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1 **Diving into bacterial dormancy: emergence of osmotically stable wall-less**
2 **forms in an aquatic environment**

3

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17

18 **Abstract**

19

20 Bacteria can respond to environmental stresses by entering a dormant state, called
21 viable but non-culturable (VBNC) state, in which they no longer grow in routine culture media.
22 VBNC pathogens pose thus a significant risk for human and animal health as they are not
23 detected by standard growth-based techniques and can “wake up” back into a vegetative and
24 virulent state. Although hundreds of species were reported to become VBNC in response to
25 different stresses, the molecular mechanisms governing this phenotypic switch remain largely
26 elusive.

27 Here, we characterized the VBNC state transition process in the Gram-positive
28 pathogen *Listeria monocytogenes* in response to nutritional deprivation. By combining
29 fluorescence microscopy, cryo-electron tomography and analytical biochemistry, we found
30 that starvation in mineral water drives *L. monocytogenes* into a VBNC state via a mechanism
31 of cell wall (CW) shedding that generates osmotically stable CW-deficient (CWD) coccoid
32 forms. This phenomenon occurs in multiple *L. monocytogenes* strains and in other *Listeria*
33 species, suggesting it may be a stress-adapting process transversal to the *Listeria* genus.
34 Transcriptomic and gene-targeted approaches revealed the stress response regulator SigB and
35 the autolysin NamA as major moderators of CW loss and VBNC state transition. Finally, we
36 show that this CWD dormant state is transient as VBNC *Listeria* revert back to a walled,
37 vegetative and virulent state after passage in embryonated eggs.

38 Our findings provide unprecedented detail on the mechanisms governing the transition
39 to a VBNC state, and reveal that dormant CWD bacterial forms can naturally arise in aquatic
40 environments without osmotic stabilization. This may represent an alternative strategy for
41 bacterial survival in oligotrophic conditions, which can potentially generate public health-
42 threatening reservoirs of undetectable pathogens.

43

44 **Introduction**

45

46 Bacteria often face less than optimal growth conditions and a variety of abiotic stresses
47 in their environment. Some species are able to produce highly resistant cellular structures,
48 called endospores, to enter a metabolically inactive state until environmental conditions are
49 adequate for resuming vegetative growth (Beskrovnaya et al., 2021). Alternatively, bacteria
50 may enter a dormant state known as the viable but non-culturable (VBNC) state, in which they
51 preserve some metabolic activity at the expense of losing the ability to grow on regular culture
52 media (Dong et al., 2020). The VBNC state is documented in over a hundred species
53 (Ayrapetyan et al., 2018; Dong et al., 2020), but our knowledge concerning the molecular
54 processes driving the transition from a vegetative lifestyle to the VBNC state, particularly in
55 Gram-positive bacteria, are still fragmentary.

56 The transition to a VBNC state is frequently accompanied by a morphological change,
57 often cell dwarfing and/or rounding (Li et al., 2014). The underlying reasons are not entirely
58 understood but an hypothesis is that a spherical shape, with a smaller surface area/volume ratio,
59 might help VBNC bacteria reduce their energy demands and optimize nutrient uptake (Baker
60 et al., 1983). Studies mostly performed in Gram-negative bacteria reported structural
61 modifications of the cell wall (CW) peptidoglycan recovered from VBNC cells that might

62 contribute to cell rounding (Costa et al., 1999; Signoretto et al., 2000, 2002). However, a direct
63 link between CW modifications, cell morphology and dormancy has not yet been clearly
64 established.

65 Cell rounding has also been observed when some bacteria switch into a CW-deficient
66 (CWD) state. This is the case of L-forms, CWD variants generated by exposure to CW-
67 targeting agents, such as wall-active antibiotics, lytic enzymes or phages (Errington et al.,
68 2016; Kawai et al., 2018; Wohlfarth et al., 2023). L-form cells remain viable and able to
69 replicate, but the absence of CW makes them sensitive to osmotic lysis, and therefore they need
70 to be cultivated in osmoprotective conditions (Errington et al., 2016). For this reason, the
71 physiological relevance of L-forms, and CWD bacteria in general, is a matter of debate
72 (Errington et al., 2016).

73 Here, we report that the Gram-positive bacterium *Listeria monocytogenes* (*Lm*)
74 undergoes a rod-to-coccus differentiation in transition to a VBNC state in a nutrient-deprived
75 natural water setting. We reveal that this cell rounding results from loss of the CW via a
76 molting-like shedding process. Remarkably, these CWD VBNC *Lm* forms are resistant to
77 osmotic lysis, likely as a result of adaptive changes in the physicochemical properties of their
78 plasma membrane. We further show that this CWD VBNC state is extensive to other *Listeria*
79 *sensu stricto* species. To our knowledge, this is the first report of CWD VBNC bacteria
80 naturally arising in a non-osmotically stabilized environment. We further identified the stress-
81 responsive transcription factor SigB and the autolysin NamA as major molecular players in the
82 formation of CWD VBNC *Lm*. Finally, we show that dormant wall-less *Lm* can revert back to
83 a walled, vegetative and fully virulent state after passage in embryonated chicken eggs. Our
84 results suggest that CW shedding is an adaptive process employed by *Listeria* to survive under
85 prolonged nutritional limitation.

86

87 **Results**

88

89 **Dynamics of VBNC *Lm* formation in mineral water**

90

91 To induce a VBNC state in *Lm*, we followed a starvation-based approach by incubating
92 *Lm* in water (Besnard et al., 2000b, 2002). We used a commercial mineral water due to its
93 natural spring origin, low mineral content and quality-controlled composition (**Supplementary**
94 **Table 1**). As the starting number of bacteria affects the dynamics of culturability loss (Besnard
95 et al., 2002), we tested initial *Lm* concentrations ranging from 10^9 to 10^6 bacteria/mL. We
96 observed that the rate and magnitude of culturability loss increased when the starting bacterial
97 concentration was reduced (**Fig. 1A**). Notably, a concentration of 10^6 *Lm*/mL resulted in
98 culturability levels of <1 colony-forming units (CFU)/mL after 28 days (**Fig. 1A**). We chose a
99 starting concentration of 10^8 *Lm*/mL as the standard condition to induce the VBNC state in
100 mineral water throughout this work, since it produces a 2-log drop in culturable *Lm* after
101 28 days while still providing sufficient material for downstream analyses.

102 To confirm the formation of VBNC *Lm*, we monitored the total number of bacteria and
103 the fraction of viable bacteria by flow cytometry. The viable population was determined using
104 carboxyfluorescein diacetate (CFDA, **Extended Data Fig. 1A**), a fluorogenic dye that is
105 enzymatically activated and retained in the cytoplasm of metabolically active bacteria with an

106 integral plasma membrane (Wideman et al., 2021). While CFU counts dropped progressively
107 to 10^6 *Lm*/mL after 28 days, the total and viable population numbers remained nearly
108 unchanged (**Fig. 1B**). The increasing difference between the viable and culturable populations
109 with time demonstrates the gradual and almost complete transition to a VBNC state (**Fig. 1C**).
110 This was also observed when using the double-dye Live/Dead assay to assess viability
111 (**Extended Data Fig. 1B**). In this case, viable population numbers slightly dropped with time,
112 which might be related with the different mode of action of these dyes (Stiefel et al., 2015).

113 ATP is only produced by live cells and quickly depleted upon cell death, constituting
114 thus a marker of cellular viability. We measured the intracellular ATP levels in mineral water-
115 incubated *Lm* cells over time, in parallel to their culturability. Variations in the ATP content
116 did not follow the changes in culturability, unlike what we observed from a dilution series of
117 freshly prepared suspensions, used to report the ATP content expected from a given number of
118 culturable cells (**Fig. 1D**). Indeed, whereas culturable *Lm* numbers declined steadily, ATP
119 levels dropped drastically after 7 days, recovering partially afterwards. Importantly, from
120 day 21, the measured ATP levels were higher than those expected from a similar number of
121 culturable cells (**Fig. 1D**), suggesting that this ATP surplus comes from the larger, non-
122 culturable *Lm* subpopulation.

123 Together, these results confirm the efficient transition of *Lm* to a VBNC state in mineral
124 water.

125

126 **VBNC *Lm* assume a coccoid morphology in mineral water**

127

128 Changes in the bacterial cell size and shape are frequently associated with the VBNC
129 state (Li et al., 2014). We thus acquired phase-contrast images of *Lm* suspensions in mineral
130 water to track the occurrence of morphological changes during VBNC cell formation. The
131 initial *Lm* population (day 0) consisted of typical rod-shaped cells, isolated or in tethered pairs
132 (**Fig. 1E**). From 7 days of incubation, coccoid forms were also observed and became more
133 abundant over time, at the expense of the rod-shaped subpopulation (**Fig. 1E**). Quantitative
134 analysis of the populational morphology confirmed this progressive rod-to-coccus shape
135 transformation (**Fig. 1F, G**). A suspension of GFP-expressing *Lm* showed GFP-positive
136 coccoid cells appearing as of day 7 and increasing in number by day 28 (**Extended Data Fig.**
137 **2**), further confirming that the spherical forms derive from the initial rod-shaped *Lm*.
138 Noteworthy, coccoid cells were sometimes found next to phase-light rod-shaped structures
139 resembling empty cell wall sacculi; in some cases, even appearing to extrude from the latter
140 (**Fig. 1E**, insets day 14 and 21).

141 These results show that incubation in mineral water triggers a rod-to-coccus transition
142 in *Lm* cells. Interestingly, the dynamics of this morphological transition closely overlap with
143 the dynamics of culturability decline and VBNC cell formation (**Fig. 1B, C**), suggesting a link
144 between the shape change and the transition to a VBNC state.

145

146 **Rod-to-coccus transition in *Lm* is caused by CW loss via a molting-like shedding process**

147

148 The switch from rod to coccoid shape has been observed in bacteria converting to L-
149 forms, with the loss of the CW as the main driving force of this morphological change (Dell’Era

150 et al., 2009; Domínguez-Cuevas et al., 2012; Errington et al., 2016). Having observed coccoid
151 *Lm* associated with “ghost” structures resembling empty cell wall sacculi (**Fig. 1E**), we
152 investigated whether coccoid *Lm* cells were CWD forms. We first performed a Gram staining
153 of the *Lm* population at day 0 and day 28. Remarkably, the usual crystal violet staining
154 displayed by rod-shaped *Lm* cells at day 0 was no longer present when the population consisted
155 of coccoid cells after 28 days in mineral water (**Fig. 1H**), indicating the absence of a typical
156 Gram-positive CW in coccoid VBNC *Lm* cells. We then compared the peptidoglycan content
157 purified from equivalent *Lm* cell numbers at different timepoints of incubation in water, by
158 performing UHPLC analysis of muropeptides. The muropeptide elution profiles showed that,
159 while their composition did not visibly change, the amount of the different muropeptide species
160 decreased with time until virtually no peptidoglycan was detected by day 28 (**Fig. 1I**).
161 Together, these results confirm the progressive depletion of the *Lm* CW during transition to a
162 VBNC state.

163 Next, we monitored the dynamics of *Lm* CW loss by fluorescence microscopy. The
164 walled *Lm* population was fluorescently stained with wheat germ agglutinin (WGA), a lectin
165 that binds to free *N*-acetylglucosamine (GlcNAc) residues present in wall teichoic acids
166 (WTAs) of serogroup 1/2 strains (Fiedler, 1988). To monitor in parallel the appearance of
167 CWD *Lm* cells, we generated anti-CWD *Lm* antibodies after immunization of rabbits with
168 CWD *Lm* recovered from mineral water suspensions after 28 days. The anti-CWD *Lm*
169 antibodies labeled the coccoid (i.e. CWD) but not the rod-shaped (i.e. walled) *Lm* cells,
170 confirming their specificity (**Extended Data Fig. 3**). On the first day, *Lm* cells were only
171 stained by WGA, indicating intact CW and a plasma membrane externally inaccessible to
172 labeling by the anti-CWD *Lm* (**Fig. 1J, K**). Two additional subpopulations emerged with time:
173 double-labelled cells, corresponding to *Lm* with a more permeable/damaged CW, and cells
174 labeled only by the anti-CWD *Lm*, representing CWD *Lm* (**Fig. 1J, K**). The fraction of CWD
175 *Lm* progressively increased with time to represent >95% of bacteria by day 28 (**Fig. 1K**).

176 Lastly, we investigated the CW loss phenomenon at the ultrastructural level in near-
177 native conditions by cryogenic electron tomography (cryo-ET) of whole *Lm* cells. Micrographs
178 and 3D rendering of segmented tomograms allowed us to reconstruct the different stages of the
179 morphological transition of *Lm* in mineral water. Starting as a rod-shaped bacterium with a
180 CW wrapped tightly around the plasma membrane (**Fig. 2, stage 0; Supplementary Movie 1**),
181 *Lm* cells start showing a substantial detachment between these two layers (**Fig. 2, stage 1**),
182 followed by the weakening and appearance of variably sized gaps in the CW mesh (**Fig. 2,**
183 **stage 2; Supplementary Movie 2**). These gaps allow the enclosed protoplast to gradually
184 egress the CW sacculus (**Fig. 2, stages 3 and 4; Supplementary Movies 3 and 4**) and escape
185 into the extracellular medium as a spherical cell (**Fig. 2, stage 5; Supplementary Movie 5**).

186 Altogether, these results reveal that *Lm* cells transitioning to a VBNC state lose their
187 CW through a molting-like shedding process that generates wall-less coccoid cell forms.

188 **A CWD VBNC state is widespread in *Listeria* species**

189 We next wondered if the CWD VBNC state induced in mineral water occurred in other
190 *Lm* strains, besides our reference strain EGDe. We monitored VBNC cell formation in the *Lm*
191 strain 10403S, another well-studied reference laboratory strain (Bishop & Hinrichs, 1987), and
192
193

194 in two clinical *Lm* strains isolated from human and bovine listeriosis cases: CLIP 63713 and
195 JF5203, respectively (Aguilar-Bultet et al., 2018; Jonquières et al., 1998). Similar to EGDe,
196 these three strains showed declining culturability over time (**Extended Data Fig. 4A–D**) and
197 formation of a VBNC subpopulation (**Extended Data Fig. 4E–H**) in mineral water. To monitor
198 the presence of CW by fluorescence microscopy, we used a commercial antibody raised against
199 *Lm* CW-specific antigens (anti-*Lm*), since WGA does not label the CW of *Lm* serogroup 4
200 strains (CLIP 63713 and JF5203) due to the lack of free GlcNAc residues in their WTAs
201 (Rismondo et al., 2020; Shen et al., 2017). The three strains displayed gradual loss of the CW
202 (**Extended Data Fig. 4I–L, Q**) and exposure of the plasma membrane (**Extended Data Fig.**
203 **4M–P, Q**), with dynamics comparable to those of VBNC cell formation. These results
204 demonstrate that the formation of CWD VBNC forms is a strain-independent property of *Lm*.

205 We then investigated other *Listeria* species, namely from the *sensu stricto* clade to
206 which also *Lm* belongs. These include the pathogenic species *L. ivanovii*, and the non-
207 pathogenic species *L. innocua*, *L. marthii*, *L. seeligeri* and *L. welshimeri* (Schardt et al., 2017).
208 Like *Lm*, they all formed VBNC subpopulations in mineral water (**Fig. 3A–J**). *L. ivanovii*
209 exhibited the greatest drop in culturability (3 log) after 7 days (**Fig. 3A**), which meant that
210 >99% of viable *L. ivanovii* cells present at day 7 were in a VBNC state (**Fig. 3F**). *L. marthii*
211 showed transition dynamics similar to *Lm*, with a slower decline in culturability after 7 days
212 (**Fig. 3C**, compare to **Fig. 1B** and **Extended Data Fig. 4A–D**). Finally, *L. innocua*, *L. seeligeri*
213 and *L. welshimeri* displayed intermediate profiles of culturability loss (**Fig. 3B, D, E**) and
214 VBNC cell formation (**Fig. 3G, I, J**). The anti-*Lm* antibody also reacted with the CW of these
215 species, so we used it to follow their CW status by fluorescence microscopy. As observed with
216 *Lm*, the other *Listeria* species lost their CW while transitioning to a VBNC state (**Fig. 3K–**
217 **O, U**). In parallel, the anti-CWD *Lm* antibody was also able to reveal the gradual exposure of
218 the plasma membrane in these species as they shed their CW (**Fig. 3P–U**).

219 Collectively, these results show that the emergence of CWD VBNC forms in mineral
220 water is a transversal phenomenon in *Listeria sensu stricto* species.

221

222 ***Lm* changes the physicochemical properties of its plasma membrane to adapt to a CWD** 223 **lifestyle in water**

224

225 The bacterial CW protects shape and counters the intracellular osmotic pressure,
226 protecting the cell from osmotic lysis. We sought to understand how CWD *Lm* cells can survive
227 in a hypotonic medium, like mineral water, without signs of lysis. We hypothesized that *Lm*
228 may change the properties of its plasma membrane to become more resistant to osmotic
229 pressure before shedding its CW. Indeed, bacteria modulate the fluidity of their plasma
230 membrane, in response to changing environmental factors, to preserve the physical and
231 functional integrity of their interface with the external environment. This is mainly
232 accomplished by changing the fatty acid (FA) composition of membrane phospholipids, which
233 adjusts their degree of packing and, consequently, the fluidity of the membrane (Yoon et al.,
234 2015).

