *Listeria monocytogenes*, and their significance for
623 extra- and intracellular growth. *Microbiology* **156**: 1069–1083.

624 Townsend, A., Strawn, L.K., Chapman, B.J., and Dunn, L.L. (2021) A systematic review of
625 *Listeria* species and *Listeria monocytogenes* prevalence, persistence, and diversity
626 throughout the fresh produce supply chain. *Foods* **10**: 1427.

627 Tsai, H.-N. and Hodgson, D.A. (2003) Development of a synthetic minimal medium for
628 *Listeria monocytogenes*. *Appl Environ Microbiol* **69**: 6943–6945.

629 Vasanthakrishnan, R.B., de Las Heras, A., Scortti, M., Deshayes, C., Colegrave, N., and
630 Vázquez-Boland, J.A. (2015) PrfA regulation offsets the cost of *Listeria* virulence
631 outside the host. *Environ Microbiol* **17**: 4566–4579.

632 Vasseur, C., Rigaud, N., Hébraud, M., and Labadie, J. (2001) Combined effects of NaCl,
633 NaOH, and biocides (monolaurin or lauric acid) on inactivation of *Listeria*
634 *monocytogenes* and *Pseudomonas spp*. *J Food Prot* **64**: 1442–1445.

635 Vimr, E.R., Kalivoda, K.A., Deszo, E.L., and Steenbergen, S.M. (2004) Diversity of microbial
636 sialic acid metabolism. *Microbiol Mol Biol Rev* **68**: 132–153.

637 Welshimer, H.J. (1963) Vitamin requirements of *Listeria monocytogenes*. *J Bacteriol* **85**:
638 1156–1159.

639 Whiteley, A.T., Garelis, N.E., Peterson, B.N., Choi, P.H., Tong, L., Woodward, J.J., and
640 Portnoy, D.A. (2017) c-di-AMP modulates *Listeria monocytogenes* central metabolism to
641 regulate growth, antibiotic resistance and osmoregulation. *Mol Microbiol* **104**: 212–233.

642 Xayarath, B. and Freitag, N.E. (2012) Optimizing the balance between host and
643 environmental survival skills: lessons learned from *Listeria monocytogenes*. *Future*
644 *Microbiol* **7**: 839–852.

645 Yamamoto, H., Serizawa, M., Thompson, J., and Sekiguchi, J. (2001) Regulation of the glv

646 operon in *Bacillus subtilis*: YfiA (GlvR) is a positive regulator of the operon that is
647 repressed through CcpA and *cre*. *J Bacteriol* **183**: 5110–5121.

648 Zeng, Z., Li, S., Boeren, S., Smid, E.J., Notebaart, R.A., and Abee, T. (2021) Anaerobic
649 growth of *Listeria monocytogenes* on rhamnose is stimulated by vitamin B(12) and
650 bacterial microcompartment-dependent 1,2-propanediol utilization. *mSphere* **6**:
651 e0043421.

652

653

654 **Tables**

655 **Table 1: Bacterial strains used in this study**

Unique ID	Strain name and resistance	Source
***Escherichia coli* strains**		
ANG1264	DH5α pKSV7; AmpR	(Smith and Youngman, 1992)
EJR149	XL10 Gold pWH844; AmpR	(Schirmer *et al.*, 1997)
EJR88	XL1-Blue pKSV7-Δ*lmo2795*; AmpR	This study
EJR121	XL1-Blue pKSV7-Δ*lmo2800*; AmpR	This study
EJR122	XL10-Gold pKSV7-Δ*lmo2799*; AmpR	This study
EJR123	XL10-Gold pKSV7-Δ*lmo2798*; AmpR	This study
EJR131	DH5α pWH844-*lmo2795*; AmpR	This study
EJR164	XL1-Blue pKSV7-Δ*lmo2797*; AmpR	This study
EJR210	XL10-Gold pKSV7-Δ*nanE*; AmpR	This study
EJR236	XL10-Gold pKSV7-Δ*lmo2796*; AmpR	This study
***Listeria monocytogenes* strains**		
ANG873	EGD-e	(Glaser *et al.*, 2001)
ANG1263	10403S; StrepR	(Bishop and Hinrichs, 1987)
BUG2214	EGD-e Δ*prfA*	(Mandin *et al.*, 2007)
LJR123	EGD-e Δ*lmo2795*	This study
LJR132	EGD-e Δ*lmo2798*	This study
LJR135	EGD-e Δ*lmo2799*	This study
LJR204	EGD-e Δ*lmo2797*	This study
LJR205	EGD-e Δ*lmo2799* Δ*lmo2797*	This study
LJR206	EGD-e Δ*lmo2800*	This study
LJR207	EGD-e Δ*nanE*	This study

