Regulatory Interplay between RNase III and Antisense RNAs in *E. coli*: the Case of AsflhD and FlhD, Component of the Master Regulator of Motility

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ABSTRACT In order to respond to ever-changing environmental cues, bacteria display resilient regulatory mechanisms controlling gene expression. At the post-transcriptional level, this is achieved by a combination of RNA-binding proteins, such as ribonucleases (RNases), and regulatory RNAs, including antisense RNAs (asRNAs). Bound to their complementary mRNA, asRNAs are primary targets for the double-strand-specific endoribonuclease, RNase III. Taking advantage of our own and previously published transcriptomic data sets obtained in strains inactivated for RNase III, we selected several candidate asRNAs and confirmed the existence of RNase III-sensitive asRNAs for crp, ompR, phoP, and flhD genes, all encoding global regulators of gene expression in *Escherichia coli*. Using FlhD, a component of the master regulator of motility (FlhD4C2), as our model, we demonstrate that the asRNA AsflhD, transcribed from the coding sequence of flhD, is involved in the fine-tuning of flhD expression and thus participates in the control of motility.

IMPORTANCE The role of antisense RNAs (asRNAs) in the regulation of gene expression remains largely unexplored in bacteria. 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 flhD gene, encoding the transcription factor serving as the primary regulator of bacterial swimming motility (FlhD4C2), controls flhD expression, which in turn affects the expression of other genes of the motility cascade. The role of AsflhD highlights the importance of fine-tuning mechanisms mediated by asRNAs in the control of complex regulatory networks.

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

Bacteria efficiently adapt to changes in their environment by sensing various signals and adjusting their genetic expression accordingly. Gene regulation occurs at all steps from DNA transcription to protein synthesis via a wide range of regulatory factors (both proteins and RNAs). *trans*-Encoded small RNAs (sRNAs) are regulators acting by imperfect base-pairing, often supported by RNA-binding protein (RBP) chaperones such as Hfq and ProQ (1). In contrast, antisense RNAs (asRNAs) are encoded in *cis* to their complementary target. Fewer asRNAs have been described compared to sRNAs, probably because of their high lability (as unprotected RNAs), their low conservation among species (e.g., asRNAs identified in a single study in *Escherichia coli* and *Salmonella enterica* revealed only 14% overlap [2]), and their low levels of expression (reviewed in references 3 and 4).

Initially, asRNAs were identified on mobile genetic elements (prophages and plasmids), in which their role is to control replication and partitioning. The importance of asRNAs was later demonstrated to extend to almost all kinds of biological processes (5), as in the case of type I toxin-antitoxin systems, in which the toxin mRNA is neutralized by an asRNA that induces degradation and/or inhibition of translation (6). 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 (7, 8).

The mechanisms of action of asRNAs are diverse. They can negatively regulate transcription by interference due to the collision of two converging RNA polymerases (RNAPs) or by attenuation due, in some cases, to the stabilization of a terminator structure in the mRNA upon binding of the asRNA (9, 10). Moreover, despite their complementarity, the interaction of an asRNA with its target requires, in some cases, formation of an intermediate called “kissing complex” (7, 11). These interactions can have negative or positive consequences on gene expression since they induce modifications to the RNA secondary structure and/or physically interfere with the activity of other regulators (12, 13). In a surprisingly large number of cases, the mechanism by which a specific asRNA regulates its target remains unclear due, in part, to the impossibility of modifying the sequence of the asRNA independent of its target.

More recently, various genomic approaches have been used leading to the identification of hundreds to thousands of asRNAs and/or antisense transcription start sites (TSSs) in the transcriptome of *E. coli*. These approaches include genomic library overexpression (14), inhibition of Rho-dependent termination (15), mapping of transcriptional units (16), capture of double-stranded RNAs (17, 18) and enrichment of primary (19–21) or small transcripts (22). It is interesting to note that comparison of some of these data sets revealed only a modest overlap (19), enforcing the idea that asRNAs are difficult to identify and may not be well conserved even between related bacterial species.

Taking advantage of available transcriptomic data sets and of our previous study, during which a tailored transcriptome sequencing (RNA-seq) analysis was performed (23), we compared transcriptomes of an *rnc* mutant to its isogenic wt strain. We identified and validated the expression of four new asRNAs complementary to the coding sequence of genes *crp*, *ompR*, *phoP*, and *flhD*. Their coordinates are indicated in Table 1, and their expression was confirmed by northern blotting (Fig. 1). Of note, the identification of RNase III processing sites in the wt strain was usually not obvious since

**RESULTS**

**Characterization of asRNAs upon RNase III inactivation.** We previously performed a transcriptomic approach in *E. coli* K-12 (wt) and its *rnc*105 derivative strain (*rnc*) by tagging transcripts according to their 5′-phosphorylation status, allowing us to distinguish between 5′-triphosphate fragments (primary transcripts, TSS), 5′-monophosphate fragments (processed transcripts, PSS), and internal fragments resulting from the fragmentation (INT) (23). From this data set, we sorted the antisense reads to open reading frames (ORFs) that were enriched upon RNase III inactivation and checked whether they have been detected in independent data sets (as described in Text S1) (14, 16, 17, 19, 21). Four asRNAs complementary to gene coding for major transcriptional regulators were selected, i.e., asRNAs to *crp*, *ompR*, *phoP*, and *flhD*. Their coordinates are indicated in Table 1, and their expression was confirmed by northern blotting (Fig. 1). Of note, the identification of RNase III processing sites in the wt strain was usually not obvious since

| Sense gene | asRNA | Genomic coordinates | Detected in previous studies |
|------------|-------|---------------------|----------------------------|
|            |       | Starting from the TSS relative to *E. coli* genome | asRNA reads | TSS only |
|            | Name  | Genomic coordinates | (14) | (16) | (17) | (19) | (21) |
|            | Name  | Starting from the TSS relative to *E. coli* genome |       |       |       |       |
| crp        | Ascrp | 3,486,231 to 3,485,953 | N | N | Y | N | N |
| ompR       | AsompR| 3,536,438 to 3,536,775 | N | N | Y | Y | Y |
| phoP       | AsphoP| 1,190,165 to 1,190,508 | N | N | Y | Y | Y |
| flhD       | AsflhD| 1,978,175 to 1,978,395 | Y | N | Y | Y | Y |

*Identical transcription start sites (TSSs) at ±1 nt.
cleavage by RNase III presumably provoked the subsequent rapid degradation of the asRNA.

The *crp* gene encodes the major regulator of carbon catabolite repression, and it was shown previously to be transcriptionally regulated by the recruitment of the RNAP to a divergent and overlapping TSS located 3 nt upstream from the *crp* TSS (24, 25)

encoding the divergently expressed gene *yfhA*. We observed antisense reads validating this previously characterized TSS in the wt strain. In addition, antisense reads complementary to the ORF and 5′-untranslated region (5′-UTR) of the *crp* mRNA accumulate in the *rnc* mutant (Fig. 1A; Table 1), and we predicted an additional TSS to be located 112 nt downstream from the start codon of *crp*. Northern blot analysis revealed that...
Antisense RNA Control of flhD Expression

Searching the 35 promoters for the motif recognized by RNase III led to the identification of two potential promoters: the putative P_{AsphoP} and the mutated P_{AsphoP} (P_{AsphoP(m)}). To validate these promoters, we constructed transcriptional fusions containing 150 nt before and 15 nt after the putative TSS of AsphoP, with the wt sequence (P_{AsphoP(wt)}) or mutations (P_{AsphoP(m)}) decreasing the agreement with the consensus in the predicted 35 boxes (Fig. 2B). The mutated AsphoP promoter (P_{AsphoP(m)}) strongly decreased the expression of P_{AsphoP-lacZ} (20-fold), confirming it as an endogenous AsphoP promoter (Fig. 2C). Furthermore, inactivation of RNase III led to an increase (1.6-fold) of phoP mRNA stability (Fig. 2D) and revealed that AsphoP is much more stable than phoP mRNA in the mc mutant (Fig. 2E). In summary, we confirmed that AsphoP is transcribed from the predicted promoter and that RNase III negatively affects the expression of phoP and AsphoP.

