1   **_Listeria monocytogenes_ requires DHNA-dependent intracellular redox**

2   **homeostasis facilitated by Ndh2 for survival and virulence**

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18   homeostasis, extracellular electron transfer, Ndh2

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20   **RUNNING TITLE:** Ndh2 utilizes DHNA to maintain redox homeostasis

ABSTRACT

Listeria monocytogenes is a remarkably well-adapted facultative intracellular pathogen that can thrive in a wide range of ecological niches. *L. monocytogenes* maximizes its ability to generate energy from diverse carbon sources using a respiro-fermentative metabolism that can function under both aerobic and anaerobic conditions. Cellular respiration maintains redox homeostasis by regenerating NAD^+ while also generating a proton motive force (PMF). The end products of the menaquinone (MK) biosynthesis pathway are essential to drive both aerobic and anaerobic cellular respiration. We previously demonstrated that intermediates in the MK biosynthesis pathway, notably 1,4-dihydroxy-2-naphthoate (DHNA), are required for the survival and virulence of *L. monocytogenes* independent of their role in respiration. Furthermore, we found that restoration of NAD^+/NADH ratio through expression of water-forming NADH oxidase (NOX) could rescue phenotypes associated with DHNA deficiency. Here we extend these findings to demonstrate that endogenous production or direct supplementation of DHNA restored both the cellular redox homeostasis and metabolic output of fermentation in *L. monocytogenes*. Further, exogenous supplementation of DHNA rescues the *in vitro* growth and *ex vivo* virulence of *L. monocytogenes* DHNA-deficient mutants. Finally, we demonstrate that exogenous DHNA restores redox balance in *L. monocytogenes* specifically through the recently annotated NADH dehydrogenase Ndh2, independent of the extracellular electron transport (EET) pathway. These data suggest that the production of DHNA may represent an additional layer of metabolic adaptability by *L. monocytogenes* to drive energy metabolism in the absence of respiration-favorable conditions.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen that is exceptionally well-adapted to survive and replicate in the restrictive mammalian host cytosol (1-3). Bacteria that lack the specific adaptations required to survive or replicate in the host niche are effectively cleared (4-7), often by triggering host defense mechanisms comprised of innate immune pathways (8-13). *L. monocytogenes* utilizes its internalin proteins to facilitate invasion into the host cell where it becomes captured in a phagosome (14, 15). The pore-forming cytolysin listeriolysin O (LLO) then facilitates escape from the phagosome into the cytosol (14, 16), where *L. monocytogenes* can utilize ActA to mediate actin-based motility by hijacking the host's actin machinery (17-20). Using this motility, *L. monocytogenes* moves into adjacent cells where they again invade the cytosol by expressing LLO and two phospholipase Cs, PlcA and PlcB, enabling it to restart its life cycle (14, 21).

L. monocytogenes can also thrive in a diverse range of ecological niches that contain highly variable pools of fermentable and non-fermentable carbon sources (2, 22). *L. monocytogenes* employs both fermentative and respiratory metabolic mechanisms to maximize its energy output from scavenged nutrients (22, 23). In contrast to canonical respiratory organisms however, *L. monocytogenes* contains an incomplete tricarboxylic acid (TCA) cycle and is therefore unable to fully oxidize its carbon substrates (24). Accordingly, *L. monocytogenes* utilizes a respiro-fermentative metabolism characterized by glycolysis-derived pyruvate that is funneled into the fermentative production of acetate, generating ATP through substrate-level phosphorylation (SLP) via the activity of acetate kinase (24, 34). During the respiro-

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67 fermentative process, the activity of *L. monocytogenes*' respiratory electron transport

68 chain (ETC) enables it to regenerate NAD^+, without having to rely upon lactate

69 dehydrogenase, while also producing a functional proton motive force (PMF) (22, 24,

70 34). Further lending to its diverse metabolic adaptability, *L. monocytogenes* possesses

71 two distinct respiratory ETCs that allow it to respire both aerobically and anaerobically

72 (25). The aerobic ETC in *L. monocytogenes* mediates electron transfer from a type II

73 NADH dehydrogenase, Ndh1, to a membrane-bound menaquinone (MK) and

74 subsequently to terminal cytochrome oxidases QoxAB (aa3) or CydAB (bd) for final

75 transfer to O_2 (26, 27). In contrast, the recently annotated anaerobic respiratory

76 pathway in *L. monocytogenes* uses a flavin-based ETC to drive extracellular electron

77 transfer (EET) to extracytosolic acceptors such as fumarate or ferric ion using a novel

78 NADH dehydrogenase (Ndh2) and an alternative demethylmenaquinone (DMK)

79 intermediate (25, 28). Both of the respiratory ETC in *L. monocytogenes* rely upon the

80 MK biosynthesis pathway to generate their respective quinone electron acceptors, with

81 the biosynthetic intermediate 1,4-dihydroxy-2-naphthoate (DHNA) functioning as a

82 mutual branching point (**Fig. S1**) (25).

83   The requirement for *L. monocytogenes* to perform cellular respiration during

84 infection has been well documented (29-32). However, understanding the specific

85 contributions of maintaining cellular redox homeostasis via NAD^+ regeneration versus

86 the production of a functional PMF to achieve virulence has remained elusive. Further

87 complicating our ability to dissect the specific contributions that cellular respiration may

88 have during infection, the MK intermediates DHNA-CoA and DHNA have recently been

89 reported to be required for the survival and virulence of *L. monocytogenes* independent

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90   of MK synthesis and aerobic respiration (29, 31, 32). Importantly, although it was

91   observed that the supplementation of exogenous DHNA could rescue the *in vitro* growth

92   of a DHNA-deficient *L. monocytogenes* mutant, this rescue did not coincide with the

93   restoration of its PMF (31). Therefore, although DHNA-deficient strains of *L.*

94   *monocytogenes* possess the downstream enzymes to produce MK or DMK, these data

95   suggest that exogenous DHNA is not utilized to promote either aerobic or anaerobic

96   cellular respiration. Recent work from Rivera-Lugo *et al.* sought to dissect the relative

97   importance of maintaining redox homeostasis versus PMF generation for the

98   pathogenesis of *L. monocytogenes* using a water-forming NADH oxidase (NOX) that

99   specifically regenerates NAD^+ independent of respiration and PMF function (34).

100   Through the heterologous expression of NOX in respiration-deficient strains of *L.*

101   *monocytogenes*, it was concluded that the regeneration of NAD^+ represents a major role

102   for cellular respiration during pathogenesis.