656

657 **Table 2: Primers used in this study**

Number	Name	Sequence
AK004	pWG844-*lmo2795* fw	AAAGGATCCATGACGATTTTAAATGAAATCCAACAAAATTA
AK005	pWG844-*lmo2795* rev	TTTGTCGACTTAATGAAATTCATCGGTAACAGTATTGA
JR142	*lmo2797* up fw	CGCGGATCCGTTGCAGACCGAGAACAAAAG
JR143	*lmo2797* up rev	GTGAAATTATTATAACATGCCCGTACCCCC
JR144	*lmo2797* down fw	ACGGGCATGTTATAATAATTTCACTAAAGAGGTGAAG
JR145	*lmo2797* down rev	CCGGAATTCCTGCTGTATCGAACATCGGC
JR211	*lmo2800* up fw	CGCGGATCCGTTAGTGGTTTCCTGCCAAG
LMS169	qRT-PCR *gyrB* fw	GGTGGCTCATGGATAACGTCT
LMS170	qRT-PCR *gyrB* rev	GCGAACTTGCTTTCTTGAATCGT
LMS346	*lmo2795* down rev	AAAGAGCTCCGGTGAAATCTAGGTCCATTCC
LMS347	*lmo2795* down fw	CAACAAAATTACATCAATACTGTTACCGATGAATTTCATTAA
LMS348	*lmo2795* up rev	AACAGTATTGATGTAATTTTGTTGGATTTCATTTAAAATCGTCAT
LMS349	*lmo2795* up fw	AAATCTAGACGAAGCAAATTCAAGCCAAGCA
LMS369	qRT-PCR *nanE* fw	GTGGGGGTGATAAATACGTCCG
LMS375	qRT-PCR *rplD* fw	ATGCCTTGTAAGTTGCGTGC
LMS376	qRT-PCR *rplD* rev	GGTTTGACTTTCGATGCACCT
LMS377	qRT-PCR *nanE* rev	TTCAAGGTGGTGCTGTTGGT
LMS384	qRT-PCR *lmo2796* fw	AGCCCACTTCACATGCTGTA
LMS385	qRT-PCR *lmo2796* rev	CTTGGCGAGTTTTGGTTGGG

LMS395	*lmo2796* up fw	AAAGGATCCCCGTGAATTAAATGAGAAGAATCAAATGG
LMS396	*lmo2796* up rev	ATCTTTTTCGGCGTCGACACAAAGATAATTTTCCATTTCTTC
LMS397	*lmo2796* down fw	CTTTGTGTCGACGCCGAAAAAGATGACGATTTTAAATGA
LMS398	*lmo2796* down rev	TTTCTGCAGGTGAAAAAGGCATCTGGGTCC
LMS401	*nanE* up fw	AAAGGATCCAATAATGAGCTAAAATATACGGTATAAGCC
LMS402	*nanE* up rev	TTGAATTTTTTGGATTTTTTCCATAACAGAATTGCCCAC
LMS403	*nanE* down fw	ATGGAAAAAATCCAAAAAATTCAAGAAGAAAGAGGTAAGTGA
LMS404	*nanE* down rev	TTTCTGCAGCCAATCATATGTGTGGCGCTATTTT
LMS408	*lmo2800* up rev	TCCTTCAGCAATAATTACGCCAACTTTGATTTGCTTTG
LMS409	*lmo2800* down fw	GTTGGCGTAATTATTGCTGAAGGAAAAGAAGTATCACTATAA
LMS410	*lmo2800* down rev	TTTCTGCAGATGTGGTCGCCGGGTG
LMS411	*lmo2799* up fw	AAAGGATCCCAAAGCAAATCAAAGTTGGCGTAATT
LMS412	*lmo2799* up rev	GTCTGCAACTATCTTTTGTACATGTACCCGAATAGACAT
LMS413	*lmo2799* down fw	CATGTACAAAAGATAGTTGCAGACCGAGAACAAAAG
LMS414	*lmo2799* down rev	TTTCTGCAGCATCAGCCAATGTTGCAAAGGT
LMS417	*lmo2798* up fw	AAAAAGCTTCCCACTAACGGCTTTAATCATTGAG
LMS418	*lmo2798* up rev	CATTATTTCTGTGTCAAAGTCCATCACAATCGCTTT