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 the compared genomes (Fig. 2A). This, in turn, implies that, if AsphoP has any function, it could be limited to E. coli K-12 and has been counterselected in these other species or, more likely, represents a novel, evolving trait.

Physiological expression of AsflhD. Candidate -10 and -35 boxes were identified upstream from the putative TSS of AsflhD and sequence alignment of this region in other enterobacteria shows a good conservation of a promoter with an extended
210 5
 9
-TG box (37), suggesting that As
fl
hD expression is conserved among enterobac-
teria (Fig. 3A). To validate the predicted promoter, a PAs
fl
hD-lacZ transcriptional fusion (PAs
fl
hD(wt)) was constructed containing 165 nt before and 15 nt after the putative TSS of
As
fl
hD (Fig. 3B). This fusion showed a relatively low level of 
β-galactosidase activity (10MU; Fig. 3C). Its expression was strongly increased (34-fold) when the 210 motif was improved toward the RpoD consensus (PAs
fl
hD(11)), while mutating the 235 and 210 to less consensus sequences (PAs
fl
hD(22), PAs
fl
hD(21), and PAs
fl
hD(23)) decreased expres-
sion (3.7- to 8.7-fold), hence validating the predicted promoter (Fig. 3C). It should be
noted that mutations were designed to be used in the endogenous
fl
hD locus and
chosen to minimally affect the coding sequence of
fl
hD and to avoid introduction of
rare codons. However, the PAs
fl
hD(11) mutation produces an aspartate to asparagine
change at position 12 of FlhD (D12N), which may affect FlhD function (see below). The
low level of expression made us wonder whether AsflhD may be expressed using an al-
ternative sigma factor. Heat shock increased P
As
phoP-lacZ expression 1.8-fold after 15 min and 4.3-fold after 60 min (Fig. 3D). Comparison of P
As
phoP with consensus sequences for the two heat-shock sigma factors, σH (RpoH) and σE (RpoE) (38, 39), shows better correlation with the σE consensus than with σH (Fig. 3A), suggesting that RpoE could be involved in the transcription from the P
As
phoP.

Hence, we examined whether P
As
fl
hD is under the control of RpoE by using a strain deleted for rseA (anti-sigma factor inhibitor of RpoE), which leads to the strong induc-
tion of the RpoE regulon (40, 41). Deletion of rseA increased the expression of the wt P
As
fl
hD-lacZ fusion (Fig. 3E, 1.7-fold), comparable with the effect of the heat shock at

FIG 2 Regulation of AsphoP and phoP levels by RNase III. (A) Genetic structure of the phoP locus and alignment of the promoter sequence of AsphoP from selected bacterial species. P
AsphoP (orange bent arrow) is indicated relative to the translation start of phoP mRNA (+282). Sequences correspond to the following genomes: Eco, Escherichia coli MG1655 (NC_000913.3); Sen, Salmonella enterica LT2 (CP014051.2); Pst, Pantoea stewartii ZL-FGXX1 (CP049115.1); Ec, Enterobacter cloacae NHT7 (CP040827.1); But, Butiauxella sp. 3AFRM03 (CP033076.1); Erw, Erwinia sp. J780 (CP046509.1); Pge, Pluralibacter gergoviae (LR699009.1); Cce, Clostridium cellulovorans 7438 (CP002160.1); and Yre, Yokenella regensburgeri W13 (CP050811.1). Nucleotides mutated to inactivate P
AsphoP are shown in red, stars represent conserved nt relative to Eco, and the −35 and −10 motifs of AsphoP are highlighted in gray. (B) Genetic structure of the transcriptional P
AsphoP-lacZ reporter fusion (P
AsphoP in MG2118). Mutations repressing activity of the AsphoP promoter (P
AsphoP(-4) in MG2120) are in red. (C) Expression of β-galactosidase activity (given as Miller units [67]) was determined from the P
AsphoP-lacZ and P
AsphoP(-4)-lacZ fusions in the wt strain. The
values are the means of three biological replicates for each strain, and the bars indicate the standard deviations. Statistical significance was determined by
analysis of variance (ANOVA). *** P ≤ 0.001. (D and E) Total RNA was prepared from samples taken from the wt strain (N3433) and its
rnc
derivative (IBPC633) at different times after addition of rifampicin and was subjected to northern blot analysis. The membranes were probed successively for phoP or AsphoP and 5S. The decay rate of phoP mRNA was calculated as described in the Materials and Methods section.
FIG 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 (38, 39, 74) and with eight Eubacterial species showing 49 to 92% FlhD identity with E. coli (51). The position of the promoter of AsflhD (orange bent arrow) is indicated relative to the flhD translation start of flhD (+22). Sequences correspond to the following bacteria: Eco, Escherichia coli MG1655 (NC_000913.3); Sen, Salmonella enterica Typhimurium (D43640); Eca, Erwinia carotovora (AF130387); Sma, Serratia marcescens (AF077334); Sli, Serratia liquefaciens (Q7M0S9); Yen, Yersinia enterocolitica (AF081587); Xne, Xenorhabdus nematophilus (AJ012828); Pmi, Proteus mirabilis (U96964); and Bbr, Bordetella bronchiseptica (U17998). Bases identical to the E. coli sequence are shown with asterisks. Gray highlighting indicates the bases corresponding to the consensus for a TG-extended rpoD promoter (37).

(B) Genetic structure of the transcriptional PAsflhD-lacZ reporter fusion and location of mutations altering the AsflhD promoter. (C) Expression of b-galactosidase from PAsflhD-lacZ fusions, wt (PAsflhD(wt), MG2114-PAsflhD), and derivatives carrying the PAsflhD mutations (PAsflhD(-2), ML239; PAsflhD(-1), ML604; PAsflhD(+1), ML605; and PAsflhD(+3), ML218) at 37°C. Of note, PAsflhD(+1) leads to the replacement of the 12th (Continued on next page)
46°C, known to induce the RpoE regulon (42). Thus, our results suggest that AsflhD is more expressed at high temperature and when the RpoE sigma factor is activated.

To further characterize AsflhD, the P_{AsflhD(-2)}, P_{AsflhD(-1)}, P_{AsflhD(3)}, and P_{AsflhD(-1)} mutations were introduced at the endogenous flhD locus, and the expression of AsflhD was examined by northern blotting (Fig. 3F). No AsflhD was detected in the rnc derivatives of strains with the three mutations reducing the expression of AsflhD. Conversely, AsflhD overexpression from the P_{AsflhD(+1)} mutation led to the detection of a faint smear in the wt strain and to the much greater accumulation of AsflhD in the rnc mutant (4.6-fold relative to the rnc mutant containing the P_{AsflhD(0)}). In summary, we have identified the promoter of AsflhD and shown that mutations in the promoter of AsflhD can be used as tools to study the function of AsflhD at the flhD locus.

**Processing of AsflhD.** Circular RT-PCR (cRT-PCR) experiments confirmed that AsflhD is expressed in both the wt and rnc strains from P_{AsflhD} with heterogeneous AsflhD 3′-ends extending up to 345 nt in the rnc mutant (Fig. 4A). Surprisingly, no 220-nt-long RNA was detected in the mutant by cRT-PCR, while a 149-nt-long fragment was found several times exclusively in the wt strain, which might be an intermediate in the degradation of AsflhD, e.g., via RNase E (see below). Surprisingly, we did not detect 220-nt RNAs observed by northern blotting in the rnc strain. As this species is likely to be present as double-stranded RNA with the processed flhD mRNA (as described below), we suspect that it is less efficiently ligated during the initial step of the cRT-PCR, as previously reported (see Materials and Methods) (43). We investigated the degradation of AsflhD by...

