LuxS/AI-2 signaling in *Salmonella* Typhimurium regulates *phoP/phoQ* to protect against acidic pH for enhanced survival in macrophages

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Abstract

Salmonella quorum sensing involves producing and detecting Autoinducer-2 (AI-2) signaling molecules. Our research reveals that *Salmonella* Typhimurium enhances AI-2 biosynthesis and transport under acidic conditions, aiding in environmental adaptation and facilitating pathogenesis in macrophages. AI-2 signaling regulates the pH-sensing two-component system genes, *phoP/phoQ*, ensuring cytosolic pH homeostasis, survival, and acid tolerance. It also involves regulating the Lysine/Cadaverine-mediated acid tolerance response and maintaining bacterial cytosolic pH. In this study, we show that LsrR protein, via its Y25 and R43 amino acids, apart from the *lsr* promoter, binds to the *phoP* promoter to negatively modulate the expression of *phoP*. This signaling ameliorates intracellular survival by modulating *Salmonella* Pathogenicity Island-2 (SPI-2) regulators (*ssrA/ssrB*) and SPI-2 effector expression via PhoP. Mouse models demonstrate that AI-2 signaling is essential for colonizing primary and secondary infection sites, showcasing its critical role in pathogenesis and low pH survival mechanisms in *Salmonella*.

Keywords: Autoinducer-2, acidic pH, macrophage, PhoP/PhoQ, *Salmonella* pathogenicity island-2

38 **Introduction**

39 *Salmonella* is considered a major food-borne pathogen worldwide (1) which
40 gains access to the host through contaminated food and water. The bacteria
41 stealthily survive the acidic pH of the stomach and the hostile environment
42 of the host macrophages. Within the host cells, *Salmonella* resides in the
43 *Salmonella*-containing vacuole (SCV), a membrane-bound compartment
44 that bestows with an acidic niche and utilizes the SPI-2 encoded genes for
45 its intracellular proliferation (2-4).

46 The community behaviour of bacteria, known as quorum sensing, protects
47 them from various stress factors such as exposure to ultraviolet light, acids,
48 detergents, or antimicrobial agents (5, 6). Also, AI-2 signaling is linked to
49 bacterial survival and pathogenesis, as in *Lactobacillus sp.,* where AI-2
50 helps in survival under acidic conditions (7) (8). While, *Salmonella*
51 recognizes a chemically distinct quorum sensing signal called autoinducer
52 2 (AI-2), synthesized by the enzyme LuxS (9) which it maximally produces
53 during its exponential growth phase(10). As bacterial quorum increases,
54 extracellular AI-2 accumulates and, upon reaching a threshold, is sensed by
55 LsrB protein in *Salmonella*. Then AI-2 is transported into the cytosol by an
56 ABC transporter (LsrA, LsrB, LsrC, and LsrD) encoded by the *lsr* operon and
57 is phosphorylated by the LsrK kinase. This phosphorylated AI-2 binds to the

58 LsrR repressor protein, relieving LsrR's repression on the *lsr* operon and
59 enabling further AI-2 import (11) (12).

60 The LuxS/AI-2 signaling regulates the expression of genes related to oxidative
61 stress responses such as *sodA*, *sodCI*, and *sodCII* , which play an essential
62 role in survival of *Salmonella* within macrophages (13). Further, the
63 inactivation of LsrR by AI-2 is essential for SPI-1 transcription and flagella
64 expression in *Salmonella* (14) (15). However, the molecular basis of this
65 remains unclear, not only because of the diverse and distinct survival
66 strategies of *Salmonella* but also due to the limited understanding of the AI-
67 2 regulatory mechanism and its role in enhancing pathogenesis, beyond the
68 *lsr* operon.

69 Counteracting and conquering the acidic pH of the stomach and acidic
70 intravacuolar environment is an indispensable survival strategy for
71 *Salmonella*. We contemplated that, the pH sensing and tolerance
72 responses might be controlled by the interbacterial communication and
73 signaling systems. Here, we show that LuxS/AI-2 signaling regulates a two-
74 component system *phoP*/*phoQ* to activate the acid tolerance system in
75 *Salmonella* and tune the SPI-2 encoded genes, enhancing its survival in *in*
76 *vitro* and *in vivo* models. We found that LsrR, the regulator of LuxS/AI-2

signaling, binds to the *phoP/phoQ* promoter via its Y25 and R43 amino acid residues, apart from its known regulation of the *lsr* operon.

Results

LuxS/AI-2 quorum sensing is required by *Salmonella* Typhimurium to survive in the macrophages.

We first investigated the AI-2 levels at different growth phases from STM WT grown at 37℃ in LB media. Consistent with previous studies, AI-2 dependent light production by *V. harveyi* BB-170 (which produces luminescence responses to AI-2) is maximum at the mid-log phase and gradually reduces till the late stationary phase **(Fig.1A, Extended Data Fig.1A)** (15). This gradual reduction of AI-2 may be due to the intracellular accumulation of AI-2 in *Salmonella*. Since the LuxS/AI-2 system is a well-researched class of quorum sensing, we were curious to know how *Salmonella* Typhimurium tunes the expression of the *lsr* operon and AI-2 production genes and observed that the *lsr* operon genes(*lsrB*, *lsrK*, *lsrR*) are upregulated during the mid-log phase to the early stationary phase, while the *luxS* expression was unchanged throughout the growth phases *in vitro* **(Fig.1 B, C)**.

The deletion of *luxS* and *lsr* operon genes (*lsrK*, *lsrB*, *lsrR*) in STM did not affect the *in vitro* growth in LB broth and minimal media. **(Extended Data**

Fig.1B,C). Next, we observed that in comparison to the STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK* displayed increased phagocytosis in RAW 264.7 macrophages **(Fig.1D)**. At the same time, STM *ΔlsrR* behaved as STM WT. Interestingly, quorum-sensing mutants STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* showed a decreased ability to proliferate in RAW264.7 cells **(Fig .1E)**. Immunofluorescence study validated the compromised proliferation of the mutants **(Extended Data Fig. 1E,F)**.

Further, we noted that all the *lsr* and the *luxS* genes showed a 1.5–2 fold upregulation post 6 h of infection into RAW 264.7 macrophages till 16 h **(Fig.1F,G**). These results indicate that *Salmonella* Typhimurium uses LuxS/AI-2 signaling for intracellular survival in the macrophages. We corroborated our results in primary macrophages derived from the peritoneal lavage of C57BL/6J mice. Similarly, the mutants *luxS, lsrB*, and *lsrK* exhibited an increased uptake by macrophages, while their proliferation was reduced in primary macrophages **(Fig.1H,I)**. Furthermore, we checked AI-2 activity upon STM WT, STM Δ*luxS*, and STM Δ*lsrB* infection into RAW 264.7 cells and observed the AI-2 activity till 16h post-infection **(Extended Data Fig.1D)**. These findings imply that *Salmonella* Typhimurium requires LuxS/AI-2 signaling to survive in the hostile environment of macrophages.

115 **STM Δ*luxS* acquires AI-2 from STM WT during its *in vitro* growth but may**
116 **not within its intravacuolar niche**

117 To find out whether STM Δ*luxS* can use AI-2 molecules from STM WT; we co-
118 infected RAW 264.7 macrophages with STM WT and STM Δ*luxS* (1:1 ratio).
119 We observed that STM WT outcompetes STM Δ*luxS* when both strains infect
120 together **(Fig.2A)**. However, when STM WT and STM Δ*luxS* were cocultured
121 in LB media prior to infection in RAW 264.7 macrophages, STM WT and STM
122 Δ*luxS* similarly proliferated in the macrophages **(Fig.2B)**. To figure out
123 whether STM Δ*luxS* can utilize AI-2 molecule produced by STM WT during *in*
124 *vitro* conditions, we treated STM Δ*luxS* with the STM WT spent media and
125 exogenous DPD molecule. Upon infection into RAW 264.7 macrophages
126 with these treated strains, we found that STM Δ*luxS* can use AI-2 and
127 synthetic DPD molecules under *in vitro* conditions, and STM Δ*luxS* survival
128 in the intracellular environment of RAW 264.7 macrophages is recovered
129 similarly to STM WT **(Fig. 2C,D, Extended Data Fig. 2A,B,C,D).** To further
130 validate, we used a commercially available inhibitor (Z)-4- bromo-5-(
131 bromomethyllene)-3-methylfran-2(5H) (BF-8) of LuxS/AI-2 signaling(16)
132 (17)at different concentrations with STM WT and observed that BF-8 did not
133 affect STM WT growth *in vitro*, while it attenuated its survival in RAW 264.7
134 macrophages **(Extended Data Fig.2E, Fig. 2E, F)**. We also confirmed the

135 light production and inhibition upon synthetic DPD and the inhibitor BF-8
136 treatments, respectively, by autoinducer assay **(Extended Data Fig. 2F,G).**
137 Also, results upon infection into peritoneal macrophages suggested that
138 exogenous DPD enhanced STM Δ*luxS* survival while BF-8 attenuated STM
139 WT proliferation **(Extended Data Fig.2H,I).** These findings imply that
140 *Salmonella* Typhimurium requires LuxS/AI-2 signaling to survive in
141 macrophages, and signaling inhibition attenuates the infection.

142 **The loss of a functional AI-2 or its receptor-mediated sensing attenuates**
143 ***Salmonella* survival in acidic pH.**

144 Quorum sensing pathways monitor changes in the density of the bacterial
145 population and control the expression of genes involved in critical cellular
146 processes, such as survival and pathogenicity(18). The mechanisms
147 underlying bacterial quorum and pH sensing have been thoroughly
148 investigated, primarily as two distinct and unconnected gene regulatory
149 systems. Our study shows that environmental acidification is the critical
150 physiological signal that upregulates AI-2 production by *Salmonella*
151 **(Fig.2G).** Hence, we hypothesize that LuxS/AI-2 signaling coordinates with
152 the pH sensing system of *Salmonella* to regulate survival in an acidic
153 environment. First, we assessed the *luxS* and *lsr* operon gene expression in
154 F-media (pH 5 mimics the acidic vacuolar condition) and found that the

155 mRNA expression of *luxS* is 1.5-fold increased at the mid-log phase to the
156 late-log phase **(Extended Data Fig.3A),** and *lsrB*, *lsrK* and *lsrR* mRNA
157 expression were similarly upregulated at the mid-log phase during their *in*
158 *vitro* growth in the acidic F-media **(Extended Data Fig.3B)**. Moreover, we
159 observed that STM stably produces AI-2 even during its growth in F-media at
160 pH 5 **(Fig.2G).** Our result showed that the light production was higher at the
161 mid-log phase to the late-log phase in the case of STM WT spent media,
162 while STM *ΔluxS* showed no light production. As expected, in the case of STM
163 *ΔlsrB* spent media, luminescence production continued because there was
164 no accumulation of AI-2 inside the STM *ΔlsrB*.
165 To determine whether communication between *Salmonella* by AI-2
166 regulates their survival in acidic environments, we performed an acid
167 survival assay with a pH range of 3 to 8 and noted that STM WT, STM *ΔluxS*,
168 STM *ΔlsrB*, STM *ΔlsrK*, and STM *ΔlsrR* survive similarly at pH 6 to pH 8
169 **(Extended Data Fig. 3C)**. However, at pH 3, pH 4, and pH 5, STM WT survives
170 significantly better than STM *ΔluxS*, STM *ΔlsrB* and STM *ΔlsrK*. **(Fig. 2 H-J,**
171 **Extended Data Fig.3 D-G)**. Here, we can conclude that LuxS/AI-2 signaling
172 helps *Salmonella* to communicate and regulate survival strategies in acidic
173 environments under *in vitro* conditions.