103   The studies presented here sought to define the respiration-independent

104   mechanisms of DHNA utilization to promote the survival and virulence of *L.*

105   *monocytogenes*. Consistent with observations from Rivera-Lugo *et. al*, in the absence of

106   respiration, the *ex vivo* and *in vivo* virulence defects associated with DHNA-deficiency

107   were a result of impaired redox homeostasis which could be rescued upon ectopic NOX

108   expression. Similarly, exogenous DHNA supplementation rescues the *in vitro* and *ex*

109   *vivo* growth and cytosolic survival of DHNA-deficient mutants. Indeed, DHNA-dependent

110   rescue by direct supplementation resulted in a restored cellular redox homeostasis with

111   a concurrent shift of fermentative flux from lactate production to acetate in *L.*

112   *monocytogenes*, independent of respiration. We further go on to show that the recently

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113   annotated anaerobic-specific Ndh2 is essential for DHNA-deficient *L. monocytogenes*

114   mutants to utilize exogenous DHNA for growth in defined medium, independent of its

115   canonical role in EET, suggesting that Ndh2 is the NADH dehydrogenase specifically

116   required for the restoration of redox homeostasis via DHNA. Taken together, these data

117   suggest that the endogenous production of DHNA can be utilized by *L. monocytogenes*

118   to restore both its intracellular redox homeostasis and fermentative metabolic flux

119   through an undefined mechanism requiring Ndh2.

120

121   **RESULTS**

122   **Redox homeostasis via NOX shifts fermentative output and rescues *in vitro***

123   **growth of DHNA-deficient *L. monocytogenes.***

124   Two main outcomes of cellular respiration include 1) maintaining intracellular

125   redox homeostasis by regenerating NAD^+ from NADH and 2) the generation of a PMF

126   to drive oxidative phosphorylation and various other aspects of bacterial physiology. A

127   recent study employed a water-forming NADH oxidase (NOX) expression system in *L.*

128   *monocytogenes* to dissect the relative importance of cellular respiration in maintaining

129   redox homeostasis versus PMF generation (34). We had previously demonstrated that

130   *L. monocytogenes* mutants lacking the key MK biosynthetic intermediate DHNA were

131   attenuated, in part, independent of loss of respiration (29, 31, 32). We hypothesized that

132   restoration of NAD^+ pools might rescue these virulence defects similar to the rescue

133   observed for mutants lacking components of the respiratory chains (34). To test this

134   hypothesis, we assessed NAD^+/NADH levels in Δ*menB*, Δ*menI* and Δ*menA* mutants +/-

135   expression of NOX *in trans*. The inability to generate endogenous DHNA by the Δ*menB*

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136   mutant results in a severely diminished redox homeostasis as measured by the ratio of

137   oxidized NAD^+ to reduced NADH. This imbalance was significantly restored by ectopic

138   expression of NOX to a level similar to the $\Delta menA$ mutant (**Fig. 1A**). The $\Delta menI$ mutant,

139   which can generate DHNA-CoA, displays an intermediate phenotype between $\Delta menB$

140   and $\Delta menA$ levels, which is similarly rescued upon NOX expression (**Fig. 1A**),

141   consistent with possible respiration independent roles for DHNA in NAD^+/NADH redox

142   balancing.

143   *L. monocytogenes* employs a respiro-fermentative metabolism due to an

144   incomplete TCA cycle, characterized by the funneling of pyruvate towards the

145   fermentative production of acetate (23, 24). Respiration-deficient mutants of *L.*

146   *monocytogenes* are impaired in their ability to maintain cellular redox homeostasis and

147   as a result nearly exclusively produce lactate rather than acetate as a metabolic

148   byproduct (34). To test whether impaired redox homeostasis due to DHNA-deficiency

149   would similarly result in the predominant production of lactate, we analyzed

150   fermentation byproducts in bacterial supernatants using high-performance liquid

151   chromatography (HPLC). As expected, wild-type *L. monocytogenes* predominantly

152   generated acetate whereas DHNA-deficient $\Delta menB$ had a drastic shift to lactate

153   production (**Fig. 1B**). Heterologous NOX expression rescued $\Delta menB$ acetate production

154   back to wild-type levels, consistent with restored redox homeostasis driving acetate

155   production to generate ATP (**Fig. 1B**). Consistent with the results seen in our

156   NAD^+/NADH experiments, the $\Delta menI$ mutant displayed an intermediate phenotype by

157   producing similar levels of acetate and lactate, which was also fully restored to wild-type

158   upon NOX expression (**Fig. 1B**). The $\Delta menA$ mutant produced slightly more lactate and

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159   less acetate when compared to wild-type, likely attributed to the difference in redox

160   homeostasis observed previously (**Fig. 1A, B**).

161   Finally, we have previously shown that the production of DHNA is critical for *L.*

162   *monocytogenes in vitro* growth in chemically defined medium (29, 31, 32). To test

163   whether restoration of redox homeostasis can rescue this growth defect, we've assayed

164   for *in vitro* growth of the above mutants complemented with NOX in defined medium. As

165   expected, Δ*menB* showed the largest growth defect followed by Δ*menI*, and both

166   mutants showed wild-type level growth upon NOX complementation (**Fig. 1C**).

167   Together, these data suggest that metabolic defects associated with DHNA deficiency

168   in *L. monocytogenes* are due to NAD$^+$/NADH redox imbalances and that restoration of

169   this balance can rescue Δ*menB* mutant growth and carbon metabolism in *L.*

170   *monocytogenes*.

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172   **Restoration of redox homeostasis rescues virulence defects associated with**

173   **DHNA-deficiency.**

174   Based on the restoration of *in vitro* growth of Δ*menB* mutants via expression of

175   NOX, we hypothesized that restoration of NAD$^+$ pools would similarly rescue virulence

176   defects of DHNA-deficient mutants. DHNA-deficient mutants are susceptible to cytosolic

177   killing in the macrophage cytosol, therefore we assessed cytosolic survival of Δ*menB*,

178   Δ*menI*, and Δ*menA* with or without expression of NOX *in trans* (29, 35). As

179   hypothesized, Δ*menB* and Δ*menI* displayed increased cytosolic killing and NOX

180   expression rescued their survival in the macrophage cytosol (**Fig. 2A**). Rescue by NOX

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181   expression was specific to mutants with disrupted NAD^+/NADH redox homeostasis as

182   NOX expression was unable to rescue cytosolic survival of a Δ*glmR* mutant susceptible

183   to cytosolic killing due to cell wall defects (**Fig 2A**) (33, 35, 36). Consistent with NAD^+

184   pool restoration supporting cytosolic survival, Δ*menB* mutant replication in the

185   macrophage cytosol was also rescued upon expression of NOX *in trans* (**Fig. 2B**).