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**FIG 3** Legend (Continued)

codon GAC (aspartic acid) to the codon AAC (asparagine), P_{AsflhD(-2)} leads to the replacement of the 19th codon CUA to the synonymous codon UUG, and P_{AsflhD(-1)} leads to the replacement of the 11th codon UAU to the synonymous codon UUG, while P_{AsflhD} combines the effect of the two previous mutations. (D) Expression of β-galactosidase in P_{AsflhD(-1)} lacZ before (30°C, t = 0) and after 15 and 60 min of upshift (46°C). (E) Expression of β-galactosidase in P_{AsflhD(0)} lacZ in the rseA mutant derivative (ML729) at 37°C. The values are means of three biological replicates for each strain, and the bars indicate the standard deviations. Statistical significance was determined by ANOVA. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. (F) Mutations in red were introduced in the endogenous flhD-AsflhD locus to reduce the activity of the AsflhD promoter (P_{AsflhD(-2)}, ML73; P_{AsflhD(-1)}, ML609; and P_{AsflhD}, ML610) and to increase its activity in green (P_{AsflhD(0)}, ML241). Strains wt (MG1655-8), P_{AsflhD(-2)}, (ML73), P_{AsflhD(-1)}, (ML609), P_{AsflhD(0)}, (ML610), P_{AsflhD(-1)}, (ML241), and their rnc derivatives (ML65, ML75, ML613, ML614, and ML341), respectively were grown at 37°C until mid-log phase. Total RNA was analyzed by northern blotting. The membrane was probed successively for AsflhD and for M1 RNA (377 nt, highly stable catalytic component of the RNase P, used as a loading control [75]). n.d., not determined.
other RNases. Inactivation of the major endonuclease RNase E allowed the detection of a 300-nt fragment (independently of the presence of RNase III), whereas the loss of the exoribonuclease polynucleotide phosphorylase (PNPase) had no effect on AsflhD degradation (Fig. 4B). Hence, RNase III and RNase E are independently involved in the rapid turnover of AsflhD.

To understand the role of RNase III in the degradation of flhD and AsflhD RNAs, we analyzed the effect of RNase III inactivation on the stability of both RNAs. In the rnc mutant, the major 220-nt-long AsflhD transcript and minor 160-nt-long transcript were highly stable, while both the amount and the stability of the full-length flhD mRNA (here referred to as flhDp) increased 2-fold (Fig. 5A and B). In addition, the 5'-UTR probe used (Fig. 1D and 5B, top; Table S1) detected a 220-nt-long 5'-UTR flhD RNA fragment (here referred to as flhDp), complementary to AsflhD, which is also highly stable in the rnc strain. The interaction of flhDp with AsflhD is expected to generate a double-stranded RNA (Fig. 4A), the degradation of which depends on RNase III. Supporting this hypothesis, we could not detect flhDp RNA in a strain with RNase III inactivated and in which the endogenous AsflhD expression was reduced (PAsflhD(2)), while flhDp RNA accumulates when AsflhD expression was increased (PAsflhD(1)); Fig. 6A; Fig. S1B).

We further confirmed the interaction and cleavage by RNase III of AsflhD and flhD in vitro. A 308-nt-long flhD transcript corresponding to the 5'-UTR and part of the ORF of the flhD mRNA and a 256-nt-long AsflhD asRNA were synthesized and labeled at their 5'-ends. These two RNAs form a duplex when present in equimolar concentrations (Fig. S2A), which is completely degraded upon addition of RNase III (Fig. S2B). Remarkably, under the same condition, RNase III cleaves the individual RNAs independently at two sites on AsflhD and at four major sites on flhD (Fig. S2C and D). These cleavage sites are located within regions able to form a secondary structure on each molecule (44, 45) (Fig. S2C and D). RNase III is thus able to cleave both AsflhD and flhD RNAs in vitro, at specific sites but is also able to drive the complete degradation of the
flhD/AsflhD duplex. These results support a dual role of RNase III in the processing of flhD mRNA via the binding of AsflhD or independently of AsflhD.

**Regulation of flhD expression by AsflhD.** We next determined the effect of increased or decreased expression of AsflhD, in cis, on flhD expression by following flhD mRNA abundance and stability in strains carrying the endogenous PAsflhD mutations described above. While a decrease in flhD mRNA abundance results from both decreased AsflhD (1.5-fold in PAsflhD(-2), 1.6-fold in PAsflhD(-1), and 1.2-fold in PAsflhD(-3)) and increased AsflhD expression (1.8-fold in PAsflhD(+1)) (Fig. 6A and B; Fig. S3A), the stability of flhD mRNA was not significantly affected in either the PAsflhD(-2) or PAsflhD(+1) mutant (Fig. S1A to C).

A transcriptional/translational (PAsflhD-flhD-lacZ) and a translational flhD-lacZ reporter fusion under the control of a constitutive promoter (Ptet-flhD-lacZ) encompassing the 5′-UTR and the first 34 amino acids of FlhD (which includes PAsflhD) were introduced at the lacZ chromosomal locus (Fig. 6C). Mutations in PAsflhD resulting in decreased (PAsflhD(-2)) and increased expression (PAsflhD(+1)) of AsflhD were also introduced into both fusions. Both mutations reduced expression of both fusions, but the effect of the AsflhD-overexpressing

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**FIG 6** AsflhD regulates the expression of flhD. (A, top) The RNA probe used to detect flhD mRNA (S′-probe) is represented in black relative to the flhD locus (flhD in purple and AsflhD promoter in orange). (A, bottom) Total RNA was extracted from the wt (MG1655-B) and endogenous AsflhD promoter mutants (PAsflhD(-2) in ML73 and PAsflhD(+1) in ML241) and their rnc mutant derivatives (ML65, ML75, and ML341, respectively) and subjected to northern blot analysis. The membrane was probed successively for flhD and for M1 RNA. (B) Average flhD mRNA abundance in the PAsflhD(-2) (red) and PAsflhD(+1) (green) mutants relative to the wt strain (gray), as shown in panel A, was calculated as the mean of five biological replicates. (C) Genetic structures of the PAsflhD-flhD-lacZ (ML219) and Ptet-flhD-lacZ reporter fusions (ML233) and their derivatives containing the mutations leading to either decreased expression (PAsflhD(-2), ML221 and ML235, respectively, in red) or increased expression (PAsflhD(+1), ML226 and ML237, respectively, in green) of AsflhD. (D, E) Expression of PAsflhD-flhD-lacZ (D) and Ptet-flhD-lacZ (E) reporter fusions (gray) and their derivatives are given as β-galactosidase activity. The values are means of 10 biological replicates for each strain, and the bars indicate standard deviations. Statistical significance was determined by ANOVA. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
mutation (P_{As\text{fl}\text{hD}(1)}) was greater (4.9-fold decrease of the fusion driven by the wt flhD promoter and 2.3-fold on the P_{tet}-driven version) than the effect of the promoter-down mutation (1.4-to 1.2-fold in P_{As\text{fl}\text{hD}(2)}; Fig. 6D and E). The results, obtained during the exponential phase of growth, were confirmed by measurements in late exponential phase (A_{600})_{C25} (Fig. S3B and C) when flhD expression, from the P_{flhD} promoter, increases (as previously reported [46]). It can also be noted that As\text{fl}\text{hD} overexpression has a greater impact on flhD-lacZ expression from the native flhD promoter (Fig. 6D) than on flhD mRNA abundance (Fig. 6A) or flhD-lacZ expression from the P_{tet} promoter (Fig. 6E).

