Characterization of the growth behavior of *Listeria monocytogenes* in *Listeria* synthetic media

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

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, both *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* 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.

Originality-Significance Statement

Current knowledge of growth and survival of the human pathogen *Listeria monocytogenes* under diverse stress conditions is mostly generated in complex medium, a condition that is rarely found in the environment or host of the pathogen. Our work contributes to the characterization of the physiology of *L. monocytogenes* grown under nutrient limiting conditions and its growth requirements with regards to metabolizable carbon sources.

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

The survival of *L. monocytogenes* in food processing facilities and within the host is enabled due to its ability to grow under diverse stress conditions (reviewed in (Gaballa *et al.*, 2019; Wiktorczyk-Kapischke *et al.*, 2021; Osek *et al.*, 2022)). Previous studies reported that *L. monocytogenes* can grow at temperatures between 1 and 45°C, in a pH range from 4.0 to 9.5 or in the presence of up to 10% salt (Patchett *et al.*, 1992; Arizcun *et al.*, 1998; Vasseur *et al.*, 2001). *L. monocytogenes* is also able to use a variety of carbon sources ranging from simple monosaccharides (glucose, fructose) to more complex sugars (maltodextrin) and polyols (glycerol, D-arabitol, D-xylitol) (Gopal *et al.*, 2010; Deutscher *et al.*, 2014; Kentache *et al.*, 2016). The ability to take up such a wide range of carbon sources is facilitated by a diverse set of transporters, which are encoded in the genome of *L. monocytogenes*. Most of the carbon sources are imported into the cell via the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). The genome of *L. monocytogenes* wildtype strain EGD-e contains 86 PTS genes, which encode for 29 complete and 12 incomplete PTS complexes (Glaser *et al.*, 2001; Stoll and Goebel, 2010). These PTS transporters are involved in the transport of diverse carbon sources such as glucose, fructose, D-arabitol and D-xylitol

72 (Deutscher *et al.*, 2014; Kentache *et al.*, 2016). Some of these PTS transporters have also
73 overlapping substrate specificities. For instance, the two PTS systems ManLMN (MptACD)
74 and MpoABCD, encoded by *lmo0096-0098* and *lmo0784-0781*, respectively, are both involved
75 in the transport of glucose and mannose (Stoll and Goebel, 2010; Aké *et al.*, 2011). Inactivation
76 of all PTS systems, e.g. by deleting the phosphotransferase system enzyme I encoding gene
77 *ptsI*, did not abolish fermentation of some sugars such as glucose, fructose or mannose,
78 suggesting that *L. monocytogenes* possesses also other sugar transporters (Deutscher *et al.*,
79 2014). Indeed, *L. monocytogenes* encodes three GlcU-like non-PTS permeases (Lmo0169,
80 Lmo0176, Lmo0424), which could be involved in the import of these sugars (Aké *et al.*, 2011;
81 Deutscher *et al.*, 2014). The import of L-rhamnose is accomplished by the major facilitator
82 superfamily protein Lmo2850, which uses a proton symport mechanism (Fieseler *et al.*, 2012;
83 Deutscher *et al.*, 2014; Zeng *et al.*, 2021). Within the host, *L. monocytogenes* uses mainly
84 glucose-1-phosphate, glucose-6-phosphate and glycerol as carbon sources (Ripio *et al.*, 1997;
85 Grubmüller *et al.*, 2014). The transporter Hpt, also named UhpT, takes up hexose phosphates
86 from the host cell cytosol (Chico-Calero *et al.*, 2002), while the glycerol uptake facilitator GlpF
87 facilitates the import of glycerol (Joseph *et al.*, 2008). The expression of *hpt* thereby depends
88 on PrfA, the main virulence regulator of *L. monocytogenes* (Ripio *et al.*, 1997; Chico-Calero *et*
89 *al.*, 2002). *L. monocytogenes* possesses eight predicted carbohydrate ATP-binding cassette
90 (ABC) transporters. One of these transporters, which is encoded by *lmo2123-2125*, is required
91 for the import of maltose and maltodextrin (Gopal *et al.*, 2010). The remaining seven ABC
92 transporters have not yet been studied.

93 For many studies on the physiology and stress tolerance of *L. monocytogenes*, bacteria
94 were cultured in complex medium such as brain heart infusion (BHI) or tryptic soy broth (TSB).
95 In their natural habitat or within their host, *L. monocytogenes* likely encounters more limiting
96 growth conditions. Within the last decades, several minimal media have been developed which
97 can be used to grow *L. monocytogenes* (Welshimer, 1963; Premaratne *et al.*, 1991; Phan-
98 Thanh and Gormon, 1997; Tsai and Hodgson, 2003; Jarvis *et al.*, 2016; Whiteley *et al.*, 2017).
99 In this study, we aimed to characterize the growth of the two widely used *L. monocytogenes*

100 wildtype strains EGD-e and 10403S, which belong to serotype 1/2a, in the recently developed
101 *Listeria* synthetic medium (LSM) (Whiteley *et al.*, 2017). *L. monocytogenes* strains belonging
102 to serotype 1/2a are often recovered from food, environmental and clinical samples (Ward *et*
103 *al.*, 2008; Orsi *et al.*, 2011) and thus encounter diverse environmental conditions. We thus
104 determined the growth behavior of EGD-e and 10403S in LSM under stress conditions, which
105 they encounter in food or the environment or within the host during infection, namely high salt
106 and low pH stress. To our knowledge, growth of *L. monocytogenes* in LSM was so far only
107 assessed with glucose as a sole carbon source. We therefore determined, which other carbon
108 sources can facilitate growth of *L. monocytogenes* EGD-e and 10403S in LSM. While 95% of
109 the open reading frames are conserved between EGD-e and 10403S, the genomes differ by
110 over 30,000 single nucleotide polymorphisms (Glaser *et al.*, 2001; Bécavin *et al.*, 2014). It is
111 thus not surprising that we could observe differences in the growth behavior between these
112 two strains.
113

114 **Experimental procedures**

115 **Bacterial strains and growth conditions.** All strains and plasmids used in this study are
116 listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium and *Listeria*
117 *monocytogenes* strains in brain heart infusion (BHI) medium or *Listeria* synthetic medium
118 (LSM) at 37°C unless otherwise stated. Where required, antibiotics and supplements were
119 added to the medium at the following concentrations: for *E. coli* cultures, ampicillin (Amp) at
120 100 µg ml^{-1}, kanamycin (Kan) at 50 µg ml^{-1}, and for *L. monocytogenes* cultures,
121 chloramphenicol (Cam) at 7.5 µg ml^{-1} and Kan at 50 µg ml^{-1}.

