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1 **Regulatory interplay between RNase III and asRNAs in *E. coli*; the case of AsflhD and the master**
2 **regulator of motility, *flhDC***

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7
8 *Running Head: Antisense RNA control of flhD expression*

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16

17 Abstract

18 In order to respond to ever-changing environmental cues, bacteria have evolved resilient regulatory
19 mechanisms controlling gene expression. At the post-transcriptional level, this is achieved by a
20 combination of RNA-binding proteins, such as ribonucleases (RNases) and RNA chaperones, and
21 regulatory RNAs including antisense RNAs (asRNAs). AsRNAs bound to their complementary mRNA
22 are primary targets for the double-strand-specific endoribonuclease, RNase III. By comparing
23 primary and processed transcripts in an *rnc* strain, mutated for RNase III, and its isogenic wild type
24 strain, we detected several asRNAs. We confirmed the existence of RNase III-sensitive asRNA for
25 *crp*, *ompR*, *phoP* and *flhD* genes, encoding master regulators of gene expression. AsflhD, the asRNA
26 to the master regulator of motility *flhDC*, is slightly induced under heat-shock conditions in a
27 sigma24 (RpoE)-dependent manner. We demonstrate that expression of AsflhD asRNA is involved in
28 the transcriptional attenuation of *flhD* and thus participates in the control of the whole motility
29 cascade. This study demonstrates that AsflhD and RNase III are additional players in the complex
30 regulation ensuring a tight control of flagella synthesis and motility.

32 Importance

33 The importance of asRNAs in the regulation of gene expression has long been underestimated.
34 Here, we confirm that asRNAs can be part of layered regulatory networks since some are found
35 opposite to genes encoding global regulators. In particular, we show how an antisense RNA (AsflhD)
36 to the gene expressing a transcription factor serving as the primary regulator of bacterial swimming
37 motility (FlhD₄C₂) is involved in the transcriptional attenuation of *flhD*, which in turn impacts the
38 expression of other genes of the motility cascade. The role of AsflhD highlights the importance of
39 discrete fine-tuning mechanisms in the control of complex regulatory networks.

40 **Keywords**

41 asRNAs, RNase III, transcriptional attenuation, *flhD*, motility, *phoP*, *E. coli*

42 **Abbreviations**

43 Antisense RNA (asRNA); RNA-binding protein (RBP); untranslated region (UTR); small RNA (sRNA):
44 open reading frame (ORF); nucleotide (nt); circular RT-PCR (cRT-PCR); RNA polymerase (RNAP)

45 Introduction

46 In eukaryotes, hundreds of RNA-binding proteins (RBPs) and multiple classes of regulatory RNAs are
47 involved in the complex regulation of gene expression (splicing, editing...). In bacteria, the relative
48 scarcity of RBPs and regulatory RNAs, led to the supposition that they provided only accessory
49 contributions to the major bacterial gene regulatory mechanisms. An important obstacle in
50 deciphering regulatory networks is their multi-component nature and the existence of “missing
51 links” between regulators and their targets. These intermediates can have both positive and
52 negative impacts on gene expression, leading to compensatory effects upon removal of one of them
53 (1-6). Hence, the fine-tuning of gene expression is far from fully understood in many if not most
54 cases.

55 Many bacterial small RNAs (sRNAs) are regulators that base-pair with RNA, with their genes located
56 in *trans* to their targets and acting by short, imperfect regions of base-pairing. This property allows
57 them to act on multiple targets. In contrast, the genes for antisense RNAs (asRNAs) are located in *cis*
58 to their complementary target and thus have, in most cases, a single dedicated target. Fewer
59 asRNAs have been described as compared to sRNAs probably because of their high lability, their low
60 conservation among species and because they were usually considered the products of pervasive
61 transcription arising from leaky terminators (7-10).

62 Initially, asRNAs were identified on mobile genetic elements (prophages and plasmids), with their
63 only purpose to control their replication and partition. The importance of asRNAs was later
64 demonstrated to extend to almost all kinds of biological processes (11), as in the case of type I
65 toxin-antitoxin systems, involved in persistence, in which the toxin mRNA is neutralized by an asRNA
66 that induces degradation and/or translation inhibition (12). Furthermore, the double-strand-specific
67 RNase III has been known to be an important player in asRNA regulation, as in the case of the
68 regulation of plasmid copy number and toxin-antitoxin systems (13-14).

69 The mechanisms of action of asRNAs are diverse. They can negatively regulate transcription by
70 interference due to the collision of two converging RNA polymerases or by attenuation due, in some
71 cases, to the stabilization of a terminator structure in the mRNA upon binding of the asRNA (15, 16).
72 However, despite complete complementarity, the interaction of asRNA and its target requires, in
73 some cases, formation of an intermediate called "kissing complex" (13, 17). These interactions can
74 have negative or positive consequences on gene expression since they induce modifications to the
75 RNA secondary structure and/or physically interfere with the activity of other regulators (18, 19).
76 Very often, the mechanism by which a specific asRNA regulates its target remains unclear due to the
77 impossibility to modify the sequence of the asRNA independently of its target.

78 Various approaches have been used to enrich the *E. coli* transcriptome for double-stranded RNAs,
79 which are the presumed intermediates in asRNA regulation. Studies using inhibition of Rho-
80 dependent transcription termination demonstrated that pervasive antisense transcription is
81 common in almost all loci in *E. coli* (20). In 2010, one thousand asRNAs were identified suggesting
82 their importance in the control of gene expression (9). Another study focusing on the mapping of
83 transcriptional units highlighted the presence of 498 asRNAs mostly from overlapping untranslated
84 regions (UTRs) within convergent or divergent operons (21). Immunoprecipitation of double-
85 stranded RNAs using specific antibodies allowed the identification of 200 asRNAs of which 21 were
86 validated as RNase III-degraded asRNAs (22). In a fourth study, primary transcripts were isolated by
87 selective tagging allowing the identification of 212 asRNA transcription start sites (asTSSs) (23).
88 More recently, p19 viral protein capture of double-stranded RNAs identified 436 asRNAs (24).
89 Unexpectedly, there is a little overlap between the identified asRNAs from these different studies,
90 which may be due to technical bias, but may also depend on the genetic context and/or the
91 environmental conditions.

92 While previously published works focused on the identification of asRNAs, we aimed to characterize
93 physiologically relevant asRNAs. Some time ago we performed a transcriptome analysis of an *rnc*
94 mutant compared to its isogenic wt strain. We used a tailored RNA-seq approach described
95 previously (25). In agreement with other published genomics experiments (22-23, 26) several
96 candidate asRNA were detected, which were stabilized in the strain lacking RNase III activity. We
97 were surprised to see that many were asRNA to genes of important regulators which raised the
98 question of whether or not they could have a physiological impact on the expression and function of
99 their target regulator and hence on the downstream regulon. We first confirmed that RNase III
100 modulates the level of 4 of these antisense transcripts and then concentrated on the asRNA to *flhD*.
101 *flhD* is the first gene of the *flhDC* operon encoding the master regulator of swimming motility. We
102 find that AsflhD is involved in the direct repression of the transcription elongation of *flhD*, which
103 provides an additional regulatory layer to the complex cascade of motility in enterobacteria.

105 **Results**

106 *Characterization of asRNAs stabilized upon RNase III inactivation*

107 An RNA-seq analysis in a wt and its *rnc105* derivative strain was performed by tagging transcripts
108 according to their 5'-phosphorylation status, allowing to distinguish between 5'-triphosphate
109 fragments (primary transcripts TSS), monophosphate 5'-fragments (processed transcripts, PSS) and
110 internal fragments resulting from the fragmentation (INT) (25). The depth of sequence coverage
111 was not sufficient for a compilation of all asRNAs. Instead we looked manually for antisense reads
112 covering the translation signals of mRNAs that were enriched upon RNase III inactivation. Potential
113 asRNA promoter regions were deduced by examination of the TSS and PSS fractions in the *rnc*
114 strain. The RNase III processing sites in the wild-type were usually not obvious since they
115 presumably provoked the rapid degradation of the asRNA. We selected 4 asRNAs to the *crp*, *ompR*,

116 *phoP* and *flhD* transcripts encoding important global regulators for verification by northern blot (Fig.
117 1). We note that they had all been proposed as antisense transcripts in one or more of the previous
118 genomic studies (9, 21-24).

119 The *crp* gene encodes the major regulator of carbon catabolite repression and it was shown
120 previously to be transcriptionally regulated by a transcript initiated from a divergently expressed
121 promoter 3 base-pairs upstream and on the opposite strand (27-28). This transcript, now known to
122 express the *yhfA* gene, was detected in the wt strain (Fig. 1-A). Additional asRNAs, were stabilized in
123 the *rnc* strain in the 3 fractions. The TSS of one species is located 20 nts upstream of the *crp*
124 translation start on the opposite strand (shown by an orange dotted arrow on Fig. 1A) and in
125 addition there is extensive asRNA, complementary to the *crp* coding sequence and 5'-UTR (INT). We
126 performed northern blots using complementary probes hybridizing to positions 13 to 441 of the *crp*
127 ORF to detect both the *crp* mRNA and its antisense transcript. An asRNA, named *Ascrp*, of about 350
128 nts accumulates only in the mutant (Fig. 1-A).

129 The *ompR* gene encodes the response regulator of a two-component system involved in cell wall
130 homeostasis and response to low pH, EnvZ-OmpR (29-32). We observed an asTSS 147 nts
131 downstream from the AUG (Fig. 1-B) in wt and enhanced in the *rnc* mutant. Northern blotting with
132 complementary probes corresponding to the 5'-end of the *ompR* ORF confirmed the presence of the
133 *ompR-envZ* transcript and a long asRNA transcript, *AsompR*, of about 2000 nts, which is likely to also
134 encode the divergently expressed *greB* gene. In addition, in the mutant, smaller fragments (less
135 than 500 nts) are detected for both *ompR* and *AsompR* (Fig. 1-B), likely corresponding to a stable
136 duplex between the sense and asRNA transcripts in the absence of RNase III.

137 The *phoP* gene encodes the response regulator of the PhoQ-PhoP two-component system, involved
138 in cell wall homeostasis and in response to low magnesium (33-34). We observed an asRNA upon
139 RNase III inactivation in the INT fraction about 282 nts downstream from the *phoP* AUG. Northern

140 blot with complementary probes corresponding to the 5'-end of the *phoP* ORF confirmed the
141 accumulation of two fragments (AsphoP) about 300-320 nts long in the mutant (Fig. 1-C).

142 The *flhDC* genes are co-transcribed and together they encode the master regulator of motility,
143 FlhD₄C₂ (35). We detected an asRNA to *flhD* mRNA (AsflhD) accumulating in the mutant, initiated
144 22 nts downstream from the *flhD* AUG. Northern blot analysis with probes hybridizing to the 5'-UTR
145 of *flhD* confirmed the accumulation of AsflhD, with a major fragment about 220 nts and one minor
146 fragment about 160 nts upon RNase III inactivation. Together, there was an increase in the amount
147 of the full-length *flhD* mRNA in the mutant and the stabilization of a *flhD* fragment of approximate
148 size 220 nts (Fig. 1-D).

