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1 **Fis family members synergistically control the virulence of *Legionella pneumophila***

2

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23

24 **ABSTRACT (238 words/250)**

25 *Legionella pneumophila* virulence is controlled in a growth phase-dependent manner by a  
26 complex regulatory network involving several two-component systems, small regulatory RNAs  
27 and the translational CsrA regulator. Here, we address the additional role of Nucleoid-  
28 associated proteins (NAP) regulators in this network, by investigating the regulatory functions  
29 of the three Fis paralogs (Fis1, Fis2, Fis3), a unique feature among bacteria, in the infection  
30 cycle of *L. pneumophila*. Specifically, we show that deletion of *fis1* has a major impact on *L.*  
31 *pneumophila* virulence, and that deletion of *fis2* enhances the intensity of this phenotype.  
32 Consistently, RNA-seq analysis and reporter gene fusions demonstrate the predominant role of  
33 Fis1 in the regulation of many virulence-related genes, including those involved in the  
34 flagellum, pili biosynthesis, and Dot/Icm type 4 secretion machinery, as well as several genes  
35 encoding Dot/Icm effectors. Both Fis1 and Fis2 bind to AT-rich motifs upstream their target  
36 genes, but Fis1 with higher affinity than Fis2. Importantly, Fis1 and Fis2 would be capable of  
37 forming heterodimers that could bind with variable affinity to this AT-rich motif. It is also  
38 important to note that the three Fis proteins are not produced at the same time and in the same  
39 amounts. We therefore hypothesize that the duplication of *fis* genes in *L. pneumophila* is not  
40 simply a back-up system to compensate for potentially deleterious mutations in a *fis* gene, but  
41 rather a means to fine-tune the expression of targeted genes, particularly virulence genes.

42

43 **IMPORTANCE (149/150)**

44 Appropriate control of virulence gene expression is crucial to the success of bacterial infection.  
45 Nucleoid-associated protein regulators, including Fis proteins, have been shown to participate  
46 in the virulence of several human pathogens. The importance of our discovery lies in the fact  
47 that *L. pneumophila* possesses three non-homologous Fis proteins instead of just one. We  
48 demonstrate that Fis1 and Fis2 are not functional duplicates of each other. On the contrary, Fis1

49 and Fis2 are synthesized neither simultaneously nor in equal amounts during the bacterial  
50 growth phase, and they cooperate to regulate virulence gene expression by targeting similar  
51 AT-rich motifs, albeit with distinct affinity, and by being capable of forming heterodimers.  
52 Taken together, our data suggest that the high conservation of *fis* gene duplication results from  
53 the need for fine-tuned control of *Legionella* virulence in response to its different environmental  
54 and human hosts, rather than from functional redundancy to circumvent deleterious *fis*  
55 mutations.

56

57 **KEYWORDS:** *Legionella pneumophila*; Nucleoid-associated proteins (NAPs), Factor for  
58 Inversion Stimulation (FIS), effectors; Dot/Icm T4SS

59

## 60 INTRODUCTION

61 Nucleoid-associated proteins (NAPs), such as Fis proteins, are conserved small basic proteins  
62 capable of binding and bending DNA, therefore influencing DNA topology and the expression  
63 of many genes, either positively or negatively, depending on the location of their binding sites  
64 relative to the gene promoter sequence (1, 2). Originally identified as Factor for  
65 the Inversion Stimulation promoting site-specific recombination reactions catalysed by Hin and  
66 Gin DNA recombinases of *Salmonella* and phage Mu respectively (3, 4), Fis has also been  
67 shown to play a versatile role in the regulation of many diverse physiological processes, such  
68 as the initiation of DNA replication (5), DNA supercoiling (6, 7), the site-specific  
69 recombination of bacteriophage lambda (8, 9) and the transcription of rRNA and tRNA operons  
70 (10–12). It is also considered as a global transcriptional regulator that participates in the control  
71 of quorum sensing (13), biofilm formation (14, 15), antibiotic resistance (16) and virulence of  
72 diverse pathogens (17–25). Strikingly, Fis protein is encoded by a single chromosomal copy of  
73 the *fis* gene in all bacterial species, with the exception of all *Legionella* species, which have  
74 three non-homologous copies of the *fis* gene in their genome. This unique feature among  
75 bacteria is presumably the result of two duplication events that occurred prior to the divergence  
76 of the *Legionella* genus (26).

77 *L. pneumophila* is a facultative intracellular parasite able to replicate within many different  
78 free-living freshwater *protozoa* and to survive into the extracellular environment until the  
79 infection of a new host (27–29). It is also an accidental human pathogen replicating within  
80 alveolar macrophages, thus causing a severe pneumonia termed Legionnaires' disease (30–34).  
81 *L. pneumophila* infects its phagocytic, environmental and human hosts in a similar biphasic  
82 cycle, comprising a replicative phase and a transmissive phase. Crucial for establishing this  
83 cycle is the Dot/Icm type 4 secretion system (T4SS) (35, 36) which translocates more than 300  
84 effector proteins that modulate host cell functions into infected cells (34, 37–41). Specifically,

85 following phagocytosis, the bacteria escape endocytic degradation and promote the biogenesis  
86 of an endoplasmic-reticulum (ER)-derived vacuole, called *Legionella*-containing vacuole  
87 (LCV), in which the bacteria replicate (42–46). During intracellular replicative phase, genes  
88 involved in bacterial replication and central metabolism are expressed while those involved in  
89 virulence, cytotoxicity, stress resistance and motility (also called transmissive traits) are  
90 repressed, mainly due to the post-transcriptional control exerted by the RNA-binding protein  
91 CsrA (47–49). After extensive multiplication within the LCV, *L. pneumophila* undergoes a  
92 global change in gene expression leading to the derepression of transmissive traits in order to  
93 promote host cell lysis and the infection of new phagocytic cells (50–52). The switch between  
94 replicative and transmissive phases results from nutritional stress, in particular depletion in  
95 amino acids and perturbation in fatty acids biosynthesis that leads to the accumulation of the  
96 alarmone (p)ppGpp. This accumulation triggers the two-component system (TCS) LetA/LetS  
97 (GacA/S orthologues) and increases the amount of the alternative sigma factor of stress  
98 response, RpoS. Both transcriptional regulators, LetA and RpoS, are required for the full  
99 expression of the regulatory RNAs RsmX, RsmY and RsmZ that bind and sequester the  
100 repressor CsrA, thus relieving the expression of transmissive traits and about 40 Dot/Icm  
101 effector-encoding genes (47, 53–56). Additional regulatory systems control virulence in *L.*  
102 *pneumophila*, including the *Legionella* Quorum Sensing system LqsST/R (57, 58) and the TCSs  
103 PmrA/B (59–61) and CpxR/A (62–66) All these systems were shown to control the expression  
104 of both Dot/Icm translocated effectors encoding genes and several *dot/icm* secretion machinery  
105 genes, at least for CpxR/A (63, 64) and PmrA /B TCSs (61). PmrA response regulator is also  
106 required for CsrA transcription while LqsR expression depends on CsrA activity (47), thus  
107 connecting all these regulatory pathways the ones with the others.

108 Here, we address the question of the role of the three Fis1, Fis2, and Fis3 proteins in the control  
109 of the virulence of *L. pneumophila* Paris strain, with a particular attention paid to potential

110 specific, redundant or complementary roles of each copy of *fis* genes. In a previous study, Fis1  
111 and Fis3, but not Fis2, were shown to directly repress the expression of 18 Dot/Icm effector-  
112 encoding genes in the Philadelphia strain (26). More recently, three additional effector-  
113 encoding genes and their cognate regulators, located on two distinct genomic islands found in  
114 only a few *Legionella* species, have also been reported to be silenced by Fis1 and Fis3 (67, 68).  
115 In the present study, we demonstrate the predominant role of Fis1 in regulating genes at the  
116 onset of the post-exponential/transmissive phase, by directly targeting genes that encode  
117 transmissive traits such as flagella and type IV pili, but also genes that encode major players of  
118 the complex regulatory network described above. We also show that Fis2 complete this role by  
119 synergically targeting the same set of genes, thus demonstrating a novel role of Fis2 in  
120 regulating of key virulence factors. We also demonstrate that Fis1 and Fis2 control the genes  
121 encoding the components of the Dot/Icm T4SS, as well as many effector genes, thus  
122 contributing to the fine regulation of *L. pneumophila*'s main virulence factors. Finally, our data  
123 reveal that Fis1 and Fis2, which are synthesized neither simultaneously, nor in equal amounts  
124 during bacterial growth phase, bind to the same regulatory sequences on promoter regions of  
125 target genes, albeit with distinct affinity. This suggests that Fis2 plays a crucial role in finely  
126 regulating the expression of targeted genes, particularly those involved in *L. pneumophila*  
127 virulence, rather than merely serving as a redundant copy of Fis1. It's worth noting that despite  
128 our inability to obtain a *fis3* mutant in the Paris strain, probably due to its essentiality in this  
129 genetic context, we have obtained insights showing that Fis3 exhibits more polymorphism  
130 within the *L. pneumophila* species and the genus *Legionella* and is distinguished by a slightly  
131 different structural organization from Fis1 and Fis2, which might suggest a different functional  
132 role from its paralogs Fis1 and Fis2.

133

## 134 **RESULTS**

135 **Occurrence and diversity of the three Fis paralogs among *Legionella* species.**

136 Zusman et al. (26) demonstrated that the three *Fis* paralogs are present in four distinct  
137 *Legionella* species and hypothesized that these paralogs originated from two gene duplication  
138 events prior to the divergence of the *Legionella* genus. To further investigate the evolutionary  
139 conservation of *Fis* paralogs across the genus, we conducted a comparative genomic analysis  
140 examining their presence in 64 non-*Legionella pneumophila* species and 6 *L. pneumophila*  
141 strains (**Fig. 1A and Table S1**). Phylogenetic reconstructions confirm that Fis1, Fis2, and Fis3  
142 are conserved across all species analyzed, with Fis2 and Fis3 displaying greater genetic  
143 diversity compared to Fis1. Notably, Fis1 shows a high degree of conservation with an average  
144 amino acid identity exceeding 92%, whereas Fis2 and Fis3 exhibit substantial sequence  
145 variation, with identities ranging from 73–100% for Fis2 (average 84%) and 72–100% for Fis3  
146 (average 86%). This increased sequence variability of Fis2 and Fis3 suggests that that these  
147 paralogs may be under more relaxed selective pressures than Fis1, potentially allowing for  
148 adaptive divergence in certain species.

149 To further explore the genetic diversity of *Fis* paralogs at the species level, we analyzed the  
150 protein sequence variability in 5,216 *L. pneumophila* genomes currently available from the  
151 NCBI (**Fig. S1**). Several distinct allelic variants have been identified for each *Fis* proteins, but  
152 the proportion of these variants vary according to the protein (**Fig.1B**). Fis1 and Fis2 are highly  
153 conserved within the *L. pneumophila* species, with at least 99.4% of strains possessing identical  
154 protein sequences. The other alleles are present in a very small number of strains (between 1  
155 and 5). In contrast, Fis3 displays higher polymorphism. The predominant allelic variant was  
156 found in 91.4% of the strains (4,768 strains), while alleles 2 and 3 were detected in 360 and 52  
157 other strains, respectively. This pattern suggests increased sequence diversity of Fis3 within the  
158 *L. pneumophila* population, which could potentially lead to functional variability.

159



160 **Deleting *fis1* and *fis2* impairs LCV biogenesis and intracellular replication within**  
161 **eukaryotic cells.**

162 To establish the respective role of the three Fis proteins in *Legionella* pathogenesis, single  $\Delta fis1$ ,  
163  $\Delta fis2$ , and double  $\Delta fis1/\Delta fis2$  (named hereafter  $\Delta fis1-2$ ) mutants of *L. pneumophila* Paris strain  
164 were constructed. Repeated attempts to obtain the  $\Delta fis3$  mutant in either wild-type strain or  
165 single mutants failed, suggesting this gene is essential in this genetic context.

166  $\Delta fis1$ ,  $\Delta fis2$  and  $\Delta fis1-2$  mutants were assessed for their intracellular growth kinetics. Wild-type  
167 strain and mutants expressing the mCherry-fluorescent protein were used to infect either  
168 *Acanthamoeba castellanii* amoebae or U937 macrophages, and the mCherry fluorescence,  
169 reflecting bacterial intracellular growth, was monitored over a period of 72h (**Fig. 2A and 2B**).  
170 As expected, fluorescence increases for wild-type strain but not for the avirulent  $\Delta dotB$  mutant.

171 Deleting *fis2* has minimal impact on virulence, while the  $\Delta fis1$  mutant is impaired in both  
172 eukaryotic hosts, showing a predominant role for Fis1 in intracellular replication. The  $\Delta fis1-2$   
173 double mutant showed delayed and decreased intracellular growth, compared with the  $\Delta fis1$   
174 mutant, revealing that Fis2 act synergistically with Fis1 to control intracellular replication of *L.*  
175 *pneumophila*. Overexpressing *fis1* or both *fis1* and *fis2* partially restores mutant phenotypes  
176 (**Fig. 2C**), confirming that Fis1 and, to a lesser extent, Fis2, play a role in the intracellular  
177 growth of *L. pneumophila*.

178 Biogenesis of the endoplasmic reticulum (ER)-derived vacuole called LCV (*Legionella*  
179 Containing Vacuole), essential for *L. pneumophila* intracellular replication, was assessed by  
180 infecting GFP-calnexin producing *Dyctiostelium discoideum* amoeba with mCherry-expressing  
181 wild-type and mutant strains. Confocal microscopy showed that 57% of vacuoles with wild-  
182 type strain were ER-calnexin positive at 90 min post-infection (**Fig. 2D**). As expected, the  
183 avirulent  $\Delta dotB$  mutant failed to recruit ER vesicles. 47% of vacuoles with  $\Delta fis2$  mutant were  
184 calnexin-positive, which is not significantly different from the wild-type, while only 36% and

185 27% of vacuoles with  $\Delta fis1$  and  $\Delta fis1-2$  mutants were calnexin-positive, indicating significant  
186 impairment in LCV biogenesis. Finally, ER-recruitment increases when the  $\Delta fis1-2$  mutant is  
187 complemented with a plasmid co-expressing both, *fis1* and *fis2* (**Fig. 2D**).

188 Overall, these results confirm the key role of Fis1, but also the synergistic activity of Fis2, in  
189 controlling the virulence of *L. pneumophila*.

190

### 191 **Concomitant deletions of *fis1* and *fis2* have synergistic effects on global gene expression.**

192 To further characterize the respective role of Fis1 and Fis2 proteins in *L. pneumophila*  
193 virulence, we compared the global gene expression profiles of the  $\Delta fis1$ ,  $\Delta fis2$  and  $\Delta fis1-2$   
194 mutants. Importantly, we analyzed their transcriptome by RNA-Seq at the transition between  
195 the replicative and the transmissive phase of the infectious cycle (corresponding to early  
196 stationary phase broth cultures).

197 The deletion of *fis1* leads to significant expression changes, with 279 genes differentially  
198 expressed whereas the  $\Delta fis2$  mutant shows a less pronounced transcriptional impact, with only  
199 59 gene expression changes compared to the wild-type strain (fold change (FC) >2) (**Fig. 3A**).