122 LSM was prepared in accordance with the recipe from Whiteley et al. with minor
123 modifications (Whiteley *et al.*, 2017). L-leucine, L-methionine and L-valine were used instead
124 of the corresponding DL-enantiomers. L-arginine·HCl, riboflavin, $Na_2HPO_4 \cdot 7\ H_2O$, $CoCl_2 \cdot 7\ H_2O$
125 and L-cysteine·2·HCl were substituted for L-arginine, riboflavin 5' monophosphate sodium salt
126 hydrate, $Na_2HPO_4 \cdot 12\ H_2O$, $CoCl_2 \cdot 6\ H_2O$ and L-cysteine·HCl·H_2O, respectively (see Table 2 for
127 detailed LSM composition and preparation). The pH of LSM was adjusted with HCl, where

128 indicated. Sodium chloride was added to LSM for characterization of the growth of *L.*
129 *monocytogenes* strains under salt stress at the following concentrations: 1% (0.17 M), 2%
130 (0.34 M), 3% (0.51 M), 4% (0.68 M), 5% (0.86 M) and 6% (1.03 M). To test the ability of *L.*
131 *monocytogenes* to metabolize different carbon sources, 1% glucose, which is usually
132 contained in LSM, was replaced by 1% of the following carbon sources: glycerol, glucosamine
133 (Glc*N*), *N*-acetylglucosamine (Glc*N*Ac), *N*-acetylmannosamine (Man*N*Ac), glucose-1-
134 phosphate (Glc-1-P), glucose-6-phosphate (Glc-6-P), mannose, maltose, mannitol, sucrose,
135 rhamnose, trehalose, succinate, cellobiose, galactose and *N*-acetylneuraminic acid (Neu5Ac).
136 A previous study showed that the expression of *prfA* is induced in the presence of low
137 levels of branched chain amino acids (Lobel *et al.*, 2015). For growth of *L. monocytogenes*
138 under PrfA-activating conditions, the concentration of branched chain amino acids was 10-fold
139 reduced in freshly prepared LSM yielding 80 µM L-isoleucine, 80 µM L-leucine and 90 µM L-
140 valine.
141
142 **Growth assays.** Overnight cultures of the indicated *L. monocytogenes* strains were diluted in
143 fresh BHI medium to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.3 was reached.
144 Cells of 2 ml culture were collected per condition, washed twice with 1 ml of the corresponding
145 LSM and re-suspended in 1 ml LSM. The cultures were adjusted to an OD_{600} of 0.2 and 100 µl
146 of the cell suspension were transferred into wells of a 96-well plate containing 100 µl of the
147 corresponding LSM. The plate was incubated at 37°C with orbital shaking in the BioTek Epoch
148 2 microplate reader and the OD_{600} was measured every 15 min for at least 36 h. Averages and
149 standard deviations of at least three independent growth assays were plotted.
150 To test whether growth of *L. monocytogenes* was possible in LSM Glc-1-P and Glc-6-
151 P after pre-growth of the indicated strains under PrfA-activating conditions, overnight cultures
152 of the indicated *L. monocytogenes* strains were diluted in LSM glucose with low concentrations
153 of BCAA to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.3 was reached. Cells
154 were collected, washed and used to inoculate a 96-well plate containing the corresponding
155 LSM with low concentrations of BCAA as described above.

156

157 **Strain and plasmid construction.** All primers used in this study are listed in Table 3. For
158 markerless deletion of *lmo2795*, 1-kb fragments up- and downstream of *lmo2795* were
159 amplified by PCR using primer pairs LMS346/LMS347 and LMS348/LMS349, respectively.
160 The resulting PCR fragments were fused by PCR using primers LMS346/LMS349. The
161 deletion fragment was subsequently digested with *Xba*I and *Sac*I and ligated into plasmid
162 pKSV7 that had been cut with the same enzymes. Plasmid pKSV7-Δ*lmo2795* was recovered
163 in *E. coli* XL1-Blue yielding strain EJR88. For markerless deletion of *lmo2796*, *lmo2799*,
164 *lmo2800* and *nanE*, 1-kb fragments up- and downstream of the corresponding gene were
165 amplified by PCR using primer pairs LMS395/LMS396 and LMS397/LMS398 (*lmo2796*),
166 LMS411/LMS412 and LMS413/LMS414 (*lmo2799*), JR211/LMS408 and LMS409/LMS410
167 (*lmo2800*), LMS401/LMS402 and LMS403/LMS404 (*nanE*). The resulting PCR fragments
168 were fused by PCR using primers LMS395/LMS398 (*lmo2796*), LMS411/414 (*lmo2799*),
169 JR211/LMS410 (*lmo2800*) and LMS401/LMS404 (*nanE*). The Δ*lmo2799*, Δ*lmo2800* and
170 Δ*nanE* deletion fragments were digested with *BamH*I and *Pst*I and ligated into *BamH*I/*Pst*I cut
171 pKSV7. Plasmids pKSV7-Δ*lmo2796,* pKSV7-Δ*lmo2799* and pKSV7-Δ*nanE* were recovered in
172 *E. coli* XL10-Gold yielding strains EJR238, EJR122 and EJR210, respectively. pKSV7-
173 Δ*lmo2800* was recovered in *E. coli* XL1-Blue yielding strain EJR121. For the construction of
174 pKSV7-Δ*lmo2798* and pKSV7-Δ*lmo2797*, 1-kb fragments up- and downstream of the
175 corresponding gene were amplified by PCR using primer pairs LMS417/LMS418 and
176 LMS419/LMS420 (*lmo2798*) and JR142/JR143 and JR144/JR145 (*lmo2797*). The resulting
177 PCR fragments were fused by PCR using primers LMS417/LMS420 (*lmo2798*) and
178 JR142/JR145 (*lmo2797*), cut with *BamH*I and *EcoR*I and ligated into *BamH*I/*EcoR*I cut pKSV7.
179 pKSV7-Δ*lmo2798* was transformed into *E. coli* XL10-Gold yielding strain EJR123. Plasmid
180 pKSV7-Δ*lmo2797* was recovered in *E. coli* XL1-Blue yielding strain EJR164. Plasmids pKSV7-
181 Δ*lmo2795*, pKSV7-Δ*lmo2796*, pKSV7-Δ*lmo2797*, pKSV7-Δ*lmo2798*, pKSV7-Δ*lmo2799*,
182 pKSV7-Δ*lmo2800* and pKSV7-Δ*nanE* were transformed into *L. monocytogenes* strain EGD-e

183 and genes *lmo2795*, *lmo2797*, *lmo2798*, *lmo2799*, *lmo2800* and *nanE* deleted by allelic
184 exchange as previously described (Camilli *et al.*, 1993) yielding strains EGD-e Δ*lmo2795*
185 (LJR123), Δ*lmo2796* (LJR264), EGD-e Δ*lmo2797* (LJR204), EGD-e Δ*lmo2798* (LJR132),
186 EGD-e Δ*lmo2799* (LJR135), EGD-e Δ*lmo2800* (LJR206) and EGD-e Δ*nanE* (LJR207). To
187 construct strain EGD-e Δ*lmo2799* Δ*lmo2797* (LJR205), pKSV7-Δ*lmo2797* was transformed
188 into *L. monocytogenes* strain LJR204 and *lmo2797* deleted by allelic exchange.
189 For the purification of Lmo2795 with an N-terminal His-tag, *lmo2795* was amplified
190 using primer pair AK004/AK005. The resulting PCR product was digested with *BamH*I and *Sal*I
191 and ligated into pWH844. Plasmid pWH844-*lmo2795* was recovered in *E. coli* DH5α yielding
192 strain EJR131.
193

194 **RNA extraction.** RNA of the indicated *L. monocytogenes* strains was isolated following a
195 previously published method with minor modifications (Hauf *et al.*, 2019). Briefly, a single
196 colony was used to inoculate 10 ml LSM medium with 1% glucose as sole carbon source and
197 cultures were grown overnight at 37°C. Next day, cultures were used to inoculate 30 ml of
198 fresh LSM medium with 1% glucose to an OD_{600} of 0.1. When the cultures reached an OD_{600}
199 of 0.5 ± 0.05, 25 ml of the cell suspensions were harvested by centrifugation for 15 min at
200 4,000 rpm and 4°C and the pellet was snap frozen in liquid nitrogen and stored at -80°C. To
201 isolate the RNA, pellets were re-suspended in 1 ml killing buffer (20 mM Tris, pH 7.5, 5 mM
202 $MgCl_2$, 20 mM NaN_3), transferred to 1.5 ml microtubes and centrifuged at 13,000 rpm for 60
203 sec. Cells were then re-suspended in 1 ml lysis buffer I (25 % sucrose; 20 mM Tris-HCl pH 8,
204 0.25 mM EDTA) and 2 µl lysozyme (100 mg ml^{-1}) and incubated for 5 min on ice, followed by
205 centrifugation at 5,000 rpm and 4°C for 5 min. Pellets were then re-suspended in 300 µl lysis
206 buffer II (3 mM EDTA; 200 mM NaCl) and added to pre-heated (95°C) lysis Buffer III (3 mM
207 EDTA; 200 mM NaCl; 1 % SDS). Samples were incubated for exactly 5 min at 95°C and 600
208 µl phenol/chloroform/isoamylalcohol (25:24:1) (PCI) was added. After shaking the samples at
209 700 rpm for 5 min, the two phases were separated by centrifugation at 13,000 rpm for 5 min