186   Finally, we had previously demonstrated that DHNA-deficient mutants are more

187   attenuated *in vivo* than respiration-deficient mutants, suggesting that DHNA contributes

188   to virulence in a respiration independent manner (29, 31, 32). To determine if the

189   respiration independent function of DHNA during *in vivo* infection is due to NAD^+/NADH

190   homeostasis defects, we assessed virulence of Δ*menB*, Δ*menI*, and Δ*menA* mutant *L.*

191   *monocytogenes* with and without expression of NOX *in trans*. Ectopic NOX expression

192   rescued the *in vivo* burden of Δ*menB* mutants by ~100-fold in the spleen and liver (**Fig.**

193   **2C**) and a similar rescue for Δ*menI* mutants in the liver following NOX expression is also

194   observed (**Fig. 2C**). Interestingly, there was little to no change in the *in vivo* virulence of

195   Δ*menA* upon the introduction of NOX (**Fig 2C**). This is in agreeance with our previous

196   results that showed both redox homeostasis and acetate production of the Δ*menA*

197   mutant was also not significantly altered upon NOX expression (**Fig 1A, B**). Taken

198   together, these data suggest that in *L. monocytogenes* maintaining cellular redox

199   homeostasis in the absence of DHNA is sufficient to promote survival and virulence

200   both *ex vivo* and *in vivo*.

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202   **DHNA production or supplementation promotes similar effects to NOX**

203   **complementation in *L. monocytogenes*.**

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204   We have previously demonstrated that exogenous addition of either purified

205   DHNA or culture supernatant from DHNA sufficient strains of *L. monocytogenes* could

206   rescue the *in vitro* growth of DHNA-deficient *L. monocytogenes* in defined media (31),

207   suggesting that *L. monocytogenes*, like other bacteria including *Propionibacterium* spp.

208   and *Lactobacillus* spp., may secrete DHNA (43, 45). To test the hypothesis that *L.*

209   *monocytogenes* secretes DHNA, we assayed culture supernatants for DHNA via mass

210   spectrometry. As hypothesized, wild-type *L. monocytogenes* contained abundant levels

211   of DHNA, while Δ*menB* mutants contained no detectable extracellular DHNA (**Fig. S2**).

212   Given that exogenous DHNA could rescue the *in vitro* growth of DHNA-deficient *L.*

213   *monocytogenes* mutants and that DHNA-deficient mutants could similarly be rescued by

214   NAD$^+$ regeneration through NOX expression, we hypothesized that exogenous DHNA

215   could act to restore NAD$^+$ levels in Δ*menB* mutants. To test this hypothesis, we

216   measured cellular NAD$^+$/NADH with or without DHNA supplementation. Consistent with

217   the results observed with NOX expression, the exogenous supplementation of DHNA

218   rescued redox homeostasis of Δ*menB* mutants to levels similar to those seen with

219   Δ*menA* mutants, suggesting that exogenous DHNA might be utilized in a similar fashion

220   to DHNA produced endogenously (**Fig. 3A**). Consistent with DHNA supplementation of

221   Δ*menB* rescuing cellular redox homeostasis, exogenous DHNA also shifted the

222   metabolic flux of Δ*menB* back towards acetate production, similar to Δ*menA* levels (**Fig.**

223   **3B**). Importantly, we had previously demonstrated that exogenous DHNA does not

224   restore respiration and membrane potential (31). Taken together, these data suggest

225   that DHNA, independent of its role in respiration, restores cellular redox homeostasis,

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226   subsequently shifting the fermentative output from lactate back towards acetate that

227   likely drives ATP production through acetate kinase (24, 34).

228   Having previously observed that DHNA can restore NAD^+ redox homeostasis

229   and that NOX-dependent NAD^+ restoration could restore virulence defects of Δ*menB*

230   mutants, we hypothesized that exogenous DHNA supplementation during infection may

231   similarly rescue the cytosolic survival and intracellular growth of DHNA-deficient *L.*

232   *monocytogenes*. Indeed, the addition of exogenous DHNA during macrophage infection

233   with Δ*menB* or Δ*menI* mutants restored their cytosolic survival back to wild-type and

234   Δ*menA* levels (**Fig. 3C**). Importantly, as observed with NOX expression, DHNA

235   supplementation did not rescue the cytosolic survival of Δ*glmR* mutants whose

236   virulence phenotypes are due to cell wall stress response defects (**Fig. 3C**) (33, 36),

237   demonstrating that the rescue of cytosolic survival by DHNA is specific to DHNA-

238   deficient *L. monocytogenes*. Accordingly, supplementing DHNA during macrophage

239   infection also rescued the ability of Δ*menB* mutants to replicate intracellularly to levels

240   similar of that during Δ*menA* infection (**Fig 3D**). Taken together, these results

241   demonstrate that exogenously provided DHNA can balance NAD^+/NADH redox

242   homeostasis thereby potentiating *L. monocytogenes* virulence.

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244   ***ndh2* is conditionally essential for DHNA utilization *in vitro*.**

245   Although DHNA can drive regeneration of NAD^+ in *L. monocytogenes* upon

246   exogenous supplementation, it does not restore membrane potential suggesting that it

247   is not simply imported and used to synthesize MK as described in Streptococci (44, 53).

248   We hypothesized that the two annotated *L. monocytogenes'* NADH dehydrogenases

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249   encoded by *ndh1* (*LMRG_02734*) and *ndh2* (*LMRG_02183*), respectively, may utilize

250   DHNA independent of the respiratory pathways to facilitate NAD^+/NADH homeostasis

251   (23, 25). To test this hypothesis, we generated Δ*ndh1*/*menB*::Tn and *ndh2*::Tn/Δ*menB*

252   mutants and assayed for growth with or without 5μM exogenous DHNA in defined

253   medium. As expected, both double mutants were unable to grow without exogenous

254   DHNA due them being a Δ*menB* mutant (**Fig. 4A**). DHNA supplementation rescued

255   growth of the Δ*ndh1*/*menB*::Tn mutant suggesting that Ndh1 is not required for DHNA-

256   dependent NAD^+/NADH redox homeostasis. In contrast, the *ndh2*::Tn/Δ*menB* mutant

257   was unable to grow in the presence of exogenous DHNA (**Fig. 4B**). *ndh2* is required for

258   the function of the recently described EET pathway in *L. monocytogenes* (25), therefore

259   we hypothesized that EET may be necessary to utilize DHNA for NAD^+/NADH redox

260   homeostasis. To test this hypothesis, we transduced *pplA*::Tn, *dmkA*::Tn, *eetA*::Tn, and

261   *fmnA*::Tn mutations into a Δ*menB* background. The growth of all four of these double

262   mutants were rescued upon DHNA supplementation in defined medium (**Fig. S3**).

263   Finally, exogenous MK supplementation can restore not only growth of DHNA deficient

264   mutants but also their membrane potential (31, 32), likely through direct insertion of MK

265   in the membrane and subsequent restoration of the aerobic respiratory chain. To ensure

266   that *ndh2*::Tn/Δ*menB* mutants are not more generally incapable of growing in defined

267   media, we supplemented *ndh2*::Tn/Δ*menB* mutants with either DHNA or MK directly.