174 **LuxS/AI-2 system tunes *phoP/phoQ* expression, thereby assisting in pH**
175 **sensing and adaptation in *Salmonella***

176 Jesudhasan et al.,2010 determined that there is a differential gene
177 expression between the STM WT and STM Δ*luxS* (19) pertaining to virulence
178 genes, flagellar genes, and motility genes. A well-known two-component
179 system (TCS) known as Phop/PhoQ is essential in mediating the acid
180 tolerance response in several Gram-negative bacteria, including *Salmonella*
181 (20) (21). Thus, we conjectured that LuxS/AI-2 signaling modulates the
182 *phoP/phoQ* gene expression and thereby aids in the survival of *Salmonella*
183 under an acidic environment. The LuxS/AI-2 signaling cascade involves the
184 accumulation of the exogenous AI-2(DPD molecules) within the bacterial
185 cytosol and also phosphorylation by the LsrK kinase enzyme **(Fig.3 A)**. The
186 intriguing question is whether this phosphorylation of AI-2 is essential to
187 regulate the expression of the downstream gene targets apart from the *lsr*
188 operon. To answer this, we assessed the mRNA expression of the *phoP* gene
189 in STM WT, STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* during the growth in *in vitro*
190 LB media. We observed that *phoP* expression was downregulated in STM
191 *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* compared to STM WT **(Fig. 3 B, Extended**
192 **Data Fig. 4 A)**. Interestingly, this was rescued when *STM ΔluxS* was grown in
193 STM WT spent media**(Fig. 3C)**. We further validated the LuxS/AI-2 mediated

194 *phoP* gene regulation in F- media and found similar observation **(Extended**
195 **Data Fig. 4 B, D).** Next, upon infection of RAW 264.7 macrophages with STM
196 *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK*, the mRNA expression of *phoP* and *phoQ*
197 genes were down-regulated compared to STM WT **(Fig. 4 D, E, Extended**
198 **Data Fig. 4 C)**. Considering these findings, we conclude that AI-2 sensing
199 and the signaling cascade orchestrate the *phoP/phoQ* expression in
200 *Salmonella* Typhimurium within macrophages.
201 We further hypothesized that this AI-2 signaling might also control the acid
202 tolerance system in *Salmonella*. In comparison to STM *ΔluxS*, STM *ΔlsrB*,
203 and STM *ΔlsrK*, pH 5 adapted cultures of STM WT and STM *ΔlsrR* were more
204 resilient and showed better survival upon exposure to pH 3 **(Fig. 3 F)**. Hence,
205 prior adaptation to pH 5 potentiates the acid tolerance response only in STM
206 WT and STM *ΔlsrR*. As the STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* exhibit
207 diminished ability to mount an acid-tolerance response, we determined the
208 intracellular pH of all the strains. Upon determination of the intracellular pH
209 of *Salmonella* by using a pH-sensitive pHuji plasmid, we observed that all
210 the *Salmonella* strains maintained their cytosolic pH in the neutral LB media
211 (pH 7). However, when grown in acidic F-media (pH 5), STM WT and STM
212 *ΔlsrR* retain their cytosolic pH within the near-neutral range with a pH of 6.2
213 and 6.1, respectively while STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* are unable

214 to sustain their intracellular pH in the neutral range and drops to pH 5.1, 5.2,
215 and 5.0, respectively **(Fig. 3G, Extended Data Fig. 4 E-I)**. Simultaneously,
216 upon infection of all these mutant strains in RAW 264.7 macrophages, STM
217 WT and STM *ΔlsrR* maintained their cytosolic pH near 6.4 (±0.2). At the same
218 time, STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* maintained cytosolic pH around
219 5.6, 5.4, and 5.2 (±0.2) **(Fig.3 G)**.
220 The cadC/BA system regulated by the PhoP/PhoQ two-component system
221 functions to increase the pH both inside and outside, thereby promoting
222 bacterial survival in mildly acidic environments and aiding in pH
223 homeostasis (22). CadB encodes a lysine/cadaverine antiporter, CadC
224 encodes a transcriptional regulator, and CadA encodes a lysine
225 decarboxylase(23). Our mRNA expression study indicates that *cadC*, *cadB*,
226 and *cadA* expression in STM Δ*phoP* is diminished upon infection in RAW
227 264.7 macrophages compared to STM WT **(Extended Data Fig.5A-C)**. Thus,
228 we have quantified the cadaverine/ lysine ratio in response to acidic pH, and
229 noted that the cadaverine/lysine ratio was low in acidic pH 5 in STM *ΔluxS*,
230 STM *ΔlsrB*, and STM *ΔlsrK* compared to STM WT **(Fig.3H).** CadA, lysine
231 decarboxylase uses H^+ and lysine to synthesize cadaverine and export
232 cadaverine out of cells to maintain the bacterial cytosolic pH(22). Moreover,
233 we observed that *cadA*, *cadB*, and *cadC* gene expression was

234 downregulated at 2h, 6h, and 16h post-infection into RAW 264.7 cells in the
235 case of the mutants compared to STM WT **(Fig.3J,K,L).** Together, these
236 results conclude that LuxS/AI-2 signaling regulates ATR and maintains the
237 cytosolic pH of *Salmonella* via regulating *cadC/AB* operon.

238 **LuxS/AI-2 signaling regulates *phoP/phoQ* expression through LsrR**
239 **interaction with the *phoP* promoter in *Salmonella***

240 We explored whether LsrR, the downstream regulator of LuxS/AI-2 signaling
241 pathway, could directly interact with the *phoP* promoter to influence its
242 activity. AlphaFold3 structural models revealed that LsrR interacts with
243 both the *phoP* and *lsr* promoters (24). The interactions were mediated by
244 residues Y25, T31, Q32, S33, R43, L44, K45, S47, and R48, all within a
245 distance of <5 Å and a predicted aligned error (PAE) of <14 across multiple
246 replicates **(Fig.4A,B Extended Data Fig.6A,B)**. These residues are located in
247 the N-terminal domain, spanning positions 25-48, between alpha helices 1,
248 2, and 3. Notably, Y25, T31, and R43 contributed the most significant
249 interactions, with R43 from helix 3 displaying the highest number of contact
250 points(25). A covariance analysis of the multiple sequence alignment
251 revealed that R43 most frequently co-occurs with Y25, an interacting
252 residue located in helix 1, with the highest probability among all analyzed
253 residues (26). Given the strong interactions of R43 and Y25 with the *lsr* and

254 *phoP* promoters, we generated Y25A, R43A, and Y25A/R43A mutants and
255 used AlphaFold3 to predict structural impacts. The AlphaFold structures
256 indicated that the mutations severely disrupted promoter interactions, with
257 PAE values exceeding 18 and pLDDT scores for all the DNA-contacting
258 residues dropping below 30. Additionally, we performed NPT ensemble
259 molecular dynamics simulations for the mutant protein-DNA complex,
260 which revealed a significant weakening of interactions. We validated our
261 findings *in vitro* through electrophoretic mobility shift assays (EMSA) using
262 purified wild-type LsrR and its Y25A, R43A, and Y25A/R43A mutants to
263 assess their binding to HPLC-purified *lsr* and *phoP* promoters **(Fig.4D,4E,**
264 **Extended Data Fig.6C,D)**. WT LsrR bound both promoters, Y25A showed
265 reduced binding, R43A further reduced it, and the double mutant lost nearly
266 all binding at 200 mM NaCl **(Extended Data Fig.7A,B)**. A similar result was
267 observed for the interaction of LsrR with a single-stranded promoter
268 sequence **(Extended Data Fig.7D,E,F,G),** and random DNA sequence did
269 not show any interaction (**Extended Data Fig.7E)**. To assess regulatory
270 effects during infection, we generate a luxS/lsrR double knockout STM and
271 complemented with *lsrR* and mutant *lsrR*. While the double knockout strain
272 proliferated similarly to WT STM, *lsrR* complementation reduced
273 proliferation. The Y25A/R43A mutant restored survival to knockout levels

274 **(Fig.4F,G)**, indicating LsrR's binding to *lsr* and *phoP* promoters acts as a
275 negative regulator of *Salmonella* pathogenicity.

276 **AI-2 orchestrates the *Salmonella* Pathogenicity Island-2 gene**
277 **expression by sensing low pH**

278 PhoP has been shown to control the two-component system SsrA/SsrB at
279 the transcription level, which are the master regulators of the SPI-2 genes
280 (27). We also validated that *ssrA* and *ssrB* gene expression were
281 downregulated in STM Δ*phoP* upon infection into RAW 264.7 cells **(Extended**
282 **Data Fig.8A,B)**. Thus, we hypothesized that LuxS/AI-2 signaling might
283 control the two-component SsrB/SsrA system via PhoP/PhoQ. Upon
284 infection into RAW 264.7 macrophages, the mRNA expression of *ssrB* was
285 downregulated from initial stage post-infection to late stage post-infection
286 in macrophages **(Fig.5A)**. On the other hand, *ssrA* was downregulated at a
287 late stage post-infection **(Fig.5B)**. Furthermore, we observed expression of
288 *ssaV* a Type 3 secretion system (T3SS) needle complex protein, encoded by
289 SPI-2 and *spiC* an SPI-2 encoded effector molecule were downregulated in
290 the absence of *luxS, lsrB,* and *lsrK* genes in STM upon infection into RAW
291 264.7 macrophages **(Fig.5 C, D).**

292 So far, our findings convey that the LuxS/AI-2 signaling regulates *phoP/phoQ*
293 genes and, thereby, the SPI-2 cluster in *Salmonella*. However, whether AI-2

294 directly or the AI-2-PhoP/PhoQ axis controls the SPI-2 genes remains
295 unclear. Thus, we cloned the *phoP*/*phoQ* operon under a non-native
296 promoter in the pQE60 plasmid and transformed it into STM WT and
297 knockout strains. We confirmed the *phoP* mRNA overexpression level in
298 STM WT:pQE60-*phoP*/*phoQ* and STM *ΔluxS:*pQE60-*phoP*/*phoQ* upon
299 infection in RAW 264.7 **(Extended Data Fig.8C,D)**. Using these transformed
300 *Salmonella* strains, we performed an acid survival assay at pH 3, pH 4, and
301 pH 5 and observed STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* with a *phoP*/*phoQ*
302 cloned under non-native promoter survived similarly to STM WT **(Fig.5.E-G)**.
303 Furthermore, the STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* with cloned
304 *phoP*/*phoQ* showed enhanced ATR response **(Fig.5H)**. Thus, when
305 *phoP/phoQ* is not under their native promoter, the regulation by LuxS/AI-2 is
306 undetected. These results further validated the binding of LsrR protein to the
307 promoter of *phoP*. Also, *ssrB* and *ssrA* expression are significantly higher in
308 *phoP*/*phoQ* complemented knockout strains compared to STM *ΔluxS*, STM
309 *ΔlsrB*, and STM *ΔlsrK* strain **(Fig.5I-K, Extended Data Fig.7E-G)**.
310 Cumulatively, these results suggest that indeed, the LuxS/AI-2 signalling
311 controls ATR by regulating the *phoP/phoQ* genes.