210 and the upper aqueous phase was transferred to a new 1.5 ml microtube containing 600 µl
211 PCI. The extraction was repeated with 600 µl chloroform/isoamylalcohol (25:1). Finally, the
212 upper phase was transferred into an RNAse free tube and RNA was precipitated by addition
213 of 0.1 x volume 3 M sodium acetate (pH 5.2) and 1.5 volumes 96 % ethanol and incubated at
214 20°C overnight. To precipitate the RNA, samples were centrifuged at 13,000 rpm for 15 min,
215 washed in 70 % ethanol and dried under the fume hood. Finally, samples were re-suspended
216 in 25 µl RNAse free H_2O.

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

222

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

232

233 **Purification of His-Lmo2795.** For the overexpression of His-Lmo2795, plasmid pWH844 was
234 used, which contains the *lacI* gene, enabling the strictly IPTG-dependent overproduction of
235 His-tagged proteins (Schirmer *et al.*, 1997). pWH844-*lmo2795* was transformed into *E. coli*
236 BL21 and grown in 2x LB. When cultures reached an OD_{600} of 0.6-0.8, expression was induced
237 by the addition of IPTG at a final concentration of 1 mM and the cells were grown for 2 h at

238 37°C. Cells were collected by centrifugation, washed once with 1x ZAP buffer (50 mM Tris-
239 HCl, pH 7.5, 200 mM NaCl) and the cell pellet stored at -20°C. The next day, cells were re-
240 suspended in 1x ZAP (50 mM Tris-HCl, pH 7.5, 200 mM NaCl) and lysed by three passages
241 (18,000 lb/in2) through an HTU DIGI-F press (G. Heinemann, Germany). The resulting crude
242 extract was centrifuged at 46,400x g for 60 min followed by protein purification using a Ni^{2+}
243 nitrilotriacetic acid column (IBA, Göttingen, Germany). His-Lmo2795 was eluted using
244 imidazole and elution fractions analyzed using SDS-PAGE. Selected elution fractions were
245 subsequently subjected to dialysis against 1x ZAP buffer at 4°C overnight. The protein
246 concentration was determined according to the method of Bradford (Bradford, 1976) using the
247 Bio-Rad protein assay dye reagent concentrate. Bovine serum albumin was used as standard.
248 The protein samples were stored at -80°C until further use.
249

250 **Electrophoretic mobility shift assay (EMSA).** EMSA was performed as previously described
251 with some modifications (Dhiman *et al.*, 2014). Briefly, the upstream region of the *lmo2795-*
252 *nanE* operon containing a putative promoter site was amplified using primer pair
253 LMS430/LMS431. A 204 bp region within the operon, which served as an unspecific control,
254 was amplified using primers LMS428/LMS429. Binding reactions were performed at 25°C for
255 20 min in 20 µl binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% glycerol,
256 5 mM EDTA, 20 mM DTT and varying amounts of His-Lmo2795 (3.5 – 14 µg), as well as 250
257 pmol of DNA. To ease loading of the samples into the wells, 1 µl DNA loading dye was added
258 to each sample. A 60-min pre-run was carried out at 70 V prior to sample loading. The samples
259 were separated on an 8% polyacrylamide native gel in 0.5x TBE buffer (50 mM Tris-HCl, pH
260 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
261 0.5x TBE buffer containing 5 µl HDGreen™ Plus DNA dye (INTAS, Göttingen, Germany) for 3
262 min, followed by a 5 min washing step in 0.5x TBE buffer, three 20 sec washing steps in H_2O
263 and an additional 30 min washing step in H_2O. DNA bands were visualized using a GelDoc™
264 XR+ (BioRad, Munich, Germany).
265

266 **Results and Discussion**

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

268 For most physiological studies, which aimed at the characterization of salt and pH resistance
269 of *L. monocytogenes*, bacteria were grown in complex media such as BHI or TSB. In nature
270 as well as within the host, *L. monocytogenes* likely encounters limited access to nutrients, such
271 as carbon and nitrogen sources or vitamins, which likely affects their ability to adapt to
272 environmental stress conditions. We therefore assessed the ability of the two widely used *L.*
273 *monocytogenes* wildtype strains EGD-e and 10403S to grow under high salt and low pH stress
274 in the chemically defined medium LSM. Growth of EGD-e was barely affected in the presence
275 of 1% NaCl. In the presence of 2, 3 and 4 % NaCl, growth of EGD-e was delayed as compared
276 to LSM. At a concentration of 5% salt, EGD-e was still able to grow, but only reached an optical
277 density of around 0.3 after 36 h. The *L. monocytogenes* wildtype strain 10403S showed a lower
278 salt resistance as compared to EGD-e. Growth of 10403S was already impaired in LSM
279 containing 3% salt. In the presence of 4% and 5% salt, 10403S could only reach an optical
280 density of around 0.2 after 36 h (Fig. 1A). In contrast, *L. monocytogenes* is able to grow in the
281 presence of up to 10% salt, when a complex medium is used (Patchett *et al.*, 1992; Vasseur
282 *et al.*, 2001). To assess the pH resistance, we compared the growth of *L. monocytogenes*
283 strains grown in standard LSM, which has a pH of 8.7, and LSM adjusted to a pH of 8, 7, 6
284 and 5.5. Growth of the *L. monocytogenes* wildtype strain EGD-e was similar in LSM with a pH
285 of 7 as compared to standard LSM, while growth of 10403S was slightly reduced. Both strains
286 had a severe growth defect in LSM with a pH of 6 and were unable to grow in LSM with a pH
287 of 5.5 (Fig. 1B). Overall, we observed that the *L. monocytogenes* wildtype strain 10403S was
288 less tolerant to salt and pH stress than EGD-e (Fig. 1). Interestingly, 10403S grew better in
289 BHI medium, which was adjusted to a low pH, than EGD-e (Cheng *et al.*, 2015). Resistance of
290 *L. monocytogenes* to pH stress is mainly conferred by the activity of the glutamate
291 decarboxylase activity (GAD) system, a process, which depends on the availability of
292 extracellular glutamate (Cotter *et al.*, 2001; Feehily *et al.*, 2014). The two *L. monocytogenes*

293 wildtype strains EGD-e and 10403S seem to use two distinct GAD systems, which confer a
294 different degree of acid resistance. The GAD system used by 10403S was shown to give a
295 higher acid tolerance (Feehily *et al.*, 2014; Cheng *et al.*, 2015). However, complex medium
296 such as BHI likely contains higher glutamate levels than LSM, which can be used by the GAD
297 system. Thus, the GAD system of 10403S might be less efficient when the cells are grown in
298 LSM and are therefore less resistant to acid stress.
299