LMS419	*lmo2798* down fw	ATGGACTTTGACACAGAAATAATGGCTGAATATAA TAAATAAGGG
LMS420	*lmo2798* down rev	TTTCTGCAGAGCCCACTTCACATGCTGTAA
LMS428	EMSA unspecific fw	CTTTAATATCCTCTAAGCCAGCATTTTTG
LMS429	EMSA unspecific rev	CAAGATGCAGCGAAGAAAATGGAA
LMS430	EMSA *lmo2795* rev	CTAACCCACCTTTGATTTTTTCCATAAC
LMS431	EMSA *lmo2795* fw	CGGAACACCCAACAAATTACCAAT

658

659 **Table 3: Carbon sources supporting growth of *L. monocytogenes* in chemically defined**

660 **media.**

	(Pine *et al.*, 1989)		(Tsai and Hodgson, 2003)		(Premaratne *et al.*, 1991)		This study	
	ABCD medium[3]		HTM[3]		MWB[3]		LSM[3]	
Carbon source[1]	Conc.[2]	Growth	Conc.[2]	Growth	Conc.[2]	Growth	Conc.[2]	Growth
Glucose	0.25%	yes	1%	yes	1%	yes	1%	yes
Fructose			1%	yes	1%	yes		
Galactose	0.25%	no	1%	no	1%	no	1%	no
Mannose			1%	yes	1%	yes	1%	yes
Mannitol			1%	no	1%	no	1%	no
Arabinose			1%	no	1%	no		
Ribose			1%	no	1%	no		
Xylose	0.25%	no	1%	no	1%	no		
Lactose	0.25%	yes	1%	no	1%	no		
Rhamnose	0.25%	yes	1%	no			1%	weak
Maltose			1%	no	1%	weak	1%	weak
Sucrose			1%	no	1%	no	1%	no
Cellobiose					1%	yes	1%	yes
Trehalose					1%	yes	1%	yes
Glc-1-P					1%	no	1%	yes[4]
Glc-6-P					1%	no	1%	yes[4]
Glc*N*					1%	yes	1%	yes
Glc*N*Ac					1%	yes	1%	yes
Mur*N*Ac					1%	yes		

Man*N*Ac							1%	yes
Neu5Ac							1%	no
Glycerol			1%	yes	1%	weak	1%	yes
Succinate					1%	no	1%	weak

661 [1] The following carbon sources do not support growth: Melibiose, Raffinose, Sorbose, Sorbitol,
662 Galactose-1-phosphate, Galactose-6-phosphate, Lactate, Citrate, Isocitrate, α-Ketoglutarate,
663 Fumarate, Malate, Pyruvate, Acetate, Chitin (Premaratne *et al.*, 1991). Abbreviations: Glc-1-P =
664 Glucose-1-phosphate, Glc-6-P = Glucose-6-phosphate, Glc*N* = glucosamine, GlcNAc = *N*-
665 acetylglucosamine, Mur*N*Ac = *N*-acetylmuramic acid, Man*N*Ac = *N*-acetylmannosamine, Neu5Ac = *N*-
666 acetylneuraminic acid.

