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Synthesis of the NarP response regulator of nitrate respiration in *Escherichia coli* is regulated at multiple levels by Hfq and small RNAs

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ABSTRACT

Two-component systems (TCS) and small RNAs (sRNA) are widespread regulators that participate in the response and the adaptation of bacteria to their environments. TCSs and sRNAs mostly act at the transcriptional and post-transcriptional levels, respectively, and can be found integrated in regulatory circuits, where TCSs control sRNAs transcription and/or sRNAs post-transcriptionally regulate TCSs synthesis. In response to nitrate and nitrite, the paralogous NarQ-NarP and NarX-NarL TCSs regulate the expression of genes involved in anaerobic respiration of these alternative electron acceptors to oxygen. In addition to the previously reported repression of NarP synthesis by the $SdsN_{137}$ sRNA, we show here that RprA, another Hfq-dependent sRNA, also negatively controls *narP*. Interestingly, the repression of *narP* by RprA actually relies on two independent mechanisms of control. The first is via the direct pairing of the central region of RprA to the *narP* translation initiation region and presumably occurs at the translation initiation level. In contrast, the second requires only the very 5′ end of the *narP* mRNA, which is targeted, most likely indirectly, by the full-length or the shorter, processed, form of RprA. In addition, our results raise the possibility of a direct role of Hfq in *narP* control, further illustrating the diversity of post-transcriptional regulation mechanisms in the synthesis of TCSs.

INTRODUCTION

Bacteria have the ability to sense, respond and adapt to a wide diversity of environments and their capacity to regulate gene expression plays a key role in this process. Examples of control have been reported at multiple steps of gene expression. Transcription initiation is, for instance, commonly regulated by proteins that bind to the promoter regions of genes and can activate or repress their transcription (1). Regulators falling in this category are involved in the response to diverse input signals, often via a change in their activity in response to a cognate signal, by phosphorylation in the case of two-component systems (TCS). TCSs are widely used in bacteria and consist of a sensor kinase that can auto-phosphorylate in response to specific stimuli and transfer the phosphate group to a cognate response regulator (RR). In most cases, the phosphorylated form of the RR is the biologically active form and regulates transcription by binding to DNA.

While undoubtedly crucial for bacterial adaptation, transcriptional regulation is not the only form of control. Indeed, many bacterial genes can also be regulated at the post-transcriptional level and, in these cases, translation is often the regulated step. Although translational control by proteins was described many decades ago, the observation that a myriad of small RNAs (sRNAs), most of which acting as post-transcriptional regulators, exist in virtually all bacteria has confirmed the importance of post-transcriptional control in bacterial adaptation. In the vast majority of cases, sRNAs act by pairing to target mRNAs via imperfect base-pairing interactions and repress, or more rarely increase, their translation and/or stability (2). Based on the examples studied so far, the most common scenario is that sRNAs of this category pair at or in the vicinity of the ribosome binding site of their target and repress translation initiation by directly competing with binding of the 30S ribosomal subunit. Several other mechanisms have been described, however, including translation activation or repression by sRNAs pairing outside of the translation initiation region (TIR), or the stabilization or destabilization of target mRNAs as

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a direct consequence of sRNAs binding (3,4). In enteric bacteria such as *Escherichia coli* and *Salmonella*, for which many of the details of sRNAs action have been elucidated so far, these imperfectly pairing sRNAs require an RNA chaperone, Hfq or the more recently identified ProQ, for stability and duplex formation with their targets (5,6). Consistent with this, Hfq is involved in the regulation of a multitude of genes whose expression is under the control of sRNAs. Furthermore, Hfq has also been shown to be involved in the direct control of gene expression, independently of sRNAs (7,8).

Interestingly, transcriptional and post-transcriptional controls do not form completely independent regulatory networks in bacterial cells but rather result in mixed regulatory circuits relying on both proteins and sRNAs acting mostly at the transcriptional and post-transcriptional level, respectively (9,10). Transcription of sRNAs is most often controlled by transcriptional regulators, while sRNAs in turn post-transcriptionally regulate the synthesis of transcriptional regulators. One example is the stress response alternative sigma factor RpoS, which directs transcription of SdsR and SdsN sRNAs in enterobacteria (11,12), while RpoS synthesis is directly up-regulated by at least three sRNAs, namely DsrA, RprA and ArcZ (13–16). This control of, and by, sRNAs is also true for regulators of TCSs, including OmpR and PhoP, two of the most studied RRs in enterobacteria, as well as the LuxO RR involved in quorum-sensing in *Vibrio* species (17–23).