312 **The absence of AI-2 mediated signaling compromises the *in vivo***
313 **colonization of *Salmonella* in mice**

314 AI-2, being a universal quorum sensing molecule, is an excellent choice to
315 mediate interactions between different bacterial species in the mammalian
316 gut, where hundreds of bacterial species live and interact. It has already
317 been determined that several gut-associated bacteria encode LuxS (the AI-
318 2 synthase) or generate AI-2 (28). Thus, we aimed to understand the role of
319 LuxS/AI-2 signaling in regulating *Salmonella* pathogenesis in *in vivo* mice
320 models.
321 We infected C57BL/6J mice by orally gavaging with STM WT and knockout
322 strains at a CFU of 10^7 per mouse **(Fig. 6A**). We observed that STM *ΔluxS*, STM
323 *ΔlsrB*, and STM *ΔlsrK* showed a significantly lower organ burden in the
324 intestine, MLN, spleen, and liver and lower bacteremia upon oral gavage
325 **(Fig.6B–F)**. Oral gavage mimics the physiological route of *Salmonella*
326 infection into its host and *Salmonella* is required to be able to breach the
327 intestinal barrier successfully. The lower organ colonization in STM *ΔluxS*,
328 STM *ΔlsrB*, and STM *ΔlsrK* can be explained by their inability to cross the gut
329 epithelial barrier. Next, we bypassed the gut epithelial barrier by infecting
330 C57BL/6 mice intraperitoneally and found that STM *ΔluxS,* STM *ΔlsrB, and*
331 STM *ΔlsrK* exhibited reduced colonization in the spleen and liver and less
332 dissemination in blood than STM WT **(Extended Data Fig.8A-D).**
333 Furthermore, upon infection of mice by oral gavaging at a CFU of 10^8 per

334 mouse, we noted that the mice infected with STM WT and STM *ΔlsrR*
335 succumbed to death as early as the 6th day of post-infection, while STM
336 *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* infected mice survived longer **(Fig.6G-H**
337 with less weight reduction**(Extended Data Fig.8E)**. We further observed that
338 the BF-8 inhibitor at 4 mg/kg reduced the STM WT colonization to different
339 sites of infection in C57BL/6 mice **(Fig.6I-N**) and improved the survival of
340 mice with delayed weight reduction compared to untreated mice **(Fig.6O,P**
341 **Extended Data Fig.9F).** Further, the histopathological sections show
342 significantly less liver tissue damage with BF-8 inhibitor treatment
343 (**Extended Data Fig.9G)**. Our results show that LuxS/AI-2 signaling is critical
344 for the *in vivo* pathogenesis and virulence of *Salmonella* Typhimurium.

345 **Discussion**

346 Every aspect of the bacterial lifestyle and pathogenesis relies on the
347 bacterial quorum and community attributes, like for *Salmonella*, the
348 infectious dose to cause the illness in humans is more than 10,000 CFU(29).
349 Autoinducers are chemical signal molecules that underpins bacterial
350 communication and differ amongst species, just like human languages
351 exist. Here, we report that AI-2 synthesis and *lsr* operon are concurrently
352 regulated under neutral and acidic conditions in *Salmonella,* aiding in its
353 survival. The phosphorylation of AI-2 by LsrK is crucial for its function (30)

354 and is validated by our study, where we observe that deletion of even *lsrK*
355 renders *Salmonella* incapable of surviving in macrophages and does not
356 upregulate *phoP* expression.
357 The gastrointestinal tract is a dynamic environment where microbes
358 compete for space and nutrients through various mechanisms. Many gut-
359 associated bacteria encode LuxS or produce AI-2, including a significant
360 proportion of Firmicutes and Proteobacteria, and some species of
361 Bacteroidetes and Actinobacteria(31). *Salmonella* might benefit from the AI-
362 2 produced by the gut commensal in the intestinal lumen. Our *in vivo* studies
363 justify it to some extent with compromised colonization of the primary and
364 secondary sites of infection upon abrogation of LuxS/AI-2 signaling in
365 *Salmonella*. Although the neighboring bacteria might produce AI-2 in the
366 intestinal niche, STM Δ*lsrB* and STM Δ*lsrK* cannot utilize AI-2 to mediate the
367 downstream signaling cascades. Thus, we underscore the critical function
368 of individual members of this cascade, the AI-2 synthesis enzyme, receptor,
369 and kinase enzyme. During intestinal colonization, a prime strategy of this
370 pathogen, is to activate the ATR systems to endure this harsh environment.
371 The four major regulators that control relevant stress responses in
372 *Salmonella* are RpoS, PhoPQ, Fur, and OmpR/EnvZ (32). We describe a
373 mechanism that AI-2 signaling tunes the expression of the two-component

374 system *phoP/Q*, which helps in ATR. Relating to intravacuolar survival,
375 studies show that the acidic pH of the vacuole is necessary for the assembly
376 of T3SS encoded by SPI-2, but not for the effector molecule's secretion (33).
377 Yet, it is essential to maintain the neutral pH of the bacterial cytoplasm to
378 secrete the effector molecule, which we found to be lost upon abrogation of
379 AI-2 signaling (34). Thus, *Salmonella* regulates its cytosolic pH in acidic
380 environments via the AI-2 signalling and tries to sustain its intracellular pH
381 near neutral to maintain the regular cellular functions and secretion of SPI-
382 2 effectors for its survival and proliferation within the macrophages.
383 Moreover, *Salmonella* AI-2 phosphorylation by LsrK is a key process to
384 control the downstream targets. While our study elucidates the mechanism
385 of acid tolerance and macrophage survival via AI-2 signaling in *Salmonella*,
386 it has some limitations. We cannot conclusively determine STM Δ*luxS*
387 utilizes AI-2 from STM WT in the coinfection study in RAW 264.7 cells **(Fig.**
388 **2A)**. Furthermore, the study does not confirm whether both bacterial strains
389 infect the same cell, as observations reflect a global phenotype. But, it might
390 be possible that STM *ΔluxS* cannot utilize AI-2 even when infecting the same
391 cell due to an inactive *lsr* operon.
392 We broaden the horizon of LsrR regulation of gene expression beyond the *lsr*
393 operon by unraveling the bind of LsrR to the *phoP* promoter via its Y25 and

394 R43 amino acid residues. Overall, we identify a novel regulatory network via
395 AI-2 and PhoP/PhoQ TCS, leading to an astonishing survival strategy for
396 *Salmonella* under acidic pH. Our findings highlight and open avenues to
397 target the AI-2 signaling, thereby abrogating PhoP/PhoQ mediated signaling
398 to limit *Salmonella* infection.

399 **Materials and Methods**

400 **Bacterial strains and Growth conditions**-

401 The wild-type *Salmonella enterica* serovar Typhimurium strain 14028S (STM
402 WT) used in all experiments of this study was a kind gift from Professor
403 Michael Hensel of the Max Von Pettenkofer-Institute for Hygiene und
404 Medizinische Mikrobiologie in Germany. The bacterial strains were revived
405 on LB agar with or without antibiotics. The LB broth culture of wild-type,
406 knockout, and complemented strains was cultured at 37˚C (170 rpm) in an
407 orbital shaker incubator. Antibiotics Kanamycin (50µg/ml),
408 Chloramphenicol (25µg/ml) and Ampicillin(50µg/ml) were used wherever
409 required. *Vibrio campbellii* ATTC BAA-1117 strain was used for autoinducer
410 Bioassay. This strain was grown in ATCC Medium: 2746 Autoinducer
411 Bioassay (AB) Medium.

412 A growth kinetics study was conducted in LB and M9 minimal media (35) (36)
413 (**Table S2**). Briefly, STM WT and mutants were inoculated in fresh LB broth

414 (1:100 ratio from overnight primary culture). The cultures were incubated at
415 37˚C (170 rpm) in an orbital shaker incubator. The OD at 600nm was
416 measured at regular intervals until 24 hours post-inoculation.

417 **Bacterial gene knockout and strain generation**

418 *Salmonella enterica* serovar Typhimurium strain 14028S and isogenic
419 mutants were used for all assays. *luxS*, *lsrB*, *lsrK,* and *lsrR* gene knockout
420 strains were made by λ-Red recombination system as described previously
421 in one gene step chromosomal gene inactivation method demonstrated by
422 Datsenko and Wanner (37). Briefly, STM WT strains were transformed with
423 pKD46 plasmid expressing λ-red recombinase system under Arabinose
424 inducible plasmid. The pKD3 and pKD4 plasmid were used as the template
425 to amplify the chloramphenicol (Chl) and kanamycin (Kan) resistance
426 cassette with Knockout primers **(Table S3).** The amplified reaction products
427 were purified using chloroform isopropanol precipitation. The purified PCR
428 product was transformed into pKD46- containing STM WT expressing λ-Red
429 recombinase system, which is required for homologous recombination. The
430 transformed cells were plated on LB agar with chloramphenicol and
431 kanamycin accordingly. The confirmation for knockout strains has been
432 done by using PCR with primers **(Table S3)** designed corresponding to the
433 ~100bp upstream and downstream of the genes for the knockout strains to

434 observe a difference in exchange gene size upon STM *ΔluxS,* STM *ΔlsrB,* STM
435 *ΔlsrK,* and STM *ΔlsrR* knockout generation.