In brief, reducing or increasing the expression of As\text{fl}\text{hD} reduces flhD expression at the translational and mRNA levels, while not appreciably affecting the stability of the flhD mRNA. Hence, this suggests that a native intermediate level of As\text{fl}\text{hD} expression is required for optimal flhD expression.

**As\text{fl}\text{hD} represses flhD expression in trans.** Increasing the endogenous As\text{fl}\text{hD} expression leads to the repression of flhD expression. Hence, we next investigated the ability of As\text{fl}\text{hD} to repress the expression of flhD when expressed in trans. As\text{fl}\text{hD} was overexpressed from a plasmid, under the control of a P_{lac} promoter inducible by isopropyl-\beta-D-thiogalactopyranoside (IPTG). The short (242 nt) As\text{fl}\text{hD} is transcribed from the 1 to the +220 nucleotides (nt) relative to the TSS of As\text{fl}\text{hD} (i.e., +220 to +1 relative to the TSS of flhD) with a rm\beta\text{T2} terminator to stabilize the transcript. Under inducing conditions, trans-overexpression of As\text{fl}\text{hD} is stronger than cis-overexpression (9-fold in the rnc mutant; Fig. 7A) and can be directly observed in the wt strain. In agreement with

*FIG 7* As\text{fl}\text{hD} represses the expression of flhD in trans. (A, B) The wt strain (MG1655-B) and its derivative containing the endogenous As\text{fl}\text{hD} promoter mutant (P_{As\text{fl}\text{hD}(1)} ML241) and their rnc mutant derivatives (ML65 and ML341, respectively) containing the control pCA24N (Ctl) or the pCA24N As\text{fl}\text{hD} (As) plasmids were grown in the presence of 10^{-4} M isopropyl-\beta-D-thiogalactopyranoside (IPTG). Total RNA was extracted and subjected to northern blot analysis. The membranes were probed for (A) As\text{fl}\text{hD} and M1 RNA or (B) for flhD (using the 3’-probe as represented) and M1 RNA. (C) Average flhD mRNA abundance upon trans-overexpression of As\text{fl}\text{hD} (dark green), as shown in (B), was calculated as the mean of six biological replicates. (D, E) Expression of P_{lac}-flhD-lacZ (ML219) (D) and P_{tet}-flhD-lacZ (ML233) (E) reporter fusions (gray) containing the plasmid pCA24N control (Ctl, in gray) or the plasmid pCA24N As\text{fl}\text{hD} (As, in dark green) was determined in the presence of 10^{-4} M IPTG. The values are the means of three biological replicates, and the bars indicate standard deviations. Statistical significance was determined by ANOVA. ***, P ≤ 0.01; ****, P ≤ 0.0001.
results presented in the previous section (Fig. 6B), the trans-overexpression of AsflhD decreased the abundance of flhD mRNA both in the wt strain and in the rnc mutant (Fig. 7B and C). Furthermore, the short flhD$_{rnc}$ RNA, accumulating in the rnc mutant upon cis-overexpression of AsflhD, was even more abundant upon trans-overexpression of AsflhD (Fig. S4B). It is not observed with the 3'-probe (Fig. 7B), confirming that it is derived from the region complementary to AsflhD. It is noteworthy that despite a higher expression in trans, plasmid-borne AsflhD is not as efficient at repressing the expression of flhD. This difference may be due to the presence of the ramB2T2 terminator and/or due to pervasive plasmid transcription, including transcripts antisense to the AsflhD insert, which can be observed when probing for the S'-UTR of flhD in a strain deleted for the endogenous flhD locus (Fig. S4A). Of note, a similar problem has been reported for other vectors (both in eukaryote and bacteria) in which spurious expression of multiple overlapping transcripts was detected (18, 47).

We confirmed these results on flhD mRNA levels by measuring the expression of the flhD-lacZ reporters under conditions of trans-overexpression of AsflhD and observed a small repression of flhD expression in both P$_{rnc}$-flhD-lacZ and P$_{aw}$-flhD-lacZ fusions (1.4 and 1.6-fold; Fig. 7D and E). It should be emphasized that these effects are independent of the flhD promoter (native P$_{rnc}$ or P$_{aw}$). A combination of cis-overexpression (endogenous to the reporter) and trans-overexpression (from the plasmid) of AsflhD had little additive effect on the final repression when flhD was expressed from its own promoter (Fig. S4C), consistent with AsflhD repressing flhD expression by a common mechanism when expressed in cis or in trans. In summary, we show that trans-overexpression of AsflhD can repress flhD expression at both mRNA and translational levels.

Control of the motility cascade by AsflhD. The flhDC operon encodes the FlhD$_4$C$_2$ transcriptional regulator, main activator of the cascade of motility-related genes, which are divided into three classes (48). The flhDC operon encodes the only class I protein complex, FlhD$_{C_2}$, which is essential for expression of class II genes, which in turn control class III genes. Thus, we next investigated the impact of changing AsflhD levels on representative class II and III genes. We selected the following class II genes: flaA that encodes FlrA, the sigma factor for class III motility genes, and flgB that encodes FlgB, a component of the flagella proximal rod and the class III gene flgC gene, encoding the main component of flagella, FlIC. The amounts of flaA, flgB, and flgC mRNAs are reduced (from 1.2- to 1.9-fold) when AsflhD expression is reduced (mutations P$_{Aw}$flhD$(–2)$, P$_{Aw}$flhD$(–1)\psi$ and P$_{Aw}$flhD$(–3)\psi$) and strongly reduced (from 5- to 100-fold) upon cis-overexpression of AsflhD (P$_{rnc}$flhD$(+1)\psi$; Fig. 8A to C; Fig. S5A to C). The effects were strongest for the class III gene flgC. Using a P$_{rnc}$-lacZ transcriptional reporter fusion, we confirmed the reduced expression of flgC when AsflhD levels decreased (from 1.6- to 2.4-fold; Fig. 8D; Fig. S5D). Surprisingly but consistent with the northern blot (Fig. 8C; Fig. S5C), cis-overexpression of AsflhD by the P$_{Aw}$flhD$(+1)\psi$ mutation produced a very large decrease in flgC-lacZ expression (218-fold; Fig. 8D). It has previously been observed that a modest reduction of flhD transcription could lead to a strong repression of the motility cascade (49, 50), and this could be the case for flgC, which depends upon the FlhD$_{C_2}$-dependent class II sigma factor, FlrA, for its expression. We had measured a reduction in flhD expression and mRNA and a greater repression of class II gene flaA, but the very strong repression of flgC-lacZ exerted by the cis-overexpression of AsflhD raised the question of whether the P$_{Aw}$flhD$(+1)\psi$ mutation affected the activity of FlhD since it leads to the mutation of the 12th amino acid (FlhD$_{D12A}$). Thus, we cannot exclude that this change might affect FlhD activity. However, it was previously shown that the FlhD$_{D12A}$ mutation did not affect motility (51), and D12 is not in a region involved in contacting FlhC in the FlhD$_{C_2}$ complex (52). Moreover, several pieces of evidence show that the FlhD$_{D12A}$ (P$_{Aw}$flhD$(–1)\psi$) protein is still active, in particular because the P$_{Aw}$flhD$(–1)\psi$ mutant strain is still motile (see below).

To verify that the changes in AsflhD gene expression were reflected in bacterial behavior, we analyzed the effect of AsflhD on motility using low-agar plates and observed a reduction in the swimming speed when AsflhD expression was either reduced (1.3-fold in P$_{Aw}$flhD$(–2)$) or increased (5.7-fold in P$_{Aw}$flhD$(+1)$), while a strain deleted...
for the flhD locus (ΔflhD) showed no motility (Fig. 8E and F). Swimming motility was measured in super optimal broth in the presence of magnesium (SOB1Mg). This medium (as opposed to LB used for other studies) was preferred to allow measurements within 12 h, since flhD expression and motility are increased (see Materials and Methods).