235 We thus analyzed the FA composition of the *Lm* membrane by gas chromatography
236 coupled to mass spectrometry. Between day 0 and day 28, we observed a decrease in the
237 relative abundance of anteiso branched-chain species (a-BFA) a-C15:0 and a-C17:0 (**Extended**

238 **Data Fig. 5**), which comprise the majority of the *Lm* FA population and are key regulators of
239 membrane fluidity in Gram-positive bacteria (Yoon et al., 2015). In contrast, linear saturated
240 (SFA) and unsaturated (UFA) FAs showed increased relative levels, mainly on account of
241 C16:0 and C16:1 species (**Extended Data Fig. 5**). Due to their minor representation in the
242 initial FA population, the fold increase in the SFA and UFA levels was substantial, when
243 compared to the fold change of the more abundant a-BFAs (**Fig. 4A**). We then investigated if
244 these changes in FA composition were associated with an alteration in the *Lm* membrane
245 fluidity by measuring the generalized polarization (GP) of laurdan, a ratiometric probe that
246 shifts its fluorescence emission peak in response to local membrane phase transitions caused
247 by fluidity changes (Scheinflug et al., 2017). An increase of the laurdan GP in labeled *Lm*
248 cells during the first 14 days was suggestive of decreased membrane fluidity (**Fig. 4B**). To
249 confirm this observation, we directly measured the fluidity-dependent diffusion of the
250 fluorogenic dye Nile red in the *Lm* membrane using total internal reflection fluorescence
251 correlation spectroscopy (TIR-FCS). This technique has been recently implemented to quantify
252 bacterial membrane fluidity in Gram-positive bacteria (Barbotin et al., 2023). TIR-FCS showed
253 a significant reduction of the diffusion coefficient of Nile red in rod-shaped cells between 7
254 and 14 days (**Fig. 4C**), suggestive of membrane rigidification. This corresponds to the period
255 when *Lm* is most severely impacted by CW damage and loss (**Fig. 1K**). Notably, the diffusion
256 coefficient in CWD coccoid cells was similar after 7 or 14 days of incubation in water
257 (**Fig. 4C**), consistent with increased membrane packing to adapt to a wall-less lifestyle.
258 Furthermore, after 14 days in water, the diffusion coefficient was similar in rod-shaped and
259 coccoid cells, suggesting that the reduction in membrane fluidity occurs prior to CW loss.

260 Altogether, these findings indicate that *Lm* alters the physicochemical properties of its
261 plasma membrane while transiting to a CWD VBNC state in mineral water. As a result of
262 changes in FA composition, although not excluding the contribution of other membrane
263 components, the membrane becomes more rigid, which may protect the wall-less bacterial cell
264 from osmotic lysis. In agreement with this hypothesis, total *Lm* numbers were notably reduced
265 (2 log) when freshly prepared suspensions were immediately treated with mutanolysin, which
266 digests the *Listeria* CW, without allowing the *Lm* cells to adapt to the hypotonic medium
267 (**Fig. 4D**). In contrast, bacteria from 28-day-old suspensions were insensitive to this treatment
268 (**Fig. 4D**).

269 270 **Transcriptomics highlight the role of stress response in the formation of CWD VBNC *Lm***

271
272 Despite several studies reporting the induction of a VBNC state in *Lm* under different
273 stressful conditions (Besnard et al., 2002; Bremer et al., 1998; Cunningham et al., 2009;
274 Highmore et al., 2018; Lindbäck et al., 2010; Noll et al., 2020; Robben et al., 2018), the
275 molecular factors and pathways involved in this transition remain elusive.

276 To identify early effectors required for VBNC state transition in mineral water, we
277 analyzed the transcriptional changes in *Lm* cells after 7 days, when loss of culturability and
278 CW alterations are first observed (**Fig. 1**). RNA-seq analysis identified a total of 1229
279 differentially expressed genes (q-value ≤ 0.05 , absolute \log_2 fold change ≥ 1), of which 593
280 were downregulated and 636 were upregulated (**Fig. 5A; Supplementary Table 2**). Gene set
281 enrichment analysis revealed the most prevalent upregulated and downregulated biological

282 processes and pathways. Downregulated genes were found associated with biosynthesis of
283 nucleotides and coenzymes (biotin, pyridoxal phosphate, coenzyme A), transcription
284 regulation, uptake of phosphate and carbohydrates (maltose/maltodextrin and trehalose
285 phosphotransferase systems), cell envelope assembly (biosynthesis of glycerophospholipids
286 and teichoic acids) and maintenance (peptidoglycan catabolism), cell division (division septum
287 assembly), energy production (pyruvate metabolism, ATP synthesis-coupled proton transport),
288 and protein secretion (**Fig. 5B, Extended Data Fig. 6A–C**). Upregulated genes were linked
289 with acquisition and/or metabolism of amino acids (aspartate, glutamate, methionine, cysteine,
290 isoleucine, valine, leucine, threonine, arginine); biosynthesis of pyrimidine nucleotides, uptake
291 of carbohydrates (glucose/mannose phosphotransferase systems) and metal ions (iron, zinc);
292 protein translation and folding, and response to osmotic (transport of compatible solutes
293 carnitine/glycine betaine) and oxidative stress (glutathione metabolism) (**Fig. 5C; Extended
294 Data Fig. 6D–F**). These results are consistent with a physiological transition taking place in a
295 population of mixed culturable states. Most downregulated genes likely reflect the transition
296 from a vegetative growth state to a VBNC state, whereas most upregulated genes possibly
297 mirror bacterial responses to nutritional and hypoosmotic stresses.

298 Interestingly, prophage loci were almost completely activated and among the most
299 strongly upregulated genes (**Supplementary Table 3**), in line with previous reports linking
300 prophage activation with environmental stress (Argov et al., 2019; Duru et al., 2021; Ivy et al.,
301 2012; Wang et al., 2010). In agreement with a stress response activation, nearly half of the
302 regulon controlled by the stress-responsive sigma factor SigB (181 out of 455 genes) was
303 induced (**Supplementary Table 4**). This elevated number of upregulated SigB-controlled
304 genes prompted us to investigate its involvement in the transition of *Lm* to a VBNC state. We
305 found that SigB-deficient *Lm* cells transitioned considerably faster than wild-type cells, with a
306 2-log decline in CFU counts resulting in >90% of viable cells in a non-culturable state after
307 7 days (**Fig. 5D, E**). Importantly, >90% of the *Lm* population had already converted to CWD
308 coccoid forms (**Fig. 5F, G**). These results reveal a major modulating role for SigB in *Lm*
309 adaptation to nutritional deprivation and generation of CWD VBNC forms in mineral water.
310 The unaffected viability of $\Delta sigB$ cells indicates, however, that SigB is not essential for *Lm*
311 survival in this situation (**Fig. 5D**).

312 The stringent response is an important stress signaling mechanism that regulates
313 adaptation to starvation via the alarmone (p)ppGpp (Irving et al., 2021). The production of
314 (p)ppGpp was shown to be promoted during transition to a VBNC state, and (p)ppGpp-
315 deficient bacteria were found to lose culturability at a higher rate than their wild-type
316 counterparts (Bai et al., 2021; Boaretti et al., 2003). Although our transcriptomic data showed
317 no upregulation of the alarmone synthase-encoding genes (*relA*, *relP* and *relQ*)
318 (**Supplementary Table 2**), we examined if the enzymatic activity of these proteins could
319 impact the transition to a VBNC state. Compared to wild type *Lm*, a $\Delta relAPQ$ strain displayed
320 a significantly faster transition after 7 days (**Fig. 5H, I**), which was correlated with a faster
321 decline of the walled population in the same period (**Fig. 5J**). This result suggest that the
322 stringent response plays a role in the early phase of VBNC *Lm* formation.

323

324 **The autolysin NamA is a major mediator of *Lm* CW loss and VBNC state entry**

325

326 To gain a molecular insight into the *Lm* CW remodeling dynamics involved in VBNC
327 *Lm* formation in water, we then focused on genes involved in CW metabolism. These genes
328 presented a heterogeneous expression profile, showing either down/upregulation or no change
329 (**Supplementary Table 5**). Peptidoglycan maturation and turnover is carried out by a family
330 of peptidoglycan hydrolases, commonly called autolysins, that cleave different bonds within
331 the peptidoglycan structure (Höltje, 1998). *Lm* encodes around 20 proteins with confirmed or
332 predicted autolytic activity (Bierne & Cossart, 2007; Popowska & Markiewicz, 2006). Our
333 transcriptomic data indicated that many known and putative *Lm* autolysin-coding genes were
334 strongly downregulated after 7 days (e.g. *lmo0394*, *p60*, *aut*, *lmo1215*, *lmo1521*, *lmo2522*, *ami*,
335 *namA*) (**Supplementary Table 5**), suggesting that degradation of the CW prior to shedding is
336 carried out efficiently by an existing autolytic activity, without need for additional protein
337 synthesis. We thus tested *Lm* mutants of genes encoding autolysins with different classes of
338 bond-cleaving activity: the DL-endopeptidase p60/Iap, the *N*-acetylmuramoyl-L-alanine
339 amidase Ami, the *N*-acetylglucosaminidases Auto and NamA, and the putative *N*-
340 acetylmuramidases/lytic transglycosylases and resuscitation-promoting factor (Rpf)-like
341 proteins Lmo0186 and Lmo2522 (Bierne & Cossart, 2007; Carroll et al., 2003; Pinto et al.,
342 2013).

343 *Lm* deficient in p60 (**Extended Data Fig. 7A–C**) or in both Rpf proteins (**Extended**
344 **Data Fig. 7D–F**) showed culturability, VBNC cell formation and CW loss profiles largely
345 similar to those of wild type bacteria. Ami-deficient *Lm* showed a higher proportion of walled
346 bacteria at day 14, associated with a delay in culturability decline and formation of VBNC cells
347 at day 7 (**Extended Data Fig. 7G–I**). Interestingly, in the absence of Auto, *Lm* presented a
348 significant drop in culturable and walled cell numbers – and thus larger VBNC population – at
349 day 7 (**Extended Data Fig. 7J–L**).

350 The most striking phenotype was observed with NamA-deficient *Lm*, which displayed
351 strongly delayed dynamics of transition to a VBNC state (**Fig. 6A–C**). Indeed, the culturability
352 of $\Delta namA$ bacteria was barely affected at day 7 and showed 10-fold higher values than wild
353 type bacteria at day 14 (**Fig. 6A**). Importantly, more than 90% of the $\Delta namA$ population still
354 conserved their CW after 14 days, compared to 44% of wild type *Lm* (**Fig. 6C**). As the export
355 of NamA to the bacterial surface is specifically mediated by the accessory Sec system ATPase
356 SecA2 (Lenz et al., 2003), we investigated whether SecA2-deficient *Lm* exhibited a phenotype
357 similar to NamA-deficient *Lm*. Indeed, $\Delta namA$ and $\Delta secA2$ bacteria demonstrated very similar
358 dynamics with respect to culturability loss, VBNC cell formation and CW loss, despite
359 substantially lower initial culturable $\Delta secA2$ counts (**Fig. 6D–F**). This initial difference is likely
360 due to a division/scission defect of the $\Delta secA2$ strain that results in a chaining phenotype
361 (**Fig. 6G**), as SecA2 mediates the secretion of both NamA and p60 autolysins (Machata et al.,
362 2005). However, the lack of a role for p60 in CW shedding during VBNC *Lm* formation
363 (**Extended Data Fig. 7A–C**) suggests that the $\Delta secA2$ phenotype is caused by the absence of
364 exported NamA. Supporting this hypothesis, CW loss by $\Delta secA2$ cells was delayed to the same
365 extent as in $\Delta namA$ cells (**Fig. 6C, F**).

366 Altogether, these results reveal the *Lm* autolysin NamA as a major player in VBNC *Lm*
367 formation, with an important role in the events tied to CW breakdown and shedding.

368

369 **CWD VBNC *Lm* can revert back to a vegetative, walled and virulent state**

370

371 Some bacteria, mostly Gram-negative species, have been shown to exit the VBNC state
372 and regain culturability under specific “resuscitation” conditions (Ayrapetyan et al., 2018;
373 Dong et al., 2020). Reversion of the VBNC state in *Lm* and other Gram-positive bacteria
374 remains a challenge (Dong et al., 2020; Lotoux et al., 2022). Revival attempts through nutrient
375 supplementation (e.g. inoculation into fresh or conditioned medium, pure or diluted) were
376 unsuccessful in our hands (our unpublished results). We thus turned to the chicken embryo
377 model, which was previously used to effectively revive VBNC bacteria, including *Lm* (J. M.
378 Cappelier et al., 1999; Jean Michel Cappelier et al., 2007; Chaveerach et al., 2003; Talibart et
379 al., 2000). We inoculated embryonated chicken eggs with a suspension of GFP-expressing
380 VBNC *Lm* containing 10^6 viable cells/mL, of which <1 cells/mL were culturable. In parallel,
381 eggs inoculated with mineral water or with vegetative *Lm* from an overnight broth culture
382 served respectively as negative and positive controls. Two days after inoculation, eggs were
383 processed to assess the presence of culturable *Lm*. All embryonated eggs inoculated with
384 VBNC *Lm* scored positive for bacterial growth (24 out of 24 eggs), similarly to embryonated
385 eggs inoculated with vegetative *Lm* (8 out of 8 eggs). As expected, no bacterial growth was
386 observed in eggs inoculated with mineral water (**Supplementary Table 6**).

387 A critical point with resuscitation of VBNC bacteria is whether the recovered culturable
388 cells resulted from a true revival of VBNC forms or from regrowth of a trace number of
389 culturable cells. To rule out the latter possibility, VBNC *Lm* were inoculated in parallel in BHI,
390 a rich medium that does not support VBNC *Lm* resuscitation (Besnard et al., 2002). This
391 resulted in bacterial growth in only 9.52% (8 in 84) of inoculated wells, which largely
392 contrasted with growth obtained from 100% of inoculated embryonated eggs. This significantly
393 different proportion of *Lm* growth before and after passage of VBNC cells in embryonated
394 eggs ($p=1.63\times 10^{-17}$) attests the successful resuscitation of *Lm* from the VBNC state
395 (**Supplementary Table 6**). As a further control, VBNC cells were also inoculated into non-
396 embryonated eggs, which were shown to fail in promoting VBNC *Lm* revival (Jean Michel
397 Cappelier et al., 2007). Whereas vegetative *Lm* were able to proliferate in non-embryonated
398 eggs, we observed no growth coming from VBNC *Lm*-inoculated eggs (**Supplementary**
399 **Table 6**), underlining the requirement of an embryo for VBNC *Lm* resuscitation and further
400 supporting that the revival of VBNC *Lm* in embryonated eggs was not due to residual culturable
401 bacteria in the inoculum.

402 To confirm if these awakened *Lm* were phenotypically equivalent to vegetative *Lm*, we
403 investigated their morphology and virulence. Fluorescence microscopy of two revived *Lm*
404 clones revealed populations consisting of GFP-expressing, walled, rod-shaped cells that are
405 indistinguishable from vegetative bacteria grown in broth medium (**Fig. 7A**). We then assessed
406 the virulence of the revived clones by infecting human trophoblastic (JEG-3) and hepatocytic
407 (HepG2) cell lines. Quantification of the intracellular bacterial load over time revealed no
408 differences between vegetative and revived *Lm* (**Fig. 7B**). Microscopy analysis of infected cells
409 showed that both revived *Lm* clones produced foci of infected cells after 6 h and spread to the
410 rest of the cell monolayer by 72 h post-infection as efficiently as vegetative *Lm* (**Fig. 7C**),
411 supported by an equal capacity of polymerizing host actin into propulsive comet-like tails
412 (**Fig. 7D**).

413 Altogether, these results provide strong evidence that the CWD VBNC state of *Lm* is
414 fully reversible and bacteria are able to switch back to a walled and cell-infecting state.

415

416 **Discussion**

417

418 *Lm* is a ubiquitous bacterium known to be tolerant to several biotic and abiotic insults.
419 A key factor explaining the presence and survival of this non-sporulating species in a
420 multiplicity of harmful environments is the ability to phase into a dormant VBNC state. First
421 investigated over 20 years ago (Besnard et al., 2000a), the VBNC state of *Lm* remains however
422 largely uncharacterized. In this work, we showed that *Lm* switches from rod-shaped to coccoid
423 cell as it transitions to a VBNC state in mineral water. We further revealed that this coccoid
424 cell form represents a CWD variant that is generated by a molting-like process of CW shedding.
425 Our findings suggest that generation of CWD cells is key for the transition of *Lm* into a non-
426 culturable dormant state in a natural water environment.