268   Supplementation of MK rescued growth of *ndh2*::Tn/Δ*menB* in defined medium unlike

269   DHNA, showing that this mutant is specifically dysfunctional in the use of DHNA as a

270   redox homeostasis substrate (**Fig. 4C**). Taken together, these data suggest that Ndh2

271   facilitates DHNA-dependent NAD$^+$/NADH redox homeostasis in the absence of

272   respiration in *L. monocytogenes*.

273   **DISCUSSION**

274   Cytosolic pathogens require specific adaptations to survive and replicate within

275   the host. In *L. monocytogenes*, MK biosynthetic intermediate DHNA is among those

276   factors necessary for cytosolic survival, independent of its known role in MK synthesis

277   and cellular respiration (29, 31). In the present study, we sought to address the

278   respiration-independent mechanism by which DHNA is required for the survival and

279   virulence of *L. monocytogenes*. Utilizing a heterologous NOX expression system, we

280   demonstrated that virulence defects associated with loss of DHNA could be rescued by

281   restoration of NAD$^+$/NADH homeostasis (**Fig. 1, 2**). We then found that exogenous

282   DHNA supplementation restores NAD$^+$/NADH balance, cytosolic survival, and

283   intracellular replication of the DHNA-deficient mutant Δ*menB* (**Fig.3**). Balancing of redox

284   homeostasis also coincided with a marked shift in fermentative flux from lactate to

285   acetate upon DHNA production or supplementation (**Fig. 3B**) to maximize ATP

286   production via SLP through the activity of acetate kinase (34, 37). Lastly, we provide

287   evidence that Ndh2 is the NADH dehydrogenase responsible for restoring redox

288   homeostasis during extracellular DHNA utilization, independent of its role in EET (**Fig.**

289   **4**).

290   Although we've demonstrated that Ndh2 is conditionally essential for DHNA

291   utilization in *L. monocytogenes*, it is still unclear how Ndh2 utilizes DHNA to maintain

292   intracellular redox homeostasis. One possibility is that DHNA, or one of its derivatives,

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293   may be used as an alternative quinone to directly accept electrons from Ndh2,

294   regenerating NAD^+ similar to the system recently described in *Shewanella oneidensis*

295   MR-1. Mevers *et al.* recently demonstrated that a derivative of DHNA, 2-amino-3-

296   carboxy-1,4-naphthoquinone (ACNQ), could serve as a novel electron shuttle that

297   functioned to promote redox balance and energy metabolism (38). The authors went on

298   to show that ACNQ is produced non-enzymatically from extracellular DHNA under

299   oxidizing conditions in the presence of a nitrogen donor (i.e. ammonium or amino acids)

300   (38). We have confirmed that indeed, DHNA is secreted by wild-type *L. monocytogenes*

301   (**Fig. S2**) and extracellular DHNA is readily converted to ACNQ in our defined medium

302   based on mass spectrometry analysis (data not shown). Based on this model, it is

303   possible that DHNA produced by *L. monocytogenes* is secreted outside of the cell to

304   shuttle electrons away where it is then freely oxidized non-enzymatically in the local

305   environment to form ACNQ. Newly formed ACNQ would then be imported back into *L.*

306   *monocytogenes* to be reduced again through the activity of Ndh2. The repeated

307   oxidation and reduction of DHNA and/or ACNQ is the hallmark of an "electron shuttle"

308   and is one of the proposed mechanisms of EET in *S. oneidensis* (38, 39). A strikingly

309   similar model has been described in *Pseudomonas aeruginosa* in which endogenous

310   production of phenazine is cyclically reduced intracellularly, shuttled outside of the cell,

311   and oxidized by a terminal electron acceptor where it is then imported again by the cell

312   (41). Studies to determine whether DHNA/ACNQ fuels an Ndh2-dependent electron

313   shuttle to maintain intracellular redox homeostasis or whether DHNA works via an

314   alternative mechanism are currently ongoing.

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315   It has been proposed that in addition to serving as an electron shuttle by *P.*

316   *aeruginosa,* secreted phenazine may be used as a shared resource by the surrounding

317   microbial community to fuel their own redox shuttling (42). The function of phenazine as

318   a shared metabolite is also similar to what has been previously documented with the

319   secretion of DHNA being used as a shared resource to fuel metabolic processes of

320   other localized microbes (31, 43–45). Furthermore, a recent study by Tejedor-Sanz *et*

321   *al.* reported that the homofermentative lactic acid bacteria *Lactiplantibacillus plantarum*

322   contains the EET gene locus previously annotated in *L. monocytogenes*, however it is

323   missing the upstream genes necessary for quinone biosynthesis (40). Upon addition of

324   exogenous DHNA, *L. plantarum* was observed to employ an Ndh2-dependent form of

325   EET that functioned to increase intracellular redox homeostasis by enhancing metabolic

326   flux through fermentative pathways, generating additional lactate, while increasing ATP

327   generation through SLP (40). Importantly, the capacity of DHNA supplementation to

328   induce EET in *L. plantarum* did not coincide with the generation of a PMF to drive

329   oxidative phosphorylation, similar to the phenotypes observed in *L. monocytogenes*.

330   Whether there are functions of *L. monocytogenes* secreted DHNA as a shared

331   metabolite in complex microbial communities such as those found in the intestine during

332   the early stages of infection will require additional future studies.

333   Overall, we've shown that *L. monocytogenes* can utilize DHNA to maintain redox

334   homeostasis through the anaerobic-specific NADH dehydrogenase Ndh2, independent

335   of other EET proteins. Utilization of extracellular DHNA can aid DHNA-deficient *L.*

336   *monocytogenes* mutants to restore their ability to grow and replicate within the cytosol

337   by potentially driving a yet unclear method of energy metabolism. Pathways involved in

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338   unique energy metabolism by various pathogens are increasingly viewed as attractive

339   drug targets and as such future studies utilizing the important model pathogen *L.*

340   *monocytogenes* to understand the mechanisms of DHNA-dependent redox homeostasis

341   could provide novel insights into the generation of new antimicrobials.

