1   **Cell shape and antibiotic resistance is maintained by the activity of multiple**

2   **FtsW and RodA enzymes in *Listeria monocytogenes***

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22   Abstract word count: 250

23   Main text word count: 6801

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28   Running Title: Functional analysis of multiple SEDS in *Listeria*

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Abstract

Rod-shaped bacteria have two modes of peptidoglycan synthesis: lateral synthesis and synthesis at the cell division site. These two processes are controlled by two macromolecular protein complexes, the elongasome and divisome. Recently, it has been shown that the *Bacillus subtilis* RodA protein, which forms part of the elongasome, has peptidoglycan glycosyltransferase activity. The cell division specific RodA homolog FtsW fulfils a similar role at the divisome. The human pathogen *Listeria monocytogenes* encodes up to six FtsW/RodA homologs, however their functions have not yet been investigated. Analysis of deletion and depletion strains led to the identification of the essential cell division-specific FtsW protein, FtsW1. Interestingly, *L. monocytogenes* encodes a second FtsW protein, FtsW2, which can compensate for the lack of FtsW1, when expressed from an inducible promoter. *L. monocytogenes* also possesses three RodA homologs, RodA1, RodA2 and RodA3 and their combined absence is lethal. Cells of a *rodA1/rodA3* double mutant are shorter and have increased antibiotic and lysozyme sensitivity, probably due to a weakened cell wall. Results from promoter activity assays revealed that expression of *rodA3* and *ftsW2* is induced in the presence of antibiotics targeting penicillin binding proteins. Consistent with this, a *rodA3* mutant was more susceptible to the β-lactam antibiotic cefuroxime. Interestingly, overexpression of RodA3 also led to increased cefuroxime sensitivity. Our study highlights that *L. monocytogenes* encodes a multitude of functional FtsW and RodA enzymes to produce its rigid cell wall and that their expression needs to be tightly regulated to maintain growth, cell division and antibiotic resistance.

Importance

The human pathogen *Listeria monocytogenes* is usually treated with high doses of β-lactam antibiotics, often combined with gentamicin. However, these antibiotics only act bacteriostatically on *L. monocytogenes* and the immune system is needed to clear the infection. Therefore, individuals with a compromised immune system are at risk to develop a severe form of *Listeria* infection, which can be fatal in up to 30% of cases. The development of new strategies to treat *Listeria* infections is therefore necessary. Here we show that the expression of some of the FtsW and RodA enzymes of *L. monocytogenes* is induced by the presence of β-lactam antibiotics and their combined absence makes bacteria more susceptible to this class of antibiotics. The development of antimicrobials that inhibit the activity or production of FtsW/RodA enzymes might therefore help to improve the treatment of *Listeria* infections and thereby lead to a reduction in mortality.

Introduction

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65 Bacterial cells are surrounded by a mesh of peptidoglycan (PG) that determines their shape

66 and also protects the cells from lysis due to their high internal turgor pressure (Weidel and

67 Pelzer, 1964, Vollmer et al., 2008, de Pedro and Cava, 2015). Peptidoglycan is comprised of

68 glycan strands that are crosslinked by short peptides (Rogers et al., 1980). The glycan strands

69 are composed of alternating N-acetylglucosamine and N-acetylmuramic acid residues that are

70 connected by a β-1,4 glycosidic bond (Ghuysen and Strominger, 1963). The synthesis of

71 peptidoglycan begins in the cytoplasm with the production of the PG precursor lipid II by the

72 proteins MurABCDEF, MraY and MurG (Blumberg and Strominger, 1974, van Heijenoort,

73 2001, Scheffers and Pinho, 2005, Pinho et al., 2013). Lipid II is then transported across the

74 cytoplasmic membrane by the flippase MurJ and Amj (Meeske et al., 2015, Sham et al.,

75 2014, Ruiz, 2008) and subsequently incorporated in the growing glycan strand by

76 glycosyltransferases. The polymerization and the crosslinking of the glycan strands are

77 facilitated by the activity of glycosyltransferases and transpeptidases, respectively. Class A

78 penicillin binding proteins (PBPs) are bifunctional enzymes that possess glycosyltransferase

79 and transpeptidase activity, whereas class B PBPs only contain a transpeptidase domain

80 (Höltje, 1998, Sauvage et al., 2008, Goffin and Ghuysen, 1998). In addition, some species

81 such as *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* encode

82 monofunctional glycosyltransferases (MGTs) that can also incorporate lipid II into the

83 growing glycan strand (Park and Matsuhashi, 1984, Park et al., 1985, Karinou et al., 2018,

84 Wang et al., 2001, Hara and Suzuki, 1984).

85 *B. subtilis* encodes four class A PBPs and no MGT, however, deletion of all class A PBPs

86 only manifests in small PG changes (McPherson and Popham, 2003). Recently, it has been

87 shown that members of the SEDS (shape, elongation, division, sporulation) family of

88 proteins, namely RodA and FtsW, also act as glycosyltransferases (Meeske et al., 2016, Cho

89 et al., 2016, Emami et al., 2017, Taguchi et al., 2019). Both, RodA and FtsW, form

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90   complexes with cognate class B PBPs to enable polymerization and crosslinking of glycan

91   strands (Cho et al., 2016, Taguchi et al., 2019, Leclercq et al., 2017, Fraipont et al., 2011,

92   Reichmann et al., 2019). Interestingly, SEDS proteins and the class B PBPs are more

93   conserved among different bacterial species than class A PBPs (Meeske et al., 2016).

94   In rod-shaped bacteria, peptidoglycan is synthesized by two multiprotein complexes, the

95   elongasome that is essential for the cell elongation and the divisome that is crucial for the

96   formation of the division septum (Nanninga, 1991, Carballido-Lopez and Formstone, 2007,

97   Typas et al., 2012, Errington and Wu, 2017). RodA is part of the elongasome and is essential

98   in many bacteria including *B. subtilis* and *S. pneumoniae* (Liu et al., 2017, Henriques et al.,

99   1998). Depletion of RodA results in the production of enlarged, spherical cells in *B. subtilis*

100   (Henriques et al., 1998). In contrast, FtsW is essential for cell division and cells depleted for

101   FtsW grow as long filaments (Kobayashi et al., 2003, Boyle et al., 1997, Gamba et al., 2016).

102   *B. subtilis* harbors a sporulation specific member of the SEDS family, SpoVE in addition to

103   RodA and FtsW. SpoVE is dispensable for growth, however, it is essential for the synthesis

104   of the spore cortex peptidoglycan (Henriques et al., 1992, Ikeda et al., 1989). Other *Bacillus*

105   species such as *B. cereus* and *B. anthracis* possess 4 to 5 FtsW/RodA proteins and strains of

106   different serotypes of the human pathogen *Listeria monocytogenes* encode even up to 6

107   FtsW/RodA homologs in their genome. However, their functions have not yet been

108   investigated.

109   Here, we determined the role of the different FtsW and RodA homologs for the growth and

110   cell morphology of *L. monocytogenes*. Our results show that *L. monocytogenes* encodes two

111   FtsW and three RodA enzymes. Absence of either FtsW1 or of all three RodA proteins is

112   lethal under standard laboratory conditions. *L. monocytogenes* infections are usually treated

113   with high doses of β-lactam antibiotics such as ampicillin, which inhibit the transpeptidase

114   activity of PBPs (Swaminathan and Gerner-Smidt, 2007). We demonstrate that the expression

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115   of two SEDS proteins, FtsW2 and RodA3, is induced in the presence of β-lactam antibiotics

116   likely to compensate for the inhibition of PBPs and that a *rodA3* mutant is more sensitive to

117   the β-lactam antibiotic cefuroxime. Antimicrobials inhibiting the activity of proteins of the

118   SEDS family could therefore potentially improve the treatment of *Listeria* infections in the

119   future.

120

121   **Materials and Methods**

122   **Bacterial strains and growth conditions**

123   All strains and plasmids used in this study are listed in Table S1. Strain and plasmid

124   constructions are described in the supplemental materials and method section and all primers

125   used in this study are listed in Table S2. *E. coli* strains were grown in Luria-Bertani (LB)

126   medium and *L. monocytogenes* strains in brain heart infusion (BHI) medium at 37°C unless

127   otherwise stated. If necessary, antibiotics and supplements were added to the medium at the

128   following concentrations: for *E. coli* cultures, ampicillin (Amp) at 100 µg/ml and kanamycin

129   (Kan) at 30 µg/ml, and for *L. monocytogenes* cultures, chloramphenicol (Cam) at 10 µg/ml,

130   kanamycin (Kan) at 30 µg/ml and isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM.

131   We used the *L. monocytogenes* strain 10403S and derivatives thereof. However, we refer to

132   *L. monocytogenes* EGD-e gene and locus tag numbers as this was the first fully sequenced *L.*

133   *monocytogenes* strain.

134

135   **Growth curves**

136   Overnight cultures of wildtype *L. monocytogenes* 10403S and the indicated deletion strains

137   were diluted to an OD_{600} of 0.01 or 0.05 in 15 ml BHI medium and the cultures were

138   incubated at 37°C with shaking. Growth was monitored by determining OD_{600} readings at

139   hourly intervals. For growth curves with the IPTG-inducible depletion strains 10403SΔ*ftsW1*

140   i*ftsW* (ANG4314), 10403SΔ*ftsW1* i*ftsW2* (ANG5119) and 10403SΔ*rodA1-3* i*rodA1*

5

141   (ANG5192), the strains were cultivated overnight in the presence of 1 mM IPTG. The next

142   day, cells were washed once with fresh medium, diluted 1:50 in 5 ml BHI medium and grown

143   for 8-10 h in the absence of the inducer. The cultures were diluted 1:100 into fresh BHI

144   medium and grown until the next morning at 37°C. The depleted cells were then diluted to an

145   OD_{600} of 0.01 and grown in the presence or absence of 1 mM IPTG at 37°C. Averages and

146   standard deviations from three independent experiments were plotted.

147

148   **RNA extraction and quantitative real-time PCR (qRT-PCR)**

149   For the extraction of RNA from *rodA* complementation strains, overnight cultures of *L.*

150   *monocytogenes* strains 10403S, 10403S*ΔrodA1ΔrodA3*, 10403S*ΔrodA1ΔrodA3* pIMK3-

151   *rodA1,* 10403S*ΔrodA1ΔrodA3* pIMK3-*rodA2* and 10403S*ΔrodA1ΔrodA3* pIMK3-*rodA3*

152   were diluted in BHI medium (with 1 mM IPTG for the plasmid-containing complementation

153   strains) to an OD_{600} of 0.05 and incubated at 37°C until the cultures reached an OD_{600} of 1.

154   For the extraction of RNA from strain 10403S*ΔrodA1-3* i*rodA1* (ANG5192), bacteria were

155   grown as described for the growth curve assay to deplete RodA1. Next, 10403S and depleted

156   cells of 10403S*ΔrodA1-3* i*rodA1* were diluted to an OD_{600} of 0.01 and grown in BHI medium

157   in the presence or absence of IPTG until an OD_{600} of 0.5. Twenty mls of the cultures were

158   mixed with 47 ml guanidine thiocyanate (GTC) buffer (5 M GTC, 0.5% N-lauryl sarcosine,

159   0.1 M β-mercaptoethanol, 0.5% Tween-80, 10 mM Tris-HCl pH 7.5), bacteria were harvested

160   by centrifugation and subsequently lysed using the FastRNA^{TM} Pro Blue kit (MP

161   Biomedicals). The total RNA was isolated by chloroform extraction and ethanol precipitation

162   and further purified using the RNeasy mini kit (Qiagen) and finally treated with TURBO

163   DNase (Invitrogen). cDNA was synthesized from 10 ng of RNA using the Superscript III first

164   strand synthesis kit (Invitrogen). The expression of *rodA1*, *rodA2* and *rodA3* in the different

165   strains was assessed using the TaqMan$^®$ probe-based gene expression assay (Applied

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166   Biosystems). Expression of *gyrB* was used as control. The cycle threshold (Ct) values

167   obtained for *rodA1*, *rodA2* and *rodA3* were normalized to the values obtained for *gyrB*. The

168   fold changes of gene expression for the different strains were calculated using the ΔΔCt

169   method.

170

171   **Determination of minimal inhibitory concentration (MIC)**

172   The minimal inhibitory concentration for bacitracin, penicillin and moenomycin and

173   lysozyme was determined using a microbroth dilution assay in 96-well plates. Approximately

174   10^4 *L. monocytogenes* cells were used to inoculate 200 µl BHI containing two-fold dilutions

175   of the different antimicrobials. The starting antibiotic concentrations were: 1 mg/ml for

176   bacitracin A, 1 µg/ml for penicillin G, 0.8 or 1.6 µg/ml for moenomycin, 8 µg/ml cefuroxime

177   and 10 mg/ml for lysozyme. The OD_{600} readings were determined after incubating the 96-

178   well plates for 24 h at 37°C with shaking at 500 rpm in a plate incubator (Thermostar, BMG

179   Labtech). The MIC value refers to the antibiotic concentration at which bacterial growth was

180   inhibited by >90%.

