



HAL
open science

The iron-sulfur cluster sensor IscR is a negative regulator of Spi1 Type III Secretion System in *Salmonella enterica* Cellular Microbiology

Alexandra Vergnes, Julie Viala, Ouadah-tsabet Rabah, Bérengère Pocachard, Laurent Loiseau, Stephane Meresse, Frédéric Barras, Laurent Aussel

► To cite this version:

Alexandra Vergnes, Julie Viala, Ouadah-tsabet Rabah, Bérengère Pocachard, Laurent Loiseau, et al.. The iron-sulfur cluster sensor IscR is a negative regulator of Spi1 Type III Secretion System in *Salmonella enterica* Cellular Microbiology. Cellular Microbiology, 2017, 19 (4), pp.e12680. 10.1111/cmi.12680 . hal-01542927

HAL Id: hal-01542927

<https://amu.hal.science/hal-01542927v1>

Submitted on 23 Jun 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **The iron-sulfur cluster sensor IscR is a negative regulator of Spi1 Type III**
2 **Secretion System in *Salmonella enterica***

3

4 Running title: Regulation of Spi1 TTSS by IscR

5

6

7 Alexandra Vergnes¹, Julie P.M. Viala^{1,#}, Rabah Ouadah-Tsabet¹, Bérengère
8 Pocachard¹, Laurent Loiseau¹, Stéphane Méresse², Frédéric Barras¹, and Laurent
9 Ausseil^{1*}

10

11

12

13 ¹Aix Marseille Université, CNRS, LCB UMR 7283, IMM, 13402, Marseille,
14 France ²Aix Marseille Université, CNRS, INSERM, CIML, Marseille, France.

15

16

17

18

19 *Corresponding author. Mailing address: Laboratoire de Chimie Bactérienne, 31
20 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France.

21 Phone: +33 4 91 16 44 31. Fax +33 4 91 71 89 14. E-mail: ausseil@imm.cnrs.fr

22

23

24 # Present address: Laboratoire d'Ingénierie des Systèmes Macromoléculaires, 31
25 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France.

26 **Summary**

27

28 Iron-sulfur (Fe-S)-containing proteins contribute to various biological processes,
29 including redox reactions or regulation of gene expression. Living organisms have
30 evolved by developing distinct biosynthetic pathways to assemble these clusters,
31 including ISC (Iron Sulfur Cluster) and SUF (Sulfur mobilization). *Salmonella enterica*
32 serovar Typhimurium is an intracellular pathogen responsible for a wide range of
33 infections, from gastroenteritis to severe systemic diseases. *Salmonella* possesses all
34 known prokaryotic systems to assemble Fe-S clusters, including ISC and SUF.
35 Because iron starvation and oxidative stress are detrimental for Fe-S enzyme
36 biogenesis and because such environments are often met by *Salmonella* during its
37 intracellular life, we investigated the role of the ISC and SUF machineries during the
38 course of the infection. The *iscU* mutant, which is predicted to have no ISC system
39 functioning, was found to be defective for epithelial cell invasion and for mice infection,
40 whereas the *sufBC* mutant, which is predicted to have no SUF system functioning, did
41 not present any defect. Moreover, the *iscU* mutant was highly impaired in the
42 expression of Spi1 TTSS that is essential for the first stage of *Salmonella* infection.
43 The Fe-S cluster sensor IscR, a transcriptional regulator matured by the ISC
44 machinery, was shown to bind the promoter of *hilD*, which encodes the master
45 regulator of Spi1. IscR was also demonstrated to repress *hilD* and subsequently Spi1
46 gene expression, consistent with the observation that an *iscR* mutant is hyperinvasive
47 in epithelial cells. Collectively, our findings indicate that the ISC machinery plays a
48 central role in *Salmonella* virulence through the ability of IscR to down-regulate Spi1
49 gene expression. At a broader level, this model illustrates an adaptive mechanism used

50 by bacterial pathogens to modulate their infectivity according to iron and oxygen
51 availability.

52 **Introduction**

53

54 Iron-sulfur (Fe-S) cluster-containing proteins play major roles in a wide variety of
55 process, including redox reactions, regulation of gene expression or metabolic
56 pathways (Kiley and Beinert, 2003). Assembly and delivery of Fe-S clusters are
57 catalysed by multi-protein systems, ISC (Iron Sulfur Cluster) and SUF (Sulfur
58 mobilization), conserved in most living organisms. Briefly, sulfur is mobilized from L-
59 cysteine by IscS/SufSE (Hidese *et al.*, 2011), and scaffold proteins (IscU/SufBC) bind
60 both sulfur and iron to assemble a cluster before transferring it to the apo-protein *via*
61 the A-type carriers IscA/SufA (Bonomi *et al.*, 2005; Vinella *et al.*, 2009; Wollers *et al.*,
62 2010) (Figure S1). In *Escherichia coli* and most Gram-negative bacteria, the ISC
63 system is encoded by the *iscRSUA-hscBA-fdx* operon while the SUF system is
64 encoded by the *sufABCDSE* operon (Fig. S1). Other so-called "non-ISC non-SUF"
65 proteins intervene at the sulfur production step (CyaY), at the transfer steps (ErpA,
66 NfuA, Mrp), or, possibly, at the repair step (YggX).

67

68 In the last decade, several studies reported the importance of ISC and/or SUF systems
69 in sustaining virulence of various bacteria. Indeed, iron starvation and oxidative stress
70 are detrimental for Fe-S enzyme biogenesis, and such environments are often met by
71 bacterial pathogens upon infection of their host. Thus, the SUF and the ISC systems
72 are required for full virulence of the plant pathogen *Dickeya dadantii* (Nachin *et al.*,
73 2001; Rincon-Enriquez *et al.*, 2008). In *Shigella flexneri*, inactivation of the ISC system
74 prevents plaques formation because the strain is non-invasive (Runyen-Janecky *et al.*,
75 2008). The SUF system is essential for *Mycobacterium tuberculosis* survival and

76 therefore required for virulence (Huet *et al.*, 2005). In this context, the case of the *iscR*
77 gene deserves specific attention.

78

79 In *E. coli*, IscR is a [2Fe-2S]-containing protein whose maturation is primarily catalyzed
80 by the ISC system. The holo-form of IscR acts as a repressor of a large number of
81 genes, including the *isc* operon. The apo-form of IscR acts as an activator of a few
82 genes, including the *suf* operon. Hence, under iron limitation or in the presence of
83 reactive oxygen species (ROS), IscR exists primarily under its apo-form that activates
84 expression of SUF and no longer represses expression of *isc*, the amount of Fe-S
85 clusters produced being at its highest level. As IscR maturation is catalyzed by the ISC
86 system, a defect in this latter leads to the accumulation of apo-IscR and activation of
87 the SUF system. This regulatory connection between ISC and SUF must be taken into
88 account when interpreting the phenotype of ISC-defective strains. For instance, a *D.*
89 *dadantii iscR* mutant is non-invasive on *Saintpaulia ionantha* plants because the SUF
90 system can't be activated by IscR, but it presents a wild-type phenotype on *Arabidopsis*
91 *thaliana* presumably because the demand in Fe-S clusters to be satisfied for the
92 bacterium to grow is met by ISC in this case (Rincon-Enriquez *et al.*, 2008). In fact,
93 IscR appears to be important for full virulence of several pathogens. Mutations in *iscR*
94 reduce virulence of *Pseudomonas aeruginosa* and *Xanthomonas campestris* (Kim *et*
95 *al.*, 2009; Fuangthong *et al.*, 2015). Moreover, recent studies have shed new light on
96 the role of IscR by expanding its zone of influence beyond the usual Fe-S cluster
97 containing proteins; namely, in enterotoxigenic *Escherichia coli*, IscR was shown to
98 bind upstream *cfaA* and to activate CFA/I fimbriae production in response to iron
99 starvation (Haines *et al.*, 2015). In *Yersinia pseudotuberculosis*, IscR was found to be
100 involved in the regulation of the Type III secretion system (TTSS) master regulator LcrF

101 and was demonstrated to be essential for the virulence of *Yersinia* in a mouse model
102 of infection (Miller *et al.*, 2014).

103

104 *Salmonella enterica* serovar Typhimurium is a facultative intracellular pathogen that is
105 associated with a wide range of infections in mammals, ranging from self-limiting
106 gastroenteritis to severe systemic diseases. During the first stage of infection, the
107 bacterium invades intestinal epithelial cells using a TTSS encoded by *Salmonella*
108 Pathogenicity Island 1 (Spi1) (Galán and Curtiss, 1989). Afterwards, it survives and
109 replicates in macrophages within a membrane-bound compartment called the
110 *Salmonella*-containing vacuole (SCV) using a TTSS encoded by Spi2 (Figueira and
111 Holden, 2012). Synthesis of the Spi1 TTSS apparatus and most of its effectors is
112 controlled by a complex regulatory scheme. HilA activates directly the expression of
113 the *inv* and *prg* operons, which encode the TTSS apparatus (Ellermeier *et al.*, 2005).
114 Expression of *hilA* is under the control of many environmental conditions and three
115 transcriptional activators: HilC, HilD and RtsA (Ellermeier *et al.*, 2005).

116

117 Among the key points leading to the establishment of infection is the ability of the
118 pathogen to adapt to various environments and to resist different stresses, including
119 iron limitation and ROS production. Recently, CyaY and YggX, both involved in iron-
120 sulfur biogenesis, were demonstrated to be important for *Salmonella* virulence in mice
121 (Velayudhan *et al.*, 2014). Here, we investigated the involvement of ISC and SUF in
122 *Salmonella*. Whereas no major role was attributed to the SUF system, the ISC
123 machinery was found to be essential for epithelial cell invasion and we explained this
124 by revealing a molecular link between Fe-S cluster and Spi1 TTSS functioning. In
125 particular, we showed that IscR represses the synthesis of the Spi1-carried regulator

126 HilD, decreasing the expression of genes encoding the TTSS apparatus. Thus, the
127 IscR regulator can be exploited by *Salmonella* as a sensor to modulate its invasiveness
128 depending on the stage of the infection and the compartment infected.