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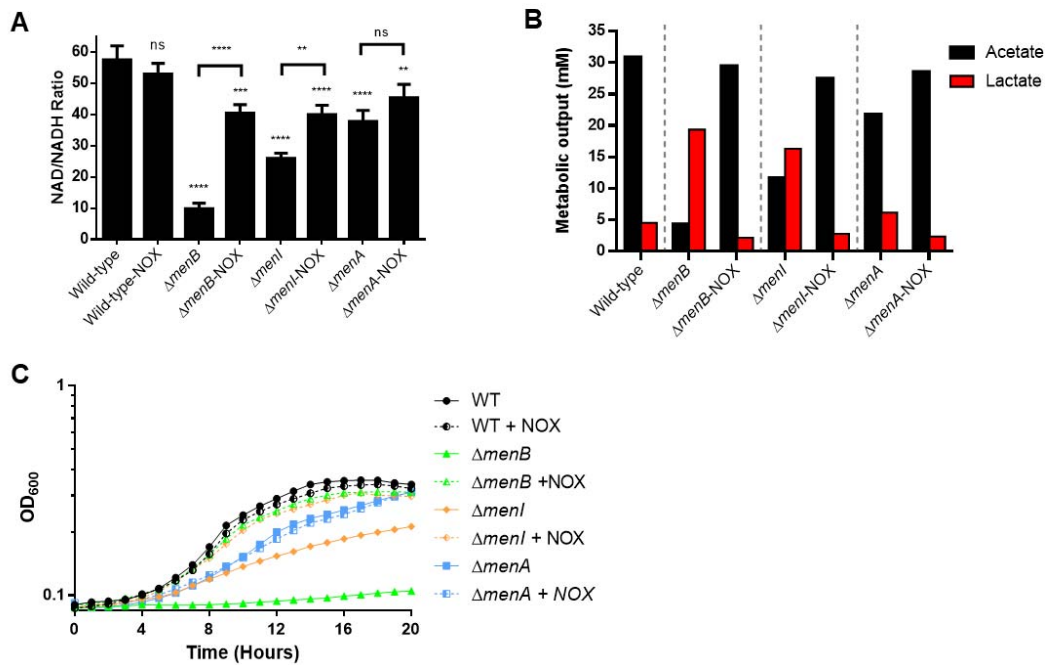
Figure 1. Redox homeostasis via NOX shifts fermentative output and rescues *in vitro* growth of DHNA-deficient *L. monocytogenes*. (A) NAD^+/NADH ratios of indicated *L. monocytogenes* strains +/- NOX plasmid complementation grown aerobically at 37°C in defined medium to mid-logarithmic phase (OD_{600} 0.4-0.6). Δ*menB* mutant fails to grow in defined medium, thus these culture samples were spiked with 2×10^8 total CFU from an overnight BHI culture during experimental setup. (B) HPLC quantification of fermentation products (Lactate and Acetate) produced and secreted by indicated *L. monocytogenes* strains +/- NOX plasmid complementation grown in BHI media aerobically at 37°C to stationary phase. (C) *L. monocytogenes* strains +/- NOX plasmid complementation were grown in defined medium at 37°C. OD_{600} was monitored for 20 hours. Data are representative of three (A, C) or two (B) independent experiments. ns, not significant; WT, wild-type

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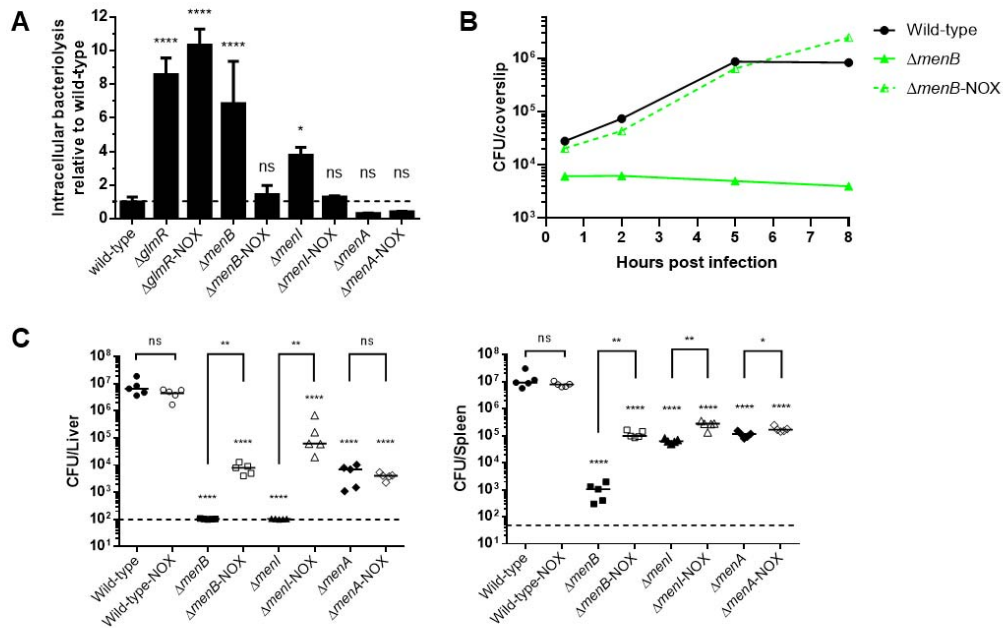
Figure 2. Restoration of redox homeostasis rescues virulence defects associated with

DHNA-deficiency. (A) Indicated *L. monocytogenes* strains (MOI of 10) +/- NOX plasmid

complementation were tested for cytosolic survival in immortalized IFNAR$^{-/-}$ bone

marrow-derived macrophages (BMDM) over a 6 hr infection. Data are normalized to

wild-type levels of bacteriolysis and presented as the standard deviation of the means

from three independent experiments. (B) Intracellular growth of wild-type, Δ*menB,* or

Δ*menB*-NOX was determined in BMDMs following infection at an MOI of 0.2. Growth

curves are representative of at least three independent experiments. Error bars

represent the standard deviation of the means of technical triplicates within the

representative experiment. (C) Bacterial burdens from the spleen and liver were

enumerated at 48 hr post-intravenous infection with 1 × 10^5 total CFU of indicated *L.*

monocytogenes strains +/- NOX plasmid complementation. Data are representative of

results from two independent experiments. Horizontal bars represent the limits of

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370   detection and the bars associated with the individual strains represents the mean of the

371   group. ns, not significant.