181

182   **Determination of antibiotic susceptibility using a spot plating assay**

183   Overnight cultures of the indicated *L. monocytogenes* strains were adjusted to an OD_{600} of 1

184   and 5 µl of serial dilutions were spotted on BHI agar plates or BHI agar plates containing 1

185   µg/ml cefuroxime and where indicated also 1 mM IPTG. Plates were photographed after

186   incubation at 37°C for 24 h.

187

188   **Fluorescence and phase contrast microscopy**

189   For bacterial cell length measurements, 100 µl of mid-log cultures were mixed with 5 µl of

190   100 µg/ml nile red solution to stain the cell membrane. Following incubation at 37°C for 20

7

191 min, the cells were washed once with PBS and resuspended in 50 µl PBS. 1.5 µl of the

192 different samples were spotted on microscope slides covered with a thin agarose film (1.5 %

193 agarose in distilled water), air-dried and covered with a cover slip. Phase contrast and

194 fluorescence images were taken using a Zeiss Axio Imager.A1 microscope coupled to a

195 AxioCam MRm and a 100x objective and processed using the Zen 2012 software (blue

196 edition). For the detection of nile red fluorescence signals, the Zeiss filter set 00 was used.

197 For the cell length determinations, 300 cells were measured for each experiment and the

198 median cell length was calculated. Averages and standard deviations of the median cell

199 length of three independent experiments were plotted.

200

Peptidoglycan isolation and analysis

202 Overnight cultures of *L. monocytogenes* 10403S, 10403S*ΔrodA1ΔrodA3* and

203 10403S*ΔrodA1ΔrodA3* pIMK3-*rodA1* were used to inoculate 1 L BHI broth (with 1 mM

204 IPTG for the complementation strain 10403S*ΔrodA1ΔrodA3* pIMK3-*rodA1*) to a starting

205 OD_{600} of 0.06. The cultures were grown at 37°C until they reached an OD_{600} of 1, at which

206 point the cultures were cooled on ice for 1 h. The bacteria were subsequently collected by

207 centrifugation and peptidoglycan was purified and digested with mutanolysin as described

208 previously (de Jonge et al., 1992, Corrigan et al., 2011). Digested muropeptides were

209 analyzed by high-performance liquid chromatography (HPLC) and recorded at an absorption

210 of 205 nm as described previously (de Jonge et al., 1992). For quantification, the areas of the

211 main muropeptide peaks were integrated using the Agilent Technology ChemStation

212 software. The sum of the peak areas was set to 100% and individual peak areas were

213 determined. Averages and standard deviations from three independent extractions were

214 calculated.

215

216 **β-galactosidase assay**

217 For the determination of the β-galactosidase activity, overnight cultures of strains 10403S

218 pPL3e-P$_{lmo2689}$-*lacZ*, 10403SΔ*rodA1* pPL3e-P$_{lmo2689}$-*lacZ* and 10403SΔ*rodA1*Δ*rodA2* pPL3e-

219 P$_{lmo2689}$-*lacZ* were diluted 1:100 in fresh BHI medium and grown for 6 h at 37°C. Sample

220 collection and preparation were performed as described previously (Gründling et al., 2004).

221 Briefly, OD$_{600}$ readings were determined (for the final β-galactosidase unit calculations) for

222 the different cultures after 6 h of growth and cells from 1 ml culture were pelleted by

223 centrifugation for 10 min at 13,200 x g, resuspended in 100 µl ABT buffer (60 mM K$_2$HPO$_4$,

224 40 mM KH$_2$PO$_4$, 100 mM NaCl, 0.1% Triton X-100, pH 7.0), snap frozen in liquid nitrogen

225 and stored at -80°C until use. For the identification of substances inducing the expression of

226 the *lmo2689-lmo2686* operon, an overnight culture of strain 10403S pPL3e-P$_{lmo2689}$-*lacZ* was

227 diluted 1:100 in fresh BHI medium and the culture incubated with shaking at 37°C until an

228 OD$_{600}$ of 0.5-0.6. The culture was divided into several flasks and incubated for two hours at

229 37°C in the presence or absence of the following substances: 0.5 µg/ml ampicillin, 0.05

230 µg/ml penicillin, 0.5 µg/ml vancomycin, 4 µg/ml cefuroxime, 0.05 µg/ml moenomycin, 0.5

231 mg/ml lysozyme, 1% ethanol, 300 µg/ml MgSO$_4$ or 300 µg/ml EDTA. Bacteria were pelleted

232 and samples frozen as described above.

233 Samples were thawed and 1:10 dilutions were prepared in ABT buffer. Fifty µl of the 1:10

234 diluted samples were mixed with 10 µl of 0.4 mg/ml 4-methyl-umbelliferyl-β-D-

235 galactopyranoside (MUG) substrate prepared in DMSO and incubated for 60 min at room

236 temperature (RT). A reaction with ABT buffer alone was used as negative control. Following

237 this incubation step, 20 µl of each reaction was diluted into 180 µl of ABT buffer in a black

238 96-well plate and fluorescence values were measured using an HIDEX Sense Microplate

239 Reader at 355 nm excitation and 460 nm emission wavelengths. 0.125-20 µM of the

240 fluorescent 4-methylumbelliferone (MU) standard were used to obtain a standard curve. β-

9

241   galactosidase units were calculated as (pmol of substrate hydrolyzed x dilution factor)/(ml

242   culture volume x OD_{600} x minute). The amount of hydrolyzed substrate was determined from

243   the standard curve as (emission reading – y intercept)/slope.

244

245   **Bacterial two-hybrid assays**

246   Protein-protein interactions between the different FtsW/RodA homologs and class B PBPs

247   were analyzed using the bacterial adenylate cyclase two-hybrid (BACTH) assay (Karimova et

248   al., 1998). The indicated pUT18/pUT18c and pKT25 derivatives were co-transformed into *E.*

249   *coli* strain BTH101. Transformants were selected on LB agar plates containing 100 µg/ml

250   ampicillin, 30 µg/ml kanamycin, 0.1 mM IPTG and 50 µg/ml X-Gal. Images were taken after

251   incubation for 24 h and 48 h at 30°C.

252

253   **Results**

254   ***L. monocytogenes* 10403S encodes six FtsW/RodA homologs**

255   So far, FtsW and RodA proteins of the human pathogen *L. monocytogenes* have not been

256   studied. FtsW and RodA are members of the SEDS (shape, elongation, division, sporulation)

257   family of proteins and are multispanning membrane proteins with 8-10 transmembrane

258   helices and a large extracellular loop (Fig. 1A). Using BLAST, six proteins with homology to

259   the *B. subtilis* FtsW and RodA proteins could be identified in the genome of *L.*

260   *monocytogenes* 10403S (Table 1). The protein encoded by *lmo0421* has the weakest

261   homology to *B. subtilis* FtsW and RodA (Fig. S1, Table 1). *lmo0421* is part of the *sigC*

262   operon, which is comprised of *lmo0422* encoding the PadR-like repressor LstR and *lmo0423*

263   coding for the ECF-type sigma factor SigC (Fig. 1B). The *sigC* operon acts as a lineage II

264   specific heat shock system (Zhang et al., 2005) and is therefore not encoded in all *L.*

10

265   *monocytogenes* genomes. Due to the weak homology to FtsW and RodA and its absence in *L.*

266   *monocytogenes* strains of lineage I and III, Lmo0421 was excluded from further analysis.

267   The *L. monocytogenes* protein Lmo1071 is the closest homolog to *B. subtilis* FtsW with a

268   sequence identity of 48% (Table 1). Furthermore, *L. monocytogenes lmo1071* and *B. subtilis*

269   *ftsW* are found in the same chromosomal context. More specifically, *lmo1071* is located

270   between genes *lmo1070*, which encodes a protein with homology to the *B. subtilis* YlaN

271   protein, and *pycA* coding for the pyruvate carboxylase, which are also adjacent to *ftsW* in *B.*

272   *subtilis*. This analysis suggests that gene *lmo1071* encodes the cell division protein FtsW.

273   However, *L. monocytogenes* encodes a second protein, Lmo2688, that shares a higher degree

274   of homology to the *B. subtilis* FtsW as compared to the *B. subtilis* RodA protein (Table 1).

275   Due to these similarities and additional data presented in this study, we refer to Lmo1071 and

276   Lmo2688 as FtsW1 and FtsW2, respectively.

277   The BLAST search with the *B. subtilis* RodA sequence as a query sequence yielded the *L.*

278   *monocytogenes* protein Lmo2428 as the closest homolog with a sequence identity of 40%

279   (Table 1). In addition to Lmo2428, two additional RodA homologs are present in *L.*

280   *monocytogenes*, namely Lmo2427 and Lmo2687. As presented below, Lmo2427, Lmo2428

281   and Lmo2687 are likely bona-fide RodA homologs and were therefore renamed RodA1,

282   RodA2 and RodA3, respectively. *rodA1* is located adjacent to *rodA2*, but despite their

283   proximity, *rodA1* and *rodA2* are likely not transcribed as part of the same operon (Toledo-

284   Arana et al., 2009). In contrast, *rodA3* and *ftsW2* are part of the four-gene operon *lmo2689-*

285   *lmo2686*. Lmo2689 is similar to a Mg^{2+}-type ATPase, whereas *lmo2686* encodes a protein of

286   unknown function. Analysis of around 2000 genomes of *L. monocytogenes* strains presently

287   available at NCBI revealed that the five FtsW/RodA homologs named here FtsW1, FtsW2,

288   RodA1, RodA2 and RodA3 are conserved in the different strains with one exception; *L.*

289   *monocytogenes* 4b strains of the sequence type ST2 lack homologs of RodA3 and FtsW2.

290

lmo1071 encodes FtsW1 and is essential for the survival of *L. monocytogenes*

The cell division protein FtsW is essential for growth in the Gram-negative and Gram-positive model organisms *E. coli* and *B. subtilis* (Boyle et al., 1997, Ikeda et al., 1989, Khattar et al., 1994, Kobayashi et al., 2003). Depletion of FtsW in these organisms leads to a block in cell division and formation of elongated cells (Boyle et al., 1997, Gamba et al., 2016). All our attempts to delete the *ftsW1* gene in *L. monocytogenes* 10403S remained unsuccessful, suggesting that FtsW1 is also essential for growth in *Listeria*. Next, strain 10403S*ΔftsW1* i*ftsW1* was constructed, in which the expression of *ftsW1* is controlled by an IPTG-inducible promoter. While no difference in the growth was observed between the wildtype and FtsW1-depletion strain (likely due to leakiness of the inducible promoter) (Fig. 2A), cells depleted for FtsW1 were significantly elongated (Fig. 2B-C). Bacteria depleted for FtsW1 had a median cell length of 3.41±0.16 µm, while wildtype and 10403S*ΔftsW1* i*ftsW1* bacteria grown in the presence of 1 mM IPTG had a median cell length of 1.85±0.16 µm and 1.93±0.07 µm, respectively (Fig. 2C). These data indicate that *lmo1071* encodes the cell division specific SEDS protein FtsW.

306

L. monocytogenes encodes a second FtsW protein

To our knowledge, all bacteria analyzed to date possess only one FtsW protein that is essential for cell survival. We identified a second potential FtsW protein, Lmo2688, in *L. monocytogenes*. In contrast to *ftsW1,* a *L. monocytogenes ftsW2* deletion strain could be constructed and no significant growth or cell morphology phenotypes could be observed for the *ΔftsW2* deletion strain (Fig. S2). In a previous study, it was reported that the operon comprised of genes *lmo2689-lmo2686* is only minimally expressed when *L. monocytogenes* 10403S is grown in BHI broth (Lobel and Herskovits, 2016). We reasoned that if *ftsW2* does

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315   indeed code for a second FtsW protein, it should be possible to delete *ftsW1* in a strain in

316   which *ftsW2* is artificially expressed from an IPTG-inducible promoter. Indeed, strain

317   10403SΔ*ftsW1* i*ftsW2* could be generated in the presence of IPTG. In contrast, we were

318   unable to generate strain 10403SΔ*ftsW1* when any of the other FtsW/RodA homologs

319   Lmo2427 (RodA1), Lmo2428 (RodA2) or Lmo2687 (RodA3) were expressed from the same

320   IPTG-inducible promoter system. While prolonged depletion of FtsW2 in strain

321   10403SΔ*ftsW1* i*ftsW2* had again no impact on the growth, the cells were significantly

322   elongated in the absence of the inducer compared to wildtype or bacteria grown in the

323   presence of inducer (Fig. 3). These data strongly suggest that *ftsW2* encodes a second FtsW

324   enzyme while the remaining three proteins Lmo2427, Lmo2428 and Lmo2687 likely function

325   as RodA proteins.