427 The major finding of this work is that CWD VBNC bacteria can naturally emerge and
428 persist in a hypotonic (i.e. osmotically hostile) environment. Following an adaptation period of
429 the initial walled cells to mineral water, the CWD *Lm* forms described in this work are
430 relatively robust in this environment without osmoprotection. This contrasts with other CWD
431 bacterial types, such as L-forms or the recently reported actinomycete S-cells (Ramijan et al.,
432 2018), which are formed in an osmoprotective (i.e. hypertonic) environment, or require one
433 during their formation, to avoid explosive cell lysis after CW loss (Claessen & Errington,
434 2019). We also showed that, as *Lm* transitions to a CWD VBNC state, it fine-tunes the
435 physicochemical characteristics of its plasma membrane to become more rigid and potentially
436 more resistant to lysis. It is also possible that physical changes in the cytoplasm could account
437 for the increased mechanical resistance to the extracellular hypoosmotic conditions. To this
438 regard, low metabolic activity has been shown to induce a “glassy” behavior of the cytoplasm
439 that might help bacteria to preserve their cellular architecture (Parry et al., 2014).

440 The CWD *Listeria* described in this study are also distinct from L-forms in their
441 mechanism of formation. Whereas *in vitro*-generated L-forms are typically induced by
442 artificial weakening/breakdown of the CW (e.g. exposure to antibiotics or lytic enzymes), we
443 show that CWD VBNC *Listeria* arise naturally in mineral water. S-cells were also shown to be
444 naturally formed in response to hyperosmotic conditions; however, unlike CWD VBNC
445 *Listeria*, they did not survive in a hypotonic medium (Ramijan et al., 2018). Notably, *Lm* is
446 ubiquitously found in oligotrophic aquatic environments and its occurrence in environmental
447 surface water samples, for example, is estimated at 10–30% after culturing in selective rich
448 medium (Lyautey et al., 2007; Raschle et al., 2021; Sharma et al., 2020; Weller et al., 2015).
449 The presence of unculturable CWD *Lm* in these environmental niches may therefore be vastly
450 underestimated. In this regard, the antibodies generated in this study that specifically recognize
451 wall-less VBNC forms of different *Listeria* species, including the two pathogenic *Lm* and
452 *L. ivanovii*, constitute a novel biomolecular tool that can be potentially used in the detection of
453 dormant pathogens, otherwise untraceable by standard growth-based techniques.

454 Cryo-ET characterization of the *Lm* CW shedding process showed the extrusion of the
455 bacterial protoplast through one of many breaches in the CW sacculus. A similar process was
456 notably first observed in *Bacillus subtilis* cells transitioning to an L-form state (Domínguez-

457 Cuevas et al., 2012), which suggests common mechanisms underlying the CW loss event in
458 both species. Indeed, perturbations in CW loss affected the production of *B. subtilis* L-forms
459 (Domínguez-Cuevas et al., 2012) as well as of VBNC *Lm* in water. Mutations in *B. subtilis*
460 genes resulting in sustained autolysin activity and/or septum malformation were found to
461 promote CW extrusion and L-form emergence (Domínguez-Cuevas et al., 2012). Interestingly,
462 we identified the *Lm* autolysin NamA as an important player in this process, since NamA-
463 deficient cells were strongly delayed in CW shedding and VBNC state entry. Among the other
464 tested autolysins, only Ami promoted the *Lm* CW shedding process, although at a less
465 significant level compared to NamA. An in-depth screening of the *Lm* autolysin collection
466 should reveal the full list of peptidoglycan-degrading enzymes involved in the formation of
467 CWD VBNC *Lm*.

468 Inactivation of the *Lm* general stress response or the stringent response, via genetic
469 deletion of the transcription factor SigB or the (p)ppGpp synthetases (RelAPQ), resulted in
470 unexpectedly faster dynamics of VBNC cell formation. This phenotype was particularly strong
471 in SigB-deficient cells, almost completely wall-less after just 7 days. Intriguingly, the
472 absence/misregulation of these important stress response systems did not affect *Lm* cell
473 viability, indicating that they are not required for bacterial survival in a rather stressful context.
474 These results suggest that stress response regulators may secure a balance between vegetative
475 and dormant states; in mineral water, the absence of these regulators might break the balance
476 in favor of transition to the VBNC state. It will be interesting to understand whether the lack
477 of SigB or a functional stringent response in these mutant bacteria has deleterious
478 consequences, for example in their capacity to successfully exit the VBNC state.

479 We then showed that CW shedding during transition to a VBNC state is not limited to
480 *Lm* and can occur in other pathogenic and non-pathogenic *Listeria* species. This may thus
481 represent an evolutionary strategy within the *Listeria* genus to withstand prolonged nutritional
482 deficiency. It has been hypothesized that the bacterial CW primarily evolved as a structure for
483 storage of sugar- and amino acid-rich components (Claessen & Errington, 2019). Breakdown
484 and salvage of CW components could constitute a bacterial mechanism to secure nutrients to
485 sustain a minimal metabolic flux through the unknown duration of a VBNC state. Whether this
486 ability to form wall-less dormant forms in similar conditions also extends to other species from
487 phylogenetically related genera, including sporulating species, remains to be investigated.

488 Many pathogenic bacteria have been reported to transition to a VBNC state and, for
489 some, this is associated with a loss of virulence (Zhao et al., 2017). We showed that CWD
490 VBNC *Lm* reverted to a culturable state and recovered its CW and virulence, after passing
491 through the chicken embryo. This indicates that one or more yet-unidentified signals present
492 in this host environment can “wake up” these dormant *Lm* forms. Similar resurrection signals
493 might be present in other eukaryotic hosts and in nature, and their identification constitutes a
494 challenging but exciting research avenue.

495 It is becoming clear that a CWD state is an alternative lifestyle that enables bacteria to
496 survive under stress and even proliferate without the mechanical protection of a rigid CW
497 (Claessen & Errington, 2019; Ramijan et al., 2018). The emergence of CWD forms in
498 phylogenetically distant bacterial species (Dannenberg et al., 2022; Ramijan et al., 2018;
499 Slavchev et al., 2013) raises the possibility that transient CW loss may be a more common
500 phenomenon among bacteria than expected. In a world dominated by walled microbes, a

501 temporary wall-less state of dormancy might represent a strategy to promote bacterial
502 persistence under harsh environmental contexts.

503

504 **Methods**

505

506 **Bacteria, cell lines, growth media and conditions**

507

508 Bacteria used in this work are listed in Supplementary Table 7 and were grown at 37 °C
509 in brain and heart infusion (BHI) broth (with agitation) and agar media (BD Difco). Cell lines
510 of human origin used in this work included JEG-3 trophoblasts (ATCC HTB-36), cultivated in
511 MEM (Gibco, Thermo Fisher Scientific) supplemented with 10% (v/v) fetal calf serum
512 (Eurobio Scientific), and HepG2 hepatocytes (ATCC HB-8065), cultivated in DMEM (Gibco,
513 Thermo Fisher Scientific) supplemented with 10% (v/v) fetal calf serum. Cells were incubated
514 at 37 °C in a humidified (90–95%) atmosphere with CO₂ at 5% (for cell propagation) or 10%
515 (for infected cells).

516

517 **Preparation of bacterial suspensions in mineral water**

518

519 Bacterial suspensions in mineral water were prepared with bacteria from overnight-
520 grown stationary-phase cultures. For each tested species, the bacterial concentration of
521 stationary-phase cultures was determined beforehand by enumeration of colony-forming units
522 (CFU) after plating in agar media. Bacteria were pelleted by centrifugation (3,000 × g, 3 min)
523 and washed with 1 volume of sterile-filtered (0.22 μm) mineral water (henceforth referred
524 simply as “mineral water”) for three times before resuspension in 1 volume of mineral water.
525 Washed bacteria were then set to the desired concentration in a final volume of 30 mL of
526 mineral water, and incubated statically at room temperature in an upright-standing, sterile
527 tissue-culture flask (25 cm², vented cap). Samples were collected for downstream analyses
528 immediately after preparation (“day 0”) and after 7, 14, 21 and 28 days.

529

530 **Bacterial culturability and viability assays**

531

532 Bacterial suspensions were regularly sampled for monitorization of the total, viable and
533 culturable cell populations. The culturable population was quantified through enumeration of
534 CFU following the plating of serial dilutions of the suspension on agar media. The total and
535 viable populations were quantified by flow cytometry using a CytoFLEX S analyzer (Beckman
536 Coulter) equipped with three excitation lasers (405, 488 and 561 nm) and operated by the
537 CytExpert software (Beckman Coulter).

538

539 Suspensions prepared at 10⁸ cells/mL were ten-fold diluted in mineral water before
540 acquisition at a flow rate of 10 μL/min. Bacteria-associated events were detected in a forward
541 scatter (FSC) versus side scatter (SSC) plot (**Extended Data Fig. 1A**) and the total population
542 was quantified by enumeration of FSC/SSC-gated events in a defined sample volume (10 μL).
543 For determination of viable population using viability dyes, diluted suspensions were incubated
544 in the dark either with 5(6)-carboxyfluorescein diacetate (CFDA, 30 μM) (Sigma-Aldrich) for
30 min or with a mix of SYTO 9 (3.34 μM) and propidium iodide (PI, 20 μM) from the

545 LIVE/DEAD BacLight Bacterial Viability kit (#L7012, Molecular Probes, Thermo Fisher
546 Scientific) for 15 min. Fluorescence emission by CFDA, SYTO 9 (525/40 nm bandpass) and
547 PI (690/50 nm bandpass) was detected from FSC/SSC-gated bacteria, and populations
548 containing viable (i.e. CFDA⁺ or SYTO 9⁺/PI⁻) or injured/dead bacteria (i.e. CFDA⁻ or PI⁺)
549 were gated with the help of a “dead bacteria” control sample consisting of heat-treated (95 °C,
550 30 min) bacterial suspension (**Extended Data Fig. 1A**). The viable population was quantified
551 by enumeration of CFDA⁺ or SYTO 9⁺/PI⁻-gated events in a defined sample volume (10 µL).

552

553 **Intracellular ATP quantification**

554

555 The intracellular ATP content of bacteria suspended in mineral water was determined
556 using the luciferase-based BacTiter-Glo Microbial Cell Viability Assay kit (Promega). As per
557 the manufacturer instructions, 100 µL of bacterial suspension were mixed in an opaque white
558 96-well plate with 100 µL of room temperature-equilibrated BacTiter-Glo Reagent, and
559 incubated in the dark for at least 5 min. Relative luminescence units (RLU) were then recorded
560 in an Infinite M200 microplate reader (Tecan) with a 1-second integration time per well. Wells
561 containing mineral water were used to obtain background luminescence.

562

563 **Peptidoglycan extraction and UHPLC analysis**

564

565 Peptidoglycan was extracted from *Lm* EGDe as described (Sun et al., 2021). Bacteria
566 (10^{11} cells) were harvested from mineral water suspensions (10^8 cells/mL) on the day of
567 preparation (day 0) and after 7 and 28 days by centrifugation ($4,000 \times g$, 5 min), flash-frozen
568 in liquid nitrogen and stored at -80 °C until further processing. Each bacterial cell pellet was
569 then resuspended in 40 mL of cold distilled water, boiled for 10 min, cooled, and centrifuged.
570 After suspending the cell pellet in 1 mL of distilled water, 1 mL of SDS solution (10% SDS in
571 100 mM Tris-HCl pH 7.0) at 60 °C was added and the suspension was boiled for 30 min and
572 centrifuged (20 min, $25,000 \times g$). The pellet was resuspended in 2 mL of lysis solution (4%
573 SDS in 50 mM Tris-HCl pH 7.0), boiled for 15 min, and washed six times with 60 °C-heated
574 distilled water. Next, the pellet was treated with 2 mg/mL of pronase from *Streptomyces*
575 *griseus* (Roche) in 50 mM Tris-HCl pH 7.0 for 1.5 h at 60°C, and afterwards with 10 µg/mL
576 of DNase (Thermo Fisher Scientific), 50 µg/ml of RNase (Thermo Fisher Scientific) and
577 50 µg/mL lipase from *Aspergillus niger* (Sigma-Aldrich) in a buffer solution (20 mM Tris-HCl
578 pH 7.0, 1 mM MgCl₂, 0.05% sodium azide) for 4 h at 37 °C. The suspensions were washed
579 with distilled water and treated with 200 µg/mL of trypsin (Sigma-Aldrich) in 20 mM Tris-
580 HCl pH 8.0 overnight at 37°C with agitation. Finally, after inactivating trypsin (3-min boil),
581 the suspensions were incubated with 48% hydrofluoric acid (Merck) overnight at 4 °C. After
582 centrifugation (20 min, $25,000 \times g$), the pellet was washed twice with 250 mM Tris-HCl pH
583 7.0 and four times with distilled water to raise the pH to 5. The extracted peptidoglycan was
584 lyophilized and resuspended in distilled water.

585

586 Muropeptides were prepared from purified peptidoglycan by overnight digestion with
587 2500 U/mL mutanolysin (Sigma-Aldrich) in 25 mM NaHPO₄ pH 5.5, at 37°C with shaking.
588 After reduction with sodium borohydride, muropeptide originating from peptidoglycan
589 extracted from the same number of cells (1.5×10^9) were analyzed by reverse phase-ultra high-

589 pressure liquid chromatography (RP-UHPLC) using a 1290 chromatography system (Agilent
590 Technologies) equipped with a Zorbax Eclipse Plus C18 RRHD column (100×2.1 mm, 1.8- μ m
591 particle size; Agilent Technologies). Elution was performed at 50 °C with 10 mM ammonium
592 phosphate pH 5.6 and a linear gradient (0–20%, 270 min) of methanol, at a flow rate of
593 0.5 mL/min. Eluted muropeptides were detected by absorbance (202 nm).

594

595 **Gentamicin protection assay**

596

597 JEG-3 and HepG2 cell lines were seeded in 24-well plates, with or without coverslips,
598 to reach 90–100% confluency on the day of infection. Prior to seeding HepG2 cells, wells and
599 coverslips were surface-coated with collagen (type I, rat tail) (Sigma-Aldrich) in a 50 μ g/mL
600 solution in Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Thermo Fisher Scientific)
601 for 30 min and washed once with DPBS. On infection day, bacterial inocula were prepared by
602 washing bacteria from overnight-grown, stationary-phase BHI cultures with DPBS and diluting
603 them in serum-free medium. Cell monolayers were washed once with serum-free medium and
604 infected for 1 h with the inocula at a multiplicity of infection (MOI) of 0.01 bacteria/JEG-3
605 cell or 5 bacteria/HepG2 cell. The inocula were removed from the wells and replaced with
606 serum-supplemented medium containing 25 μ g/mL of gentamicin (Sigma-Aldrich), to kill
607 non-internalized bacteria. At 2 h, 6 h, 24 h and 72 h post-infection, cells were processed for
608 immunofluorescence (see below) or for quantification of intracellular viable bacteria. In the
609 latter case, cells were lysed in cold distilled water and serial dilutions of the lysates in DPBS
610 were plated on BHI agar and incubated at 37 °C for at least 24 h for CFU enumeration.

611

612 **Generation of a polyclonal antiserum against CWD *Lm***

613

614 A rabbit polyclonal antiserum was raised against CWD *Lm* as follows. *Lm* 10403S were
615 suspended in mineral water (10⁸ bacteria/mL), as described above, and incubated for 42 days.
616 Bacteria were harvested by centrifugation (3,000 × *g*, 5 min), resuspended and incubated in
617 fixative solution (1% (v/v) paraformaldehyde (PFA) in PBS) at 32 °C for 2 h, washed three
618 times and resuspended in PBS. Bacterial neutralization was confirmed after plating on BHI
619 agar and incubation at 37°C for several days.

620 Animal immunizations and serum recovery were performed by Covalab (Bron, France).
621 White New Zealand female rabbits were inoculated with 1 mL of a 1:1 mixture of 10⁸ PFA-
622 fixed CWD *Lm* and incomplete Freund's adjuvant, and received boosts every three weeks for
623 a total of three boosts. Immune serum was harvested at 53 and 74 days post-immunization and
624 its reactivity and specificity towards CWD *Lm* was assessed by immunofluorescence
625 microscopy.

626

627 **Immunofluorescence microscopy**

628

629 Bacterial cells spotted onto poly-L-lysine-treated coverslips or coverslip-attached
630 eukaryotic cells were fixed in a 4% (v/v) paraformaldehyde (PFA) solution in PBS for 20 or
631 30 min, respectively. Cells were washed in PBS, incubated in a blocking solution (2% bovine
632 serum albumin in PBS) for 20 min and, in the case of eukaryotic cells, permeabilized (in a

633 0.4% (v/v) Triton X-100 solution in PBS for 4 min, followed by three washes in PBS) before
634 proceeding with fluorescent labeling.