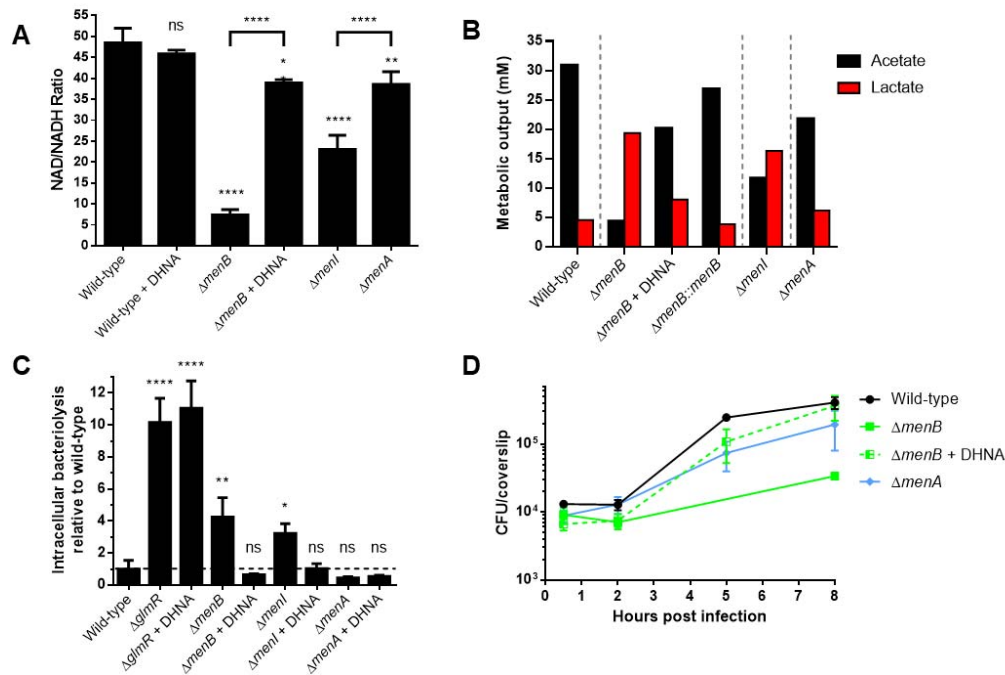372

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Figure 3. DHNA production or supplementation promotes similar effects to NOX

complementation in *L. monocytogenes*. (A) NAD$^+$/NADH ratios of indicated *L.*

monocytogenes strains +/- exogenous DHNA supplementation grown aerobically at

37°C in defined medium to mid-logarithmic phase. Again, Δ*menB* were spiked with 2 ×

10^8 total CFU from an overnight BHI culture during experimental setup. Data are

presented as the standard deviation of the means from three independent experiments.

(B) HPLC quantification of fermentation products (Lactate and Acetate) produced and

secreted by indicated *L. monocytogenes* strains +/- exogenous DHNA supplementation

grown in BHI media aerobically at 37°C to stationary phase. Data are representative of

two independent experiments. (C) Indicated *L. monocytogenes* strains (MOI of 10) +/-

DHNA supplementation were tested for cytosolic survival in primary IFNAR -/- BMDMs

over a 6 hr infection. Data are normalized to wild-type levels of bacteriolysis and

presented as the standard deviation of the means from three independent experiments.

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398   (D) Intracellular growth of wild-type, Δ*menB*, or Δ*menA* was determined in BMDMs

399   following infection at an MOI of 0.2. Growth curves are representative of at least three

400   independent experiments. Error bars represent the standard deviation of the means of

401   technical triplicates within the representative experiment. ns, not significant.
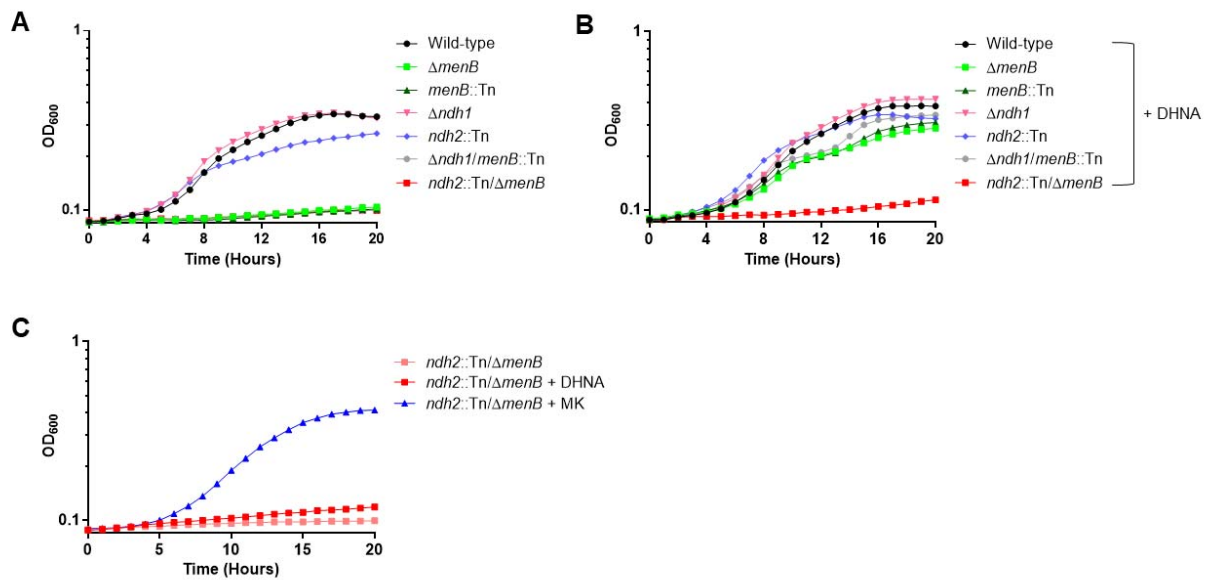
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Figure 4. *ndh2* is conditionally essential for DHNA utilization *in vitro.* Indicated strains of *L. monocytogenes* were grown in defined medium without (A) or with (B) 5µM DHNA supplementation aerobically at 37°C and monitored for OD_{600} over 20 hr. (C) *ndh2*::Tn/Δ*menB L. monocytogenes* was grown aerobically in defined medium with either 5µM DHNA or 5µM MK and monitored for growth (OD_{600}) over 20 hr. All data represent one representative out of three biological replicates.