200 Notably, there is no overlap between the genes regulated by Fis2 and those controlled by Fis1,  
201 indicating that these 59 genes form a Fis2-specific regulon (**Fig. 3B**). Intriguingly, the

202 simultaneous deletion of both *fis1* and *fis2* results in a higher number of differentially expressed  
203 genes (609) than those observed in the  $\Delta fis1$  and  $\Delta fis2$  single mutants (279+59=338 genes). The

204 genes exclusively identified in the double mutant might be targets of the combined action of

205 Fis1 and Fis2 or might have been undetected in the single mutants due to an FC below the  
206 threshold of 2. Consistently, 60% of the genes targeted by Fis1 show a greater FC in the double

207  $\Delta fis1-2$  mutant. These data are consistent with the respective virulence phenotypes of the  $\Delta fis1$

208 and  $\Delta fis2$  mutants described above. They highlight the predominant role of Fis1 and show that

209 Fis1 and Fis2 share common target genes they synergistically regulate.

210 The virulence defects of the *Δfis1* and *Δfis1-2* mutants are reflected in their global gene  
211 expression profiles. Many transmissive genes such as flagellar and type IV pili genes or several  
212 effector-encoding genes (51) are downregulated in mutants (**Fig. 3C**). T4SS-independent  
213 virulence factors like HtpB (Hsp60), LadC, RtxA toxin, as well as the enhanced entry proteins  
214 EnhABC and their homologues are also repressed in *Δfis1-2* mutant. Moreover, key post-  
215 exponential regulators are modulated in a Fis1/Fis2-dependent manner. The transmission trait  
216 enhancer LetE, the RNA chaperone Hfq, sigma factors RpoN ( $\sigma_{54}$ ), RpoE ( $\sigma_{24}$ ), and FliA  
217 ( $\sigma_{28}$ ), along with TCS-response regulators PilR, CpxR and PmrA, are all down-regulated in  
218 the *Δfis1-2* mutant strain (**Table S2**). In addition, 9 of 24 GGDEF/EAL regulatory proteins  
219 which play a key role in the control of virulence, biofilm formation and flagellar regulation  
220 (69–72) show lower expression in the *Δfis1-2* mutant. Overall, these data suggest that Fis1 and  
221 Fis2 are part of the regulatory circuit that governs the biphasic life cycle of *Legionella*.

222

### 223 **Fis1 and Fis2 synergistically control the expression of flagellum and type IV pili.**

224 To further investigate the role of Fis1 and Fis2 in controlling the biphasic infectious cycle of *L.*  
225 *pneumophila*, we deciphered in detail their role in the expression of two important transmissive  
226 traits associated with *Legionella* virulence, namely the flagellum and type IV pili.

227 The biogenesis of functional flagella involves 45 flagellar genes organized into four distinct  
228 classes, controlled by a hierarchical cascade initiated by the major regulators FleQ and RpoN  
229 (87). Remarkably, 24 flagellar genes, including RpoN and FliA sigma factors required for the  
230 transcription of flagellar genes, are downregulated in the *Δfis1-2* double mutant (**Fig. 4A**). To  
231 confirm that Fis1 and Fis2 control flagella biogenesis, the production of the flagellin FlaA was  
232 quantified by immunoassay using an anti-FlaA antibody (**Fig. 4B**). Flagellin is not detected in  
233 exponential phase but synthesized by wild-type strain, *Δfis1* and *Δfis2* in post-exponential  
234 phase. More importantly, FlaA synthesis is strongly decreased in *Δfis1-2*, similar to non-

235 flagellated *ΔfleR* mutant (73), showing that Fis1 and Fis2 cooperate to control synthesis of  
236 flagellin in *L. pneumophila*. Overexpressing *fis1* or *fis2* in *Δfis1-2* mutant restores flagellin  
237 production, but Fis3 does not complement this phenotype suggesting a distinct regulatory  
238 function. Consistent with immunoassay results, electron transmission microscopy observations  
239 show that the wild-type strain, *Δfis1* and *Δfis2*, but not the *Δfis1-2* double mutant, display  
240 flagella (**Fig. 4C**). Together, these data demonstrate that Fis1 and Fis2 jointly control the  
241 expression of flagellar genes and the biogenesis of the flagellum.

242 Type IV pili are also considered an important transmissive trait (50, 51). The operon *pilBCD*,  
243 the type IV prepilin gene *pilE1*, the major type IV pilin *pilE2* and the operon clustering *pilE3*  
244 (*lpp0686* to *lpp0681*) are all down-regulated in the *Δfis1-2* double mutant compared to the wild-  
245 type strain (**Table S2**). To confirm the RNAseq data, a transcriptional fusion containing the  
246 300-bp promoter region of the pilus operon *lpp0686-lpp0681* fused to the firefly luciferase gene  
247 *luc* (designated  $P_{pilE-luc}$ ) was constructed in the wild-type strain and mutants. Noteworthy, 4  
248 putative Fis binding sites were identified in this regulatory region, based on similarities with  
249 the 15-bp consensus sequence established for *E. coli* (74, 75) (**Fig. 4D**). Maximum  
250 luminescence was observed after bacteria enter the stationary phase, confirming that pili are  
251 transmissive traits. Importantly,  $P_{pilE-luc}$  expression was repressed in *Δfis1* and *Δfis2* mutants  
252 and nearly abolished in *Δfis1-2* double mutant, indicating that pilus gene expression depends  
253 on both Fis1 and Fis2 (**Fig. 4E**).

254 Taken together, these results strongly support a significant role of Fis1 and Fis2 in the  
255 regulation of key virulence factors known to be induced during the transmissive phase.

256

### 257 **Fis1 and Fis2 are essential for optimal Dot/Icm-dependent translocation.**

258 To better understand the roles of Fis1 and Fis2 in *L. pneumophila* virulence, we examined their  
259 impact on the regulation of T4SS Dot/Icm synthesis which secretes over 300 effector proteins

260 into the host cell and is crucial for intracellular replication. Among the 27 *dot/icm* genes  
261 organized into 14 transcription units (92) (**Fig. 5A**), *icmPO* (*dotML*), *icmDJB* (*dotPNO*), *icmV-*  
262 *dotA*, *icmWX* operons, as well as the *dotV* gene are downregulated, whereas the *dotKJIHGF*  
263 operon is upregulated in the  $\Delta fisI-2$  double mutant. Moreover, several putative *fis* binding  
264 motifs are identified in the promoter regions of these transcriptional units (**Fig. 5A**). To analyse  
265 the effect of Fis1 and Fis2 on Dot/Icm synthesis, we examined the expression of four  
266 transcriptional *luc*-fusions with the promoter regions of gene or operon (i) *icmTS* (designated  
267  $P_{icmT-luc}$ ), (ii) *dotKJIHGF* ( $P_{dotK-luc}$ ), (iii) *dotV* ( $P_{dotV-luc}$ ) and iv) *icmV-dotA* ( $P_{icmV-luc}$ ), during  
268 axenic growth. The expression of  $P_{dotV-luc}$  and  $P_{icmV-luc}$  decreased, while  $P_{dotK-luc}$  increased in  
269 the  $\Delta fisI-2$  double mutant compared to the wild-type strain (**Fig. 5B**), confirming the RNAseq  
270 data and the synergistic effect of Fis1 and Fis2. Interestingly,  $P_{icmT-luc}$  expression was 2-fold  
271 higher in all three *fis* mutants, which was not apparent in the RNAseq data (**Fig. 5A and 5B**).  
272 This discrepancy is likely because  $P_{icmT-luc}$  expression differences between  $\Delta fisI-2$  and wild-  
273 type strain are most pronounced during the exponential growth phase, while RNAseq was  
274 performed on post-exponential phase cultures. These results demonstrate that Fis proteins  
275 negatively regulate the *icmTS* operon, likely through binding to the putative regulatory regions  
276 identified.

277 Since Fis1 and Fis2 controlled *dot/icm* gene expression, we tested T4SS functionality in  $\Delta fis$   
278 mutants, by measuring translocation of several Dot/Icm effectors (LepA, DrrA, LidA) using  
279 the  $\beta$ -lactamase reporter (71, 76). U937 phagocytes were infected with Paris strain,  $\Delta dotB$ , or  
280  $\Delta fis$  mutants expressing TEM-effector fusions, and effector secretion was quantified by CCF4  
281 fluorescence change from green to blue. As expected, TEM-LepA, TEM-DrrA, and TEM-LidA  
282 were efficiently secreted by the wild-type strain (460nm/530nm ratio >1.5), but not by  $\Delta dotB$ .  
283 Translocation decreased in  $\Delta fisI$  (50%),  $\Delta fis2$  (45%), and  $\Delta fisI-2$  (65%) mutants (**Fig. 5C**).  
284 Importantly, TEM fusion proteins are produced in similar and stable amounts across different

285 genetic backgrounds (**Fig. 5D**), indicating that the reduced TEM fusion protein detected in the  
286 host cell is due to lower translocation efficiency by the Dot/Icm machinery, rather than reduced  
287 synthesis by the bacteria. These data confirm Fis1 and Fis2 impact both *dot/icm* gene expression  
288 and T4SS functionality, thus contributing to *Legionella* virulence.

289

290 **Fis proteins positively or negatively regulate the expression of numerous T4SS effectors.**

291 The Dot/Icm T4SS secretes over 300 effector proteins into the host cell during infection, with  
292 secretion controlled over time to match their biological function. This temporal regulation is  
293 achieved by controlling the Dot/Icm machinery's functionality (69) and effector gene  
294 expression. Most effectors are synthesized in the post-exponential growth phase, similar to the  
295 transmission phase of the *Legionella* infection cycle, though some are produced during the  
296 exponential phase (77, 78).

297 Transcriptomics of  $\Delta fis1$ ,  $\Delta fis2$ , and  $\Delta fis1-2$  mutants identified 17 upregulated and 44  
298 downregulated effector genes (fold-change > 1.5) (**Table 1**). Many genes altered in  $\Delta fis1$  are  
299 more affected in  $\Delta fis1-2$ , showing Fis1 and Fis2 synergistic action. Among the 17 effector genes  
300 repressed by Fis1 and Fis2, *ankC*, *legK3* and *cegK3* were previously identified as being  
301 inhibited by Fis1 and Fis3 in the Philadelphia strain of *L. pneumophila* (26, 67). Conversely,  
302 the *sidC* gene, strongly repressed by Fis3 in Philadelphia (26), appears to be activated by Fis1  
303 and Fis2 in Paris strain. Among the 44 effector genes activated by Fis1 and Fis2, 29 have been  
304 identified as typical virulence factors of the transmissive phase (51, 78). Interestingly, this  
305 group includes 11 effectors known to be repressed by the PmrA/B TCS (60, 61), excepted *sidG*  
306 which is activated by PmrA and *sidH* whose expression is enhanced by both PmrA and CpxR.  
307 Additionally, *ralF*, *sidC* and *sdcA* are also under the positive regulation of the *Legionella*  
308 quorum sensing (Lqs) system (57, 58). These data suggest that effector gene promoters are

309 subject to multiple transcriptional controls, including the three Fis proteins, to fine-tune the  
310 synthesis of each effector during the infectious cycle.

311 To experimentally confirm the activating or repressive regulatory role of Fis1 and Fis2, we  
312 selected 4 differently regulated effector genes, namely *RalF*, *SidG*, *AnkG*, and *MarB* to analyze  
313 the expression of the related transcriptional *luc*-fusions in the Paris wild-type strain, *Δfis1*,  
314 *Δfis2*, and *Δfis1-2* mutants over 90h of growth in axenic medium. Expression profiles of these  
315 fusions confirm the RNAseq data, showing that 2 genes are positively controlled by Fis1 and  
316 Fis2 (*ralF* and *sidG*, **Fig. 6A**) and 2 genes are negatively controlled (*ankG* and *marB*, **Fig. 6B**).  
317 Interestingly, the positively regulated genes are strongly expressed in post-exponential phase  
318 while the negatively regulated genes are mainly expressed during exponential phase, suggesting  
319 a growth phase-dependent regulatory role of Fis. Notably, the absence of Fis2 alone affects the  
320 expression levels of  $P_{ralF-luc}$  and  $P_{sidG-luc}$ . This effect was not detected in RNAseq, likely due  
321 to the higher sensitivity of *luc*-reporter fusions compared to the global transcriptomic approach.  
322 Overall, these results highlight the regulatory function of Fis1 and, for the first time, uncover  
323 the role of Fis2 in effector gene expression. Additionally, they reveal that the repertoire of  
324 effector genes regulated by Fis proteins is broader than previously described (26).

325

### 326 **Fis1 and Fis2 bind Fis-consensus regulatory elements in the promoter of their targeted** 327 **genes**

328 As previously noted, we identified putative Fis-binding sites in the promoters of genes targeted  
329 by Fis1 and Fis2 proteins. Positions 1 (G) and 15 (C) of these sites, indicated by asterisks in  
330 **Fig. 7A**, were substituted using site-directed mutagenesis and the resulting mutated promoter  
331 regions were fused to the luciferase gene, creating  $P_{pilE\_mut-luc}$ ,  $P_{ralF\_mut-luc}$  and  $P_{icmT\_mut-luc}$   
332 fusions. Mutations introduced into Fis consensus binding sites of *ralF* and *pilE* fusions result  
333 in a sharp decrease of luciferase, and conversely in a significant increase of expression of the



334 *P<sub>icmT<sub>mur</sub></sub>*-*luc* fusion, thus confirming the direct regulation of the 3 genes by Fis1 and Fis2  
335 proteins, likely through Fis binding to the sites identified in their promoters (**Fig. 7B**). To  
336 confirm this binding, Electrophoresis Mobility Shift Assays (EMSA) were performed using  
337 native purified Fis1 and Fis2 proteins and PCR fragments encompassing the putative Fis  
338 regulatory elements of each promoter as binding targets. As the amount of Fis1 or Fis2  
339 increased, shifted DNA bands were observed, indicating that both Fis1 and Fis2 can bind to the  
340 *pilE*, *ralF*, and *icmT* promoters (**Fig. 7C**). Notably, at equal protein concentration (100  $\mu$ M),  
341 all the DNA molecules are shifted with Fis1 but not with Fis2, demonstrating that Fis1 binds  
342 the promoters with greater affinity than Fis2, which is consistent with the lesser impact of Fis2  
343 on the expression of these genes, as observed by RNAseq and *luc*-fusions. Moreover, while  
344 only a single high-molecular-weight shifted band is observed for Fis1, indicating its  
345 simultaneous binding to multiple sites, distinct lower molecular weight shifted bands appear as  
346 the Fis2 concentration increases. These bands correspond to different Fis2-DNA complexes,  
347 suggesting that Fis2 binds to fewer sites and in a more gradual manner compared to Fis1.  
348 Finally, the specificity of Fis1 and Fis2 binding to the *lpp0686*, *ralF*, and *icmT* promoters was  
349 demonstrated, as both proteins bind with much less efficiency to the mutated target DNA (**Fig.**  
350 **7C**). In the cases of the mutated-*pilE* and -*ralF* promoters, low-intensity shifted bands were  
351 observed, suggesting that Fis1 and Fis2 can still bind to other putative sites in these promoter  
352 regions (**Fig. 7A**).

353

354 **Fis1 and Fis2 display redundant activity and partially distinct growth-dependent**  
355 **expression pattern.**

356 We sought to investigate whether the cooperation of Fis1 and Fis2 in controlling virulence traits  
357 is due to their complementarity, interchangeability, or more complex interactions. To address  
358 the question of the role of *Legionella* Fis proteins in a simple way, we tested their ability to



359 complement phenotypes characteristic of an *E. coli*  $\Delta$ *fis* mutant, namely (i) the lack of mobility  
360 due to the absence of activation of flagellum synthesis (79), and (ii) a dysregulation of DNA  
361 supercoiling due to the absence of repression of the DNA gyrase gene (80, 81)(**Fig. 8A**). We  
362 also assayed each *Legionella* Fis protein to control the expression of the *E. coli* *fis* gene, i.e. to  
363 reproduce the autoregulation set up by the *E. coli* Fis protein (82). We first deleted the *fis* gene  
364 in *E. coli* BW25113 to obtain the  $\Delta$ *fis* mutant strain BWL0, and then replaced the *E. coli* *fis*  
365 gene by *fis1*, *fis2* or *fis3* from *L. pneumophila* under the *E. coli* *fis* promoter (**Fig. 8B**).  
366 Noteworthy, we were unable to obtain *E. coli* strain expressing *fis3* gene, as genetic  
367 reconstruction led to mutations in the 3' end of *fis3* gene. As expected, deletion of *E. coli* *fis*  
368 results in both reduced motility (**Fig. 8C**), increased expression of *fis* (**Fig. 8D**), and activation  
369 of *PgyrA* (**Fig. 8E**), when compared to the *E. coli* wild-type strain. All 3 phenotypes are  
370 complemented by the expression of *Legionella* *fis1* or *fis2* genes, with Fis1 being better able to  
371 complement all phenotypes. This suggests that Fis1, and to a lesser extent Fis2, have similar  
372 activities to *E. coli* Fis. Better complementation by Fis1 could be consistent with its higher  
373 activity and its predominant role in the control of phase transition and virulence of *L.*  
374 *pneumophila*. Furthermore, the inability to express *fis3* in *E. coli* likely highlights a different  
375 role for Fis3.