129 **Results**

130

131 **The ISC machinery is essential for HeLa cell invasion and virulence in mice**

132 In order to evaluate the contribution of the ISC and SUF systems to *Salmonella*
133 intracellular proliferation, we infected activated RAW 264.7 mouse macrophages with
134 *Salmonella* wild-type strain, *iscU* and *sufBC* mutants. Bacterial proliferation was
135 assayed by calculating the proliferation index as a ratio of the intracellular bacteria
136 between 16 and 2 hours post-infection. The proliferation index of the wild-type strain
137 and the *iscU* mutant were very close (8.8 and 9.4, respectively), whereas it was slightly
138 higher for the *sufBC* mutant (12.2) (Fig. 1A), reflecting no major role for Fe-S proteins
139 during the intracellular proliferation of macrophages. Next, HeLa cell invasion was
140 carried out. Each strain was incubated for ten minutes with HeLa cells followed by one
141 hour of gentamicin treatment. We found the *iscU* mutant to invade epithelial cells much
142 less efficiently than the wild-type (Fig. 1B), whereas the *sufBC* mutant invasion showed
143 no defect compared to the wild-type (Fig. 1B). Next, mixed infections of mice were
144 carried out using wild-type and mutants in order to determine the competitive index
145 (CI) and to compare virulence of the strains (Beuzón and Holden, 2001). The injection
146 of the inoculum was either intraperitoneal (IP), during which bacterial cells can rapidly
147 reach their target organs (Fig. 1C & Table S1), or oral to force the pathogen to cross
148 the intestinal barrier before accessing the blood system (Fig. 1D & Table S2). By
149 performing intraperitoneal infections, we found the *iscU* mutant to be affected for its
150 virulence (CI = 0.304) (Fig. 1C & Table S1). In contrast, the CI of the *sufBC* mutant did
151 not show any significant alteration of virulence compared with the wild-type strain (Fig.
152 1C & Table S1). Lastly, mice oral infections were carried out. We observed a severe

153 virulence defect of the *iscU* mutant (CI = 0.011) (Fig. 1D & Table S2), 30-fold less
154 compared to intraperitoneal infection and 90-fold less compared to the wild-type. The
155 *sufBC* mutation had no effect (CI = 0.758) (Fig. 1D & Table S2). Taken together, these
156 results indicate that the ISC machinery plays a major role in *Salmonella* virulence
157 during the invasion of epithelial cells and in the mouse model.

158

159 **The ISC system plays a key role in the regulation of Spi1 Type III Secretion**

160 **System gene expression**

161 To test the involvement of Fe-S biosynthesis machineries in Spi1 TTSS regulation, the
162 promoter of *hilD* - which was shown to be activated to allow Spi1 TTSS biogenesis
163 (Fig. 2A) - was fused to GFP and inserted into the pFPV25 plasmid, yielding the *PhilD-*
164 *gfp* transcriptional fusion. Such constructs were previously used in *Salmonella* to report
165 gene expression within host cells (Valdivia and Falkow, 1997; Aussel *et al.*, 2011).
166 Cells were grown in LB and the fusion expression levels were measured after 16 hours
167 of culture. Expression of the *PhilD-gfp* fusion was found to be 22% lower in an *iscU*
168 mutant compared with the wild-type, whereas the *sufBC* mutation had no effect on the
169 expression of this fusion (Fig. 2B). We next tested whether the effect of the *iscU*-
170 mediated reduction had an impact on the expression of the *hilD*-regulated genes such
171 as *rtsA*, *prgH* and *invF*. Transcriptional fusions were constructed for each gene and
172 their expression assayed in *iscU* and *sufBC* backgrounds. Compared to the wild-type
173 strain, the *iscU* mutant exhibited a decrease in the expression of *rtsA* (4.1-fold), *prgH*
174 (5.3-fold) and *invF* (7-fold) (Fig. 2C, 2D and 2E). The *sufBC* mutation had no effect on
175 the expression level of these genes. Altogether, these results showed that Spi1 TTSS

176 genes are down-regulated in an *iscU* mutant, conferring a key role to the ISC
177 machinery in the synthesis of this secretion apparatus.

178

179 **IscR binds on the promoter of *hilD***

180 Because the ISC machinery was found to play a role in the regulation of Spi1 TTSS
181 gene expression, we aimed at identifying a protein matured by ISC and involved in the
182 control of the Spi1 TTSS apparatus. Two putative binding sites of IscR - a [2Fe-2S]-
183 containing regulator matured by the ISC system - were found in the *hilD* promoter
184 region. Only three amino acids differ between *E. coli* and *Salmonella* IscR proteins
185 (98% identity) and none of these residues are in the DNA binding domain, supporting
186 the idea that identical motifs can be recognized by both proteins (Rajagopalan *et al.*,
187 2013). The two putative IscR binding sites upstream of *hilD* resemble Type 2 motifs
188 which can be bound by both holo- and apo-IscR (Giel *et al.*, 2006). Sites 1 and 2 lie,
189 respectively, from -105 to -81 (ATTACCCAATTTGGGTTCTTTTGG) and from -65 to
190 -41 (ATTGCCAACACACGCTAATAAAGAG) upstream of the *hilD* gene transcription
191 start site (Fig. 3A). Comparison of these two sites with the IscR sites from *E. coli hyaA*
192 and *Yersinia pseudotuberculosis IcrF* shows that they contain five and four of the nine
193 bases in this binding motif, respectively (Fig. 3B) (Giel *et al.*, 2006). Importantly, Site 1
194 overlaps with a previously identified HilD binding site (Fig. 3A) (Olekhovich and
195 Kadner, 2002). To test the interaction between IscR and the promoter of *hilD* (*PhilD*),
196 electrophoretic mobility shift assays (EMSA) were carried out. When incubated with
197 increasing concentrations of purified His₆-IscR, the *PhilD* probe shifted gradually (Fig.
198 3C). IscR was able to form four different complexes, consistent with the presence of
199 two Type 2 motifs on *PhilD* and with the ability of IscR to form dimers (Fig. 3C). Next,

200 three nucleotides were mutated at the positions 1, 5 and 6 of the Type 2 motifs on the
201 *hilD* promoter (ATTACC was changed to CTTAGG on the site 1 and ATTGCC was
202 changed to CTTGGG on the site 2). When incubated with increasing concentrations of
203 IscR, the resulting DNA fragment, called *PhilD**, displayed a more modest shift
204 compared to *PhilD*, highlighting the importance of these conserved nucleotides in the
205 interaction between IscR and the promoter of *hilD* (Fig. 3D and 3E). Finally, to confirm
206 the direct interaction between IscR and *PhilD*, DNA-protein interaction experiments
207 were carried out using Bio-Layer Interferometry (BLI), a technique previously used to
208 study protein-protein interactions (Arlet *et al.*, 2014). IscR was also found to interact
209 with *PhilD* in this assay, whereas no interaction was detected with the promoter of
210 *ahpC* used as a negative control (Fig. 3F). Interestingly, this experiment allowed us to
211 measure a dissociation constant (Kd) of 390 nM between IscR and *PhilD*, consistent
212 with previous values of 93 and 112 nM measured between *E. coli* IscR and the *iscR*
213 promoter (Giel *et al.*, 2013). Taken together, the results presented in this panel
214 demonstrate a direct interaction between IscR and the promoter of *hilD*.

215

216 **IscR represses the expression of *hilD* gene and HilD targets**

217 Next, to investigate the regulatory role of IscR on *hilD*, the expression of the *hilD* fusion
218 was measured and found to be about 1.5-fold up-regulated in an *iscR* mutant (Fig. 4A),
219 suggesting that IscR acts as a negative regulator. Interestingly, the expression level of
220 *hilD* was reduced to a similar extent (2.5-fold) in the *hilD* and in the *hilD iscR* mutants,
221 consistent with a scenario where IscR could act as an anti-activator on HilD. Because
222 the extent of the regulatory effect was only 1.5-fold, we tested whether the IscR-
223 mediated regulation of *hilD* had any effect on the expression of downstream HilD-

224 regulated genes. The expression of the *rtsA*, *prgH* and *invF* fusions was measured in
225 a wild-type, *iscR*, *hilD* and *hilD iscR* mutant strains. A *hilD* mutation abrogated the
226 expression of *rtsA*, *prgH* and *invF* (Fig. 4B, 4C and 4D). In contrast, the *iscR* mutant
227 exhibited a 1.5-fold increase in *rtsA* expression (Fig. 4B) and a 2-fold increase in *prgH*
228 and *invF* expression (Fig. 4C and 4D). In the *hilD iscR* mutant, the expression level of
229 the *rtsA*, *prgH* and *invF* fusions was found to be lower than 10 units but higher than the
230 *hilD* mutant values (Fig. 4B, 4C and 4D). The *iscR* and *hilD* mutant strains were also
231 complemented by plasmid-borne wild-type copies of *iscR* and *hilD* under an arabinose-
232 inducible promoter, respectively (Fig. S2). To investigate further the regulatory effect
233 of IscR, quantitative reverse transcription PCR (qRT-PCR) experiments were carried
234 out with the genes previously analysed. The *iscR* mutation led to a 5.5-fold increase in
235 *hilD* mRNA level compared to the wild-type strain, confirming that IscR acts as a
236 repressor of *hilD* (Fig. 4E). Consistently, *rtsA*, *prgH* and *invF* mRNA levels were found
237 to increase significantly in the *iscR* mutant (2.5-, 7- and 5.5-fold, respectively) whereas
238 they decreased in the *hilD* mutant (Fig. 4F, 4G and 4H). Noticeably, IscR was not found
239 to shift the *PinvF* probe in EMSA, supporting the notion that expression of *invF* is
240 indirectly regulated by IscR (Fig. S3). In the *hilD iscR* mutant, the *rtsA*, *prgH* and *invF*
241 mRNA levels were 360-, 60- and 460-fold lower compared to the wild-type strain,
242 showing that the expression of *rtsA*, *prgH* and *invF* is totally abrogated in the absence
243 of both *iscR* and *hilD* and suggesting a possible Spi1 gene expression reprogramming
244 process in this background (Fig. 4F, 4G and 4H). Collectively, these results indicate
245 that IscR acts as a repressor of *hilD* gene expression, leading to the down-regulation
246 of HilD targets.