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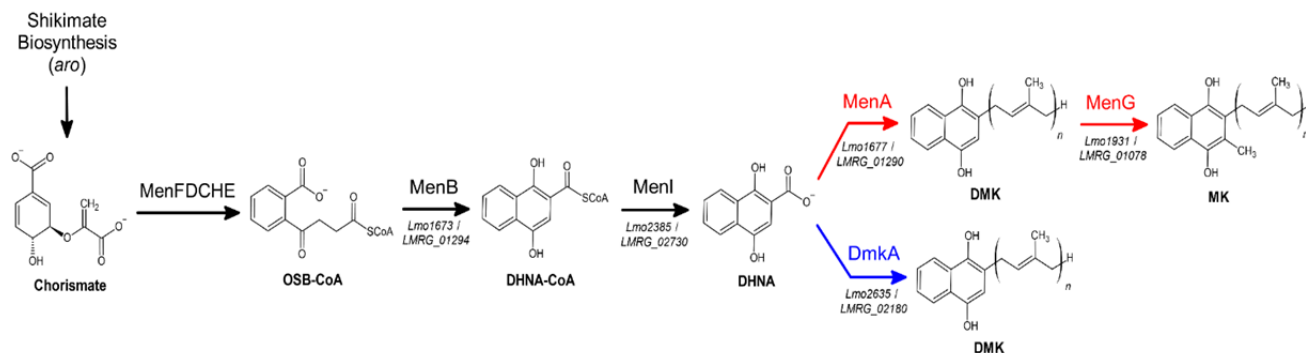
Figure S1. Menaquinone biosynthetic pathway in *Listeria monocytogenes*. Chorismate is generated by the upstream shikimate biosynthesis pathway and is converted to DHNA by the series of listed enzymes (MenFDCHEBI). Red arrows indicate DHNA branching point towards aerobic respiration. Blue arrow indicates DHNA branching point towards anaerobic respiration. Corresponding gene locus numbers for *L. monocytogenes* strains EGD-e (*Lmo*) and 10403S (*LMRG*; parental strain used in this study) are listed underneath reaction arrows. OSB, *o*-succinylbenzoate; DMK, demethylmenaquinone.
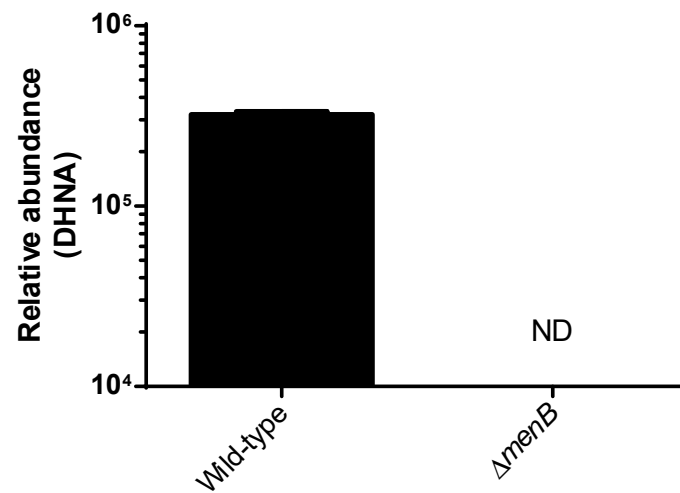
Figure S2. Detection of secreted DHNA by mass spectrometry. Detection of DHNA from the cell-free supernatants of overnight aerobic cultures of wildtype or Δ*menB* strains by mass spectrometry. Data were analyzed via MAVEN. Error bars represent the standard deviation of the means from two independent experiments.
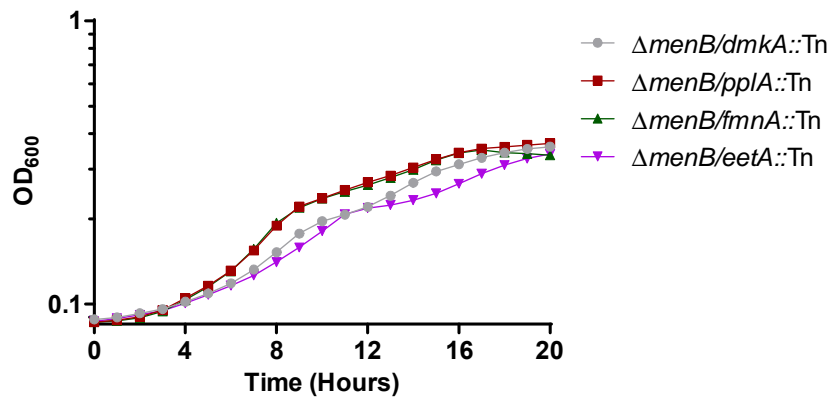
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Figure S3. Four separate genes other than *ndh2* that are part of the *L. monocytogenes* EET locus did not display growth defects. Indicated strains were grown in defined medium at 37°C with the addition of 5μM DHNA. OD_{600} was monitored for 20 hours. Data represents one representative out of three biological replicates.

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461 **MATERIALS AND METHODS**

462 **Bacterial strains, plasmid construction, and growth conditions *in vitro*.** *L.*

463 *monocytogenes* strain 10403S is referred to as the wild-type strain, and all other strains

464 used in this study are isogenic derivatives of this parental strain. Vectors were

465 conjugated into *L. monocytogenes* by *Escherichia coli* strain S17 or SM10 (47). The

466 integrative vector pIMK2 was used for constitutive expression of *L. monocytogenes*

467 genes for complementation (48).

468   *L. monocytogenes* strains were grown at 37°C or 30°C in brain heart infusion

469 (BHI) medium (237500; VWR) or defined medium supplemented with glucose as the

470 sole carbon source. Defined medium is identical to the formulation described by Smith

471 *et al.* (220). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C.

472 Antibiotics were used at concentrations of 100 μg/ml carbenicillin (IB02020; IBI

473 Scientific), 10 μg/ml chloramphenicol (190321; MP Biomedicals), 2 μg/ml erythromycin

474 (227330050; Acros Organics), or 30 μg/ml kanamycin (BP906-5; Fisher Scientific) when

475 appropriate. Medium, where indicated, was supplemented with 5 μM 1,4-dihydroxy-2-

476 naphthoate (DHNA) (281255; Sigma) or 5 μM menaquinone (MK) (V9378; Sigma).

477

478 **Phage Transduction**

479 Phage transductions were performed as previously described (310). Briefly, MACK *L.*

480 *monocytogenes* was grown overnight in 3mL LB at 30°C stationary to propagate U153

481 phage stocks. MACK cultures were pelleted and resuspended in LB + 10mM $CaSO_4$ +

482 10mM $MgCl_2$ and added into LB + 0.7% agar + 10mM $CaSO_4$ + 10mM $MgCl_2$ at 42°C.

483 This mixture was immediately poured on BHI plates and incubated overnight at 30°C.

484 U153 phage plaques were collected and soaked out with 10mM Tris (pH7.5) + 10mM

485 CaSO$_4$ + 10mM MgCl$_2$. Donor plaque soak-outs were propagated the same way and

486 were filter-sterilized using a 0.2µm syringe filter (09-740-113; Fisher Scientific) and

487 additionally kept sterile by adding 500µL chloroform. Recipient Δ*menB* strain was

488 infected with these donor soak-outs for 30 minutes at room temperature and

489 subsequently plated on BHI agar with erythromycin for selection at 37°C.

490

491 **Intracellular bacteriolysis assay.** Standard intracellular bacteriolysis assays were

492 performed as previously described (29). Briefly, primary or immortalized bone marrow-

493 derived *IFNAR*$^{-/-}$ macrophages (5×10^5 per well of 24-well plates) were grown in a

494 monolayer overnight in 500 µL volume. *L. monocytogenes* strains carrying the

495 bacteriolysis reporter pBHE573 (35) were grown at 30°C without shaking overnight.