149 All these asRNAs are partially or completely processed by RNase III since they are only visible in the
150 *rnc* strain. Crp, OmpR, PhoP and FlhD are major regulators of gene expression in *E. coli*, all involved
151 in the control of large regulons (RegulonDB v 10.5 (36)). We wondered whether these asRNAs and
152 RNase III have a functional regulatory role and thus affect cell physiology. We studied in more
153 details asRNAs to *phoP* and *flhD*, two regulators tightly controlled at both the transcriptional and
154 post-transcriptional levels.

156 *Regulation of phoP and AsphoP by RNase III*

157 The RNA-seq profiles suggested that AsphoP may be transcribed from an asTSS located 282 nts
158 downstream from the translation start of *phoP* mRNA. An asRNA derived from this TSS was
159 confirmed by northern blot, probing for the 5'-region of the *phoP* ORF (Fig 1-C). In addition, the *rnc*
160 mutation slightly increases *phoP* stability and amount but induces very large increases in the
161 stability and level of AsphoP (Fig. 2-AB). Candidate consensus -10 and -35 sequences are located just
162 upstream of AsphoP TSS (Fig. 2-C). To validate this potential promoter, we constructed a P_{AsphoP}-*lacZ*

163 transcriptional fusion containing 150 nts before and 15 nts after the putative TSS of AsphoP, with
164 the wt sequence (P_{AsphoP}^{wt}) and also with mutations decreasing the agreement with the consensus in
165 the predicted -35 and -10 boxes (P_{AsphoP}^-) (Fig. 2-D). The mutated AsphoP promoter (P_{AsphoP}^-) strongly
166 decreased the expression of $P_{AsphoP}-lacZ$ (20-fold), confirming it as the endogenous AsphoP
167 promoter (Fig. 2-E). The activity of the $P_{AsphoP}^{wt}-lacZ$ fusion decreased 2-fold in the mutant implying
168 that RNase III positively regulates AsphoP. In summary, RNase III positively controls the
169 transcription of AsphoP and also participates to the degradation of both *phoP* and AsphoP
170 transcripts.

171 Sequence comparison with other bacterial species showed that although the region of the AsphoP
172 promoter is moderately well conserved, there are several A to G substitutions in the -10 box at
173 positions -9 and -12, suggesting that this promoter may be inactive in these genomes (Fig. 2-C). This,
174 in turn, implies that, if AsphoP has any function, it could be limited to *E. coli* K-12 and have been
175 counter-selected in these other species or more likely, represents a novel, evolving trait.

176 *Physiological expression of AsflhD*

177
178 Figure 1 shows a corresponding increase in the amounts of *flhD* and the appearance of a smaller
179 mRNA fragment in the *rnc* mutant. Intriguingly 40 years ago it was noted that RNase III was involved
180 in the swimming activity of *E. coli* and *rnc* mutants were immotile (37). In this work we have
181 investigated whether RNase III could exert this effect *via* AsflhD.

182 The RNA-seq profiles revealed an asTSS 22 nts downstream from the translation start of *flhD* as
183 reported by Dornenberg *et al.* (9). A candidate promoter exists upstream of this asTSS. Sequence
184 alignment of this region in other enterobacteria shows a good conservation of a promoter with an
185 extended -10 5'-TG box (38), suggesting that this promoter is conserved and active (Fig. 3-A).

186 To validate the presence of a functional promoter, a P_{AsflhD} -*lacZ* transcriptional fusion (P_{AsflhD}^{wt}) was
187 constructed containing 165 nts before and 15 nts after the putative TSS of *AsflhD* (Fig 3-B). This
188 fusion showed a relatively low level of β -galactosidase activity (Fig. 3-C). Its expression was strongly
189 increased when the -10 motif was improved towards the RpoD consensus (P_{AsflhD}^{+}) while mutating
190 the -35 to a less consensus sequence (P_{AsflhD}^{-}) decreased expression 2-fold (Fig. 3-C) confirming that
191 we had identified the *AsflhD* promoter. It should be noted that mutations were designed to be used
192 in the endogenous *flhD* locus, and chosen to minimally affect the coding sequence of *flhD* and to
193 avoid introduction of rare codons. The low level of expression made us wonder if *AsflhD* was
194 expressed using an alternative sigma factor. A heat-shock increased P_{AsflhD} -*lacZ* expression 2-fold
195 after 15 minutes and 5-fold after 60 minutes and also increased the level of the *AsflhD* RNA in the
196 *rnc* strain (Fig. 3-DE). Comparison of the *asflhD* promoter with the consensus sequences for the two
197 heat-shock sigma factors, σ^H and σ^E (*rpoH* and *rpoE*) shows better correlation with the σ^E consensus
198 than with σ^H (Fig. 3-A) (39-40).

199 We then examined whether the P_{AsflhD} promoter is under the control of RpoE by using a strain
200 deleted for *rseA* (anti- σ factor inhibitor of RpoE), which leads to strong induction of the RpoE
201 regulon (41-42). Deletion of *rseA* increased 2-fold the expression of the wt P_{AsflhD} -*lacZ* fusion and of
202 the improved P_{AsflhD}^{+} -*lacZ* construct (Fig. 3-FG) comparable with the effect of the heat-shock at 46°C,
203 known to induce the RpoE regulon (43). To test for an effect of *rpoH*, we introduced the P_{lac}
204 promoter in front of the endogenous *rpoH* gene and compared *AsflhD* RNA levels in wt and *rnc*
205 mutant cells after 15 minutes of heat-shock at 45°C, in the presence or absence of IPTG. *rpoH* mRNA
206 expressed from its own promoter or from the P_{lac} promoter in the presence of IPTG was strongly
207 increased by the heat-shock (Fig. 3-E). *AsflhD* was only detected in the *rnc* mutant and there was a
208 correlation between the levels of *rpoH* and *AsflhD* transcripts at 30°C and 45°C with and without
209 IPTG (Fig. 3-E). However, *RpoH* overexpression at 37°C revealed a slight repression of the

210 transcription of *AsflhD* on the wt P_{AsflhD} -*lacZ* fusion (Fig. S1-A) even though RNase III-stabilized
211 *AsflhD* accumulated when RpoH was induced at 45°C. This would seem to rule out a direct role for
212 RpoH in *AsflhD* transcription. We also tested RpoS overexpression but found it had no effect on the
213 transcription of *AsflhD* (Fig. S1-B). These experiments indicate that P_{AsflhD} is functional and induced
214 during a heat-shock due primarily to the activity of RpoE, acting directly or indirectly on the
215 promoter of *AsflhD*.

216 To confirm the identification of the *AsflhD* promoter and to allow variation of the *AsflhD* expression,
217 P_{AsflhD}^- and P_{AsflhD}^+ were introduced at the endogenous *flhDC* locus and the expression of the *AsflhD*
218 asRNA was examined by northern blot. The inactivation of the native promoter prevented the
219 detection of *AsflhD* in the *rnc* mutant bacteria. Conversely, the mutation overexpressing *AsflhD*, led
220 to the detection of a faint smear in the wt strain and to the accumulation of a high level of *AsflhD* in
221 the mutant (Fig. 3-H). In summary, we have identified the *AsflhD* promoter and shown that the
222 mutations in the promoter of *AsflhD* can be used as tools to study the function of *AsflhD* at the
223 genomic locus of *flhD*.

224

225 *Characterization of the 3'-end of AsflhD*

226 *AsflhD* RNA is only detected in the *rnc* strain, implying that it is very labile when RNase III is active
227 (Fig. 3-E). Circular RT-PCR experiments (cRT-PCR) confirmed that *AsflhD* is indeed expressed in both
228 the wt and *rnc* strains from the predicted asTSS promoter but that the 3'-extremities of the
229 different *AsflhD* transcripts are highly heterogeneous and can extend up to 345 nts in the mutant
230 (Fig. 4-A). Surprisingly, no 220 nts long RNA (Figs. 1-C, 3-EH) was detected in the mutant by cRT-PCR
231 while a 149 nts long fragment was found several times exclusively in the wt strain, which might
232 suggest that it is an intermediate in the degradation of *AsflhD*. It should be noted that the
233 requirement for a ligation step during the cRT-PCR may lead to a bias towards more accessible

234 single-stranded RNA fragments and could have excluded double-stranded RNAs from this analysis.
235 The stable AsflhD 220 nts transcript detected in *rnc* (Fig. 5-A) should correspond to duplex RNA
236 formation between transcripts of the convergent *flhD* and AsflhD promoters. The equivalent sense-
237 transcript is also detected (Figs. 1-D, 5-B below). RNase III is clearly a major factor in the
238 degradation of AsflhD. We also investigated the role of RNase E, the major endonuclease involved in
239 mRNA turnover in *E. coli*, and of PNPase an exoribonuclease negatively controlled by RNase III. We
240 found that the low stability of AsflhD is independent of the activity of PNPase but depends on
241 RNase E, since a longer transcript of an approximate size of 300 nts is detected upon RNase E
242 inactivation (Fig. 4-B). Hence, RNase III and RNase E are both involved in the rapid turnover of
243 AsflhD but they act independently.

244 245 *Independent degradation of flhD and AsflhD transcripts by RNase III*

246 We further investigated the role of RNase III in the degradation of *flhD* mRNA and AsflhD asRNA.
247 First, we analyzed the stability of both AsflhD and *flhD* transcripts upon RNase III inactivation. In the
248 mutant the 220 nts long AsflhD transcript and also a somewhat shorter about 160 nts long
249 transcript were strongly stabilized, while both the amount and the stability of the long *flhDC* mRNA
250 increased only 2-fold (Fig. 5-AB). In addition, a 220 nts long *flhD* RNA fragment was highly stabilized
251 in the *rnc* strain. It is derived from the 5'-UTR, where the probe used in this study to detect *flhD*
252 mRNA is located (Table S2). It presumably corresponds to a fragment of the *flhD* mRNA extending
253 from its promoter to the AsflhD promoter located in the beginning of the *flhD* ORF (Fig. 3-A) and
254 thus is complementary to AsflhD. The interaction of the 5'-UTR of *flhD* mRNA with AsflhD should
255 generate an RNA duplex (Fig. 4-A) whose degradation depends on cleavage by RNase III.

256 We examined the interaction between AsflhD and *flhD* RNAs and their cleavage by RNase III *in vitro*.
257 A 308 nts long *flhD* transcript corresponding to the 5'-UTR and part of the ORF of the *flhD* mRNA

258 and a 256 nts long AsflhD asRNA were synthesized and labeled at their 5'-extremity. These two
259 RNAs form a duplex when present in equimolar concentrations, which is completely degraded upon
260 addition of RNase III (Fig. S2-AB). Remarkably, under the same condition, RNase III cleaves the
261 individual RNAs independently at 2 sites on AsflhD and 4 sites on *flhD* (Fig. S2-CD). These cleavage
262 sites are located within regions able to form secondary structure on each molecule (6, 44) (Fig. S2-
263 C). RNase III is thus able to process both AsflhD and *flhD* RNAs *in vitro*, at specific sites but is also
264 able to drive the complete degradation of the 5'-UTR of *flhD* when it is bound to the asRNA AsflhD.
265 As AsflhD is never detected in the wt strain, this implies that it immediately base-pairs with *flhD* and
266 both are degraded, so changes in AsflhD expression will directly modulate the level of *flhD* mRNA.