326

L. monocytogenes encodes three RodA homologs

328   We were able to assign roles for two of the FtsW/RodA homologs as FtsW-like proteins.

329   However, *L. monocytogenes* encodes three additional homologs, which show a higher

330   similarity to the *B. subtilis* RodA protein as compared to the *B. subtilis* FtsW protein (Table

331   1). As described above, expression of none of the enzymes Lmo2427, Lmo2428 or Lmo2687

332   was able to rescue the growth of an *ftsW1* deletion strain, indicating that these enzymes likely

333   function as RodA proteins in *L. monocytogenes* and hence they were renamed RodA1,

334   RodA2 and RodA3, respectively. All attempts to construct a *rodA1-3* triple mutant failed

335   further corroborating that these proteins function as RodA proteins and at least one of them

336   needs to be present for cell viability. To determine whether the different RodA homologs

337   have distinct functions or whether they are merely duplications, single and double mutant

338   strains were generated. No significant differences with regards to growth and cell length

339   could be observed between the wildtype strain 10403S and single *rodA1*, *rodA2* or *rodA3*

340   deletion strains (Fig. S3). Similar observations were made with the *rodA* double mutant

341   strains 10403S*ΔrodA1ΔrodA2* and 10403S*ΔrodA2ΔrodA3* (Fig. 4). However, cells lacking

342   RodA1 and RodA3 were shorter (1.3±0.03 μm) compared to wildtype cells (1.9±0.06 μm,

343   Fig. 4B-C), indicating that either RodA1 or RodA3 needs to be present for *L. monocytogenes*

344   to maintain its rod shape. RodA3 is part of the *lmo2689-lmo2686* operon that is only

345   minimally expressed when *L. monocytogenes* is grown in BHI broth (Lobel and Herskovits,

346   2016). The fact that we observe differences in cell morphology between a *rodA1* single and

347   the *rodA1/rodA3* double mutant, suggests that the function of the two proteins could be

348   additive or that *rodA3* expression, which is only minimally expressed in a wildtype strain

349   under standard laboratory growth conditions, might increase upon deletion of *rodA1*. To test

350   if *rodA3* expression is increased in the absence of other RodA proteins, we fused the

351   promoter upstream of *lmo2689* and driving *rodA3* expression to *lacZ* and inserted this fusion

352   into the chromosome of wildtype 10403S, the *rodA1* and the *rodA1/rodA2* deletion strains.

353   The promoter activity was indeed 1.5- to 2-fold higher in the *rodA1* and *rodA1/rodA2* mutant

354   strains as compared to the wildtype, as assessed by the increase in the β-galactosidase activity

355   (Fig. 4G). This result indicates that expression of the *lmo2689-lmo2686* operon, which

356   encodes FtsW2 and RodA3, is induced in the absence of RodA1, suggesting a coordination of

357   the expression of the different RodA homologs.

358   To confirm that the decrease in cell length of the double mutant strain 10403S*ΔrodA1ΔrodA3*

359   depends on the absence of RodA1 and RodA3, complementation strains with IPTG-inducible

360   expression of *rodA1* or *rodA3* were constructed. Expression of RodA1 restored the cell length

361   to 1.84±0.1 μm, which is comparable to the cell length of wildtype cells (1.76±0.15 μm, Fig.

362   4E). On the other hand, expression of *rodA3* from an ectopic locus and IPTG-inducible

363   promoter in strain 10403S*ΔrodA1ΔrodA3* led to the formation of longer cells with an average

364   cell length of 2.47±0.01 μm (Fig. 4D-E). These results indicate that induction of RodA3 from

365 the IPTG-inducible promoter likely results in an overproduction of the protein as compared to

366 the expression from the native promoter. To confirm that *rodA3* expression is increased when

367 expressed from the IPTG-inducible promoter as compared to its native promoter, *rodA3*

368 transcript levels were assessed by qRT-PCR in the wildtype strain, the

369 10403SΔ*rodA1*Δ*rodA3* deletion strain and strain 10403SΔ*rodA1*Δ*rodA3* i*rodA3* grown in the

370 presence of IPTG (Fig. 4H). Significant higher *rodA3* transcript levels were detected in the

371 inducible strain in the presence of IPTG as compared to the wildtype strain. Similarly,

372 expression of *rodA3* from an IPTG-inducible promoter in wildtype 10403S led to the

373 formation of elongated cells with an average cell length of 2.49 ± 0.08 μm, whereas additional

374 expression of *rodA1* or *rodA2* using the same inducible system had no impact on the cell

375 length of 10403S (Fig. S4). These results highlight that in particular fine-tuning of RodA3

376 production is essential for cell-length determination in *L. monocytogenes*.

377 The observation that the *rodA1/rodA3* double mutant forms shorter cells suggests that RodA2

378 is not sufficient to maintain the cell length of *L. monocytogenes*. There are several possible

379 explanations for this: RodA2 might have a reduced activity as compared to RodA1 or RodA3.

380 RodA2 might have a function that is different from RodA1 and RodA3 or the protein levels

381 of RodA2 might be sufficient to maintain cell viability but too low to maintain the rod shape.

382 To investigate this further, a strain was constructed which lacks *rodA1* and *rodA3*, but carries

383 pIMK3-*rodA2* to allow for IPTG-inducible expression of *rodA2* in addition to the expression

384 of *rodA2* from its native locus (10403SΔ*rodA1*Δ*rodA3* i*rodA2*). In the absence of the inducer,

385 the cells had a median cell length of 1.2 ± 0.03 μm (data not shown). However, the cell length

386 of strain 10403SΔ*rodA1*Δ*rodA3* i*rodA2* increased to 1.71 ± 0.8 μm when the strain was grown

387 in the presence of IPTG (Fig. 4F). Therefore, additional expression of *rodA2*, which was

388 verified by qRT-PCR (Fig. 4I), can partially complement the cell length phenotype of the

389 *rodA1/rodA3* deletion strain, suggesting that RodA2 has a similar function as RodA1 and

390   RodA3, but that it has either a lower activity or is not expressed in sufficient amounts from

391   its native promoter for proper cell length maintenance.

392   As stated above, several attempts to construct a strain inactivated for all three RodA

393   homologs remained unsuccessful, suggesting that at least one of the proteins RodA1, RodA2

394   or RodA3 needs to be present for the viability of *L. monocytogenes*. The results presented so

395   far indicate that RodA1 is the most important RodA homolog considering that RodA2 alone

396   is not sufficient to maintain the rod shape and that RodA3 is only minimally expressed under

397   standard laboratory conditions (Lobel and Herskovits, 2016) and also lacking in *L.*

398   *monocytogenes* 4b strains of the sequence type ST2. To understand the impact of RodA

399   enzymes on cell growth and cell division in *L. monocytogenes*, a strain was constructed

400   which lacks all three *rodA* genes from its genome, but harbors pIMK3-*rodA1* to enable IPTG-

401   inducible expression of RodA1. Prolonged depletion of RodA1 in strain 10403SΔ*rodA1-3*

402   i*rodA1* led to a growth defect that was not seen when the strain was grown in the presence of

403   the inducer (Fig. 5A). However, the depletion was not efficient enough to see a complete

404   growth inhibition, which would be expected for a strain lacking all three RodA homologs.

405   This is likely caused by residual *rodA1* expression from the inducible promoter even in the

406   absence of IPTG. Consistent with this notion, even after prolonged depletion, *rodA1*

407   transcripts could still be detected in strain 10403SΔ*rodA1-3* i*rodA1* as assessed by qRT-PCR

408   (Fig. S5). However, cells of the *L. monocytogenes* strain 10403SΔ*rodA1-3* i*rodA1* that were

409   grown without IPTG were significantly shorter with a cell length of 1.18±0.08 μm as

410   compared to cells of the double mutant 10403SΔ*rodA1*Δ*rodA3* or the wildtype strain 10403S

411   (Fig. 5B, D). Interestingly, different cell morphologies could be observed for strain

412   10403SΔ*rodA1-3* i*rodA1* after prolonged RodA1 depletion (Fig. 5C). The placement of the

413   division septum was affected in some cells and daughter cells of different size or cells with

414   two septa were observed (Fig. 5C). These morphological defects could be complemented, and

16

415   the cell length increased to 1.95±0.04 µm upon addition of IPTG and expression of RodA1

416   (Fig. 5D). These data highlight that RodA1 alone is sufficient to maintain the cell shape of *L.*

417   *monocytogenes*.

418

419   **Decreased moenomycin and lysozyme resistance in the absence of RodA homologs**

420   Next, we wondered whether the absence of FtsW or RodA proteins affects the resistance of *L.*

421   *monocytogenes* towards the antibiotics penicillin, bacitracin and moenomycin, which target

422   different steps in the peptidoglycan biosynthesis process. Penicillin binds to the

423   transpeptidase domain of PBPs and inhibits their function, leading to a reduced crosslinking

424   of the peptidoglycan (Nakagawa et al., 1979, Korsak et al., 2010). Bacitracin inhibits the

425   dephosphorylation of the bactoprenol carrier leading to a block in lipid II synthesis (Stone

426   and Strominger, 1971). The phosphoglycolipid antibiotic moenomycin inhibits the

427   glycosyltransferase activity of bifunctional PBPs and thereby prevents the polymerization of

428   the glycan chain (van Heijenoort et al., 1987).

429   No significant differences could be observed in terms of resistance against penicillin,

430   bacitracin or moenomycin for the FtsW1 depletion strain 10403SΔ*ftsW1* i*ftsW1*. This is

431   presumably due to basal level expression of *ftsW1* even in the absence of the inducer.

432   Simultaneous deletion of *rodA1* and *rodA3* resulted in a slight decrease in the MIC for

433   penicillin, however, this difference was not significant (Fig. 6A). On the other hand, strain

434   10403SΔ*rodA1*Δ*rodA3* was 2-4-fold more sensitive to the antibiotic bacitracin (Fig. 6B). This

435   phenotype could be complemented by expressing either RodA1, RodA2 or RodA3 from an

436   IPTG-inducible promoter (Fig. 6B).

437   As described above, moenomycin inhibits the transglycosylase activity of PBPs leading to a

438   decreased activity of these enzymes. In the absence of RodA1 and RodA3, cells are more

439   susceptible to a reduced activity of PBPs manifesting in a 4-fold reduced resistance to

17

440 moenomycin (Fig 6C). Induction of RodA1 expression in strain 10403SΔ*rodA1*Δ*rodA3*

441 resulted in a significantly higher resistance to moenomycin as compared to the wildtype

442 strain 10403S and expression of RodA2 or RodA3, led to partial or complete

443 complementation of the moenomycin sensitivity (Fig. 6C).

444 Moreover, resistance to lysozyme, an enzyme that cleaves the linkage between N-acetyl

445 muramic acid and N-acetylglucosamine residues of the peptidoglycan, was drastically

446 decreased in strain 10403SΔ*rodA1*Δ*rodA3* and could be fully restored by expression of

447 RodA1, RodA2 or RodA3 (Fig. 6D). Lysozyme resistance in *L. monocytogenes* is mainly

448 accomplished by two modifications of the peptidoglycan; deacetylation of N-

449 acetylglucosamine residues by PgdA or O-acetylation of N-acetylmuramic acid residues by

450 OatA (Boneca et al., 2007, Aubry et al., 2011). To determine whether the activity of PgdA is

451 changed in the absence of RodA1 and RodA3, peptidoglycan was purified from the

452 10403SΔ*rodA1*Δ*rodA3* mutant strain, digested with mutanolysin and the resulting

453 muropeptides analyzed by HPLC. Peptidoglycan samples isolated from the wildtype strain

454 10403S and the complementation strain 10403SΔ*rodA1*Δ*rodA3* i*rodA1*, that had been grown

455 in the presence of IPTG, were analyzed as controls (Fig. 6E). The main muropeptide peaks

456 were assigned as described previously (Rismondo et al., 2015, Burke et al., 2014). Peaks 1

457 and 2 correspond to the acetylated and deacetylated monomeric muropeptides, respectively,

458 whereas peak 3 and peaks 4-6 are acetylated and deacetylated muropeptide dimers,

459 respectively. Deletion of *rodA1* and *rodA3* led to a reduction of both monomeric

460 muropeptides and therefore to an increase in crosslinked peptidoglycan fragments by

461 approximately 2% as compared to the wildtype strain 10403S, in which 65% of the

462 peptidoglycan was cross-linked (Fig. 6F). However, no significant difference with regards to

463 the deacetylated muropeptides could be observed between wildtype 10403S, strain

464 10403SΔ*rodA1*Δ*rodA3* and the 10403SΔ*rodA1*Δ*rodA3* i*rodA1* complementation strain (Fig.