436 For complement strain, the *luxS* gene was amplified with gene-specific
437 primers (containing BamH1 and HindIII restriction site sequence) **(Table S3)**
438 by colony PCR to generate complement strain. Empty pQE60 vector
439 backbone was subjected to BamH1(NEB) and HindIII (NEB) digestion in cut-
440 smart buffer (NEB) at 37°C for 1 hour. The double-digested insert and vector
441 were subjected to ligation by T4DNA ligase in ligation buffer (NEB) overnight
442 at 16°C. The respective ligated vector was transformed into STM Δ*luxS*
443 strains to generate the complemented strain. Complement strain was
444 initially confirmed by colony PCR with cloning primers and internal primers
445 of the genes and finally by restriction digestion of recombinant plasmid
446 isolated from complement strains to insert release.

447 **Cell culture and maintenance**

448 Murine macrophage cell line RAW 264.7 was maintained in Dulbecco's
449 Modified Eagle Medium (DMEM, Lonza), supplemented with 10% FBS
450 (Gibco) and 1% Penicillin-streptomycin (Sigma-Aldrich) in a humidified
451 incubator with 5% CO2 at 37 °C. The cells were seeded into the respective
452 cell culture plate for each experiment to conduct intracellular survival
453 assays and intracellular gene expression experiments.

454 **Peritoneal macrophages isolation**

455 The primary macrophage isolation was performed as previously described
456 (38). Briefly, C57BL/6J mice (5-6 weeks old) were injected with Brewer's thio-
457 glycolate medium (8% w/v, HIMEDIA). At four days of treatment, 5ml of cold
458 PBS was injected into the peritoneal cavity, followed by aspiration of the fluid
459 from the cavity and dispensed into the 15 ml centrifuge tube. The centrifuge
460 was done at 300g for 10 minutes, and the resuspended cell pellet in Roswell
461 Park Memorial Institute 1640 (RPMI 1640) was supplemented with 10% FBS
462 and an antibiotic cocktail (Penicillin-Streptomycin). Cells were counted by
463 hemocytometer and seeded onto 24 well cell culture plates.

464 **AI-2 bioassay**

465 The culture of *Salmonella* Typhimurium WT and mutants was obtained at 0h,
466 1h, 3h, 6h, 9h, and 12h, and centrifuged at 6000rpm for 10 minutes. After
467 being filtered using a 0.22 μm filter, the supernatant was stored at -20 °C
468 until needed again. Using the sensor strain *Vibrio campbellii* ATTC BAA-1117
469 (Strain designation *V. harveyi* BB-170, which only responds to AI-2), the AI-2
470 activity was determined. With minor modifications, the AI-2 bioassay was
471 performed as previously described (39) (5). A single colony of *V. harveyi* BB-
472 170 was incubated in 5 ml of Autoinducer Bioassay medium (ATCC Medium:
473 2746 AB Medium) at 30°C and 200 rpm. A 1: 1000 dilution was obtained by

474 transferring a 10 µl sensor strain culture to 10 mL of fresh AB media.
475 *Salmonella* cell-free culture supernatant (20 ul) was incubated with a 180
476 ml diluted sensor strain. The mixture was added to a white, flat-bottomed
477 96-well microtiter plate (Corning, USA), followed by shaking in a rotary
478 shaker at 200 rpm, at 30°C. Luminescence was measured post 3 h in a
479 fluorescent microtiter plate reader. Autoinducer signal calculated as –
480 (Luminescence produced by *Vibrio* in the presence of *Salmonella*
481 Typhimurium free supernatant -Luminescence in media control)/
482 Luminescence produced by *Vibrio* with sterile media. To validate the activity
483 of exogenous DPD molecule and BF-8 inhibitor, an autoinducer bioassay
484 was performed in the presence of DPD and BF-8 inhibitors(Cat# 53796,
485 purchased from Sigma).

486 **Gentamicin protection assay**

487 RAW 264.7 macrophages and peritoneal macrophages isolated from
488 C57BL/6J mice were seeded in tissue culture plates for infection. Cells were
489 infected with STM WT, STM Δ*luxS,* STM Δ*lsrB,* STM Δ*lsrK,* STM Δ*lsrR,* and
490 *STMΔluxS:luxS* (stationary phase culture growing overnight in LB broth). The
491 Multiplicity of Infection (MOI) of 10 was used for the intracellular survival
492 assay. MOI of 20 was used for the *Salmonella* Typhimurium gene expression
493 study (qRT-PCR). Following the infection of STM strains into the cell line, the

494 plate was spun for 5 minutes at 600 rpm using a Rota-Superspin R–NV swing
495 bucket centrifuge. After that, the plate was incubated for 25 minutes at 37
496 °C with 5% CO_2 in a humidified incubator. Then, the infected cells were
497 washed twice with 1X PBS and treated with a 100 µg/ mL concentration of
498 gentamycin. After 1 hour, the media were changed with a reduced
499 concentration of gentamycin (25 µg/ mL) and incubated until the desired
500 time point. The time points 2h, 6h, and 16h were taken for the qRT-PCR
501 samples and 2h and 16h for the intracellular survival assay.

502 Also, for studying the effect of exogenous DPD in the recovery of the
503 phenotype of mutant *luxS,* were grown *in vitro* with supplementation of 25
504 µM, 50 µM,100 µM, and 200µM DPD molecule and 10%, 20% , 30% and 50%
505 spent media of STM WT collected at 3h in LB growing. For inhibition, the
506 inhibitor (Z)-4- bromo-5-(bromomethyllene)-3-methylfran-2(5H) known as
507 BF-8 treatment, was given to STM WT in LB media. DPD or spent media
508 supplemented and BF-8 treated culture was used for gentamycin protection
509 assay.

510 **Intracellular survival assay and phagocytosis assay**

511 The cells were lysed with 0.1% triton-X 100 in PBS at specific time points
512 post-infection. For the intracellular survival assay, 2h and 16h post-infection
513 samples were collected and plated, and the corresponding CFU at 2h and

514 16h was determined. Fold proliferation was determined using the formula [
515 CFU at 16 hours]/ [CFU at 2 hours]. For phagocytosis, the bacterial number
516 was determined in the inoculum and 2h time point post-infection. Percent
517 phagocytosis was calculated by using the formula- [(CFU at 2 hours)/ (CFU
518 of pre-inoculum)]×100.

519 **Confocal microscopy**

520 To study the *Salmonella* proliferation and intracellular vacuolar status of
521 STM strains, all strains STM WT, STM Δ*luxS,* STM Δ*lsrB,* STM Δ*lsrK,* and STM
522 Δ*lsrR* were transformed with pFPV 25.1-mCherry-Amp^R plasmid. Briefly,
523 1×10^5 RAW264.7 cells were seeded on the coverslip and infected with
524 different strains of bacteria at MOI 20. After appropriate hours post-
525 infection, cells were washed twice with PBS and, fixed with 3.5%
526 paraformaldehyde (PFA), and incubated with a specific antibody (anti-
527 mouse LAMP-1) in a blocking buffer containing 2% BSA and 0.01% saponin
528 for 2 hours at RT or overnight at 4℃. The cells were washed twice with PBS
529 and incubated with appropriate secondary antibody tagged with
530 fluorochrome for 1 hour at RT. The coverslips were then mounted onto a
531 clean glass slide and imaged under a confocal microscope (Zeiss LSM-710)
532 using a 63X objective.

533 ***In vitro* Cell culture competition Assay**

534 RAW 264.7 macrophages were infected at a 1:1 ratio with STM WT and STM
535 Δ*luxS*. At 16h, post-infection cells were lysed, and the sample was collected
536 as described above. The competition was also done with the coculture of
537 STM WT and STM Δ*luxS*. This involved the co-cultivation of STM WT and STM
538 Δ*luxS* together, followed by infection in RAW264.7 macrophages. The
539 competitive index was calculated for a WT strain and Δ*luxS* by dividing the
540 ratio between CFU (STM Δ*luxS*) and CFU (STM WT) by the ratio of both strains
541 in the inoculum.

542 **RNA isolation and RT-qPCR**

543 For gene expression study in Luria Bertani (LB) media and F-media (Acidic
544 media mimics the cell vacuole environment) (40) (36), overnight-grown
545 culture in LB media was subjected to subcultured (at 1:100) in LB or F-media
546 (**Table S1**). At 3h, 6h,9h, and 12h, bacterial cell pellets were resuspended in
547 TRIzol (from TaKaRa, RNA isoPlus- 9109) and kept at -80˚C. Total RNA was
548 isolated by chloroform extraction followed by isopropanol precipitation. To
549 evaluate the quality, the amount of RNA was quantified in nanodrop(
550 ThermoFischer) and examined on a 2% agarose gel.
551 To produce cDNA, 2 µg of RNA sample was treated with DNase I at 37˚C for
552 1 hour (TaKaRa) followed by heating for 10 minutes at 65˚C. Then, DNA-free

553 RNA samples were used to make cDNA by using the PrimeScript RT reagent
554 Kit provided by TaKaRa (Cat# RR037A).
555 For gene expression of *Salmonella* Typhimurium upon infection in RAW
556 264.7 macrophages, infection was given at MOI 20. At 2h, 6h, and 16h post-
557 infection, the cells were lysed using TRIzol (from TaKaRa, RNA isoPlus- 9109)
558 and kept at -80˚C. Total RNA isolation and cDNA were synthesized as a
559 similar protocol described by the manufacturer, TRIzol (from TaKaRa, RNA
560 isoPlus-9109) and TaKaRa (Cat# RR037A) reagent according to the
561 manufacturer's protocol. The list of expressions Primer is provided in **Table**
562 **S3**.

563 **Acid survival assay**

564 Overnight culture of STM WT, STM Δ*luxS,* STM Δ*lsrB,* STM Δ*lsrK,* and STM
565 Δ*lsrR* was adjusted to OD_{600} 0.3. For survival in 1XPBS, pH was adjusted to
566 3,4,5,6,7,8 using concentrated HCl, 10^7 bacteria/ml of each strain of STM
567 was exposed to different pH as mentioned above in 1X PBS and incubated
568 for 2h. At 2 hours post-treatment, CFU was enumerated by plating on LB agar
569 media. The same protocol was followed for survival in LB media with the
570 similar pH ranges. For the strain of STM harboring plasmids pQE60
571 complemented with *phoP*/*phoQ*, a survival assay was done at pH of 3, 4, and
572 5 in 1X PBS.

