

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

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bioRxiv preprint doi: https://doi.org/10.1101/2021.05.11.443715; this version posted May 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license. Regulatory interplay between RNase III and asRNAs in E. coli; the case of AsflhD and the master regulator of motility, flhDC Maxence Lejars,^{a,*,#} Joël Caillet,^a Maude Guillier,^a Jacqueline Plumbridge,^a Eliane Hajnsdorf,^{a,#} ^aUMR8261, CNRS, Université de Paris, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France Running Head: Antisense RNA control of flhD expression [#]Address correspondence to Eliane.Hajnsdorf@ibpc.fr, maxence.lejars@md.tsukuba.ac.jp ^{*}Present address; Division of Biomedical Science, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan.

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17 Abstract

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

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32 Importance

The importance of asRNAs in the regulation of gene expression has long been underestimated. Here, we confirm that asRNAs can be part of layered regulatory networks since some are found opposite to genes encoding global regulators. In particular, we show how an antisense RNA (AsflhD) to the gene expressing a transcription factor serving as the primary regulator of bacterial swimming motility ($FlhD_4C_2$) is involved in the transcriptional attenuation of *flhD*, which in turn impacts the expression of other genes of the motility cascade. The role of AsflhD highlights the importance of discrete fine-tuning mechanisms in the control of complex regulatory networks.

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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)

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45 Introduction

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

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).

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

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

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

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

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105 Results

106 Characterization of asRNAs stabilized upon RNase III inactivation

An RNA-seq analysis in a wt and its *rnc*105 derivative strain was performed by tagging transcripts 107 according to their 5'-phosphorylation status, allowing to distinguish between 5'-triphosphate 108 fragments (primary transcripts TSS), monophosphate 5'-fragments (processed transcripts, PSS) and 109 internal fragments resulting from the fragmentation (INT) (25). The depth of sequence coverage 110 was not sufficient for a compilation of all asRNAs. Instead we looked manually for antisense reads 111 covering the translation signals of mRNAs that were enriched upon RNase III inactivation. Potential 112 asRNA promoter regions were deduced by examination of the TSS and PSS fractions in the rnc 113 strain. The RNase III processing sites in the wild-type were usually not obvious since they 114 presumably provoked the rapid degradation of the asRNA. We selected 4 asRNAs to the crp, ompR, 115

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

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

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

The *phoP* gene encodes the response regulator of the PhoQ-PhoP two-component system, involved in cell wall homeostasis and in response to low magnesium (33-34). We observed an asRNA upon 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 <i>flhDC</i> genes are co-transcribed and together they encode the master regulator of motility,
143	$FlhD_4C_2$ (35). We detected an asRNA to <i>flhD</i> mRNA (AsflhD) accumulating in the mutant, initiated
144	22 nts downstream from the <i>flhD</i> AUG. Northern blot analysis with probes hybridizing to the 5'-UTR
145	of <i>flhD</i> 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 <i>flhD</i> mRNA in the mutant and the stabilization of a <i>flhD</i> 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

details asRNAs to *phoP* and *flhD*, two regulators tightly controlled at both the transcriptional and post-transcriptional levels.

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156 Regulation of phoP and AsphoP by RNase III

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

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163	transcriptional fusion containing 150 nts before and 15 nts after the putative 155 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} - <i>lacZ</i> 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.

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

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177 Physiological expression of AsflhD

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

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

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

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

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

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

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225 Characterization of the 3'-end of AsflhD

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

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

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245 Independent degradation of flhD and AsflhD transcripts by RNase III

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

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

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

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268 AsflhD represses the expression of flhD

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

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

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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 284 overexpressing AsflhD (P_{AsflhD}^{+}). Northern blot confirms that, as expected, the abundance of *flhD* 285 mRNA increases in the rnc strains overexpressing or not AsflhD (Fig. 6-D). Remarkably, the 220 nts 286 287 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 288 promoter drives the accumulation of the small fragment corresponding to the 5'-UTR of *flhD* mRNA, 289 presumably as a duplex with AsflhD and that both are rapidly degraded by RNase III. This short *flhD* 290 fragment could either be generated by processing of longer *flhD* mRNA or correspond to premature 291 transcriptional termination of *flhD* mRNA. In summary, we show that AsflhD is involved in the 292 repression of the expression of *flhD* at the post-transcriptional level. 293