247

248 **Apo- and holo-IscR down-regulate Spi1 TTSS gene expression**

249 To investigate whether IscR acts on Spi1 TTSS gene expression under its apo- or holo-
250 form, we built a plasmid encoding an IscR variant (IscR-3CA). This IscR variant has all
251 three cluster-coordinating cysteine residues (Cys92, Cys98 and Cys104) converted
252 into alanine, which prevents binding of the [2Fe-2S] cluster (Rajagopalan *et al.*, 2013).
253 The *iscR* mutant was transformed with an empty plasmid, with a plasmid encoding
254 IscR or with a plasmid encoding IscR-3CA. In an *iscR* mutant, expression of the *prgH*
255 gene was down-regulated 2.4-fold in the presence of *piscR* while it was down-regulated
256 1.6-fold in the presence of *piscR*-3CA (Fig. 5A, left). This indicated that both forms
257 have a negative effect on *prgH* expression. Similarly, the *invF* gene was down-
258 regulated 1.7-fold in an *iscR/piscR* strain and 1.3-fold in an *iscR/piscR*-3CA strain,
259 again indicating that apo- and holo-IscR modified *invF* gene expression (Fig. 5A, left).
260 The experiment was also carried out in a wild-type strain in which the overexpression
261 of *iscR* reduced the expression of *prgH* and *invF* (2.9- and 2.7-fold, respectively) (Fig.
262 5A, right). The same observation was made when the *iscR*-3CA variant was
263 overexpressed (1.9- and 2.1-fold repression compared to the empty plasmid). Taken
264 together, these results show that both apo- and holo-IscR down-regulate the
265 expression of the Spi1 TTSS gene.

266

267 **IscR mediates iron-dependent control of Spi1 TTSS genes**

268 It was previously shown that iron limitation down-regulates the expression of Spi1
269 TTSS genes (Ellermeier and Slauch, 2008; Teixidó *et al.*, 2011). Therefore, we tested
270 whether IscR could have a role in mediating this regulation. Expression of *prgH* and
271 *invF* genes was monitored in bacteria grown in the presence of 2,2'-dipyridyl (dip), an
272 iron chelator (Fig. S4). In the presence of dip, the level of *prgH* and *invF* expression

273 dropped 8.8- and 5.7-fold compared with untreated cells (Fig. 5B). In an *iscR* mutant,
274 the addition of dip was still inhibiting expression of the two genes but to a much reduced
275 extent, i.e. 3.2-fold for *prgH* and 1.7-fold for *invF* fusions (Fig. 5B). These data indicate
276 that IscR contributes significantly to the iron-mediated regulation of Spi1 TTSS gene
277 expression.

278

279 **IscR reduces the invasion of HeLa cells by *Salmonella***

280 Because IscR was shown to decrease the expression of Spi1 TTSS genes, we
281 evaluated its contribution to *Salmonella* virulence. After ten minutes of incubation with
282 HeLa cells, the *iscR* mutant presented an efficiency of entry 1.44-fold higher than the
283 wild-type strain (Fig. 6A). Finally, the wild-type and the *iscR* mutant strains were used
284 in mice co-infection experiments. The inoculum was either injected intraperitoneally or
285 ingested orally. Virulence of the *iscR* mutant was reduced as compared with the wild-
286 type, using both methods: a CI of 0.144 was obtained using intraperitoneal infections
287 (Fig. 6B left & Table S3), whereas a value of 0.255 was found after oral ingestion (Fig.
288 6B right & Table S3). These results show that during the first step of the infection, IscR
289 can reduce HeLa cell invasion by *Salmonella*, consistent with its negative role in Spi1
290 TTSS expression.

291 Discussion

292

293 In this study, we showed that the Iron Sulfur Cluster (ISC) machinery is required for
294 the virulence of *Salmonella enterica*. The reduced invasion of an *iscU* mutant in HeLa
295 cells combined to its drastic virulence attenuation during oral mouse infections confer
296 a central role to the ISC system in the establishment of the *Salmonella* infectious
297 process. One reason underlying the reduced virulence of an *iscU* mutant could be a
298 decrease in Spi1 TTSS gene expression. Indeed, we showed that apo-IscR, which
299 prevails in an *iscU* mutant, downregulates the expression of the master regulator *hilD*
300 and thereby of several downstream HilD-regulated genes. Our findings support the
301 idea that the ISC machinery is important for *Salmonella* virulence through the ability of
302 the Fe-S cluster sensor IscR to regulate Spi1 gene expression (Fig. 7). They also
303 reflect an adaptive mechanism used by *Salmonella* to favour its infectivity in the gut
304 lumen and tissues, where oxygen is rare and iron available to the bacterium (Kortman
305 *et al.*, 2014; Jennewein *et al.*, 2015).

306

307 In a strain lacking the ISC machinery, the expression of *iscR* increases significantly
308 (Fig. S5) and the synthesized IscR is mostly under its apo-form. Apo-IscR binds on
309 Type 2 DNA-binding motifs and such motifs were found in the promoter region of *hilD*
310 (Fig. S6). Therefore, the increase in *iscR* expression observed in an *iscU* mutant
311 should lead to a decrease in Spi1 gene expression. This prediction was demonstrated
312 experimentally as Spi1 TTSS genes were found to be down-regulated in an *iscU*
313 mutant and up-regulated in an *iscR* mutant. Moreover, our results using an Fe-S
314 cluster-free variant indicated that the apo-form of IscR negatively regulates the
315 expression of the Spi1 TTS genes. Recently, *Yersinia* IscR was shown to bind

316 upstream of the operon encoding the TTSS master regulator LcrF, which endowed
317 IscR with a positive role in transcription of *Yersinia* TTSS genes (Miller *et al.*, 2014).
318 Thus, both pathogens appear to have recruited IscR with a primary role in their
319 virulence program. Based on the phenotypes exhibited by *iscR* mutants, it appears that
320 IscR targets TTSS regulation in opposite ways, as an activator in *Yersinia* and a
321 repressor in *Salmonella*. Further studies are required to characterize the physiological
322 significance of the TTSS regulation by IscR in both species.

323

324 In recent years, studies from different groups reported that the presence of iron in the
325 environment induced the expression of *hilA* (Ellermeier and Slauch, 2008) and *hilD*
326 (Teixidó *et al.*, 2011). The question arose whether the iron sensing regulator Fur was
327 involved in this iron-mediated activation (Ellermeier and Slauch, 2008; Teixidó *et al.*,
328 2011). Slauch and colleagues proposed two models to explain Fur activation of Spi1:
329 either by indirect control of the HilD protein or by affecting the threshold value of HilD
330 concentration required to activate the *hilD* promoter (Ellermeier and Slauch, 2008).
331 More recently, Campoy and colleagues proposed a different model in which Fur would
332 bind upstream of the *hilD* promoter and directly stimulate the expression of *hilD*
333 (Teixidó *et al.*, 2011). In the present study, we also found iron to be required for Spi1
334 gene expression. Expression of *prgH* and *invF* decreased 9- and 6-fold under iron
335 limited conditions, this regulatory mechanism being largely mediated by Fur. In an *iscR*
336 mutant, the expression of these two genes was also reduced under iron limited
337 conditions compared to LB, but to a much reduced extent (3- and 2-fold, respectively).
338 Thereby, we wondered if IscR could be involved in the iron regulation of Spi1 genes.
339 Our results show that under iron replete conditions, IscR represses Spi1 expression
340 1.5-fold only (WT vs. Δ *iscR* in LB), whereas under iron limited conditions, IscR exerts

341 a 5-fold repression effect (WT vs. $\Delta iscR$ in LB + dip). So going from iron limited to iron
342 replete conditions will shift the equilibrium from apo- to holo-IscR, thereby repressing
343 *iscR* and inducing Spi1 gene expression by alleviating IscR repression. Altogether,
344 these results suggest that IscR could act as an additional iron-sensor in the regulation
345 of Spi1 TTSS.

346

347 Expression of Spi1 TTSS is one of the most important factors leading to the success
348 of *Salmonella* invasion of intestinal epithelial cells. The composition of such an
349 environment is a complex issue because *in vivo* studies in the gut are extremely
350 challenging and because bacterial infections trigger an inflammatory response which
351 modifies the nature of this environment. Nevertheless, recent studies shed light on
352 oxygen and iron availability. Except for a relatively aerobic zone adjacent to the
353 mucosal surface, the gut lumen contains low levels of oxygen (Marteyn *et al.*, 2010;
354 Jennewein *et al.*, 2015). In contrast, iron is present in sufficient amounts to sustain
355 bacterial replication in the gut, although the production of siderophores by enteric
356 pathogens suggests a limited availability (Kortman *et al.*, 2014). Both limited oxygen
357 tensions and available iron define optimal conditions for Fe-S cluster stability. Hence,
358 IscR is predicted to be mostly under its holo-form and at low cellular concentrations,
359 favoring Spi1 TTSS gene expression (Fig. S5) (Schwartz *et al.*, 2001; Vinella *et al.*,
360 2013). In previous studies, we have shown that ROS were produced by macrophages
361 and that intracellular *Salmonella* were capable of sensing these ROS (Aussel *et al.*,
362 2011). Moreover, an increase in iron efflux was observed in *Salmonella*-infected
363 phagocytes, reducing both the cytoplasmic labile iron and the ferritin storage within
364 macrophages, thus restricting the acquisition of iron by intracellular *Salmonella* (Nairz
365 *et al.*, 2007). Under such environmental conditions - iron starvation and oxidative stress

366 -, the apo-IscR form is predicted to dominate, decreasing Spi1 TTSS gene expression
367 and limiting energetic expenses for the pathogen during macrophage infection. This
368 model is supported by transcriptomic and deep-sequencing analysis showing reduced
369 expression for most of the invasion-associated Spi1 genes in macrophages (Eriksson
370 *et al.*, 2003; Srikumar *et al.*, 2015). Indeed, in macrophages, *hilD* gene expression
371 decreased around 3-fold whereas *iscR* gene expression increased around 5-fold
372 compared to LB medium (Eriksson *et al.*, 2003). Thus, this scenario describes an
373 adaptive mechanism used by *S. enterica* to favor its virulence in the gut and to succeed
374 in epithelial cell invasion. From this viewpoint, IscR is predicted to play a key role in
375 sensing oxygen and iron levels since its expression and maturation can be modulated
376 as a function of the host environmental conditions.