376 As Fis1 and Fis2 have redundant functions and target the same DNA regions in *L. pneumophila*,  
377 we aimed to determine the relative abundance of the 3 Fis proteins throughout axenic growth.  
378 We analyzed samples extracted at OD<sub>600</sub> = 1; 2; 3; 4 and 5 from 3 independent liquid growth  
379 of Paris strain at 30°C using quantitative mass spectrometry (**Fig. 9**). Fis1 and Fis3 are highly  
380 expressed early in growth (OD<sub>600</sub> = 1), then decrease to a minimum in the stationary phase  
381 (OD<sub>600</sub> = 5). Conversely, Fis2 levels are low initially, peak at the end of the exponential growth  
382 phase (OD<sub>600</sub> = 3), and then decrease. In Philadelphia strain grown at 37°C Fis1-Fis3 are highly,  
383 and Fis2 exclusively, expressed during the exponential phase (77), similarly to the unique Fis

384 protein in *E. coli* and other enteric bacteria that peaks at the beginning of the logarithmic growth  
385 phase (23, 83–85).

386 The dynamic presence and absence of the 3 *Legionella* Fis proteins likely reflect distinct and  
387 specific regulatory roles. Noteworthy, despite their disparate production profiles, Fis proteins  
388 coexist at specific points in time and are likely to collaborate during the *Legionella* infection  
389 cycle.

390

391 **Fis1 and Fis2 could form heterodimers to cooperatively control *L. pneumophila* virulence**

392 From a biochemical perspective, Fis1, Fis2, and Fis3 proteins of *L. pneumophila* have limited  
393 amino acid sequence similarities with each other and with the canonical Fis protein of *E. coli*  
394 or *Salmonella*, however, they show remarkable structural similarities (**Fig. 10A**). According to  
395 AlphaFold models, their quaternary structure as homodimers is similar. The  $\alpha$ -helices from  
396 each subunit ( $\alpha1$ - $\alpha2$  and  $\alpha1'$ - $\alpha2'$ ) assemble into a four-helix bundle forming the Fis dimer core.  
397 Additionally, the helix  $\alpha3$ - $\alpha4$  and  $\alpha3'$ - $\alpha4'$  at the C-terminal end forming a helix-turn-helix  
398 (HTH) motif involved in DNA recognition and binding (26) is also well conserved. Conversely,  
399 the N-terminal end exhibits greater variability. Fis1 and Fis2 display a highly flexible end while  
400 the N-terminus of Fis3 forms a continuous helix with helix  $\alpha1$ , both different from the N-  
401 terminus of Fis of *E. coli* which folds into 2  $\beta$ -hairpins required for controlling Hin-catalyzed  
402 DNA inversion (86–88) (**Fig. 10B**). Importantly, critical residues for DNA invertases activation  
403 in *E. coli*, are not conserved in *Legionella* Fis proteins, which raises questions about the role of  
404 *Legionella* Fis N-terminus in DNA inversion regulation or other DNA transactions.  
405 Interestingly, AlphaFold modelling also yielded very reliable predictions of Fis1-Fis2, Fis1-  
406 Fis3, Fis2-Fis3 heterodimeric complexes (**Fig. 10B**), supported by pLDDT and idTT scores  
407 similar to the homodimer *E. coli* Fis model.

408 To confirm that *Legionella* Fis proteins can form homo- and heterodimers, we performed  
409 bacterial two-hybrid assays between Fis1, Fis2, Fis3, and *E. coli* Fis. Briefly, Fis proteins were  
410 fused to the T25 and T18 fragments of the adenylate cyclase (CyaA) catalytic domain. *E. coli*  
411 *cya* mutant BTH101 was co-transformed with plasmids bearing T25 and T18 fusion proteins.  
412 Interaction between hybrid proteins restores CyaA enzyme function, leading to cAMP  
413 synthesis, which activates the *lac* operon via the cAMP-CAP complex. Protein-protein  
414 interaction was thus assessed by measuring  $\beta$ -galactosidase activity on X-Gal plates (**Fig. 10D**)  
415 and in ONPG - liquid assays (**Fig. 10E**). *E. coli* Fis, and *Legionella* Fis1 and Fis2 can form  
416 homodimers, as the corresponding strains form blue colonies on X-Gal and significant  $\beta$ -  
417 galactosidase activity is detected in ONPG - liquid assays. Conversely, Fis3 transformed strain  
418 displays white/pale blue colonies and low  $\beta$ -galactosidase activity, indicating it does not form  
419 homodimers or the complex is unstable. More interestingly, Fis1 and Fis2 are able to form  
420 heterodimers with each other and with *E. coli* Fis. These interactions are specific, as shown by  
421 negative controls using LssA, a Type 1 secretion complex protein, and the Zip segment (**Fig.**  
422 **10D and Figure S3**). It is tempting to speculate that Fis1 and Fis2 heterodimers might  
423 recognize regulatory sequences with different affinities compared to homodimers. Given the  
424 varying abundance of Fis1 and Fis2 during the *Legionella* infection cycle, it can be postulated  
425 that the proportion of hetero- and homodimers is likely to vary during intracellular growth. This  
426 introduces a further layer of complexity and subtlety to the regulation of Fis.

427

## 428 **DISCUSSION**

429 The virulence of pathogenic bacteria is based on complex strategies involving the finely  
430 regulated expression of different virulence genes. Appropriate control of this expression is  
431 achieved by sophisticated regulatory networks at the transcriptional, post-transcriptional,  
432 translational and post-translational levels. These targeted controls, specific to each virulence

433 gene, are complemented by the action of global regulators, including NAPs, whose role in  
434 virulence has been somewhat neglected. Our study contributes to this field by demonstrating  
435 the role of Fis1 and Fis2 in controlling *L. pneumophila* virulence. Fis proteins have already  
436 been shown to participate to the virulence of several human pathogens such as *E. coli*  
437 pathogenic strains (18, 19), *Shigella flexneri* (18), *Haemophilus ducreyi* (20), *Yersinia*  
438 *pseudotuberculosis* (21), *Pseudomonas aeruginosa* (17) and its role been extensively studied  
439 in the human pathogen *Salmonella enterica* where it regulates the sequential expression of the  
440 *Salmonella* pathogenicity islands SPI-1 and SPI-2, both of which encode type 3 secretion  
441 systems (T3SS) and effector proteins required for invasion of host tissues or macrophages (23,  
442 24, 89). The significance of our discovery lies in the fact that *L. pneumophila* has three non-  
443 homologous Fis proteins instead of just one, making it a unique model for dissecting the role  
444 of the Fis network in virulence. Specifically, we have shown that deletion of *fis1* has a major  
445 impact on *L. pneumophila* virulence, leading to defects in LCV biogenesis and subsequent  
446 intracellular replication, and that deletion of *fis2* increases the intensity of these phenotypes.  
447 Consistently, Fis1 plays a predominant role in gene regulation, as the transcriptome of the  $\Delta fis1$   
448 mutant shows 279 deregulated genes, the  $\Delta fis2$  mutant only 59, and the  $\Delta fis1-2$  double mutant  
449 more than 600 genes. Noteworthy, we were unable to dissect the role of Fis3 in the virulence  
450 of *L. pneumophila* Paris due to our inability to delete the *fis3* gene. This suggests that *fis3* is  
451 essential in the genetic background of the Paris strain, whereas it is not essential in the  
452 Philadelphia background (26). Consistent with our results, it is interesting to note that the  
453 concomitant deletion of the *fis1* and *fis3* genes is not viable in the *L. pneumophila* Philadelphia  
454 strain (68). In addition, the roles of Fis1, Fis2 and Fis3 appear to differ between strains. In the  
455 Philadelphia strain, Fis1 and Fis3 have been found to directly regulate effector gene expression,  
456 whereas Fis2, which we have shown to play a role in the Paris strain, does not (26).

457 Among the 329 downregulated genes in the  $\Delta fis1-2$  double mutant, approximately one third  
458 encode typical transmissive phase traits such as the flagellar regulon, pili-encoding genes and  
459 other Dot/Icm independent virulence factors that are predicted to affect the first step of cell  
460 invasion (EnhABC and their homologues, RtxA, LidL), but also several regulators involved in  
461 the transition between the replicative and the transmissive phase, such as the transmission trait  
462 enhancer LetE (bacham and Swanson, 2004), the sigma factors RpoN and FliA (51, 90–92), the  
463 RNA chaperone Hfq (93, 94), the two-component system CpxR/A (63, 65, 66), PilS/R and  
464 lpp2523/2524 (51), and several secondary messenger cyclic-diGMP metabolizing enzymes  
465 known to coordinate cell differentiation, virulence (70, 71) and multicellular behaviour like  
466 biofilms (72). Interestingly, other genes previously defined as "late transmission genes" for  
467 their strong stationary phase induction, such as the sigma factor-encoding gene *rpoE*, the pilus  
468 operon (*lpp0686-0681*), the *enhA*-like gene (*lpp1341*) or the effector genes *sidA* and *sidE* are  
469 also strongly down-regulated in the  $\Delta fis1-2$  mutant strain. Strikingly, an AT-rich DNA motif,  
470 very similar to the Fis binding site consensus sequence, was detected upstream of their  
471 respective start codons (51). Our results suggest that this set of late transmission genes is  
472 actually directly regulated by Fis regulators. Overall, the *Legionella* Fis proteins appear to  
473 regulate, directly or indirectly, transmissive associated traits and thus should be considered as  
474 a novel class of transcriptional regulators that govern the biphasic life cycle of *L. pneumophila*.  
475 Transcriptomic analysis of  $\Delta fis1$ ,  $\Delta fis2$ , and  $\Delta fis1-2$  mutants also demonstrate that Fis regulators  
476 control the expression of several *icm/dot* genes that encode the main virulence factor Dot/Icm  
477 T4SS. Consequently, the secretion machinery's functionality is directly affected. This explains,  
478 at least in part, the strongly attenuated virulence phenotype of the  $\Delta fis1-2$  mutant strain. Little  
479 is known about the regulatory factors that control the expression of Dot/Icm-encoding genes,  
480 except for CpxR that binds and activates the expression of four *dot/icm* genes (*icmR*, *icmV*,  
481 *icmW*, and *lvgA*)(63, 64). Interestingly, transcriptomic analysis, confirmed by transcriptional

482 fusions, shows that the *IcmPO* (*dotML*), *icmDJB* (*dotPNO*), *icmV-dotA*, *icmWX* operons as well  
483 as *dotV* gene are downregulated while the *dotKJIHGF* (*IcmNMLKEG*) operon is up-regulated  
484 in the  $\Delta fis1-2$  double mutant. Importantly, the promoters of these loci include Fis putative  
485 binding sites, which suggests that they would be directly controlled by Fis1 and/or Fis2  
486 proteins.

487 Fis1 and Fis2 not only control the secretion machinery but also regulate the expression of 61 of  
488 the 330 genes that encode the Dot/Icm effectors. Specifically, Fis1 and Fis2 repress certain  
489 genes at the start of infection (exponential phase) and activate others at later times (post-  
490 exponential and stationary phases), thereby contributing to the regulatory network that controls  
491 the fine coordination of the expression of Dot/Icm effectors, which includes i) the CpxR/A two  
492 component system (TCS) that directly activates or represses about 30 effectors genes(62–66),  
493 ii) PmrA/B TCS which activates 42 effector-encoding genes (59–61), and iii) the regulatory  
494 cascade LetA/S-RsmY/Z whose role is to relieve CsrA repression in order to activate the  
495 expression of about 40 effector genes during the transmissive phase (47, 53). Whether the  
496 control of the effector genes is direct or indirect remains unclear, as Fis1 and Fis2 activate the  
497 *cpxR/cpxA* and *pmrA/pmrB* genes, as indicated above. Nevertheless, some of the genes  
498 repressed by Fis1 and Fis2 that we have identified in this study are those described by Zusman  
499 as direct transcriptional targets of Fis1 and Fis3 in the Philadelphia strain, suggesting that at  
500 least some of the effector genes are directly controlled by Fis proteins (26).

501 Although the Fis1 and Fis2 proteins of *L. pneumophila* have limited amino acid sequence  
502 similarities to each other and to the canonical Fis protein of enteric bacteria, their quaternary  
503 structure is conserved. Both Fis1 and Fis2 bind to an AT-rich region of DNA upstream of their  
504 target genes, but Fis1 has a higher affinity for this motif than Fis2. Consistently, Fis1 plays a  
505 predominant role in controlling the replicative/transmissive transition, virulence and gene  
506 regulation. Importantly, Fis1, Fis2 and even Fis3 are able to interact with each other, suggesting

507 that they are capable of forming heterodimers that could bind with variable affinity to the AT-  
508 rich region they target. It is important to note that the three Fis proteins are not produced at the  
509 same time or in the same quantities. While large quantities of Fis1 and Fis3, are present at the  
510 start of bacterial growth, Fis2 synthesis peaks when the bacteria enter the PE phase. Variations  
511 in the intracellular concentration of each Fis protein probably contribute to the sequential  
512 regulation of target genes in order to optimize their expression according to the different stages  
513 of the *Legionella* life cycle. Consistently, the synergistic effect of the *fis1-2* double mutation  
514 on gene expression argues that Fis1 and Fis2 proteins are not simply interchangeable but rather  
515 cooperate to modulate gene expression for the benefit of the bacterium. We therefore  
516 hypothesize that the duplication of *fis* genes in *L. pneumophila* is not simply a back-up system  
517 to compensate for potential deleterious mutations in one *fis* gene, but rather a means to fine-  
518 tune the expression of targeted genes, in particular virulence genes.

519

## 520 MATERIAL AND METHODS

### 521 Bacteria, cells and culture conditions.

522 The *L. pneumophila* wild-type strain used in this work is the strain Paris (CIP 107629T)(95).  
523 All the mutant strains, derived from Paris wild-type, are listed in the supplemental material  
524 **Table S4**. The *E. coli* strains used in this study are also listed in **Table S4**. Bacterial media,  
525 antibiotics and culture conditions were as previously described (69, 96). Axenic *Acanthamoeba*  
526 spp. cells were grown on PYG medium (proteose peptone, yeast extract, glucose) at 30°C and  
527 *Dictyostelium discoideum* cells were grown in HL5 medium at 22°C. Human monocyte cell  
528 line U937 was maintained at 37°C in 5% CO<sub>2</sub> and RPMI 1640 medium supplemented with 10%  
529 heat-inactivated fetal bovine serum (FBS) (Hyclone, GE Healthcare). Differentiation of U937  
530 monocytes in macrophages was induced with 100 ng/ml phorbol 12-myristate 13-acetate  
531 (PMA, Sigma) for 48h.