573 **Acid tolerance Assay**

574 A standard Acid tolerance assay was performed as previously described
575 with slight modification (20). Briefly, Acid tolerance assay (ATR) was
576 conducted with strains grown overnight at 37°C in LB broth containing the
577 appropriate antibiotic. The culture corresponding to 0.3 OD_{600} of the
578 overnight culture (unadapted) was centrifuged and the pellet was
579 resuspended into 2 ml of LB broth (pH 5) and incubated at 37°C with shaking
580 for 2 h (known as adapted culture). The acid challenge of unadapted and
581 adapted cultures (0.3 OD_{600}) involved readjusting the pH to 3. CFU was
582 determined for adapted and unadapted cultures after exposure to pH 3 (2 h
583 of pH 3 treatment). ATR tolerance assay was also done with the strain of
584 *Salmonella* containing plasmid pQE60 complemented with *phoP/phoQ*
585 gene.

586 ***Salmonella* intracellular pH measurement by pHuji plasmid**

587 The plasmid pBAD:pHuji (Plasmid #61555) was used to transform STM WT,
588 STM Δ*luxS*, STM Δ*lsrB*, STM Δ*lsrK*, and STM Δ*lsrR*. pHuji is a pH-sensitive red
589 fluorescent protein demonstrating a more than 20-fold fluorescent intensity
590 change from pH 5.5 to 7.5 (41). Overnight-grown bacterial strains were
591 subjected to a pH range of 3.0 to 8.0 in phosphate buffer(PBS) in 40μM of
592 sodium benzoate to ensure equilibration to the desired pH. The resulting

593 fluorescence intensity ratio, obtained from flow cytometric analysis, was
594 plotted as a function of pH and fitted to get a standard curve. The standard
595 curve was used to interpolate ratios measured within the intracellular pH of
596 bacteria in LB media, F-media, and RAW 264.7 macrophages.

597 **Mass-spectrometry for determination of Lysine and cadaverine**

598 Overnight grown STM WT, STM Δ*luxS*, STM Δ*lsrK,* STM Δ*lsrB,* and STM Δ*lsrR*
599 were sub-cultured in acidic pH 5 Lb media and incubated at 37℃ until mid-
600 log phase. Thereafter, bacteria were spun down, and the spent media was
601 collected. Spent media were kept with an equal volume of acetone at -20℃
602 overnight. Next, samples were collected after centrifugation at 13k rpm for
603 10 minutes. Samples were analysed using the Orbitrap fusion instrument
604 connected to UHPLC Vanquish (Thermo Scientific). Water and acetonitrile
605 with 0.1% formic acid were used as a mobile phase. The flow rate was kept
606 at 0.3ml/min linear gradient was started with 5% to 95% using Hypersil Gold
607 C18 column (2.1X100mm & particle size 1.9µ).

608 **Plasmid construction and site-directed mutagenesis**

609 The vector pET28a(+) was used to construct plasmids p*lsrR* for the
610 overexpression of LsrR as a C-terminal 6xHis-tagged fusion protein. The
611 DNA fragment containing *the lsrR* coding region was amplified by
612 polymerase chain reaction (PCR) from the chromosome of *Salmonella*
613 Typhimurium 14028s using primers (F-lsrR and R-lsrR containing restriction

site NcoI and XhoI, respectively). The PCR products were digested with NcoI and XhoI and ligated with NcoI and XhoI digested pET28a(+). The inserts were checked by DNA sequencing.

For Y25A and R43A site-directed mutagenesis in LsrR protein, PCR for SDM was set up with template DNA from pETa(+)- *lsrR* and amplified with Q5 polymerase (NEB). The PCR products were digested with DpnI and transformed into *E. coli* DH5-alpha. Y25A and R43A double mutants were generated using pET28a(+) *lsrR* R43A plasmid as a template for SDM using primers specific to the second set of mutations. All plasmids were confirmed by DNA sequencing.

Overexpression and purification of LsrR

pET28a(+)-*lsrR* and its mutants containing C-terminal 6xHis tag were transformed into *E. coli* Rosetta (DE3). The transformed colonies were inoculated into 5 ml LB containing Kana and Chl and grown overnight. Inoculum (1%) was added to 1.2 L LB containing Kana and Chl, grown at 37°C to an OD600 of 0.4 under shaking, and thereafter supplemented with 0.1 mM IPTG, and grown at 16℃ overnight. Cells were harvested by centrifugation at 4℃, resuspended in 10 ml buffer A [20 mM Tris–HCl pH 8, M NaCl, 10% glycerol (v/v), 1.5 mM -mercaptoethanol, 1 mM PMSF) lysed by sonication, and centrifuged at 4℃ in a pre-cooled centrifuge at 10000 rpm

634 for 10 minutes. The supernatant was loaded onto a 1 ml Ni-NTA column
635 equilibrated with buffer A, washed with 20 ml buffer A, and eluted with a
636 gradient of imidazole (20–500 mM) in the same buffer. The fractions were
637 analysed on 12% SDS-PAGE. Fractions enriched for LsrR were pooled,
638 loaded onto a Superdex75 gel filtration column, and eluted in buffer B [20
639 mM Tris–HCl pH 8, 1M NaCl, 10% glycerol (v/v) and 1.5 mM 2-
640 mercaptoethanol]. The purity of LsrR was checked on 12% SDS-PAGE.
641 Fractions with apparent homogeneity were pooled, concentrated using a 10
642 kDa cut-off Centricon (Millipore), and estimated by Bradford's method using
643 bovine serum albumin (BSA) as standard. The proteins were dialyzed against
644 buffer A containing 50% glycerol (v/v) and stored at –20 ˚C.

645 **Gel Shift Assay**

646 HPLC-purified 90bp double or single-stranded DNA oligos of the *lsrR* and
647 *phoP* operon promoters were used as substrates for the EMSA
648 (Supplementary Table S3). Binding reactions involved incubation of
649 promoter DNA with varying concentrations of WT LsrR or the mutants. The
650 incubation buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 3 mM
651 magnesium acetate, 0.1 mM EDTA, and 0.1 mM DTT. The reaction mix were
652 incubated at room temperature for 15 minutes, added native loading buffer
653 to a final concentration of 1x (60% 0.25× TBE, 40% glycerol, 0.2%

654 bromophenol blue) and resolved on 6% native PAGE. The gel was stained for
655 DNA with SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen) and imaged for
656 analysis.

657 ***In vivo* animal experiment**

658 Oral gavavging of 10^7 CFU of STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK*, STM
659 *ΔlsrR*, and STM *ΔluxS:luxS* was used to infect 5- to 6-week-old C57BL/6J
660 mice. Post five days of infection, the intestine (Peyer's patches), MLN(
661 mesenteric lymph node), spleen, liver, and blood were aseptically extracted
662 (in a Biosafety level 2 cabinet) to examine the colonization in the organs. The
663 CFU was enumerated on differential and selective *Salmonella-Shigella* (SS)
664 agar.

665 **Mice survival assay**

666 Male C57BL/6, aged 5 to 6 weeks, were obtained from the Central Animal
667 Facility, IISc. The mice were given 10^8CFU of each strain (overnight grown
668 cultures) orally to compare the survival and weight alterations of mice post-
669 infection. Following infection, the mice were observed every day to
670 determine their survival and weight, and the results were expressed as a
671 percentage of survival (Kaplan-meier curve) and weight reduction.
672 All experiments comply with the rules set forth by the Indian Institute of
673 Science, Bangalore's IEAC. The approved protocol number is CAF/

674 Ethics/852/2021. The Institutional Animal Ethics Committee approved every
675 animal experiment, and the National Animal Care Guidelines were
676 scrupulously followed.

677 **Statistical analysis**

678 As mentioned in figure legends, each experiment has been independently
679 repeated two to five times. GraphPad Prism 8.4.3 was utilized for all
680 statistical analyses. As the figure legends state, the statistical analyses
681 included an unpaired, two-tailed Student's t-test, One-way ANOVA with
682 Dunnet's post hoc test, and Two-way ANOVA with Tukey's post hoc test. A
683 non-parametric one-way ANOVA (Kruskal Wallis) test with Dunn's post-hoc
684 test was performed for the animal experiment. P-values less than 0.05 were
685 regarded as significant. The analysis is presented as mean ±SD or mean
686 ±SEM with information on group sizes and p values mentioned in the
687 respective figure legends.

688 **Funding**

689 This work was funded by the Department of Biotechnology (DBT), Ministry of
690 Science and Technology, the Department of Science and Technology (DST),
691 Ministry of Science and Technology. DC acknowledges DAE-SRC
692 (DAE00195) outstanding investigator award and funds and ASTRA Chair
693 Professorship funds. The authors jointly acknowledge the DBT-IISc

partnership program. Infrastructure support from ICMR (Center for Advanced Study in Molecular Medicine), DST (FIST), UGC-CAS (special assistance), and TATA fellowship is acknowledged. AS duly acknowledges UGC-SRF for the financial assistance. AVN duly acknowledges the IISc-MHRD for the financial assistance. SA duly acknowledges the CSIR for the financial assistance. SD and SK duly acknowledge IISc-MHRD for their financial assistance.

Ethics statement

All experiments comply with the rules set forth by the Indian Institute of Science, Bangalore's IEAC. The approved protocol number is CAF/ Ethics/852/2021. The Committee for Control and Supervision of Experiments on Animals (CPCSEA), a statutory committee established under Chapter 4, Section 15 (1) of the Prevention of Cruelty to Animals Act 1960, and National Animal Care provided guidelines that were meticulously adhered to during all animal experiments, all of which were approved by the Institutional Animal Ethics Committee. (Registration No. 435 48/1999/ CPCSEA). The Institutional Animal Ethics Committee approved every animal experiment, and the National Animal Care Guidelines were meticulously followed.

Author contribution

AS and DC conceived the study. AS and DC designed experiments. AS and AVN performed experiments. UV supervised, and SA performed bioinformatics and simulation work. SM supervised the synthetic DPD molecule synthesis. SD and SK synthesized the synthetic DPD molecule. AS analyzed the data, prepared the figures, and wrote the manuscript draft. AS, AVN, SA, and DC reviewed and edited the manuscript. DC supervised the work. All the authors read and approved the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgment

Dr. Shashank Tripathi (CIDR, IISc Bangalore) and Dr. Sandeep M Eswarappa (Dept. of Biochemistry, IISc Bangalore)are duly acknowledged for the Luminometer. Divisional Mass Spectrometry facility IISc and Mrs. Sunita Joshi for the MS analysis. The Departmental Confocal Facility, Departmental Real-Time PCR Facility, Divisional Flowcytometry Facility, and Central Animal Facility at IISc are duly acknowledged. Mr Sumith and Ms Navya are acknowledged for their help in image acquisition. Dr. Ritika Chatterjee and Ms. Sagrika are acknowledged for technical help.

Figure1: LuxS/AI-2 quorum sensing is required by *Salmonella* Typhimurium to survive in the macrophages.