377

378 Iron-sulfur clusters are used by a large number of protein species - over 130 in *E. coli*
379 and *Salmonella* - which participate in diverse biological processes (Py and Barras,
380 2010). In this study, the defect of the *iscU* mutant in HeLa cell invasion and oral mice
381 infection can be partially attributed to the down-regulation of Spi1 TTSS gene
382 expression by IscR. Other cellular processes dependent on enzymes carrying Fe-S
383 clusters, like motility, adhesion or respiration, could also be affected as shown in other
384 pathogens (Lim and Choi, 2014). Conversely, the *iscR* mutant was shown to be more
385 invasive in HeLa cells and less virulent in mice compared to the wild-type, consistent
386 with a possible regulation of other genes involved in *Salmonella* pathogenesis by IscR.
387 Analysis of *iscR* and *iscU* contributions to *Salmonella* virulence at the genomic level
388 might identify additional actors and will constitute the scope of future studies.

389 **Experimental Procedures**

390

391 *Ethics Statement*

392 Animal experimentation was conducted in strict accordance with good animal practice
393 as defined by the French animal welfare bodies (Law 87–848 dated 19 October 1987
394 modified by Decree 2001-464 and Decree 2001-131 relative to European Convention,
395 EEC Directive 86/609). All animal work was approved by the Direction Départementale
396 des Services Vétérinaires des Bouches-du-Rhône (authorization number 13.118 to
397 SM, Application number AR 1A09382857717).

398

399 *Bacterial strains and growth conditions*

400 The bacterial strains used in this study are described in Table 1. Strains were routinely
401 grown at 37°C in Luria-Bertani (LB) medium. Ampicillin (50 µg/ml), kanamycin (25
402 µg/ml) and chloramphenicol (25 µg/ml) were added when necessary. Deletions of
403 various genes and concomitant insertion of an antibiotic resistance cassette was
404 carried out using Lambda-Red-mediated recombination (Datsenko and Wanner,
405 2000). Mutations were moved to 12023 wild-type strain by P22 transductions.

406

407 *Plasmid constructions*

408 The cloning vector used to monitor gene expression was pFPV25, carrying
409 promoterless *gfpmut3a* gene (Valdivia and Falkow, 1997). The inserts carrying 300 bp
410 upstream *prgH*, *invF*, *rtsA* or *hilD* start codon were PCR-amplified from *S. enterica*

411 12023 by using the primers listed in Table S4. PCR products were digested using XbaI
412 and KpnI, and cloned into pFPV25 vector, yielding *PprgH-gfp*, *PinvF-gfp*, *PrtsA-gfp*
413 and *PhilD-gfp* plasmids (Table 1). For *iscR* and *hilD* complementation, the coding
414 sequence of each gene was digested using XbaI and NcoI and cloned into pKI*,
415 yielding *piscR* and *philD* (Table 1). For IscR purification, the coding sequence of *iscR*
416 was digested using NdeI and XhoI and cloned into pet22b(+), yielding pHis₆-*iscR*
417 (Table 1). The plasmids expressing IscR variant (*piscR*-3CA and pHis₆-*iscR*-3CA) were
418 PCR-amplified in two steps using two central oligonucleotides carrying the mutations
419 (C92A/C98A/C104A), the *iscR*-3CA fw and *iscR*-3CA-rv oligonucleotides (Table S4).
420 All the inserts were verified by DNA sequencing.

421

422 *Bacterial infection of macrophages*

423 RAW 264.7 macrophages were seeded at a density of 4×10^5 cells per well in 6-well
424 tissue culture plates containing DMEM with 10% fetal bovine serum (FBS) (HyClone).
425 Macrophages were supplemented with IFN- γ (10 U/ml, ImmunoTools) 24 hours before
426 use when indicated. Bacteria were cultured over-night at 37°C with shaking, and
427 opsonized in DMEM containing FBS and normal mouse serum (10%, Perbio) for 30
428 minutes. The macrophages were activated with 0.2 μ M phorbol 12-myristate 13-
429 acetate (PMA) (Sigma Aldrich) before infection when indicated. Bacteria were added
430 to the monolayers at a multiplicity of infection 10:1, centrifuged at 400 g for 5 min at
431 4°C, and incubated for 30 min at 37°C in 5% CO₂. The macrophages were washed
432 three times, and incubated with DMEM containing FBS and 100 μ g/ml gentamicin for
433 60 minutes, after which the gentamicin concentration was decreased to 10 μ g/ml for
434 the remainder of the experiment. For enumeration of intracellular bacteria,

435 macrophages were washed two times with PBS, lysed with 0.1% Triton X-100, and a
436 dilution series was plated on LB agar.

437

438 *Invasion assay of HeLa cells*

439 HeLa cells were seeded at a density of $5 \cdot 10^4$ cells/ml in 6-well plates and incubated
440 overnight at 37°C with 5% CO₂ in DMEM with 10% FBS. Bacterial strains were cultured
441 aerobically overnight at 37°C in LB. Bacteria were diluted 100-fold and sub-cultured
442 for 3 h at 37°C. HeLa cells were infected for 10 minutes with bacterial strains at MOI
443 100:1. Cells were washed three times with PBS and incubated for 1 h in DMEM
444 supplemented with 100 µg/ml gentamicin. After incubation, HeLa cells were washed
445 two times with PBS and lysed with 0.2% Triton X-100. Colony-forming units (cfu) of
446 intracellular bacteria were counted by plating the appropriate dilution on LB agar
447 plates.

448

449 *Competition assays*

450 Eight- to 10-week-old C57/B6 mice were inoculated intraperitoneally or orally with
451 equal amounts of two bacterial strains for a total of 10^5 bacteria per mouse. The
452 spleens were harvested 48 hours after inoculation (i.p. infection) or 5 days after
453 ingestion (oral infection), then homogenized. Bacteria were recovered and enumerated
454 after plating a dilution series onto LB agar and LB agar with the appropriate antibiotics.
455 Competitive indexes (CI) were determined for each mouse (Beuzón and Holden,
456 2001). The CI is defined as the ratio between the mutant and wild-type strains within
457 the output (bacteria recovered from the mouse after infection) divided by their ratios

458 within the input (initial inoculum). A one-sample *t* test was used to determine whether
459 the CIs were significantly different. All statistical analyses were performed by using
460 Prism (GraphPad, San Diego, CA). The two-tailed *P* value was calculated.

461

462 *Purification of His₆-iscR and His₆-iscR-3CA*

463 *E. coli* strain BL21(DE3) / *piscR*-His₆ was grown at 37°C in LB medium. At an OD_{600nm}
464 = 0.6, 0.5 mM IPTG was added and growth was carried on for 2 h. Cells were harvested
465 by centrifugation, and the pellet was resuspended in 8 ml of buffer A (25 mM Tris-HCl,
466 pH 7.5, 0.5 M NaCl, 10 mM imidazole). Resuspended cells were broken by two
467 passages through an ice-chilled French pressure cell at 0.5 tons. The resulting crude
468 extract was centrifuged twice at 10000 x *g* for 30 min at 4°C. The supernatant was
469 applied to a 1-ml His-trap column prepacked with nickel (GE Healthcare life Sciences)
470 and equilibrated with buffer A. Proteins were eluted with a 40-ml gradient running from
471 10 to 500 mM imidazole. Fractions were analysed by a sodium dodecyl sulfate-15%
472 polyacrylamide gel electrophoresis (SDS-PAGE). After elution, the His₆-IscR-
473 containing fractions were pooled and the solution was concentrated by ultrafiltration on
474 Amicon ultra 10 K (Millipore) (2500 x *g*, 4°C). Protein concentration was determined
475 spectrophotometrically at 280 nm (NanoDrop 1000; Thermo Fisher Scientific). Identical
476 protocol was used to purify the His₆-IscR-3CA protein.

477

478 *Mutagenesis and preparation of DNA substrates*

479 Substrates for EMSA were produced using 5'-Cy5 labelled primers (Eurogentec) at one
480 extremity. The resulting DNA fragments include 200 nucleotides upstream and 40
481 nucleotides downstream the start codon of *hilD* and *invF* genes, yielding *PhilD* and
482 *PinvF*. *PhilD** was obtained in two steps using two central oligonucleotides carrying the

483 mutations (See Table S4 for details). Sequence accuracy of the PCR products was
484 checked by sequencing.

485

486 *Electrophoretic Mobility Shift Assays (EMSA)*

487 Reaction mixes (10 μ l) contained Cy5 labelled DNA (100 nM) in Tris-HCl, pH 7.5, 0.5
488 M KCl buffer containing 0.3 mg/ml sonicated calf thymus DNA. Appropriate protein
489 concentrations indicated in figure legends were added prior to incubation at 25 °C for
490 30 min. Samples were loaded on a 6% native PAGE in TBE 1 \times at 90 V, and ran for 5
491 hours at 135 V. Migration profiles were analysed by scanning the gel using a 635 nm
492 laser and a LPR filter (FLA5100, Fujifilm).

493

494 *BioLayer Interferometry (BLI)*

495 Protein-DNA interaction experiments were conducted at 25°C with the BLItz instrument
496 from ForteBio (Menlo Park, CA, USA). The BLI consists in a real time optical
497 biosensing technique exploits the interference pattern of white light reflected from two
498 surfaces to measure biomolecular interactions (Concepcion *et al.*, 2009). Purified His₆-
499 IscR protein ligand was immobilized onto two different Ni-NTA biosensors (ForteBio)
500 in duplicate at 6 μ M. PCR amplified DNA fragment of *hilD* and *ahpC* promoters were
501 used as the analytes throughout the study at the 240 nM. The assay was conducted in
502 25 mM TRIS-HCl, pH 7.5, 0.5M NaCl. The binding reactions were performed with an
503 initial baseline for 30 seconds, an association step at 120 seconds and a dissociation
504 step of 120 seconds with lateral shaking at 2200 rpm. A double reference subtraction
505 (sensor reference and His₆-IscR) was applied to account for non-specific binding,
506 background, and signal drift to minimize sensor variability.