267 268 *AsflhD represses the expression of flhD*

269 To investigate the function of AsflhD we determined the effect of AsflhD silencing and
270 overexpression on *flhD* expression by following *flhD* mRNA abundance and stability using the
271 endogenous P_{AsflhD} mutations described above. While a slight decrease (35%) of *flhD* mRNA
272 abundance results from both silencing (P_{AsflhD}^-) and overexpression (P_{AsflhD}^+) of AsflhD, the stability of
273 *flhD* mRNA was not significantly affected in either P_{AsflhD} mutants (Table 1).

274 Two translational *lacZ* reporter fusions encompassing the 5'-UTR and the first 34 amino-acids of
275 FlhD (including P_{AsflhD}) were introduced at the *lacZ* chromosomal locus. The P_{flhD} -*flhD-lacZ* fusion
276 allows simultaneous monitoring of the transcriptional and translational regulation of *flhD* and the
277 P_{tet} -*flhD-lacZ*, monitors only the post-transcriptional regulation (Fig. 6-A). The mutations in the
278 AsflhD promoter producing silencing and overexpression of AsflhD, were also introduced into both
279 fusions. While loss of AsflhD (P_{AsflhD}^-) had no impact on the expression of FlhD, overexpression of
280 AsflhD (P_{AsflhD}^+) resulted in decreased expression of *flhD-lacZ* expression from both the native and
281 P_{tet} promoters (Fig. 6-BC). Hence, overexpression of AsflhD leads to the reduction of *flhD* expression

irrespective of its promoter which suggests that AsflhD is involved in the direct regulation of *flhD* mRNA and/or translational levels and not *via* an effect on the *flhD* promoter.

Finally, we determined the effect of RNase III inactivation on *flhD* mRNA in the mutant overexpressing AsflhD (P_{AsflhD}^+). Northern blot confirms that, as expected, the abundance of *flhD* mRNA increases in the *rnc* strains overexpressing or not AsflhD (Fig. 6-D). Remarkably, the 220 nts long fragment observed upon RNase III inactivation (Figs. 4-B, 5-B) strongly accumulates when AsflhD is overexpressed (Fig. 6-D). Hence this suggests that the increase in strength of the AsflhD promoter drives the accumulation of the small fragment corresponding to the 5'-UTR of *flhD* mRNA, presumably as a duplex with AsflhD and that both are rapidly degraded by RNase III. This short *flhD* fragment could either be generated by processing of longer *flhD* mRNA or correspond to premature transcriptional termination of *flhD* mRNA. In summary, we show that AsflhD is involved in the repression of the expression of *flhD* at the post-transcriptional level.

Mutual repression of transcriptional elongation by AsflhD and flhD in vitro

To determine whether AsflhD can repress the transcription of *flhD*, we performed *in vitro* transcription experiments using a DNA template corresponding to the *flhD* gene from 76 nts before to 388 nts after the transcription start site of *flhD*, which allows the transcription of a 388 nts *flhD* RNA and of a 335 nts AsflhD RNA (Fig. 7-A). We compared the abundance of both transcripts synthesized from this latter DNA fragment to those generated from templates carrying the promoter mutations leading to either silencing (P_{AsflhD}^-) or overexpression (P_{AsflhD}^+) of AsflhD. *In vitro* transcription assays were performed in a single round of elongation in the presence of heparin and with RNA polymerase (RNAP) pre-bound to templates in the absence of RNA, hence observed effects are restricted to the elongation step and should be independent of the initiation of transcription. The results correlate with *in vivo* data even though the amplitude of the effects is

306 different. Figure 7 shows that expression of AsflhD is strongly impaired (10-fold) on the template
307 carrying the silencing (P_{AsflhD}^-) mutation and increased (2.5-fold) from the template carrying the
308 overexpression (P_{AsflhD}^+) mutation of AsflhD (Fig. 7-B orange bars). Also, as *in vivo* (Fig. 6-B), AsflhD
309 silencing does not affect the level of the *flhD* RNA, while its overexpression in *cis* results in a
310 decrease of the transcription of the *flhD* RNA (40%) (Fig. 7B purple bars). cAMP/CAP is known to
311 activate the transcription of *flhD* by binding to a sequence located 72 nts upstream from the TSS of
312 *flhD* (45). As expected, its addition increased the transcription of *flhD*, which was still reduced by
313 AsflhD overexpression (Fig. 7-B left). The role of AsflhD in the repression of *flhD* transcription
314 elongation, was further confirmed by using a template where the P_{tet} promoter replaced the P_{flhD}
315 promoter (Fig. S3-A) producing the same 388 nts *flhD* RNA but a shorter (260 nts) AsflhD RNA.
316 Similar results were observed from the templates carrying the P_{AsflhD} silencing and overexpression
317 mutations (Fig. S3-B). We conclude that overexpression of AsflhD *in cis* leads to the repression of
318 transcription elongation of *flhD*, which seems to be independent of the transcription level and of
319 the promoter expressing *flhD*.

320 We then determined the effect of purified AsflhD or *flhD* RNA addition on the transcription of both
321 *flhD* and AsflhD using the same linear DNA templates. Figure 7-C shows that addition of increasing
322 amount of AsflhD led to a linear decrease of *flhD* while not affecting the accumulation of AsflhD.
323 The reciprocal assay by adding increasing concentrations of purified *flhD* RNA decreased linearly the
324 amount of AsflhD synthesized, while the amount of *flhD* was not affected. We performed the same
325 assay with the shorter template carrying the P_{tet} promoter and observed similar results (Fig. S3-C).
326 In summary, AsflhD represses the transcription elongation of *flhD* both in *cis* and in *trans*,
327 independently of the promoter and its expression level. Thus, we propose that AsflhD asRNA and
328 *flhD* mRNA are involved in their mutual transcriptional attenuation in which the interaction of one

329 molecule with the other leads to a reduction in transcription *via* an alteration of transcription
330 elongation.

331
332 *AsflhD* represses the transcription of *flhD* in trans in vivo

333 To confirm *in vivo* the ability of AsflhD to repress the transcription of *flhD* in trans, AsflhD was
334 overexpressed from a plasmid, under the control of a P_{tac} promoter inducible by IPTG. The short 242
335 nts long AsflhD is transcribed from the +1 to the +220 nt relative to the TSS of AsflhD with a *rrnBT2*
336 terminator to enable its stabilization. In the *rnc* mutant, this transcript is further processed with the
337 appearance of the characteristic 160 nts intermediate (Figs. 8-A, 1-D). It is noteworthy that this
338 smaller fragment is slightly longer than in the endogenous AsflhD suggesting that it may correspond
339 to a 3'-fragment of AsflhD. Overexpression of AsflhD upon addition of IPTG decreases the
340 abundance of *flhD* mRNA both in the wt strain (30%) and in the mutant (20%) (Fig. 8-B), in
341 agreement with our *in vitro* data (Figs. 7-C, S3-C). Thus, consistent with previous experiments, trans
342 overexpression of AsflhD *in vivo* reduces the abundance of *flhD* mRNA but it is less effective than
343 AsflhD expressed from the *flhD* locus *in cis* and this effect is mostly independent of RNase III activity.

344 To further confirm that AsflhD can impact *flhD* expression *in vivo*, we measured the expression of
345 the *flhD-lacZ* reporter fusions when AsflhD is overexpressed from the plasmid. The overexpression
346 induces about a 30-40% decrease in the expression of *flhD* both from the P_{flhD} -*flhD-lacZ* and the P_{tet} -
347 *flhD-lacZ* fusions (Fig. 8-CD, dark grey). However, overexpression of AsflhD from the plasmid in the
348 strain where expression of AsflhD is already upregulated, by the presence of P_{AsflhD}^+ mutation in *cis*,
349 has no or little additive effect on the final repression (Fig. 8-CD, dark green). It should be
350 emphasized that these effects are independent of the *flhD* promoter (native P_{flhD} or P_{tet}). In
351 summary, we demonstrate that AsflhD RNA is involved in the transcriptional attenuation of *flhD*
352 both in *cis* and in *trans*, and that it does not involve the native P_{flhD} promoter.

353

354 *AsflhD* controls the motility cascade

355 The *flhDC* operon encodes the FlhD₄C₂ transcriptional master regulator of the swimming motility, so
356 we next investigated the effect of *AsflhD* overexpression on the expression of key factors belonging
357 to the motility cascade. This cascade of gene activation is divided into three classes of genes (46).
358 The *flhDC* operon encodes the only Class I protein, FlhD₄C₂, which is required for expression of class
359 II genes, which in turn control class III genes. We tested the effect of *AsflhD* on representative Class
360 II and Class III genes. The selected class II genes are *fliA* that encodes FliA, the sigma factor for class
361 III motility genes, and *flgB* that encodes FlgB, the main component of the flagella rod. The *fliC* gene
362 is a class III gene, located upstream from the *fliA* gene, and it encodes the main component of
363 flagella, FliC. The amounts of *fliA*, *flgB* and *fliC* mRNAs are all very strongly reduced upon
364 overexpression of *AsflhD* from its endogenous locus in the mid-log phase (Fig. 9-ABC). As the
365 reduction is considerably stronger than the effect on *flhD* itself, this implies that the effect of *AsflhD*
366 overexpression is amplified compared to that on *flhD*, similarly to what was previously reported for
367 transcriptional regulators of *flhD* expression (47-48). Furthermore, a bioinformatics search
368 (TargetRNA2 (49)) for possible direct *trans* targets of *AsflhD* found no candidates amongst genes
369 from the motility cascade. In addition, the abundance of *flgB* and *fliC* mRNAs in late-log phase
370 slightly increases relatively to mid-log phase (Fig. S4-AB) therefore this is consistent with the
371 hypothesis that *cis*-overexpression of *AsflhD* limits the level but also delays the timing for the
372 induction of the motility cascade.

373 Finally, we analyzed the effect of *AsflhD* on the motility of bacteria on low-agar plates and observed
374 that overexpression of *AsflhD* from both the *flhD* locus (P_{AsflhD}^+) or from the plasmid decreases the
375 swimming speed on plates (50% and 30% respectively) while the combined *cis* and *trans*
376 overexpression of *AsflhD* induces a stronger reduction of the swimming speed (70%) (Fig. 9-D).

377 These results enforce our hypothesis that the observed strong reduction of the motility cascade
378 effectors (*fliA*, *flgB* and *fliC* genes) in mid-log phase reflects a delayed induction rather than actually
379 inhibiting expression.

380 In summary, both *cis* and *trans* overexpression of AsflhD reduces transcription of *flhD*, which in turn
381 leads to repression of the whole cascade of motility and a reduction in swimming speed. Of note,
382 *cis*-expressed AsflhD appears to be more effective than the *trans*-expressed to control *flhD*
383 expression (Fig. 8-CD) and bacterial motility (Fig. 9-D). We hypothesize that the overexpression of
384 AsflhD delays the induction of *flhD* to maintain the timing of the motility program.