635 Rabbit *Listeria* O Antiserum Poly (anti-*Lm*; #223021, BD Difco) was used to label the
636 *Listeria* CW. Oregon Green 488- or TRITC-conjugated WGA (Molecular Probes, Thermo
637 Fisher Scientific) was used (25 µg/mL) to label the CW of serogroup 1/2 *Lm* strains (EGDe,
638 10403S). Rabbit anti-CWD *Lm* antiserum was used to label the exposed protoplast membrane
639 of CW-shedding or CWD *Listeria*. Secondary antibodies consisted of goat and alpaca anti-
640 rabbit antibodies conjugated with Alexa Fluor 488 (Molecular Probes, Thermo Fisher
641 Scientific), Cy3 or Cy5 (Jackson ImmunoResearch). Alexa Fluor 647-conjugated phalloidin
642 and Hoechst 33342 (Molecular Probes, Thermo Fisher Scientific) were respectively used to
643 label F-actin and DNA, and, as with WGA, were added together with secondary antibodies.
644 All incubations were made in blocking solution for 1 h in the dark.

645 Samples were mounted onto microscope glass slides with Fluoromount-G medium
646 (Interchim) and examined on a ZEISS Axio Observer.Z1 epifluorescence microscope equipped
647 with Plan-Apochromat 20×/0.8 NA (non-immersion), 40×/1.3 NA Oil and 100×/1.4 NA Oil
648 (immersion) objectives, an AxioCam 506 Mono camera and operated with ZEN software (Carl
649 Zeiss AG). Three to seven fields were acquired per coverslip and images were processed for
650 quantification (see below) and/or figure montage with Fiji software.

651

652 **Image quantifications**

653

654 Bacterial cell morphology was analyzed using Fiji software as follows: automatic
655 thresholding was applied to phase-contrast images to select objects, and particle length and
656 roundness parameters were selected (“Fit ellipse” and “Shape descriptors” options in “Set
657 Measurements” menu) to be measured on thresholded objects (“Limit to threshold” option in
658 “Set Measurements” menu). Outlier objects (i.e. too small/big, irregularly shaped) were
659 excluded from the particle analysis (size: 0.5–1.5 µm², circularity: 0.1–1.0). The length and
660 roundness values per measured object were retrieved from the results table under the “Major”
661 and “Round” columns, respectively.

662 To quantify the fraction of bacterial populations with CW and/or exposed plasma
663 membrane (i.e. single or double) labeling, phase-contrast channel images were thresholded to
664 select phase-contrast dense objects. Object outlines were then first laid over the DNA
665 fluorescence channel to select bacterial cells, and afterwards over the fluorescence channels
666 associated with CW and/or exposed plasma membrane to enumerate bacteria with
667 single/double labeling. As co-labeling with the anti-*Lm* and anti-CWD *Lm* antibodies was not
668 possible (same host species), only single-labeling quantifications were performed from
669 separately labeled samples of the same bacterial population.

670

671 **Cryo-electron tomography**

672

673 A solution of bovine serum albumin-coated gold tracer containing 10-nm colloidal gold
674 fiducial particles (Aurion) was mixed with bacterial suspensions at a 2:1 ratio. This mixture
675 was applied to the front (3.7 µL) and to the back (1.3 µL) of carbon-coated copper grids (R2/2,
676 Cu 200 mesh; Quantifoil) previously glow-discharged (2 mA, 1.8×10⁻¹ mbar, 1 min) in an

677 ELMO system (Cordouan Technologies). Excess liquid was removed by blotting the grid
678 backside with filter paper (9 sec, 18 °C, 95% humidity) and the sample was immediately frozen
679 in liquid ethane in an EM GP automatic plunge freezer (Leica Microsystems). Grids were
680 stored in liquid nitrogen until image acquisition.

681 Tilt series were acquired in a 300 kV Titan Krios G3 transmission electron microscope,
682 equipped with a Cold FEG tip, a Selectris X energy filter with slit width set to 20 eV, single-
683 tilt axis holder and a Falcon 4i direct electron detector (Thermo Fisher Scientific), and operated
684 with the SerialEM software (version 4.0.13, U. Colorado Boulder, USA) (Mastronarde, 2003).
685 Tilt series acquisition was performed in batches using a dose-symmetric scheme. One batch
686 was obtained with an angular range of $\pm 42^\circ$ (3° increment), a defocus range of -3 to -8 μm , a
687 pixel size of 4.8 \AA (26,000x magnification), an exposure time of 8 s, a dose rate of
688 13.7 e/pixel/s and a total electron dose of about 140 e/ \AA^2 . Another batch was acquired with an
689 angular range of $\pm 50^\circ$ (2° increment), a defocus range of -3 to -8 μm , a pixel size of 6.4 \AA
690 (19,500x magnification), an exposure time of 10 s, a dose rate of 12 e/pixel/s and a total
691 electron dose of about 150 e/ \AA^2 . Tilt series were saved as separate stacks of frames, motion-
692 corrected and restacked in order using the *alignframes* module in SerialEM.

693 3D reconstructions of tomograms were calculated in IMOD software (version 4.9.10,
694 U. Colorado Boulder, USA) (Mastronarde & Held, 2017) by weighted back projections with
695 dose weighting and a SIRT-like filter. The IMOD drawing tools and interpolator module were
696 used to manually trace and produce a 3D surface of the bacterial plasma membrane. This
697 surface was then imported to ChimeraX software (version 1.6, UC San Francisco, USA)
698 (Pettersen et al., 2021) and used as a mask to extract slabs of subvolumes corresponding to the
699 bacterial plasma membrane and CW. For the plasma membrane subvolume, the slab was
700 produced using the *volume onesmask* function. For the CW, the *volume mask* function was
701 used instead, cropping a larger slab beyond the CW limits, and the subvolume was visualized
702 using the isosurface representation with an appropriate intensity threshold. Final visualizations
703 and rendering were performed in ChimeraX using homemade scripts for video production.

704

705 **Fatty acid extraction and GC-MS analysis**

706

707 Bacteria were harvested by centrifugation ($3000 \times g$, 5 min), flash-frozen in liquid
708 nitrogen and stored at -80 °C until further processing. Extraction and methylation of fatty acids
709 (FA) were carried out directly on bacterial pellets as described (Touche et al., 2023). Whole-
710 cell FA were first saponified and esterified by methanolic NaOH (1 mL of 3.75 M NaOH in
711 50% (v/v) methanol for 30 min at 100 °C) followed by methanolic HCl (addition of 2 mL of
712 3.25 M HCl in 45% (v/v) methanol solution and incubation for 10 min at 80°C). FA methyl
713 esters (FAME) were then extracted with a 1:1 (v/v) diethyl ether/cyclohexane solution, and the
714 organic phase was washed with dilute base (0.3 M NaOH).

715 Analytical gas chromatography of FAME was carried out in a GC-MS Trace 1300 /
716 ISQ 7000 system (Thermo Fisher Scientific) equipped with a BPX70 capillary column (25 m,
717 0.22-mm internal diameter) (SGE, Victoria, Australia). Column temperature was set at 100 °C
718 for 1 min and then increased to 170 °C at a rate of 2 °C/min. FA species were identified using
719 MS databases (Replib, Mainlib, FAME2011). The relative abundance of FA species was
720 expressed as the percentage of the total FAME peak area. Identified FA species were grouped

721 in the following classes: iso and anteiso branched-chain FA (i-BFA and ai-BFA), saturated FA
722 (SFA), and unsaturated FA (UFA).

723

724 **Laurdan generalized polarization**

725

726 The generalized polarization of the lipophilic dye laurdan (6-dodecanoyl-2-
727 dimethylaminonaphthalene), when bound to the *Lm* plasma membrane, was used as a measure
728 of the bacterial membrane fluidity (Scheinflug et al., 2017). Bacteria were sampled (1 mL)
729 from mineral water suspensions (10^8 cells/mL) at 0, 7, 14 and 28 days and incubated for 10 min
730 in the dark with 10 μ M of laurdan (Sigma-Aldrich) from a 1 mM stock in dimethylformamide
731 (DMF). Unbound laurdan was washed off of bacterial cells with four cycles of centrifugation
732 ($8,000 \times g$, 5 min) and resuspension (vortex) in 1% (v/v) DMF in mineral water. After a final
733 resuspension (vortex) in 1 mL of the washing solution, technical replicates (200 μ L) were
734 added to a clear-bottom black 96-well plate, which was then equilibrated to 25 $^{\circ}$ C in a Spark
735 microplate reader (Tecan). After equilibration, laurdan fluorescence emission was induced at
736 350 nm and recorded at 450 and 500 nm. The generalized polarization (GP) of laurdan was
737 determined by the formula: $GP = (I_{450} - I_{500}) / (I_{450} + I_{500})$, where I corresponds to the fluorescence
738 intensity value at the recorded emission wavelength (Scheinflug et al., 2017). The GP values
739 obtained for every timepoint were normalized to those obtained from bacterial suspensions
740 prepared on the same day (i.e. day 0). An increase of normalized laurdan GP values can be
741 interpreted as a reduction of plasma membrane fluidity.

742

743 **Total internal reflection fluorescence correlation spectroscopy (TIR-FCS)**

744

745 The diffusion of the lipophilic dye Nile red in the membrane of *Lm* was measured using
746 total internal reflection fluorescence correlation spectroscopy (TIR-FCS) (Barbotin et al.,
747 2023). In short, bacterial cells were labeled for 10 min with 0.1 μ g/mL of Nile red from a 50
748 μ g/mL stock in DMSO. FCS acquisitions were performed with a ZEISS Elyra PS1 TIRF
749 microscope equipped with a Plan-Apochromat 100 \times /1.46 NA Oil immersion objective (Carl
750 Zeiss AG). Fluorescence excitation at 561 nm was set to a power of ~ 70 nW/ μ m². Each FCS
751 acquisition consisted of a stack of 50,000 frames with a frame acquisition time of 1.26 ms,
752 maximized by the use of only 10 lines of the camera chip. Pixels were binned 2 by 2 to increase
753 signal levels to an effective pixel size of 320 nm. The resulting intensity timetraces were
754 correlated and fitted as described (Barbotin et al., 2023).

755 TIR-FCS estimation of diffusion coefficients in small cells, such as bacteria, is biased
756 by the cell morphology (Barbotin et al., 2023). To correct for this bias, the cell width and length
757 of Nile red-stained bacteria were measured in epifluorescence images, using ImageJ. The
758 average cell morphology values in each condition were then used to simulate TIR-FCS
759 experiments to determine the diffusion coefficient bias (Barbotin et al., 2023). The corrected
760 diffusion coefficient was obtained by dividing the experimentally measured diffusion
761 coefficient value by the corresponding bias value.

762

763 **RNA extraction, RNA sequencing and gene set enrichment analysis**

764

765 Bacteria (10^9 cells) were harvested ($5000 \times g$, 3 min) from mineral water suspensions
766 (biological triplicates at 10^8 cells/mL) in the first day and after 7 days of incubation, and
767 immediately flash-frozen in liquid nitrogen and stored at -80°C until further processing. Total
768 RNAs were recovered from bead-beaten bacterial cells using a phenol/chloroform extraction
769 method. Purified RNA samples were further prepared for sequencing at the I2BC sequencing
770 platform (Gif-sur-Yvette, France)

771 RNA sample quality was assessed in an Agilent Bioanalyzer 2100, using the RNA 6000
772 pico kit (Agilent Technologies). Total RNAs (450 ng) were treated with DNase (Baseline-
773 ZERO DNase, Epicentre) and ribosomal RNA was removed using the Illumina Ribo-Zero
774 Magnetic Kit (Bacteria), according to the manufacturer recommendations. Directional RNA
775 sequencing libraries were constructed using the TruSeq Stranded Total RNA Library Prep kit
776 (Illumina) and sequenced (paired-end 2×75 -bp) in a NextSeq500 instrument (Illumina).
777 Alignment to the reference genome sequence of *Listeria monocytogenes* EGDe (RefSeq:
778 NC_003210.1) was done using Bowtie2 (v2.4.4) (Langmead & Salzberg, 2012). For the
779 detection of differentially expressed genes (DEGs), relative library sizes, fold changes ($\log_2\text{FC}$)
780 and p-values were estimated using the R package “DESeq2” (v1.38.3) (Love et al., 2014), and
781 p-values were then converted to q-values using the R package “fdrtool” (v1.2.17) (Strimmer,
782 2008). Genes with q-value ≤ 0.05 and absolute $\log_2\text{FC} \geq 1$ were considered as DEGs.

783 Gene set enrichment analysis was performed on DEGs using the FUNAGE-Pro web
784 server (<http://funagepro.molgenrug.nl>) (de Jong et al., 2022). A single-list analysis (gene locus
785 tags) was done against the *Listeria monocytogenes* EGD-e reference genome (RefSeq:
786 NC_003210.1). Gene ontology (GO) and KEGG pathway terms with a p-value ≤ 0.05 were
787 considered to be statistically enriched.

788

789 **Chicken embryo infection assay**

790

791 The chicken embryo model was used to test the recovery of *Lm* from a VBNC state in
792 mineral water suspensions, as previously described (Andersson et al., 2015; Jean Michel
793 Cappelier et al., 2007). Embryonated eggs from white Leghorn chickens raised under specific
794 pathogen-free (SPF) conditions were obtained from the Infectiology of Farm, Model and
795 Wildlife Animals Facility (PFIE) at the INRAE Centre Val de Loire (Nouzilly, France). Intact
796 eggs were placed in an incubator (FIEM; Guanzate, Italy) at 37.7°C and 47% humidity, under
797 gentle rocking motion, to initiate embryo development. After 6 days, eggs were candled to
798 check for signs of developing embryos, such as a strong vascularized network and embryo
799 movement. Eggs showing underdeveloped or collapsed blood vessels were discarded, while
800 eggs with no signs of embryo development (absence of blood vessels) were set aside to be used
801 as “non-embryonated” eggs. Egg shells were sterilized with 70% ethanol and perforated just
802 above the border of the air sac to allow the injection of $100\ \mu\text{L}$ of bacterial suspension into the
803 allantoic cavity (or albumen in non-embryonated eggs), using a 25G (0.5×16 mm) needle. Shell
804 punctures were sealed with a sticky tag and the eggs were returned to the incubator. At 48 h
805 post-inoculation, embryonated eggs were candled to discard dead embryos, and viable embryos
806 were euthanized by incubation at 4°C for 2 h. Embryos were recovered in aseptic conditions
807 and placed into a tube with 4 mL of sterile DPBS to undergo mechanical homogenization (T-
808 25 Ultra-Turrax, IKA). Serial dilutions of the embryo homogenate (or albumen from non-

809 embryonated eggs) in DPBS were plated (500 μ L) on BHI agar and incubated at 37 °C for at
810 least 24 h to assess the presence of culturable *Lm*.

811 For inoculation of eggs with VBNC *Lm*, suspensions of EGDe-GFP at 10⁶ bacteria/mL
812 were prepared 28 days before, and their viability and culturability were checked to select the
813 one with the lowest residual culturability. As a result, we used an inoculum containing 10⁶
814 viable *Lm*/mL and 0.5 *Lm* CFU/mL, which corresponded to an inoculated dose containing 10⁵
815 viable and 0.05 culturable bacteria. Injections of 100 μ L of mineral water or a suspension of
816 vegetative EGDe-GFP – prepared by washing and diluting bacteria from an overnight-grown
817 culture to approximately 5,000 CFU/mL – were included as negative and positive controls,
818 respectively.

819 To assess whether bacterial growth recovered from inoculated eggs resulted from the
820 revival of VBNC cells or solely from the regrowth of residual culturable bacteria, we compared
821 the frequency of bacterial growth obtained before and after egg inoculation with the VBNC *Lm*
822 suspension. The frequency before inoculation was determined by serially inoculating wells of
823 a 96-well plate containing 100 μ L of BHI broth with 100 μ L of the VBNC *Lm* suspension, and
824 calculating the fraction of inoculated wells showing bacterial growth after 48 h of incubation
825 at 37 °C. Similarly, the frequency after inoculation was determined from the fraction of
826 inoculated eggs scored positive for bacterial growth on BHI agar.

827

828 **Statistics**

829

830 Data from experiments comprising biological replicates (n=3) are graphically presented
831 as mean \pm standard deviation or median \pm interquartile range. Prism software (version 9.0;
832 GraphPad) was used to generate graphs and to perform statistical analyses (tests reported in
833 figure legends). Differences between means were considered statistically significant for
834 p values \leq 0.05.

835

836 **Data availability**

837

838 The RNA sequencing data generated in this study have been deposited in NCBI's Gene
839 Expression Omnibus and are accessible through GEO Series accession number GSE246157
840 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246157>). Additional data and
841 material generated by this work are available upon request.

842

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844

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867

868 **Author contributions**

869

870 FC, HB and AP conceived the work plan and methodology. EM conceived and performed pilot
871 experiments. FC, AC, ASR, ST, ADG, PC, PN, FDB, AB, ED, KG, CS, MPCC, EM and AP
872 performed experiments. FC, ASR, ADG, PN, FDB and AB analyzed collected data. ASR, ST,
873 PN, FDB, KG, RCL, MPCC, HB and AP provided resources (reagents, equipment and/or
874 analysis tools). FC, HB and AP wrote the original draft. FC, HB and AP reviewed and edited
875 the draft. FC prepared and organized data visualization. HB and AP provided funding and
876 supervision. All authors contributed to this work and approved the submitted version.