507

508 *RNA Preparation and Reverse Transcription*

509 RNAs were prepared from 1 ml culture of *S. enterica* grown in LB until $OD_{600nm} = 1.5$.
510 The cells were harvested and frost at $-80^{\circ}C$. Total RNAs were isolated from the pellet
511 using the SV Total RNA Isolation System (Promega). The RNA quality was assessed
512 by an Experion chip (Bio-Rad) and the absence of DNA contamination was confirmed
513 by PCR. For cDNA synthesis, 650 ng total RNA and 0.5 μg random primers were used
514 with the GoScript™ Reverse transcriptase (Promega).

515

516 *Quantitative Real-Time-PCR for Transcriptional Analyses*

517 Quantitative real-time PCR (qPCR) analyses were performed on a CFX96 Real-Time
518 System (Bio-Rad). The reaction volume was 15 μl and the final concentration of each
519 primer was 0.5 μM . The cycling parameters of the qRT-PCR were $98^{\circ}C$ for 2 min,
520 followed by 45 cycles of $98^{\circ}C$ for 5 s, $56^{\circ}C$ for 10 s, $72^{\circ}C$ for 1s. A final melting curve
521 from $65^{\circ}C$ to $95^{\circ}C$ is added to determine the specificity of the amplification. The *yejA*
522 gene was used as a reference for normalization. For each point, a technical duplicate
523 was performed. The amplification efficiencies for each primer pairs were comprised
524 between 75 and 100%. All of the primer pairs used for qRT-PCR are reported in the
525 table S4.

526 **Table 1. Bacterial strains and plasmids.**

527

528

Strain	Relevant genotype	Source or reference
12023	Wild-type	Laboratory stock
ST360	$\Delta iscU::Kn$	This study
ST332	$\Delta sufB-sufC::Cm$	This study
ST491	$\Delta iscR::Kn$	This study
ST532	$\Delta iscR (Kn^S)$	This study
ST520	$\Delta hilD::Cm$	This study
ST533	$\Delta hilD::Cm \Delta iscR::Kn$	This study
Plasmids		
pFPV25	GFP reporter fusion vector (Ap ^r)	(Valdivia and Falkow, 1997)
<i>PprgH-gfp</i>	pFPV25 derivative carrying the <i>prgH-gfp</i> promoter (Ap ^r)	This study
<i>PinvF-gfp</i>	pFPV25 derivative carrying the <i>invF-gfp</i> promoter (Ap ^r)	This study
<i>PrtsA-gfp</i>	pFPV25 derivative carrying the <i>rtsA-gfp</i> promoter (Ap ^r)	This study
<i>PhilD-gfp</i>	pFPV25 derivative carrying the <i>hilD-gfp</i> promoter (Ap ^r)	This study
pET-22b(+)	Expression vector carrying a C-terminal His tag	Novagen
pHis ₆ - <i>iscR</i>	pET-22b(+) derivative carrying the His ₆ - <i>iscR</i> (Ap ^r)	This study
pHis ₆ - <i>iscR</i> -3CA	pET-22b(+) derivative carrying the His ₆ - <i>iscR</i> -3CA (Ap ^r)	This study
pKI*	pBAD24 derivative carrying a kanamycin resistance cassette (Kn ^r)	This study
<i>piscR</i>	pKI* derivative carrying <i>iscR</i> (Kn ^r)	This study
<i>philD</i>	pKI* derivative carrying <i>hilD</i> (Kn ^r)	This study

<i>piscR</i> -3CA	pKI* derivative carrying <i>iscR</i> -C92A/C98A/C104A (Kn')	This study
-------------------	---	------------

529

530

531 **Figure legends**

532

533 **Figure 1. The ISC machinery is essential for HeLa cell invasion and mice**
534 **infection.**

535 (A) Opsonized bacteria (wild-type, $\Delta iscU$, $\Delta sufBC$) were phagocytosed by RAW 264.7
536 cells activated with IFN- γ and PMA. Two and 16 hours post-infection, mouse
537 macrophages were lysed for enumeration of intracellular bacteria (gentamicin-
538 protected) determined by colony-forming unit (cfu) counts. The values shown represent
539 the proliferation index calculated as a ratio of the intracellular bacteria between 16 and
540 2 hours post-infection. (B) Wild-type, $\Delta iscU$ or $\Delta sufBC$ strains were inoculated with
541 HeLa cells for 10 minutes, then treated with gentamicin for one hour. Epithelial cells
542 were lysed for enumeration of intracellular bacteria determined by cfu counts. The
543 values shown represent the invasion of the strains relative to WT, which was set at 1.
544 Results presented in the panels A and B are the means \pm standard deviation of at least
545 three independent experiments each in triplicate. Asterisks indicate a statistically
546 significant difference between mutants and the WT. ns, not significant; * $P \leq 0.05$
547 (Mann-Whitney U test). (C & D) C57BL/6 mice were inoculated intraperitoneally (C) or
548 orally (D) with a 1:1 mixture of mutant and wild-type *Salmonella* strains. 48 h (C) or five
549 days (D) post-inoculation, spleens were harvested for bacterial counts. Competitive
550 indexes of wild-type versus mutant strains in mice were determined. Each circle
551 represents one mouse and horizontal bars correspond to the mean. A one-sample *t*
552 test was used to determine whether the CI was significantly different from 1. ns, not
553 significant; *** $P \leq 0.001$.

554

555 **Figure 2. The ISC system is required for the expression of Spi1 Type III Secretion**
556 **System.**

557 **(A)** Simplified model for the regulation of Spi1. The arrows indicate direct activation of
558 gene expression. For clarity, the genes encoding HilD, HilC, RtsA, and HilA are not
559 shown. See reference 24 for a more complete model of Spi1 regulation. **(B-E)** Wild-
560 type, *iscU* and *sufBC* mutant strains were transformed with a plasmid carrying the
561 *PhilD-gfp* **(B)**, the *PrtsA-gfp* **(C)**, the *PprgH-gfp* **(D)** and the *PinvF-gfp* **(E)** fusions.
562 Strains were grown aerobically in LB for 16 hours and GFP synthesis was measured
563 using a fluorimeter. The fluorescence levels shown on the graphics are calculated as
564 the GFP values normalised to the OD_{600nm}. Results are the means ± standard deviation
565 of at least five independent experiments. Asterisks indicate a statistically significant
566 difference between mutants and the WT. ns, not significant; **P* ≤ 0.05 (Mann-Whitney
567 U test).

568

569 **Figure 3. IscR binds to the *hilD* promoter *in vitro*.**

570 **(A)** Key features of the *hilD* promoter, including HilD binding site (bold bar), -10 and -
571 35 sites, the transcription start site (+1, arrow) and the translation initiation codon of
572 the *hilD* gene (ATG) (Olekhovich and Kadner, 2002). The IscR Type 2 binding sites
573 are represented by the two boxes, the arrow indicating the orientation from 5' to 3'. **(B)**
574 Sequence alignment of the two putative IscR-binding sites from *S. enterica hilD* with
575 the *Escherichia coli hyaA* and *Yersinia pseudotuberculosis lcrF* domains. **(C-E)**
576 Electrophoretic mobility shift assays using Cy5-DNA fragments (240 pb) corresponding
577 to the promoters of *hilD* (*PhilD*, **C**) and *hilD* mutated in the IscR Type 2 binding sites
578 (*PhilD**, **D**). In each experiment, 100 nM of Cy5-DNA fragments were incubated with

579 increasing concentrations of IscR (1, 1.9, 2.8, 3.7, 4.6 and 5.5 μ M) or BSA as a control
580 (5.5 μ M) and run on a 6% native PAGE. (E) Quantification of the shifted DNA (reflecting
581 the bound DNA) in panels C and D. The values shown on this figure represents the
582 means \pm standard deviation of at least three independent experiments. (F). Average
583 binding curves (light colours) and duplicates (dark colours) of immobilized IscR
584 incubated with *PhilD* (red) and *PahpC* (blue) at a concentration of 240 nM obtained by
585 biolayer interferometry.

586

587 **Figure 4. IscR represses *hilD* gene expression.**

588 (A-D) Wild-type, *iscR*, *hilD* and *hilD iscR* mutant strains were transformed with a
589 plasmid carrying the *PhilD-gfp* (A), *PrtsA-gfp* (B), *PprgH-gfp* (C) and *PinvF-gfp* (D)
590 fusions. Strains were grown aerobically for 16 hours and the fluorescence was
591 measured afterwards. Levels shown on the graphics are calculated as the GFP values
592 normalised to the OD_{600nm}. (E-H) Expression pattern of *hilD* (E), *rtsA* (F), *prgH* (G) and
593 *invF* (H) encoding genes in the *iscR*, *hilD* and *hilD iscR* mutants compared to the wild-
594 type strain. RNA was extracted from the wild-type, *iscR* and *hilD* mutant strains grown
595 in LB to an OD_{600nm} = 1.5. Quantitative real-time PCR was performed to amplify the
596 *hilD*, *rtsA*, *prgH* and *invF* genes. ND, Not Determined. Results are the means \pm
597 standard deviation of at least three independent experiments. Asterisks indicate a
598 statistically significant difference between mutants and the WT. * $P \leq 0.05$ (Mann-
599 Whitney U test).

600

601 **Figure 5. Apo- and holo-IscR negatively regulates the expression of Spi1 TTSS** 602 **genes.**

603 (A) *iscR* mutants (left) or wild-type cells (right) carrying the *PprgH-gfp* or the *PinvF-gfp*
604 fusion were transformed with an empty plasmid (green bars), with a plasmid encoding
605 IscR (orange bars) or with a plasmid encoding the IscR-3CA variant (grey bars). The
606 fluorescence was measured after 16 hours of aerobic growth in LB. (B) Wild-type or
607 *iscR* mutant strains carrying the *PprgH-gfp* fusion (left) or the *PinvF-gfp* fusion (right)
608 were grown for 6 hours in LB with or without 2,2'-dipyridyl (dip), and the fluorescence
609 was measured afterwards. The values shown on this figure were calculated as the
610 GFP values normalised to the OD_{600nm} and represents the means \pm standard deviation
611 of at least five independent experiments. Asterisks indicate a statistically significant
612 difference between two strains. ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$ (Mann-Whitney
613 U test).