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295 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* 296 transcription experiments using a DNA template corresponding to the *flhD* gene from 76 nts before 297 to 388 nts after the transcription start site of *flhD*, which allows the transcription of a 388 nts *flhD* 298 RNA and of a 335 nts AsflhD RNA (Fig. 7-A). We compared the abundance of both transcripts 299 synthesized from this latter DNA fragment to those generated from templates carrying the 300 promoter mutations leading to either silencing (P_{AsflhD}) or overexpression (P_{AsflhD}) of AsflhD. In vitro 301 transcription assays were performed in a single round of elongation in the presence of heparin and 302 with RNA polymerase (RNAP) pre-bound to templates in the absence of RNA, hence observed 303 effects are restricted to the elongation step and should be independent of the initiation of 304 transcription. The results correlate with in vivo data even though the amplitude of the effects is 305

306 different. Figure 7 shows that expression of AsflhD is strongly impaired (10-fold) on the template carrying the silencing (P_{AsflhD}) mutation and increased (2.5-fold) from the template carrying the 307 overexpression (P_{AsflhD}⁺) mutation of AsflhD (Fig. 7-B orange bars). Also, as in vivo (Fig. 6-B), AsflhD 308 309 silencing does not affect the level of the flhD RNA, while its overexpression in cis results in a decrease of the transcription of the flhD RNA (40%) (Fig. 7B purple bars). cAMP/CAP is known to 310 activate the transcription of *flhD* by binding to a sequence located 72 nts upstream from the TSS of 311 flhD (45). As expected, its addition increased the transcription of flhD, which was still reduced by 312 AsflhD overexpression (Fig. 7-B left). The role of AsflhD in the repression of flhD transcription 313 elongation, was further confirmed by using a template where the P_{tet} promoter replaced the P_{flhD} 314

promoter (Fig. S3-A) producing the same 388 nts *flhD* RNA but a shorter (260 nts) AsflhD RNA. Similar results were observed from the templates carrying the P_{AsflhD} silencing and overexpression mutations (Fig. S3-B). We conclude that overexpression of AsflhD *in cis* leads to the repression of transcription elongation of *flhD*, which seems to be independent of the transcription level and of the promoter expressing *flhD*.

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

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

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332 AsflhD represses the transcription of flhD in trans in vivo

To confirm in vivo the ability of AsflhD to repress the transcription of flhD in trans, AsflhD was 333 overexpressed from a plasmid, under the control of a P_{tac} promoter inducible by IPTG. The short 242 334 nts long AsflhD is transcribed from the +1 to the +220 nt relative to the TSS of AsflhD with a *rrnB*T2 335 terminator to enable its stabilization. In the rnc mutant, this transcript is further processed with the 336 appearance of the characteristic 160 nts intermediate (Figs. 8-A, 1-D). It is noteworthy that this 337 smaller fragment is slightly longer than in the endogenous AsflhD suggesting that it may correspond 338 to a 3'-fragment of AsflhD. Overexpression of AsflhD upon addition of IPTG decreases the 339 abundance of flhD mRNA both in the wt strain (30%) and in the mutant (20%) (Fig. 8-B), in 340 agreement with our in vitro data (Figs. 7-C, S3-C). Thus, consistent with previous experiments, trans 341 overexpression of AsflhD in vivo reduces the abundance of *flhD* mRNA but it is less effective than 342 AsflhD expressed from the *flhD* locus in cis and this effect is mostly independent of RNase III activity. 343

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

353

354 AsflhD controls the motility cascade

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

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

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

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

385

386 Discussion

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

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

408

409 Conservation of the promoters of AsflhD and AsphoP

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

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

20

promoter activity. In the case of AsphoP, the promoter located in the central region of the *phoP*ORF is not conserved in other enterobacteria. In *E. coli*, the two G to A changes in the -10 sequence
of the promoter of AsphoP, compared to other bacteria make a relatively good consensus -10
(CATAAT) which can account for the high level of expression of AsphoP (Fig. 2-E) compared to
AsflhD (Fig. 3-C). The lack of conservation in other bacteria suggests that, on the contrary to AsflhD,
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 same time it is also involved in the *flhD* degradation. The formation of an intermolecular RNA 434 duplex can trigger the RNase III-mediated degradation of a mRNA as in the case of the *puf* mRNA 435 upon binding to asPcrL in Rhodobacter sphaeroides or a sRNA upon binding to its target, as in the 436 case of the sRNA RyhB upon binding to the sodB mRNA (53). However, RNase III cleavage may 437 generate a shorter but more stable mRNA and so can have a positive role on gene expression (19, 438 54). In the case of AsphoP the *rnc* mutation reduces promoter activity (Fig. 2-E) but greatly increases 439 AsphoP stability (Fig. 2-A). In the case of AsflhD, RNase III allows the recycling of the products of 440 transcriptional attenuation between *flhD* and AsflhD (Fig. 7-A). 441

442

443 Mechanism of regulation by AsflhD

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

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

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

- 472 not stabilize a terminator structure but could rather modify the stability of the elongating RNAP 473 leading to heterogenous 3'-termini as observed for AsflhD *in vivo* by cRT-PCR (Fig. 4-A).
- 474

475 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.
- 490

491 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

- single cell RNA-seq (68) are likely to pave the way for the elucidation of the role of the widely
- 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.