NarP is another example of a RR whose synthesis is under sRNA control. Together with its paralog NarX-NarL, the NarQ-NarP TCS regulates the expression of genes involved in the anaerobic respiration on nitrate, the energetically most favourable electron acceptor in the absence of oxygen, and on nitrite, the reduction product of nitrate (24). The translation of *narP* mRNA is repressed in *E. coli* by $SdsN_{137}$, one isoform of a set of RpoS-dependent sRNAs (12). In addition to this control, we report here that expression of *narP* is also regulated by Hfq and the RprA sRNA, whose synthesis is primarily controlled by the Rcs phosphorelay, but also responds to the CpxAR TCS and the LrhA regulator of flagellar synthesis (15,25,26). RprA regulates expression of multiple genes involved in diverse stress responses, biofilm formation, formate metabolism, conjugation as well as the gene encoding LrhA, one of its transcriptional regulators (14,27–29). By adding *narP* to the list of RprA and Hfq targets, our results expand the connections between sRNAs and TCSs, two classes of widespread bacterial regulators, and highlights the high level of integration of the diverse pathways that regulate gene expression. In addition, they demonstrate a dual level of control by a single sRNA, acting both canonically at the TIR of *narP* and, via a distinct sRNA site, and most likely indirectly, at the distant 5′end of the same mRNA.

MATERIALS AND METHODS

Strains, plasmids and general microbiology techniques

All strains and plasmids used in this study are listed in Supplementary Table S1. Cells were grown at 37°C, either in LB medium or in a defined medium (MMGly) composed of potassium phosphate buffer (100 mM) adjusted to pH 7.4, ammonium sulfate (15 mM), sodium chloride (9 mM), magnesium sulfate (2 mM), sodium molybdate (5 μM), Mohr's salt (10 μM), calcium chloride (100 μM), casaminoacids (0.5%) and thiamine (0.01%), supplemented with 140 mM of glycerol as sole carbon source. When needed, nitrate was added at a final concentration of 5 mM. Anaerobic growth was performed in gas tight Hungate tubes under Argon atmosphere. When necessary, antibiotics were used at the following concentrations: 100 or 150 μg/ml ampicillin (Amp_{100} or Amp_{150} in figure legends), 10μg/ml tetracycline, 25 or 50 μg/ml kanamycin and 10μg/ml chloramphenicol. 100 μM of IPTG was also added when required to induce expression of sRNAs from pBRplac derivatives. Amplification of DNA fragments was performed with either Phusion DNA polymerase or LongAmp DNA polymerase (NEB).

Except for cloning, strains are all derivatives of *E. coli* MG1655, modified by recombineering or P1 transduction when needed. Mutant *rprA::tet* (from strain NM667, from N. Majdalani, unpublished; the *tet* resistance gene replaces *rprA* sequence from –10 to + 75 relative to the TSS, hence the RprA terminator is still present) and *sdsN::kan* (strain GS0762 (12)) were obtained from S. Gottesman's and G. Storz's laboratories, respectively, while the *ΔnarP::kan* allele was taken from the Keio collection (strain JW2181, (30)). For the construction of strains carrying *hfq* point mutants, a *Δhfq::cat-sacB purA::kan* mutant (strain DJS2604, from D. Schu) was first transduced into the recipient strain, and the different *hfq* alleles (from strains DJS2927 (wt), DJS2609 (*Δhfq*), KK2561 (R16A), KK2562 (Y25D) or KK2560 (Q8A), from D. Schu carrying alleles from (31,32)) were then moved into the resulting strain, allowing selection on glucose minimal medium; mutant alleles were then checked by sequencing. The various fusions with the *lacZ* reporter gene were made as follows: a PCR fragment encompassing the sequence to be placed upstream of *lacZ*, flanked by homology regions, was recombined into the strain MG1508 (or MG2114 for construction of the P_{napF}-lacZ fusion), carrying the genes for recombineering on a mini-lambda and where a P_{tet}-*cat-sacB* cassette has been introduced upstream of *lacZ*. Recombinant cells were selected on sucrose-containing medium, checked for chloramphenicol sensitivity and the fusion was sequenced. For fusions whose transcription originates from P_{narP} or P_{napF}, an *rrnBt2* transcription terminator was introduced upstream, either on the PCR fragment (P_{narP}), or by recombineering into MG2114 strain carrying an *rrnBt2-cat-sacB-lacZ* construct (for P_{napF} promoter fusion). Fusions $P_{narP\text{-}207+50}$-*lacZ* and $P_{tet+1+50narP}$-*lacZ* have a mutation in the second codon of *lacZ* (ACC is changed to AAC) which prevents the formation of an inhibitory structure for translation.

For the insertion of a 3x-Flag (sequence DYKDHDGDYKDHDIDYKDDDDK) just upstream of the *narP* stop codon, a PCR fragment carrying the 3x-Flag sequence preceded by a linker (protein sequence GAGAGAGA) and followed by a kanamycin resistance gene flanked by FRT sites was amplified from plasmid pSUB11 (33) with homology regions to the end of *narP*. This fragment was then recombined into strain MG1433

and checked by sequencing after selection and purification on LB-Kan. A control adding only the linker sequence was constructed as well. This *narP*-3xFlag -FRT-KanR-FRT allele was then transduced into recipient strains as needed.