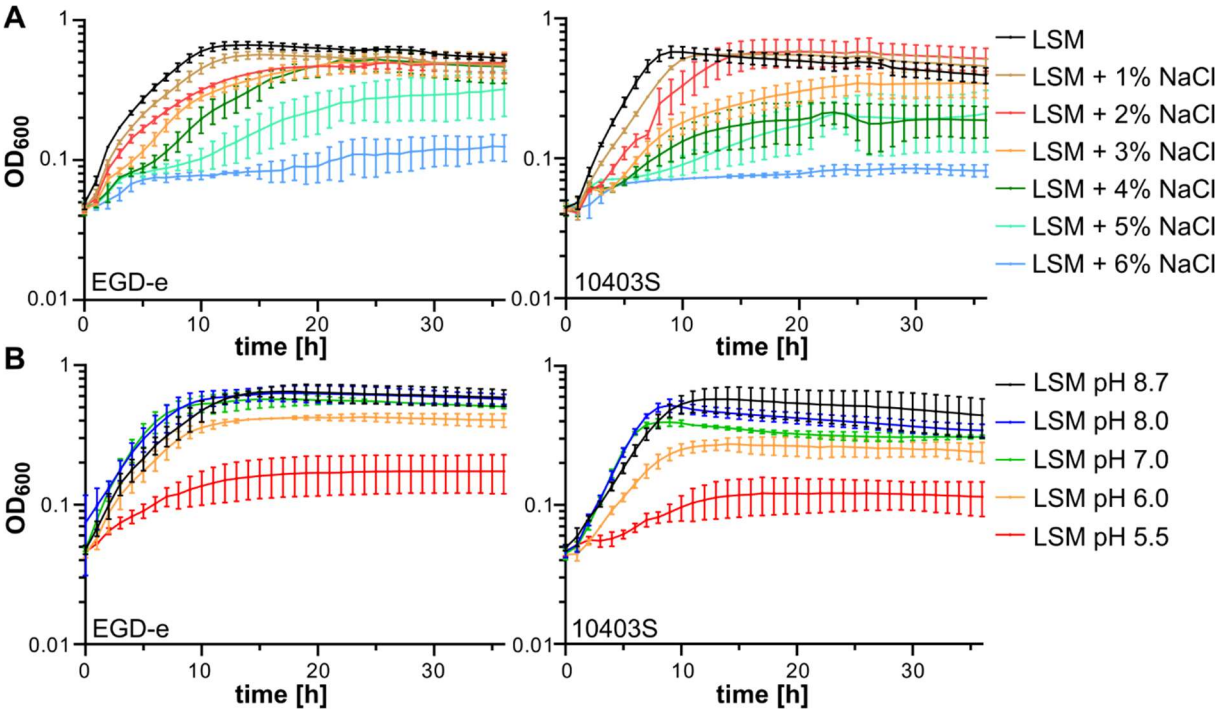
667 [2] Conc. = concentration of carbon source

668 3 ABCD medium = *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered, chemically defined
669 medium; HTM = Hsiang-Ning Tsai medium, MWB = Modified Welshimer's broth, LSM = *Listeria* synthetic
670 medium

671 [4] Pre-growth in LSM glucose with low concentrations of BCAA, a PrfA-activating condition, is required.