386 Discussion

387 Regulatory RNA molecules are often part of complex genetic networks in bacteria. They correspond
388 to a heterogeneous class of molecules that differ in gene organization, size and function. Our goal
389 was to detect, identify and investigate the function of some antisense transcripts in *E. coli*. We
390 compared the transcriptomes of an *rnc* mutant to that of a wt strain and selected candidate
391 asRNAs. We validated the presence of discrete transcripts, only detected in the *rnc* mutant, which
392 were complementary to *crp*, *ompR*, *phoP* and *flhD* genes. We identified the promoters encoding
393 AsphoP and AsflhD, both producing transcripts convergently expressed towards the promoters of
394 their target genes *phoP* and *flhD*. We show that RNase III is involved in the decay of both AsphoP
395 and *phoP*. AsflhD is highly unstable and it is degraded independently by RNase E and RNase III. The
396 promoter of AsflhD is induced by a heat-shock and is partially dependent on RpoE. We reveal that
397 AsflhD is involved in transcriptional attenuation of *flhD* by being able to repress *flhD* transcription
398 both *in vitro* and *in vivo* when overexpressed in *cis* or in *trans* (Figs. 7-8). Remarkably, *in vitro*, *flhD*
399 RNA in *trans* could also drive the transcriptional attenuation of AsflhD asRNA (Fig. 7), suggesting
400 that the interaction of the two RNAs can perturb the transcription elongation of the other transcript

401 *in vivo*. The relatively small effect of AsflhD on *flhD* expression has important consequences for the
402 FlhD₄C₂ regulon since, the *cis* overexpression of AsflhD lead to decreased expression of three
403 representative genes of the motility cascade; *fliA*, *flgB* and *fliC*, and reduced the swimming speed
404 (Fig. 9). In conclusion, this work demonstrates that AsflhD is an additional player acting in the
405 already complex regulatory process controlling *flhDC*. Our view of how AsflhD, by co-
406 transcriptionally fine-tuning the expression of *flhD*, reinforces the control of the motility cascade, is
407 shown in Figure 10.

408 409 *Conservation of the promoters of AsflhD and AsphoP*

410 asRNAs are poorly conserved among bacteria (50), but if this can be expected for asRNAs from
411 intergenic regions with low sequence constraints, nucleotide changes within the coding region of
412 the target risk to upset the function of the ORF and could be counterselected. AsflhD corresponds
413 almost entirely to the 5'-UTR of *flhD* but with the promoter located in the ORF, which is fairly well
414 conserved in enterobacteria (Fig. 3-A). The presence of multiple critical amino-acids along the
415 protein, whose modification leads to decreased motility, could explain the conservation of the
416 amino sequence of FlhD among Gram-negative bacteria (51). Thus, the conservation of the
417 promoter of AsflhD could be the result of direct selection for FlhD activity or for the regulatory
418 function of AsflhD controlling the expression of FlhD in these bacteria. In spite of the close similarity
419 between the AsflhD promoter regions of *Salmonella enterica* sv Typhimurium and that of *E. coli* (Fig.
420 3-A), it would be interesting to examine whether AsflhD is also expressed and controls *flhD* levels in
421 *S. enterica*, since it was reported that *E. coli flhDC* operon is expressed in *S. enterica* but at
422 significantly lower levels (52).

423 The promoter of AsflhD shows low activity *in vivo* despite a -10 consensus with an extended -10 5'-
424 TG-3' element and a -35 element which is functional since the P_{AsflhD}⁻ mutation decreased the

425 promoter activity. In the case of AsphoP, the promoter located in the central region of the *phoP*
426 ORF is not conserved in other enterobacteria. In *E. coli*, the two G to A changes in the -10 sequence
427 of the promoter of AsphoP, compared to other bacteria make a relatively good consensus -10
428 (CATAAT) which can account for the high level of expression of AsphoP (Fig. 2-E) compared to
429 AsflhD (Fig. 3-C). The lack of conservation in other bacteria suggests that, on the contrary to AsflhD,
430 any function of AsphoP may be unique to *E. coli* where it was most likely acquired.

431 432 *Role of RNase III in the degradation of asRNAs-mRNAs*

433 We demonstrate that RNase III initiates the rapid degradation of AsphoP and AsflhD RNAs, at the
434 same time it is also involved in the *flhD* degradation. The formation of an intermolecular RNA
435 duplex can trigger the RNase III-mediated degradation of a mRNA as in the case of the *puf* mRNA
436 upon binding to asPcrL in *Rhodobacter sphaeroides* or a sRNA upon binding to its target, as in the
437 case of the sRNA RyhB upon binding to the *sodB* mRNA (53). However, RNase III cleavage may
438 generate a shorter but more stable mRNA and so can have a positive role on gene expression (19,
439 54). In the case of AsphoP the *rnc* mutation reduces promoter activity (Fig. 2-E) but greatly increases
440 AsphoP stability (Fig. 2-A). In the case of AsflhD, RNase III allows the recycling of the products of
441 transcriptional attenuation between *flhD* and AsflhD (Fig. 7-A).

442 443 *Mechanism of regulation by AsflhD*

444 *Cis*-encoded regulatory elements and asRNAs have the advantage of their close location facilitating
445 their access to their target. Binding of AsflhD to the 5'-UTR of *flhD* mRNA can have multiple
446 consequences on the interaction of other *trans*-acting regulators, known to affect *flhD* expression.
447 For example, after removal of the 5'-triphosphate, CsrA binds two regions of the *flhD* mRNA and
448 protects it by competing with RNase E (6, 55). Closer to the translational start, the binding of the

449 McaS sRNA is required to expose the ribosome binding site and activate translation. On the
450 contrary, binding of the sRNAs OxyS, ArcZ, OmrA and OmrB represses translation (5, 56). AsflhD
451 binding can interact at any of these sites along the *flhD* mRNA to compete with the RNase E-
452 mediated degradation pathway and/or the sRNA control of translation. Our results clearly
453 demonstrated that AsflhD represses the transcription of *flhD* but without excluding that AsflhD may
454 also be involved in the control of the translation of *flhD* mRNA. However, it is difficult to assess
455 whether AsflhD may have a direct effect on translation or an indirect effect *via* the competition with
456 the other positive and negative post-transcriptional regulators of translation of *flhD*.

457 AsflhD and *flhD* mutually repress their transcription elongation. AsflhD interacting with the 5'-UTR
458 of *flhD* mRNA, could either provoke the termination of transcription by transcriptional interference
459 or by transcriptional attenuation (57), or drive the processing of *flhD* mRNA (*e.g.*, *via* RNase III) or
460 inhibit translation. Diverse mechanisms of transcriptional attenuation have been described which
461 are dependent on regulatory RNAs. For example, sRNA binding drives the premature Rho-
462 dependent transcription termination of the *rpoS* mRNA (58). The asRNA RNA β in *Vibrio anguillarum*
463 promotes transcription termination on the *fatDCBA* polycistronic mRNA before RNA polymerase
464 reaches the end of the *fatDCBAangRT* operon. Remarkably, the region where termination occurs
465 does not contain any canonical terminator motif (59). The asRNA RnaG was also shown to stabilize a
466 terminator structure upon binding to the *icsA* mRNA in *Shigella flexneri* (16). Similarly, the asRNA
467 RNAIII binds to the leader region of the *repR* mRNA and favors the formation of a terminator
468 structure in *Bacillus subtilis* (60). The asRNA anti-Q in *Enterococcus faecalis* is responsible for both
469 transcriptional interference due to RNAPs collisions and attenuation by an uncharacterized
470 mechanism (61). Our experiments do not detect the accumulation of a shorter transcript *in vitro*
471 upon addition of one or the other of the transcripts suggesting that binding of AsflhD to *flhD* does

not stabilize a terminator structure but could rather modify the stability of the elongating RNAP leading to heterogenous 3'-termini as observed for *AsflhD* *in vivo* by cRT-PCR (Fig. 4-A).

Physiology of AsflhD

Motility depends on the growth-rate due to the regulation of *flhD* (62). The expression of *flhD* peaks at the end of the exponential phase then decreases and is stabilized to an intermediate level during the stationary phase in *E. coli* (63). The temporal control of the motility cascade is maintained *via*, among other mechanisms, the expression of anti- σ factor, FlgM, at the same time as FliA, in order to allow the expression of class II genes without inducing class III genes expression prematurely. When the basal part of the flagellum is assembled, FlgM is exported from the cytoplasm and FliA induces the expression of class III genes.

We show here that the P_{AsflhD} promoter is induced during a heat-shock likely *via* RpoE. Remarkably, the swimming motility behavior is down-regulated during a heat-shock. This was proposed to be due to both a lowered level of FlhD and the inefficient export of FlgM (64-65). Our results show that the up-regulation of *AsflhD* during the heat-shock reducing *flhD* expression could also contribute to the decrease in swimming motility. Thus, as well as providing a fine-tuning mechanism to coordinate the expression of *flhD*, a function of *AsflhD* could be help to maintain the motility cascade off in conditions where motility would be detrimental and/or too costly.

Outlook

Our results demonstrate that the asRNA *AsflhD* is involved in a mutual transcriptional attenuation mechanism with its target *flhD* mRNA. Regulatory RNAs are far from being fully understood in bacteria and new mechanisms of action are likely to be discovered. Development of global approaches able to capture RNA-RNA and RNA-protein interaction (66-67) as well as prokaryotic

496 single cell RNA-seq (68) are likely to pave the way for the elucidation of the role of the widely
497 distributed asRNAs which were, until recently mostly considered as pervasive transcriptional noise
498 but for which the study of individual cells and molecules could be critical for the understanding of
499 their function.

500

501

502 **Materials and methods**

503 *Bacterial strains and culture conditions*

504 Strains and plasmids used in this work are listed in table S1. Constructions and mutations were
505 made by using primers given in table S2 and are described in Supplementary Materials and
506 methods. Strains were grown in LB Miller medium at 37°C, or at 30°C and shifted to 42°C, 45°C or
507 46°C for the heat-shock experiments. Appropriate antibiotics were added when required. IPTG was
508 used at the indicated concentrations for induction of AsflhD from the pCA24N AsflhD plasmid and
509 arabinose for RpoS from the pBAD18 plasmid.

511 *Northern blotting and RNA-seq analysis*

512 Total RNA was prepared from bacteria grown to the A_{600} 0.4 using the hot-phenol procedure (69).
513 Five μ g of total RNA were electrophoresed either on 1% agarose, 1xTBE or 6% polyacrylamide gels
514 (19/1), 7M urea, 1xTBE for analysis by northern blotting (70-71) along with RiboRuler High-Range
515 marker (ThermoFisher) or radio-labeled Msp1-digested pBR322 (NEB). Membranes were hybridized
516 with complementary RNA probes. Templates for the synthesis of the RNA probes were obtained by
517 PCR amplification using the pair of “m” and “T7” oligonucleotides (Table S1). Probes were
518 synthesized by T7 RNAP with [α -³²P]-UTP yielding uniformly labeled RNAs (72). Membranes were
519 also probed with M1 or 5S as loading control by using 5'-end labeled primers (Table S2). An RNA-seq
520 analysis was performed to compare the transcriptomes of the wild-type (N3433) and the RNase III
521 deficient strain (IBPC633). Sample preparation for RNA-seq, 5'-RNA tagging and RNA-seq analysis
522 were performed as in (25). Data have been deposited in the ArrayExpress database at EMBL-EBI
523 under accession number [E-MTAB-9507](#) (M. Lejars, L.Kuhn, A. Maes, P. Hammann, E. Hajnsdorf
524 manuscript in preparation).

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β-galactosidase assays

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Cultures were initiated at A_{600} 0.05 and sampled at A_{600} 0.4 or in the case of the results presented in figure S2 also at A_{600} 1.2. Samples (200 μ L) were lysed in 800 μ L PBS buffer with 10 μ L 0.1% SDS and 20 μ L chloroform. β -galactosidase activity was assayed as described (73), results are the mean of at least three biological replicates.