In addition, we verified that expression of the PiC-flhD-lacZ and PiC-lacZ reporter fusions were also repressed by the cis-overexpression of AsflhD (PAsflhD+) in SOB+Mg (5.7- and 81-fold, respectively; Fig. S6). Importantly in SOB+Mg the expression of flIC-lacZ in the PAsflhD+ mutant is 15-fold higher than in a strain deleted for flhD (Fig. S6B), confirming that the FlhD(D12N) protein is at least still partially functional.

We also attempted to confirm that AsflhD expressed in trans from the plasmid reduced flIC expression. As shown above, the trans-overexpression of AsflhD modestly represses the expression of flhD (Fig. 7B to E), while we found a small reduction of flIC expression at both mRNA (1.6-fold; Fig. S7A) and translational levels (2-fold; Fig. S7B) compared to much stronger effects on flIC by the PAsflhD+ mutation. Expression of AsflhD in trans did, however, lead to a slight reduction of the swimming speed (1.2-fold; Fig. S7D).

It is possible that the effect of AsflhD could also be partially due to independent regulatory events on other targets within the motility cascade. However, a bioinformatics search (TargetRNA2 [53] and CopraRNA [54]) for possible direct trans targets of AsflhD found no candidates among genes from the motility cascade. Furthermore, as shown previously (48) and above (in SOB+Mg; Fig. S7C), flIC expression is dependent...
upon flhD via the sigma factor flaA. trans-overexpression of AsflhD had no effect on the expression of flIC-lacZ in a flhD mutant strain (Fig. S7C). Thus, all results are congruent with the notion that AsflhD affects the expression of flIC and motility through the repression of flhD (Fig. 8; Fig. S7D). In summary, both reduced and increased expression of AsflhD repress the expression of flhD, which in turn leads to repression of the whole cascade of motility and a reduction of the swimming speed.

**Transcriptional repression of flhD by AsflhD in vitro.** We next investigated the mechanism of action of AsflhD. The overexpression of AsflhD represses the expression of flhD at the mRNA and translation level without affecting the stability of the flhD mRNA. Hence, we reasoned that AsflhD could be involved in the transcriptional repression of flhD. To test this hypothesis, we performed in vitro transcription experiments using a DNA template corresponding to the flhD gene from 76 nt before to 388 nt after the TSS of flhD, which allows the transcription of a 388-nt flhD RNA (flhD1) and of a 335-nt AsflhD RNA (AsflhD) (Fig. 9A). We compared the abundance of both transcripts synthesized from the latter DNA fragment to those generated from templates carrying the promoter mutations, leading to either decreased (P_{AsflhD(−2)}, P_{AsflhD(−1)}, and P_{AsflhD(−3)}) or increased expression (P_{AsflhD(+)}) of AsflhD. We confirmed that expression of AsflhD is strongly impaired when transcribed from the template carrying mutations reducing AsflhD repression (around 10-fold in P_{AsflhD(−2)}, P_{AsflhD(−1)}, and P_{AsflhD(−3)}) and AsflhD expression increases from the template carrying the mutation enhancing the expression of AsflhD (2.7-fold in P_{AsflhD(+1)}; Fig. 9B, left panel, orange bars). At the same time, AsflhD overexpression resulted in a decrease in the transcription of flhD RNA (1.7-fold in P_{AsflhD(−1)}), while the loss of AsflhD led to an increase of the transcription of flhD RNA (1.1-fold in P_{AsflhD(−2)}, 1.2-fold in P_{AsflhD(−1)} and 1.5-fold in P_{AsflhD(−3)}; Fig. 9B, left panel, purple bars).

The transcription factor CAP promotes the transcription of flhD by binding to a sequence located 72 nt upstream from the TSS of flhD (SS). As expected, the presence of cAMP/CAP increased the transcription of flhD, which was still reduced when expression of AsflhD was increased (1.9-fold) but only slightly increased when AsflhD expression was reduced from the down-mutations (maximum 1.3-fold for P_{AsflhD(−3)}; Fig. 9B, right panel, purple bars). In vitro transcription assays were performed in a single round of elongation in the presence of heparin and with RNAP prebound to templates; hence, the observed effects are restricted to the elongation step and should be independent of the initiation of transcription.

We also investigated the effect of AsflhD on flhD transcription using a template in which the P_{flhD} promoter replaced the P_{mlc} promoter (Fig. S8A). This template produces the same 388-nt flhD RNA but a shorter (260 nt) AsflhD RNA. Using these templates with the strong P_{flhD} promoter, we observed only a slight reduction of flhD mRNA accumulation from the template carrying the P_{AsflhD(−1)} up- or P_{AsflhD(−2)} down-mutations (maximum 1.2-fold; Fig. S8B, purple bars). In summary, in vitro enhanced expression of AsflhD from the P_{AsflhD(−1)} fragment leads to the repression of transcription elongation of flhD from the P_{flhD} in the presence or absence of CAP/AMPc, while reduction in AsflhD expression has only a modest positive effect on flhD expression.

**Mutual repression of flhD and AsflhD transcription in trans.** We also investigated the effect of including purified AsflhD or flhD RNA on the transcription of both flhD and AsflhD using the same linear DNA templates. The addition of increasing amounts of AsflhD led to a linear decrease of flhD (up to 1.9-fold in the presence of 120 nM AsflhD; Fig. 9C) without affecting the level of AsflhD expression. The reciprocal assay (addition of increasing concentrations of purified flhD RNA) led to a linear decrease of the amount of AsflhD synthesized (up to 2.8-fold in the presence of 120 nM flhD), while the amount of flhD was not affected. We performed the same assay with the template carrying the P_{flhD} promoter (Fig. S8A) and observed similar results (Fig. S8C).

In summary, AsflhD can repress the transcription elongation of flhD both in cis and in trans. Thus, we propose that AsflhD antisense and flhD mRNA are involved in their mutual transcriptional attenuation in which the interaction of one molecule with the other...
Asf1hD represses the transcription of \( \text{flhD} \) in vitro. (A) Schematic representation of the template used for the in vitro transcription assay carrying the \( P_{\text{md}} \) promoter driving the expression of a 388-nt transcript (purple) and the \( P_{\text{Asf1hD}} \) promoter driving the expression of a 335-nt transcript (orange). The linear DNA template was constructed using the oligonucleotides LM191 and LM9 (Table S1) and corresponds to –76 to +388 of the \( \text{flhD} \) transcript relative to its TSS, with a 40-nt extension carrying the \( \text{rrnB T2} \) terminator (fragment length, 504 bp). This fragment carries the native \( \text{flhD} \) promoter (–10 and –35 sites) and includes the cAMP/CAP site at –72 compared to the \( \text{flhD} \) TSS, at its upstream extremity. (B, C) In vitro transcription assays were performed on templates carrying wt, \( P_{\text{Asf1hD}(1)} \), \( P_{\text{Asf1hD}(2)} \), \( P_{\text{Asf1hD}(21)} \), and \( P_{\text{Asf1hD}(23)} \) mutations as described in the supplemental Materials and Methods section (Text S1), with or without the addition of 100 nM CAP and 0.2 mM cAMP for 15 min at 37°C before addition of RNA polymerase (RNAP). (8) or with 100 nM CAP and 0.2 mM cAMP and the addition of in vitro purified Asf1hD or (Continued on next page)
leads to a reduction in transcription at the level of transcription elongation (see discussion).

**DISCUSSION**

Regulatory RNA molecules are often part of complex genetic networks in bacteria. They correspond to a heterogeneous class of molecules that differ in gene organization, size, and function. Our goal was to detect, identify, and investigate the function of antisense transcripts in *E. coli*. In this work, we provide evidence that asRNAs can be important players in the expression of transcriptional factors despite their low level of expression. In particular, we have shown that changes in the level of the asRNA to *flhD* can affect the expression of its target and lead to defects in swimming motility as recapitulated in Fig. 10.