18

465 6F). These results suggest that the lysozyme sensitivity phenotype of strain

466 10403S*ΔrodA1ΔrodA3* is not caused by changes in the peptidoglycan deacetylation, but

467 rather due to general defects in the peptidoglycan structure.

468

469 **Cell wall-acting antibiotics induce the promoter of *lmo2689***

470 The operon *lmo2689-lmo2686*, that contains the genes encoding FtsW2 and RodA3, is only

471 minimally expressed under standard laboratory conditions (Lobel and Herskovits, 2016). A

472 genome-wide transcriptional analysis performed in *L. monocytogenes* strain LO28 has shown

473 that *lmo2687, lmo2688* and *lmo2689* are part of the CesR regulon (Nielsen et al., 2012). The

474 <u>c</u>ephalosporin <u>s</u>ensitivity response regulator CesR is part of the CesRK two-component

475 system that regulates the transcription of several cell envelope-related genes in response to

476 changes in cell wall integrity, such as caused by the presence of cell wall-acting antibiotics or

477 alcohols including ethanol (Gottschalk et al., 2008, Kallipolitis et al., 2003, Nielsen et al.,

478 2012). Therefore, we next used the *lmo2689* promoter-*lacZ* fusion described above, to assess

479 if expression of the *lmo2689-lmo2686* operon is induced in the presence of antibiotics that

480 target different processes of the PG biosynthesis or ethanol. Indeed, increased β-galactosidase

481 activity could be measured for cells that had been grown in the presence of sub-inhibitory

482 concentrations of the β-lactam antibiotics ampicillin, penicillin and cefuroxime and the

483 phosphoglycolipid moenomycin (Fig. 7A). In contrast, no increase in β-galactosidase activity

484 could be detected upon addition of vancomycin, lysozyme or ethanol as compared to

485 untreated control cells (Fig. 7A). We also tested whether the presence of MgSO$_4$ or EDTA

486 has an impact on the *lmo2689* promoter activity since *lmo2689* encodes a putative Mg^{2+}-type

487 ATPase. However, the β-galactosidase activity of cells grown in the presence of MgSO$_4$ or

488 EDTA was comparable to the β-galactosidase activity seen for untreated cells (Fig. 7A).

489 These results indicate that the expression of *ftsW2* and *rodA3*, that are part of the *lmo2689-*

19

490   *lmo2686* operon, are induced in the presence of various cell wall-acting antibiotics,

491   suggesting that FtsW2 and RodA3 might be important for the intrinsic resistance of *L.*

492   *monocytogenes* against these antibiotics. However, no significant differences in MICs for

493   penicillin and moenomycin could be observed between wildtype 10403S, the *ftsW2* or *rodA3*

494   single mutant strains or the *ftsW2/rodA3* double mutant (Fig. S6). However, there was a slight

495   reduction in the resistance of the *rodA3* single mutant against cefuroxime as compared to the

496   wildtype (Fig. S6). To further assess whether there is a difference in the cefuroxime

497   resistance between the *L. monocytogenes* wildtype strain 10403S and the *rodA1*, *rodA2* and

498   *rodA3* single mutant strains, dilutions of overnight cultures were spotted on BHI agar plates

499   with or without 1 µg/ml cefuroxime. Deletion of *rodA1* or *rodA2* results in a slightly reduced

500   ability of these strains to grow on BHI plates supplemented with 1 µg/ml cefuroxime as

501   compared to the wildtype 10403S strain (Fig. 7B). However, deletion of *rodA3* leads to a

502   stronger reduction of growth on BHI plates containing 1 µg/ml cefuroxime as compared to

503   the *rodA1* and *rodA2* single mutants (Fig. 7B). Interestingly, overexpression of RodA3, but

504   not of RodA1 or RodA2, also resulted in decreased resistance towards cefuroxime as

505   compared to the wildtype strain 10403S (Fig. 7C). Our results therefore suggest that *L.*

506   *monocytogenes* induces the expression of *rodA3* and *ftsW2* in the presence of β-lactam

507   antibiotics and moenomycin to compensate for the inhibition of the glycosyltransferase and

508   transpeptidase activity of PBPs. In particular RodA3 seems to play an important function for

509   the intrinsic cephalosporine resistance in *L. monocytogenes* and its expression needs to be

510   finely balanced as both its absence as well as increased expression have detrimental effects

511   on resistance against this antibiotic.

512

513   **FtsW and RodA proteins interact with class B PBPs**

20

514   Previous studies have shown that members of the SEDS protein family act together with a

515   cognate class B PBP to synthesize and crosslink peptidoglycan (Henriques et al., 1998, Wei

516   et al., 2003, Reichmann et al., 2019, Gamba et al., 2009, Daniel et al., 1996). *L.*

517   *monocytogenes* encodes three class B PBPs, namely PBP B1, PBP B2 and PBP B3. To

518   identify potential protein-protein interactions between the *L. monocytogenes* class B PBPs

519   and the FtsW/RodA proteins, a bacterial adenylate cyclase two-hybrid (BACTH) analysis

520   was performed. Interactions were detected between the three *L. monocytogenes* PBP B1, PBP

521   B2 and PBP B3 and all FtsW and RodA homologs (Fig. 8). These results suggest that the

522   FtsW and RodA proteins also form a complex with class B PBPs in *L. monocytogenes*.

523   However, using this bacterial two hybrid approach it was not possible to determine specific

524   SEDS protein and class B PBP pairs.

525

526   **Discussion**

527   Bacterial cell elongation and cell division need to be tightly regulated to maintain the cell

528   shape. This is accomplished by two multiprotein complexes, the elongasome and the

529   divisome, which are coordinated by the actin homolog MreB and the tubulin homolog FtsZ,

530   respectively (Carballido-Lopez and Formstone, 2007, Typas et al., 2012, Jones et al., 2001,

531   Errington and Wu, 2017, den Blaauwen, 2018). The SEDS protein FtsW is part of the

532   divisome and essential for growth as shown for many bacteria including *E. coli, B. subtilis*

533   and *S. aureus* (Boyle et al., 1997, Ikeda et al., 1989, Khattar et al., 1994, Kobayashi et al.,

534   2003, Reichmann et al., 2019). Our experiments suggested that FtsW1 is also essential in *L.*

535   *monocytogenes*, however, a second FtsW protein, FtsW2, can compensate for the loss of

536   FtsW1 if it is expressed from an inducible promoter. FtsW2 is encoded in the *lmo2689-*

537   *lmo2686* operon that appears to be only minimally expressed when *L. monocytogenes* 10403S

538   is grown under standard laboratory conditions (Lobel and Herskovits, 2016). The expression

21

539 of the *lmo2689-lmo2686* operon is regulated by the two-component system CesRK that is

540 activated by cell envelope stress (Kallipolitis et al., 2003, Gottschalk et al., 2008, Nielsen et

541 al., 2012). Using an *L. monocytogenes* strain carrying a P$_{lmo2689}$-*lacZ* promoter fusion, we

542 could detect increased β-galactosidase activity after incubation with sub-inhibitory

543 concentrations of different β-lactam antibiotics including penicillin, cefuroxime, and

544 moenomycin. However, the expression of the *lmo2689-lmo2686* operon was not induced by

545 other cell wall-targeting antibiotics such as vancomycin or the hydrolase lysozyme. This

546 suggests that inhibition of the glycosyltransferase or transpeptidase activity of PBPs leads to

547 activation of the *lmo2689-lmo2686* operon, and hence, to the expression of *ftsW2* as well as

548 *rodA3*.

549 The rod-shape determining protein RodA is part of the elongation machinery. The data

550 presented in this study suggest that *L. monocytogenes* encodes not one but three RodA

551 proteins and depletion of the three RodA enzymes leads to a decreased cell length (Fig. 5).

552 Simultaneous deletion of *rodA1* and *rodA3* already results in the formation of shorter cells,

553 whereas cells of strains deleted for *rodA1/rodA2* or *rodA2/rodA3* have a cell length that is

554 comparable to the wildtype strain 10403S. Taking into consideration that *rodA3* is only

555 minimally expressed under standard laboratory growth conditions in *L. monocytogenes*

556 10403S (Lobel and Herskovits, 2016), the results presented in this study suggest that *rodA3*

557 expression gets induced upon inactivation of RodA1, since we observed morphological

558 differences between the *rodA1* single and the *rodA1/3* double mutant strains. Indeed, β-

559 galactosidase assays confirmed that deletion of *rodA1* or *rodA1/2* increases the activity of the

560 promoter from which *rodA3* is expressed. The data presented in this study also indicate that

561 RodA1 is the "main" RodA enzyme in *L. monocytogenes* as no significant phenotypic

562 changes with regards to growth and cell division could be observed as long as RodA1 was

563 present. On the other hand, RodA2 was only able to compensate for the loss of RodA1 and

564   RodA3, when overproduced from an inducible promoter. Interestingly, cells of strains

565   10403S and 10403SΔ*rodA1*Δ*rodA3* in which *rodA3* is overexpressed from an ectopic locus

566   have an increased cell length as compared to the wildtype. An explanation for this could be

567   that elevated levels of RodA3 lead to the depletion of proteins needed at the cell division site,

568   resulting in an extended synthesis of PG on the lateral wall. Another possibility could be that

569   RodA3 directly inhibits FtsW1 or displaces FtsW1 at the cell division site, leading to a block

570   in cell division and therefore resulting in the formation of elongated cells.

571   Recent studies have shown that SEDS proteins act as glycosyltransferases (Meeske et al.,

572   2016, Emami et al., 2017). The glycosyltransferase activity of PBPs and MGT can be

573   inhibited by moenomycin, whereas RodA/FtsW enzymes are not affected by moenomycin

574   and are therefore important for moenomycin resistance (Tamura et al., 1980, Emami et al.,

575   2017). In good agreement with the importance of SEDS proteins for the intrinsic

576   moenomycin resistance, deletion of the genes encoding two of the three RodA enzymes,

577   RodA1 and RodA3, resulted in an increased moenomycin sensitivity of *L. monocytogenes*

578   (Fig. 6C).

579   In *B. subtilis*, RodA is in a complex with the class B PBP, PBP 2A (also named PbpH), and

580   these two proteins act together to polymerize and crosslink the glycan strands (Wei et al.,

581   2003, Henriques et al., 1998). Similarly, FtsW and PBP 2B form a subcomplex as part of the

582   divisome (Daniel et al., 1996, Gamba et al., 2009). Recently it was shown that RodA-PBP3

583   and FtsW-PBP1 act as cognate pairs in the coccoid bacterium *S. aureus* (Reichmann et al.,

584   2019). Depletion of all three RodA enzymes in *L. monocytogenes*, RodA1, RodA2 and

585   RodA3, leads to a drastic reduction in cell length (Fig. 5). A similar phenotype was observed

586   for a *L. monocytogenes* strain depleted for the essential class B PBP, PBP B1 (Rismondo et

587   al., 2015). In contrast, the absence of either FtsW1 (Fig. 2) or the class B PBP, PBP B2, in *L.*

588   *monocytogenes* results in the formation of elongated cells (Rismondo et al., 2015). These

589   observations suggest that RodA and FtsW might work in a complex with the cognate PBPs

590   PBP B1 and PBP B2 during cell elongation and cell division, respectively. Indeed, protein-

591   protein interactions between FtsW1 and FtsW2 with PBP B2 and between RodA1, RodA2

592   and RodA3 with the PBP B1 could be observed (Fig. 8). However, interactions were also

593   detected between the FtsW proteins and PBP B1 and PBP B3 as well as between the RodA

594   proteins and PBP B2 and PBP B3 (Fig. 8). While these data provide the first line of evidence

595   that SEDS proteins and class B PBPs also form complexes in *L. monocytogenes,* additional

596   work is necessary to determine if specific SEDS-bPBP pairs are formed in *L. monocytogenes*.

597   Taken together, *L. monocytogenes* has a repertoire of PBPs and multiple members of the

598   SEDS family of proteins to produce its rigid cell wall. The expression and the activity of

599   these enzymes need to be tightly regulated in *L. monocytogenes* to maintain its cell shape.

600   Our results suggest that *L. monocytogenes* adapts the expression of a second set of

601   FtsW/RodA enzymes, FtsW2 and RodA3, to environmental stresses such as the presence of

602   β-lactam antibiotics, thereby preventing defects in the peptidoglycan synthesis and

603   subsequent cell lysis. In *L. monocytogenes* strains that lack this back-up system, RodA2

604   might in part fulfil a similar role.

605

606   **Acknowledgements**

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Table 1: Sequence homology between *B. subtilis* and *L. monocytogenes* FtsW/RodA proteins as determined by BLAST.