The plasmids used to overexpress the different Hfq-dependent sRNAs are mostly from (Mandin & Gottesman, 16), with the addition of pCsrB (from N. de Lay, unpublished), pMcaS, pMicL and pSdsN_{137} (from G. Storz, (12,34,35)) and pCpxQ, pDapZ and pNarS (this study, based on (36–38); see Supplementary Figure S1 for Northern-blot validation of their overexpression). Mutant plasmid pSdsN_{137}-1 (12) was obtained from G. Storz and pRprAmut2 was constructed by amplification of the pRprA plasmid with mutagenic primers using the Pfu enzyme (Agilent), followed by DpnI digestion and transformation into the cloning strain NEB5-alpha F'I^q. Supplementary Table S2 summarizes the main oligonucleotides used in this study to construct strains or plasmids.

Measure of β-galactosidase activity

Cells were diluted at least 250-fold into fresh medium from an overnight culture, grown to mid-exponential phase and β-galactosidase activity was measured following Miller's protocol (39). When indicated, polymyxin B nonapeptide (PMBN, Sigma #P2076) was added to a final concentration of 20 μg/ml when cells were diluted in fresh medium. Cells were lysed either with toluene (for aerobic extracts) or SDS-chloroform (anaerobic extracts). Results presented here correspond to the average of at least two independent experiments and error bars correspond to the standard deviation. Statistical significance of the results was systematically assessed with a bilateral heteroscedastic student t-test.

RNA extraction and northern-blot analysis

The RNAs were extracted with hot phenol as previously described (19) from the same cultures as those used for β-galactosidase assays. A constant amount of total RNA (between 3.5 and 14 μg) was loaded on 8% urea acrylamide gel in 1× TBE and transferred to a nitrocellulose membrane (GE Healthcare). For detection, we used specific 5'-end biotinylated probes (see Supplementary Table S2 for sequence), hybridized to the membrane overnight in Ultrahyb buffer (Ambion). After two washes in SSC 2×–SDS 0.1% and two washes in SSC 0.1×–SDS 0.1% at 42°C, the membrane was incubated in nucleic acid detection blocking reagent (ThermoScientific), and then in the same solution in presence of a streptavidin-alkaline phosphatase conjugate (Life Technologies). Membrane was then washed three times in wash buffer (Na_2HPO_4 29 mM, NaH_2PO_4 8.5 mM, NaCl 34 mM and SDS 0.05%), equilibrated in assay buffer (NaCl 0.1 M, Tris 0.1M pH 9.5) and chemiluminescence was detected using the CDP-star substrate (Applied Biosystems).

Phos-tag electrophoresis and Western-blot

The whole procedure for protein extraction, gel electrophoresis and western-blot detection from Phos-tag containing gels was performed as described previously in (17). Briefly, cells were diluted 250-fold from an overnight culture into fresh medium, grown to mid-exponential phase and cooled down in ice. After centrifugation of 5 ml of culture, the pellet was resuspended in Bugbuster reagent (Novagen) at a concentration equivalent to 40 OD_{600}/ml. 1/4 volume of 5× SDS loading buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 25% glycerol, 572 mM β-mercaptoethanol, 0.10% bromophenol blue) was then added and supernatant was collected after centrifugation. 20 μl of this clarified lysate were loaded on 12.5% precast gels containing 50 μM PhosTag (Wako), and proteins were separated during a 7-h electrophoresis at 80V in migration buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). After migration, the gel was equilibrated twice in transfer buffer (50 mM Tris, 40 mM glycine, 0.037% SDS and 20% ethanol) supplemented with 10 mM EDTA, and then rinsed in transfer buffer without EDTA. Proteins were transferred overnight onto an Hybond-C super membrane; a 1/1000 dilution of a monoclonal anti-Flag antibody conjugated to alkaline phosphatase (Sigma) was used for detection of flagged proteins following manufacturer's instructions and CDP-star reagent as a substrate for chemiluminescence.

For standard western blots, bacterial cells were pelleted and resuspended in SDS-loading buffer containing DTT (Biolabs) and the equivalent of 0.15 OD_{600} was loaded on precast TGX gels (Biorad), prior to transfer to nitrocellulose membrane as above.