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Circular RT-PCR

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Circular RT-PCR was performed with total RNA extracted from N3433 and IBPC633 treated with 5'-polyphosphatase. After circularization with T4 RNA ligase, mflhD2 was used to prime reverse transcription and mflhD6 and masflhD10 to generate PCR products (Table S2), which were cloned (74).

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RNA band-shift assay and in vitro processing by RNase III

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DNA templates carrying a T7 promoter sequence were generated by PCR using the Term and T7 oligonucleotides (Table S2). They allow the transcription of the first 308 nts *flhD* and of the first 256 nts of *AsflhD*. RNAs were synthesized by T7 RNAP with [α - 32 P]-UTP as a tracer and were gel purified. Transcripts 5'-end-labelling, hybridization, RNase III digestion and sample analysis were described in (74-76). Briefly, radioactive *AsflhD* was incubated with increasing concentrations of *flhD* mRNA under two conditions referred to as "native" (incubation in TMN buffer (20 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 100 mM sodium acetate for 5 min at 37°C) and "full RNA duplex" (initial denaturation at 90°C for 2 min, then incubation in 1xTE at 37°C for 30 min). The complexes were loaded on native polyacrylamide gels to control for hybridization efficiency or

548 submitted to *in vitro* processing by RNase III of *E. coli*. RNase III digestion of free 5'-radiolabeled
549 AsflhD, *flhD* or complexed AsflhD with *flhD* was performed at 37°C in TMN buffer containing 1 µg
550 tRNA for 15 min with RNase III (Epicentre). Samples were loaded on denaturing polyacrylamide gels
551 together with an RNA alkaline ladder as in (75).

552 553 *In vitro* transcription assay

554 Single-round *in vitro* transcription experiments were carried out on linear templates as described in
555 Supplementary materials and methods.

556 557 *Motility assay*

558 Stationary phase bacterial cultures (MG1655-B, ML241 (P_{AsflhD}^+) carrying the pCA24N control (Ctl) or
559 the pCA24N AsflhD (As) plasmid) were inoculated (2 µL) on soft-agar (0.2 g/L) SOB motility plates
560 (containing 10^{-4} M IPTG and 2.4 g/L $MgSO_4$) at 37°C and pictures were taken using a Gel Doc
561 (Biorad) imager at the beginning and the end of the linear swimming motility period (from 5 to 8
562 hours). Swimming speed was then calculated as a function of time by comparing motility diameters.

563
564 *Image treatment, quantifications and statistical analysis* are given in Supplementary Materials and
565 Methods

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Table 1: Effect of *AsflhD* silencing and overexpression on *flhD* expression and stability

Promoter	Relative abundance <i>flhD</i> at t0 (%)	<i>flhD</i> Half-life (min)
P _{AsflhD}	100	0.57 ± 0.15
P _{AsflhD} ⁻	65	0.54 ± 0.19
P _{AsflhD} ⁺	64	0.45 ± 0.13

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MG1655-B (P_{AsflhD}), ML73 (P_{AsflhD}⁻) and ML241 (P_{AsflhD}⁺) were grown to mid-log phase (A₆₀₀ 0.4) at

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37°C. Sampling was performed at different times after addition of rifampicin (500 µg/mL) (t0) and

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total RNA was subjected to northern blot analysis. The membranes were probed successively for

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flhD and M1. The decay-rate of *flhD* mRNA was calculated as described in the “quantification and

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statistical analysis” section of the supplementary materials and methods.

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780 **Legends**

781 *Figure 1: RNase III inactivation stabilizes asRNAs*

782 The RNA-seq reads were aligned to the genome of reference (MG1655, GenBank identifier
783 U00096.3, <https://www.ncbi.nlm.nih.gov/nucore/U00096.3>) and visualized with the Integrative
784 Genomic Viewer (IGV) 2.4.2 software (<http://software.broadinstitute.org/software/igv/>). The
785 fractions isolated during the RNA-seq analysis are color-coded; strand of the target gene in wt (dark
786 blue) and in *rnc* mutant (light blue); strand of the asRNA gene in wt (red) and in *rnc* mutant
787 (orange). Reads corresponding to transcription start site (TSS), processing sites (PSS) and internal
788 fragments (INT) are indicated. The scale for the absolute number of reads identified is indicated on
789 the top right of each lane. The schemes indicate the localization of the ORFs and known promoters
790 (plain bent arrows) and the putative antisense promoters deduced from TSS data (dashed bent
791 arrows).

792 Detection of asRNAs (orange triangle) to *crp* (A), *ompR* (B), *phoP* (C) and *flhD* (D). RNAs extracted
793 from exponentially grown N3433 (wt) and IBPC633 (*rnc*) strains were analyzed on agarose or
794 denaturing acrylamide gels and northern blots were probed by using pairs of complementary
795 uniformly radio-labeled RNA probes to the same region of each target and a primer complementary
796 to the 5S rRNA. A scheme of the probed loci is shown under each panel. The position of the probes
797 relative to the DNA sequence is indicated by a dashed box. The putative asRNA promoter is
798 indicated by a dashed orange bent arrow when it could be predicted from RNA-seq data while
799 known promoters are indicated by plain arrows (purple for genes located on the opposite strand
800 from the detected asRNA). To note, *crp* and *AsompR* were successively probed on the same
801 membrane, thus they share the same loading control. The membranes shown for *phoP* and *AsphoP*
802 correspond to zero time points of the stability experiment presented in figure 2-A. It should be
803 noted that the *flhD* mRNA detected corresponds in size to the co-transcript *flhDC* (1200 nts).

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Figure 2: AsphoP and phoP levels are regulated by RNase III

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Figure 3: Transcriptional regulation of AsflhD

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(A) Genetic structure of the *flhD* locus and alignment of the promoter sequence of AsflhD with the consensus sequences for the RpoD, RpoE, RpoH and RpoS-dependent promoters (39, 40, 77) and

830 with 8 Eubacterial species showing between 49-92 % identity of FlhD with *E. coli* (51). The position
831 of the promoter of AsflhD (orange bent arrow), is indicated relative to the *flhD* translation start of
832 *flhD* (+22). Nucleotide sequences correspond to the following bacteria, Eco, *Escherichia coli*
833 MG1655 ([NC_000913.3](#)), Sen, *Salmonella enterica typhimurium* ([D43640](#)), Eca, *Erwinia carotovora*
834 ([AF130387](#)), Sma, *Serratia marcescens* ([AF077334](#)), Sli, *Serratia liquefaciens* ([Q7M0S9](#)), Yen, *Yersinia*
835 *enterocolitica* ([AF081587](#)), Xne, *Xenorhabdus nematophilus* ([AJ012828](#)), Pmi, *Proteus mirabilis*
836 ([U96964](#)), Bbr, *Bordetella bronchiseptica* ([U17998](#)). (B) Genetic structure of the transcriptional
837 AsflhD-*lacZ* reporter fusion (MG2114 P_{AsflhD}). Mutations in red and in green were introduced to
838 inactivate the promoter of AsflhD (P_{AsflhD}⁻ in the strain ML239) and and to increase its activity
839 (P_{AsflhD}⁺ in the strain ML218) respectively. (C) Effect of mutations in AsflhD promoter on expression
840 of P_{AsflhD}-*lacZ* fusion. (D) Expression of P_{AsflhD}-*lacZ* fusion in the wt (strain (MG2114 P_{AsflhD}) before
841 (30°C t=0) and after 15 and 60 minutes of upshift (46°C). (E) DJ624, DJ624-*rnc105* and their P_{lac}-*rpoH*
842 derivatives were grown at 30 °C. At mid-log phase, part of the cultures were shifted to 45°C with or
843 without simultaneous addition of 0.1 mM IPTG. Sampling was performed 15 min later. Total RNA
844 was analyzed by Northern blot, the membrane was probed for *rpoH*, AsflhD and M1. (F) Expression
845 of P_{AsflhD}-*lacZ* fusion in the wt strain (MG2114 P_{AsflhD}) and *rseA* mutant (ML279) at 37°C. (G)
846 Expression of P_{AsflhD}-*lacZ* fusion in the strain carrying the P_{AsflhD}⁺ fusion (ML218) and its *rseA*
847 derivative strain (ML312) at 37°C. Values are means of three biological replicates for each strain,
848 and error bars are standard deviations. Statistical significance was determined by a heteroscedastic
849 two-tailed t test (** for p-values ≤0.01 and *** for p-values ≤0.001). (H) MG1655-B (wt), ML73
850 (P_{AsflhD}⁻) and ML241 (P_{AsflhD}⁺) and their *rnc* derivatives (respectively ML65, ML75 and ML341) were
851 grown at 37°C until mid-log phase. Total RNA was analyzed by northern blotting. The membrane
852 was probed successively for AsflhD and 5S or M1.

854 *Figure 4: Characterization of AsflhD*

855 (A) The sequenced reads following cRT-PCR are shown relative to the *flhD* locus. Each line
856 represents one transcript. Transcripts were identified from both the wt strain (N3433) (in black) and
857 from the *rnc* mutant (IBPC633) (in blue). The 5' and 3'-end positions are indicated relative to the TSS
858 of *AsflhD*. The scheme shows the localization of the P_{AsflhD} transcript relative to P_{flhD} . (B) N3433 (wt)
859 and its derivatives, *pnp*, *rnc*, *pnp-rnc*, *rne^{ts}*, *rnc-rne^{ts}* mutants (respectively N3433-*pnp*, IBPC633,
860 IBPC633-*pnp*, N3431, IBPC637), were grown at 37°C until mid-log phase. Where indicated, cells
861 were grown at 30°C and submitted to a heat-shock at 42°C for 15 min, in order to inactivate RNase E
862 in the strain carrying the thermosensitive *rne^{ts}* allele. Total RNA was analyzed by northern blotting.
863 The membrane was probed successively for *AsflhD* and 5S.

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865 *Figure 5: RNase III is involved in the degradation of AsflhD asRNA and flhD mRNA in vivo*

866 (A) MG1655-B and its *rnc* derivative (ML65) were grown to mid-log phase at 37°C. At A_{600} 0.4
867 rifampicin (500 µg/mL) was added (t0) and sampling was performed at different times. Total RNA
868 was extracted and subjected to northern blot analysis. The membranes were probed for *AsflhD*,
869 *flhD* and 5S. The decay-rate of *flhD* mRNA was calculated as described in material and methods.

870
871 *Figure 6: AsflhD repress the expression of flhD*

872 (A) Genetic structures of the P_{flhD} -*flhD-lacZ* (ML219) and P_{tet} -*flhD-lacZ* reporter fusions (ML233) and
873 their derivatives containing the mutations leading to either silencing (P_{AsflhD}^- , in red, ML221 and
874 ML235 respectively) or *cis*-overexpression (P_{AsflhD}^+ , in green, ML226 and ML237 respectively) of
875 *AsflhD*. Expression of (B) P_{flhD} -*flhD-lacZ* and (C) P_{tet} -*flhD-lacZ* reporter fusions (gray bars) and their
876 derivatives (P_{AsflhD}^- in red and P_{AsflhD}^+ in green) are given as β-galactosidase activity. Values are
877 means of three biological replicates for each strain, and error bars are standard deviations.
878 Statistical significance was determined by a heteroscedastic two-tailed t test (*** for p-values
879 ≤0.001).