614

615 **Figure 6. Salmonella *iscR* mutant is hyperinvasive in HeLa cells.**

616 (A) Wild-type or Δ *iscR* strains were inoculated with HeLa cells for 10 minutes, then
617 treated with gentamicin for one hour. Epithelial cells were lysed for enumeration of
618 intracellular bacteria determined by cfu counts. The values shown represent the
619 invasion of the strains relative to WT, which was set at 1. These data are the means \pm
620 standard deviation of at least three independent experiments each in triplicate.
621 Asterisks indicate a statistically significant difference between the *iscR* mutant and the
622 WT. ns, not significant; * $P \leq 0.05$ (Mann-Whitney U test). (B) C57BL/6 mice were
623 inoculated intraperitoneally (IP, left) or orally (right) with a 1:1 mixture of Δ *iscR* mutant
624 and wild-type *Salmonella* strains. 48 h (IP) or 5 days (oral) post-infection, spleens were
625 harvested for bacterial counts. Competitive index of wild-type versus Δ *iscR* mutant
626 strains in mice were determined. Each circle represents one mouse and horizontal bars

627 correspond to the mean. A one-sample *t* test was used to determine whether the CI
628 was significantly different from 1. ns, not significant; ****P* ≤ 0.001.

629

630 **Figure 7. Simplified model for the regulation of Spi1 TTSS.**

631 On the promoter region of *hilD*, two IscR Type 2 binding sites flank and partially overlap
632 the HilD binding site. A competition occurs between HilD (transcriptional activator) and
633 IscR to bind *hilD* promoter, leading to a modulation in Spi1 TSSS gene expression.
634 Because IscR regulates its own expression depending upon iron and oxygen
635 availability, the *hilD* repression by IscR can be alleviated or increased in function of the
636 environment encountered by *Salmonella* during the infection.

637

638 **Acknowledgments**

639

640 Thanks are due to the members of the FB group for fruitful discussions, in particular to
641 Béatrice Py and Béatrice Roche for extremely helpful advice. We thank Sandrine
642 Ollagnier-de Choudens (LCBM Grenoble) for biochemical expertise. We are grateful
643 to Paul Hoffman (University of Virginia) and Josep Casadesús (University of Seville)
644 for providing plasmid and strain. We thank Yann Denis (Transcriptomics Facility, IMM),
645 Deborah Byrne (Protein Expression Facility, IMM), Rebecca Stevens (GAFL, Avignon)
646 and Kévin Forin for technical assistance. This work was funded by the Agence
647 Nationale de la Recherche (ANR, Programme M.I.E. "Salmo-sensor", Grant ANR-08-
648 MIEN-025-01 and ANR Blanc SPV05511), the Centre National de la Recherche
649 Scientifique (CNRS, PICS07279), and Aix-Marseille Université. BP was funded by the
650 Fondation pour la Recherche Médicale (FRM).

651 **References**

652

653 Arlet, J.-B., Ribeil, J.-A., Guillem, F., Negre, O., Hazoume, A., Marcion, G., *et al.*
654 (2014) HSP70 sequestration by free α -globin promotes ineffective erythropoiesis in
655 β -thalassaemia. *Nature* **514**: 242–246.

656 Aussel, L., Zhao, W., Hébrard, M., Guilhon, A.-A., Viala, J.P.M., Henri, S., *et al.*
657 (2011) Salmonella detoxifying enzymes are sufficient to cope with the host
658 oxidative burst. *Mol Microbiol* **80**: 628–640.

659 Beuzón, C.R., and Holden, D.W. (2001) Use of mixed infections with Salmonella
660 strains to study virulence genes and their interactions in vivo. *Microbes Infect Inst*
661 *Pasteur* **3**: 1345–1352.

662 Bonomi, F., Iametti, S., Ta, D., and Vickery, L.E. (2005) Multiple turnover transfer of
663 [2Fe2S] clusters by the iron-sulfur cluster assembly scaffold proteins IscU and
664 IscA. *J Biol Chem* **280**: 29513–29518.

665 Concepcion, J., Witte, K., Wartchow, C., Choo, S., Yao, D., Persson, H., *et al.* (2009)
666 Label-free detection of biomolecular interactions using BioLayer interferometry for
667 kinetic characterization. *Comb Chem High Throughput Screen* **12**: 791–800.

668 Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal
669 genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**:
670 6640–6645.

671 Ellermeier, C.D., Ellermeier, J.R., and Slauch, J.M. (2005) HilD, HilC and RtsA
672 constitute a feed forward loop that controls expression of the SPI1 type three
673 secretion system regulator hilA in Salmonella enterica serovar Typhimurium. *Mol*
674 *Microbiol* **57**: 691–705.

675 Ellermeier, J.R., and Slauch, J.M. (2008) Fur regulates expression of the Salmonella
676 pathogenicity island 1 type III secretion system through HilD. *J Bacteriol* **190**: 476–
677 486.

678 Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J.C.D. (2003)
679 Unravelling the biology of macrophage infection by gene expression profiling of
680 intracellular Salmonella enterica. *Mol Microbiol* **47**: 103–118.

681 Figueira, R., and Holden, D.W. (2012) Functions of the Salmonella pathogenicity
682 island 2 (SPI-2) type III secretion system effectors. *Microbiol Read Engl* **158**:
683 1147–1161.

684 Fuangthong, M., Jittawuttipoka, T., Wisitkamol, R., Romsang, A., Duang-nkern, J.,
685 Vattanaviboon, P., and Mongkolsuk, S. (2015) IscR plays a role in oxidative stress
686 resistance and pathogenicity of a plant pathogen, Xanthomonas campestris.
687 *Microbiol Res* **170**: 139–146.

688 Galán, J.E., and Curtiss, R. (1989) Cloning and molecular characterization of genes
689 whose products allow Salmonella typhimurium to penetrate tissue culture cells.
690 *Proc Natl Acad Sci U S A* **86**: 6383–6387.

691 Giel, J.L., Nesbit, A.D., Mettert, E.L., Fleischhacker, A.S., Wanta, B.T., and Kiley, P.J.
692 (2013) Regulation of iron–sulphur cluster homeostasis through transcriptional
693 control of the Isc pathway by [2Fe–2S]–IscR in Escherichia coli. *Mol Microbiol* **87**:
694 478–492.

695 Giel, J.L., Rodionov, D., Liu, M., Blattner, F.R., and Kiley, P.J. (2006) IscR-dependent
696 gene expression links iron-sulphur cluster assembly to the control of O₂-regulated
697 genes in Escherichia coli. *Mol Microbiol* **60**: 1058–1075.

698 Haines, S., Arnaud-Barbe, N., Poncet, D., Reverchon, S., Wawrzyniak, J., Nasser,
699 W., and Renaud-Mongénie, G. (2015) IscR Regulates Synthesis of Colonization

700 Factor Antigen I Fimbriae in Response to Iron Starvation in Enterotoxigenic
701 Escherichia coli. *J Bacteriol* **197**: 2896–2907.

702 Hidese, R., Mihara, H., and Esaki, N. (2011) Bacterial cysteine desulfurases:
703 versatile key players in biosynthetic pathways of sulfur-containing biofactors. *Appl*
704 *Microbiol Biotechnol* **91**: 47–61.

705 Huet, G., Daffé, M., and Saves, I. (2005) Identification of the Mycobacterium
706 tuberculosis SUF machinery as the exclusive mycobacterial system of [Fe-S]
707 cluster assembly: evidence for its implication in the pathogen's survival. *J Bacteriol*
708 **187**: 6137–6146.

709 Jennewein, J., Matuszak, J., Walter, S., Felmy, B., Gendera, K., Schatz, V., *et al.*
710 (2015) Low-oxygen tensions found in Salmonella-infected gut tissue boost
711 Salmonella replication in macrophages by impairing antimicrobial activity and
712 augmenting Salmonella virulence. *Cell Microbiol* **17**: 1833–1847.

713 Kiley, P.J., and Beinert, H. (2003) The role of Fe-S proteins in sensing and regulation
714 in bacteria. *Curr Opin Microbiol* **6**: 181–185.

715 Kim, S.-H., Lee, B.-Y., Lau, G.W., and Cho, Y.-H. (2009) IscR modulates catalase A
716 (KatA) activity, peroxide resistance and full virulence of Pseudomonas aeruginosa
717 PA14. *J Microbiol Biotechnol* **19**: 1520–1526.

718 Kortman, G.A.M., Raffatellu, M., Swinkels, D.W., and Tjalsma, H. (2014) Nutritional
719 iron turned inside out: intestinal stress from a gut microbial perspective. *FEMS*
720 *Microbiol Rev* **38**: 1202–1234.

721 Lim, J.G., and Choi, S.H. (2014) IscR is a global regulator essential for pathogenesis
722 of Vibrio vulnificus and induced by host cells. *Infect Immun* **82**: 569–578.

723 Marteyn, B., West, N.P., Browning, D.F., Cole, J.A., Shaw, J.G., Palm, F., *et al.*
724 (2010) Modulation of Shigella virulence in response to available oxygen in vivo.
725 *Nature* **465**: 355–358.

726 Miller, H.K., Kwuan, L., Schwiesow, L., Bernick, D.L., Mettert, E., Ramirez, H.A., *et al.*
727 (2014) IscR is essential for yersinia pseudotuberculosis type III secretion and
728 virulence. *PLoS Pathog* **10**: e1004194.

729 Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001) SoxR-
730 dependent response to oxidative stress and virulence of Erwinia chrysanthemi: the
731 key role of SufC, an orphan ABC ATPase. *Mol Microbiol* **39**: 960–972.