877

878 **Conflicts of interest**

879

880 The authors declare no conflict of interest.

881 **Figure legends**

882

883 **Fig. 1. *Lm* transitions into a CWD VBNC state after prolonged incubation in mineral**
884 **water.**

885 (A) Culturability profiles of *Lm* EGDe in mineral water suspensions with different starting
886 bacterial concentrations. The number of culturable bacteria was determined at each timepoint
887 by enumeration of colony-forming units (CFUs). Data are represented as the mean \pm standard
888 deviation (SD) from three independent suspensions. (B) Total, viable and culturable cell
889 profiles of *Lm* EGDe in mineral water suspensions (10^8 cells/mL). Culturable bacteria were
890 determined by enumeration of CFUs, while total and viable bacteria were quantified by flow
891 cytometry using CFDA. Data are represented as the mean \pm SD from three independent
892 suspensions. The area covered with vertical grey dashes indicates the VBNC cell population.
893 (C) Fraction of the bacterial population consisting of VBNC cells formed in mineral water.
894 Data are represented as the mean \pm SD from three independent suspensions, each indicated by
895 a dot. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc
896 multiple comparison correction. ****, $p \leq 0.0001$. (D) Comparison of the culturability and ATP
897 content of *Lm* EGDe sampled from mineral water suspensions at the indicated timepoints.
898 Culturable bacteria were determined by enumeration of CFUs, while ATP levels were
899 quantified with a luciferase-based assay. The graph on the right shows the culturability and
900 ATP levels assessed from serial dilutions of *Lm* EGDe suspensions, immediately after
901 preparation (day 0), and serves as a reference for the ATP content expected from a defined
902 number of culturable cells. Data are represented as the mean \pm SD from three independent
903 suspensions, each indicated by a dot. (E) Phase-contrast micrographs of *Lm* EGDe sampled
904 from mineral water suspensions at the indicated timepoints. Bacteria highlighted by white
905 squares are shown enlarged in bottom panels. Scale bar: 2 μ m. (F, G) Quantification of the cell
906 length (F) and roundness (G) of *Lm* EGDe sampled from mineral water suspensions at the
907 indicated timepoints. Data are represented as violin plots with the median (solid line) +
908 interquartile range (dashed lines) from three independent suspensions ($n=800-1600$ cells per
909 timepoint). Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post hoc
910 multiple comparison correction. ****, $p \leq 0.0001$. (H) Micrographs of Gram-stained *Lm* EGDe
911 sampled from mineral water suspensions at the beginning (day 0) and end (day 28) of the
912 incubation period. Scale bar: 2 μ m. (I) Reverse-phase UHPLC mucopeptide profile obtained
913 from peptidoglycan extracted from *Lm* EGDe suspensions in mineral water on the first day
914 (D0) and after 7 (D7) and 28 (D28) days. The amount of material analyzed was normalized to
915 the same bacterial cell number (1.5×10^9). Profiles are representative of two independent
916 suspensions. (J) Phase-contrast and fluorescence micrographs of *Lm* EGDe sampled from
917 mineral water suspensions (10^8 cells/mL) at the indicated timepoints. Bacteria were fixed and
918 fluorescently labelled for visualization of DNA (Hoechst 33342), CW (WGA) and exposed
919 plasma membrane (anti-CWD *Lm*). Bacteria highlighted by white squares are shown enlarged
920 in the bottom panels. Scale bar: 2 μ m. (K) Fraction of *Lm* EGDe in mineral water suspensions
921 showing single- or double-labeling of CW (WGA) and exposed plasma membrane (anti-
922 CWD *Lm*) by fluorescence microscopy at the indicated timepoints. Data are represented as
923 stacked bars (one for each labelling group) with mean \pm SD from three independent
924 suspensions. Statistical analysis was performed using a two-way ANOVA test with Tukey's

925 post hoc multiple comparison correction, and only indicated for the single-labelled groups. **,
926 $p \leq 0.01$; ****, $p \leq 0.0001$.

927

928 **Fig. 2. Cryo-electron tomography of the *Lm* CW shedding stages during transition to the**
929 **VBNC state.**

930 Ultrastructure of the different *Lm* CW shedding stages obtained by cryo-electron tomography
931 of *Lm* EGDe sampled from mineral water suspensions (10^8 *Lm*/mL) between day 0 and day 14.
932 **Stage 0:** rod-shaped bacterium with tightly connected plasma membrane and CW. In this
933 example, a septum is forming at the midcell. **Stage 1:** the detachment of the plasma membrane
934 and CW layers results in the formation of a periplasm-like space. **Stage 2:** breaches of variable
935 size are formed in the CW matrix, exposing the enclosed protoplast to the outside environment.
936 **Stage 3:** the bacterial protoplast begins bulging through a CW breach at one of the poles.
937 **Stage 4:** the protoplast continues squeezing out, leaving an empty cell wall sacculus behind.
938 **Stage 5:** the bacterium has fully molted from its rod-shaping cell wall, assuming a coccoid
939 morphology as a CWD form. Left panels display a tomogram slice, in which CW and
940 membrane (MB) are indicated by white arrowheads, a periplasm-like space is indicated by red
941 arrowheads, and CW breaches are indicated by yellow arrowheads. Right panels display a 3D
942 rendering of the CW (blue) and membrane (orange), obtained by manual segmentation of
943 tomogram slices, and middle panels display a superposition of the tomogram slice and the 3D
944 models. Scale bar: 200 nm. Movies showing all the tomogram slices used for 3D reconstruction
945 of the bacterial CW and membrane for stages 0 and 2–5 are available as supplementary Movies
946 S1 to S4.

947

948 **Fig. 3. A CWD VBNC state occurs in other *Listeria* species.**

949 (A–E) Total, viable and culturable cell profiles of mineral water suspensions (10^8 cells/mL) of
950 *Listeria sensu stricto* clade members *L. ivanovii* (A), *L. innocua* (B), *L. marthii* (C),
951 *L. seeligeri* (D) and *L. welshimeri* (E). Culturable bacteria were determined by enumeration of
952 CFUs, while total and viable bacteria were quantified by flow cytometry. Data are represented
953 as the mean \pm SD from three independent suspensions. The area covered with vertical grey
954 dashes indicates the VBNC cell population. (F–J) Fraction of the *L. ivanovii* (F), *L. innocua*
955 (G), *L. marthii* (H), *L. seeligeri* (I) and *L. welshimeri* (J) populations consisting of VBNC cells
956 formed in mineral water. Data are represented as the mean \pm SD from three independent
957 suspensions, each indicated by a dot. (K–T) Fraction of the *L. ivanovii* (K, P), *L. innocua* (L,
958 Q), *L. marthii* (M, R), *L. seeligeri* (N, S) and *L. welshimeri* (O, T) populations showing CW
959 (K–O) or exposed plasma membrane (P–T) labelling (anti-*Lm* and anti-CWD *Lm*, respectively)
960 by fluorescence microscopy at the indicated timepoints. Data are represented as the mean \pm SD
961 from three independent suspensions, each indicated by a dot. (U) Phase-contrast and
962 fluorescence micrographs of the five *Listeria* species sampled from mineral water suspensions
963 at the indicated timepoints. Bacteria were fixed and fluorescently labelled for visualization of
964 DNA (Hoechst 33342), CW (anti-*Lm*) and exposed membrane (anti-CWD *Lm*). Scale bar:
965 2 μ m.

966

967 **Fig. 4. *Lm* adjusts its plasma membrane properties to adapt to a CWD lifestyle.**

968 (A) Fold change of the relative abundance of anteiso-branched (a-BFA), iso-branched (i-BFA),
969 unsaturated (UFA) and saturated (SFA) fatty acids present in *Lm* EGDe sampled from mineral
970 water suspensions (10^8 cells/mL) at the indicated timepoints relative to the first timepoint. Fold
971 change values were calculated from the data presented in Extended Data Fig. 5, and are
972 represented as the mean \pm SD from three independent suspensions. Statistical analysis was
973 performed using a two-way ANOVA test with Tukey's post hoc multiple comparison
974 correction. ***, $p \leq 0.001$; ****, $p \leq 0.0001$. (B) Generalized polarization (GP) of laurdan-
975 labeled *Lm* EGDe sampled from mineral water suspensions (10^8 cells/mL) at the indicated
976 timepoints. GP values were measured by fluorescence spectroscopy and normalized to those
977 from overnight-grown bacteria. Increasing relative GP suggests a reduction of the bacterial
978 membrane fluidity. Data are represented as the mean \pm SD from three independent
979 suspensions, each indicated by a dot. Statistical analysis was performed using a one-way
980 ANOVA test with Dunnett's post hoc multiple comparison correction. *, $p \leq 0.05$; **, $p \leq 0.01$;
981 ***, $p \leq 0.001$. (C) Membrane diffusivity of Nile red in *Lm* EGDe cells sampled from mineral
982 water suspensions at the indicated timepoints. Diffusion coefficient values were measured in
983 Nile red-stained cells of rod and coccoid shape by total internal reflection fluorescence
984 correlation spectroscopy (TIR-FCS). Data are represented as scattered dot plots with the
985 median \pm interquartile range from three independent suspensions. Each dot represents one
986 measured bacterium and bacteria from the same suspension are indicated by the same color.
987 Statistical analysis was performed using a one-way ANOVA test with Tukey's post hoc
988 multiple comparison correction. ****, $p \leq 0.0001$. (D) Osmotic sensitivity of CWD *Lm* EGDe
989 adapted versus non-adapted to mineral water. The total bacterial population in mineral water
990 suspensions treated or not with mutanolysin at the indicated timepoints was quantified by flow
991 cytometry. Data are represented as the mean \pm SD from three independent suspensions.
992 Statistical analysis was performed using a two-way ANOVA test with Šidák's post hoc
993 multiple comparison correction. *, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

994

995 **Fig. 5. Transcriptional profiling reveals a major regulatory role for SigB and stress**
996 **response mechanisms in the onset of VBNC *Lm* formation.**

997 (A) Scatter plot of *Lm* EGDe protein-coding genes ($n=2864$) according to the statistical
998 significance (q-value) and magnitude of transcriptional change (\log_2 fold change) determined
999 at day 7 relative to day 0 by RNA-seq. Horizontal dashed lines indicate fold change thresholds
1000 ($|\log_2|=1$) and vertical dashed line indicates statistical significance threshold (q-value=0.05).
1001 Genes statistically considered to be differentially regulated ($n=1229$) are grouped into 636
1002 upregulated genes (red box) and 593 downregulated genes (blue box). (B, C) Functional
1003 analysis of statistically enriched gene ontology biological process (GOBP) terms in
1004 downregulated (B) and upregulated genes (C). GOBP terms are ranked from left to right by
1005 increasing p-value and decreasing fraction of differentially regulated genes per term. (D) Total,
1006 viable and culturable cell profiles of mineral water suspensions of wild type (WT) and SigB-
1007 deficient ($\Delta sigB$) *Lm* EGDe. Culturable bacteria were determined by enumeration of CFUs,
1008 while total and viable bacteria were quantified by flow cytometry. Data are represented as the
1009 mean \pm SD from three independent suspensions. (E) Fraction of the WT and $\Delta sigB$ populations
1010 consisting of VBNC cells formed in mineral water. Data are represented as the mean \pm SD
1011 from three independent suspensions, each indicated by a dot. Statistical analysis between

1012 mutant and WT strains was performed using a two-way ANOVA test with Šidák's post hoc
1013 multiple comparison correction. ****, $p \leq 0.0001$. (F) Fraction of the WT and $\Delta sigB$ populations
1014 showing single- or double-labeling of CW (WGA) and exposed plasma membrane (anti-
1015 CWD *Lm*) by fluorescence microscopy, at the indicated timepoints. Data are represented as
1016 stacked bars (one for each labelling group) with mean \pm SD from three independent
1017 suspensions. Statistical analysis between $\Delta sigB$ and WT strains was performed for each
1018 labelling group using a two-way ANOVA test with Tukey's post hoc multiple comparison
1019 correction. ***, $p \leq 0.001$; ****, $p \leq 0.0001$. (G) Phase-contrast and fluorescence micrographs
1020 of WT and $\Delta sigB$ bacteria sampled from mineral water suspensions at the indicated timepoints.
1021 Bacteria were fixed and fluorescently labelled for visualization of DNA (Hoechst 33342), CW
1022 (WGA) and exposed plasma membrane (anti-CWD *Lm*). Scale bar: 2 μ m. (H) Total, viable
1023 and culturable cell profiles of mineral water suspensions of wild type (WT) and RelAPQ-
1024 deficient ($\Delta relAPQ$) *Lm* EGDe. Culturable bacteria were determined by enumeration of CFUs,
1025 while total and viable bacteria were quantified by flow cytometry. Data are represented as the
1026 mean \pm SD from three independent suspensions. (I) Fraction of the WT and $\Delta relAPQ$
1027 populations consisting of VBNC cells formed in mineral water. Data are represented as the
1028 mean \pm SD from three independent suspensions, each indicated by a dot. Statistical analysis
1029 between mutant and WT strains was performed using a two-way ANOVA test with Šidák's
1030 post hoc multiple comparison correction. ****, $p \leq 0.0001$. (J) Fraction of the WT and $\Delta relAPQ$
1031 populations showing single- or double-labeling of CW (WGA) and exposed plasma membrane
1032 (anti-CWD *Lm*) by fluorescence microscopy, at the indicated timepoints. Data are represented
1033 as stacked bars (one for each labelling group) with mean \pm SD from three independent
1034 suspensions. Statistical analysis between $\Delta relAPQ$ and WT strains was performed for each
1035 labelling group using a two-way ANOVA test with Tukey's post hoc multiple comparison
1036 correction. ****, $p \leq 0.0001$.

1037

1038 **Fig. 6. The autolysin NamA promotes *Lm* CW shedding and VBNC entry.**

1039 (A, D) Total, viable and culturable cell profiles of mineral water suspensions of wild type (WT)
1040 and NamA-deficient (A) or SecA2-deficient (D) *Lm* EGDe. Culturable bacteria were
1041 determined by enumeration of CFUs, while total and viable bacteria were quantified by flow
1042 cytometry. Data are represented as the mean \pm SD from three independent suspensions. (B, E)
1043 Fraction of the WT and $\Delta namA$ (B) or $\Delta secA2$ (E) populations consisting of VBNC cells
1044 formed in mineral water. Data are represented as the mean \pm SD from three independent
1045 suspensions, each indicated by a dot. Statistical analysis between mutant and WT strains was
1046 performed using a two-way ANOVA test with Šidák's post hoc multiple comparison
1047 correction. ****, $p \leq 0.0001$. (C, F) Fraction of the WT and $\Delta namA$ (C) or $\Delta secA2$ (F)
1048 populations showing single- or double-labeling of CW (WGA) and exposed plasma membrane
1049 (anti-*Lm*CWD) by fluorescence microscopy, at the indicated timepoints. Data are represented as
1050 stacked bars (one for each labelling group) with mean \pm SD from three independent
1051 suspensions. Statistical analysis between mutant and WT strains was performed for each
1052 labelling group using a two-way ANOVA test with Tukey's post hoc multiple comparison
1053 correction. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. (G) Phase-contrast and
1054 fluorescence micrographs of WT, $\Delta namA$ and $\Delta secA2$ *Lm* sampled from mineral water
1055 suspensions (10^8 cells/mL) at the indicated timepoints. Bacteria were fixed and fluorescently

1056 labelled for visualization of DNA (Hoechst 33342), CW (WGA) and exposed plasma
1057 membrane (anti-CWD *Lm*). Scale bar: 2 μ m.

1058

1059 **Fig. 7. Chicken embryo passage restores culturability and virulence to VBNC *Lm*.**

1060 (A) Phase-contrast and fluorescence micrographs of GFP-expressing *Lm* EGDe before (WT)
1061 and after incubation in mineral water and passage through embryonated chicken eggs (clones
1062 V-E1 and VC-E2). Bacteria were fixed and fluorescently labelled for visualization of DNA
1063 (Hoechst 33342) and CW (WGA). Scale bar: 2 μ m. (B) Intracellular replication of WT and V-
1064 E1 and VC-E2 *Lm* in human epithelial JEG-3 and HepG2 cell lines. At each timepoint,
1065 intracellular bacteria were determined by enumeration of CFUs coming from the plating of
1066 epithelial cell lysates. Data are represented as mean \pm SD from two independent infection
1067 assays. (C) Low-magnification fluorescence micrographs of JEG-3 cell monolayers infected
1068 with WT, VE-1 and VC-E2 strains showing the cell-to-cell spread of intracellular bacteria
1069 between 6h (isolated clusters of infected cells) and 72h post-infection (generalized infection of
1070 the cell monolayer). Cells were fixed and fluorescently labelled for visualization of bacteria
1071 (anti-*Lm*) and F-actin (phalloidin). Bacteria-derived fluorescence signal was digitally enhanced
1072 for clarity purposes. Scale bar: 50 μ m. (D) Fluorescence micrographs of WT, VE-1 and VC-
1073 E2 *Lm* in the cytoplasm of infected JEG-3 cells at 6h post-infection. Cytosolic bacteria
1074 polymerize host actin to form comet-like tail structures that promote bacterial motility inside
1075 cells and subsequent intercellular spread. Infected cells were fixed and fluorescently labelled
1076 for visualization of DNA (Hoechst 33342), bacteria (anti-*Lm*) and F-actin (phalloidin). Bacteria
1077 highlighted by white inset squares are shown enlarged in right-side panels (nuclei are not
1078 shown for better visualization of bacteria and actin tails). Scale bars: 10 μ m (left-side panels),
1079 2 μ m (right-side panels).