532

533 **Construction and complementation of *L. pneumophila* *fis* deletion mutants.**

534 All the plasmids and primers used in this work are listed in supplemental **Tables S4 and S5**.

535 *L. pneumophila* Paris mutants defective for *fis* genes were constructed by replacing the  
536 chromosomal gene *fis1* (lpp0606), *fis2* (lpp1324) or *fis3* (lpp1707) by an antibiotic resistance  
537 cassette using double homologous recombination strategy. In brief, an approximately 1-kb  
538 fragment was amplified from upstream and downstream of the gene of interest, respectively,  
539 and cloned together into the pBluescript KS-II plasmid vector (Stratgene) to generate the  
540 plasmids pLPPA33, pLPPA34 and pLPPA35 (**Table S4**). The primers, listed in **Table S5**, were  
541 designed such that the gene to be deleted was replaced with an open reading frame consisting  
542 of the first and last 6 codons, linked by the *SalI* restriction site, allowing for the subsequent  
543 introduction of an antibiotic resistance cassette. The kanamycin (Kan<sup>R</sup>) or gentamycin (Gm<sup>R</sup>),  
544 cassettes were PCR-amplified and inserted into pLPPA33, pLPPA34 and pLPPA35 digested  
545 by *SalI* and blunted with the Klenow fragment (Biolabs), yielding plasmids pLPPA36,  
546 pLPPA37 and pLPPA38 (with Kan<sup>R</sup>) and pLPPA48, pLPPA49 and pLPPA50 (with Gm<sup>R</sup>)  
547 (**Table S4**). All the constructions were sequenced to verify for correct sequences. Then, for  
548 allelic exchange of *fis1*, *fis2* or *fis3*, the entire inserts (the flanking regions and the kanamycin  
549 or gentamycin cassette) were PCR-amplified and the resulting DNA fragments were introduced  
550 separately into the *L. pneumophila* Paris strain by natural transformation as described  
551 previously (68). Recombination events into the chromosome, leading to the replacement of *fis1*  
552 or *fis2* by the kanamycin resistance cassette, were selected by plating the cells onto BCYE  
553 plates containing the antibiotic and incubating for 4-days at 37°C. The resulting transformants  
554 were replica plated and screened for the expected allelic exchanges by PCR amplification and  
555 sequencing (**Table S5**). To construct the  $\Delta$ *fis1-2* double mutant strain, the fragment containing  
556 the two flanking regions of *fis1* with the Gm<sup>R</sup> cassette was PCR-amplified and used to transform



557 the *fis2*-Kan<sup>R</sup> mutant strain. Transformants were selected by plating cells onto BCYE plates  
558 containing both antibiotics.

559 For complementation experiments, the coding sequence of *fis1*, *fis2* and *fis3* with their  
560 cognate ribosome-binding site were amplified from genomic DNA of *L. pneumophila* Paris  
561 strain and inserted separately into the pMMB207C. The resulting plasmids pLPPA210,  
562 pLPPA211 and pLPPA212 (**Table S4**) thus contain the *fis* genes under the control of the  
563 promoter *P<sub>tac</sub>* that was induced by adding 0.5 nM of isopropyl-β-D-thiogalactopyranoside  
564 (IPTG) in the culture medium. For complementation during intracellular infections, *fis1* and  
565 *fis2* genes were cloned either separately or together into the pXDC50 encoding the fluorescent  
566 protein mCherry to monitor intracellular replication of bacteria (**Table S4**).

567

#### 568 **Construction of *E. coli fis* mutants**

569 All of our mutants were derived from BW25113 *E. coli* strain, a common laboratory strain  
570 expressing the bacteriophage lambda Red recombination system to perform gene disruptions  
571 with double-stranded PCR products (98). The *E. coli fis* gene was either deleted or replaced  
572 precisely at the same locus with *fis1*, *fis2* or *fis3* genes of *L. pneumophila* according to the  
573 following two-step protocol. First, a cassette containing the toxin-encoding gene *ccdB* under  
574 the *araBAD* promoter and a kanamycin resistance gene (patent FR3050997) was PCR-  
575 amplified from strain CR201 (**Table S5**) and introduced into the chromosome of BW25113 at  
576 the *fis* locus by the λ Red recombinase-mediated gene replacement, generating strain  
577 BW25113*fis::kan-pBADccdB* (**Table S4**). Kanamycin-resistant recombinant clones were  
578 selected and verified by sequencing, and one of them used for subsequent genetic  
579 recombination steps. The *kan-pBADccdB* cassette was then replaced with PCR product  
580 corresponding to either an in-frame deleted sequence of *E. coli fis* gene or the coding sequence

581 from start to stop codons of *L. pneumophila* *fis1*, *fis2* or *fis3*. Ara<sup>R</sup> colonies were purified and  
582 screened for loss of kanamycin cassette, and the *fis* locus was sequenced.

583

#### 584 **Intracellular multiplication in host cells**

585 Intracellular growth was performed in human macrophages U937 or in amoebae *A. castelanii*  
586 as previously described (71) with some modifications described below. Cells were seeded in  
587 96-well plates at a cell density of  $1.10^5$  cells per well and infected with *L. pneumophila* strains  
588 harboring M-cherry-expressing plasmids at a multiplicity of infection (MOI) of 1. Infections  
589 were carried out in RPMI CO<sub>2</sub>-independent medium (Gibco) with 5% FBS for U937 cells or in  
590 PY special for *A. castelanii*, both supplemented with chloramphenicol and IPTG. After  
591 inoculation, plates were centrifuged 10 min at 1500 rpm. Intracellular multiplication was  
592 monitored by measuring M-cherry fluorescence every hour with a Tecan Infinite M200 plate  
593 reader.

594

#### 595 **Endoplasmic reticulum recruitment to the LCV**

596 *D. discoideum* amoebae constitutively expressing a calnexin-GFP fusion were seeded on sterile  
597 glass coverslips in 12-well plates in HL5 medium. After one night of adhesion, HL5 was  
598 removed and replaced by MB medium ( $7.15 \text{ g.L}^{-1}$  yeast extract,  $14.3 \text{ g.L}^{-1}$  peptone, 20 mM  
599 MES [pH 6.9]) containing M-cherry-expressing bacteria at an MOI=10. The plates were  
600 centrifuged at 1500 rpm for 10 min and incubated at 25°C for 90 min. Monolayers were then  
601 washed twice with PBS 1X and cells were fixed with 4% paraformaldehyde for at least 30 min.  
602 ER-recruitment was observed with an inverted confocal microscopy (Axiovert 200M, Zeiss)  
603 with a 63x phase contrast objective lens.

604

#### 605 **Total RNAs isolation and purification.**

606 Total RNAs were extracted from *L. pneumophila* Paris wt, *fis1*, *fis2* and *fis1-2* mutant strains  
607 before RNA-Seq analysis. All experiment were performed in duplicate except for Paris wt and  
608 *fis1-2* double mutant for which two independent experiments and sequencing have been done,  
609 one in duplicate and the other in triplicate. Briefly, bacterial cultures were incubated at 37°C  
610 with shaking in AYE medium until entry in stationary phase (OD ≈5). Then, 10<sup>9</sup> cells were  
611 harvested, pelleted (1 min, 13,000 rpm at 4°C) and immediately stored at -80°C. Thawed cells  
612 were resuspended in 50 µL of RNAsnap buffer (18 mM EDTA, 0.025% SDS, 95% formamide)  
613 and lysed by incubating the suspension at 95°C for 7 min (protocol adapted from (99)). Total  
614 RNAs were then extracted by adding 1 ml of TRIzol reagent (Invitrogen) and purified with the  
615 Direct-zol<sup>TM</sup> RNA miniprep kit (Zymo-research) according to the manufacturer's  
616 instructions. Purified RNAs were eluted in Rnase-free water and quantified with a NanoDrop-  
617 2000 spectrophotometer (Thermo Fisher Scientific).

618

### 619 **RNA-seq and differential gene expression analysis**

620 Quality and integrity of RNAs were further evaluated on a fragment bioanalyser before  
621 proceeding to rRNA depletion. The cDNA libraries preparation and sequencing were performed  
622 at Genewiz/Azenta Life Science (Leipzig, Germany) using Illumina NovaSeq platform with a  
623 paired-end 150bp sequencing strategy. Differential expression analysis between the  $\Delta$ *fis*  
624 mutants and the wt Paris strains was performed using an in-house pipeline using Trimmomatic  
625 v0.36, Bowtie v2.3.0, HTseq v0.6.1 and the DESeq2 package (100–103) and the resulting *P*-  
626 value were adjusted using the Benjamini-Hochberg method. To identify differentially  
627 expressed genes, we selected those with a  $0,6 \geq \log_2 FC \geq -0,6$  and a *P*-value  $\leq 0,05$ .

628

### 629 **Construction of luciferase- or gfp-based transcriptional reporter fusions**

630 To construct luciferase-based transcriptional fusions, the 1650-bp firefly luciferase (*luc*) gene  
631 was PCR-amplified from the pLA01 (104) and cloned into the digested KpnI-BamHI pXDC91  
632 vector (105), thus replacing the *gfp*-reporter gene by luciferase gene to give the pLLA01, a luc-  
633 based reporter plasmid. Then, promoter regions, including up to 400-bp upstream and 100-bp  
634 downstream of the translational start codon, of the set of genes or operons *lpp0686-0681*  
635 (*operon pile*), *IcmTS* (*lpp0507-0508*), *dotKJIHGF* (*lpp0513-0518*), *icmV-dotA* (*lpp2741-*  
636 *2740*), *dotV* (*lpp0537*), *ralF* (*lpp1932*), *sidG* (*lpp1309*), *marB* (*lpp1761*) and *ankG* (*lpp0469*)  
637 were PCR-amplified using primers with HindIII and KpnI restriction sites (**Table S5**) and  
638 cloned into HindIII-KpnI digested pLLA01. The resulting plasmids, listed in **table S4**, were  
639 sequenced prior to their introduction by electroporation into *L. pneumophila* strains.

640 To construct substitution mutations on the putative Fis-binding sites of the regulatory regions  
641 of *lpp0686*, *ralF* and *IcmT*, site-directed mutagenesis was performed on plasmids pLPPA228,  
642 pLPPA229 and pLPPA230 using QuikChange II site-directed mutagenesis kit (Stratagene) as  
643 described previously (71). The primers used for mutagenesis are listed in **table S5**. The  
644 resulting plasmids named pLPPA228mut, pLPPA229mut and pLPPA230mut, were restrict  
645 digested with KpnI-HindIII to excise the mutated-promoter regions that were individually  
646 subcloned into pLLA01 cut with the same enzymes. The resulting plasmids, listed in **table S4**,  
647 were sequenced prior to their introduction by electroporation into *L. pneumophila* Paris strain.

648 To measure luciferase activity, freshly grown *L. pneumophila* strains carrying the *luc*-reporter  
649 fusions were resuspended in AYE containing chloramphenicol and D-luciferin (final  
650 concentration 200  $\mu\text{g}\cdot\text{mL}^{-1}$ ) to an OD600 of 0.02. Suspensions were distributed in a black with  
651 clear bottom 96-well plate (150  $\mu\text{l}$ /well) and plate was incubated at 37°C inside a plate reader  
652 (Tecan Infinite M200pro) with intermittent shaking to allow bacterial growth. Expression of  
653 luc-based transcriptional fusions was monitored by measuring the absorbance at 600 nm ( $A_{600}$ )  
654 and luminescence every 15 min.

655

### 656 **TEM translocation assay**

657 The principle of this assay was described previously (71). Briefly, *L. pneumophila* strains  
658 expressing various TEM- $\beta$ -lactamase fusions proteins (76) were used to infect U937  
659 differentiated-macrophages at an MOI of 10. After 10 min of centrifugation at 1500 rpm, plates  
660 were incubated at 37°C with 5% CO<sub>2</sub> for 90 min. Then, 20  $\mu$ L of  $\beta$ -lactamase substrate  
661 CCF4/AM substrate was added onto infected cells as described before and plates incubated for  
662 an additional two hours at room temperature. When TEM-1- $\beta$ -lactamase fused to effector is  
663 translocated into host cell cytosol, the substrate CCF4/AM is cleaved, and the fluorescence  
664 emitted turns from green fluorescence (emission at 530 nm) to blue fluorescence (emission at  
665 460 nm). The translocation efficiency of a specific TEM-effector fusion is expressed as the  
666 ratio of the blue fluorescence on green fluorescence. The cytoplasmic enzyme enoyl-CoA  
667 reductase (FabI), which is not translocated by the T4SS, is used as a control to validate the  
668 translocation assay (76).

669

### 670 **SDS-Page gel electrophoresis and Western blot analysis**

671 Flagellin detection was carried out by SDS-Page gel electrophoresis and immunoblotting  
672 using anti-FlaA antibody. Briefly, *L. pneumophila* strains grown in AYE medium to  
673 exponential and early stationary phases were lysed by sonication and cell debris were pellet by  
674 centrifugation. The protein amount of the supernatant was quantified by Bradford and 1  $\mu$ g of  
675 total protein was used for western-blot analysis as described previously (47). For TEM-effector  
676 fusion proteins detection, *L. pneumophila* strains were grown on CYE agar plate and  
677 resuspended in distilled water. Equal amounts of washed cells (10<sup>9</sup> cells) were boiled 10 min  
678 in Laemmli buffer and 15  $\mu$ L of each lysate was loaded onto SDS-polyacrylamide gel and run  
679 in 0.5X TBE buffer (50 mM Tris base, 50 mM boric acid and 1mM EDTA [pH=8]). Then

680 proteins were electrophoretically transferred to nitrocellulose membrane and subsequently  
681 incubated with anti-TEM rabbit serum as a primary antibody and an anti-rabbit peroxidase  
682 conjugate as secondary antibody. Immunoblots were revealed with the kit Pierce SuperSignal  
683 (Thermo Fisher Scientific).

684

## 685 **Proteomics**

686 Quantitative Mass Spectrometry analyses of three independent cultures of *L.*  
687 *pneumophila* Paris WT grown in AYE liquid medium at 30°C until reaching OD<sub>600nm</sub> 1, 2, 3,  
688 4, and 5 were done as previously described (96). Protein expression of Fis1, Fis2, and Fis3 were  
689 extracted from the obtained dataset (**Table S3**).

690

## 691 **Purification of native Fis proteins**

692 *Escherichia coli* BL21DE3*Afis* pLysS cells carrying pLLP21 (*fis1*) or pLPP22 (*fis2*) were  
693 grown overnight at 37°C in LB broth supplemented with ampicillin and chloramphenicol to  
694 maintain both pLysS and pLLP21 or pLPP22 plasmids. Overnight cultures were diluted in LB  
695 with antibiotics and grown until an OD<sub>600nm</sub> of 0.7. At this OD, 1 mM of IPTG was added to  
696 cultures and cells were grown for an additional 2h at 37°C to induce overproduction of Fis  
697 proteins. Cells were subsequently collected by centrifugation at 4°C and the pellets were frozen  
698 at -80°C. Cells were thawed and lysed in 15 mL of cold buffer containing 50 mM Tris (pH 8.0),  
699 5 mM EDTA, 10% glycerol, 1 M NaCl, 1 mM dithiothreitol (DTT) and proteases inhibitors by  
700 using the French press. Cell debris were removed by centrifugation at 30,000g for 40 min and  
701 the supernatant (approx. 15 ml) was diluted with 35 ml of buffer A (20 mM Tris, 1 mM EDTA,  
702 10% glycerol, 1 mM DTT) and frozen at -80°C. The following purification steps for Fis proteins  
703 were performed as previously described (106). Briefly, it consists in a two-step purification  
704 protocol combining HiTrap Heparin affinity column first, followed by a cation exchange

705 chromatography (Sulfopropyl column) for Fis2 protein or an anion exchange column (DEAE)  
706 for Fis1. At the end of the purification process the cleanest fractions were pooled and dialyzed  
707 into Fis storage buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, 50% glycerol, 5 mM  
708 DTT, and 0.5 M NaCl and was stored at -20°C.