732 Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S.M., Fritsche, G., and Weiss,
733 G. (2007) The co-ordinated regulation of iron homeostasis in murine macrophages
734 limits the availability of iron for intracellular Salmonella typhimurium. *Cell Microbiol*
735 **9**: 2126–2140.

736 Olekhovich, I.N., and Kadner, R.J. (2002) DNA-binding activities of the HilC and
737 HilD virulence regulatory proteins of Salmonella enterica serovar Typhimurium. *J*
738 *Bacteriol* **184**: 4148–4160.

739 Py, B., and Barras, F. (2010) Building Fe-S proteins: bacterial strategies. *Nat Rev*
740 *Microbiol* **8**: 436–446.

741 Rajagopalan, S., Teter, S.J., Zwart, P.H., Brennan, R.G., Phillips, K.J., and Kiley, P.J.
742 (2013) Studies of IscR reveal a unique mechanism for metal-dependent regulation
743 of DNA binding specificity. *Nat Struct Mol Biol* **20**: 740–747.

744 Rincon-Enriquez, G., Cr  t  , P., Barras, F., and Py, B. (2008) Biogenesis of Fe/S
745 proteins and pathogenicity: IscR plays a key role in allowing Erwinia chrysanthemi
746 to adapt to hostile conditions. *Mol Microbiol* **67**: 1257–1273.

747 Runyen-Janecky, L., Daugherty, A., Lloyd, B., Wellington, C., Eskandarian, H., and
748 Sagransky, M. (2008) Role and regulation of iron-sulfur cluster biosynthesis genes
749 in *Shigella flexneri* virulence. *Infect Immun* **76**: 1083–1092.

750 Schwartz, C.J., Giel, J.L., Patschkowski, T., Luther, C., Ruzicka, F.J., Beinert, H., and
751 Kiley, P.J. (2001) IscR, an Fe-S cluster-containing transcription factor, represses
752 expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins.
753 *Proc Natl Acad Sci U S A* **98**: 14895–14900.

754 Srikumar, S., Kröger, C., Hébrard, M., Colgan, A., Owen, S.V., Sivasankaran, S.K., *et*
755 *al.* (2015) RNA-seq Brings New Insights to the Intra-Macrophage Transcriptome of
756 *Salmonella Typhimurium*. *PLoS Pathog* **11**: e1005262.

757 Teixidó, L., Carrasco, B., Alonso, J.C., Barbé, J., and Campoy, S. (2011) Fur
758 activates the expression of *Salmonella enterica* pathogenicity island 1 by directly
759 interacting with the *hilD* operator in vivo and in vitro. *PloS One* **6**: e19711.

760 Valdivia, R.H., and Falkow, S. (1997) Fluorescence-based isolation of bacterial
761 genes expressed within host cells. *Science* **277**: 2007–2011.

762 Velayudhan, J., Karlinsey, J.E., Frawley, E.R., Becker, L.A., Nartea, M., and Fang,
763 F.C. (2014) Distinct roles of the *Salmonella enterica* serovar *Typhimurium* CyaY
764 and YggX proteins in the biosynthesis and repair of iron-sulfur clusters. *Infect*
765 *Immun* **82**: 1390–1401.

766 Vinella, D., Brochier-Armanet, C., Loiseau, L., Talla, E., and Barras, F. (2009) Iron-
767 sulfur (Fe/S) protein biogenesis: phylogenomic and genetic studies of A-type
768 carriers. *PLoS Genet* **5**: e1000497.

769 Vinella, D., Loiseau, L., Ollagnier de Choudens, S., Fontecave, M., and Barras, F.
770 (2013) In vivo [Fe-S] cluster acquisition by IscR and NsrR, two stress regulators in
771 *Escherichia coli*. *Mol Microbiol* **87**: 493–508.

772 Wollers, S., Layer, G., Garcia-Serres, R., Signor, L., Clemancey, M., Latour, J.-M., et
773 *al.* (2010) Iron-sulfur (Fe-S) cluster assembly: the SufBCD complex is a new type
774 of Fe-S scaffold with a flavin redox cofactor. *J Biol Chem* **285**: 23331–23341.

775

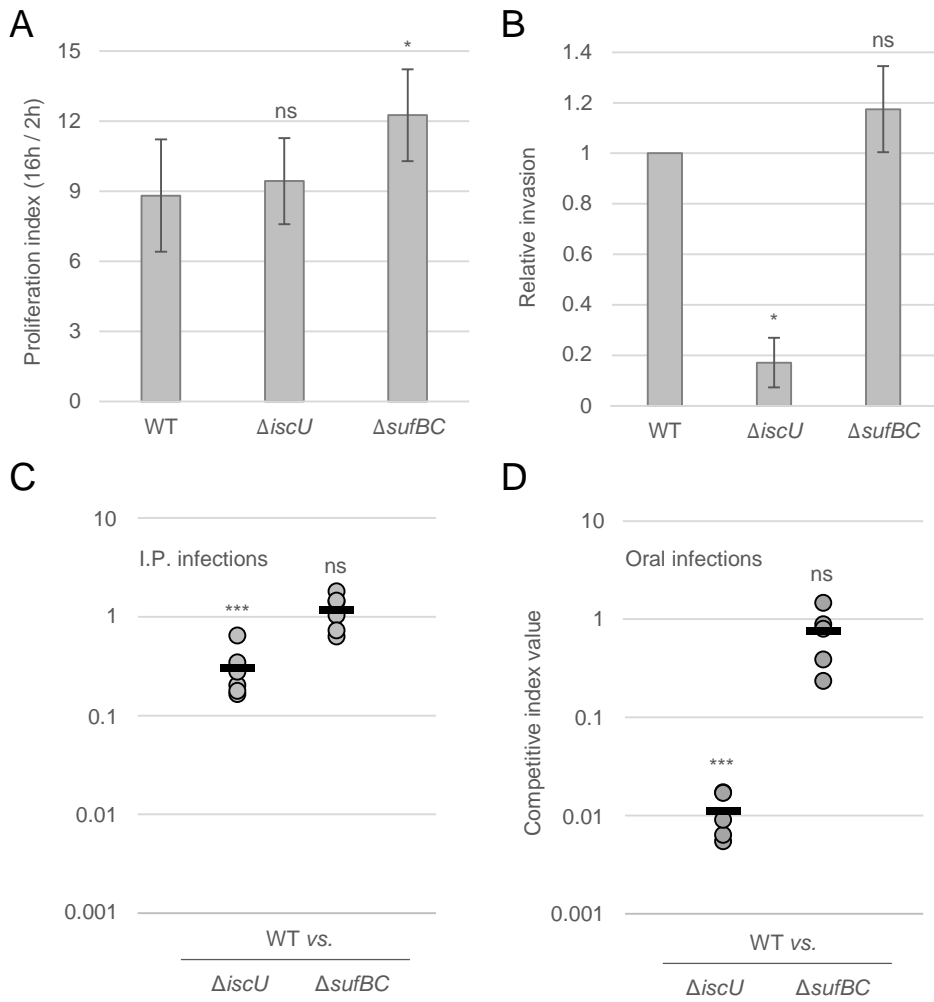


Fig 1

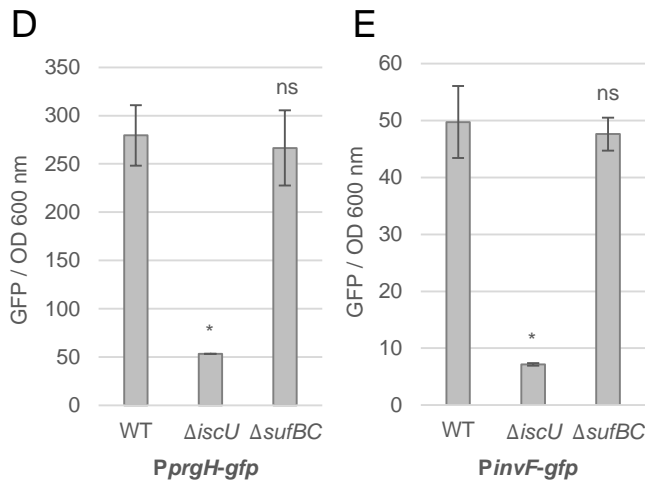
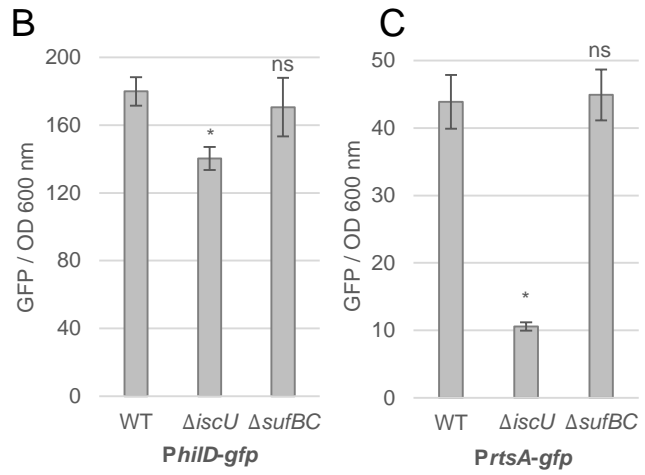
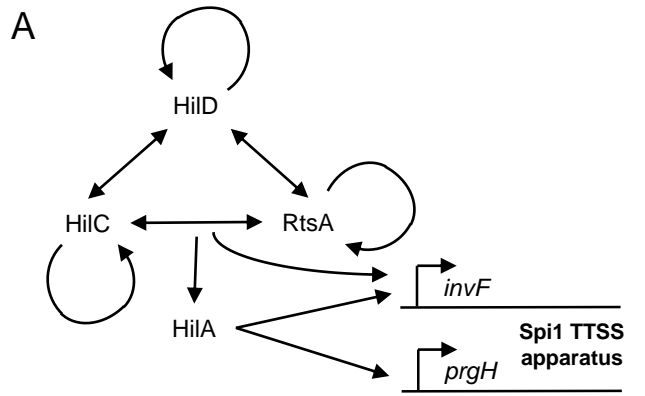