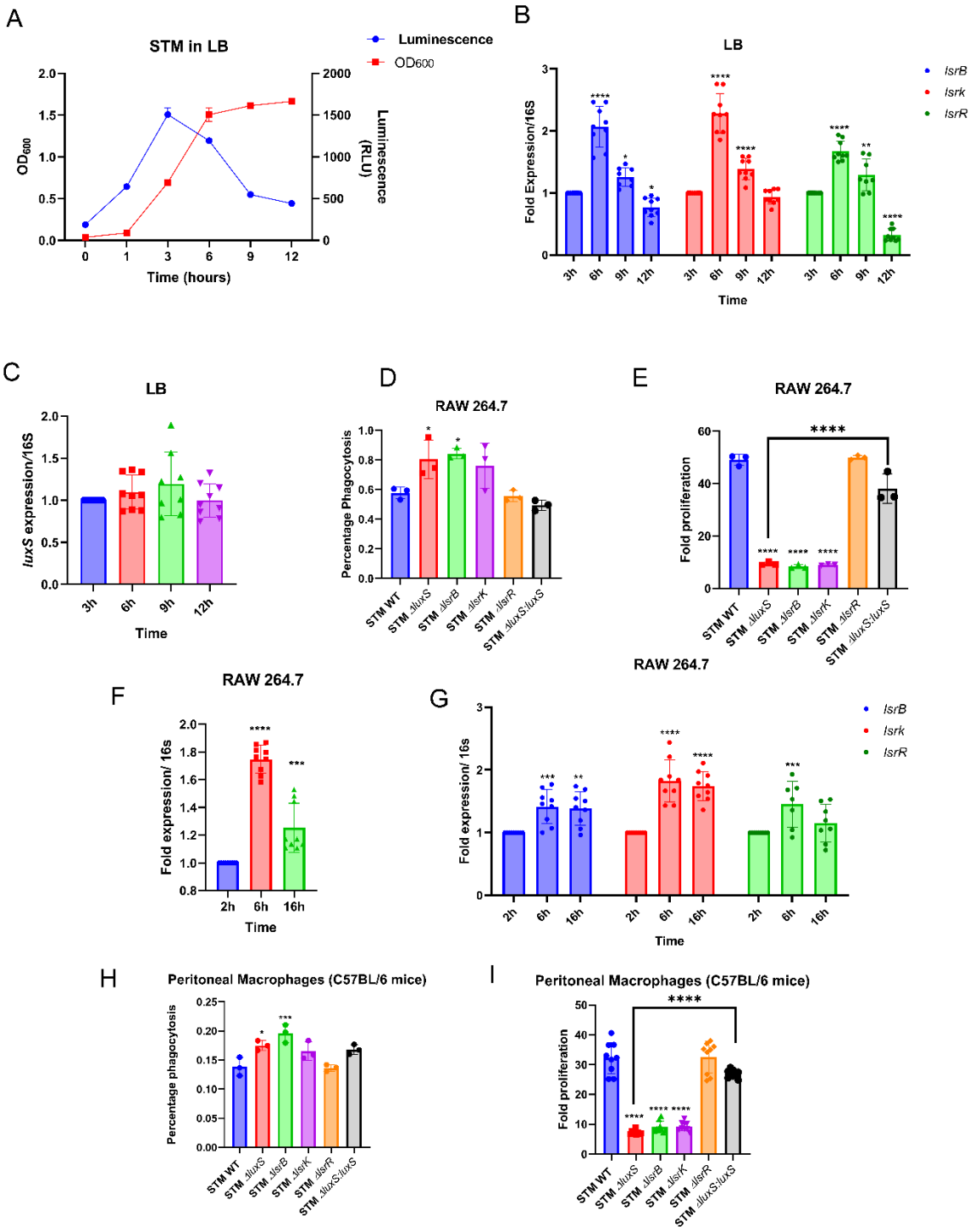

Fig.1. LuxS/AI-2 quorum sensing is required by *Salmonella* Typhimurium to survive in the macrophages. (A) *Salmonella* growth with AI-2 production in LB media. Represented as Mean+/-SD of N=3, n=4, **(B)** The mRNA expression of *lsr* operon genes *lsrB*, *lsrk*, and *lsrR* in STM WT *in vitro* growth in LB. Represented as Mean+/-SD of N=3, n=3, **(C)** *luxS* gene expression in STM WT *in vitro* growth in LB. Represented as Mean+/-SD of N=3, n=3, **(D)** Percentage phagocytosis, and **(E)** Fold proliferation upon infection in RAW 264.7 macrophages. Represented as Mean+/-SEM of N=3, n=3, **(F)** *luxS* gene expression **(G)** *lsr* operon gene *lsrB*, *lsrk*, and *lsrR* expression in STM WT upon infection in RAW264.7 macrophages. Represented as Mean+/-SD of N=3, n=3 **(H)** Percentage phagocytosis and **(I)** Fold proliferation, upon infection in peritoneal macrophages. Represented as Mean+/-SEM of N=3, n=3. One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$. Two-way Anova was used to analyze the grouped data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$

Figure 2: STM Δ*luxS* acquires AI-2 from STM WT *in vitro* but may not intravacuolarly, and AI-2 signaling loss compromises *Salmonella* survival in acidic pH

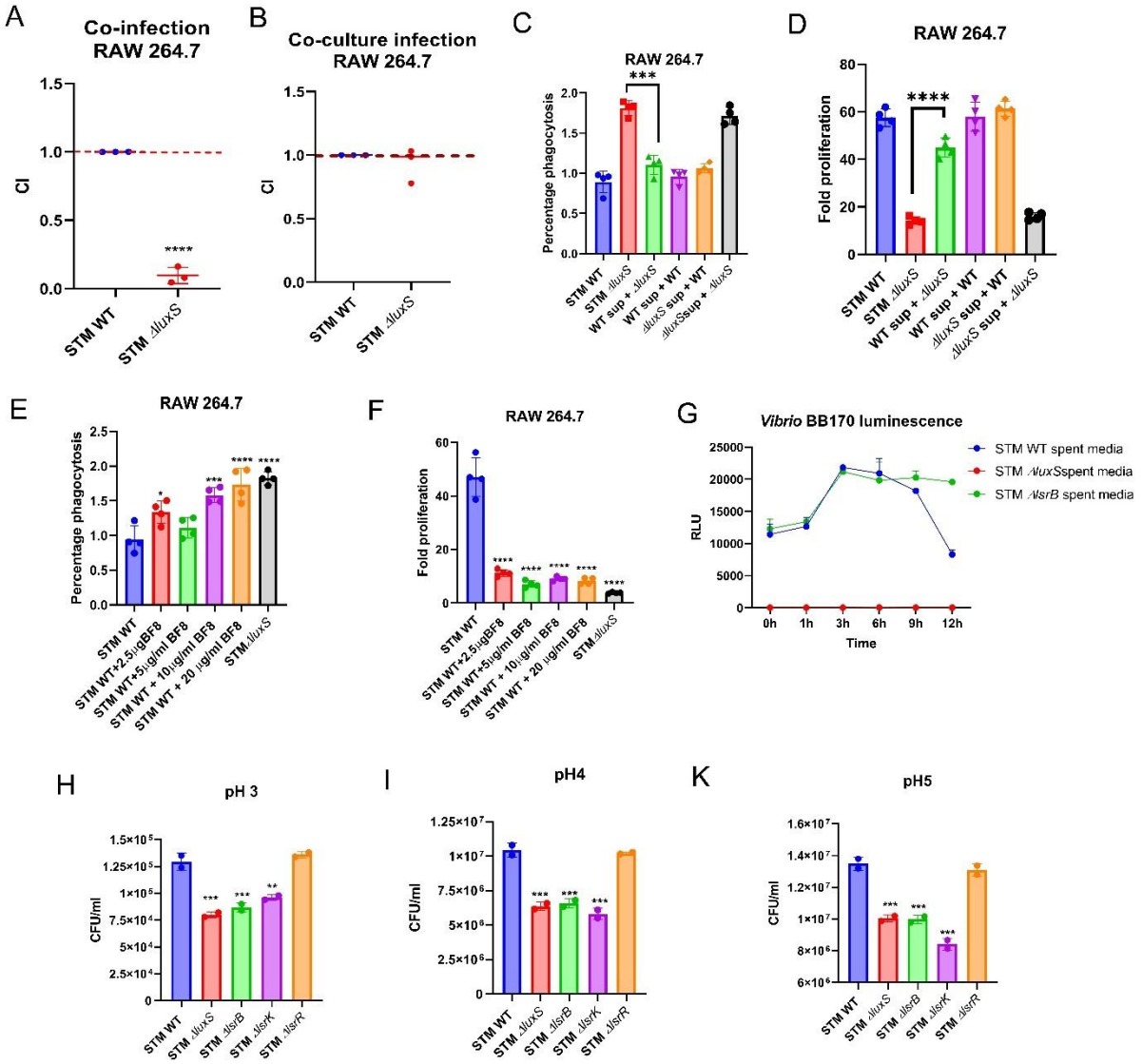

Fig. 2 STM Δ*luxS* acquires AI-2 from STM WT *in vitro* but may not intravacuolarly, and AI-2 signaling loss compromises *Salmonella* survival in acidic pH (A) Co-infection assay between STMWT and STM Δ*luxS*

764 *upon* infection in RAW 264.7 macrophages at the ratio 1:1. Represented as
765 Mean+/-SEM of N=3, n=3, **(B)** Coculture grown STM WT and STM Δ*luxS*
766 followed by infection in RAW 264.7 macrophages. Represented as Mean+/-
767 SEM of N=3, n=3, **(C)**Percentage phagocytosis, **(D)** Fold proliferation of STM
768 in RAW 264.7 macrophages upon 50% spent media treatment of STM WT
769 and STM Δ*luxS*. Represented as Mean+/-SD of N=3, n=3, **(E)**Percentage
770 phagocytosis, **(F)** Fold proliferation of STM in RAW 264.7 macrophages upon
771 treatment of BF-8 inhibitor. Represented as Mean+/-SD of N=3, n=3. **(G)** AI-
772 2 production by STM WT, STM Δ*luxS*, and STM Δ*lsrB* in F-media (pH 5).
773 Represented as Mean+/-SD of N=3, n=3. STM WT, STM *ΔluxS*, STM *ΔlsrB*,
774 STM *ΔlsrK,* and STM *ΔlsrR* survival in different range of pH(3-8) of PBS **(H)** pH
775 3 survival, **(I)** pH 4 survival, **(K)** pH 5 survival. Unpaired Student's t-test was
776 used to analyze the data; **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.05$. One-way
777 ANOVA with Dunnet's post-hoc test was used to analyze the data; p values
778 **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$
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Figure 3: LuxS/AI-2 system tunes *phoP* expression, thereby assisting in pH sensing and adaptation in *Salmonella*