RESULTS

Hfq controls the expression of several *Escherichia coli* response regulators

As mentioned above, several examples of genes encoding regulators of TCSs whose expression is post-transcriptionally regulated by Hfq-dependent sRNAs have been reported. TCSs are widespread in bacteria and >30 RR genes exist in the model bacterium *E. coli* for instance. This raises the possibility that other RRs could be subject to sRNA control. We have started to address this question by using *lacZ* reporter fusions to follow the production of 11 *E. coli* regulators of TCSs (listed in Table 1). RRs such as OmpR, PhoP or NarP, whose control by sRNAs has been previously described, have been included in this set. It is worth noting that, in agreement with their control by sRNAs, their mRNAs were found deregulated in an *hfq* null strain and/or enriched following Hfq co-immunoprecipitation (32,40,41). Other RRs included in this study were BaeR, EvgA, KdpE, RstA, UvrY and HprR, whose mRNAs were suggested to interact with Hfq and/or sRNAs in several high-throughput studies ((32,40–43), Table 1 for details), but for which no control by sRNAs has been confirmed so far. At last, we also included two RRs for which no strong indication for sRNA control has been reported to our knowledge: BasR and PhoB.

Translational fusions of *lacZ* to these 11 regulators were constructed at the *lacZ* locus by recombineering (Figure 1A). All fusions followed the same general organization. First, their transcription was systematically driven by a constitutively expressed P_{tet} promoter to exclude regulation

Table 1. TCS investigated in this study

Two-component system (kinase-regulator)[a]	Number of genes whose expression is up/down in the TCS mutant (based on (73))	Indication for sRNA regulation[d]		Portion of the RR gene present on the fusion (relative to start codon)[e]
		Deregulation in Δ*hfq*	Interaction with Hfq	
BaeS-BaeR[b] (response to membrane stress)	14/8		++ (40); + for 3′ UTR (41)	–30 + 720
BasS-BasR (response to iron)	20/12			–18 + 666
EvgS-EvgA (antibiotic resistance and acid stress)	2/3		Slight enrichment upon Hfq IP, and presence in chimeric fragments with sRNAs (42,43)	–125 + 612
KdpD-KdpE[b] (potassium transport)	10/10		++ (40); ++ in Δ*proQ* (43)	–39 + 675
NarQ-NarP[c] (nitrate metabolism)	NarQ 20/25 NarP 3/9	+++ (40)	+++ (32,40); + (41); presence in chimeric fragments with sRNAs (90)	–150 + 645
PhoR-PhoB (phosphate metabolism)	1/8			–41 + 687
RstB-RstA (acid stress response)	12/22		Presence in chimeric fragments with sRNAs (43)	–13 + 726
BarA-UvrY (global metabolism)	BarA 2/24 UvrY 86/37		+++(40); Slight enrichment upon Hfq IP, and presence in chimeric fragments with sRNAs (43)	–44 + 654
HprS-HprR (response to oxidative stress)	6/7		++ in only one out of two experiments (32)	+1 + 669
EnvZ-OmpR (response to osmotic stress and acid stress)	71/54		+ (32); presence in chimeric fragments with sRNAs in one out of two experiments (90)	–35 + 30
PhoQ-PhoP (response to magnesium)	4/27	++(40,44)	+++(32,40); + (41); Slight enrichment upon Hfq IP, and presence in chimeric fragments with sRNAs (42,43,90)	–36 + 30

[a]Unless otherwise indicated, TCSs are expressed from a bicistronic operon with the gene for the response regulator being the first cistron.
[b]In the BaeS-BaeR and KdpD-KdpE systems the kinase is the first gene in the operon
[c]The two genes of the NarQ-NarP TCS are encoded by different loci of the genome.
[d]Weak, moderate or strong indications for sRNA regulation in different studies are indicated by +, ++ or +++, respectively.
[e]Positions of the transcription start sites (TSS) were chosen based on (46); even though *baeR* and *kdpE* are not the first gene of their operon, they were introduced downstream of the P_{tet} promoter in the absence of the *baeS* or *kdpD* gene, respectively. Note that the start codon is GUG for *kdpE* and *rstA*, and UUG for *uvrY*.

at the promoter level. Second, the sequence of each RR gene covering the 5′ UTR and all of the coding region with the exception of the stop codon was placed in frame upstream of *lacZ* starting at the 10th aminoacid (aa) of β-galactosidase; for *ompR* and *phoP* however, only the 10 first aa of the coding sequence were included in the fusions as this was previously shown to be sufficient to allow regulation by sRNAs (13,14). Expression of these different fusions was then measured in an hfq^+ or hfq^- background, with the idea that a Hfq effect could indicate potential regulation by sRNAs (Figure 1B). Note however that genes whose expression is not affected by Hfq in this experiment could nonetheless be regulated by sRNAs because, for instance, these sRNAs are expressed in different experimental conditions than those used here or are Hfq-independent, or because the fusion is not a good reporter in these cases.