L. monocytogenes proteins	*B. subtilis* FtsW (403 aa)[*]	*B. subtilis* RodA (393 aa)[*]
Lmo0421	46/128 aa (36%)	77/350 aa (22%)
(416 aa)	7e-14	4e-09
FtsW1 (Lmo1071)	195/403 aa (48%)	100/333 aa (30%)
(402 aa)	1e-122	7e-35
RodA1 (Lmo2427)	124/403 aa (31%)	132/360 aa (37%)
(391 aa)	9e-29	1e-57
RodA2 (Lmo2428)	112/374 aa (30%)	159/402 aa (40%)
(389 aa)	1e-33	1e-66
RodA3 (Lmo2687)	95/324 aa (29%)	110/356 aa (31%)
(369 aa)	3e-25	3e-44
FtsW2 (Lmo2688)	147/342 aa (43%)	95/300 aa (32%)
(376 aa)	5e-67	6e-22

[*] *L. monocytogenes* proteins and their amino acid sizes (aa) are shown in bold in the left column and *B. subtilis* FtsW and RodA proteins used for the homology search are denoted in the top row. The number of amino acids, which were found to be identical between the respective *L. monocytogenes* and *B. subtilis* FtsW/RodA protein are denoted (left number) along with the amino acids that was utilized by the BLAST algorithm for this comparison (right number). The % identity is given in brackets and the corresponding e-values are indicated below.
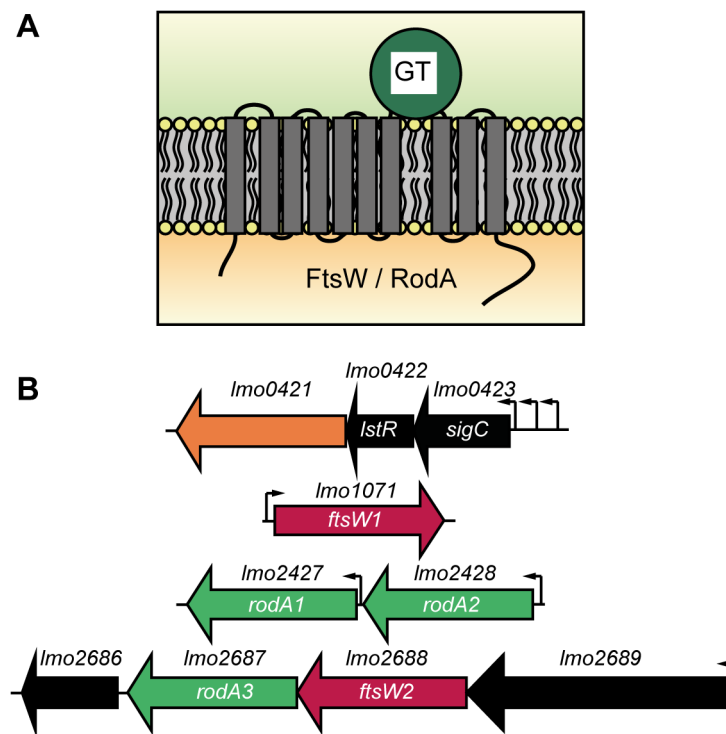
Figure 1: Topology of FtsW and RodA enzymes and their genomic arrangement in *L. monocytogenes*. (A) Topology models for FtsW and RodA enzymes of *L. monocytogenes* as predicted using the TMHMM v. 2.0 server (Sonnhammer et al., 1998). (B) Genomic organization of *ftsW/rodA* genes in *L. monocytogenes*. Arrowheads indicate the gene orientation. Small black arrows indicate promoters based on the operon structures described in Toledo-Arana *et al*. (Toledo-Arana et al., 2009). Three different promoters have been identified for the *sigC* operon (Zhang et al., 2005).
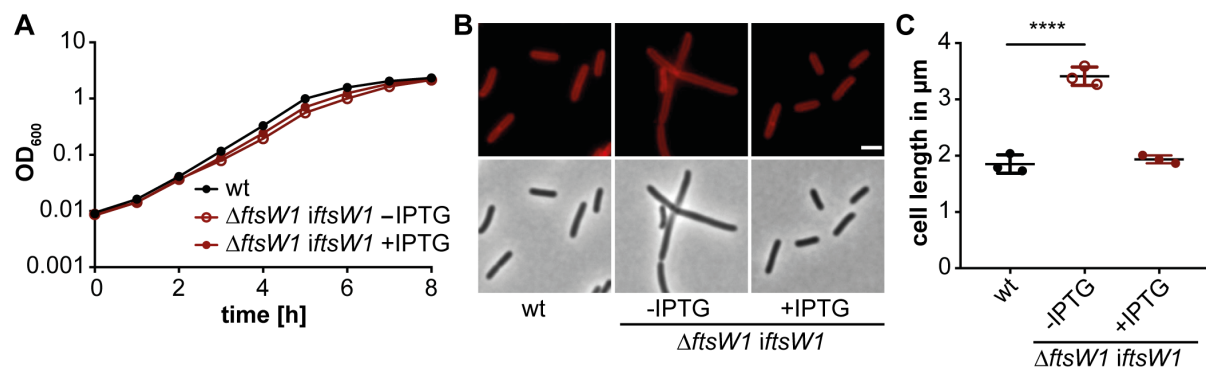
Inserted Δ*ftsW1* *in figure to have it match with the legend*

Figure 2: Depletion of FtsW1 results in cell elongation. (A) Growth of the *L. monocytogenes* FtsW1 depletion strain. Strain 10403SΔ*ftsW1* i*ftsW1* was grown overnight in BHI medium in the presence of 1 mM IPTG. The following day, the cells were washed and used to inoculate fresh BHI medium without IPTG and incubated at 37°C for 8-10h, diluted again and grown until the next morning. The depletion culture was used to inoculate BHI medium without IPTG or with 1 mM IPTG and the growth was monitored by determining OD_{600} readings at hourly intervals. Wild type *L. monocytogenes* strain 10403S (wt) was used as control. Averages and standard deviations from three independent experiments were plotted. Of note, due to the small standard deviations the actual error bars are not visible in the graph. (B) Microscopy analysis of 10403S (wt) and the *ftsW1* depletion strain grown in the presence or absence of IPTG. For depletion of FtsW1, strain 10403SΔ*ftsW1* i*ftsW1* was grown as described in panel A. Cultures of 10403S and 10403SΔ*ftsW1* i*ftsW1* were diluted 1:100 in fresh BHI medium (with 1 mM IPTG where indicated), grown for 3 h at 37°C and the cells subsequently stained with the membrane dye nile red and analyzed by fluorescence and phase contrast microscopy. The scale bar is 2 μm. (C) Cell lengths measurement of 10403S (wt) and the *ftsW1* depletion strain. The cell length of 300 cells per strain was measured and the median cell length calculated and plotted. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted. For

statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was performed (**** p≤0.0001).
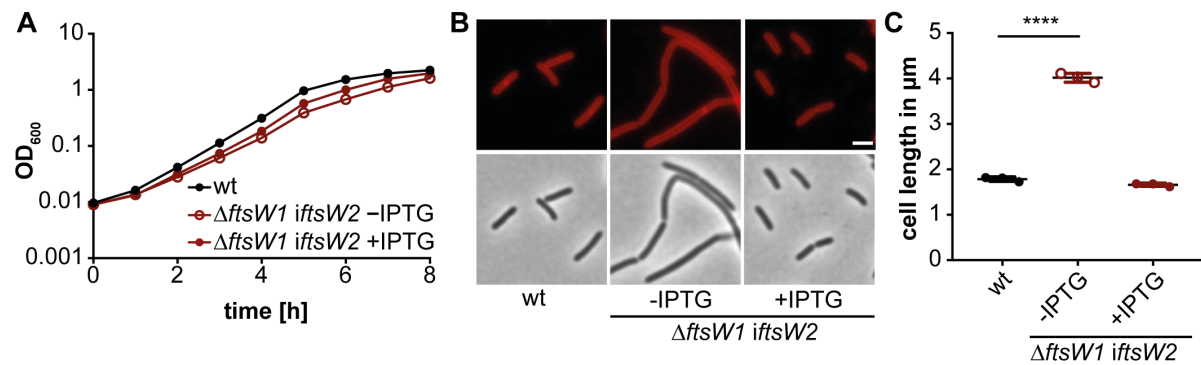
Fig. 3: Overexpression of FtsW2 can compensate for the loss of FtsW1. (A) Growth comparison of strains 10403S (wt) and 10403SΔ*ftsW1* i*ftsW2*. Growth curves were performed as described in Figure 2. (B) Microscopy images of strains 10403S (wt) and 10403SΔ*ftsW1* i*ftsW2*. For depletion of FtsW2, strain 10403SΔ*ftsW1* i*ftsW2* was grown as described in Figure 2. The next morning, cells were diluted 1:100 and grown in BHI broth (with or without IPTG as indicated) until mid-logarithmic growth phase, stained with the membrane dye nile red and subjected to fluorescence and phase contrast microscopy analysis. Scale bar is 2 μm. (C) Cell lengths measurements of 10403S (wt) and 10403SΔ*ftsW1* i*ftsW2*. The cell length of 300 cells per strain was measured and the median cell length calculated and plotted. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was performed (**** $p \leq 0.0001$).
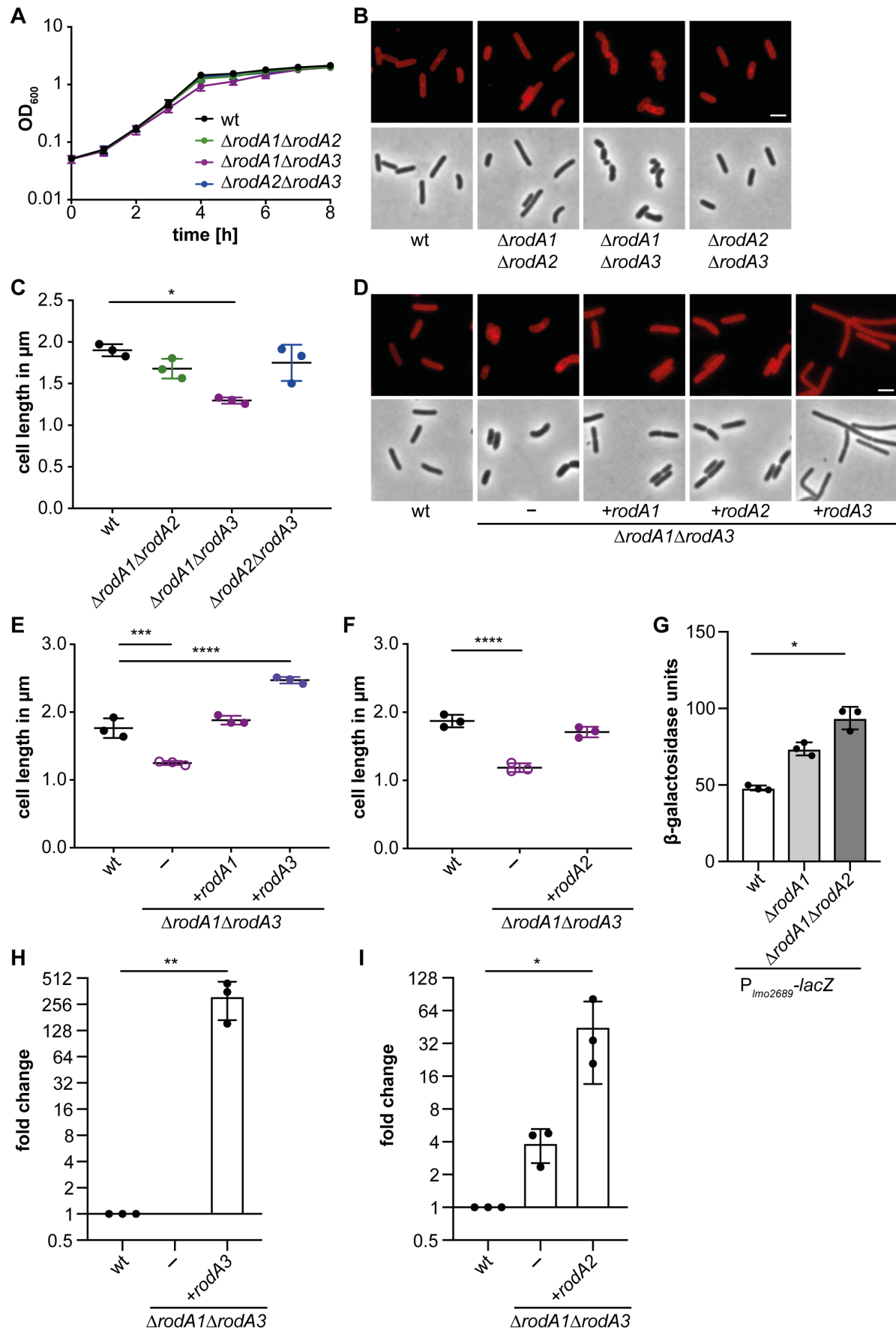
Fig. 4: RodA1 and RodA3 are essential to maintain the rod shape of *L. monocytogenes*.