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

301 Over the last decades, several chemically defined media have been developed that support
302 growth of *L. monocytogenes* (Pine *et al.*, 1989; Premaratne *et al.*, 1991; Phan-Thanh and
303 Gormon, 1997; Tsai and Hodgson, 2003; Jarvis *et al.*, 2016; Whiteley *et al.*, 2017). Most of
304 these media contain glucose as the sole carbon source. Other carbon sources such as fructose
305 and mannose were shown to support growth of *L. monocytogenes* in Hsiang-Ning Tsai medium
306 (HTM) or modified Welshimer's Broth (MWB) (Premaratne *et al.*, 1991; Tsai and Hodgson,
307 2003). Glucose could also be replaced by the amino sugars Glc*N*Ac and *N*-acetylmuramic acid
308 in MWB (Premaratne *et al.*, 1991). Interestingly, lactose and rhamnose could support growth
309 of *L. monocytogenes* in ACES-buffered chemically defined (ABCD) medium, but not in HTM
310 and/or MWB (Table 4) (Pine *et al.*, 1989; Premaratne *et al.*, 1991; Tsai and Hodgson, 2003).
311 Similarly, glycerol was shown to support growth of *L. monocytogenes* in HTM medium, while
312 only weak growth could be observed for MWB with glycerol as sole carbon source (Table 4)
313 (Premaratne *et al.*, 1991; Tsai and Hodgson, 2003). To our knowledge, no information is
314 available on the carbon sources that support growth of *L. monocytogenes* in LSM. We
315 therefore performed growth experiments with *L. monocytogenes* wildtype strains EGD-e and
316 10403S in LSM containing 1% of selected carbon sources. This analysis revealed that growth
317 of both strains was similar in LSM containing Glc*N*Ac, Glc*N*, mannose, cellobiose or trehalose
318 as sole carbon source compared to glucose (Fig. 2, Table 4). Both strains were also able to
319 use glycerol as carbon sources, while only weak growth was obtained for maltose, rhamnose
320 and succinate (Fig. 2, Table 4). No growth was observed for both strains, when glucose was

321 replaced with galactose, sucrose, glucose-1-phosphate (Glc-1-P), glucose-6-phosphate (Glc-
322 6-P) or mannitol (Fig. 3A, Table 4). The hexose phosphate transporter Hpt, which is required
323 for the import of Glc-1-P and Glc-6-P, is expressed in a PrfA-dependent manner. The
324 expression and activity of PrfA is controlled on transcriptional, translational and post-
325 translational level (reviewed in (Xayarath and Freitag, 2012; Gaballa *et al.*, 2019)). One factor
326 involved in the control of *prfA* expression is the global regulator CodY, which senses the
327 presence of branched chain amino acids (BCAAs). In the presence of high levels of BCAAs,
328 CodY acts as a *prfA* repressor, while expression of *prfA* is induced when BCAA levels drop,
329 which occurs upon invasion of host cells (Lobel *et al.*, 2015). We therefore tested whether
330 reduction of the BCAA concentration in LSM could lead to growth of *L. monocytogenes*
331 wildtype strains EGD-e and 10403S on Glc-1-P and Glc-6-P as sole carbon source. While both
332 strains grew fine in LSM with low concentrations of BCAA and glucose as sole carbon source,
333 no growth was observed in LSM with Glc-1-P or Glc-6-P (Fig. 3A). For growth analysis, *L.*
334 *monocytogenes* strains were pre-grown in BHI complex medium, in which PrfA is inactive
335 (Renzoni *et al.*, 1997). We thus wondered whether the strains have simply not sufficient time
336 to activate PrfA and by this also do not induce the expression of *hpt*, encoding the Glc-1-P and
337 Glc-6-P importer, when they are transferred into LSM containing the hexose phosphate sugars.
338 To test this, we pre-grew *L. monocytogenes* strains EGD-e and 10403S in LSM glucose with
339 low levels of BCAA and subsequently transferred them either into the same medium as a
340 control, or into LSM with low levels of BCAA and containing Glc-1-P or Glc-6-P as sole carbon
341 source. A strain lacking the virulence regulator PrfA was used as a control. Indeed, EGD-e and
342 10403S were now able to metabolize Glc-1-P and Glc-6-P, while the *prfA* mutant was unable
343 to grow (Fig. 3B). In contrast, absence of PrfA results in a growth advantage in LSM with
344 glucose as sole carbon source, suggesting that the expression and/or activity of PrfA is a
345 burden for *L. monocytogenes*, which has previously been shown (Vasanthakrishnan *et al.*,
346 2015; Friedman *et al.*, 2017). Therefore, characterization of growth and the general physiology
347 of *L. monocytogenes* in the presence of Glc-1-P and Glc-6-P as sole carbon source, requires
348 a pre-growth under PrfA-activating conditions, for instance in a chemically defined medium or

349 in BHI medium containing activated charcoal, Chelex or Amberlite XAD (Chico-Calero *et al.*,
350 2002; Portman *et al.*, 2017; Gaballa *et al.*, 2021).

351 The amino sugar Man*N*Ac, which has not been tested for any of the other chemically
352 defined media, was able to support growth of *L. monocytogenes* strains EGD-e and 10403S
353 (Fig. 2A, Table 4). Man*N*Ac is the precursor of the sialic acid *N*-acetylneuraminic acid
354 (Neu5Ac), which can serve as a carbon source for *Staphylococcus aureus* inside the host
355 (Angata and Varki, 2002; Vimr *et al.*, 2004; Olson *et al.*, 2013). However, Neu5Ac could not
356 support growth of *L. monocytogenes*, suggesting that this organism does not contain the
357 necessary catabolic pathway (Table 4).

358

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

360 In *E. coli*, Man*N*Ac is imported into the cell by the PTS transporter ManXYZ yielding Man*N*Ac-
361 6-phosphate. Man*N*Ac-6-phosphate is subsequently converted into Glc*N*Ac-6-phosphate by
362 the Man*N*Ac-6-phosphate epimerase NanE. The genome of *L. monocytogenes* strain EGD-e
363 contains one homolog of NanE encoded by *lmo2801* (37% identity, 58% similarity). Based on
364 the presence of the NanE homolog, it was already previously proposed that the *lmo2795-nanE*
365 operon might be involved in the transport of Man*N*Ac (Deutscher *et al.*, 2014). In addition to
366 NanE, the *lmo2795-nanE* operon encodes an RpiR-type regulator, a member of the repressor,
367 ORF, kinase (ROK) family, the EIIA and EIIBC components of a PTS system, a putative HAD
368 hydrolase and a putative oxidoreductase (Fig. 4A). To test whether these proteins are required
369 for the catabolism of Man*N*Ac, single deletion mutants and a mutant lacking both PTS
370 components, Lmo2797 and Lmo2799, were constructed in the EGD-e wildtype background
371 and their growth was analyzed. All deletion mutants were able to grow in LSM containing
372 glucose, however, growth was enhanced for *L. monocytogenes* strains Δ*lmo2799* and
373 Δ*lmo2797* Δ*lmo2799*, lacking one or two of the PTS components, and the *nanE* deletion mutant
374 (Fig. 4B-C). In contrast, growth on Man*N*Ac as sole carbon source was reduced for the
375 *lmo2795* and *lmo2798* deletion strains lacking the RpiR transcriptional regulator and the
376 putative HAD hydrolase, respectively, and was nearly abolished for the strain lacking NanE