**Conservation of AsflhD and AsphoP.** Sequences of intergenic regions are usually less well conserved than their neighboring coding sequences, allowing the rapid evolution of regulatory signals. This characteristic is reflected in the presence and conservation of promoters for asRNA and sRNAs. For asRNA initiated within the coding sequence of their target, nucleotide changes within the coding region risk upsetting the function of the ORF and could be counterselected. AsflhD corresponds almost entirely to the 5’-UTR of *flhD* but with the promoter located in the ORF, which is fairly well conserved in enterobacteria (Fig. 3A) (51). Thus, the conservation of the promoter of AsflhD could be the result of direct selection for FlhD activity or for the regulatory function of AsflhD (potentially) controlling the expression of FlhD in these bacteria. In *S. enterica* serovar Typhi, AsfD, a long asRNA complementary to *flhDC* and *motA* mRNAs, was observed as a 2,000-nt fragment by northern blotting in a wt strain (56). AsfD was implicated in the positive regulation of *flhDC* during stationary phase by an uncharacterized mechanism. However, this asRNA is likely to originate from a region located downstream from the *flhDC* locus that is not present in *E. coli* K-12 MG1655 (and no equivalent transcript was observed in our and independent transcriptomic data sets). To our knowledge, no transcriptomic data set is available in *Salmonella* species inactivated for RNase III. However, a low abundance asRNA to *flhD*, whose start corresponds to that of AsflhD and that was slightly enriched under nitric-oxide shock, has been previously detected (http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl?_HL) (57). Its localization is consistent with the conservation of AsflhD in *S. enterica* but we cannot exclude that this transcript is processed from AsfD in *S. enterica*.

**FIG 9** Legend (Continued)

*flhD* transcripts (C) to the reaction at the indicated concentrations before addition of RNAP. The samples were analyzed on sequencing gels. The relative intensities of the indicated bands (*flhD* in purple and AsflhD in orange) were analyzed. The values are means of four (B) and three (C) replicates, and the bars indicate standard deviations. Statistical significance was determined by ANOVA and is indicated for either AsflhD (in purple) or *flhD* (in orange) RNA. n.s., *P* > 0.05; ***, *P* < 0.0001.
In the case of AsphoP, the promoter also located in the coding region of the *phaP* ORF is not conserved in other enterobacteria. The lack of conservation in other bacteria suggests that, in contrast to AsfลhD, any function of AsphoP may be unique to *E. coli*, where it most likely arose.

**Role of RNase III in the degradation of AsfлhD and *flhD***. We showed that RNase III can cleave both AsfлhD and *flhD* and degrade the AsfлhD-*flhD* RNA duplex *in vitro* (Fig. S2B to D). In addition, RNase III affects the expression and stability of *flhD* independent of AsfлhD expression *in vivo* (Fig. S1; Fig. 6A). Hence, there appear to be two aspects to its action: first, the complete degradation of AsfлhD-*flhD* RNA duplexes, and second, the destabilization of *flhD* mRNA by cleavages within its 5′-UTR. Degradation of *flhD/*AsfлhD duplexes appears to be a stoichiometric event, removing *flhD* proportionally to the level of AsfлhD transcription, since AsfлhD is never detected free when RNase III is active in the wt strain. The second mechanism presumably involves cleavages within the 5′-UTR that likely modify the complex post-transcriptional regulation of *flhD* and may facilitate RNase E-mediated degradation.

**Mechanism of regulation by AsfлhD**. Transcriptional repression by asRNAs in bacteria has been proposed to arise either from transcriptional interference (upon colliding convergent RNAP) or from transcriptional attenuation (upon binding of the asRNA to its complementary target) (58). For example, the asRNA RNAβ promotes the premature termination of the operon *fatDCBA-angRT* in *Vibrio anguillarum* (59), while the asRNA anti-Q in *Enterococcus faecalis* is responsible for both transcriptional interference due to RNAP collisions and attenuation by an uncharacterized mechanism (60).

In this work, we show that both a decrease and an increase in AsfлhD expression reduce the abundance of *flhD* mRNA and *flhD* translation *in vivo* (Fig. 6A to D) without significantly affecting the stability of the *flhD* mRNA (Fig. S1). Using an *in vitro* system, we reveal that AsfлhD synthesized *in situ* or added exogenously can repress the transcription elongation of *flhD* (Fig. 9; Fig. S8). Since exogenous AsfлhD can repress the transcription of *flhD* *in vitro* to a similar extent as when it is synthesized in *cis* from its endogenous promoter (up to 2-fold repression; Fig. 9B and C; Fig. S8B to C), we propose that AsfлhD represses the transcription elongation of *flhD* mainly via transcriptional attenuation. Furthermore, our experiments do not detect the accumulation of a shorter transcript *in vitro* upon addition of one or the other of the transcripts, suggesting that binding of AsfлhD to *flhD* does not stabilize a terminator structure but could rather modify the stability of the elongating RNAP, leading to heterogeneous 3′-termini as observed for AsfлhD *in vivo* by crT-PCR (Fig. 4A).

The repression of *flhD* expression is weaker (1.4-fold compared to 4.9-fold) when AsfлhD is expressed in *trans* compared to in *cis* in the *P* _mcaA*-lacZ reporter fusion (Fig. 6D and E and 7D and E). This suggests that AsfлhD could also repress *flhD* by transcriptional interference when expressed from its own promoter. However, we cannot rule out that these variations are due to differences in the stoichiometry between *flhD* and AsfлhD RNAs. In addition, the transcription of AsfлhD from the same locus as *flhD* could lead to the increased local concentration of AsfлhD in the vicinity of the nascent *flhD* transcript, thus enabling AsfлhD to interfere with and terminate *flhD* transcription more efficiently.

Experiments performed *in vitro* provide evidence for the transcriptional attenuation of *flhD* expression upon overexpression of AsfлhD. However, we also observed that a reduction in AsfлhD expression leads to a decrease in *flhD* expression *in vivo*. This phenomenon was not observed *in vitro*, so it is likely that this second positive regulatory mechanism involves other factors, such as the numerous post-transcriptional regulators (RNA-binding chaperones and sRNAs) of *flhD* expression (35). Close to the translational start of *flhD*, binding of the McaS sRNA is required to expose the ribosome-binding site and activate translation (61), while the RNA-binding chaperone CsrA protects the 5′-end of *flhD* (45). On the contrary, binding of the sRNAs, OxyS, ArcZ, OmrA, and OmrB represses translation (35). AsfлhD binding could interfere with the binding of any of these sRNAs at their sites along the 5′-UTR of the *flhD* mRNA. Remarkably, most post-transcriptional regulatory events on *flhD* were shown to have weak effects (i.e., often close to 2-fold repression [35]
or activation [45, 61]). Hence, deciphering the effect of each regulator and its interference with the regulation by AsflhD will be an interesting challenge for future studies.

**Outlook.** In this work, we have demonstrated the existence of asRNAs complementary to four major regulators of gene expression in *E. coli*. As we have shown for the asRNA AsflhD, it is likely that they affect both the expression of their direct target and the downstream control of the target’s regulon. Regulatory RNAs are far from being fully understood in bacteria, and new mechanisms of action are likely to be discovered. Furthermore, as in the case of AsflhD, asRNAs demonstrate unexpected regulatory functions that raise the question as to how, when, and to what extent asRNAs participate in complex regulatory circuits.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Strains and plasmids used in this work are listed in Table S1. Constructions and mutations were made by using primers given in Table S1 and are described in the supplemental Materials and Methods (Text S1). Strains were grown in LB medium at 37 or 30°C and shifted to 42 or 46°C for the heat-shock experiments, and samples were taken in the mid-log phase (AAOO ≈ 0.4) or as indicated. Strains carrying the pCA24N control and pCA24N AsflhD (containing the 220 first nt of AsflhD relative to its TSS followed by the mrm B2 T2 terminator) were grown in the presence of chloramphenicol and induced by isopropyl β-D-1-thiogalactopyranoside (10−4 M).