510

511 Northern blotting and RNA-seq analysis

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

525

526 β -galactosidase assays

527	Cultures were initiated at A_{600} 0.05 and sampled at A_{600} 0.4 or in the case of the results presented in
528	figure S2 also at A_{600} 1.2. Samples (200 $\mu L)$ were lysed in 800 μL PBS buffer with 10 μL 0.1% SDS and
529	20 μ L chloroform. β -galactosidase activity was assayed as described (73), results are the mean of at
530	least three biological replicates.

531

532 Circular RT-PCR

533 Circular RT-PCR was performed with total RNA extracted from N3433 and IBPC633 treated with 5'-534 polyphosphatase. After circularization with T4 RNA ligase, mflhD2 was used to prime reverse 535 transcription and mflhD6 and masflhD10 to generate PCR products (Table S2), which were cloned 536 (74).

537

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

539 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 540 256 nts of AsflhD. RNAs were synthesized by T7 RNAP with $\left[\alpha^{-32}P\right]$ -UTP as a tracer and were gel 541 purified. Transcripts 5'-end-labelling, hybridization, RNase III digestion and sample analysis were 542 described in (74-76). Briefly, radioactive AsflhD was incubated with increasing concentrations of 543 flhD mRNA under two conditions referred to as "native" (incubation in TMN buffer (20 mM Tris 544 acetate, pH 7.5, 10 mM magnesium acetate, 100 mM sodium acetate for 5 min at 37°C) and "full 545 RNA duplex" (initial denaturation at 90°C for 2 min, then incubation in 1xTE at 37°C for 30 min). The 546 complexes were loaded on native polyacrylamide gels to control for hybridization efficiency or 547

26

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 μ g
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 μ L) 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

567

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 Genet 9:e1003156.
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- 768

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

Promoter	Relative abundance	flhD Half-
	<i>flhD</i> at t0 (%)	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 (A_{600} 0.4) at 37°C. Sampling was performed at different times after addition of rifampicin (500 µg/mL) (t0) and total RNA was subjected to northern blot analysis. The membranes were probed successively for *flhD* and M1. The decay-rate of *flhD* mRNA was calculated as described in the "quantification and statistical analysis" section of the supplementary materials and methods.

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780 Legends

781 Figure 1: RNase III inactivation stabilizes asRNAs

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

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

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805

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

(A) and (B) Stability of phoP and AsphoP respectively in the wt (N3433) and the rnc (IBPC633) 807 808 strains. Samples were removed at the times indicated in minutes after rifampicin (500 µg/mL) addition. Half-lives $(t_{1/2})$ were calculated using linear regression and are indicated under each 809 condition. Control of loading was assayed by probing for the 5S rRNA. (C) Genetic structure of the 810 phoP locus and alignment of the promoter sequence of AsphoP from selected bacterial species. 811 P_{AsphoP} (orange bent arrow), is positioned relative to the translation start of phoP mRNA (+282). 812 Nucleotide sequences correspond to the following genomes, Eco, Escherichia coli MG1655 813 (NC 000913.3), Sen, Salmonella enterica LT2 (CP014051.2), Pst, Pantoea stewartia ZJ-FGZX1 814 (CP049115.1), Ecl, Enterobacter cloacae NH77 (CP040827.1), But, Buttiauxella sp. 3AFRM03 815 (CP033076.1), Erw, Erwinia sp. J780 (CP046509.1), Pge, Pluralibacter gergoviae (LR699009.1), Cce, 816 Clostridium cellulovorans 743B (CP002160.1), Yre, Yokenella regensburgei W13 (CP050811.1). 817 Nucleotides in red were mutated to inactivate PAsphop, stars represent conserved nucleotides as 818 compared to Eco. The -35 and -10 motifs of AsphoP are boxed. (D) Genetic structure of the 819 transcriptional AsphoP-lacZ reporter fusion. Nucleotides in red indicate the mutations inactivating 820 the AsphoP promoter (P_{AsphoP}). (E) Expression of β -galactosidase activity was determined from the 821 PAsphop-lacZ, PAsphop-lacZ fusions in the wt strain (ML421 and ML422), and in their rnc derivatives 822 (ML424 and ML425 respectively). Values are means of three biological replicates for each strain, 823 and error bars are standard deviations. Statistical significance was determined by a heteroscedastic 824 two-tailed t test (*** for p-values ≤0.001). 825