1080

1081

1082 **Extended Data figure legends**

1083

1084 **Extended Data Fig. 1. Quantification of the viable cell populations by flow cytometry.**

1085 (A) Schematic diagram of the flow cytometry protocol (detection parameters and gating)
1086 applied for enumeration of viable bacterial cells in mineral water suspensions using the CFDA
1087 and Live/Dead viability assays. (B) Representation of the data depicted in Fig. 1A, showing an
1088 alternative quantification of viable cell population using the Live/Dead assay. The area covered
1089 with vertical grey dashes indicates the VBNC cell population.

1090

1091 **Extended Data Fig. 2. Coccoid cell forms derive from rod-shaped *Lm*.**

1092 Phase-contrast and fluorescence micrographs of GFP-expressing *Lm* EGDe sampled from
1093 mineral water suspensions (10^8 cells/mL) at the indicated timepoints. Scale bar: 2 μ m.

1094

1095 **Extended Data Fig. 3. A polyclonal antiserum raised against CWD VBNC *Lm* labels
1096 specifically bacteria with externally exposed membrane.**

1097 Phase-contrast and fluorescence micrographs of *Lm* EGDe sampled from mineral water
1098 suspensions at the indicated timepoints. Bacteria were fixed and fluorescently labelled for

1099 visualization of DNA (Hoechst 33342) and CW (WGA), and to assess the specificity of the
1100 rabbit polyclonal antiserum raised against CWD *Lm* (anti-CWD *Lm*). Scale bar: 2 μ m.

1101

1102 **Extended Data Fig. 4. Various *Lm* strains are able to reach a CWD VBNC state.**

1103 (A–D) Total, viable and culturable cell profiles of mineral water suspensions of *Lm* strains
1104 EGDe (A), 10403S (B), CLIP 63713 (C) and JF5203 (D). Culturable bacteria were determined
1105 by enumeration of CFUs, while total and viable bacteria were quantified by flow cytometry.
1106 Data are represented as the mean \pm SD from three independent suspensions. The area covered
1107 with vertical grey dashes indicates the VBNC cell population. (E–H) Fraction of the EGDe
1108 (E), 10403S (F), CLIP 63713 (G) and JF5203 (H) populations consisting of VBNC cells
1109 formed in mineral water. Data are represented as the mean \pm SD from three independent
1110 suspensions, each indicated by a dot. (I–P) Fraction of the EGDe (I, M), 10403S (J, N),
1111 CLIP 63713 (K, O) and JF5203 (L, P) populations showing CW (I–L) or exposed membrane
1112 (M–P) labelling by fluorescence microscopy, at the indicated timepoints. Data are represented
1113 as the mean \pm SD from three independent suspensions, each indicated by a dot. (Q) Phase-
1114 contrast and fluorescence micrographs of EGDe, 10403S, CLIP 63713 and JF5203 sampled
1115 from mineral water suspensions at the indicated timepoints. Bacteria were fixed and
1116 fluorescently labelled for visualization of DNA (Hoechst 33342), CW (anti-*Lm*) and exposed
1117 plasma membrane (anti-CWD *Lm*). Scale bar: 2 μ m.

1118

1119 **Extended Data Fig. 5. Changes in the fatty acid composition of the *Lm* plasma membrane
1120 during incubation in mineral water.**

1121 The fatty acid (FA) composition of the plasma membrane of *Lm* EGDe sampled from mineral
1122 water suspensions at the indicated timepoints was determined by gas chromatography-mass
1123 spectrometry (GC–MS/MS). Chain length and saturation of identified FA species is indicated
1124 in the x-axis by C#:\$, where # corresponds to the number of carbons and \$ the number of
1125 double bonds in the chain. Branched-chain FA species are indicated by the prefixes i- (iso) and
1126 a- (anteiso). Data are represented as the mean (bar) \pm SD from three independent suspensions,
1127 each indicated by a dot. Statistical analysis was performed using a two-way ANOVA test with
1128 Dunnett's post hoc multiple comparison correction. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****,
1129 $p \leq 0.0001$.

1130

1131 **Extended Data Fig. 6. Extended functional enrichment analysis of differentially regulated
1132 genes.**

1133 (A–F) Downregulated (A–C) and upregulated (D–F) *Lm* EGDe genes analyzed for statistically
1134 overrepresented gene ontology molecular function (A, D) and cellular component (GOCC)
1135 terms (B, E), and KEGG pathways (C, F). Terms and pathways are ranked from top to bottom
1136 by increasing p-value and decreasing fraction of differentially regulated genes per
1137 category/pathway.

1138

1139 **Extended Data Fig. 7. The *Lm* autolysins p60, Rpf1/2, Ami and Auto are not required for
1140 CW loss and VBNC state transition.**

1141 (A, D, G, J) Total, viable and culturable cell profiles of mineral water suspensions of wild type
1142 (WT) and isogenic *Lm* mutant strains lacking p60 (A), Rpf1 and Rpf2 (D), Ami (G), or Auto

1143 (J) autolysins. Culturable bacteria were determined by enumeration of CFUs, while total and
1144 viable bacteria were quantified by flow cytometry. Data are represented as the mean \pm SD from
1145 three independent suspensions. **(B, E, H, K)** Fraction of the WT and $\Delta p60$ (B), $\Delta rpf1-2$ (E),
1146 Δami (H) or Δaut (K) populations consisting of VBNC cells formed in mineral water. Data are
1147 represented as the mean (bar) \pm SD from three independent suspensions, each indicated by a
1148 dot. Statistical analysis between mutant and WT strains was performed using a two-way
1149 ANOVA test with Šidák's post hoc multiple comparison correction. ****, $p \leq 0.0001$. **(C, F, I,**
1150 **L)** Fraction of the WT and $\Delta p60$ (C), $\Delta rpf1-2$ (F), Δami (I) or Δaut (L) populations showing
1151 either one or both CW and exposed plasma membrane labelling by fluorescence microscopy,
1152 at the indicated timepoints. Data are represented as stacked bars (one for each labelling group)
1153 with mean \pm SD from three independent suspensions. Statistical analysis between mutant and
1154 WT strains was performed for each labelling group using a two-way ANOVA test with Tukey's
1155 post hoc multiple comparison correction. *, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.
1156

1157 **Supplementary Table 1. Composition of mineral water used in this study.**

1158

Component	mg/L
Bicarbonates (HCO ₃ ⁻)	74
Calcium (Ca ²⁺)	12
Chlorides (Cl ⁻)	15
Magnesium (Mg ²⁺)	8
Nitrates (NO ₃ ⁻)	7.3
Potassium (K ⁺)	6
Silica (SiO ₂)	32
Sodium (Na ⁺)	12
Sulfates (SO ₄ ²⁻)	9
Total dry residue (180 °C)	130

1159

1160 **Supplementary Table 6. VBNC *Lm* revert back to a culturable state after passage in**
1161 **embryonated chicken eggs.**

1162

Inoculum	Culturability before egg passage ⁽¹⁾	Culturability after egg passage ⁽²⁾			
		Embryonated eggs	Fisher's exact test (p-value) ⁽³⁾	Non-embryonated eggs	Fisher's exact test (p-value) ⁽⁴⁾
Mineral water	0/3 (0%)	0/10 (0%)	>0.999	0/10 (0%)	>0.999
VBNC <i>Lm</i>	8/84 (9.52%)	24/24 (100%)	1.63×10^{-17}	0/18 (0%)	0.344
Vegetative <i>Lm</i>	3/3 (100%)	8/8 (100%)	>0.999	2/2 (100%)	>0.999

⁽¹⁾ Number of BHI wells with bacterial growth / number of BHI wells inoculated

⁽²⁾ Number of eggs with bacterial growth / number of eggs inoculated

⁽³⁾ Comparison of culturability before egg passage and after passage in embryonated eggs

⁽⁴⁾ Comparison of culturability before egg passage and after passage in non-embryonated eggs

1163

1165 **Supplementary Table 7. Bacteria used in this study.**

1166

Species and strain	Relevant characteristics	Number	Source
<i>Listeria monocytogenes</i>			
EGDe	Wild type; CC9, ST35, sv 1/2a	BUG1600	ATCC BAA-679
EGDe-GFP	Constitutive GFP expression from chromosome-integrated plasmid, Cm ^R	BUG2538	(Balestrino et al., 2010)
EGDe-GFP (VE-1)	VBNC state revertant clone isolated after passage in chicken embryo	HBSC605	This work
EGDe-GFP (VC-E2)	VBNC state revertant clone isolated after passage in chicken embryo	HBSC606	This work
EGDe Δ ami	Deletion mutant for <i>ami</i>	DC432	D. Cabanes bacterial collection
EGDe Δ aut	Deletion mutant for <i>aut</i> , Km ^R	DC18	(Cabanes et al., 2004)
EGDe Δ namA	Deletion mutant for <i>namA</i>		P. Cossart bacterial collection
EGDe Δ secA2	Deletion mutant for <i>secA2</i>		P. Cossart bacterial collection
EGDe Δ sigB	Deletion mutant for <i>sigB</i>	BUG2215	(Mandin et al., 2007)
10403S	Wild type, Sm ^R ; CC7, ST85, sv 1/2a		(Bishop & Hinrichs, 1987)
10403S Δ p60	Deletion mutant for <i>p60</i> , Sm ^R	DP-L4611	(Lenz et al., 2003)
10403S Δ rpf1-2	Deletion mutant for <i>rpf1</i> and <i>rpf2</i> , Sm ^R	DP-L5789	(Witte et al., 2013)
10403S Δ relAPQ	Deletion mutant for <i>relA</i> , <i>relP</i> and <i>relQ</i> , Sm ^R	DP-L6294	(Whiteley et al., 2015)
CLIP 63713	Human materno-placental listeriosis isolate (France 1995); CC4, ST4, sv 4b	BUG1559	(Jonquières et al., 1998)
JF5203	Bovine rhombencephalitis isolate (Switzerland 2007); CC1, ST1, sv 4b		(Aguilar-Bultet et al., 2018)
<i>Listeria innocua</i>			
INRA 86	sv 4ab		(Van Langendonck et al., 1998)
<i>Listeria ivanovii</i> ssp. <i>ivanovii</i>			
Li 1979 ^T	Sheep isolate (Bulgaria); sv 5		ATCC 19119
<i>Listeria marthii</i>			
FSL S4-120 ^T	Forest soil isolate (USA 2010)		ATCC BAA-1595
<i>Listeria seeligeri</i>			
CHUT 861167	Unpasteurized milk isolate (France); sv 6b		(Van Langendonck et al., 1998)
<i>Listeria welshimeri</i>			
CHUT 860477	Vegetation isolate (USA); sv 6a		(Van Langendonck et al., 1998)

ATCC, American Type Culture Collection; CLIP, Collection de *Listeria* de l'Institut Pasteur; CHUT, Centre Hospitalier Universitaire de Tours; CC, clonal complex; ST, sequence type; sv, serovar; T, type strain. Antibiotic resistance: chloramphenicol (Cm^R), kanamycin (Km^R), streptomycin (Sm^R).