Fig 2

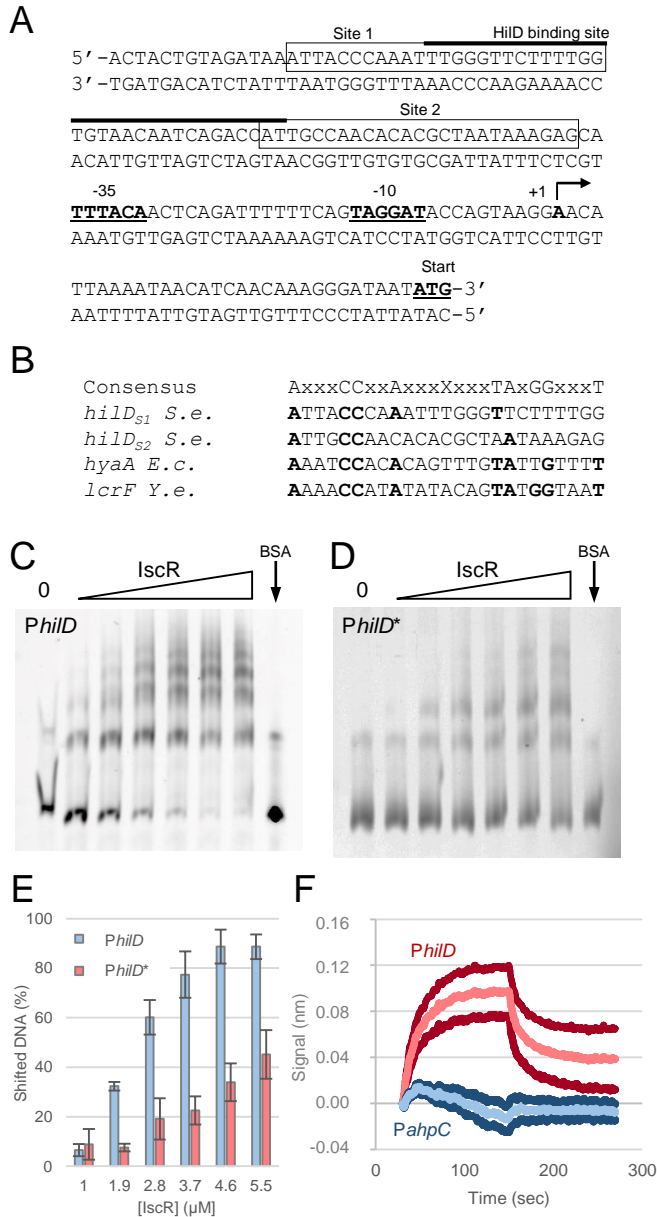


Fig 3

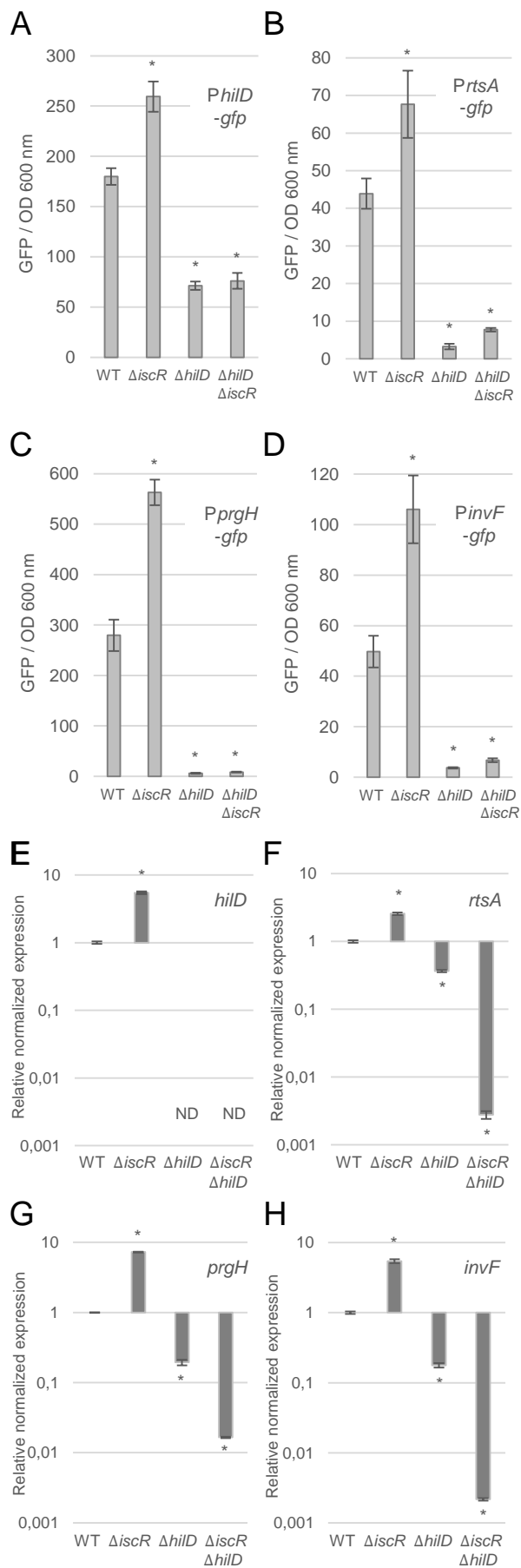


Fig 4

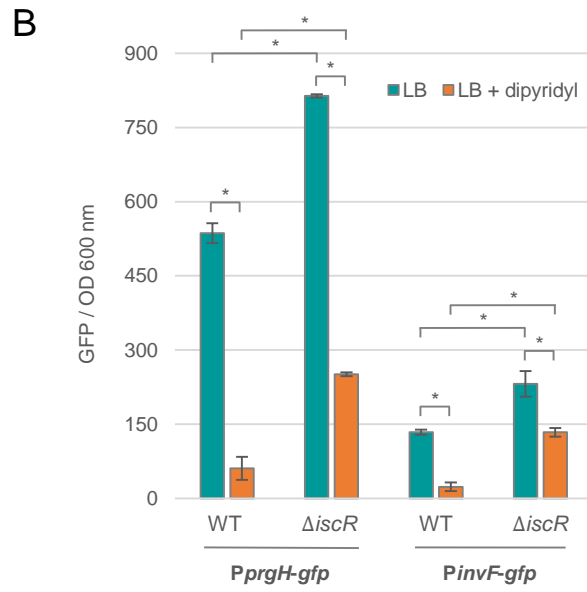
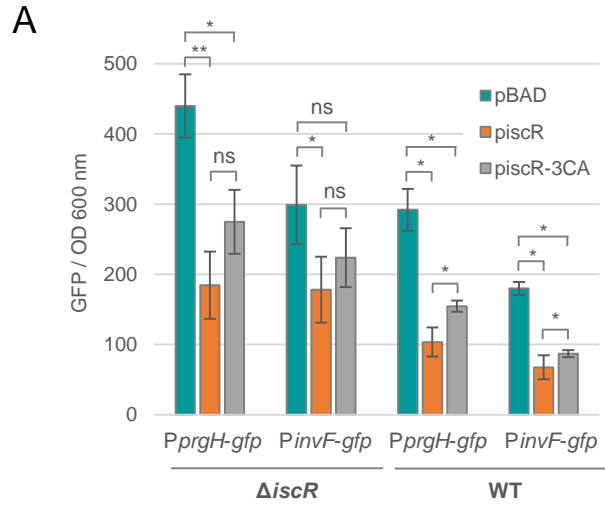


Fig 5

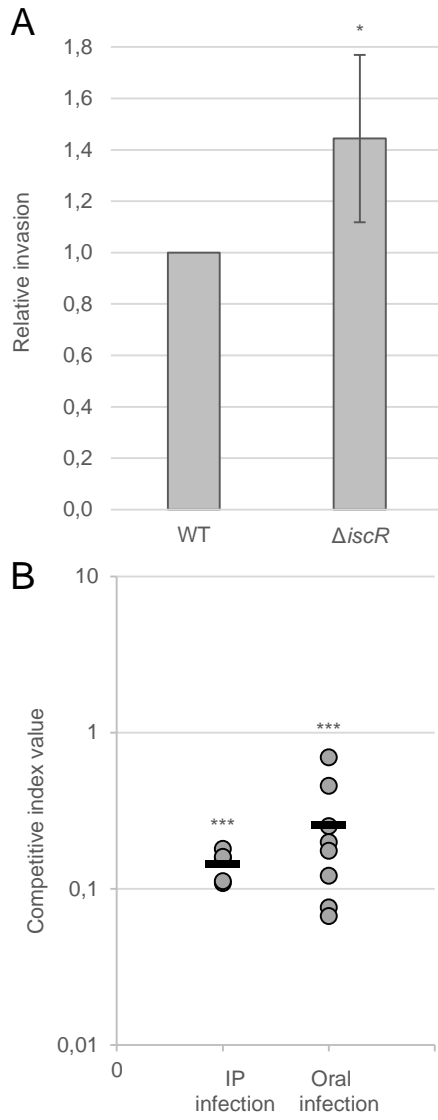


Fig 6

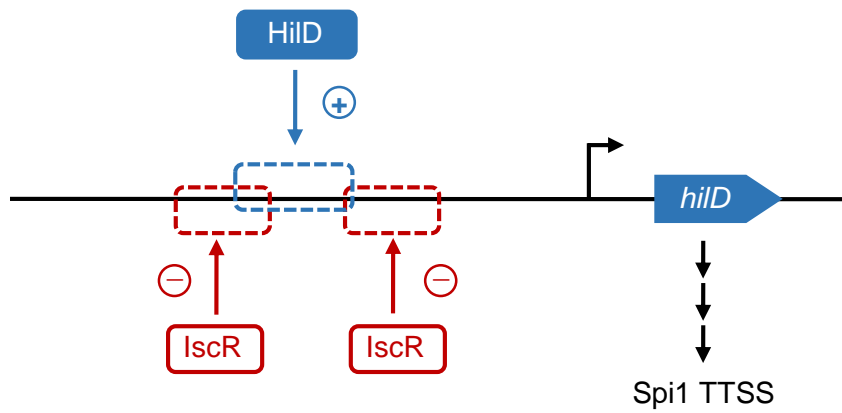


Fig 7