(D) MG1655-B (wt), ML241 (P_{AsflhD}^+) and their *rnc* mutant derivatives (ML65 and ML341 respectively) were grown to mid-log phase (A_{600} 0.4) at 37°C. Total RNA was extracted and subjected to northern blot analysis. The membrane was probed successively for *flhD* and for M1.

Figure 7: AsflhD is involved in the transcriptional attenuation of flhD

(A) Schematic representation of the template used for the *in vitro* transcription assay carrying the P_{flhD} promoter driving the expression of a 388 nts transcript (purple) and the P_{AsflhD} promoter driving the expression of a 335 nts transcript (orange). The linear DNA template was constructed using the oligonucleotides LM191 and LM9 (table S1) and corresponds to -76 to +388 of the *flhD* transcript relative to its TSS, with a 40 nts extension carrying the *rrnBT2* terminator (fragment length 504 bp). This fragment carries the native *flhD* promoter (-10 and -35 sites) and includes the cAMP/CAP site at -72 compared to the *flhD* TSS, at its upstream extremity.

In vitro transcription assays were performed as described in the Supplementary material and method (B) with or without addition of 100 nM CAP and 0.2 mM cAMP for 15 min at 37°C before addition of RNA polymerase, (C) with 100 nM CAP and 0.2 mM cAMP and the addition of *in vitro* purified AsflhD or *flhD* transcripts to the reaction at the indicated concentrations. Samples were analyzed on sequencing gels. Relative intensity of the indicated bands (*flhD* in purple and AsflhD in orange) were analyzed. Values are means of 6 (B) or 3 (C) replicates and error bars are standard deviations. Statistical significance was determined by a heteroscedastic two-tailed t test (* for p-values ≤ 0.05 , ** for p-values ≤ 0.01 and *** for p-values ≤ 0.001).

Figure 8: AsflhD repress the expression of flhD in trans

(A) MG1655-B (wt) and its *rnc* derivative (ML65) containing the control pCA24N (Ctl) or the pCA24N AsflhD (As) plasmids were grown to mid-log phase (A_{600} 0.4) at 37°C in the presence of the indicated

904 concentration of IPTG. Total RNA was extracted and subjected to northern blot analysis. The
905 membrane was probed for AsflhD and M1 RNA. (B) MG1655-B (wt) containing the control pCA24N
906 (Ctl) or the pCA24N AsflhD (As) plasmids were grown to mid-log phase (A_{600} 0.4) at 37°C in the
907 presence of 10^{-4} M IPTG. Total RNA was extracted and subjected to northern blot analysis. The
908 membrane was probed for *flhD* using a probe corresponding to the beginning of the *flhD* ORF and
909 M1 RNA. This *flhD* probe was used to detect *flhD* from the chromosomal locus and to avoid
910 detection of *flhD* RNA transcribed from a plasmid promoter. Expression of (C) P_{flhD} -*flhD-lacZ*
911 (ML219) and (D) P_{tet} -*flhD-lacZ* (ML233) reporter fusions (gray bars) and their P_{AsflhD}^+ derivatives (in
912 green, ML226 and ML237 respectively) containing the pCA24N control (Ctl, in dark grey) or the
913 pCA24N AsflhD (As, in dark green) plasmid was determined in the presence of 10^{-4} M of IPTG.
914 Values are means of three biological replicates and error bars are standard deviations. Statistical
915 significance was determined by a heteroscedastic two-tailed t test (* for p-values ≤ 0.05 , ** for p-
916 values ≤ 0.01 and *** for p-values ≤ 0.001).

917
918 *Figure 9: AsflhD overexpression represses the cascade of motility*

919 MG1655-B (wt) and ML241 (P_{AsflhD}^+) were grown at 37°C until mid-log phase. Total RNA was
920 analyzed by northern blotting. The membranes were probed successively for (A) *fliA* and M1, for (B)
921 *flgB* and M1 and for (C) *fliC* and M1. (D) Swimming motility speed is reduced upon overexpression in
922 *cis* (P_{AsflhD}^+ green bars) and in *trans* (pCA24N AsflhD dark grey and dark green bars) of AsflhD as
923 observed on motility plates. Swimming speed ($\text{cm}\cdot\text{h}^{-1}$) was calculated (see quantification and
924 statistical analysis section of the supplementary material and method) on three biological replicates
925 in the wt strain (MG1655-B) and in the P_{AsflhD}^+ strain (ML241) carrying the pCA24N control (Ctl) or
926 the pCA24N AsflhD (As) plasmid. Values are means of three biological replicates and error bars are
927 standard deviations. Statistical significance was determined by a heteroscedastic two-tailed t test (*
928 for p-values ≤ 0.05 , ** for p-values ≤ 0.01 and *** for p-values ≤ 0.001).

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Figure 10: Schematic representation of the regulatory function of AsflhD

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When the motility cascade is off, low expression from the P_{flhD} promoter (purple bent arrow) is not

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sufficient to maintain the expression of *flhDC*. The binding of AsflhD (orange) to *flhD* (purple) RNA

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may occur co-transcriptionally and perturb the elongation of both molecules reinforcing the low

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level expression of *flhD*. Upon encountering conditions where motility is required, the strong

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induction of the P_{flhD} promoter changes the balance between the sense and the asRNA allowing

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flhDC expression and activation of the motility cascade but where changes in AsflhD expression (e.g.

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in response to heat) could modulate *flhD* expression. DNA is represented by grey lines and RNAP as

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orange and purple oval shapes.

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Supplementary legends

Figure S1: The transcription of AsflhD is not activated by RpoH and RpoS

(A) Expression of *AsflhD-lacZ* fusion in the wt strain (MG2114 P_{AsflhD}) and its $P_{lac-rpoH}$ derived strain (ML310) in the presence of 10^{-3} M IPTG at 37°C was measured at OD_{600} 1.2. (B) Expression of *AsflhD-lacZ* fusion in the wt strain (MG2114 P_{AsflhD}) carrying the pBAD18 control (Ctl) or pBAD18 *RpoS* (*rpoS*) in the presence of 0.1% arabinose at 37°C was measured at OD_{600} 1.2. Values are means of three biological replicates and error bars are standard deviations. Statistical significance was determined by a heteroscedastic two-tailed t test (** for p-values ≤ 0.01).

Figure S2: In vitro cleavage of AsflhD and flhD RNAs by RNase III

5'-radiolabeled *AsflhD* RNA (308 nts) was incubated with increasing concentrations of *flhD* mRNA (256 nts) under conditions referred as Native and Full RNA duplex conditions (Materials and Methods). Native *AsflhD-flhD* complexes were formed at 37°C for 5 min in TMN buffer, and full duplexes were obtained after a denaturation-annealing treatment in TE Buffer (2 min 90°C, 30 min 37°C before loading on native polyacrylamide gels to control for hybridization efficiency (A) or (B) *in vitro* processing by RNase III. RNase III digestion of free or complexed *AsflhD* in native conditions was performed at 37°C in TMN buffer containing 1 μ g tRNA with increasing concentration of RNase III per sample. Samples were analyzed on 8% polyacrylamide-urea gels. 5'-radiolabeled *flhD* (C) and *AsflhD* (D) were cleaved by RNase III (1 unit) *in vitro* at 4 and 2 main sites respectively (represented by a numbered red arrow). Mapping of the main *in vitro* cleavage sites of RNase III on *flhD* and *AsflhD* are indicated on their predicted secondary structures with the position of the main RNase III cleavage sites indicated relative to the TSS (according to (6) and Vienna RNA websuite (44)). The localization of RNase III cleavage site (black arrows) was performed by comparing the cleavage

963 fragment relative to an alkaline RNA ladder (NaOH) obtained by partial hydrolysis in NaOH of the
964 respective labeled RNAs and radioactive markers.

965
966 *Figure S3: AsflhD repression of the transcription of flhD is independent of the P_{flhD} promoter*

967 (A) Schematic representation of the template used for the *in vitro* transcription assay carrying the
968 P_{tet-flhD} promoter driving the expression of a 388 nts transcript (purple) and the P_{AsflhD} promoter
969 driving the expression of a 260 nts transcript (orange). The DNA templates were constructed using
970 the LM213 and LM9 oligonucleotides and carry chromosomal sequences starting at the *flhD* TSS
971 with a 40 nts extension carrying the tetracycline promoter (78) so that the P_{tet} transcription starts at
972 the position of the *flhD* TSS. (B) *In vitro* transcription assays were performed on templates carrying
973 wt and P_{AsflhD}⁺ and P_{AsflhD}⁻ mutations and (C) on the wt template after addition of *in vitro* synthesized
974 AsflhD or *flhD* transcripts to the reaction at the indicated concentrations (supplementary material
975 and method). Relative intensity of the indicated bands (*flhD* in purple and AsflhD in orange) were
976 quantified as described in the “quantification and statistical analysis” with a number of sample n=6
977 (B) and n=3 (C). Values are means of 6 (B) or 3 (C) replicates and error bars are standard deviations.
978 Statistical significance was determined by a heteroscedastic two-tailed t test (* for p-values ≤0.05,
979 ** for p-values ≤0.01 and *** for p-values ≤0.001).

980
981 *Figure S4: AsflhD overexpression repress and delay the induction of the motility cascade*

982 MG1655-B (wt) and ML241 (P_{AsflhD}⁺) were grown at 37°C until mid-log phase (A₆₀₀ = 0.4) or until late-
983 log phase (A₆₀₀ = 1). Total RNA was analyzed by northern blotting. The membranes were probed
984 successively for (A) *flgB* and M1 and for (B) *fliC* and M1.