377 (Fig. 4D-E). Surprisingly, deletion strains Δ*lmo2799* and Δ*lmo2797* Δ*lmo2799* showed a better
378 growth in LSM with Man*N*Ac, similar to what was observed for the glucose containing LSM
379 (Fig. 4C-E), indicating that this PTS system is either not involved in the import of Man*N*Ac or
380 that Man*N*Ac can also be imported by other PTS systems of *L. monocytogenes*. Absence of
381 individual PTS systems can also lead to the overexpression of another PTS system (Stoll and
382 Goebel, 2010), which could explain why strains lacking the EIIB/C component Lmo2799 grew
383 better than the wildtype in LSM with glucose or Man*N*Ac.
384

385 **Expression of the *lmo2795-nanE* operon is controlled by Lmo2795**

386 RpiR-type transcriptional regulators are often involved in the control of sugar metabolic
387 pathways in Gram-positive and Gram-negative bacteria (Sørensen and Hove-Jensen, 1996;
388 Yamamoto *et al.*, 2001; Jaeger and Mayer, 2008; Li *et al.*, 2017; Aleksandrzak-Piekarczyk *et*
389 *al.*, 2019). We thus wondered whether Lmo2795 regulates the expression of the *lmo2795-*
390 *nanE* operon. First, we conducted EMSA experiments to assess whether Lmo2795 binds to
391 the promoter region of the operon. For this purpose, increasing concentrations of purified His-
392 Lmo2795 (Fig. 5A) were incubated with the DNA fragment containing the putative promoter of
393 the *lmo2795-nanE* operon. The migration of the DNA fragment was retarded in the presence
394 of His-Lmo2795 as compared to the DNA sample that did not contain the protein, suggesting
395 that a protein-DNA complex was formed. In addition, a so-called supershift could also be
396 observed, whose intensity increases with increasing protein concentrations. In contrast, no
397 retardation of the unspecific DNA control was observed in the presence of His-Lmo2795 (Fig.
398 5B). To test whether Lmo2795 activates or represses the expression of the *lmo2795-nanE*, we
399 quantified the expression of *lmo2796* and *nanE* via quantitative real-time PCR. To allow for a
400 better comparison of the gene expression, we decided to isolate RNA of the *L. monocytogenes*
401 wildtype strain EGD-e and the *lmo2795* mutant that were grown in LSM with glucose as sole
402 carbon source due to the observed growth deficit of the *lmo2795* mutant in Man*N*Ac-containing
403 LSM. This analysis revealed that the expression of both genes, *lmo2796* and *nanE*, was
404 reduced in the *lmo2795* mutant as compared to the wildtype strain (Fig. 5C), suggesting that

405 Lmo2795 acts as a transcriptional activator under the tested condition. Based on the
406 observation that a strain lacking NanE had a growth deficit in LSM with Man*N*Ac as sole carbon
407 source, we assume that Lmo2795 also activates the expression of the *lmo2795-nanE* under
408 this condition as we would expect at least wildtype-like growth of the *nanE* mutant, if Lmo2795
409 acts as a repressor. Further analysis is required to support this hypothesis.
410

411 **Conclusion**

412 The human pathogen *L. monocytogenes* is able to withstand and adapt to a variety of
413 environmental stress conditions. Most studies on physiology and stress tolerance of this
414 organism were performed in complex media without any nutrient limitation; however, this
415 condition is rarely found in their natural habitat or within the host during infection. Our study
416 focused on the characterization of the growth of two widely used *L. monocytogenes* wildtype
417 strains in the newly developed *Listeria* synthetic medium. We were able to show that both
418 strains can utilize a variety of carbon sources, when supplied as sole carbon source. To our
419 knowledge, this is the first time that the catabolism of Man*N*Ac has been investigated for *L.*
420 *monocytogenes*. We were able to show that the growth of this pathogen depends on the
421 presence of NanE, which is encoded in the *lmo2795-nanE* operon. The expression of the
422 *lmo2795-nanE* operon is regulated by the RpiR-type regulator Lmo2795, which acts as a
423 transcriptional activator under the tested conditions.
424 Our detailed characterization of the growth of *L. monocytogenes* in LSM in the presence
425 of diverse stress conditions or carbon sources can serve as a starting point for future studies
426 focusing on the adaptation of this important human pathogen on changing environmental
427 conditions.
428

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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.

References

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

Aleksandrzak-Piekarczyk, T., Szatraj, K., and Kosiorek, K. (2019) GlaR (YugA)-a novel RpiR-family transcription activator of the Leloir pathway of galactose utilization in *Lactococcus lactis* IL1403. *Microbiologyopen* **8**: e00714.

Angata, T. and Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto

456 acids: an evolutionary perspective. *Chem Rev* **102**: 439–469.

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

459 Bécavin, C., Bouchier, C., Lechat, P., Archambaud, C., Creno, S., Gouin, E., et al. (2014)
460 Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e
461 highlights genomic variations underlying differences in pathogenicity. *MBio* **5**: e00969-
462 14.

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

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

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

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

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

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

480 Deutscher, J., Aké, F., Zebre, A., Cao, T., Kentache, T., Pham, Q., et al. (2014)

481 Carbohydrate utilization by *Listeria monocytogenes* and its influence on virulence gene
482 expression. In Listeria monocytogenes*: Food Sources, Prevalence and Management*
483 *Strategies*. pp. 49–76.

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

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

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

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

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

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

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

505 Gopal, S., Berg, D., Hagen, N., Schriefer, E.-M., Stoll, R., Goebel, W., and Kreft, J. (2010)

506 Maltose and maltodextrin utilization by *Listeria monocytogenes* depend on an inducible
507 ABC transporter which is repressed by glucose. *PLoS One* **5**: e10349.

508 Grubmüller, S., Schauer, K., Goebel, W., Fuchs, T.M., and Eisenreich, W. (2014) Analysis of
509 carbon substrates used by *Listeria monocytogenes* during growth in J774A.1
510 macrophages suggests a bipartite intracellular metabolism. *Front Cell Infect Microbiol* **4**:
511 156.

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

515 Jaeger, T. and Mayer, C. (2008) The transcriptional factors MurR and catabolite activator
516 protein regulate *N*-acetylmuramic acid catabolism in *Escherichia coli*. *J Bacteriol* **190**:
517 6598–6608.

518 Jarvis, N.A., O'Bryan, C.A., Ricke, S.C., Johnson, M.G., and Crandall, P.G. (2016) A review
519 of minimal and defined media for growth of *Listeria monocytogenes*. *Food Control* **66**:
520 256–269.

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

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

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

530 Lecuit, M. (2005) Understanding how *Listeria monocytogenes* targets and crosses host

531 barriers. *Clin Microbiol Infect* **11**: 430–436.

532 Li, J., Evans, D.R., Freedman, J.C., and McClane, B.A. (2017) NanR regulates *nanI*
533 Sialidase expression by *Clostridium perfringens* F4969, a human enteropathogenic
534 strain. *Infect Immun* **85**: e00241-17.

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

538 Lourenco, A., Linke, K., Wagner, M., and Stessl, B. (2022) The saprophytic lifestyle of
539 *Listeria monocytogenes* and entry into the food-processing environment. *Front Microbiol*
540 **13**: 789801.

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

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

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

549 Orsi, R.H., den Bakker, H.C., and Wiedmann, M. (2011) *Listeria monocytogenes* lineages:
550 Genomics, evolution, ecology, and phenotypic characteristics. *Int J Med Microbiol* **301**:
551 79–96.

552 Osek, J., Lachtara, B., and Wieczorek, K. (2022) *Listeria monocytogenes* – How this
553 pathogen survives in food-production environments? *Front Microbiol* **13**: 866462.

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

556 3963.

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

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

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

564 Premaratne, R.J., Lin, W.J., and Johnson, E.A. (1991) Development of an improved
565 chemically defined minimal medium for *Listeria monocytogenes*. *Appl Environ Microbiol*
566 **57**: 3046–3048.