826

827 Figure 3: Transcriptional regulation of AsflhD

(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

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with 8 Eubacterial species showing between 49-92 % identity of FlhD with E. coli (51). The position 830 of the promoter of AsflhD (orange bent arrow), is indicated relative to the *flhD* translation start of 831 flhD (+22). Nucleotide sequences correspond to the following bacteria, Eco, Escherichia coli 832 MG1655 (NC 000913.3), Sen, Salmonella enterica typhimurium (D43640), Eca, Erwinia carotovora 833 (AF130387), Sma, Serratia marcescens (AF077334), Sli, Serratia liquefaciens (Q7M0S9), Yen, Yersinia 834 enterocolitica (AF081587), Xne, Xenorhabdus nematophilus (AJ012828), Pmi, Proteus mirabilis 835 (U96964), Bbr, Bordetella bronchiseptica (U17998). (B) Genetic structure of the transcriptional 836 AsflhD-lacZ reporter fusion (MG2114 PAsflhD). Mutations in red and in green were introduced to 837 inactivate the promoter of AsflhD (P_{AsflhD} in the strain ML239) and and to increase its activity 838 (P_{AsflbD}⁺ in the strain ML218) respectively. (C) Effect of mutations in AsflhD promoter on expression 839 of P_{AsflbD}-*lacZ* fusion. (D) Expression of P_{AsflbD}-*lacZ* fusion in the wt (strain (MG2114 P_{AsflbD}) before 840 (30°C t=0) and after 15 and 60 minutes of upshift (46°C). (E) DJ624, DJ624-rnc105 and their P_{lac}-rpoH 841 derivatives were grown at 30 °C. At mid-log phase, part of the cultures were shifted to 45°C with or 842 without simultaneous addition of 0.1 mM IPTG. Sampling was performed 15 min later. Total RNA 843 was analyzed by Northern blot, the membrane was probed for rpoH, AsflhD and M1. (F) Expression 844 of P_{AsflhD}-lacZ fusion in the wt strain (MG2114 P_{AsflhD}) and rseA mutant (ML279) at 37°C. (G) 845 Expression of P_{AsflhD}-lacZ fusion in the strain carrying the P_{AsflhD}⁺ fusion (ML218) and its rseA 846 derivative strain (ML312) at 37°C. Values are means of three biological replicates for each strain, 847 and error bars are standard deviations. Statistical significance was determined by a heteroscedastic 848 two-tailed t test (** for p-values ≤0.01 and *** for p-values ≤0.001). (H) MG1655-B (wt), ML73 849 (P_{AsflbD}⁻) and ML241 (P_{AsflbD}⁺) and their *rnc* derivatives (respectively ML65, ML75 and ML341) were 850 grown at 37°C until mid-log phase. Total RNA was analyzed by northern blotting. The membrane 851 was probed successively for AsflhD and 5S or M1. 852

853

854 Figure 4: Characterization of AsflhD

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

864

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

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

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871 Figure 6: AsflhD repress the expression of flhD

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

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

883

884 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 *rrnB*T2 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 892 method (B) with or without addition of 100 nM CAP and 0.2 mM cAMP for 15 min at 37°C before 893 addition of RNA polymerase, (C) with 100 nM CAP and 0.2 mM cAMP and the addition of in vitro 894 purified AsflhD or *flhD* transcripts to the reaction at the indicated concentrations. Samples were 895 analyzed on sequencing gels. Relative intensity of the indicated bands (flhD in purple and AsflhD in 896 orange) were analyzed. Values are means of 6 (B) or 3 (C) replicates and error bars are standard 897 deviations. Statistical significance was determined by a heteroscedastic two-tailed t test (* for p-898 values ≤ 0.05 , ** for p-values ≤ 0.01 and *** for p-values ≤ 0.001). 899

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901 Figure 8: AsflhD repress the expression of flhD in trans