985

986

Figure 1

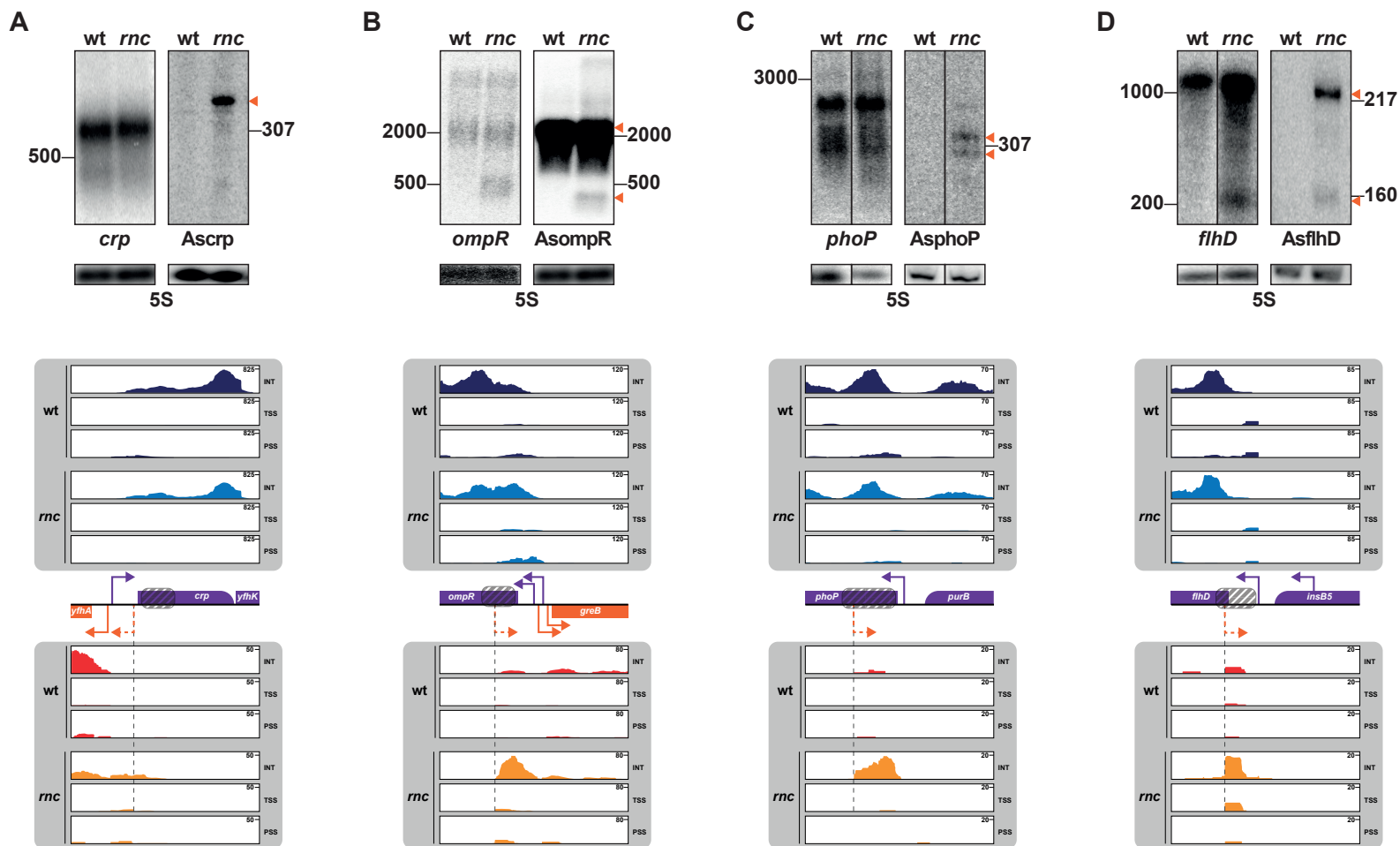


Figure 2

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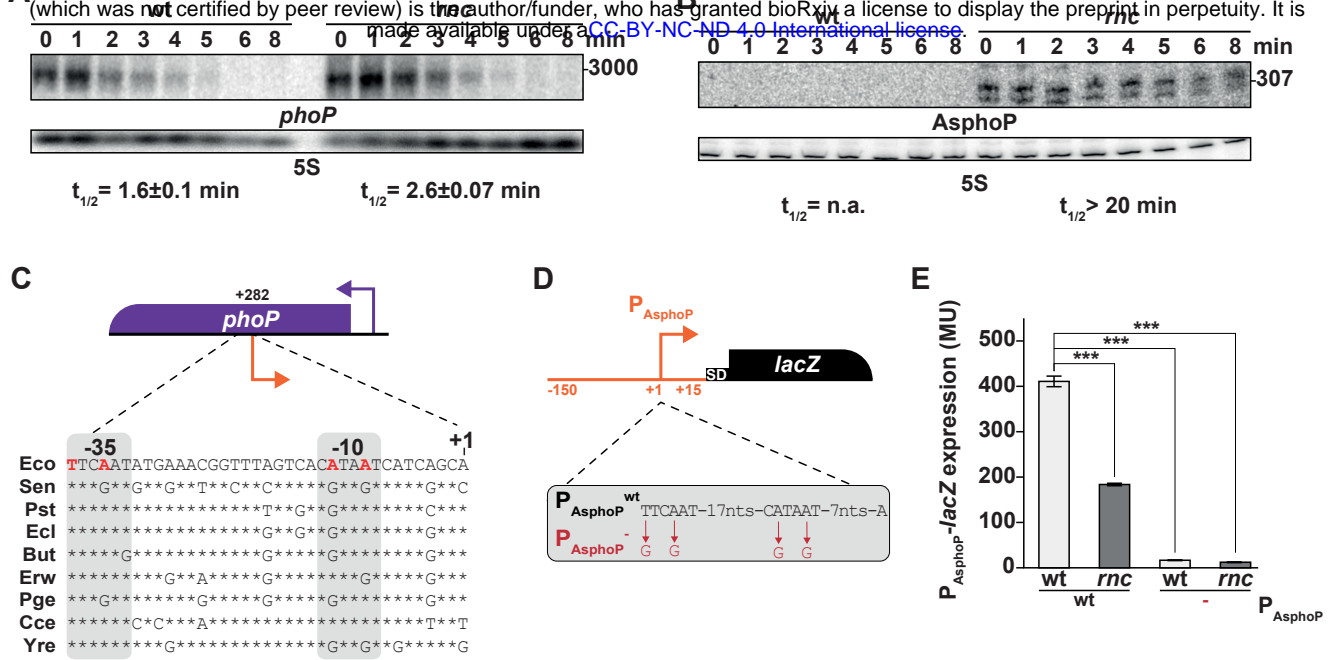


Figure 3

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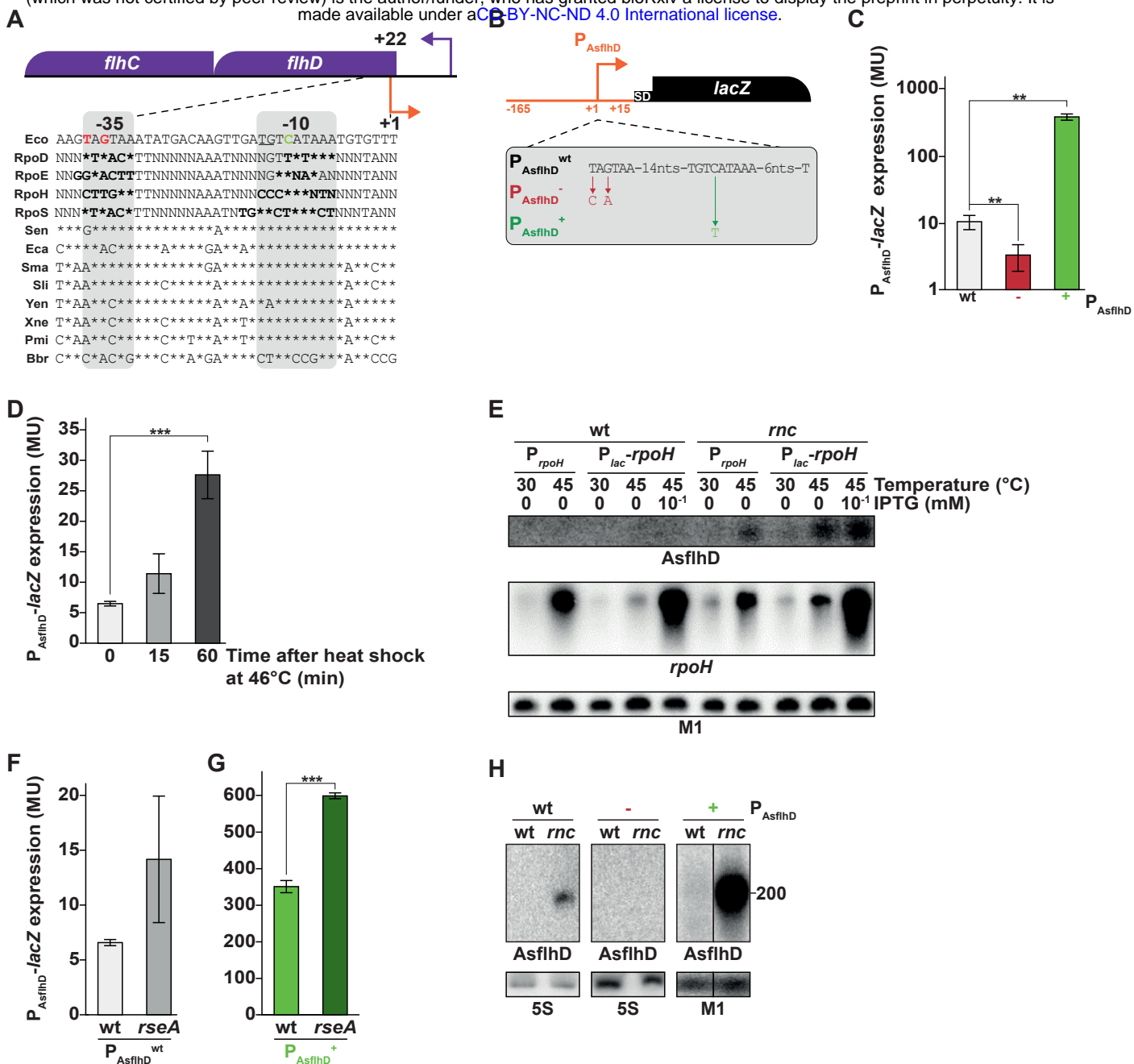


Figure 4

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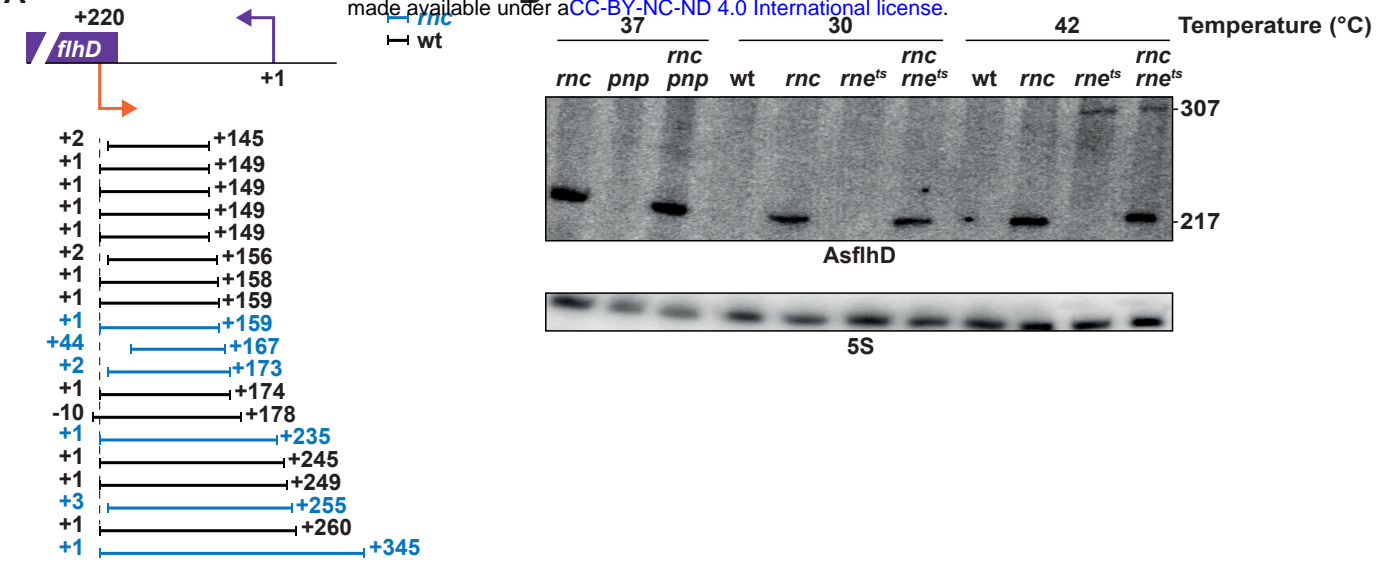