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

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

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

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

578 Smith, K. and Youngman, P. (1992) Use of a new integrational vector to investigate
579 compartment-specific expression of the *Bacillus subtilis spoIIM* gene. *Biochimie* **74**:
580 705–711.

581 Sørensen, K.I. and Hove-Jensen, B. (1996) Ribose catabolism of *Escherichia coli*:
582 characterization of the *rpiB* gene encoding ribose phosphate isomerase B and of the
583 *rpiR* gene, which is involved in regulation of *rpiB* expression. *J Bacteriol* **178**: 1003–
584 1011.

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

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

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

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

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

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

601 Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., and Bielawski, J.P. (2008) Multilocus
602 genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria*
603 *monocytogenes* isolates. *Appl Environ Microbiol* **74**: 7629–7642.

604 Welshimer, H.J. (1963) Vitamin requirements of *Listeria monocytogenes*. *J Bacteriol* **85**:
605 1156–1159.

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

609 Wiktorczyk-Kapischke, N., Skowron, K., Grudlewska-Buda, K., Wałecka-Zacharska, E.,
610 Korkus, J., and Gospodarek-Komkowska, E. (2021) Adaptive response of *Listeria*
611 *monocytogenes* to the stress factors in the food processing environment. *Front*
612 *Microbiol* **12**: 710085.

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

616 Yamamoto, H., Serizawa, M., Thompson, J., and Sekiguchi, J. (2001) Regulation of the glv
617 operon in *Bacillus subtilis*: YfiA (GlvR) is a positive regulator of the operon that is
618 repressed through CcpA and *cre*. *J Bacteriol* **183**: 5110–5121.

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

623

624

625 **Tables**

626 **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

627

628 **Table 2: Composition of *Listeria* synthetic medium**

Stock name	Stock dilution factor	Stock volume (ml)	Ingredient	Final concentration	MW (g/mol)	Final concentration (g/L)	Total in stock (g)
MOPS pH 7.5[a]	10	1000	MOPS	0.1 M	209.3	20.93	209.3
Glucose	50	1000	Glucose	55.5 mM	180.2	10	500
Phosphate	100	250	KH_2PO_4	4.8 mM	136.1	0.656	16.3
			$Na_2HPO_4 \cdot 12\ H_2O$	11.5 mM	358.14	4.12	103
Magnesium	100	250	$MgSO_4 \cdot 7\ H_2O$	1.7 mM	246.5	0.409	10.48
Micro-Nutrients[b]	100	250	Biotin	2.05 µM	244.3	0.0005	0.0125
			Riboflavin 5' monophosphate sodium salt hydrate	1.33 µM	478.33	0.0006	0.0158
			Para-Aminobenzoic acid	7.29 µM	137.1	0.001	0.025
			Lipoic acid	0.02 µM	206.3	0.000005	0.0001
			Nicotinamide	8.19 µM	122.12	0.001	0.025

			D-Pantothenic acid hemicalcium salt	4.2 μM	238.27	0.001	0.025
			Pyridoxal · HCl	4.91 μM	203.62	0.001	0.025
			Thiamine · HCl	2.96 μM	337.27	0.001	0.025
Complete amino acids[c]	50	500	L-Arginine	500 μM	174.2	0.1	2.18
			L-Histidine · HCl · H_2O	500 μM	209.6	0.1	2.62
			L-Isoleucine	800 μM	131.17	0.1	2.62
			L-Leucine	800 μM	131.17	0.1	2.62
			L-Methionine	700 μM	149.21	0.1	2.61
			L-Phenylalanine	600 μM	165.19	0.1	2.48
			L-Tryptophan	500 μM	204.23	0.1	2.55
			L-Valine	900 μM	117.15	0.1	2.64
Adenine[d]	100	250	Adenine	18.5 μM	135.13	0.0025	0.0625
Trace Metals	100	250	$FeCl_2 \cdot 4\ H_2O$	5 μM	198.8	0.00099	0.025
			$MnSO_4 \cdot H_2O$	50 μM	169	0.00845	0.212
			$ZnSO_4 \cdot 7\ H_2O$	1 μM	287.6	0.00029	0.007

			$CaCl_2 \cdot 2\ H_2O$	10 µM	147	0.00147	0.037
			$CuSO_4 \cdot 5\ H_2O$	0.1 µM	249.7	0.00002	0.0005
			$CoCl_2 \cdot 6\ H_2O$	0.1 µM	237.93	0.00003	0.0005
			H_3BO_3	0.1 µM	61.8	0.00001	0.00015
			$Na_2MoO_4 \cdot 2\ H_2O$	0.1 µM	242	0.00002	0.0005
			NaCl	8.555784 mM	58.44	0.5	12.5
			Sodium citrate tri-sodium salt	100 µM	294.1	0.2941	0.74
Added fresh[e]	10	100	L-Cysteine · HCl ·H_2O	634.44 µM	175.6	0.1	0.1
			L-Glutamine	4.106 mM	146.14	0.6	0.6

The recipe of *Listeria* synthetic medium (LSM) was adapted from Whiteley et al. (Whiteley *et al.*, 2017). All stock solutions were sterile filtrated prior use and stored at 4°C, except for Phosphate, which is stored at room temperature. LSM is prepared by combining the stock solutions in accordance with their dilution factor and in the order they are listed in the table. The final LSM medium is again sterile filtrated.

[a] Adjust pH of MOPS stock solution to pH 7.5 with 10 N NaOH.

[b] Dissolve ingredients in boiling water followed by filter sterilization.

[c] Dissolve amino acids in hot 1 N NaOH followed by filter sterilization.

[d] Dissolve adenine in 20 ml 0.2 M HCl and fill up with H_2O to 250 ml.

636 [e] These ingredients are usually freshly added to LSM according to Whiteley et al. (Whiteley *et al.*, 2017); however, a stock solution could be prepared and is stable

637 for at least 4 weeks.

638

639 **Table 3: 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

640

641 **Table 4: Carbon sources supporting growth of *L. monocytogenes* in chemically defined**

642 **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

643 [1] The following carbon sources do not support growth: Melibiose, Raffinose, Sorbose, Sorbitol,
644 Galactose-1-phosphate, Galactose-6-phosphate, Lactate, Citrate, Isocitrate, α-Ketoglutarate,
645 Fumarate, Malate, Pyruvate, Acetate, Chitin (Premaratne *et al.*, 1991). Abbreviations: Glc-1-P =
646 Glucose-1-phosphate, Glc-6-P = Glucose-6-phosphate, Glc*N* = glucosamine, GlcNAc = *N*-
647 acetylglucosamine, Mur*N*Ac = *N*-acetylmuramic acid, Man*N*Ac = *N*-acetylmannosamine, Neu5Ac = *N*-
648 acetylneuraminic acid.