672 **Figures and Figure legends**

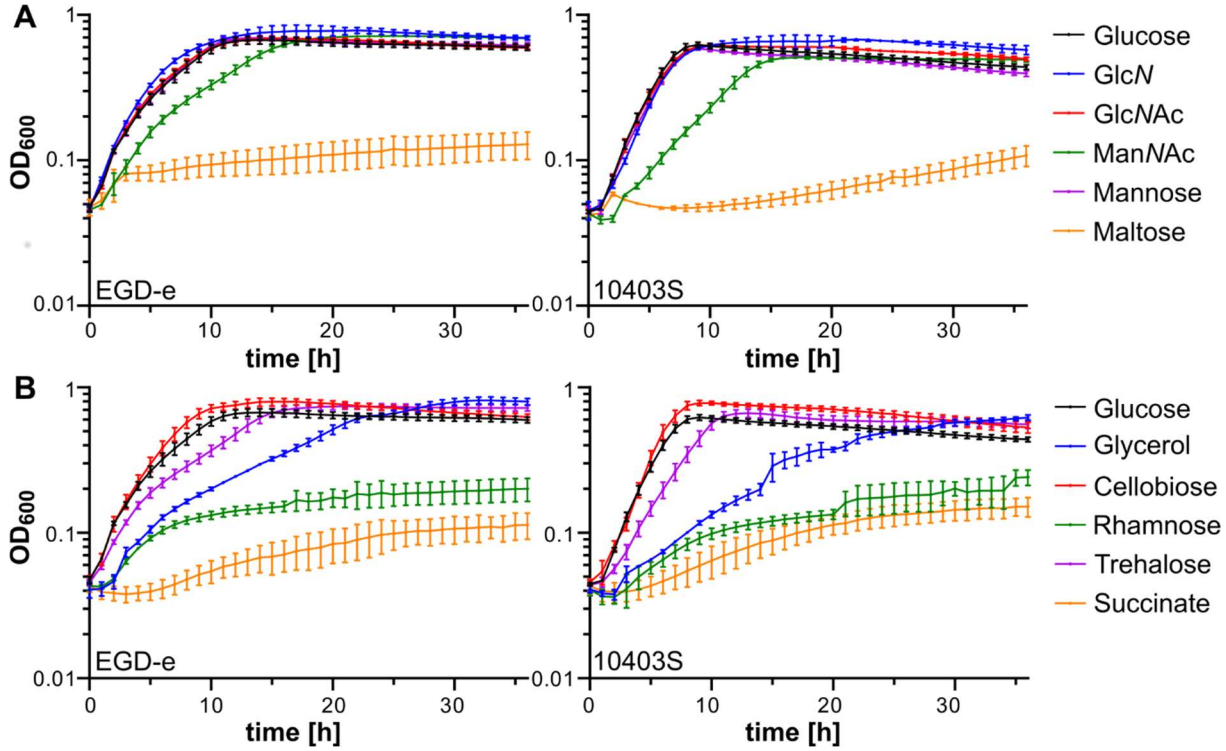
673



674

675 **Figure 1: Salt and pH resistance of *L. monocytogenes* wildtype strains.** (A) *L.*
676 *monocytogenes* strains EGD-e and 10403S were grown in LSM containing 1% glucose and
677 increasing concentrations of NaCl as described in the methods section. (B) *L. monocytogenes*
678 strains EGD-e and 10403S were grown in LSM containing 1% glucose (pH 8.7). The pH of
679 LSM was adjusted to the indicated pH-values using HCl. The average values and standard
680 deviations of three independent experiments were plotted.