Consistent with previous data (44), expression of the *phoP* fusion was up-regulated almost 2-fold in the *hfq* mutant, even if it is unclear at this stage whether this is due to sRNA control or not. In contrast, expression of *ompR*, also subject to negative regulation by sRNAs, namely OmrA and OmrB (14), was down-regulated 1.5-fold in the absence of Hfq, possibly indicating the existence of sRNAs that can activate *ompR* expression. Expression of *baeR, basR, evgA*, *kdpE, rstA* and *uvrY* fusions was not significantly changed, while that of *phoB* and *hprR* was slightly repressed, by respectively 1.6- and 1.4-fold. The strongest Hfq effect was observed on the *narP* fusion, since its activity was increased by almost 3-fold in the *hfq* deletion

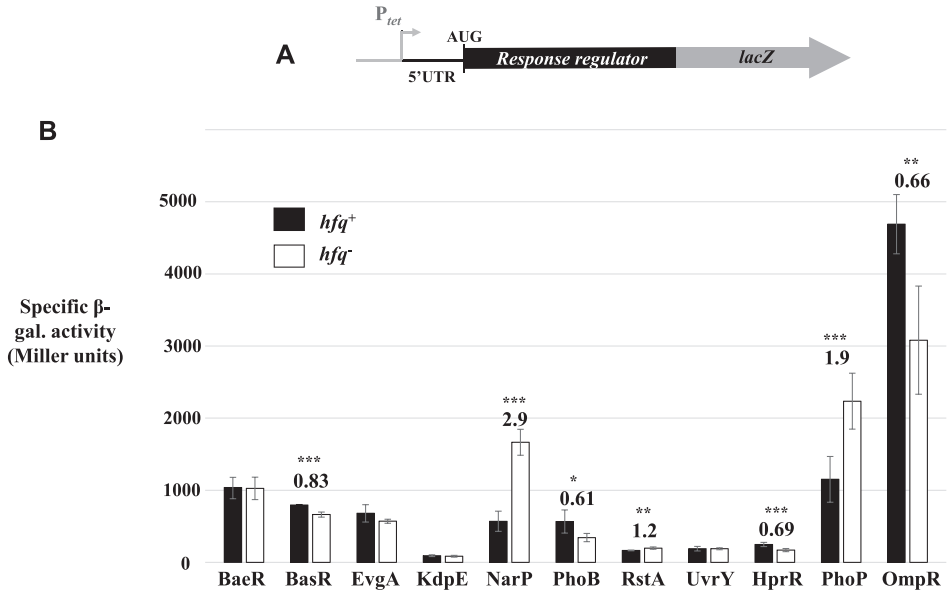

Figure 1. Hfq modulates the expression of several RR genes. (**A**) Scheme of the different translational fusions used to follow RR expression. All fusions are expressed from a P_{tet} promoter; *phoP* and *ompR* coding regions present on the fusions are limited to the 10 first aminoacids, while the entire coding regions of the RR genes, except the stop codon, are present in all other fusions. (**B**) The β-galactosidase activities of each fusion were measured in an hfq^+ of hfq^- background in exponential phase in LB medium. wt and *hfq* null strains used here were, respectively, AB1000 and AB1009 (*baeR*); AB1001 and AB1010 (*basR);* AB1002 and AB1011 (*evgA*); AB1003 and AB1012 (*kdpE*); AB1004 and AB1013 (*narP*); AB1005 and AB1014 (*phoB*); AB1006 and AB1015 (*rstA*); AB1007 and AB1016 (*uvrY*); AB1008 and AB1017 (*hprR*); MG1511 and MG1515 (*phoP*) and AB1148 and AB1149 (*ompR*). Data shown correspond to the average of three independent experiments, with error bars indicating the standard deviations. For each fusion, the numbers above the bars give the activation-fold upon *hfq* deletion, i.e. the ratio of the activity in *Δhfq* and the activity in wt cells, when considered statistically significant using a bilateral heteroscedastic Student's *t*-test; ***: *P*-value $< 5 \times 10^{-4}$, **: *P*-value < 0.005, *: *P*-value < 0.05.

strain. Interestingly, the expression of the *narP* RR gene was previously shown to be repressed at the post-transcriptional level by $SdsN_{137}$, one isoform of a set of Hfq-dependent sRNAs involved in nitrogen metabolism (12). However, SdsN sRNAs accumulate mostly in stationary phase as their transcription is dependent on the RpoS sigma factor and thus, the level of $SdsN_{137}$ is not expected to be at its maximum under the conditions used here. This makes it unlikely that the observed Hfq effect on *narP* is completely explained by the loss of regulation by $SdsN_{137}$ in the *hfq* mutant (see also below), and suggests that other Hfq-dependent sRNAs might regulate *narP* expression.