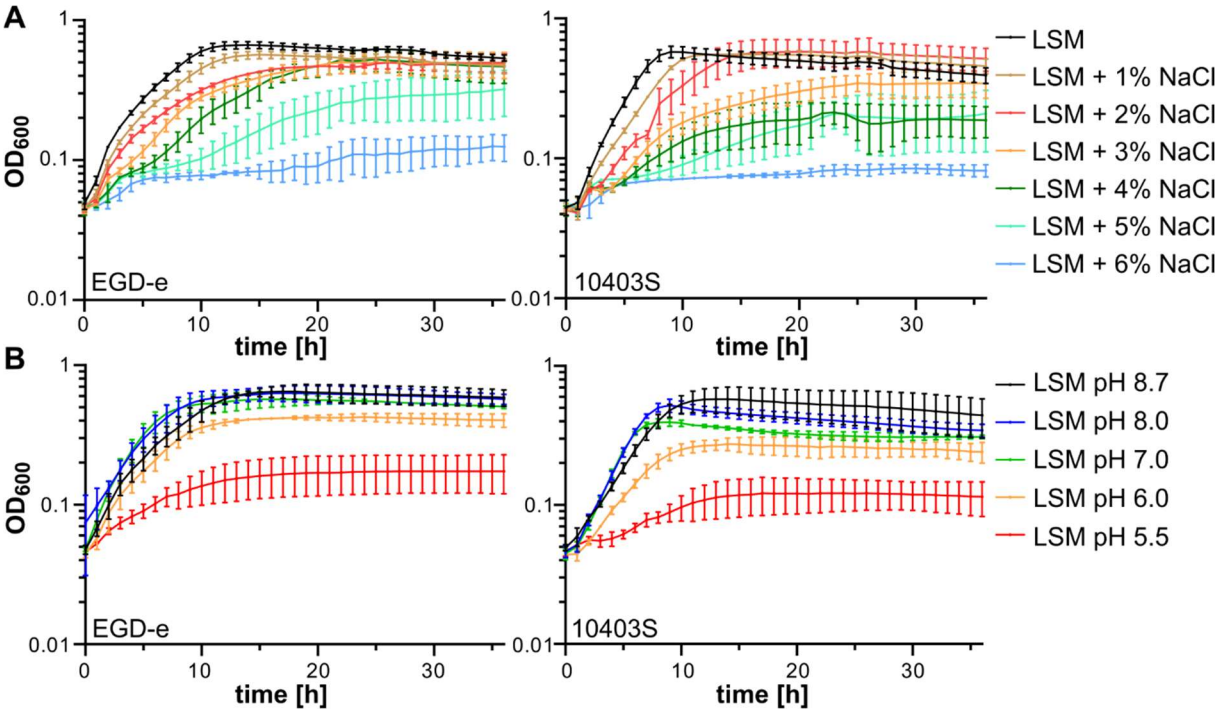
649 [2] Conc. = concentration of carbon source

650 [3] ABCD medium = *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered, chemically defined
651 medium; HTM = Hsiang-Ning Tsai medium, MWB = Modified Welshimer's broth, LSM = *Listeria* synthetic
652 medium

653 [4] Pre-growth in LSM glucose with low concentrations of BCAA, a PrfA-activating condition, is required.

654 **Figures and Figure legends**

655



656

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

663

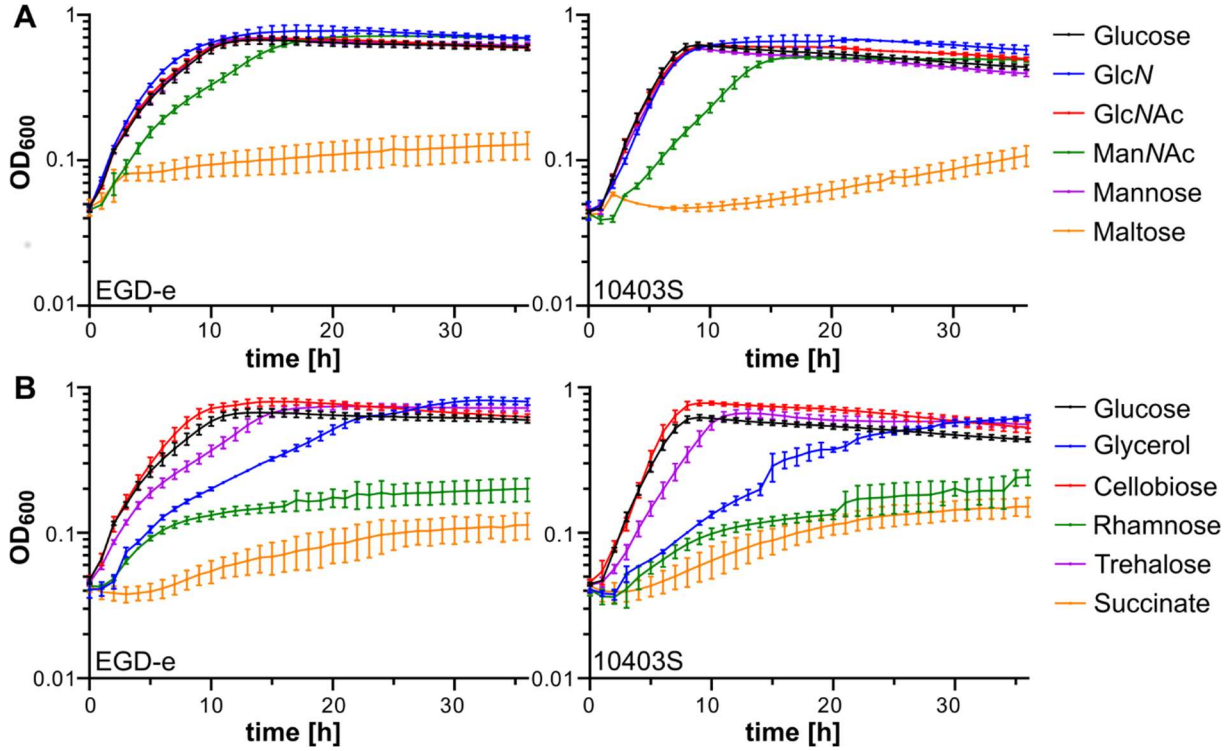
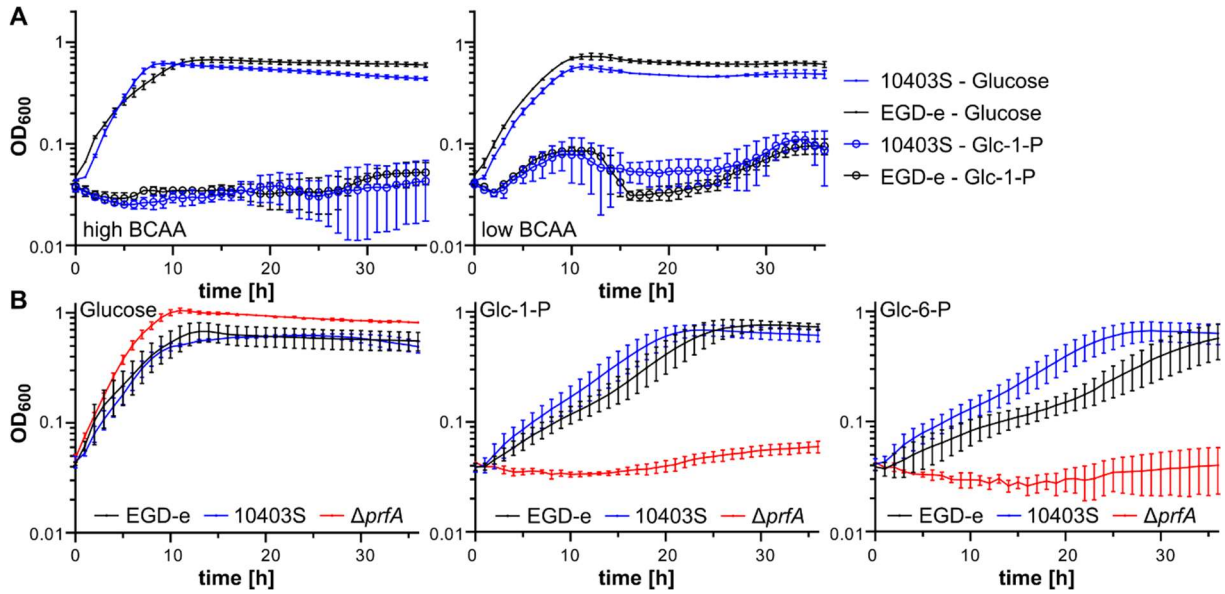
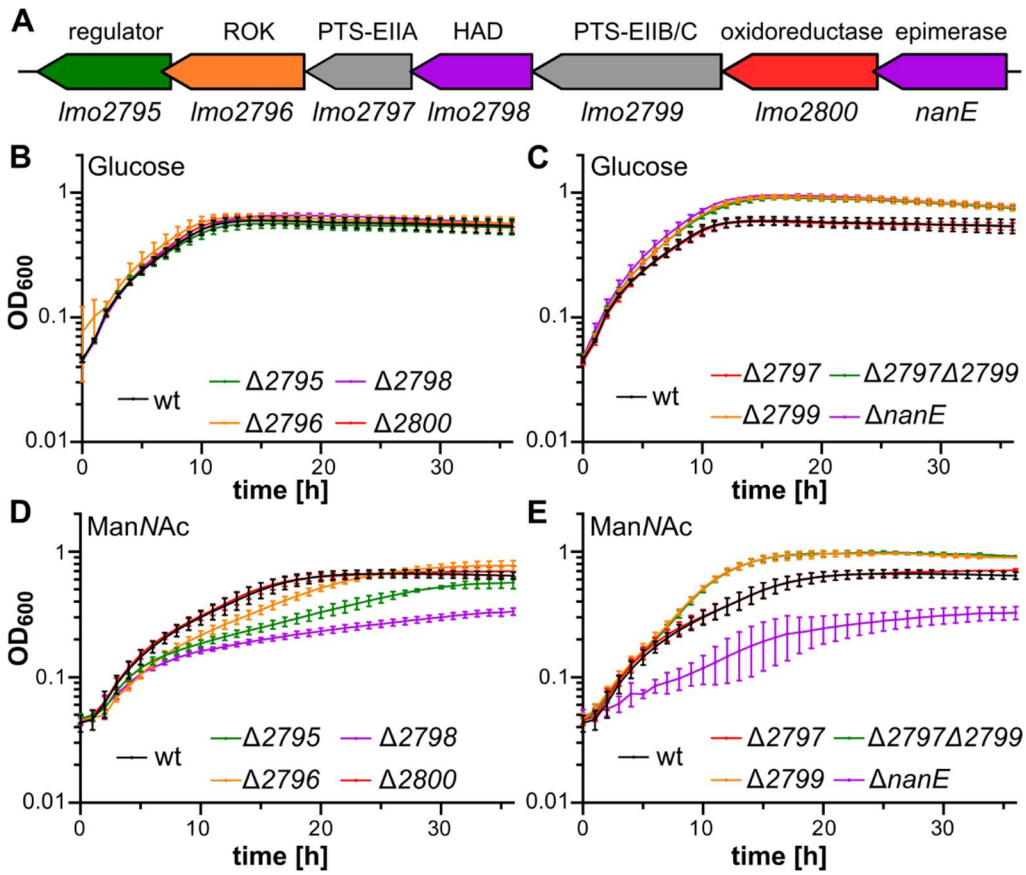