1167

1168 **References**

1169

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Fig. 1

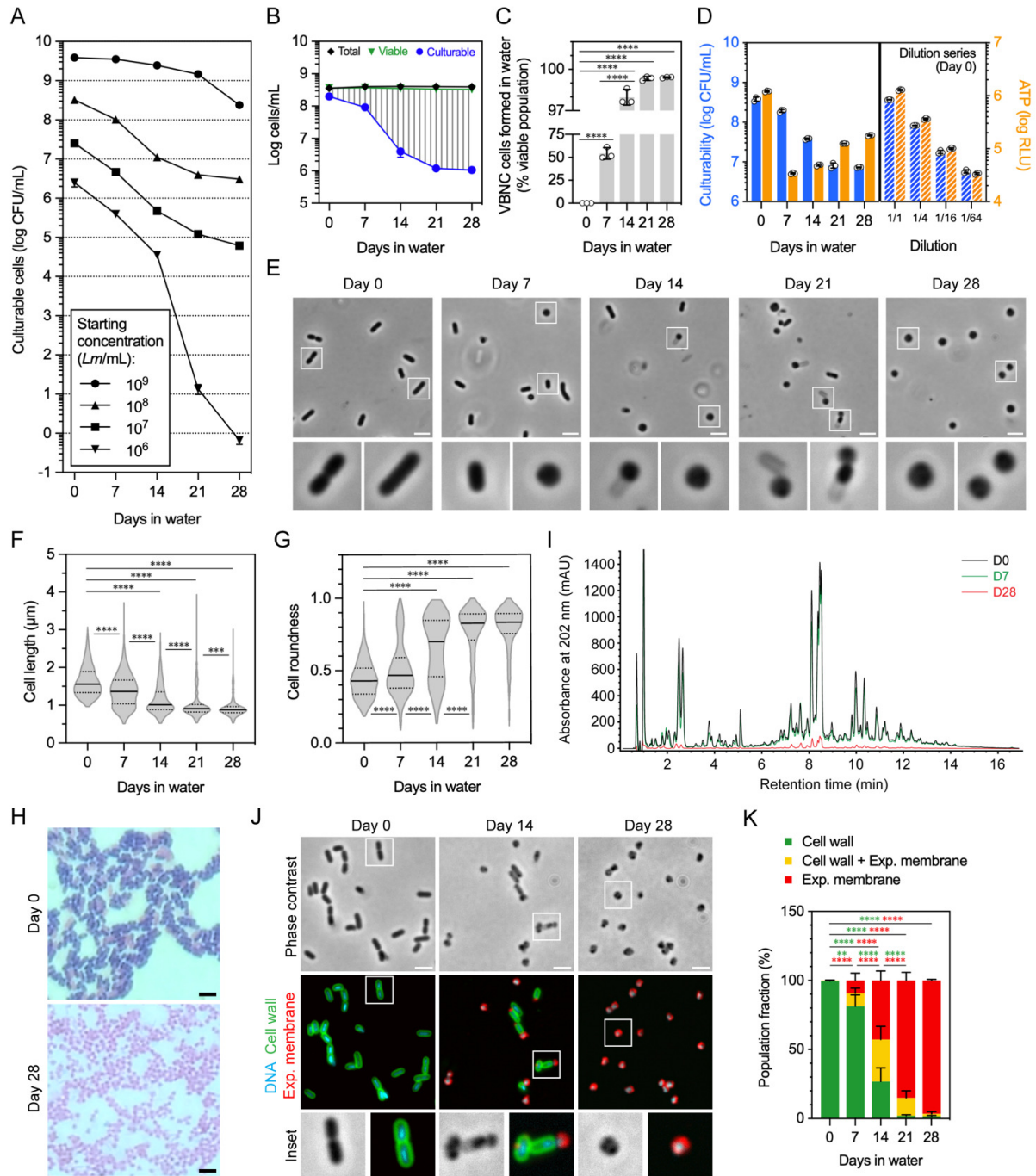


Fig. 2

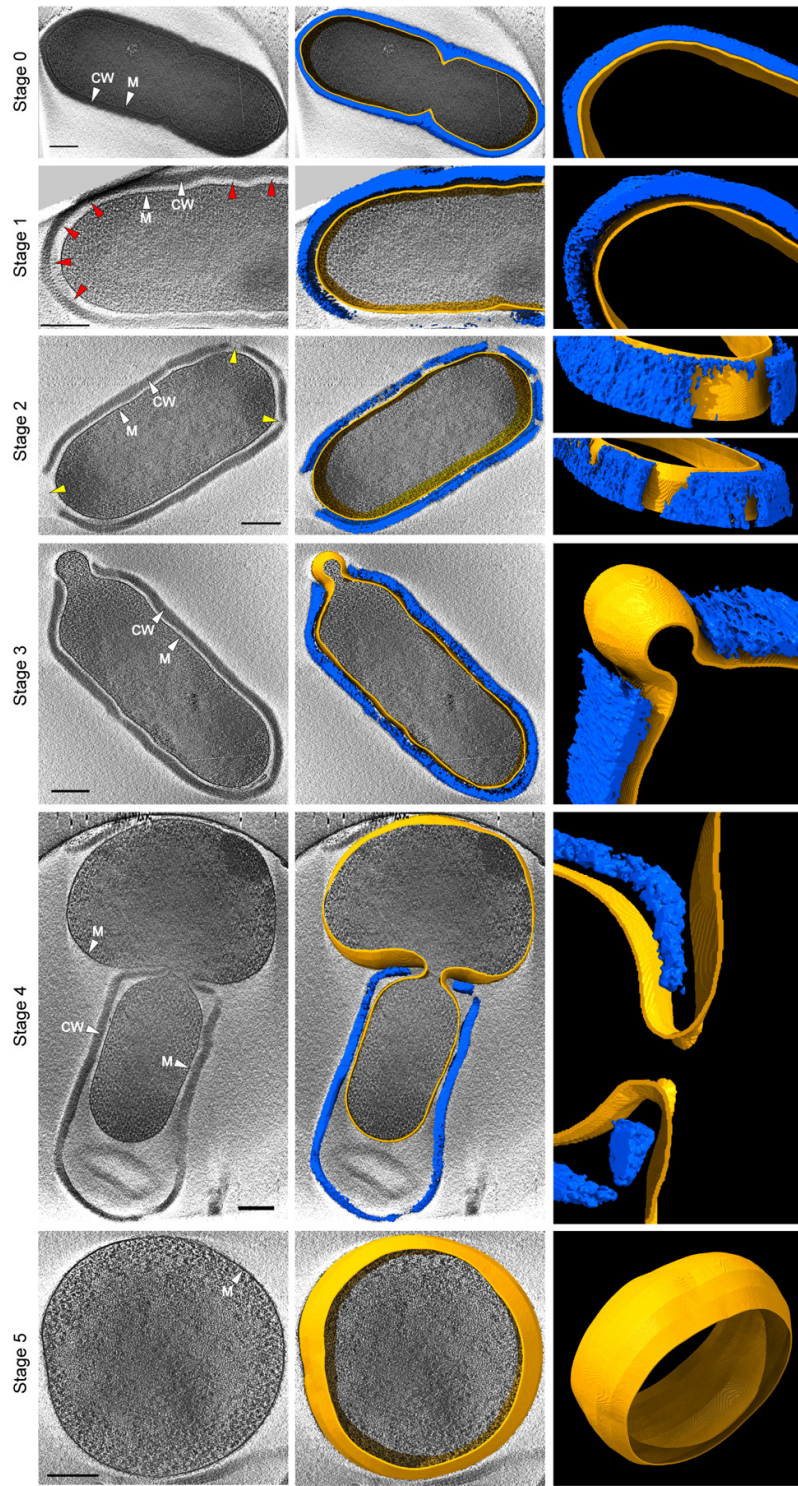


Fig. 3

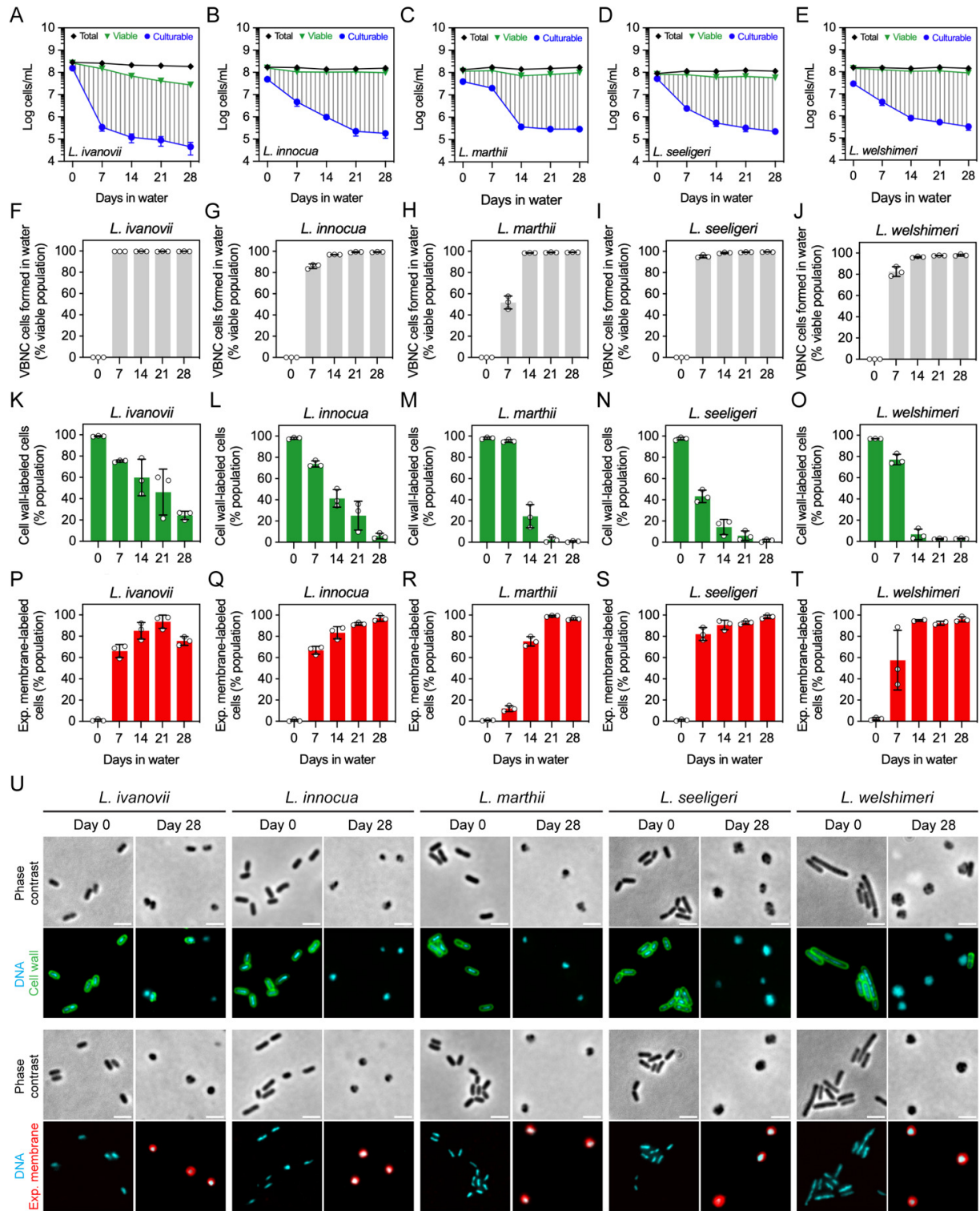


Fig. 4

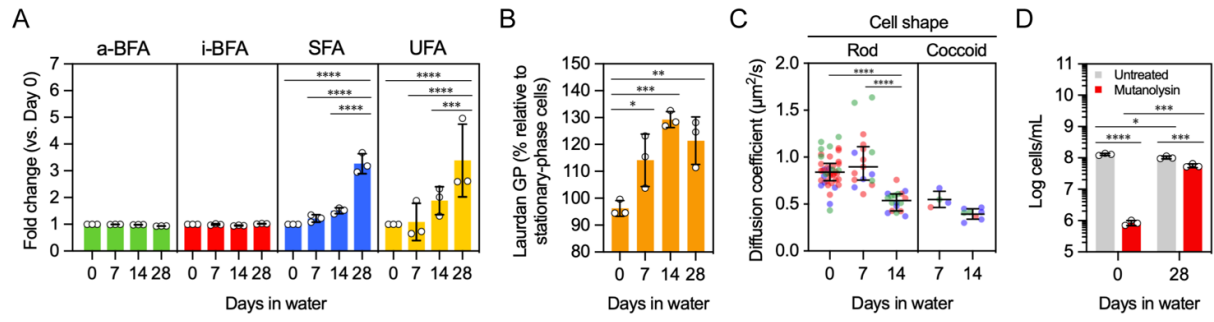


Fig. 5

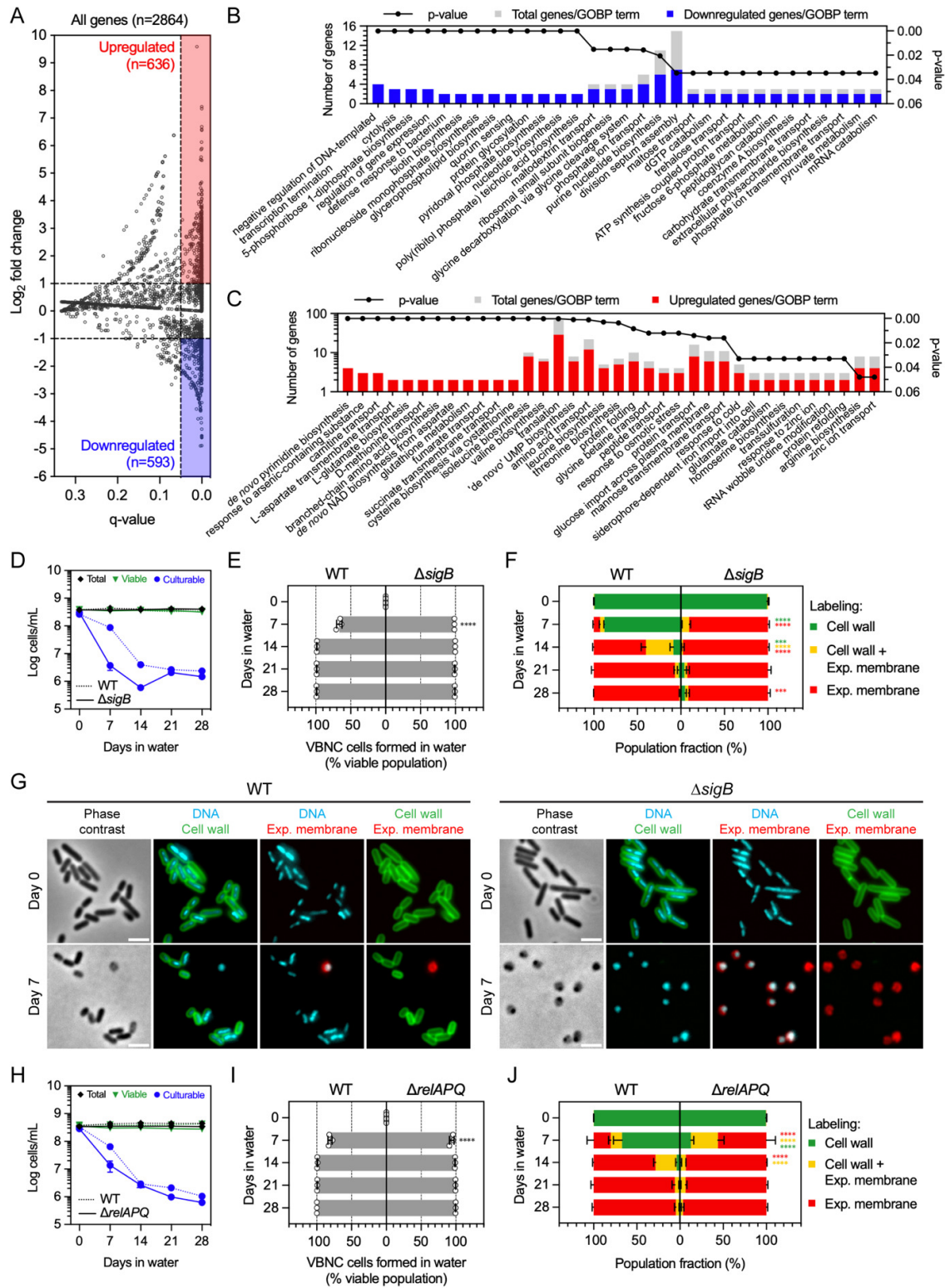


Fig. 6

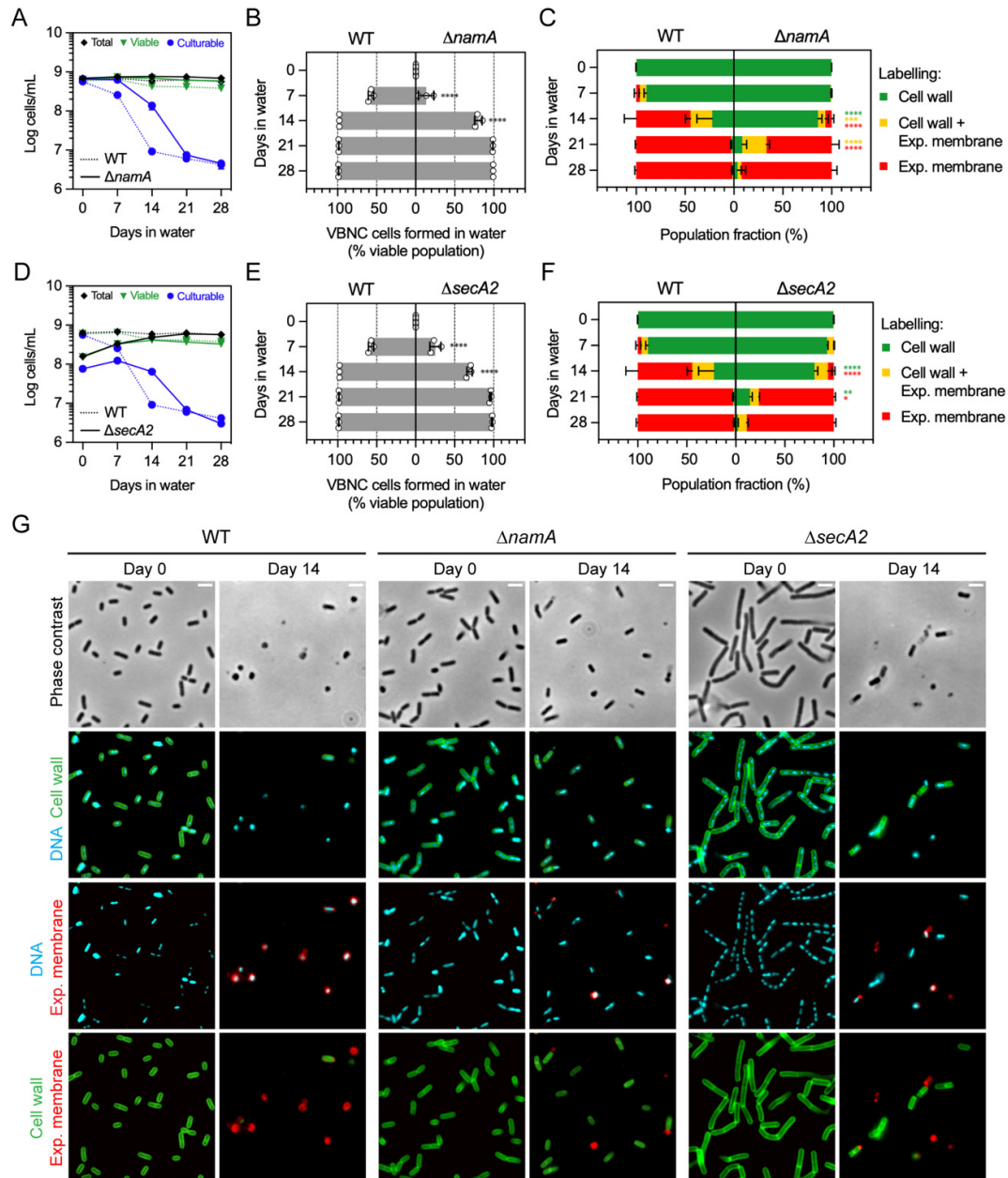
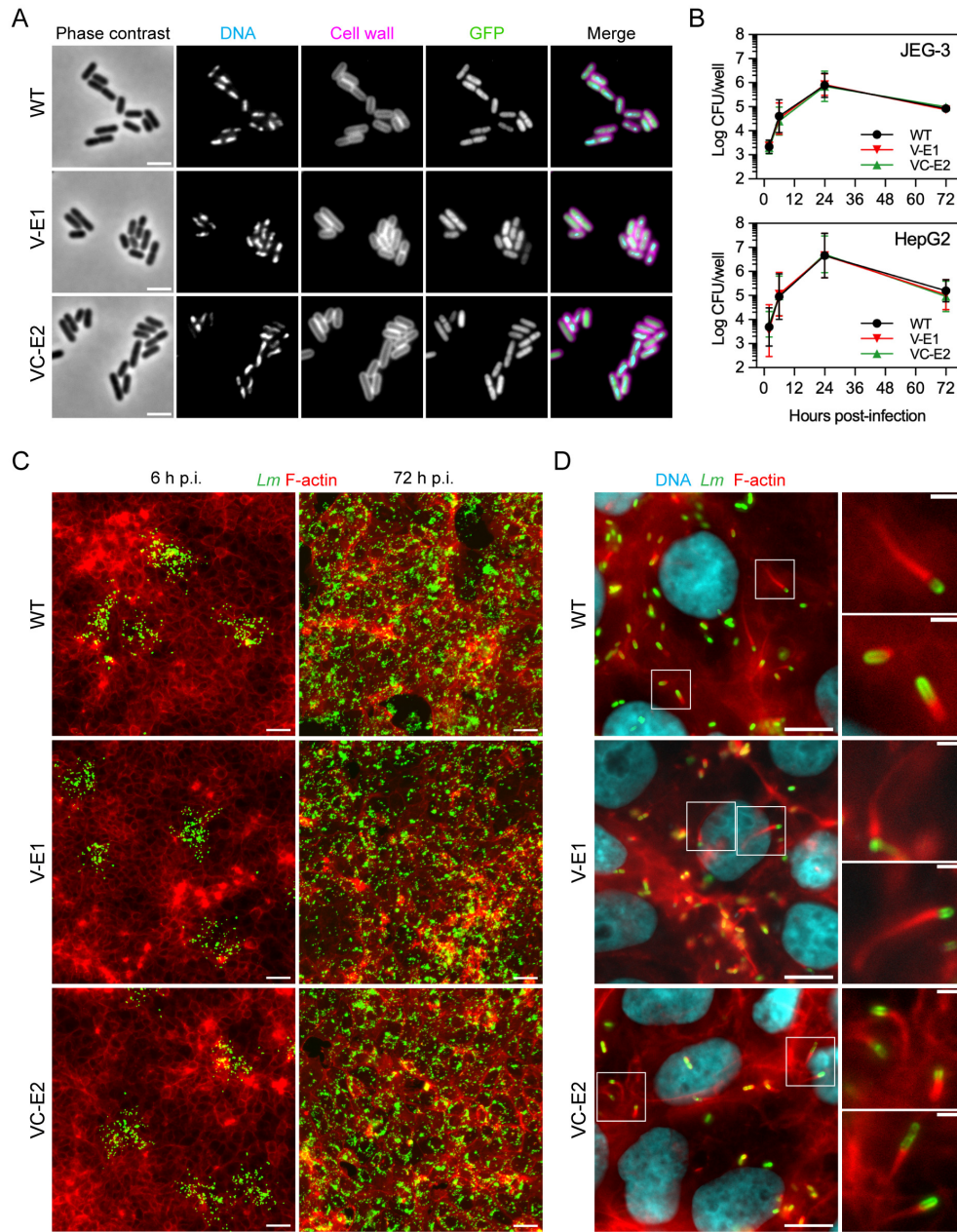
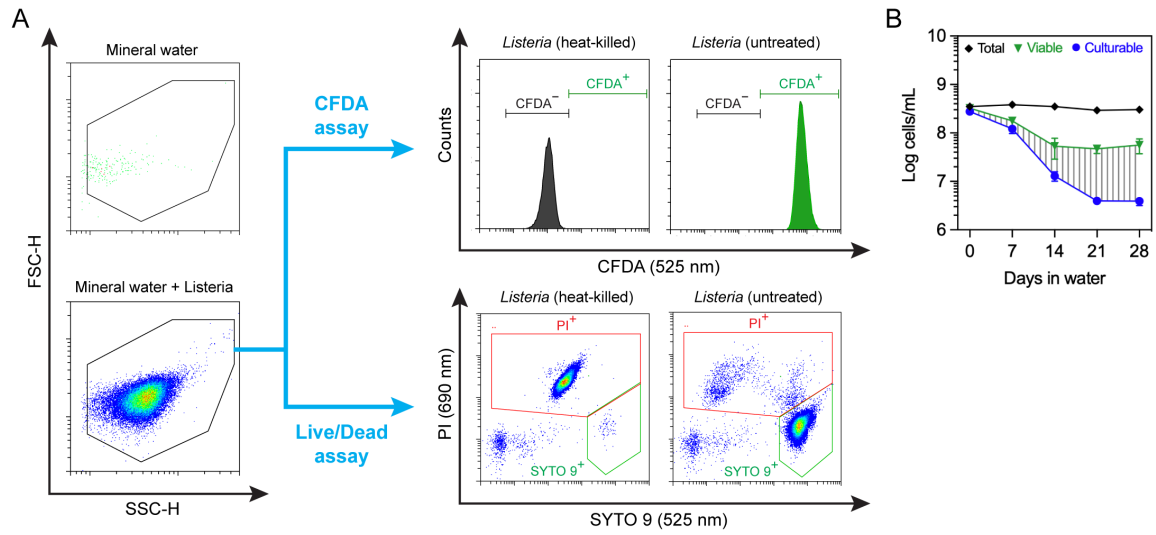