**Northern blotting and RNA methods.** Total RNA was extracted using the hot-phenol procedure (62). Five μg of total RNA were electrophoresed either on 1% agarose with 1× Tris-borate-EDTA (TBE) or 6% polyacrylamide gels (19:1) with 7 M urea and 1× TBE for analysis by northern blotting (63, 64) along with a Riboruler High-Range marker (ThermoFisher) or radiolabeled MspI-digested pBR322 (NEB). The membranes were hybridized with cRNA probes. DNA templates for the synthesis of the RNA probes were obtained by PCR amplification using the pair of “m” and “T7” oligonucleotides (Table S1). The probes were synthesized by T7 RNAp with [α-32P]UTP yielding uniformly labeled RNAs (65). The membranes were also probed with M1 RNA (or 5S) as loading control by using 5′-end-labeled primers (Table S1). DNA templates for *in vitro* processing and *in vitro* transcription assay carrying a T7 promoter sequence were generated by PCR using primers Up-T7-flhD + 308/Down-flhD and Up-T7-AsflhD/Down-AsflhD (Table S1). They allow the transcription of the first 308 nt of flhD and of the first 256 nt of AsflhD, respectively. RNAs were synthesized by T7 RNAp with [α-32P]UTP as a tracer and were gel purified. Our previously published transcriptomic data set (available in the ArrayExpress database at EMBL-EBI under accession number E-MTAB-9507) (23) was used to compare the transcriptomes of the wild-type (N3433) and the RNase III-deficient strain (ibpC633) and sorted against independently analyzed data sets (described in Table I) in which asRNAs and TSSs have been identified.

**β-Galactosidase assays.** Reporter fusions were constructed in the *lacZ* locus as described in the supplemental Materials and Methods and Text (S1). In brief, the *P*~*asphoP*-lacZ fusion contains nt −150 to +15 from the AsphoP TSS, the *P*~*asphoP-lacZ* fusion contains nt −155 to +15 relative to the AsflhD TSS, the *P*~*asphoP-lacZ* fusion contains nt −108 to +300 from the flhD TSS, and the fusion *P*~*asphoP-flhD-lacZ* contains the *P*~*asphoP* promoter sequence followed by nt +1 to +300 relative to the flhD TSS. The *fic-lacZ* fusion was described previously (66). It carries nt −79 to +96 with respect to the *ficC* TSS. The cultures were initiated at A~600~ = 0.02 and sampled at A~600~ = 0.4 to 0.5. Samples (100 or 200 μL) were lysed in Z buffer (1 mL total). β-Galactosidase activity was assayed as described (67); the results are the means of at least three biological replicates as indicated in the legends. Since the *lacZ* mRNA was previously reported to be negatively regulated by RNase III through multiple cleavages within the *lacZ* mRNA ORF (68, 69), we have not attempted to compare *lacZ* reporter fusion expression between wt and rnc mutant.

**Circular RT-PCR.** Circular RT-PCR was performed with total RNA extracted from N3433 and ibpC633 treated with 5′-polyphosphatase. After circularization with T4 RNA ligase 1 (Biolabs), mflhD2 was used to prime reverse transcription and mflhD6 and masflhD10 to generate PCR products (Table S1), which were cloned and analyzed as described (70). It should be noted that the efficiency of ligation by the RNA ligase 1 was previously shown to be affected by the presence of secondary structures, which could explain the exclusion of double-stranded RNAs (43).

**RNA band-shift assay and in vitro processing by RNase III.** AsflhD (256 nt) and flhD (308 nt) RNAs were synthesized as described in the section “Northern blotting and RNA methods.” Transcript 5′-end labeling, hybridization, RNase III digestion, and sample analysis were performed as described previously (70, 71) and are also described in the supplemental Materials and Methods (Text S1).

**In vitro transcription assay.** Single-round *in vitro* transcription experiments were carried out on linear templates as described in the supplemental Materials and Methods (Text S1). AsflhD and flhD RNAs added in *trans* were synthesized as described in the section “Northern blotting and RNA methods.”

**Motility assay.** Stationary-phase bacterial cultures (wt, MG1655-B; *P~rrnB~Δ2-T2*; ML73; and *P~rrnB~Δ1-T2* ML241), with or without the pCA24N control (Ctl) or the pCA24N AsflhD (As) plasmid, were inoculated (2 μL on soft agar (0.2 g/liter) Super optimal broth motility plates (containing 2.4 g/liter MgSO4 and 10−4 M IPTG for strains carrying pCA24N plasmids) at 37°C and pictures were taken using a Gel Doc (BioRad) imager between the beginning and the end of the linear swimming motility period (from 5 to 8 h). Representative images of swimming motility are shown at 6 and 7 h. Super optimal broth was used because bacteria are more motile (presumably due to its lower NaCl concentration, which alleviates...
ACKNOWLEDGMENTS

We thank A. Kolb for the kind gifts of purified RNAP core, α70, and CAP.

This work was supported by the Centre National de la Recherche Scientifique (UMR8261), Université Paris Cité, Agence Nationale de la Recherche (asSUPYCO, grant ANR-11-LABX-0011). M.L. is supported by Japan Society for the Promotion of Science Postdoctoral Fellowship for Research in Japan (grant P22709).