709

#### 710 **Electrophoretic mobility shift assay**

711 DNA fragments corresponding to the native or mutated promoter regions of *icmT*, *ralF* and  
712 *pilE* were amplified by PCR from plasmids listed in Table S1. DNA fragments (250 ng) were  
713 incubated with 0, 25, 50, 100  $\mu$ M of Fis1 or 0, 50, 100 and 200  $\mu$ M of Fis2. The binding reaction  
714 was performed at 25°C for 30 min in a 20- $\mu$ L volume reaction containing 4  $\mu$ L of 5X binding  
715 buffer (50 mM Tris-HCl [pH=7.5], 7 mM KCl, 1 mM DTT, 5% glycerol and 10 ng/ $\mu$ L Salmon  
716 sperm DNA). Samples were then loaded in a 6% native polyacrylamide gel that had been pre-  
717 run for one hour at 4°C in 0.5X TBE. Electrophoresis was performed at 100V and the gel was  
718 stained with 1  $\mu$ g/mL of ethidium bromide in 0.5X TBE.

719

#### 720 **Bacterial two-hybrid assay**

721 The bacterial adenylate cyclase two hybrid (BACTH) system (Euromedex, France) (107) was  
722 used to study protein–protein interactions between *L. pneumophila* Fis1, Fis2, Fis3 or the Fis  
723 protein of *E. coli* (Fis\_Ec). Each *fis* gene was PCR-amplified with specific primers (Table S2)  
724 and introduced into similarly BamHI-digested pKT25 or pUT18C plasmid to generate in-frame  
725 gene fusions with Cya T18- and Cya T25-fragment (**Table S4**). Ligation mixtures were  
726 introduced into *E. coli* DH5 $\alpha$  and transformants were selected on LB plates supplemented with  
727 100  $\mu$ g/mL Amp (for pUT18C derivatives) or 30  $\mu$ g/mL Kan (for pKT25 derivatives). All  
728 recombinant plasmids were verified by DNA sequencing. Finally, *E. coli* BTH101 (*cya* mutant,  
729 Euromedex) cells were co-transformed with a combination of one pUT18C derivative and one



730 pKT25 derivative and the transformants were selected on LB plates supplemented with both  
731 Kan and Amp. To analyze interactions between Cya T18- and Cya T25-fusion proteins, two  
732 complementary methods were conducted: (1) a screen on LB-X-Gal plates where clones  
733 positive for protein–protein interaction turned blue; and (2) a  $\beta$ -galactosidase assay providing  
734 a quantitative determination of the efficiency of the functional complementation between pairs  
735 of hybrid proteins. Several negative controls were included in each experiment: (1) pUT18C  
736 with pKT25 (empty vectors only), (2) pUT18C empty vector with each pKT25-gene fusion, (3)  
737 pUT18C-gene fusions with pKT25 empty vector and (3) pUT18C-*lssA* with each pKT25-gene  
738 fusion. A positive control (pUT18C-*zip* with pKT25-*zip*, provided with the kit) was also  
739 included in each experiment. Triplicates were performed for each pair of plasmids. *E. coli*  
740 BTH101 cultures are grown overnight at 30°C with shaking in LB supplemented with both  
741 antibiotics and 0,5 mM IPTG. 5  $\mu$ L of cell suspensions were then spotted on LB agar  
742 supplemented with ampicillin (100  $\mu$ g/ ml), kanamycin (50  $\mu$ g/ml), X-Gal (40  $\mu$ g/ml), and  
743 IPTG (0.5 mM). Plates were incubated at 30°C for 36–48 h under aerobic conditions before  
744 being imaged.

745

#### 746 **$\beta$ -galactosidase assay**

747 For  $\beta$ -galactosidase assay, 20  $\mu$ L of the overnight *E. coli* BTH101 cultures were incubated with  
748 980  $\mu$ L of Z-buffer (70mM  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 30mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 10mM KCl, 1mM  
749  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  supplemented with 100 mM  $\beta$ -mercaptoethanol just before use) at 30°C for 5 min.  
750 Then, 50  $\mu$ L of toluene and 0.001% SDS were added and mixed vigorously before being  
751 incubated for a further 10 minutes to allow for the lysis of the cells. After letting cell debris  
752 settle down at the bottom of the tube, aliquots of 20  $\mu$ L were transferred into a 96-well flat-  
753 bottom microplate containing 180  $\mu$ l of pre-warm Z buffer. Then, 10  $\mu$ L of ONPG 0.4 % were  
754 dispensed in each well and the enzymatic reaction was carried out at 30°C for 15 min with



755 measurement at OD<sub>420nm</sub> every 2 min. The relative  $\beta$ -galactosidase activity of each sample was  
756 then calculated using Beer-Lambert's law as followed Activity (mol/L of  $\beta$ -gal produced) =  
757  $[\Delta(\text{OD}_{420} - \text{OD}_{420} \text{ in control tube}) / \Delta \text{ min of incubation}] \times 5000 \times \text{dilution factor} \times \text{optical path}$   
758 (0.9 cm). The factor 5000 in the above formula is the absorption coefficient of o-nitrophenol,  
759 without Na<sub>2</sub>CO<sub>3</sub> addition (L/mol/cm). The time points were chosen to be located in the linear  
760 part of the kinetic. Results were then expressed as specific activity (mol/L/mg dry weight  
761 bacteria) as followed: AS = Activity / bacterial dry weight (~0.5 $\mu$ g per OD<sub>600</sub> unit) considering  
762 that 1ml of culture at OD<sub>600</sub> =1 corresponds to 500  $\mu$ g dry weight bacteria. Statistical tests were  
763 performed between the different conditions, including a non-parametric Kruskal-Wallis test  
764 with a p-value of 0.003669 excluding the positive control, followed with a Dunn's post-hoc  
765 pairwise test without correction carried out on each pair. Significance is represented by stars,  
766 where \* indicates a p-value < 0.5, \*\* = 0.1, \*\*\* < 0.1 and N.S indicates a p-value > 0.5.

767

#### 768 **Phylogenetic analyses to assess the inter- and intra-*Legionella* diversity of Fis paralogs**

769 All available *L. pneumophila* genomes were download from NCBI assembly database  
770 (download the May the 30<sup>th</sup> of 2024). Assembly quality was assessed using Quastv5.0.2 and  
771 assemblies with more than 100 contigs were removed of the dataset (108). Species  
772 identification was performed using fastANIv1.33, genomes mis-assigned to *L. pneumophila*  
773 were removed of the dataset (109). The remaining 5,216 genomes of the dataset were used to  
774 generate a local blast database. For phylogenetic analyses on the genus scale, one representative  
775 genome of each *L. non-pneumophila* was download from NCBI database. Assembly quality  
776 was assessed using Quastv5.0.2. *L. tunisiensis* assembly was remove from the dataset due to  
777 bad quality. The remaining 64 genomes of the dataset were used to generate a local blast  
778 database. tBlastn analyses were conducted against these databases using the amino acid  
779 sequences of the three Fis protein from the reference strain *L. pneumophila* strain Paris

780 (CIP107629T) (110). Phylogenetic trees were constructed based on amino acid sequences using

781 seaview v5.0.2 (111).

782

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790

791 **Author contributions**

792 E.K. designed research and analyzed the data; E.K., C.A. and J.B. performed all the  
793 experimental research except proteomics experiments that were designed, performed and  
794 analyzed by L.A. and K.P; C.G. performed the phylogenetic analyses; C.G. and E.K. performed  
795 and analyze transcriptomic data. A.V. provided AlphaFold models and analyzed the data. N.B.,  
796 CR and W.N. provided materials, protocols and technical assistance for confocal microscopy,  
797 construction of *E. coli* mutant strains and protein purification, respectively. A.C. contributed to  
798 discussions on signaling and virulence control in *L. pneumophila*. E.K., A.V. and P.D. wrote  
799 the paper with major input from L.A. and W.N.

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806

807 **Declaration of interest statement**

808 The authors declare no conflict of interest.

809

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- 1113
- 1114

1115 **FIGURES LEGEND**

1116

1117 **Fig. 1.** Fis paralog conservation and diversity in *Legionella* species. (A) BioNJ Tree of Fis1,  
1118 Fis2 and Fis3 from one representative genome of each *Legionella non-pneumophila* species  
1119 (n=64) and one representative sequence of each of *L. pneumophila* fis variants (n=6).  
1120 Phylogenetic tree represents the phylogenetic diversity of Fis1 (red squares), Fis2 (blue circles)  
1121 and Fis3 (green stars) (B) PhyML tree of one representative variant of fis1, 2 and 3 extracted  
1122 from 5216 *Legionella pneumophila* genomes from NCBI. Values on the right of the tree's leafs  
1123 correspond to the ratio of each variant in the genome dataset.

1124

1125 **Fig. 2.** Intracellular growth and LCV biogenesis within eukaryotic cells depend on *L.*  
1126 *pneumophila* Fis1 and Fis2 proteins. *Acanthamoeba polyphaga* (A) or U937 macrophages (B  
1127 and C) were infected at an MOI of 10 with wild-type Paris strain (dark grey),  $\Delta fis1$  (blue),  $\Delta fis2$   
1128 (yellow),  $\Delta fis1-2$  (green) and  $\Delta dotB$  (light grey) mutants harbouring the mCherry-producing  
1129 plasmid pXDC50. For complementation tests (C),  $\Delta fis1$  and  $\Delta fis1-2$  mutants carried pXDC50-  
1130 derivative plasmids expressing either *fis1* gene (*pfis1*, hatched blue) or both, *fis1* and *fis2* genes  
1131 (*pfis1\_fis2*, hatched green), respectively (see Materials and Methods). Intracellular growth was  
1132 monitored for 72 hours using a microtiter plate fluorescent reader and data, expressed in RFU  
1133 (relative fluorescent units) represent the mean values with error bars obtained from experiments  
1134 done in quadruplicate. The kinetics were performed at least three times and similar results were  
1135 obtained. (D) LCV biogenesis is assessed by monitoring endoplasmic reticulum (ER)  
1136 recruitment around the phagosome 1h post-infection. The amoeba *D. discoideum* producing  
1137 GFP-calnexin fusion was infected at an MOI of 10 with mCherry expressing bacteria. For each  
1138 strain, more than 200 vacuoles surrounded or not by green fluorescent ER-membrane were  
1139 examined by confocal microscopy and the results are expressed in percentage of ER-recruiting

1140 LCVs (averages  $\pm$  standard deviations of at least three independent experiments). Data were  
1141 analysed with R software and significant differences were determined by using Tukey's HSD  
1142 test. \*,\*\* and N.S. indicate  $P < 0.01$ ,  $P < 0.005$  and non-significant, respectively.

1143

1144 **Fig. 3.** Whole-transcriptome analysis obtained from total RNA sequencing (RNA-Seq) of  
1145  $\Delta fis1$ ,  $\Delta fis2$  and  $\Delta fis1-2$  mutants compared to wild type strain. (A) The number of genes  
1146 positively or negatively regulated in post-exponential (PE) phase is indicated for each mutant  
1147 (the count excludes genes located on plasmids). (B) Venn diagrams show the total number of  
1148 differentially expressed genes (DEGs) in absence of Fis1, Fis2 or both proteins ( $0,5 > FC > 2$  and  
1149 FDR-corrected  $P < 0,05$ ). (C) The genes positively or negatively affected in the  $\Delta fis1-2$  double  
1150 mutant were classified into 10 arbitral main functions and further divided into functional  
1151 categories (y axis) according to their annotation or prediction defined in the *L. pneumophila*  
1152 genome Paris (Cazalet et al. 2008). The percentage in each subcategory corresponds to the  
1153 number of genes upregulated (or downregulated) in this category out of the total number of  
1154 affected genes x100.

1155

1156 **Fig. 4.** Effect of *fis* genes deletion on flagellum biosynthesis and pilus gene expression in *L.*  
1157 *pneumophila* Paris. (A) Flagellar genes significantly downregulated in the  $\Delta fis1$  and  $\Delta fis1-2$   
1158 mutants (FDR-corrected  $P \leq 0,05$ ). Their expression pattern (expressed by log<sub>2</sub>FC) is depicted  
1159 in a color code based on the color scale given below. Light grey box means that the expression  
1160 does not change or is not significant. (B) Western blot analysis of whole-cell lysate of wild type  
1161 strain,  $\Delta fis$  mutants and *fleR* mutant strain (negative control), probed with a FlaA polyclonal  
1162 antibody (left panel). Cells were harvested during exponential (E) or post-exponential (PE)  
1163 growth phase. The right panel showed the expression of flagellar FlaA protein in PE-phase cell  
1164 lysates of the  $\Delta fis1-2$  double mutant complemented with either *fis1*, *fis2* or *fis3* genes and the



1165  $\Delta fis1-2$  and wild-type strain harbouring empty vector. (C) Transmission electron microscopy  
1166 pictures of post-exponential (PE)-phase *L. pneumophila* Paris wild-type,  $\Delta fis1$ ,  $\Delta fis2$  and  $\Delta fis1-$   
1167  $2$  mutants. Flagellar structure is absent only in the  $\Delta fis1-2$  double mutant strain. (D) Genomic  
1168 organization of pilus-encoding operon and the DNA sequence located upstream the operon. The  
1169  $-10$  promoter element is in italic, the transcription start is in boldface and underlined and the  
1170 putative Fis-regulatory elements are shaded in orange. (E). The transcription activity of the  
1171 pilus-related operon (*lpp0686-lpp0681*) is examined in the wild-type (orange),  $\Delta fis1$  (yellow),  
1172  $\Delta fis2$  (blue) and  $\Delta fis1-2$  (green) mutants by the means of the *lpp0686-luc* transcriptional fusion.  
1173 Luciferase activity (solid lines) and absorbance (dotted lines) were monitored for 96 hours as  
1174 described in the Experimental Procedures. Data expressed in Relative Light Units (RLU) are  
1175 means and standard deviations of technical triplicate and representative of at least three  
1176 independant experiments.

1177

1178 **Fig. 5.** The absence of Fis1 and Fis2 impact *icm/dot* genes expression and the functioning of  
1179 the T4SS apparatus in *L. pneumophila*. (A) The *icm/dot* genes are distributed in two distinct  
1180 genomic regions on the chromosome and organized in several operon structures whose  
1181 promoters are indicated by black arrows. Orange boxes located in these promoter regions  
1182 indicate the presence of putative Fis binding sites (2 boxes mean the presence of 2 or more  
1183 sites). The significant fold-change (FC) expression data of the  $\Delta fis1-2$  mutant vs. wild-type  
1184 strain is indicated above the corresponding gene, in red for repressed genes and green for  
1185 induced ones. (B) The expression level of transcriptional fusions  $P_{icmT-luc}$  (operon *icmTS*),  
1186  $P_{dotK-luc}$  (operon *dotKJIHGF*),  $P_{dotV-luc}$  and  $P_{icmV-luc}$  (operon *icmV-dotA*) were monitored for  
1187 90 hours in wild-type strain (dark grey),  $\Delta fis1$  mutant (blue),  $\Delta fis2$  mutant (yellow) and  $\Delta fis1-$   
1188  $2$  double mutant (green). Solid lines and dotted lines represent luciferase activity and  
1189 absorbance, respectively. (C) The translocation efficiency of three distinct  $\beta$ -lactam TEM-



1190 effector fusions (TEM-drrA, TEM-lidA, TEM-lepA) was monitored in the wild-type strain and  
1191 the  $\Delta fis1$ ,  $\Delta fis2$ ,  $\Delta fis1-2$ ,  $\Delta dotA$  mutant strains. The TEM-fabI fusion, coding for a cytoplasmic  
1192 protein, transformed in the wild-type strain was used as a negative control of translocation.  
1193 Differentiated-U937 cells were infected with strains at a MOI of 10 and the infection was  
1194 allowed to proceed for 1 h before  $\beta$ -lactam substrate (CCF4-AM) was added. Translocation  
1195 efficiency is calculated as the ratio of cleaved and uncleaved CCF4. (D) The expression level  
1196 of the three TEM effector fusions in the different genetic backgrounds was analyzed by  
1197 immunoblot using an anti-TEM antibody to confirm a similar expression level in all strains.