Figure 2: Growth in LSM media with different carbon sources. (A-B) *L. monocytogenes* strains EGD-e and 10403S were grown in *Listeria* synthetic medium (LSM) containing 1% of the indicated carbon sources as described in the methods section. The average values and standard deviations of three independent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



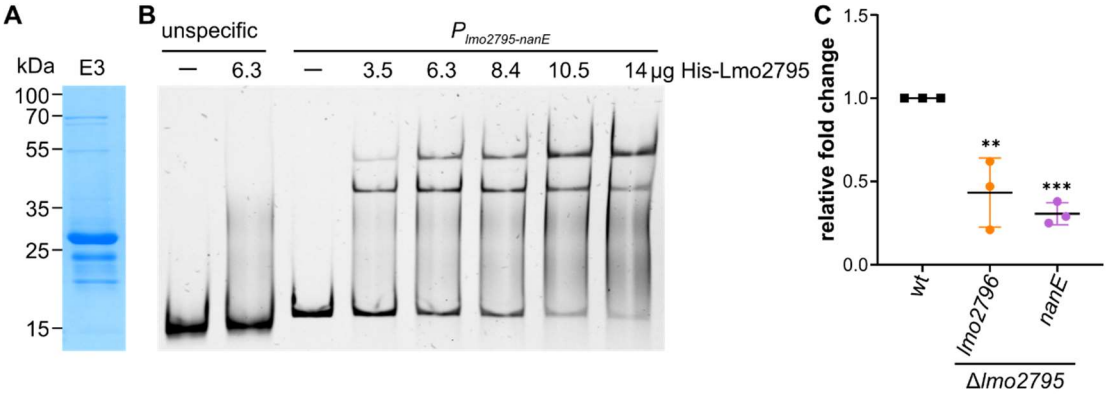
672 **Figure 3: Pre-growth of *L. monocytogenes* in LSM glucose enables growth on glucose-**
673 **1-phosphate and glucose-6-phosphate.** (A) *L. monocytogenes* strains EGD-e and 10403S
674 were grown in *Listeria* synthetic medium (LSM) containing 1% of the indicated carbon sources
675 as described in the methods section. LSM was either prepared with the standard complete
676 amino acid mix (high BCAA) or the complete amino acid mix with 10-fold less branched chain
677 amino acids (low BCAA). The average values and standard deviations of three independent
678 experiments were plotted. (B) *L. monocytogenes* strains EGD-e, 10403S and Δ*prfA* were pre-
679 grown in *Listeria* synthetic medium (LSM) containing 1% glucose and subsequently transferred
680 to LSM containing the indicated carbon sources as described in the methods section. The
681 average values and standard deviations of four independent experiments were plotted. Glc-1-
682 P = glucose-1-phosphate, Glc-6-P = glucose-6-phosphate.
683



684

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

688 EIIA and PTS-EIIB/C – components of a phosphotransferase system; HAD – member of the
689 haloacid dehydrogenase-like hydrolase superfamily. (B-G) Growth curves. The indicated *L.*
690 *monocytogenes* strains were grown in LSM containing (B, C) 1% glucose, (D, E) 1% Man*N*Ac
691 as sole carbon source. The average values and standard deviations of three independent
692 experiments were plotted.
693



694

695 **Figure 5: Lmo2795 regulates the expression of the *lmo2795-nanE* operon.** (A) SDS-PAGE
696 of elution fraction 3 containing purified His-Lmo2795. The expected molecular weight of His-
697 Lmo2795 is 31.5 kDa. (B) EMSA assay. Increasing concentrations of His-Lmo2795 were
698 incubated with the DNA fragment containing the predicted promoter region of the *lmo2795-*
699 *nanE* operon. The DNA-protein complexes were separated on an 8% polyacrylamide gel,
700 which was subsequently stained using HDGreen™. A DNA fragment containing a short region
701 from within the operon was used as the unspecific control. Reactions without protein were used
702 as negative controls (-). (C) Analysis of *lmo2796* and *nanE* expression by qRT-PCR. RNA was
703 isolated from *L. monocytogenes* strains EGD-e (wt) and Δ*lmo2795* grown in LSM containing
704 1% glucose as sole carbon source as described in the methods section. Expression of *lmo2796*
705 and *nanE* was normalized to the expression of *gyrB* and *rplD* and fold changes were calculated
706 using the $\Delta\Delta C_T$ method. Averages and standard deviations of three independent RNA
707 extractions were plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's
708 multiple comparison test was performed (** $p \leq 0.01$; *** $p \leq 0.001$).