narP expression is modulated by several sRNAs

To identify other putative sRNAs that might regulate *narP*, we made use of a plasmid library allowing overexpression of most Hfq-dependent sRNAs known to date. This library was initially created by Mandin & Gottesman (16), and was completed for the present study with plasmids overexpressing McaS (34), MicL (35), $SdsN_{137}$ (12), CpxQ (36), DapZ (37) or NarS (38), as well as the non Hfq-binding CsrB (45) sRNAs. The activity of the P_{tet}-*narP-lacZ* fusion was thus measured in presence of all plasmids of the library (Figure 2A). The *narP* fusion is the same as that used in Table 1; it carries a 150 nt-long 5′ UTR that corresponds to transcription initiating from the most distal transcription start site (TSS) based on transcriptomic data after enrichment of 5′-triphosphate containing RNAs (46). Four sRNAs had an effect >2-fold: one positively (ChiX) and three negatively (DicF, RprA and $SdsN_{137}$). Based on previous studies, the effect of ChiX is most probably due to a titration of Hfq (47,48). For the repressing sRNAs, the effect of $SdsN_{137}$ was expected and is in full agreement with the above-mentioned results (12). DicF overproduction led to a strong growth defect consistent with this sRNA inhibiting cell division (49,50), which may perturb the measurement of cell density used in the Miller assay of Figure 2A. Thus, this potentially interesting result needs to be confirmed by a different approach and the possible repression of *narP* by DicF was not investigated further in this study. The third repressing sRNA was RprA, whose transcription is under strong control by the Rcs phosphorelay (15), and for which several targets have been identified in enterobacteria such as *E. coli* or *Salmonella*. RprA is known to activate the synthesis of the alternative sigma factor σ^S and of the RicI protein that inhibits conjugation (14,28). Several negative targets of RprA have also been previously described: this sRNA represses expression of the *csgD* and *ydaM* (*dgcM*) genes involved in biofilm formation (27) and of *hdeD*, encoding an acid resistance protein (29). Interestingly, expression of other genes was found to be modulated in response to RprA pulse-overexpression in *Salmonella* (28). This includes *narP*, which was repressed, in agreement with the results reported here in *E. coli*.

Because RprA up-regulates RpoS synthesis, one might hypothesize that the pRprA plasmid used in Figure 2A could promote $SdsN_{137}$ synthesis by increasing σ^S levels,