1198  
1199 **Fig. 6.** Different effector genes are regulated by Fis proteins either positively (A and B) or  
1200 negatively (C and D). The expression level of *luc* fusions of four effector-encoding genes (*ralF*,  
1201 *sidG*, *ankG* and *marB*) was examined in wild-type strain (dark grey),  $\Delta fis1$  mutant (blue),  $\Delta fis2$   
1202 mutant (yellow) and  $\Delta fis1-2$  double mutant (green). Luciferase activity (solid lines) and  
1203 absorbance (dotted lines) were monitored for 96 hours as described in the Experimental  
1204 Procedures. Data expressed in Relative Light Units (RLU) are means and standard deviations  
1205 of technical triplicate and representative of at least three independent experiments.

1206  
1207 **Fig. 7.** *L. pneumophila* Fis1 and Fis2 proteins bind and regulate the promoter regions of *ralF*,  
1208 *pilE* and *icmT* genes. (A) The regulatory regions of *ralF*, *pilE* and *icmT* with their putative Fis-  
1209 binding sites (in orange boxes), the -10 promoter element (in blue) and transcription start (bold,  
1210 underlined) are represented. The mutations introduced in the putative Fis site are marked by  
1211 asterisks (B) The expression level of wild-type *ralF-luc*, *pilE-luc* and *icmT-luc*-fusions (in grey)  
1212 and the same fusions with mutations in the putative Fis-binding sites (*ralF\_mut-luc*, *pilE\_mut-*  
1213 *luc* and *icmT\_mut-luc*, in orange) were examined in the wild-type strain genetic context. (C)  
1214 Electrophoretic mobility shift assays were performed with purified PCR-amplified DNA

1215 fragment of promoter regions and increasing concentrations of purified native proteins Fis1 (0,  
1216 25, 50, 100  $\mu$ M) and Fis2 (0, 50, 100, 200  $\mu$ M) (left panel). On the right panel, the binding  
1217 experiments were performed by incubating the DNA fragment containing mutations in the Fis  
1218 binding site with the highest protein amount used in the left panel. The first lane of each panel  
1219 did not contain any protein (free DNA).

1220  
1221 **Fig. 8.** Ectopic expression of *Legionella* Fis1, Fis2 and Fis3 in *E. coli* BW25113. (A) Schematic  
1222 representation of regulation of motility (flagella), DNA topology (*gyrA*) and *fis* gene expression  
1223 by Fis in *E. coli*. Arrows and blunt arrows represent positive or negative regulation,  
1224 respectively. (B) Schematic representation of the different *E. coli* strain: *E. coli* BW25113 is  
1225 used as the wild-type strain; BWL0 is a *fis* null mutant and BWL1 et BWL2 express *fis1* and  
1226 *fis2* gene, respectively instead of the endogenous *fis* gene. It was not possible to construct the  
1227 BWL3 strain expressing *fis3* of *L. pneumophila*. (C) Swimming motility of *E. coli* strains on  
1228 0,3% soft agar plate. Bacteria were stabbed on the agar plate and incubated at 30°C for 16h. A  
1229 positive motility was indicated by diffused growth around the point of inoculation while growth  
1230 restricted around the stab point indicated a non-motile strain. (D and E) The expression level  
1231 of the transcriptional fusions  $P_{fis}$ -lux and  $P_{gyrA}$ -lux were examined in the *E. coli* BW25113 wild  
1232 type strain (black), BWL0  $\Delta fis$  mutant (dashed black), BWL1 mutant (yellow) and BWL2  
1233 mutant (blue). Luciferase activity was monitored for 6 hours.

1234  
1235 **Fig. 9.** Expression level of Fis1, Fis2 and Fis3 during bacterial growth. Three independent  
1236 cultures of *L. pneumophila* Paris WT were grown in liquid medium AYE (at 30°C). At the  
1237 desired OD600 (1, 2, 3, 4 and 5), samples were collected, and their protein content was analyzed  
1238 by mass spectrometry after sample-specific labelling (see Material and methods for details).

1239 The graph represents the mean relative abundance of each Fis proteins with the standard error  
1240 of the mean. Complete data set is presented in Table S3.

1241

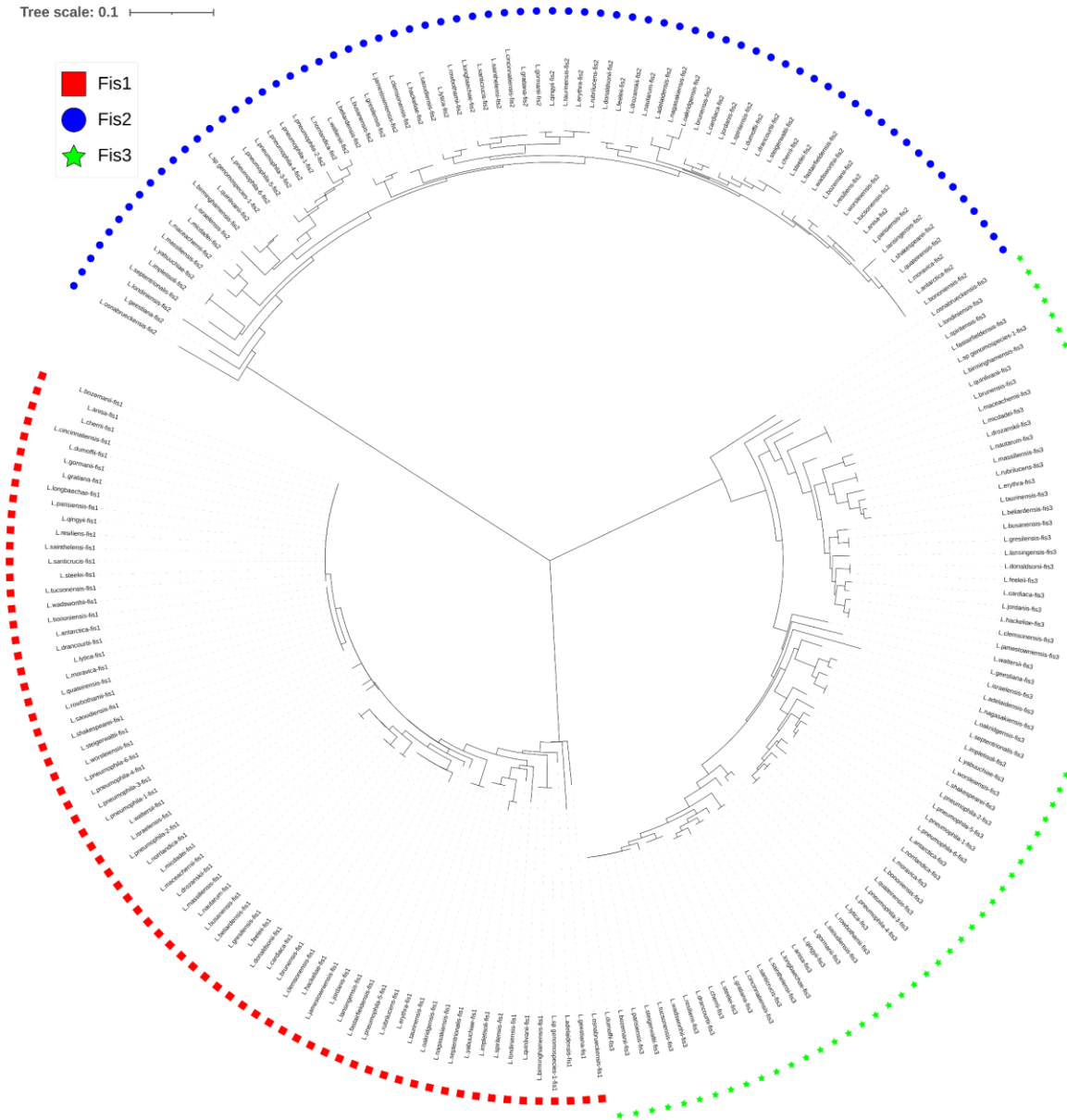
1242 **Fig. 10.** *Legionella* Fis proteins can form homodimers or heterodimers. (A) Sequence alignment  
1243 of Fis proteins from different bacteria: the three Fis proteins of *L. pneumophila* Paris, Fis from  
1244 *E. coli* MG1665 and *Salmonella enterica* serotype Thyphimurium (B) Crystal structure of Fis  
1245 from *E. coli* (Fis\_Ec) bound to 27 bp optimal binding sequence F1 (PDB  
1246 doi: <https://doi.org/10.2210/pdb3IV5/pdb>). (C) AlphaFold models of *L. pneumophila* Fis1,  
1247 Fis2, Fis3 homo- and heterodimer were generated by AlphaFold Multimer  
1248 (ColabFold/AlphaFold2.ipynb, Mirdita *et al.* 2022). The per-residue confidence (pLDDT)  
1249 metric and the interface predicted template modelling (ipTM) score were given for evaluating  
1250 the accuracy of the predictions. (D) Representative image of qualitative  $\beta$ -galactosidase assays  
1251 on agar plate. Overnight cultures of *E. coli* BTH101 co-expressing pKT25 and pUT18C  
1252 derivatives were spotted on LB-X-Gal plates, which were then incubated for at least 48 h at  
1253 30 °C. pKT25 fusions are shown in column and pUT18c fusions in rows, with the indicated  
1254 protein, empty vector or Zip positive control. The protein LssA encoded by the  
1255 *lssXYZABD* locus which constitute the core components of the *Legionella* T1SS is used as a  
1256 negative control as it is not expected to interact with Fis proteins. (E) The bacterial adenylate  
1257 cyclase two hybrid (BACTH) system was used to study protein–protein interactions between  
1258 Fis1, Fis2, Fis3 and the Fis protein of *E. coli* (Fis\_Ec). Specific  $\beta$ -galactosidase activity was  
1259 measured as described in Materials and Methods. Data represent the averages  $\pm$  standard  
1260 deviations of three technical replicates and three independent experiments. Statistical tests were  
1261 performed between the different conditions (Figure S3).

1262

1263

A

Tree scale: 0.1



B

Tree scale: 0.1

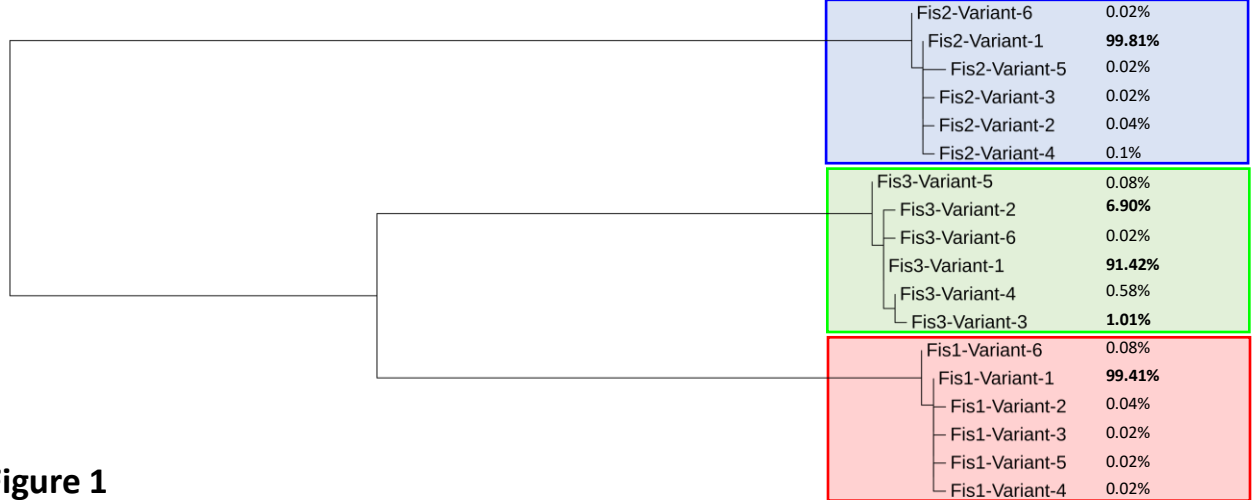
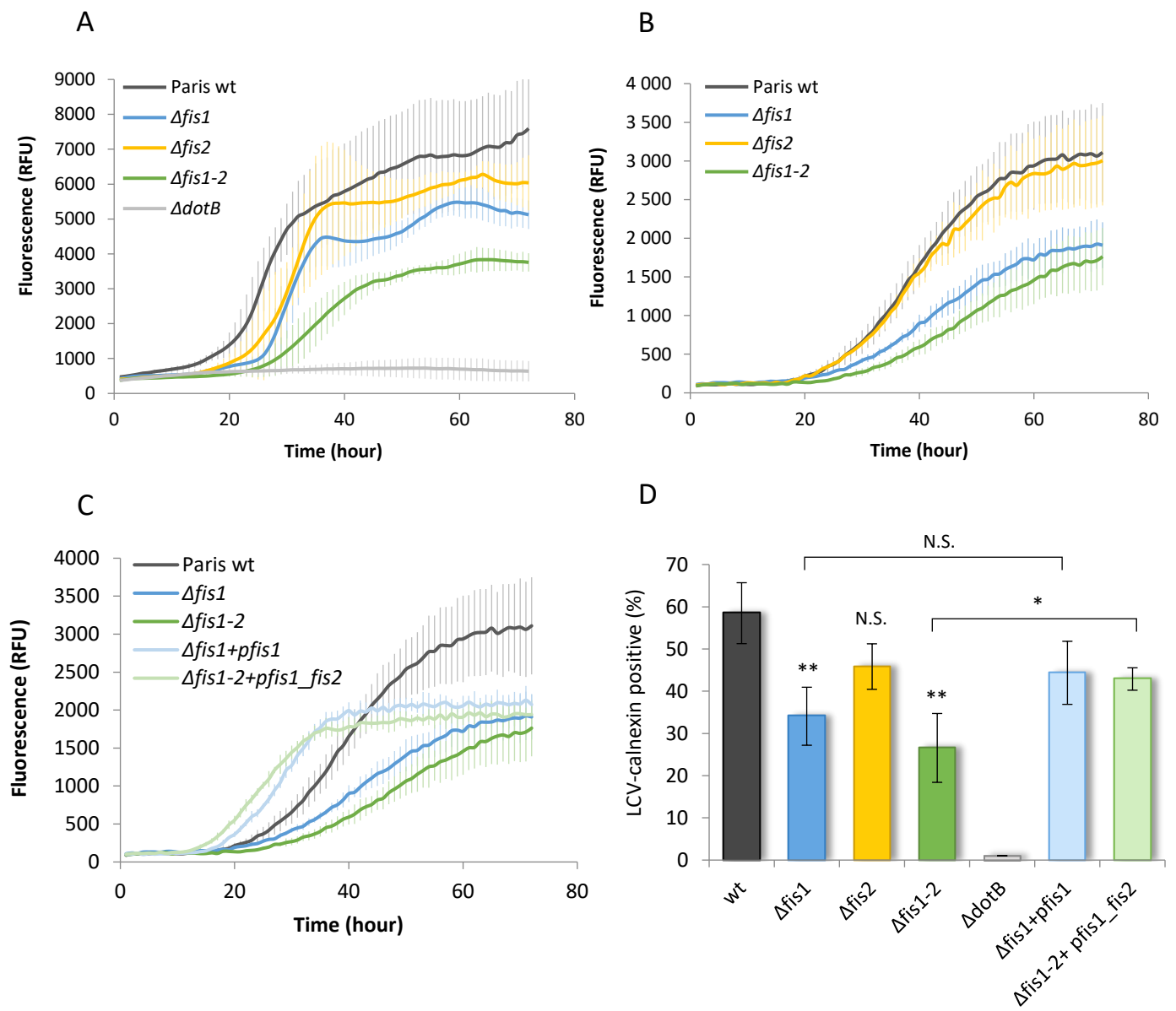