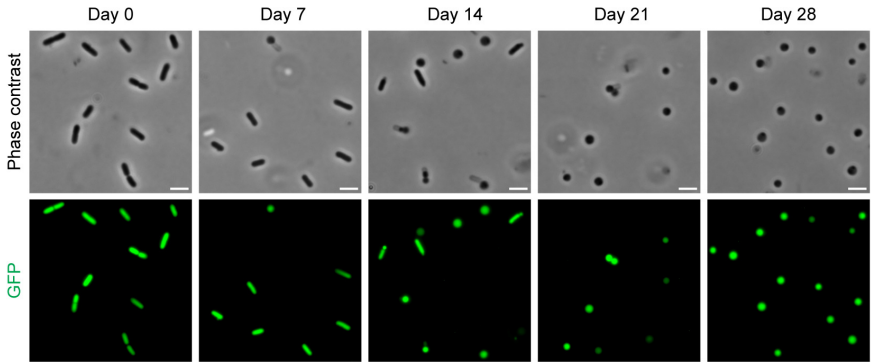
Fig. 7



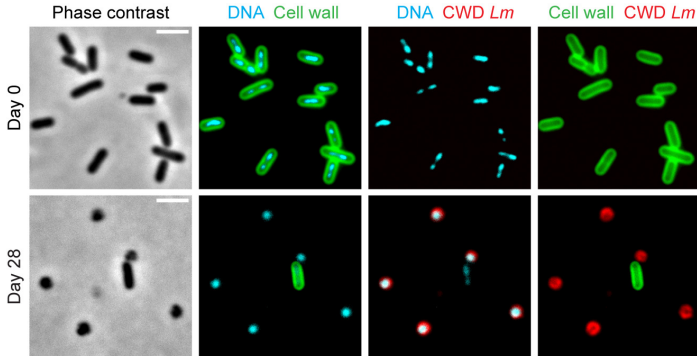
Extended Data Fig. 1



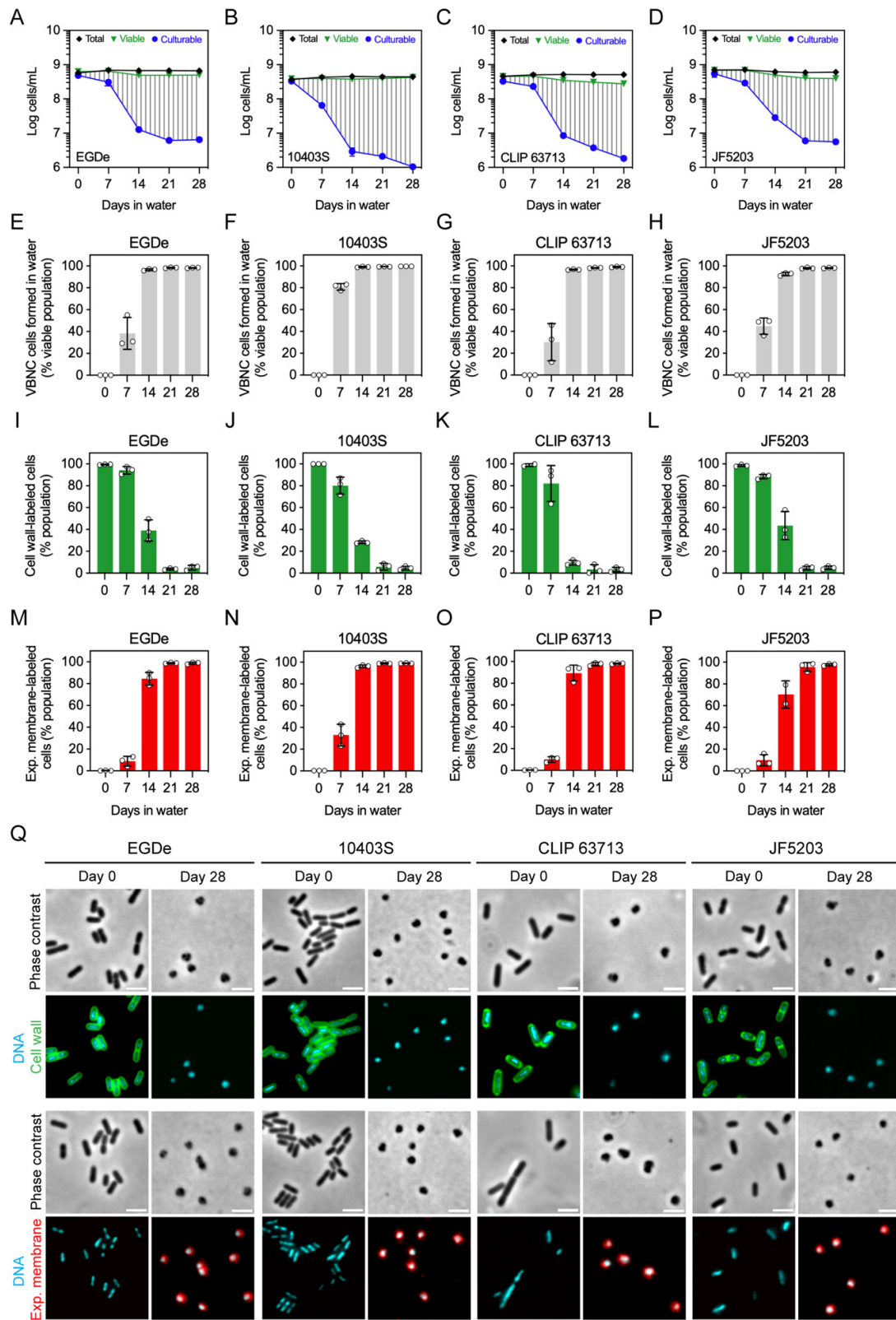
Extended Data Fig. 2



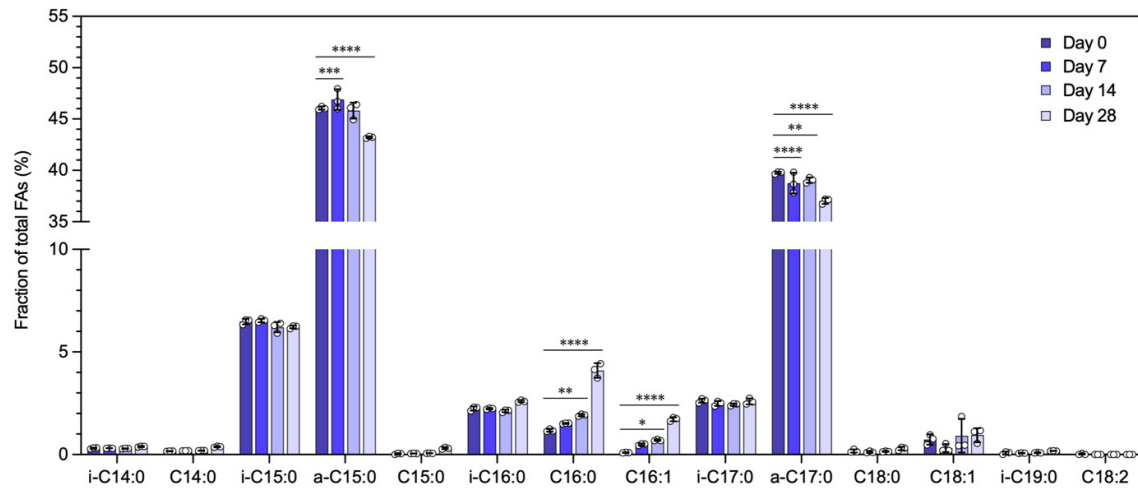
Extended Data Fig. 3



Extended Data Fig. 4

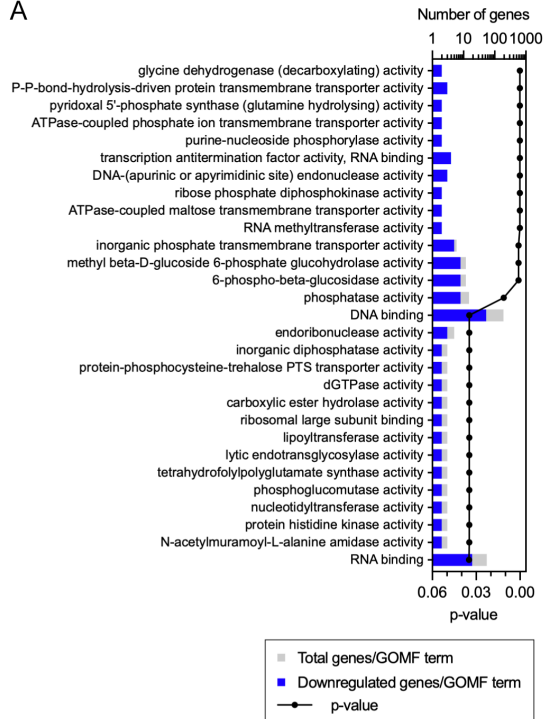


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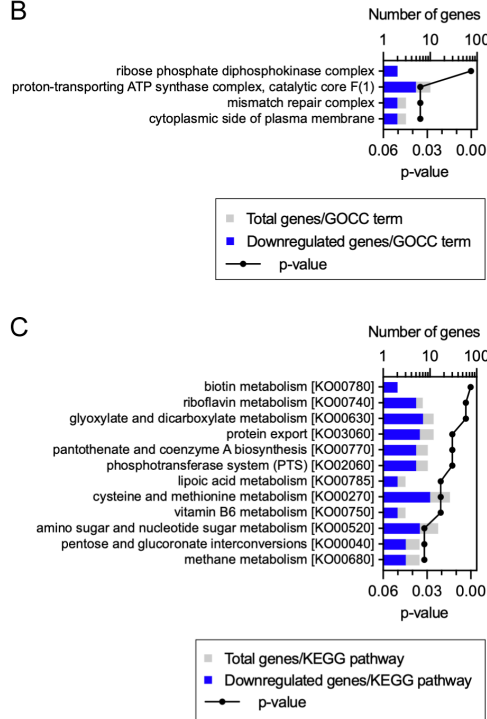


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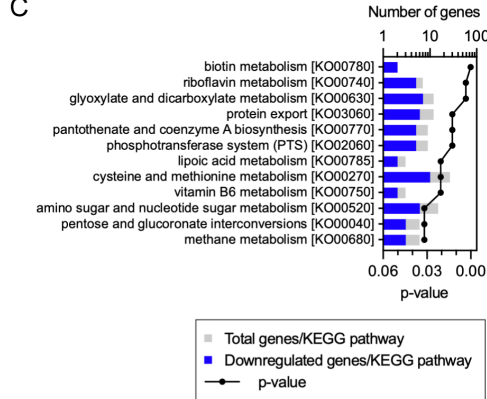
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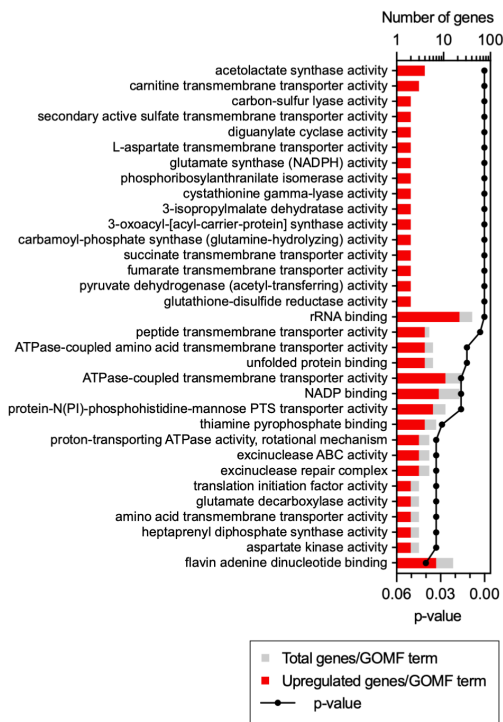
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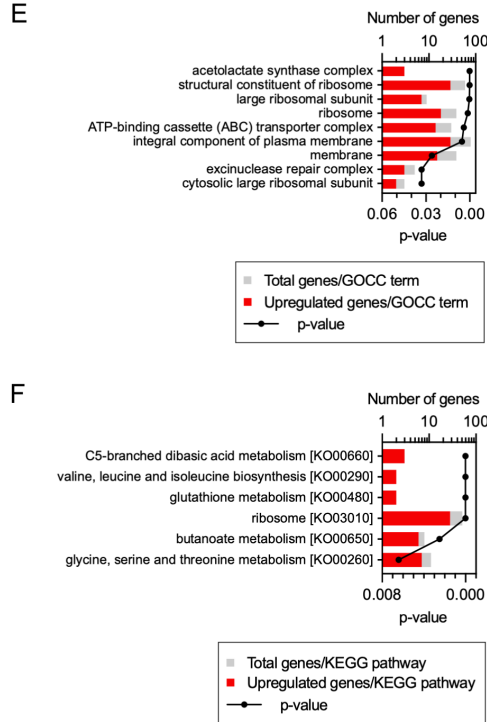
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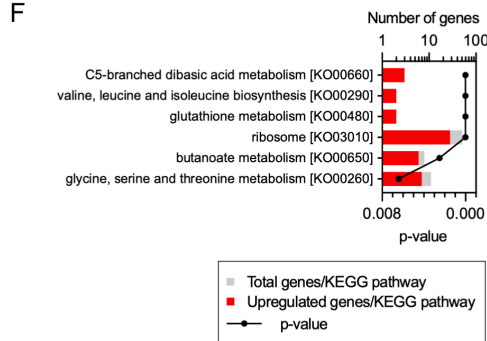
D



E



F



Extended Data Fig. 7