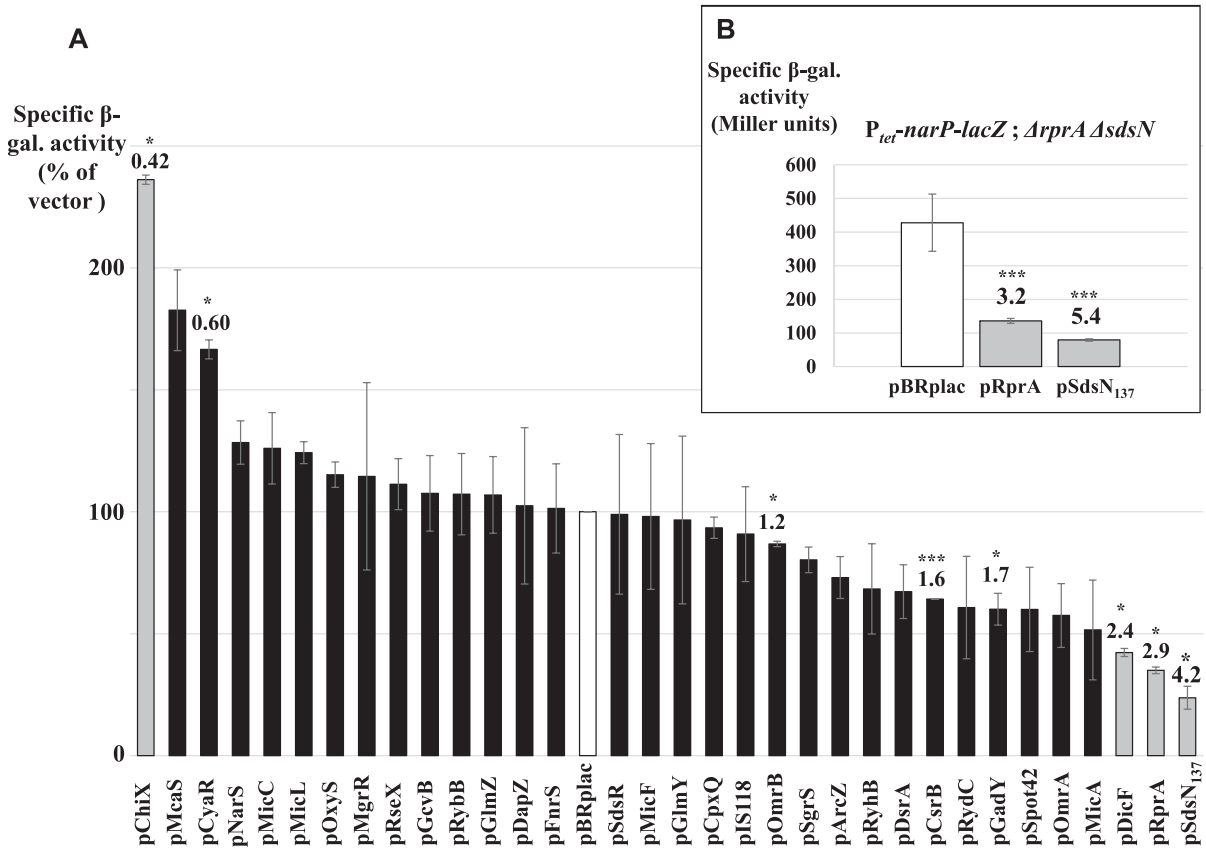

Figure 2. Hfq-dependent sRNAs modulate the expression of *narP*, most likely at the post-transcriptional level. (**A**) The β-galactosidase activity of the P_{tet}-*narP-lacZ* fusion (strain AB1004) was measured in LB-Amp_{150}-IPTG in the presence of plasmids overexpressing 33 *E. coli* sRNAs reported to date. The activity of the fusion in presence of the vector control pBRplac (white bar) was arbitrarily set at 100% and corresponds to an average value of 889 Miller units (with a standard deviation of 110). Data shown are the average of two independent experiments. When found statistically significant using a bilateral heteroscedastic student t-test (***: *P*-value $< 5 \times 10^{-4}$, **: *P*-value < 0.005, *: *P*-value < 0.05), the repression factors associated to the overproduced sRNAs compared to the pBRplac vector control are indicated above the corresponding bars. Grey bars indicate the sRNAs whose overproduction leads to a fold-change >2 in the activity of the P_{tet}-*narP-lacZ* fusion. (**B**) The β-galactosidase activity of the same P_{tet}-*narP-lacZ* fusion in a *ΔrprA ΔsdsN* background (strain AB1029) was measured in the presence of plasmids overproducing RprA or $SdsN_{137}$ in LB-Amp_{100}-IPTG. Data shown are the average of three independent experiments and statistical analysis is as in panel (A).

and thereby repress expression of the *narP-lacZ* fusion. However, the pRprA plasmid still regulated the *narP* fusion to a similar extent in a strain deleted for the chromosomal copy of *sdsN* and *rprA* (3.2-fold versus 2.9-fold repression in the $rprA^+ sdsN^+$ background, Figure 2B), indicating that its effect is independent of SdsN. We next investigated RprA control of *narP* in more detail.

RprA directly targets the *narP* mRNA

Hfq-dependent sRNAs typically regulate gene expression by pairing to their targets, and we therefore looked for possible interactions between RprA and the *narP* mRNA using IntaRNA (51). The result is shown in Figure 3A: nucleotides (nts) 31–69 of RprA can potentially imperfectly base-pair to the TIR of *narP* messenger, from nts 116 to 154 relative to the *narP* TSS (i.e. nts –35 to +4 relative to *narP* start codon). Of note, affinity purification and sequencing of RNAs associated with a tagged version of RprA identified *narP* mRNA as a potential direct target, although not among the best candidates (29).

The predicted RprA-*narP* mRNA interaction (Figure 3A) was experimentally tested by introducing mutations in RprA and/or in the previously described *narP-lacZ* fusion. Four possible base-pairs adjacent or inherent to the Shine-Dalgarno (SD) sequence were disrupted by mutating either nts 45–48 of RprA from UGAG to ACUC (RprAmut2) or nts 137 to 140 of *narP-lacZ* from CUCA to GAGU (*narP*mut2). RprAmut2 only very weakly repressed *narP-lacZ* activity (1.3-fold), even though the sRNA accumulates to a level similar to that of the wt (Figure 3B) and, similarly, the mut2 change in *narP-lacZ* strongly reduced control by RprA. Although the activity of the mutant fusion was strongly decreased, presumably because the mutation reduces the strength of the SD sequence, restoring the pairing by combining these compensatory changes partially restored control (2.7-fold repression), demonstrating that RprA sRNA directly pairs to *narP* mRNA *in vivo*. Since the validated interaction includes the SD sequence, RprA most likely inhibits *narP* translation initiation by preventing 30S ribosomal subunit binding to this mRNA.