REFERENCES

1. Hör J, Materia G, Vogel J, Gottesman S, Storz G. 2020. Trans-acting small RNAs and their effects on gene expression in Escherichia coli and Salmonella enterica. Ecotoxicology and Environmental Safety 119:16–23. https://doi.org/10.1016/j.ecoenv.2019.09.021.
2. Raghavan R, Sloan DB, Ochman H. 2012. Antisense transcription is pervasive but rarely conserved in enteric bacteria. mBio 3:e00156-12. https://doi.org/10.1128/mBio.00156-12.
3. Lejars M, Hajnsdorf E. 2020. The world of asRNAs in Gram-negative and Gram-positive bacteria. Biochim Biophys Acta Gene Regul Mech 1863:194489. https://doi.org/10.1016/j.bbagrm.2020.194489.
4. Wade JT, Grainger DC. 2014. Pervasive transcription: illuminating the dark matter of bacterial transcriptomes. Nat Rev Microbiol 12:647–653. https://doi.org/10.1038/nrmicro3316.
5. Lejars M, Kobayashi A, Hajnsdorf E. 2019. Physiological roles of antisense RNAs in prokaryotes. Biochimie 164:3–16. https://doi.org/10.1016/j.biochim.2019.04.015.
6. Masachis S, Darfeuille F. 2018. Type I toxin-antitoxin systems: regulating translation by competing with standby ribosomes. Mol Cell 26:381–392. https://doi.org/10.1016/j.molcel.2017.07.003.
7. Malmgren C, Wagner EGH, Ehresmann C, Ehresmann B, Rompy P. 1997. Antisense RNA control of plasmid R1 replication: the dominant product of the antisense RNA-mRNA binding is not a full RNA duplex. J Biol Chem 272:12508–12512. https://doi.org/10.1074/jbc.272.12.12508.
8. Darfeuille F, Unoson C, Vogel J, Wagner EG. 2007. An antisense RNA inhibits translation by competing with standby ribosomes. Mol Cell 26:381–392. https://doi.org/10.1016/j.molcel.2007.04.003.
9. André G, Even S, Putzer H, Burguière P, Croux D, Danchin A, Martin-Verstraete I, Soutourina G. 2008. S-box and T-box riboswitches and antisense RNA control a sulfur metabolic operon of Costtidi um acetobutylicum. Nucleic Acids Res 36:5955–5969. https://doi.org/10.1093/nar/gkn601.
10. Giangrossi M, Prosseda G, Tran CN, Brandi A, Colonna B, Falconi M. 2010. A novel antisense RNA regulates transcriptional level the virulence gene cspA of Shigella flexneri. Nucleic Acids Res 38:3362–3375. https://doi.org/10.1093/nar/gkw025.
11. Kolb FA, Malmgren C, Westhof E, Ehresmann C, Ehresmann B, Wagner EG, Rompy P. 2000. An unusual structure formed by antisense-target RNA binding involves an extended kissing complex with a four-way junction and a side-by-side helical alignment. RNA 6:311–324. https://doi.org/10.1017/s13558382009215x.
12. Opdyke JA, Kang J-G, Storz G. 2004. GadY, a small-RNA regulator of acid response genes in Escherichia coli. J Bacteriol 186:6698–6705. https://doi.org/10.1128/JB.186.20.6698-6705.2004.
13. Opdyke JA, Fozo EM, Hemm MR, Storz G. 2011. RNAIII participates in GadY-dependent cleavage of the gadX-gadW mRNA. J Mol Biol 406:29-43. https://doi.org/10.1016/j.jmb.2010.12.009.
14. Dornenburg JE, Devita AM, Palumbo MJ, Wade JT. 2010. Widespread antisense transcription in Escherichia coli. mBio 1:e00024-10. https://doi.org/10.1128/mBio.00024-10.
15. Peters JM, Mooney RA, Grass JA, Jessen ED, Tran F, Landick R. 2012. Rho and NusG suppress pervasive antisense transcription in Escherichia coli. Genes Dev 26:2621–2633. https://doi.org/10.1101/gad.196741.112.
16. Conway T, Creecy JP, Maddox SM, Grissom JE, Conkle TL, Shaidid TM, Teramoto J, San Miguel P, Shimada T, Ishihama A, Mori H, Wanner BL. 2014. Unprecedented high-resolution view of bacterial operon architecture revealed by RNA sequencing. mBio 5:e01442-14. https://doi.org/10.1128/mBio.01442-14.
17. Lybecker M, Zimmermann B, Bilusic I, Tukhtubaeva N, Schroeder R. 2014. The double-stranded transcriptome of Escherichia coli. Proc Natl Acad Sci U S A 111:3134–3139. https://doi.org/10.1073/pnas.1315974111.
18. Huang L, Dehghan P, Jin J, Li Y, Cheung H-C, Lee E, Mo SS, Hoover H, Abubucker S, Finkel N, McReynolds L, Hochschild A, Lieberman J. 2020. Tombusvirus p19 captures RNase III-cleaved double-stranded RNAs formed by overlapping sense and antisense transcripts in Escherichia coli. mBio 11:e00485-20. https://doi.org/10.1128/mBio.00485-20.
19. Thomason MK, Bischler T, Eisenbart SK, Forstner KU, Zhang A, Herbig A, Nieselt K, Sharma CM, Storz G. 2015. Global transcriptional start site mapping using differential RNA sequencing reveals novel antisense RNAs in Escherichia coli. J Bacteriol 197:18–28. https://doi.org/10.1128/JB.02096-14.
20. Mendoza-Vargas A, Olivera L, Olivera M, Grande R, Vega-Alvarado L, Taboada B, Jimenez-Jacinto V, Salgado H, Juárez K, Conteras-Moreira B,
60. Chatterjee A, Johnson CM, Shu C-C, Kaznessis YN, Ramkrishna D, Dunny GM, Hu W-S. 2011. Convergent transcription confers a bistable switch in Enterococcus faecalis conjugation. Proc Natl Acad Sci U S A 108:9721–9726. https://doi.org/10.1073/pnas.1101569108.

61. Thomason MK, Fontaine F, De Lay N, Storz G. 2012. A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in Escherichia coli. Mol Microbiol 84:17–35. https://doi.org/10.1111/j.1365-2958.2012.07965.x.

62. Braun F, Hajnsdorf E, Regnier P. 1996. Polynucleotide phosphorylase is required for the rapid degradation of the RNase E-processed rpsO mRNA of Escherichia coli devoid of its 3’ hairpin. Mol Microbiol 19:997–1005. https://doi.org/10.1046/j.1365-2958.1996.440971.x.

63. Hajnsdorf E, Regnier P. 1999. E. coli rpsO mRNA decay: RNase E processing at the beginning of the coding sequence stimulates poly(A)-dependent degradation of the mRNA. J Mol Biol 286:1033–1043. https://doi.org/10.1006/jmbi.1999.2547.

64. Hajnsdorf E, Carpousis AJ, Regnier P. 1994. Nucleolytic inactivation and degradation of the RNase III processed pnp message encoding polynucleotide phosphorylase of Escherichia coli. J Mol Biol 239:439–454. https://doi.org/10.1006/jmbi.1994.1387.

65. Hajnsdorf E, Regnier P. 2000. Host factor Hfq of Escherichia coli stimulates elongation of poly(A) tails by poly(A) polymerase I. Proc Natl Acad Sci U S A 97:1501–1505. https://doi.org/10.1073/pnas.040549897.

66. Maes A, Gracia C, Brechemier D, Hamman P, Chatre E, Lemelle L, Bertin PN, Hajnsdorf E. 2013. Role of polyadenylation in regulation of the flagella cascade and motility in Escherichia coli. Biochimie 95:410–418. https://doi.org/10.1016/j.biochi.2012.10.017.

67. Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

68. Shen V, Imamoto F, Schlessinger D. 1982. RNase III cleavage of Escherichia coli β-galactosidase and tryptophan operon mRNA. J Bacteriol 150:1489–1494. https://doi.org/10.1128/jb.150.3.1489-1494.1982.

69. Talkad V, Achord D, Kennell D. 1978. Altered mRNA metabolism in ribonuclease III-deficient strains of Escherichia coli. J Bacteriol 135:528–541. https://doi.org/10.1128/jb.135.2.528-541.1978.

70. Fontaine F, Gasiorowski E, Gracia C, Ballouche M, Caillet J, Marchais A, Hajnsdorf E. 2016. The small RNA SraG participates in PNPase homeostasis. RNA 22:1560–1573. https://doi.org/10.1261/rna.055236.115.

71. Maikova A, Peltier J, Boudry P, Hajnsdorf E, Kint N, Monot M, Poquet I, Martin-Verstraete I, Dupuy B, Soutourina O. 2018. Discovery of new type I toxin-antitoxin systems adjacent to CRISPR arrays in Clostridium difficile. Nucleic Acids Res 46:4733–4751. https://doi.org/10.1093/nar/gky124.

72. Li C, Louise CJ, Shi W, Adler J. 1993. Adverse conditions which cause lack of flagella in Escherichia coli. J Bacteriol 175:2229–2235. https://doi.org/10.1128/jb.175.8.2229-2235.1993.

73. Shin S, Park C. 1995. Modulation of flagellar expression in Escherichia coli by acetyl phosphate and the osmoregulator OmpR. J Bacteriol 177:4696–4702. https://doi.org/10.1128/jb.177.16.4696-4702.1995.

74. Schellhorn HE. 2020. Function, evolution, and composition of the RpoS regulon in Escherichia coli. Front Microbiol 11:560099. https://doi.org/10.3389/fmicb.2020.560099.

75. Kim K-s, Sim S, Ko J-h, Lee Y. 2005. Processing of M1 RNA at the 3’ end protects its primary transcript from degradation. J Biol Chem 280:34667–34674. https://doi.org/10.1074/jbc.M505005200.