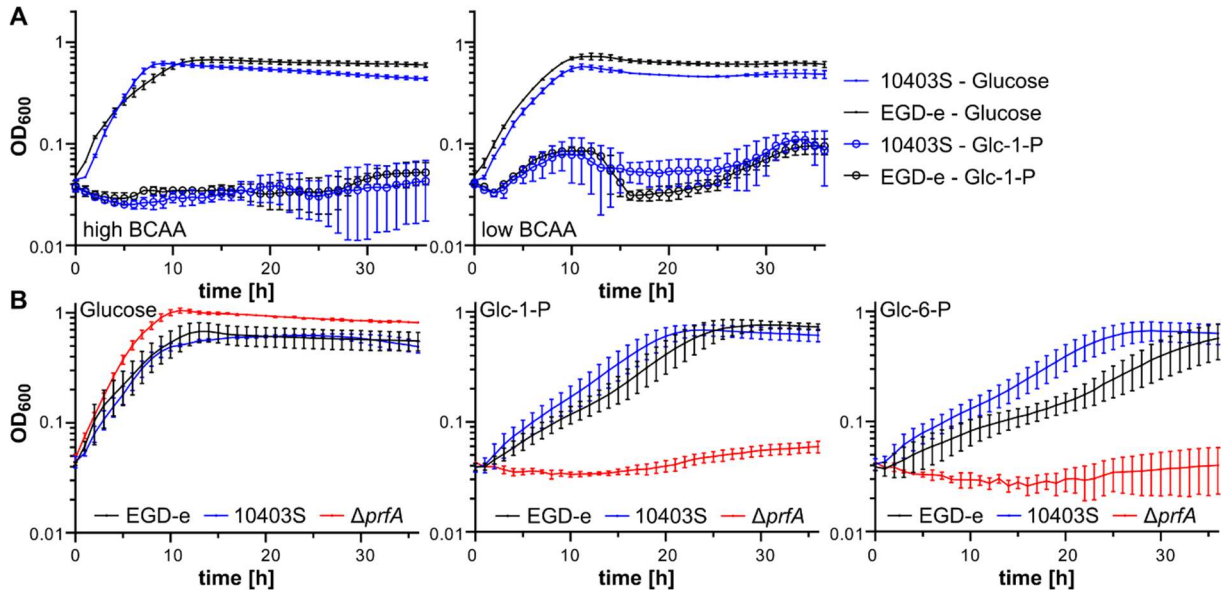
681



682

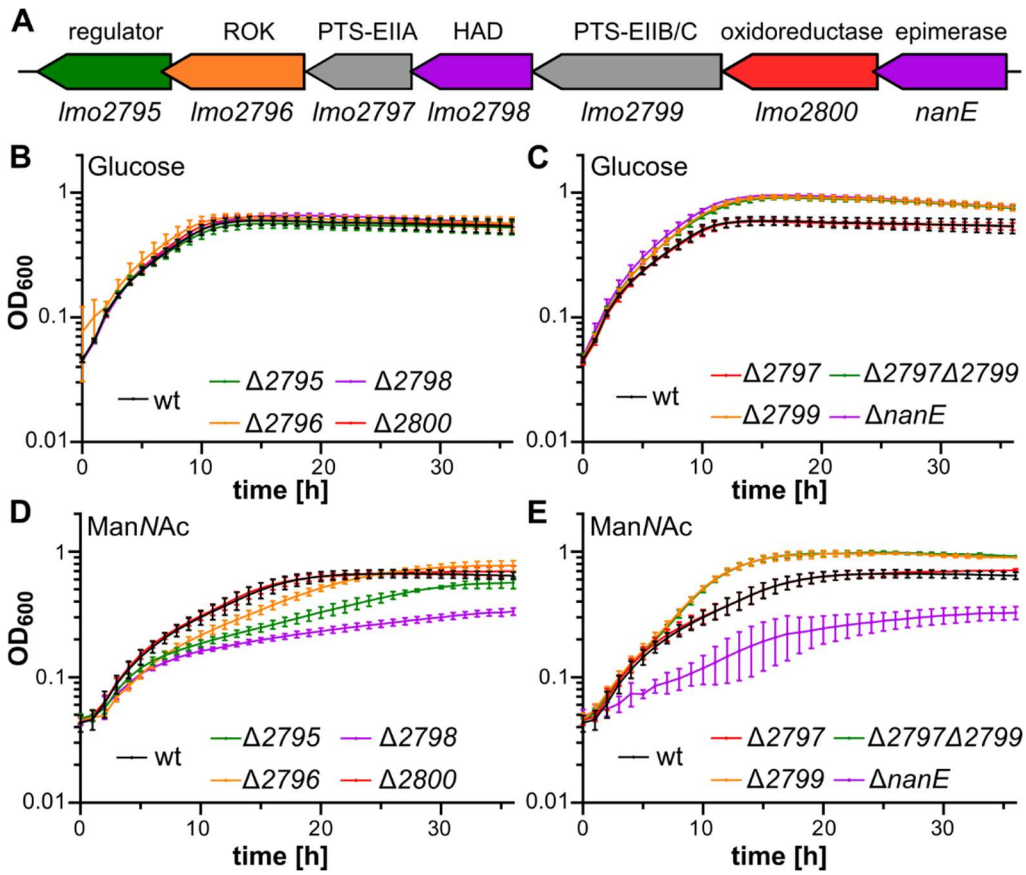
683 **Figure 2: Growth in LSM media with different carbon sources.** (A-B) *L. monocytogenes*
684 strains EGD-e and 10403S were grown in *Listeria* synthetic medium (LSM) containing 1% of
685 the indicated carbon sources as described in the methods section. The average values and
686 standard deviations of three independent experiments were plotted. Glc*N*Ac = *N*-
687 acetylglucosamine, Man*N*Ac = *N*-acetylmannosamine, Glc*N* = glucosamine.