(A) Growth of *L. monocytogenes* 10403S (wt), 10403S∆*rodA1*∆*rodA2*, 10403S∆*rodA1*∆*rodA3* and 10403S∆*rodA2*∆*rodA3* strains in BHI broth at 37°C. (B) Microscopy images of wt and mutant *L. monocytogenes* strains. Cells from the strains described in panel A were stained with the membrane dye nile red and analyzed by fluorescence and phase contrast microscopy. Scale bar is 2 μm. (C) Cell lengths measurements of wt and mutant *L. monocytogenes* strains. The cell length of 300 cells per strain was measured and the median cell length calculated and plotted. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted. For statistical analysis, one-way ANOVA t-tests for multiple comparisons were used (* $p \leq 0.05$). (D) Microscopy images of wildtype and mutant *L. monocytogenes* strains. Cells from 10403S (wt), 10403S∆*rodA1*∆*rodA3* (-) and 10403S∆*rodA1*∆*rodA3* expressing *rodA1*, *rodA2* or *rodA3* from an IPTG-inducible promoter were stained with the membrane dye nile red and analyzed by fluorescence and phase contrast microscopy. Scale bar is 2 μm. (E-F) Cell length analysis of 10403S∆*rodA1*∆*rodA3* complementation strains. Analysis was performed as described in panel C. (G) β-galactosidase assay using *L. monocytogenes* strains 10403S (wt), 10403S∆*rodA1* and 10403S∆*rodA1-2* carrying a $P_{lmo2689}$-*lacZ* fusion construct. Averages and standard deviations of three independent experiments were plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was used (* $p \leq 0.05$). (H) Analysis of *rodA3* expression by qRT-PCR. RNA was isolated from strains 10403S (wt), 10403S∆*rodA1*∆*rodA3* (-) and 10403S∆*rodA1*∆*rodA3* i*rodA3* grown in the presence of IPTG. Expression of *rodA3* was normalized to the expression of *gyrB* and fold changes calculated using the ∆∆Ct method. Averages and standard deviations of three independent experiments were plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test

was performed (** p≤0.01). (I) Analysis of *rodA2* expression using qRT-PCR. Same as panel (H) but using strains 10403S, 10403S*ΔrodA1ΔrodA3* (-) and 10403S*ΔrodA1ΔrodA3* i*rodA2* grown in the presence of IPTG. Averages and standard deviations of three independent experiments were plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was performed (* p≤0.05).
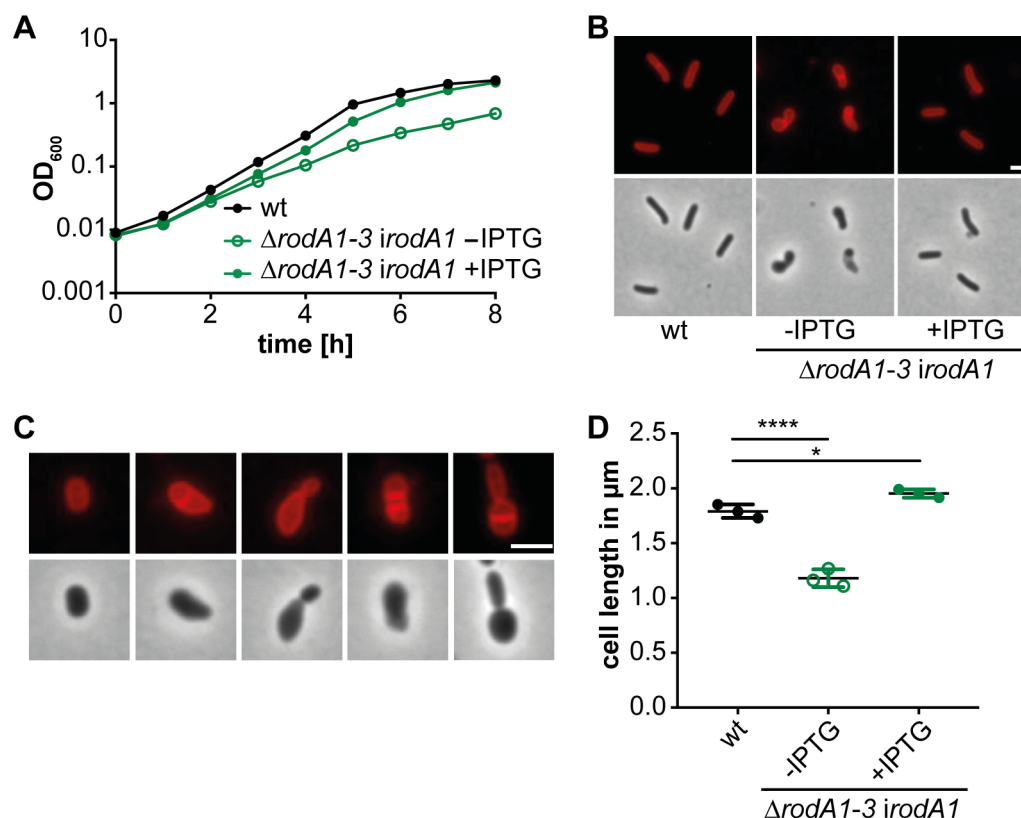
Figure 5: Absence of all three RodA proteins leads to a drastic cell shortening. (A) Growth of *L. monocytogenes* in the absence of all three RodA proteins. Growth curves were performed as described in Figure 2 using strain 10403S and strain 10403SΔ*rodA1-3* i*rodA1* grown in the presence of absence of IPTG. (B and C) Microscopy analysis of strain 10403SΔ*rodA1-3* i*rodA1*. For depletion of RodA1, strain 10403SΔ*rodA1-3* i*rodA1* was grown in the same way as described for the FtsW1 depletion in Figure 2. (B) Wildtype 10403S and depleted 10403SΔ*rodA1-3* i*rodA1* cultures were diluted 1:100, grown for 3 h at 37°C (where indicated in the presence of 1 mM IPTG), stained with nile red and phase contrast and fluorescence microscopy images were taken. Scale bar is 2 μm. (C) Microscopy images showing examples of different cell morphologies observed for strain 10403SΔ*rodA1-3 irodA1* depleted for RodA1. Scale bar is 2 μm. (D) Cell lengths measurements of 10403S (wt) and strain 10403SΔ*rodA1-3* i*rodA1*. The cell length of 300 cells per strain was measured. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted. For statistical analysis, a one-way ANOVA

coupled with a Dunnett's multiple comparisons test was performed (* p≤0.05, **** p≤0.0001).
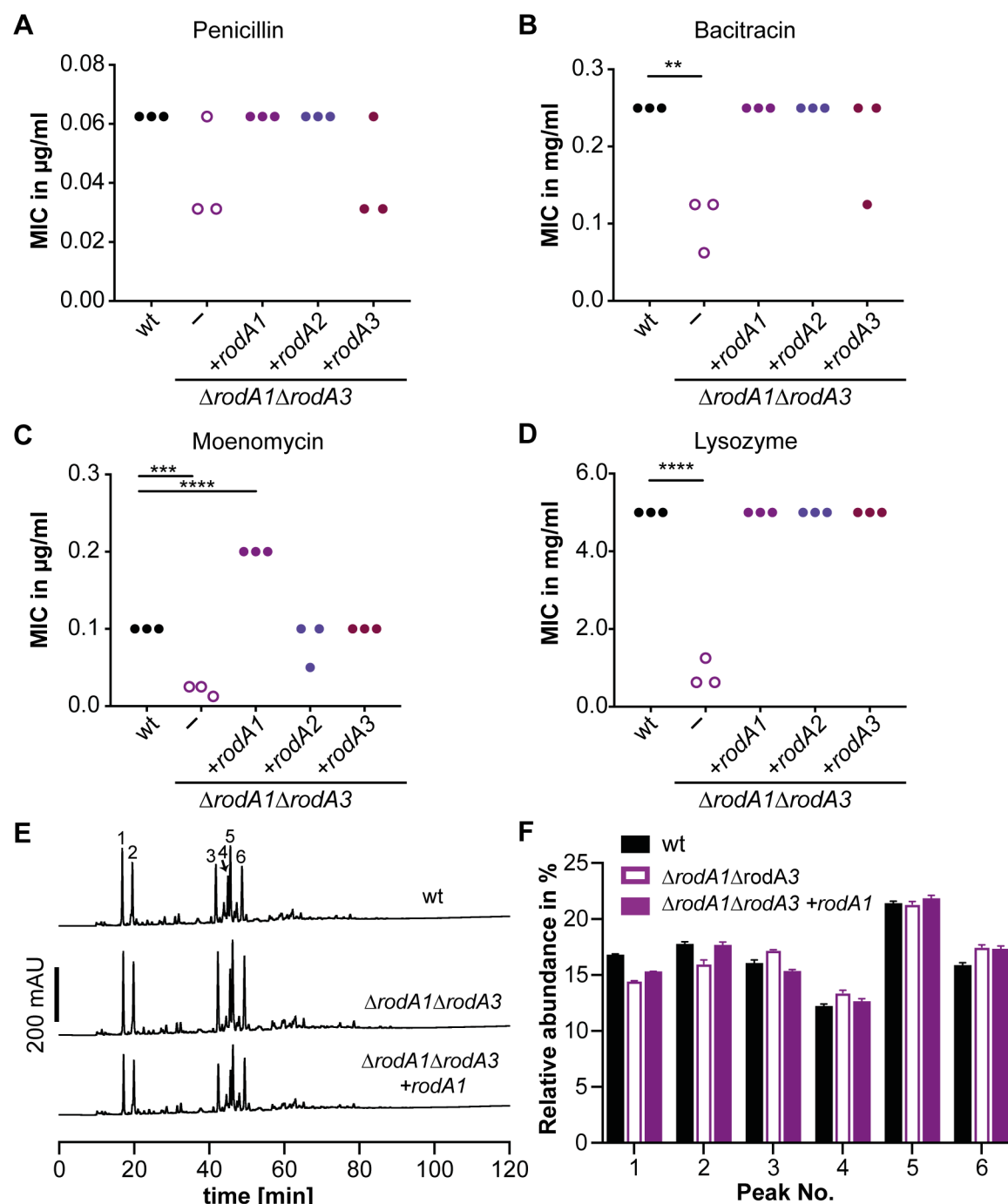
Figure 6: Impact of *rodA1* and *rodA3* deletion on the antibiotic and lysozyme resistance.

The minimal inhibitory concentrations for the antibiotics penicillin G (A), bacitracin A (B), moenomycin (C) and lysozyme (D) were determined for the wildtype *L. monocytogenes* strain 10403S, the Δ*rodA1*Δ*rodA3* deletion strain and the complementation strains 10403SΔ*rodA1*Δ*rodA3* i*rodA1,* 10403SΔ*rodA1*Δ*rodA3* i*rodA2* and 10403SΔ*rodA1*Δ*rodA3* i*rodA3* using a microbroth dilution assay. The complementation strains were grown in the