902 (A) MG1655-B (wt) and its *rnc* derivative (ML65) containing the control pCA24N (Ctl) or the pCA24N
 903 AsflhD (As) plasmids were grown to mid-log phase (A₆₀₀ 0.4) at 37°C in the presence of the indicated

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concentration of IPTG. Total RNA was extracted and subjected to northern blot analysis. The 904 membrane was probed for AsflhD and M1 RNA. (B) MG1655-B (wt) containing the control pCA24N 905 (Ctl) or the pCA24N AsflhD (As) plasmids were grown to mid-log phase (A₆₀₀ 0.4) at 37°C in the 906 presence of 10⁻⁴ M IPTG. Total RNA was extracted and subjected to northern blot analysis. The 907 membrane was probed for *flhD* using a probe corresponding to the beginning of the *flhD* ORF and 908 M1 RNA. This flhD probe was used to detect flhD from the chromosomal locus and to avoid 909 detection of *flhD* RNA transcribed from a plasmid promoter. Expression of (C) P_{flhD}-flhD-lacZ 910 (ML219) and (D) P_{tet}-flhD-lacZ (ML233) reporter fusions (grav bars) and their P_{AsflbD}⁺ derivatives (in 911 green, ML226 and ML237 respectively) containing the pCA24N control (Ctl, in dark grey) or the 912 pCA24N AsflhD (As, in dark green) plasmid was determined in the presence of 10⁻⁴ M of IPTG. 913 Values are means of three biological replicates and error bars are standard deviations. Statistical 914 significance was determined by a heteroscedastic two-tailed t test (* for p-values ≤0.05, ** for p-915 values ≤ 0.01 and *** for p-values ≤ 0.001). 916

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918 Figure 9: AsflhD overexpression represses the cascade of motility

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

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930 Figure 10: Schematic representation of the regulatory function of AsflhD

931	When the motility cascade is off, low expression from the P_{flhD} promoter (purple bent arrow) is not
932	sufficient to maintain the expression of <i>flhDC</i> . The binding of AsflhD (orange) to <i>flhD</i> (purple) RNA
933	may occur co-transcriptionally and perturb the elongation of both molecules reinforcing the low
934	level expression of <i>flhD</i> . Upon encountering conditions where motility is required, the strong
935	induction of the P_{flhD} promoter changes the balance between the sense and the asRNA allowing
936	<i>flhDC</i> expression and activation of the motility cascade but where changes in AsflhD expression (e.g.
937	in response to heat) could modulate <i>flhD</i> expression. DNA is represented by grey lines and RNAP as
938	orange and purple oval shapes.

940 Supplementary legends

941 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 OD₆₀₀ 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 OD₆₀₀ 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).

948

949 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 950 (256 nts) under conditions referred as Native and Full RNA duplex conditions (Materials and 951 Methods). Native AsflhD-flhD complexes were formed at 37°C for 5 min in TMN buffer, and full 952 duplexes were obtained after a denaturation-annealing treatment in TE Buffer (2 min 90°C, 30 min 953 37°C before loading on native polyacrylamide gels to control for hybridization efficiency (A) or (B) in 954 vitro processing by RNase III. RNase III digestion of free or complexed AsflhD in native conditions 955 was performed at 37°C in TMN buffer containing 1µg tRNA with increasing concentration of RNase 956 III per sample. Samples were analyzed on 8% polyacrylamide-urea gels. 5'-radiolabeled flhD (C) and 957 AsflhD (D) were cleaved by RNase III (1 unit) in vitro at 4 and 2 main sites respectively (represented 958 by a numbered red arrow). Mapping of the main in vitro cleavage sites of RNase III on flhD and 959 AsflhD are indicated on their predicted secondary structures with the position of the main RNase III 960 cleavage sites indicated relative to the TSS (according to (6) and Vienna RNA websuite (44)). The 961 localization of RNase III cleavage site (black arrows) was performed by comparing the cleavage 962

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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

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

MG1655-B (wt) and ML241 (P_{AsflhD}^+) were grown at 37°C until mid-log phase ($A_{600} = 0.4$) or until latelog phase ($A_{600} = 1$). Total RNA was analyzed by northern blotting. The membranes were probed successively for (A) *flgB* and M1 and for (B) *fliC* and M1.

985



Figure 2

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Figure 4

Temperature (°C) н wt flhD rnc rne^{ts} wt rnc rne^{ts} rne^{ts} rnc wt rnc rne^{ts} +1 rnc pnp pnp 307 +145 +149 +149 +149 +149 +149 217 AsflhD +156 ++158 ++159 -**5**S -+255 -+260

+345

Figure 5

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