688



689

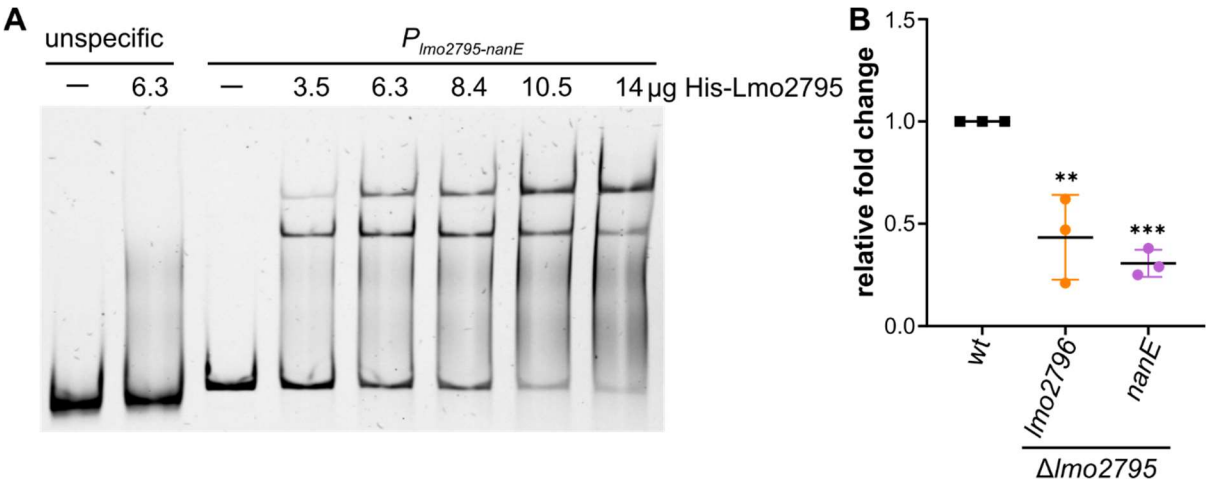
690 **Figure 3: Pre-growth of *L. monocytogenes* in LSM glucose enables growth on glucose-**
691 **1-phosphate and glucose-6-phosphate.** (A) *L. monocytogenes* strains EGD-e and 10403S
692 were grown in *Listeria* synthetic medium (LSM) containing 1% of the indicated carbon sources
693 as described in the methods section. LSM was either prepared with the standard complete
694 amino acid mix (high BCAA) or the complete amino acid mix with 10-fold less branched chain
695 amino acids (low BCAA). The average values and standard deviations of three independent
696 experiments were plotted. (B) *L. monocytogenes* strains EGD-e, 10403S and Δ*prfA* were pre-
697 grown in *Listeria* synthetic medium (LSM) containing 1% glucose and subsequently transferred
698 to LSM containing the indicated carbon sources as described in the methods section. The
699 average values and standard deviations of four independent experiments were plotted. Glc-1-
700 P = glucose-1-phosphate, Glc-6-P = glucose-6-phosphate.
701



702

703 **Figure 4: The *lmo2795-nanE* operon is required for Man*N*Ac utilization.** (A) Gene
704 arrangement of the *lmo2795-nanE* operon of *L. monocytogenes*. Predicted protein functions
705 are indicated above the genes. ROK – member of the repressor, ORF, kinase family; PTS-

706 EIIA and PTS-EIIB/C – components of a phosphotransferase system; HAD – member of the
707 haloacid dehydrogenase-like hydrolase superfamily. (B-G) Growth curves. The indicated *L.*
708 *monocytogenes* strains were grown in LSM containing (B, C) 1% glucose, (D, E) 1% Man*N*Ac
709 as sole carbon source. The average values and standard deviations of three independent
710 experiments were plotted.
711



712

713 **Figure 5: Lmo2795 regulates the expression of the *lmo2795-nanE* operon.** (A) EMSA
714 assay. Increasing concentrations of His-Lmo2795 were incubated with the DNA fragment
715 containing the predicted promoter region of the *lmo2795-nanE* operon. The DNA-protein
716 complexes were separated on an 8% polyacrylamide gel, which was subsequently stained
717 using HDGreen™. A DNA fragment containing a short region from within the operon was used
718 as the unspecific control. Reactions without protein were used as negative controls (-). (B)
719 Analysis of *lmo2796* and *nanE* expression by qRT-PCR. RNA was isolated from *L.*
720 *monocytogenes* strains EGD-e (wt) and Δ*lmo2795* grown in LSM containing 1% glucose as
721 sole carbon source as described in the methods section. Expression of *lmo2796* and *nanE*
722 was normalized to the expression of *gyrB* and *rplD* and fold changes were calculated using the
723 $\Delta\Delta C_T$ method. Averages and standard deviations of three independent RNA extractions were
724 plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple
725 comparison test was performed (** $p \leq 0.01$; *** $p \leq 0.001$).