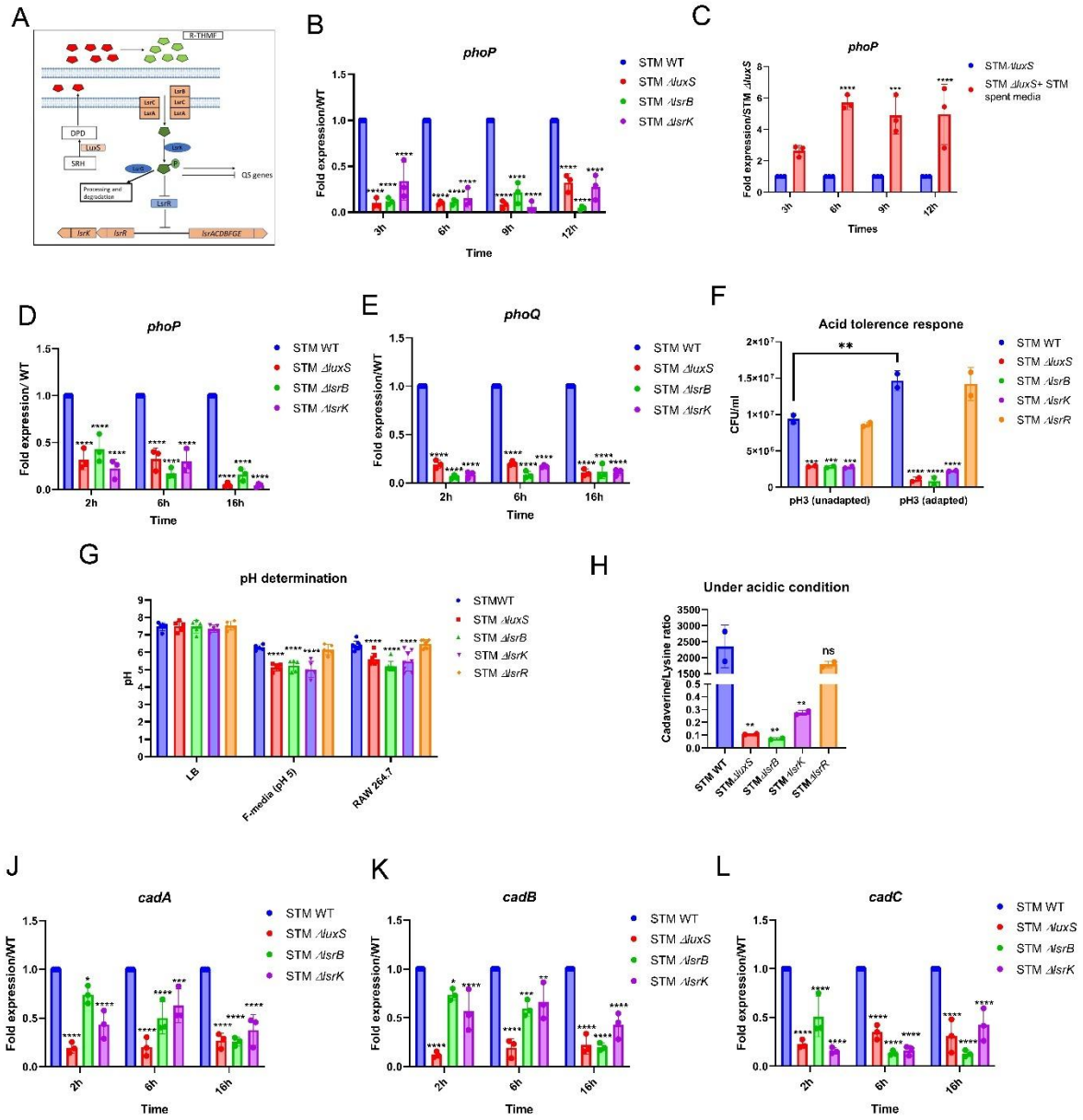

Fig. 3 LuxS/AI-2 system tunes *phoP* expression, thereby assisting in pH sensing and adaptation in *Salmonella*. (A) Schematic of AI-2 synthesis and processing in *Salmonella* Typhimurium. **(B)** *phoP* gene expression in STM WT, STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK in vitro* condition LB media.

Represented as Mean+/-SD of N=3, n=3. **(C)** mRNA expression of *phoP in* STM *ΔluxS* upon treatment of STM WT spent media. Represented as Mean+/-SD of N=3, n=3. **(D)** *phoP* **(E)** *phoQ* expression in STM WT, STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* upon infection in RAW 264.7 macrophages. Represented as Mean+/-SD of N=3, n=3. **(F)** STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK*, and STM *ΔlsrR* acid adaptation and tolerance. Represented as Mean+/-SD of N=3, n=2. **(G)** Cytosolic pH determination of STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK*, and STM *ΔlsrR* by phuji plasmid in LB Media, in F-media (pH 5) and upon infection RAW 264.7 macrophages. Represented as Mean+/-SD of N=3, n=4. **(H)** Ratio of cadaverine and lysine under acidic conditions (pH 5), N=2. **(J)** *cadA*, (**K)** *cadB,* and **(L)** *cadC*, mRNA expression upon infection in RAW 264.7 macrophages. Represented as Mean+/-SD of N=3, n=3. One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$. Two-way ANOVA was used to analyze the grouped data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$

Figure 4:LuxS/AI-2 System regulates *phoP/phoQ* expression through LsrR Interaction with the *phoP* Promoter in *Salmonella*

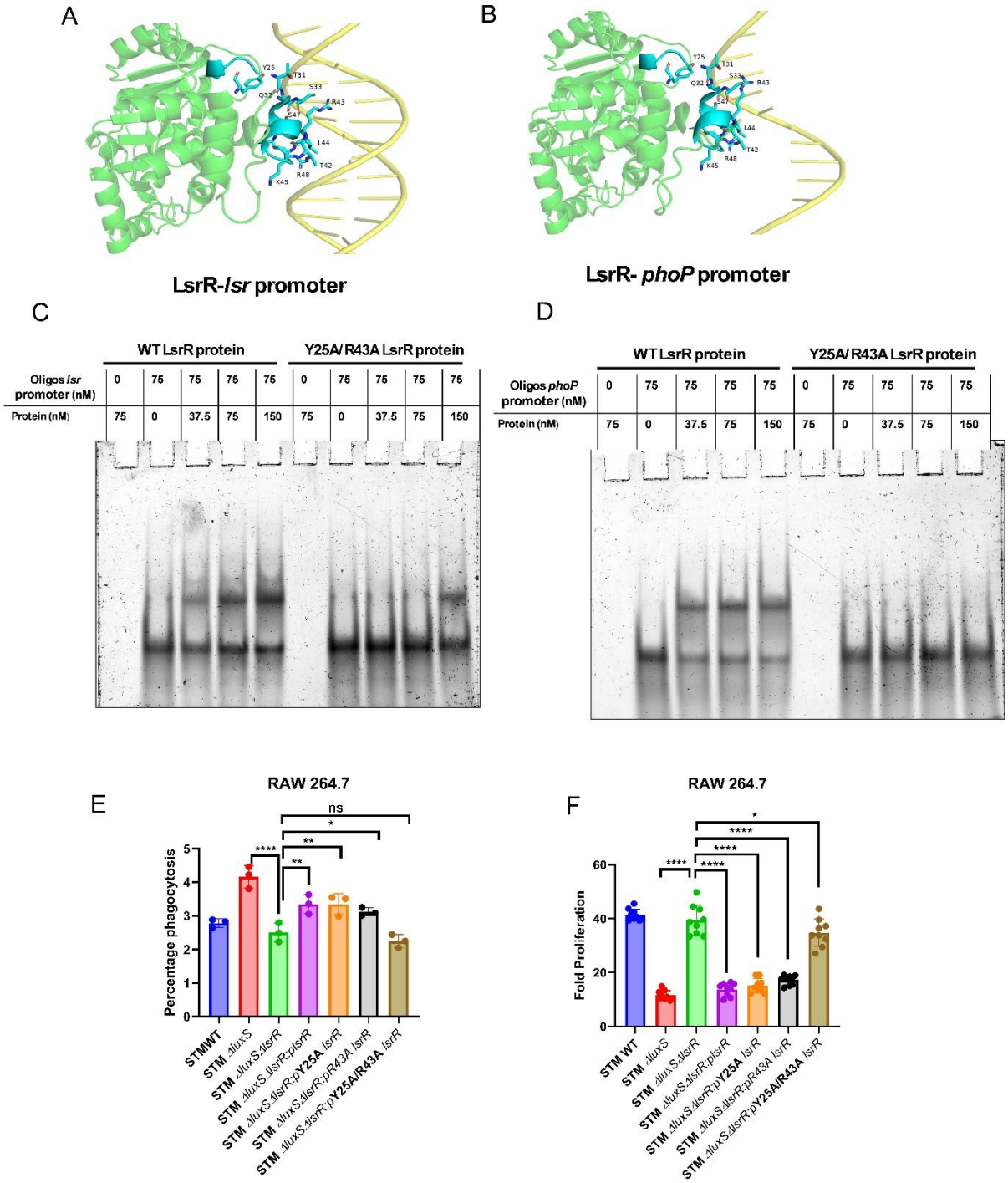

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Fig. 4 LuxS/AI-2 System regulates *phoP/phoQ* expression through LsrR Interaction with the *phoP* Promoter in *Salmonella*. AlphaFold3 structural models visualised using pymol depicting LsrR (green) interactions with the *phoP* and *lsr* promoters (yellow). The N-terminal domain residues, spanning helices 1, 2, and 3, are highlighted in blue, and the key interacting residues of LsrR are represented with a ball and stick model **(Fig. A,B)**. Electrophoretic mobility shift assays (EMSA) assessing DNA binding of wild-type LsrR and its Y25A/R43A mutant to *lsr* and *phoP* promoters (**Figs. C,D)**. Intracellular survival assay (ICSA) with mutated Y25A, R43A, and Y25A/R43A *lsrR* cloned in pQE-60 complemented strains **(E)** phagocytosis and **(F)** Fold proliferation. Represented as Mean+/-SD of N=3, n=3. One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$.