presence of 1 mM IPTG. The result of three biological replicates are shown in A-D. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was used (** p≤0.01, *** p≤0.001, **** p≤0.0001). (E) HPLC analysis of the muropeptide composition of 10403S (wt), 10403SΔ*rodA1*Δ*rodA3* and 10403SΔ*rodA1*Δ*rodA3* i*rodA1* (grown in the presence of IPTG to induce expression of RodA1). The major muropeptide peaks are numbered 1-6 as previously described (Rismondo et al., 2015, Burke et al., 2014). (F) Relative abundance of muropeptide peaks 1-6 in peptidoglycan isolated from strains 10403S (wt), 10403SΔ*rodA1*Δ*rodA3* and 10403SΔ*rodA1*Δ*rodA3* i*rodA1*. Average values and standard deviations were calculated from three independent peptidoglycan extractions.
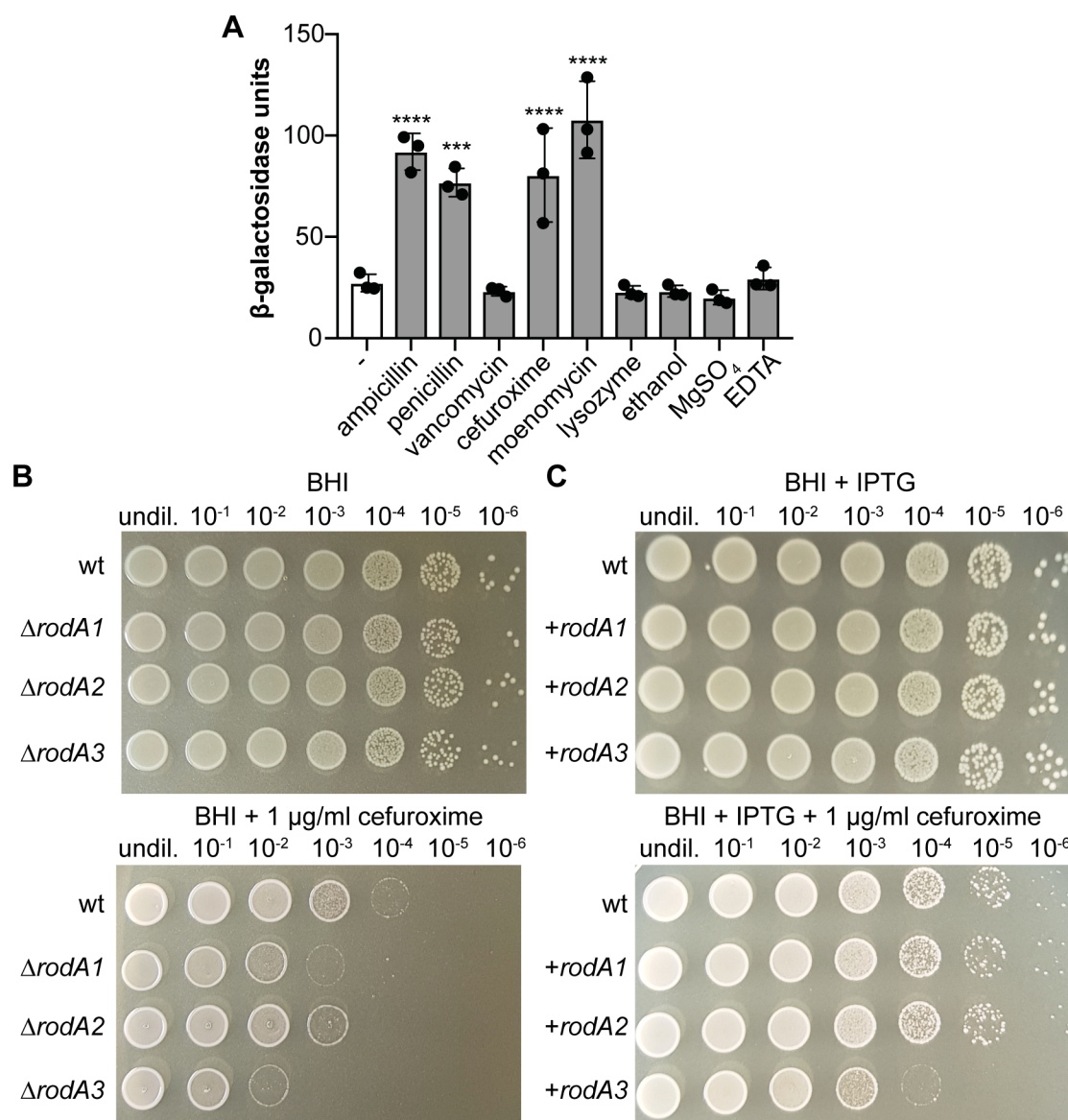
Figure 7: Cell wall-acting antibiotics increase the *lmo2689* promoter activity. (A) Bacteria from mid-logarithmic cultures of strain 10403S pPL3e-P$_{lmo2689}$-*lacZ* were exposed for 2 h at 37°C to different stressors. The activity of the *lmo2689* promoter was subsequently determined by performing β-galactosidase activity assays as described in the method section. Bacteria that had been grown in the absence of a stressor were included as negative (-) control. The averages of the β-galactosidase activity units and standard deviations from three independent experiments were plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was used (*** p≤0.001, **** p≤0.0001). (B) Absence and overexpression of RodA3 results in decreased cefuroxime resistance. Dilutions