Figure 1

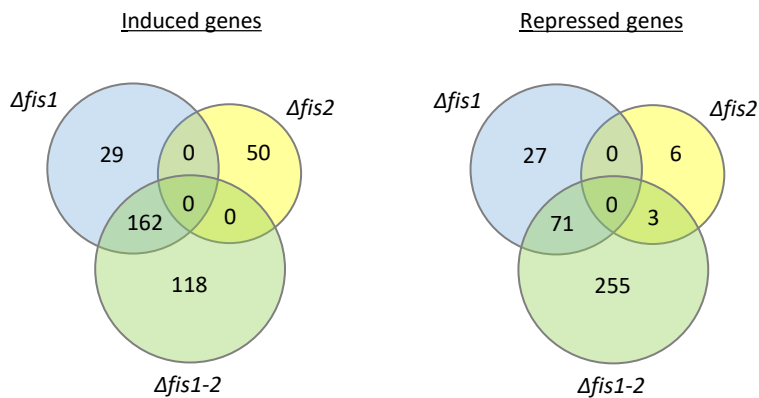


**Figure 2**

A

Growth phase	Mode of regulation	Number of genes showing altered expression in mutant		
		$\Delta fis1$	$\Delta fis2$	$\Delta fis1-2$
Post-exponential	Induced	191	50	280
	Repressed	98	9	329
	Total	279	59	609

B



C

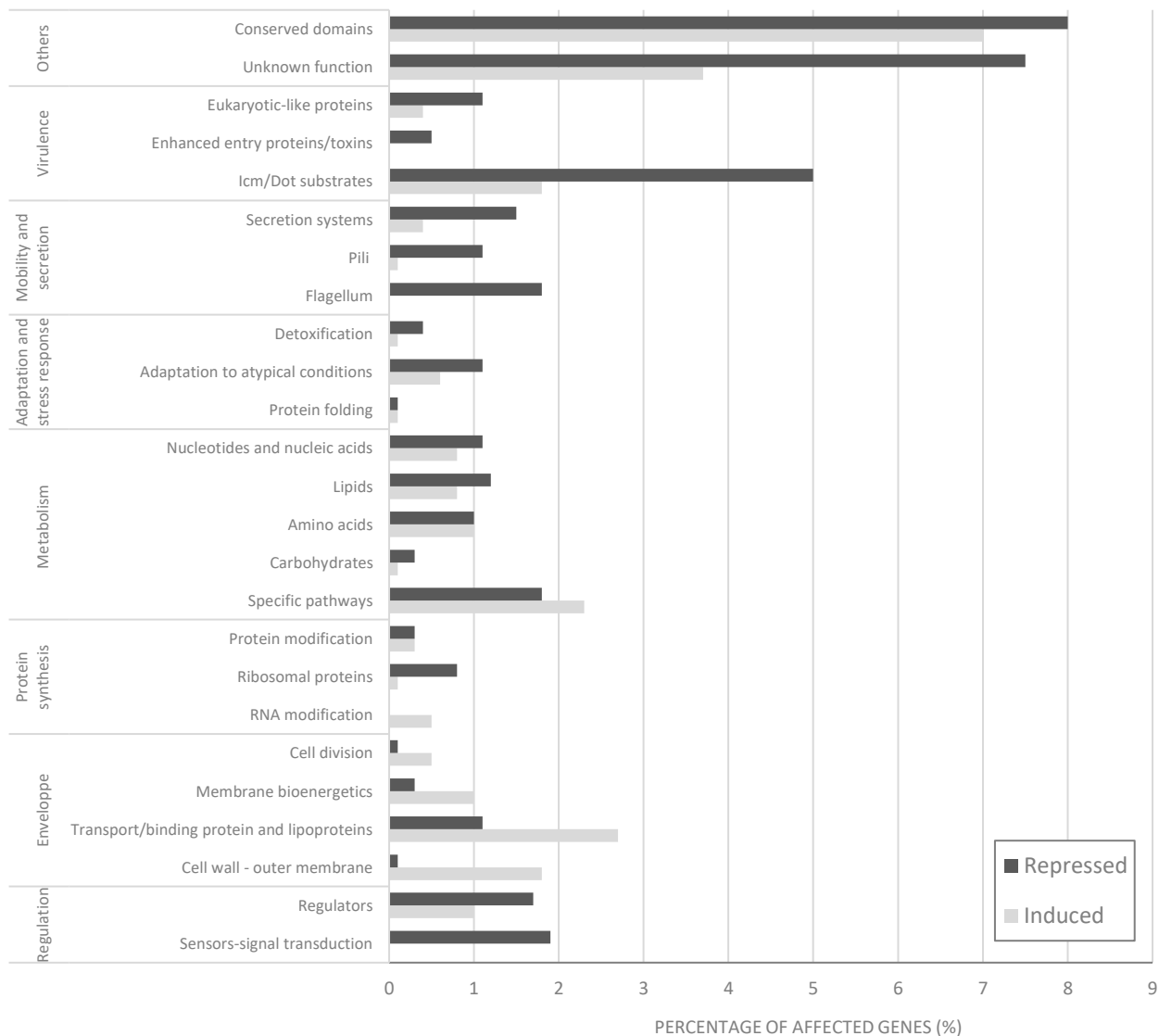
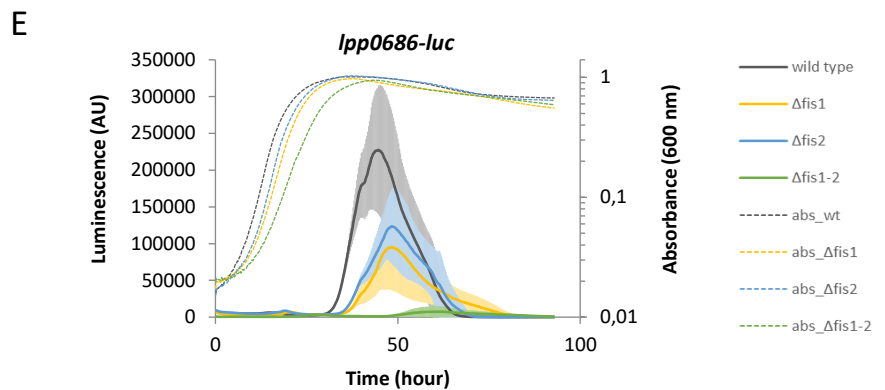
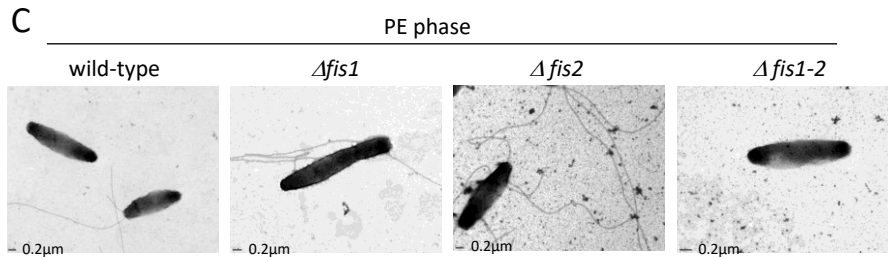
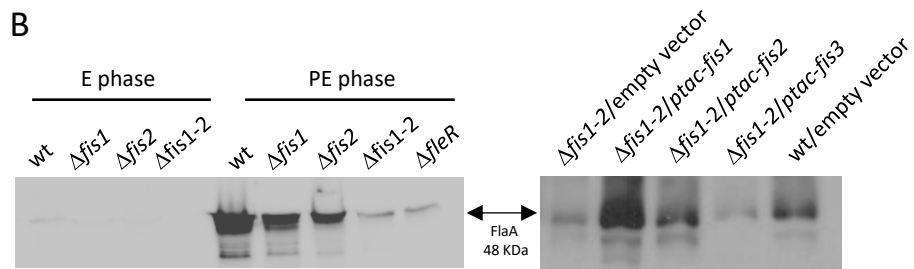
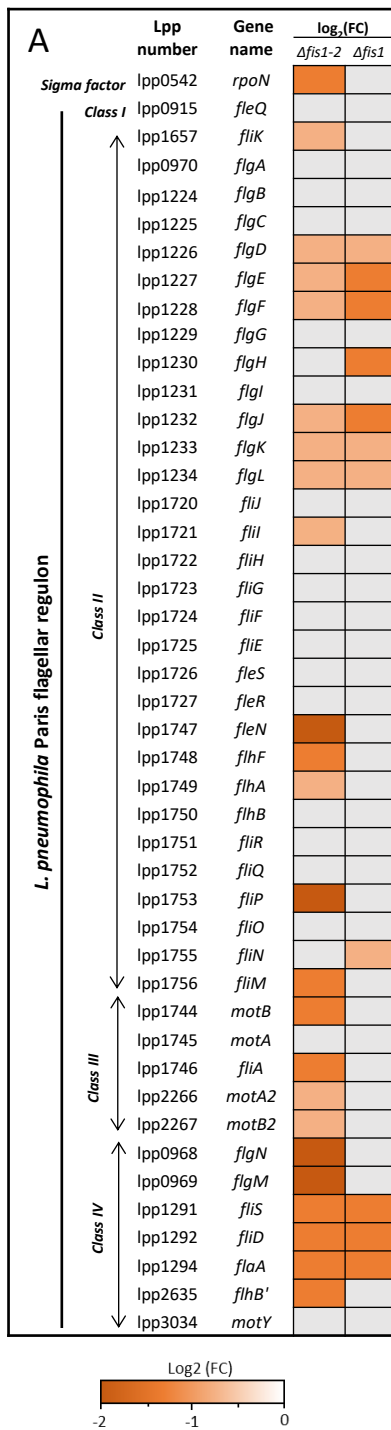
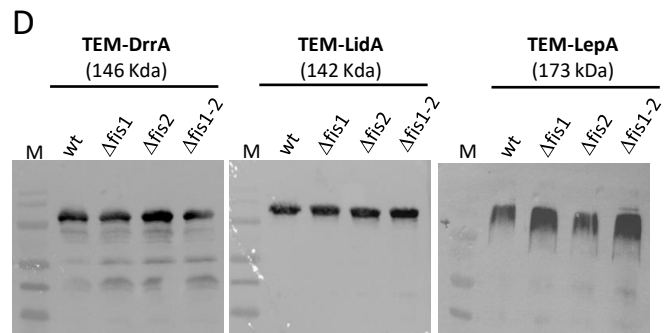
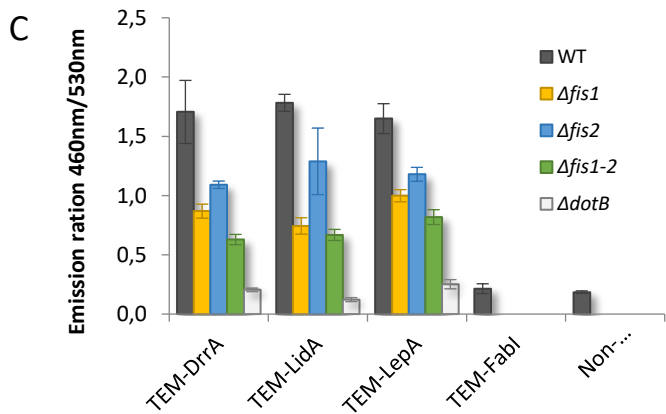
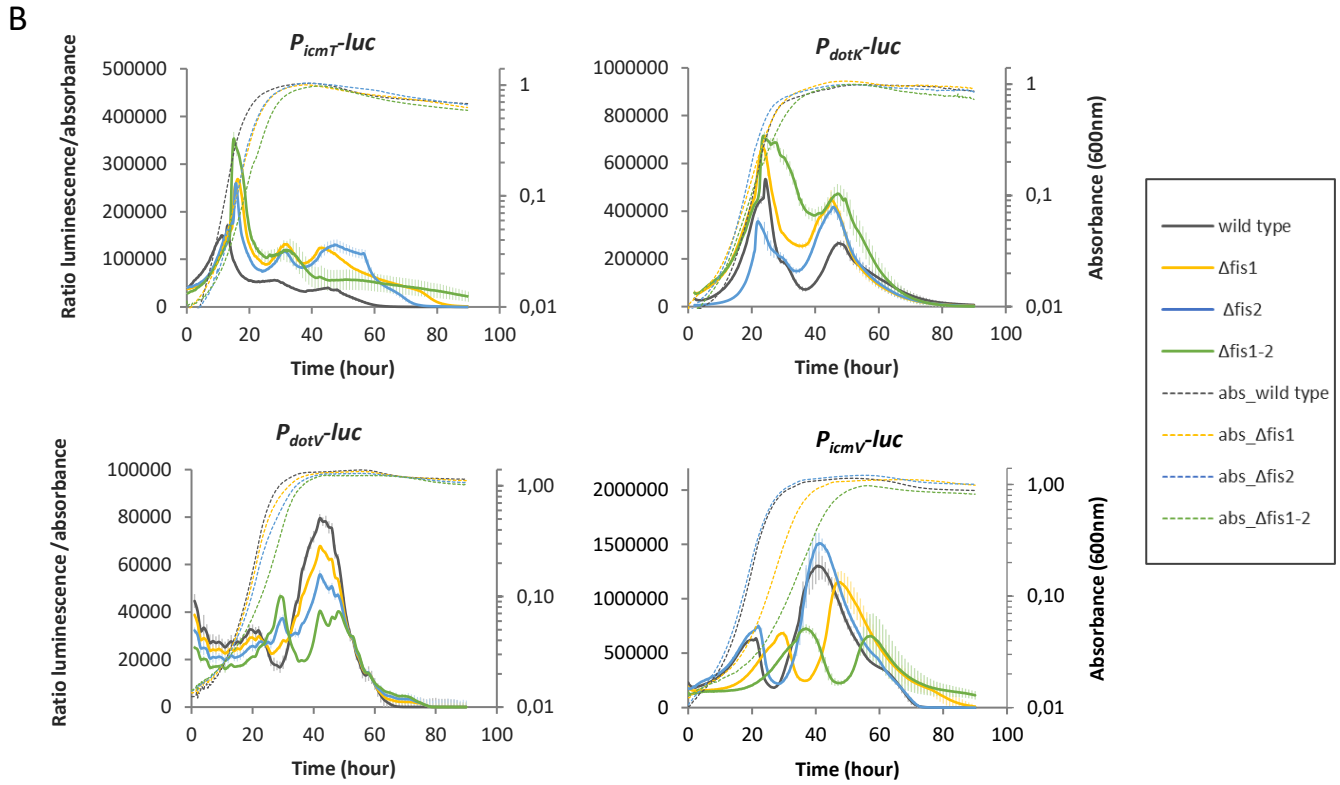
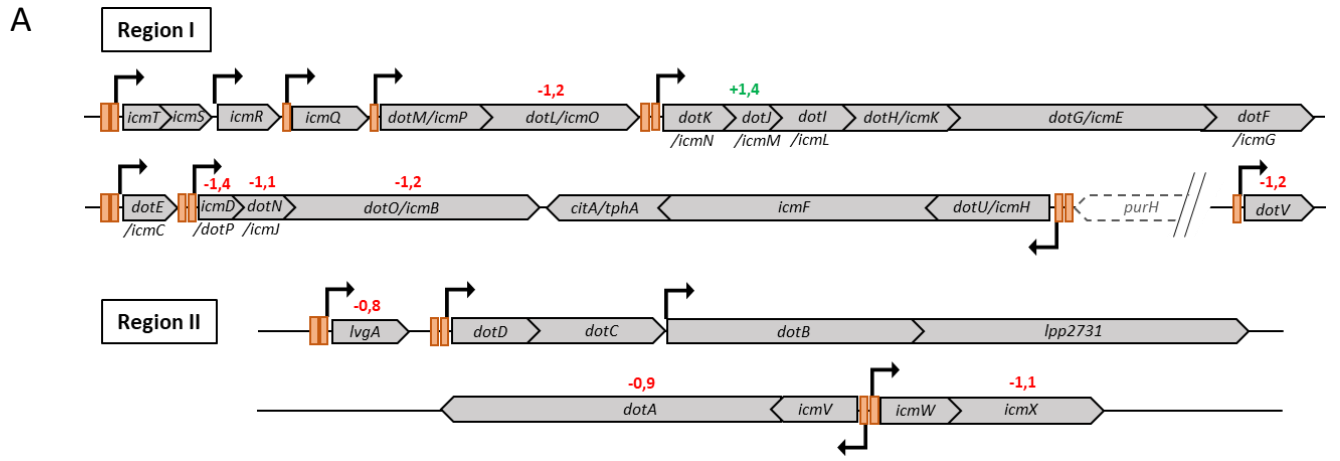