Figure 5: AI-2 orchestrates the *Salmonella* pathogenicity island-2 gene expression by sensing low pH

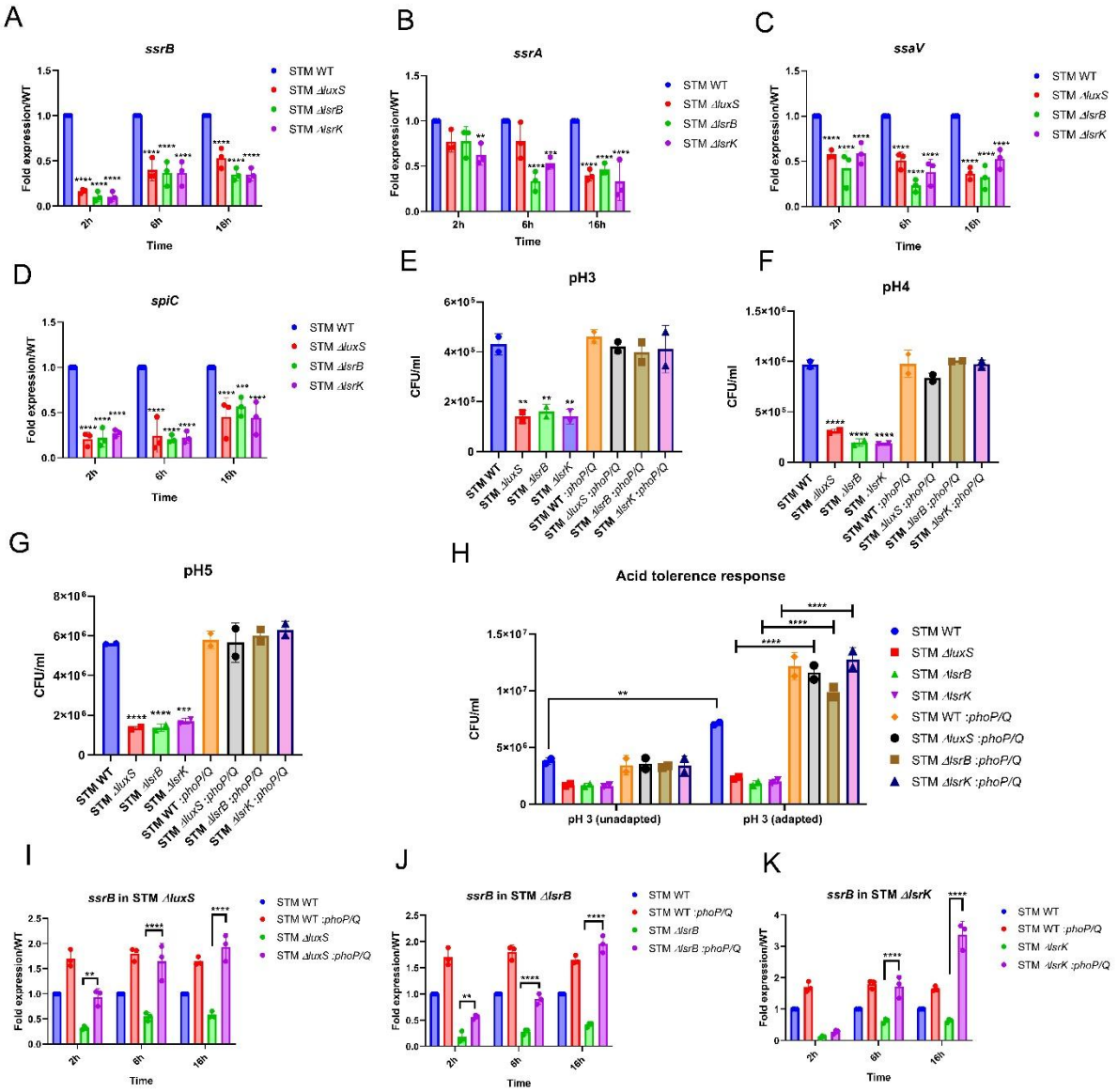

Fig.5 AI-2 orchestrates the *Salmonella* pathogenicity island-2 gene expression by sensing low pH. (A)*ssrB* **(B)** *ssrA* **(C)** *ssaV* **(D)** *spiC,* mRNA

expression in STM WT, STM *ΔluxS*, STM *ΔlsrB*, and STM *ΔlsrK* upon infection in RAW 264.7 macrophages. Represented as Mean+/-SD of N=3, n=3. STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK,* STM WT: *phoP*, STM *ΔluxS*: *phoP*, STM *ΔlsrB*: *phoP*, STM *ΔlsrK*: *phoP* survival in PBS at **(E)** pH3, **(F)** pH4, **(G)** pH5. Represented as Mean+/-SD of N=3, n=2. **(H)** STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK,* STM WT: *phoP*, STM *ΔluxS*: *phoP*, STM *ΔlsrB*: *phoP*, STM *ΔlsrK*: *phoP* acid adaptation and tolerance. Represented as Mean+/-SD of N=3, n=2. *ssrB* mRNA expression in *phoP*/*phoQ* cloned strain of **(I)** STM *ΔluxS,* **(J)** STM *ΔlsrB* and **(K)** STM *ΔlsrK*. Represented as Mean+/-SD of N=3, n=3.

One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$. Two-way ANOVA was used to analyze the grouped data; p values **** $p < 0.0001$, *** $p < 0.001$, ** $p<0.01$, * $p<0.01$

Figure 6: The absence of AI-2 mediated signaling compromises the *in vivo* colonization of *Salmonella*

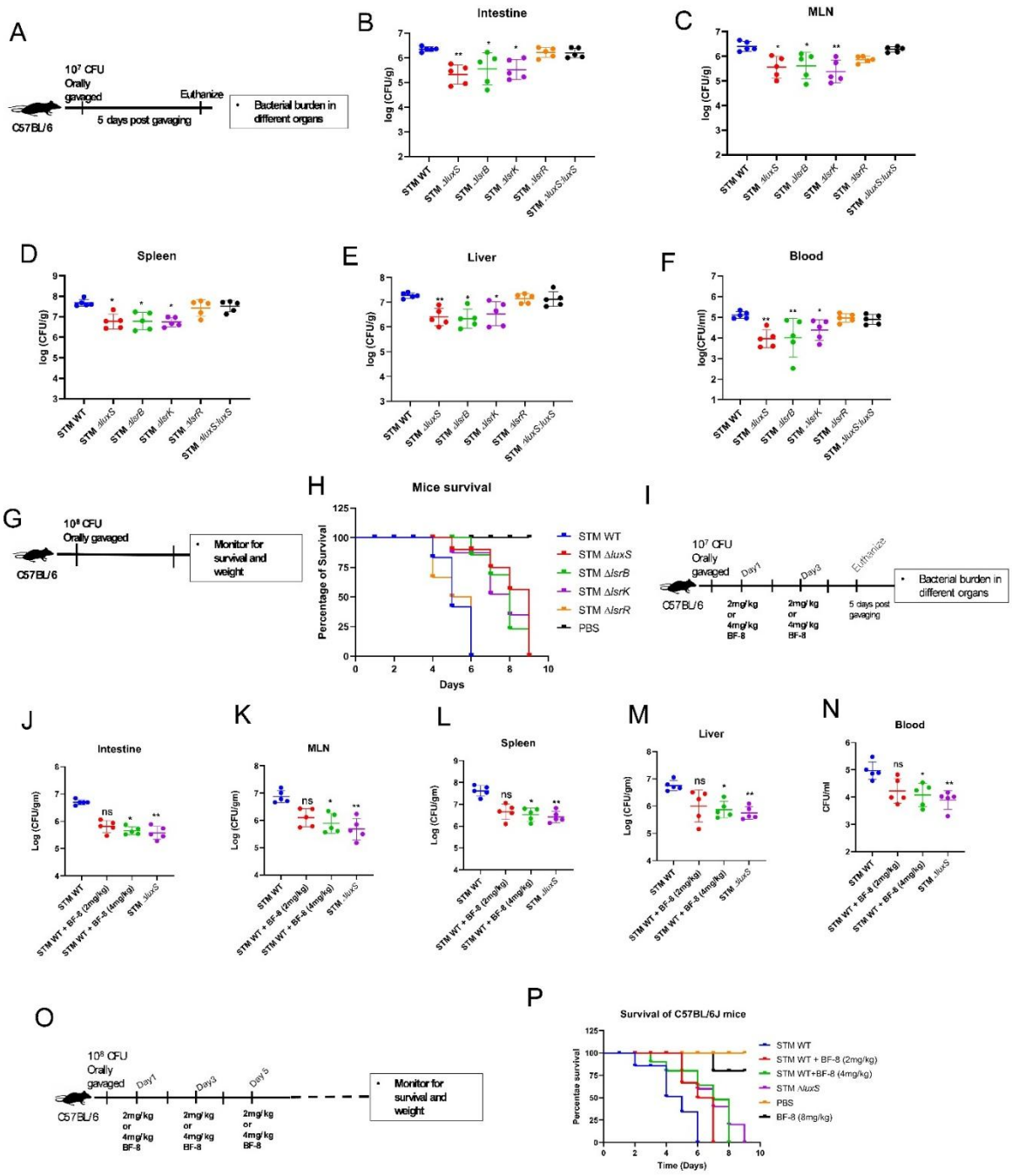

Fig. 6 The absence of AI-2 mediated signaling compromises the *in vivo* colonization of *Salmonella*. (A) The experimental protocol for organ burden in C57BL/6J mice by orally gavaging 10^7 CFU per mouse. The organ burden

post 5 days of oral gavage in **(B)** Intestine, **(C)**Mesenteric lymph node (MLN), **(D)** Spleen, **(E)** Liver, and dissemination in **(F)** Blood. Represented as Mean+/-SD of N=2, n=5 **(G)** The experimental protocol for C57BL/6J mice survival by orally gavaging 10^8 CFU of STM per mouse. **(H)** Percentage survival of Mice upon infection with STM WT, STM *ΔluxS*, STM *ΔlsrB*, STM *ΔlsrK,* STM *ΔlsrR* and STM *ΔluxS: luxS*. (**I)** The experimental protocol for organ burden in C57BL/6J mice by orally gavaging 10^7 CFU per mouse upon treatment of BF-8 inhibitor (2mg/kg and 4mg/kg through the Intraperitoneal route). The organ burden post 5 days of oral gavage in **(J)** Intestine, **(K)**Mesenteric lymph node (MLN), **(L)** Spleen, **(M)** Liver, and blood dissemination in **(N)** Blood. Represented as Mean+/-SD of N=2, n=5. **(O)** The experimental protocol of mice survival in C57BL/6J mice by orally gavaging 10^8 CFU per mouse upon treatment of BF-8 inhibitor (2mg/kg and 4mg/kg through the Intraperitoneal route). **(P)** Percentage survival of Mice upon infection with STM WT, STM WT upon treatment, and STM *ΔluxS*. Non-parametric One-way ANOVA (Kruskal Wallis) with Dunn's posthoc test was used to analyze organ burden in mice.

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