Figure 5

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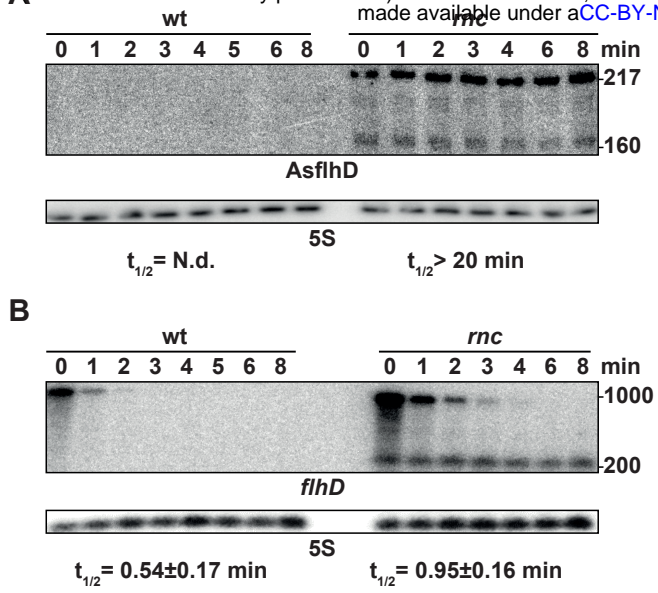


Figure 6

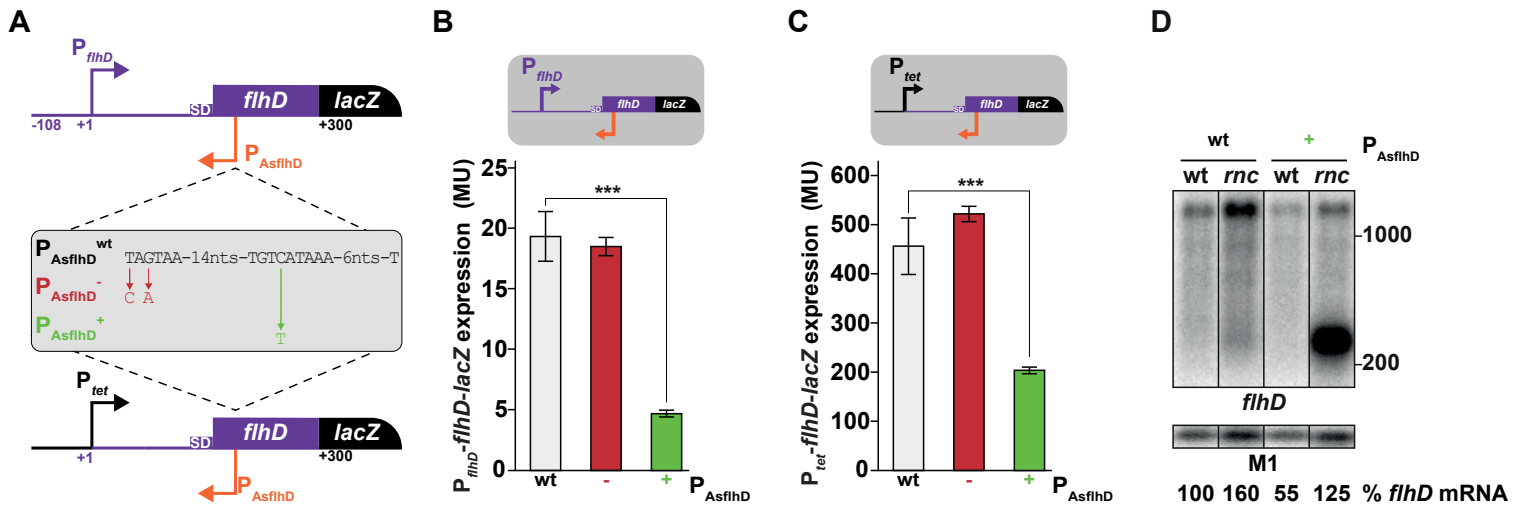


Figure 7

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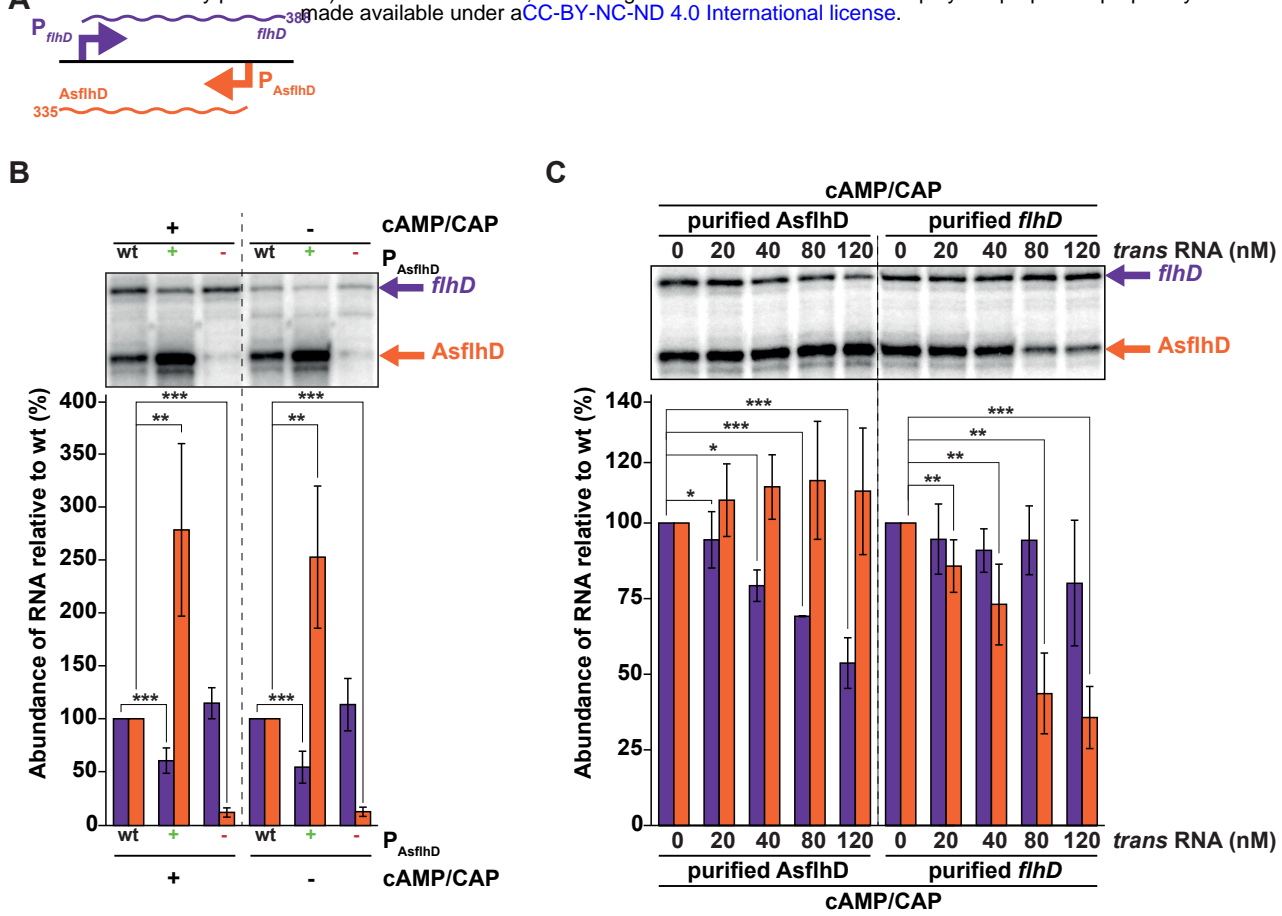


Figure 8

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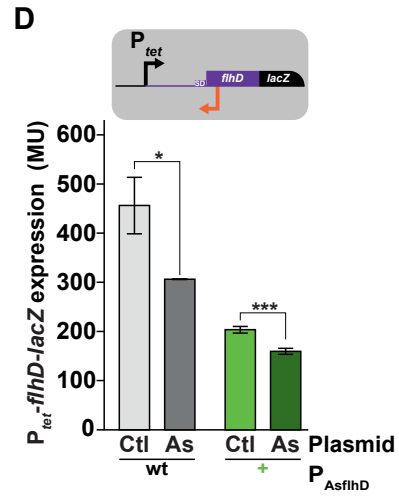
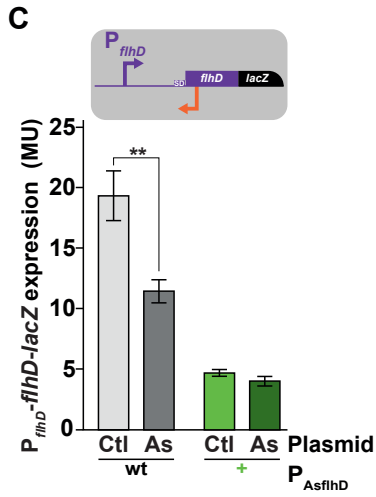
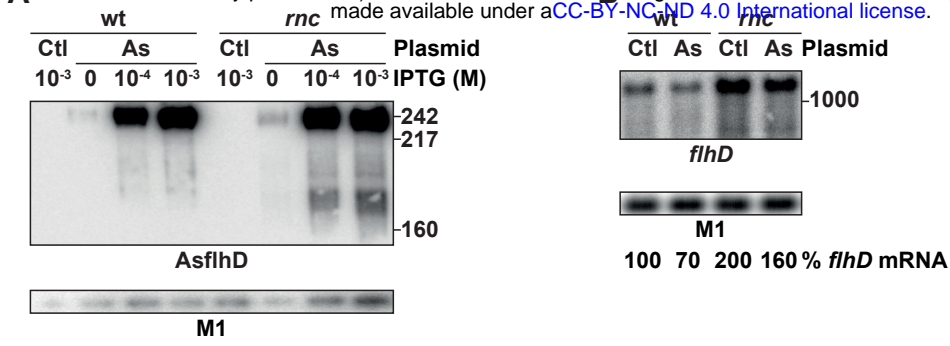


Figure 9

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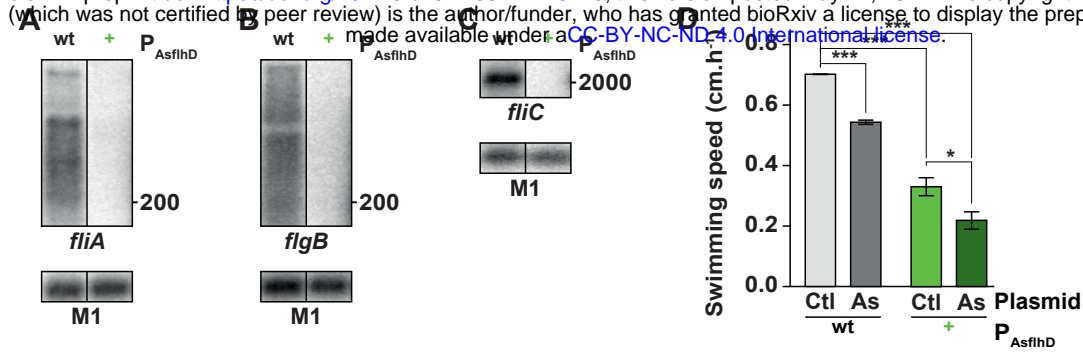


Figure 10