Figure 3

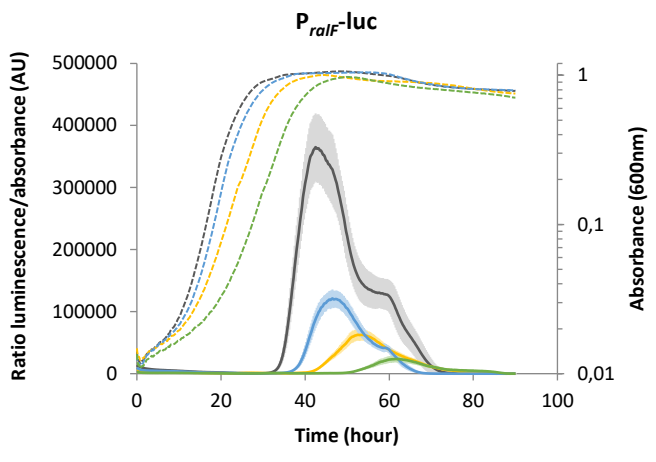
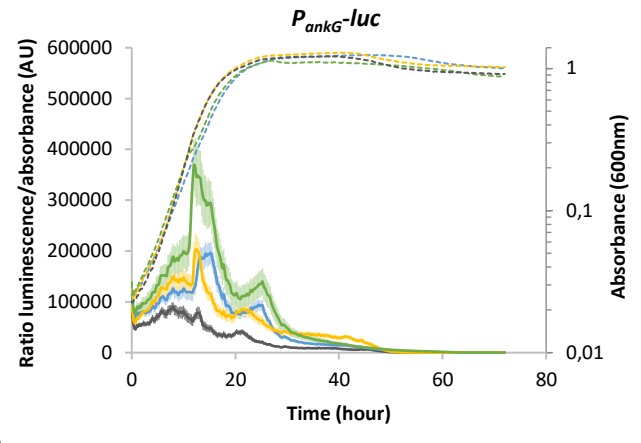
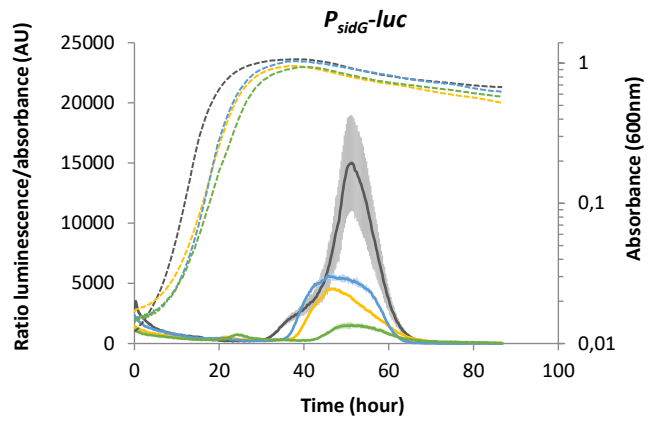
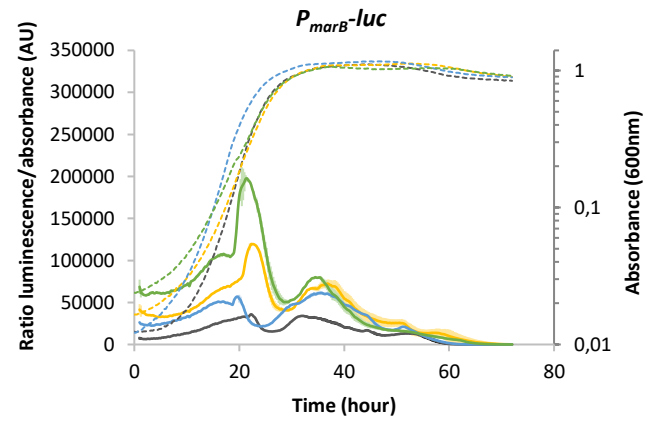


**Figure 4**



**Figure 5**



**A****C****B****D****Figure 6**

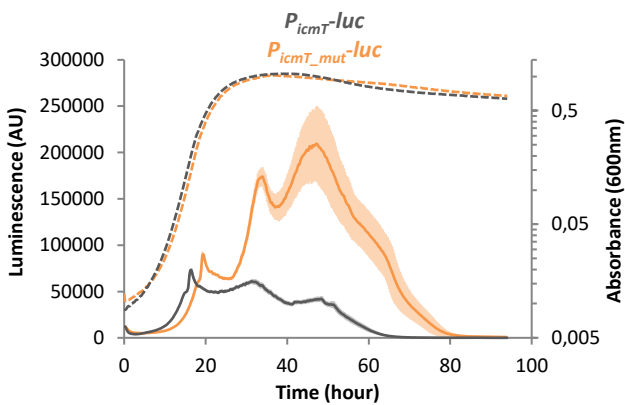
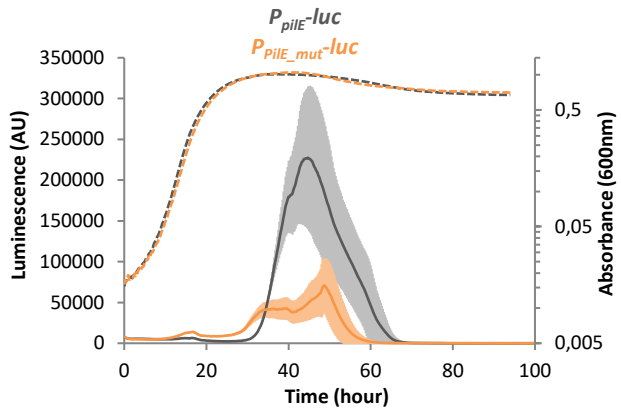
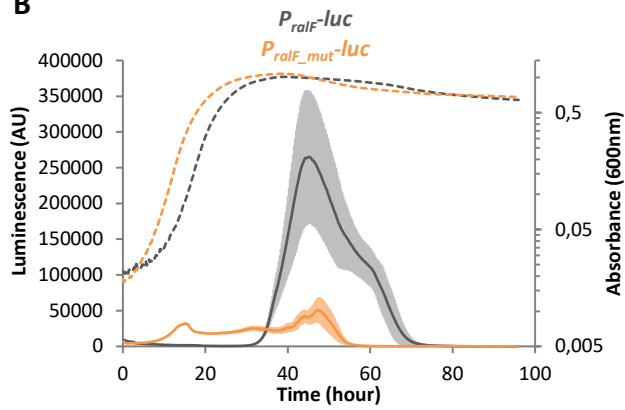
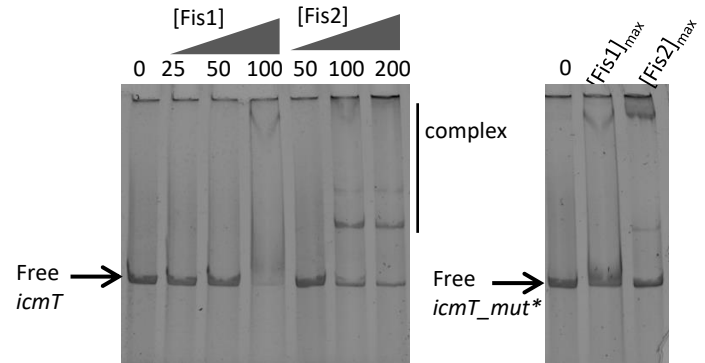
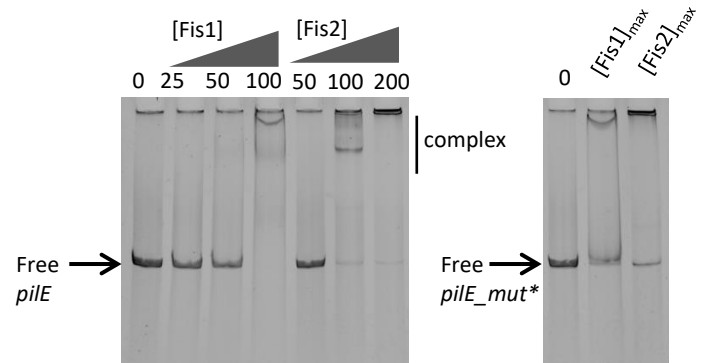
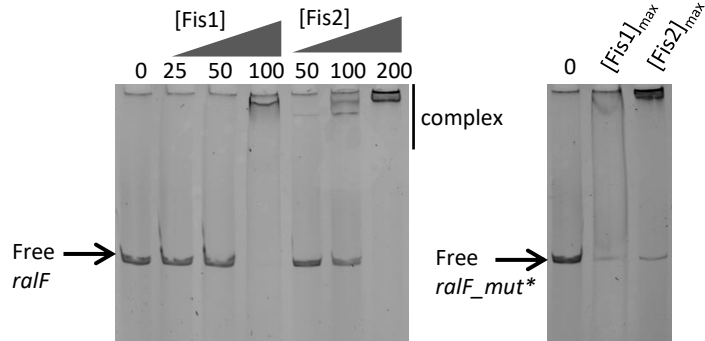
**A**

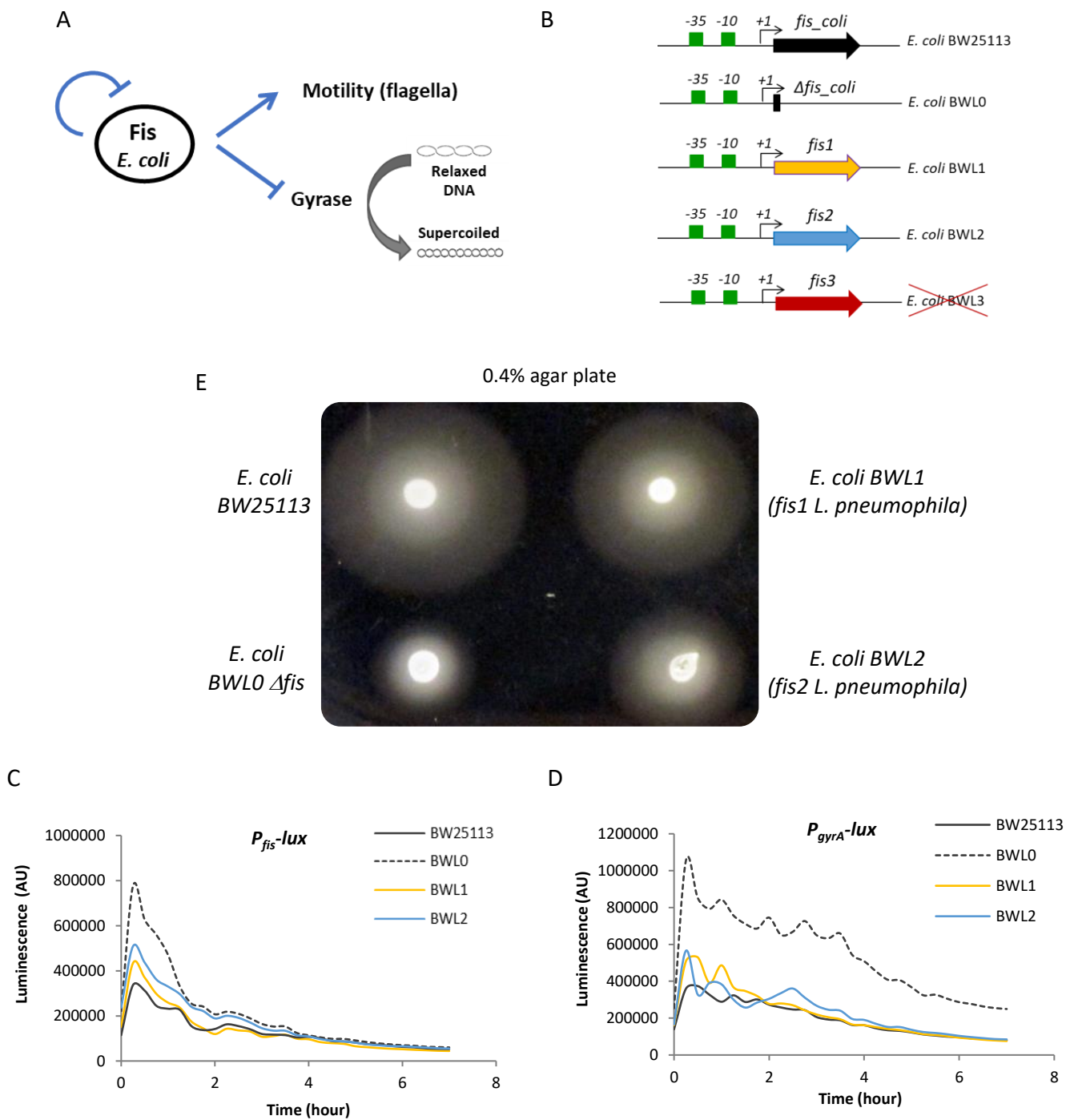
-10

*P<sub>ralF</sub>* ATCTGATTCTATATTAAGTAGTATAGAACGTATAAAGCACACTACGATATTTTGTAAATCATGAAGAAGCTAAAGCTGCAAGTATTTCTTGTCTAAAAGGCCAAAATTAGATCAATTAAGTTATAATATAATCAAAA

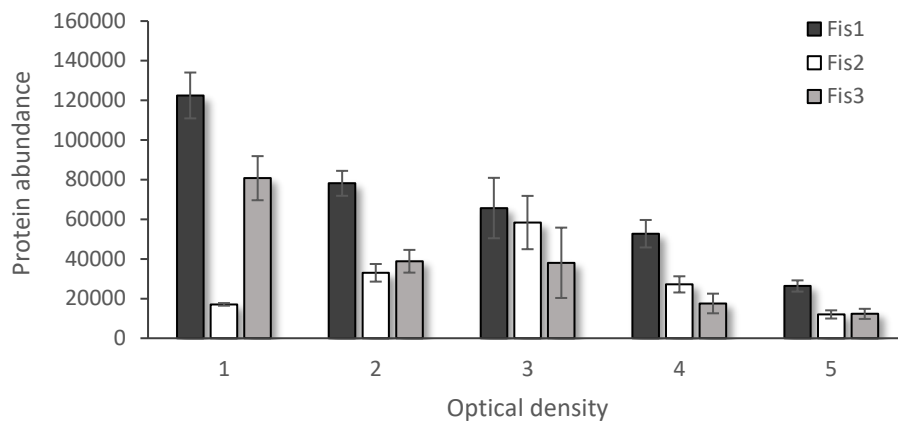
*P<sub>pilE</sub>* GCCCCTATATAATTCCTCTATACAACCTTGCTAACCGGACTATCTTAAATGCAATAGCTAACTGGACCAAGCATTGGCTAGCCAATTATTTGTAAAAATTTAACCAATTGAACTATAATTAACAATGTA

*P<sub>icmT</sub>* ATGCAAAATATTTATCTAATATGTTAGGATATCATCTAAC-70-CGTGTATGCAATATCAATTGCATAAAAAATAATCTTAATTTAAATTTCTTAAAAGTATCA-16-TTTTAAGTATATACTTTTTTTAAAG

**B****C****Figure 7**



**Figure 8**



**Figure 9**