496 Cultures were then diluted to a final concentration of 5×10^8 CFU/mL in PBS and used

497 to infect macrophages at a MOI of 10. At 1 hr postinfection, media were removed and

498 replaced with media containing 50 µg/ml gentamicin. At 6 hr post infection, media from

499 the wells were aspirated and macrophages were lysed using TNT lysis buffer (20 mM

500 Tris, 200 mM NaCl, 1% Triton [pH 8.0]). Cell lysates were transferred to opaque 96-well

501 plates, and luciferin reagent was added and assayed for luciferase activity (Synergy HT,

502 BioTek; Winooski, VT).

503

504 **Intracellular growth assay.** Bone marrow-derived macrophages (BMDMs) were

505 prepared from C57BL/6 mice as previously described (51). BMDMs were plated on

506 coverslips at 5×10^6 cells per 60mm dish and allowed to adhere overnight. BMDMs

27

507   were then infected at an MOI of 0.2 with their respective strain and infection proceeded

508   for 8 hr. At 30 min postinfection, media were removed and replaced with media

509   containing 50 µg/ml gentamicin. Total CFU were quantified at various time points as

510   previously described (50).

511

512   **NAD^+ and NADH measurements.** *L. monocytogenes* strains were grown in defined

513   medium at 37°C with shaking to mid-logarithmic phase (OD_{600} 0.4-0.6). Cultures were

514   centrifuged and then resuspended in PBS. Resuspended bacteria were then lysed

515   (2×10^8 total CFU) by a 1:1 addition of 1% dodecyltrimethylammonium bromide

516   (DTAB) (AC409310250; Fisher Scientific) for 5 min with agitation. Lysates were then

517   processed to measure NAD^+ and NADH levels using the NAD/NADH-Glo assay

518   (Promega, G9071) per the manufacturer's protocol.

519

520   **Fermentation byproduct measurements.** Cultures of *L. monocytogenes* were grown

521   in BHI at 37°C with shaking overnight. Bacteria were then centrifuged and 1 mL of the

522   resulting supernatant was filtered through a 0.2µm-pore-size syringe filter (09-740-113;

523   Fisher Scientific). Supernatant samples were next treated with 2µL of H_2SO_4 to

524   precipitate any components that might be incompatible with the running buffer. The

525   samples were then centrifuged at 16000 × g for 10 min and then 200µL of each sample

526   transferred to an HPLC vial. HPLC analysis was performed using a ThermoFisher

527   (Waltham, MA) Ultimate 3000 UHPLC system equipped with a UV detector (210 nm).

528   Compounds were separated on a 250 × 4.6 mm Rezex© ROA-Organic acid LC column

529   (Phenomenex Torrance, CA) run with a flow rate of 0.2 mL min^{-1} and at a column

530   temperature of 50 °C. The samples were held at 4 °C prior to injection. Separation was

531   isocratic with a mobile phase of HPLC grade water acidified with 0.015 N H_2SO_4 (415

532   µL L^{-1}). At least two standard sets were run along with each sample set. Standards

533   were 100, 20, 4, and 0.8mM concentrations of lactate or acetate. The resultant data was

534   analyzed using the Thermofisher Chromeleon 7 software package.

535

536   **Acute virulence assay.** All techniques were reviewed and approved by the University

537   of Wisconsin — Madison Institutional Animal Care and Use Committee (IACUC) under

538   the protocol M02501. Female C57BL/6 mice (6 to 8 weeks of age; purchased from

539   Charles River) were used for the purposes of this study. *L. monocytogenes* strains were

540   grown in BHI medium at 30°C without shaking overnight. These cultures were then

541   back-diluted the following day 1:5 into fresh BHI medium and grown at 37°C with

542   shaking until mid-exponential phase (OD_{600} 0.4-0.6). Bacteria were diluted in PBS to a

543   concentration of 5×10^5 CFU/mL and mice were injected intravenously with 1×10^5

544   total CFU. At 48 hr postinfection, spleens and livers were harvested and homogenized

545   in 0.1% Nonidet P-40 in PBS. Homogenates were then plated on LB plates to

546   enumerate CFU and quantify bacterial burdens.

547

548   **Statistical analysis.** Statistical significance analysis (GraphPad Prism, version 6.0h)

549   was determined by one-way analysis of variance (ANOVA) with a Dunnett's posttest

550   comparing wild-type to all other indicated strains or by one-way ANOVA with Tukey's

551   multiple comparisons test unless otherwise stated (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq$

552   0.001; ****, $P \leq 0.0001$).

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FUNDING INFORMATION

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575 **Table S3.1** Strains used in this study.

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Strain	Description	Reference
XL1-Blue	Competent *E. coli* strain	-
SM10	*E. coli* strain for conjugations into *Lm;* Km^R	(47)
10403S	Parental *L. monocytogenes* (*Lm*) 10403s strain [Wild-type]	-
JDS18	*Lm* with pBHE573	(35)
HS28	*Lm* with pPL2-NOX	(34)
JDS2328	*Lm* with pBHE573 and pPL2-NOX	(34)
JDS25	*Lm* Δ*glmR*	(35)
JDS21	*Lm* Δ*glmR* with pBHE573	(35)
JDS2327	*Lm* Δ*glmR* with pPL2-NOX	This work
JDS2329	*Lm* Δ*glmR* with pBHE573 and pPL2-NOX	(34)
JDS1161	*Lm* Δ*menB*	(29)
JDS1175	*Lm* Δ*menB* with pYL116	(32)
JDS1958	*Lm* Δ*menB* with pPL2-NOX	(34)
JDS1191	*Lm* Δ*menB* with pBHE573	(29)
JDS2333	*Lm* Δ*menB* with pBHE573 and pPL2-NOX	(34)
JDS2240	*Lm* Δ*menI*	(32)
JDS2155	*Lm* Δ*menI* with pBHE573	(32)
JDS2325	*Lm* Δ*menI* with pPL2-NOX	This work
JDS2326	*Lm* Δ*menI* with pBHE573 and pPL2-NOX	This work
JDS1047	*Lm* Δ*menA*	(29)
JDS813	*Lm* Δ*menA* with pBHE573	(29)
HS30	*Lm* Δ*menA* with pPL2-NOX	This work
JDS2330	*Lm* Δ*menA* with pBHE573 and pPL2-NOX	This work
JDS1213	*Lm* Δ*menD*	(52)
JDS17	SM10 *E. coli* with pBHE537	(35)
JDS1957	SM10 *E. coli* with pPL2-NOX	(34)
KL1	*Lm* Δ*ndh1*/*menB*::Tn	This work
KL4	*Lm* Δ*menB*/*ndh2*::Tn	This work
KL9	*Lm* Δ*menB*/*dmkA*::Tn	This work
KL10	*Lm* Δ*menB*/*pplA*::Tn	This work
KL11	*Lm* Δ*menB*/*fmnA*::Tn	This work
KL12	*Lm* Δ*menB*/*eetA*::Tn	This work

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578

579   **Table S3.2** Plasmids used in this study.

Plasmids	Description	Reference
pBHE573	Bacteriolysis reporter; Cam^R	(35)
pIMK2	Constitutive expression vector for complementation, P$_{help}$; Kan^R	(48)
pYL116	*menB* cloned into pIMK2	(32)
pPL2-NOX	NADH oxidase (NOX) cloned into the backbone of pPL2	(34)

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793