of overnight cultures of strains (B) 10403S (wt), 10403SΔ*rodA1*, 10403SΔ*rodA2* and 10403SΔ*rodA3* and (C) 10403S (wt), 10403S pIMK3-*rodA1*, 10403S pIMK3-*rodA2* and 10403S pIMK3-*rodA3* were spotted on BHI agar and BHI agar containing 1 µg/ml cefuroxime and incubated for 24 h at 37°C. BHI agar plates were supplemented with 1 mM IPTG for cefuroxime resistance assays shown in panel C. A representative result from three independent experiments is shown.
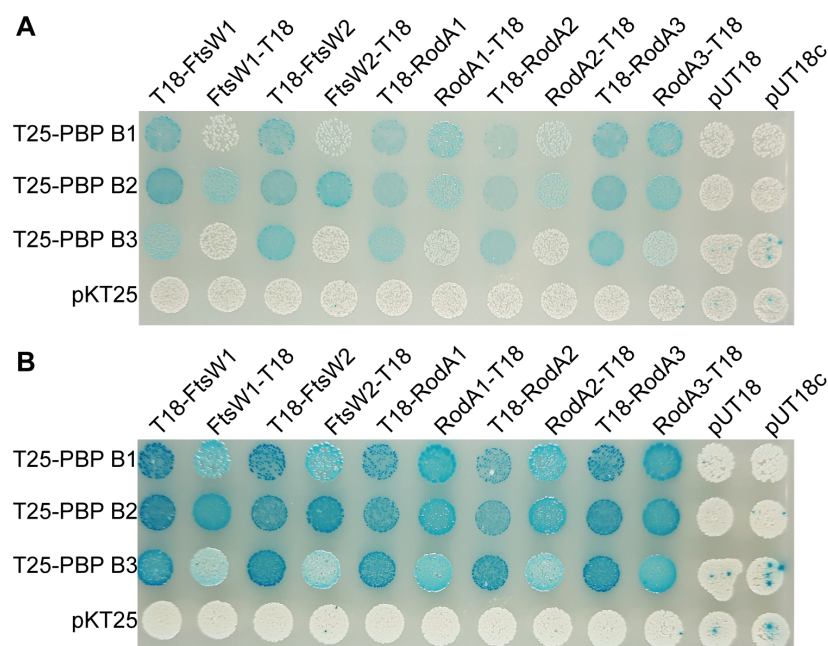
Figure 8: FtsW and RodA enzymes interact with class B PBPs. (A-B) FtsW/RodA and class B PBPs were fused to the T18 and T25-fragments of the *Bordetella pertussis* adenylate cyclase and co-transformed into the bacterial two-hybrid strain BTH101. Co-transformations with pKT25, pUT18 and pUT18c were used as negative controls. The plates were photographed after incubation at 30°C for (A) 24 h or (B) 48 h. Representative images from four independent experiments are shown.

```
Bsu RodA    ----------------------------------------------------------------MSRYKKQQSPFYQGDLIFI 19
Bsu FtsW    -------------------------------------------------------------------MLKKMLKSYDY-SLIFA 16
Lmo0421     MSSSTFEEYLGKVIANVKSKQAHSMIKKELSNHLEELSHSFQKRGLSIEDANKKAMQEMGDPSTVGRNMNQLHKPRMDW-LLIAL 84
FtsW1       ------------------------------------------------------------------MPMFKRILKSYDY-AFIAV 18
RodA1       -------------------------------------------------------------------MNQQNKLAGRIDY-GIVLS 18
RodA2       -----------------------------------------------------------------MARNRKKAVRIDY-SIIFL 18
RodA3       -------------------------------------------------------------------------MKRDVLYN-RIILS 13
FtsW2       -----------------------------------------------------------------------MRAGR-VLFVT 10
                                                                                          ::.

Bsu RodA    FGVFFIISVVSIYAAGQF------GQYGNTDWIQQIVFYLLGAVAI-TVLLYFDLEQLEKLSLYIFIIGILSLIILKISPESIAP 97
Bsu FtsW    IVLLCGFGLVMVYSSSMITAVSRYGVSSNFFFMRQLFALIAGG-ALFILMALFPYKALAHQKFQKGI--LLVSVLALISLFVFGH 98
Lmo0421     FILLAGISFLPIIGD--------VVASNSSFMKKQIVWLAIAVLAL-IGFLFFDYRKLKDLWMYFYA----AALILFTTTFLVGI 156
FtsW1       FIVLCLFGLIMIYSASWSLAIG-KGLPADYFYARQVKNFIISF-IFFILFALLPFKFYQNNKVLMLI--VFGSIGVLLLIFLVGK 99
RodA1       MMLLMIISLVSIYSAQLTN-----NQYDANFVVKQAMWFVVSTFAI-IVVMQLDYDRLTKWAYYFYGL---GLFLL-VFVLLFGK 93
RodA2       MMLLCIIGLVAIYVAGLVN-----DQYTNNFLLQQSIWIVISTGVVVVIVLFFDYDKLQWAAYYLYGI---GNLLL-VLVLIVGD 94
RodA3       VFLLSLVSCVAIYFAQQT------NQYNTNFLGMQLVFLAIGTLTCF-GVSRLPIEFLRHHAIWLYVIMVITLLGI-LIPNPLVQ 90
FtsW2       YLLLAIWSLLLVYSTSYGVAVMRYKVEPSYFFNRQLLFYGLGF-LGLLVCSRINVQLFYLRRTLRIL--AGSLLGLLLLVLLTGS 92
                ::    . : :                           *                        .    .  . :

Bsu RodA    VIKGAKSWFRIGRI-TIQPSEFMKVGLIMMLASVIGKANPKG-VRTLRDDIHLLLKIAGV---AVIPVGLILM-QDAGTAGICMF 174
Bsu FtsW    VAGNAQSWFKIGGM-SIQPGEFVKLVVILYLAAVYAKKQSYI-DHL-------LTGVAPPVVMTLIICGLIAMQPDFGTAMIIGL 174
Lmo0421     PLTGGRWMSLWGI-AIDSPAISLFLFFIAWAGIFTKANAFKG----------WKKQVVLLIL--------FWVPVISYIIINRF 222
FtsW1       TVNNANSWLVLGPR-SLQPGEFAKLAVIIYMSAIYAKKQSYI-DDF-------NRGVLPPIFFLAFVCFLIAIQPDTGTAFIIFL 175
RodA1       EVKGAKSWIVIPFLGNIQPSEVVKVILIVVLAKVIWDHNRTYKIHRFSYDAWLLLKIGLF---TLMPLILIMLQPDLGTALVFIA 175
RodA2       ERKGSKSWISIGSLGSLQPSELMKSFLILALAKVIWDHNKKYKLHTVSLDMQLLLKIGIV---SILPLGLVALQPDLGTILVFIA 176
RodA3       NINGATRWYRFAGL-SFQPSEVVKSIFIFVLAHFAVK----Y-------QAQKWKQLGILTVLTGIVLLLIMKQPDLGTTIVYGV 163
FtsW2       AANNAQRWLSIAGV-TFQPTEMVKLLLILVIATVFLKKGCGV-RVQ-------Y----WLLGFLFLTVGLVFLQPDLGTALILGV 164
                .. * : :: . .:. : . .                                        . :

Bsu RodA    IVLVMVFMSGINWKLIAIIAGSGILLISLILLVMINFPDV----------AKSVGIQDY-QIKRVTSWVSASNETQEDSNDSWQV 250
Bsu FtsW    IATCMILCSGFSGKTLVRLVILGGIVFILVSPIIYL--------------NQDKILTEG-RLARFESLEDPFKYANS---SGLQV 241
Lmo0421     VFSIM-----------------YFLCVLVMYIFYYRHNRFAIKVALGNLLVGVIFISTMLLKYPSSYLPDTSIPLK-------- 281
FtsW1       VGCCIIITSGMRLRTIMKLIGIGMGIIIGLTLILFALPDS----------VRNEIVSPT-KVARITTFMNPFEYADK---EGHQL 246
RodA1       IMSGMILISGITWKIIVPLFGSIAAIGTALIWMVIYHQNW----------LTSLGFKPY-QFERITWINPENDPQ---GGGYQV 246
RodA2       IIIGMVFISGVTWKILLPVFSSIALIGGTLIYLVMYNQEF----------LQKLGFKPY-QFKRITSWLRPEEDPL---GDGMQL 247
RodA3       TALAIILLAIKSTKLMVGIITLILTAATIGMYVVVYHISL----------LEKIGFHAY-QFSRIQTWLDPTTDPD---A-VYQL 233
FtsW2       IGVALFLTSGVGLTRLVRVAIWTFGLLLLVAMLIYFF-------------HPDFFSSA-KLGRFA-FLDPFNLDNL--DASYQL 231
                :                 ::.            .    .

Bsu RodA    DQAIMAIGSGGILGNGISNL---KVYVPESTTDFIFSIIGESFGFIGCAIVVIMFFFLIYRLVVLIDKIHPFNRFASFFCVGYTA 332
Bsu FtsW    INSYYAISSGIFGLGLGESIQKYGYLPESHTDFIMAVIAEELGIFGVLFVIFLLGFVVIKGFYIA--RKCEDPFGSLLAIGISS 324
Lmo0421     ----DILSKAGWFGKGLHN----NLILPEAHTDFVFPFLVYSLGWVFGIFLCLLLVVFILRISRNA--FKTKDLFGRLLTIGGAV 356
FtsW1       INSFYAIGSGGVSGQGLGESVQKLGYLPEAHTDFIIAVVAEELGVFGVMFIILALFFIIFKTITTG--LRAKDPFASLMCYGIAS 329
RodA1       LRAMTAIGSGQISGNGAGYD---AIAIPENHNDFIFTIVAGDYGFIGASILLAIYFLLIYQIIRVA--LDVGVPFYSYICTGVVM 326
RodA2       LRSMQAIGSGQLQGNGIGNQ---AIAIPENHNDFIFSIIGGNFGFIGGCVLIMLYFLLIYQIIRVA--LDINIPFYSYICTGVCS 327
RodA3       NLSMKAVGSGMMTGSSGT-----NAYIPESHTDMIFSTIGHQFGFVGVSLLLILFMVLIHQLIMAA--LLMKNTFSSLVLAGFAV 311
FtsW2       RNGYYAIGSGGIFGNGLGGSIQKLGYLPEPHTDFIMTVIAEELGVFGVIWTIFLLMALSFTALYIA--ISSHFIFDSMVCIGVAS 314
                :... *  .           :** .*:::  :  . *.       .               * . *

Bsu RodA    LIVIHTFQNIGMNIGIMPVTGIPLLFVSYGGSSTLSTLIGFGIVYNASVQLTKYRSYLFNS----------------- 393
Bsu FtsW    MIAIQSFINLGGVSGLIPITGVTLPFISYGGSSLVLLLGSMGILANISMFVKYSENKKKKEPLAPKGMKKKQLKKTVYL 403
Lmo0421     LFTVPACWNILMGLGIVPIMVVPLPFISYGGSMLVYAALLGLILNVYRRKDIVESTLVN------------------ 416
FtsW1       LIAIQAFINLGGASGLIPLTGVTLPFISYGGSSLMVLSMMLGIVANISMFTKYQRVYKADGSTK--ELPKKQKRR---- 402
RodA1       MLMFHVLENVGMNIGLLPITGIPLPFISYGGSALLGNMMAVGLVLGIRFNFKKSMFEVKEENHAS------------- 391
RodA2       MILFHVLENIGMTIGLLPITGIPLLFVSYGGSSLLGAFMALGLVLSARYNAPEVNLGKENRF--------------- 389
RodA3       SFAFNIFENIGMTVGLMPLTGIPLPFISYGGSAVLGNFIAVGVVLAIIRSDADLIEEK------------------- 369
FtsW2       WVSVQMFLNLGGVSGIIPLTGVPLPFISYGGSSVVMLSCAVGFVLAAARRNVL---------------AKTREVVYL 376
                . .     *:     *::*:  : * *:***** :      .*.:
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Figure S1: Sequence alignment of FtsW and RodA proteins from *B. subtilis* and *L. monocytogenes*. The alignment was created using Clustal Omega (Sievers et al., 2011). Based on a study by Meeske *et al.,* amino acid residues of *B. subtilis* RodA that are essential for RodA function are colored in blue, where conservative replacements are possible in

yellow and amino acids that can be changed to certain other amino acids are colored in
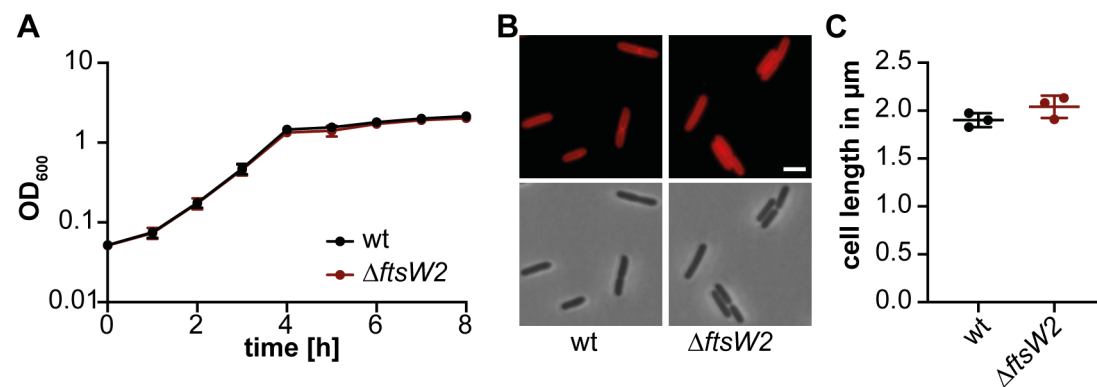
orange (Meeske et al., 2016).

Figure S2: An *L. monocytogenes ftsW2* mutant does not show a growth or cell length defect. (A) Growth of *L. monocytogenes* strains 10403S (wt) and 10403SΔ*ftsW2* in BHI broth at 37°C. (B) Microscopy images of wildtype and *ftsW2* mutant *L. monocytogenes* strains. Bacteria from cultures of the strains described in panel A were stained with the membrane dye nile red and analyzed by fluorescence and phase contrast microscopy. Scale bar is 2 µm. (C) Cell length measurement. The cell length of 300 cells of strain 10403S (wt) and 10403SΔ*ftsW2* was measured and the median cell length calculated and plotted. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted.
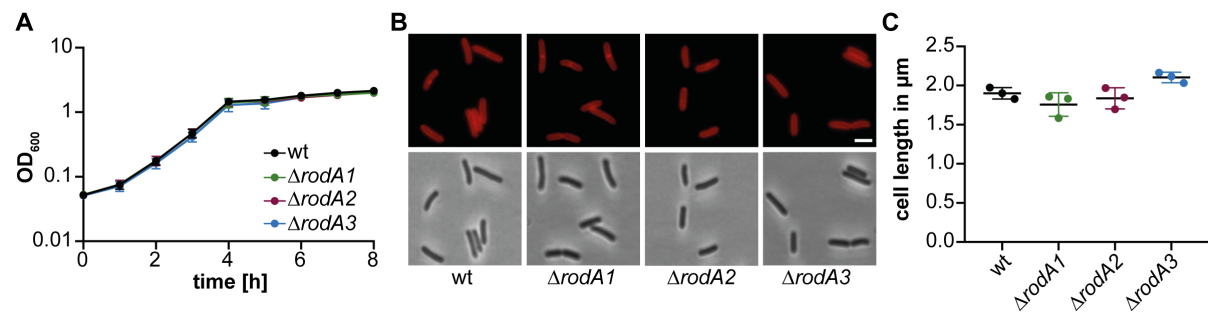
Figure S3: Single *rodA1*, *rodA2* or *rodA3* *L. monocytogenes* strains do not show a growth or cell length defect. (A) Growth of *L. monocytogenes* 10403S (wt), 10403SΔ*rodA1*, 10403SΔ*rodA2* and 10403SΔ*rodA3* in BHI broth at 37°C. (B) Microscopy images of wildtype and mutant *L. monocytogenes* strains. Bacteria from cultures of the strains described in panel A were stained with the membrane dye nile red and analyzed by fluorescence and phase contrast microscopy. Scale bar is 2 μm. (C) Cell length of *L. monocytogenes* wt and *rodA* single mutants. The cell length of 300 cells per strain was measured and the median cell length calculated and plotted. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted.
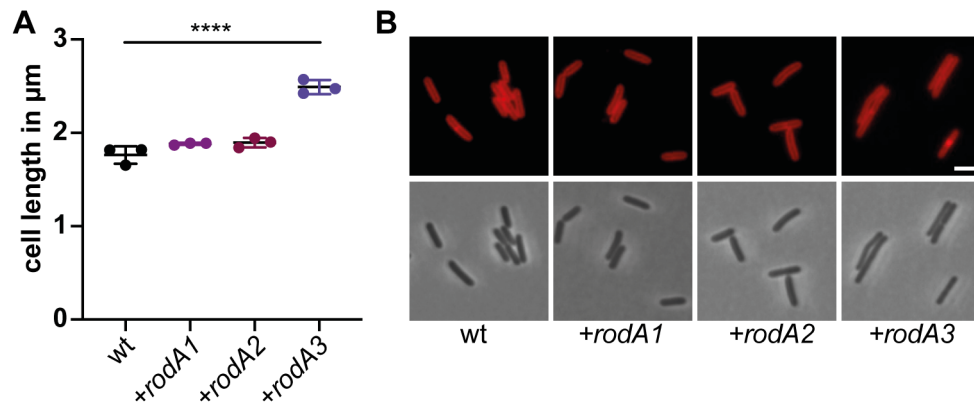
Figure S4: Overexpression of RodA3 results in cell elongation. (A) Cell lengths measurements of 10403S (wt) and strains 10403S pIMK3-*rodA1*, 10403S pIMK3-*rodA2* and 10403S pIMK3-*rodA3*. The cell length of 300 cells per strain was measured. Three independent experiments were performed, and the average and standard deviation of the median cell length plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple comparisons test was performed (**** p≤0.0001). (B) Microscopy images of *L. monocytogenes* strains. Bacteria from cultures of the strains described in panel A were stained with the membrane dye nile red and analyzed by fluorescence and phase contrast microscopy. Scale bar is 2 μm.
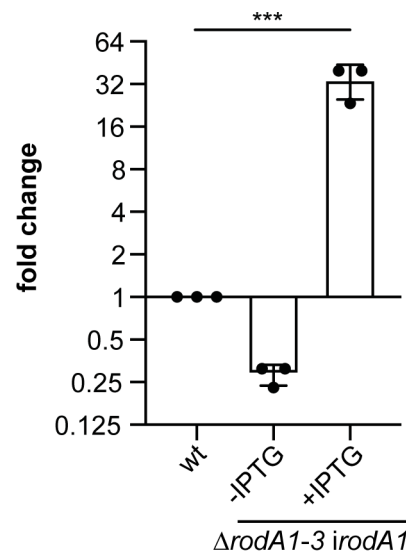
Fig. S5: *rodA1* transcripts are still detected even after prolonged RodA1 depletion.
Analysis of *rodA1* expression using qRT-PCR. RNA was isolated from strains 10403S and
10403S*ΔrodA1-3* i*rodA1,* that had been grown in absence and presence of IPTG. Expression
of *rodA1* was normalized to the expression of *gyrB* and fold changes calculated using the
ΔΔCt method. Averages and standard deviations of three independent experiments were
plotted. For statistical analysis, a one-way ANOVA coupled with a Dunnett's multiple
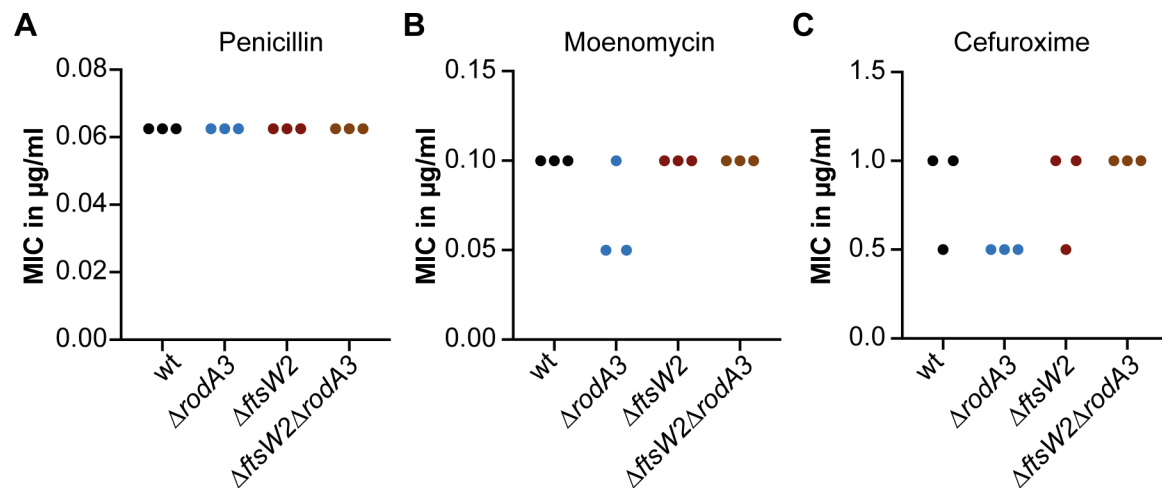comparisons test was performed (*** p≤0.001).

Figure S6: Deletion of *rodA3* but not *ftsW2* has a minor impact on the resistance towards a selected number of cell wall active antibiotics. The minimal inhibitory concentrations for the antibiotics penicillin G (A), moenomycin (B) and cefuroxime (C) were determined for the wildtype *L. monocytogenes* strain 10403S, the 10403S*ΔrodA3* and 10403S*ΔftsW2* single deletion strains and the 10403S*ΔftsW2ΔrodA3* double mutant strain using a microbroth dilution assay. The result of three biological replicates are shown in A-C.