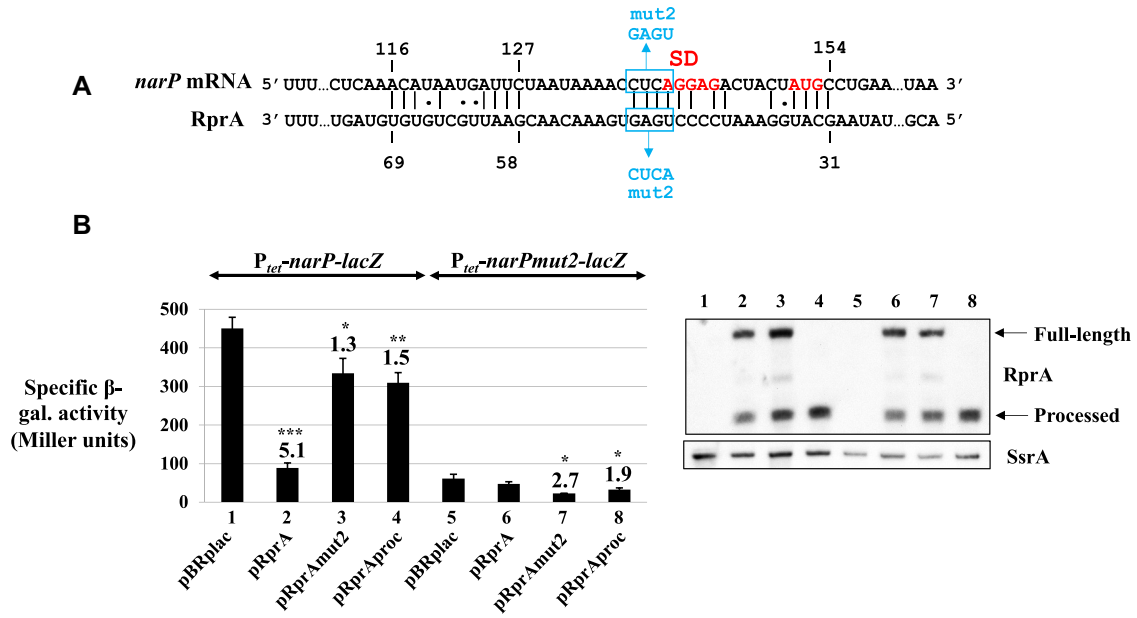

Figure 3. RprA directly pairs to the translation initiation region of *narP* mRNA. (**A**) Predicted base-pairing interaction between RprA and *narP* mRNA. The Shine-Dalgarno sequence and start codon of *narP* are indicated in red, and the compensatory mut2 changes in RprA or *narP-lacZ* fusion are shown in blue. (**B**) The β-galactosidase activity of the P_{tet}-*narP-lacZ* translational fusion, wt or carrying the mut2 change, was measured upon overproduction of RprA, RprAmut2 or RprA processed (RprAproc) in LB-Amp_{150}-IPTG. Data shown are the average of three independent experiments. When statistically significant with a bilateral heteroscedastic Student's *t*-test (***P-value $< 5 \times 10^{-4}$, **P-value < 0.005, *P-value < 0.05), the repression factors associated with the overproduced sRNAs are indicated above the corresponding bars. Strains used in this experiment are AB1018 (wt fusion) and PB65 (mut2) and are deleted for the chromosomal *rprA* copy. In parallel, RNA was extracted from the same cultures and Northern-blot analysis was performed to assess the overproduction of RprA and its derivatives. SsrA was probed from the same membrane and used as a loading control.

Overexpression of RprA or $SdsN_{137}$ decreases NarP protein levels

In most cases, the active form of RRs is the phosphorylated form. However, changing the total levels of a RR does not necessarily lead to changes in the levels of its phosphorylated form. For instance, for OmpR RR, it was shown that modulating the synthesis of the protein, either with an inducible heterogenous promoter or with sRNA control, affected OmpR levels, without affecting that of phosphorylated OmpR (17,52). We thus wondered whether controlling *narP* synthesis by RprA and $SdsN_{137}$ sRNAs would affect the levels of the phosphorylated form of the NarP RR (NarP-P). For this purpose, a tagged version of the NarP protein was constructed, where a 3xFlag sequence was added at its C-terminus after a short linker. This construction replaces the *narP* chromosomal copy. The biological activity of this tagged version of NarP was then assessed by measuring its ability to activate transcription from the *napF* promoter (Figure 4A).

The *E. coli napFDAGHBC* operon encodes the periplasmic nitrate reductase (and its accessory proteins) required for nitrate respiration in the presence of low concentration of this substrate (53). Expression of the *napF* operon is induced by FNR and NarP-P, and repressed by NarL, as binding of NarL-P, the phosphorylated form of NarL to the *napF* promoter prevents binding of NarP-P. Because NarP and NarL are preferentially phosphorylated under low and high nitrate concentration, respectively, *napF* expression is expected to be higher under low nitrate conditions (54–56).

napF expression was thus followed by using a transcriptional fusion between the *napF* promoter (from nts –85 to +19 relative to the TSS from the proximal promoter) and *lacZ* sequence starting 17 nts before the translation initiation codon. In a preliminary experiment, the activity of this fusion was measured in cells grown under anaerobic conditions at different nitrate concentrations and was found to peak at around 5 mM nitrate (data not shown), in agreement with its control by NarL and NarP. The same minimal medium with glycerol as the sole carbon source and supplemented with 5 mM nitrate (hereafter MMGly+Ni) was thus used in the next experiments. As expected, expression of the P_{napF}-*lacZ* fusion was strongly decreased in the *narP* deleted strain (Supplementary Figure S2A). Furthermore, expression was partially restored in presence of the NarP-3xFLAG protein, showing that this tagged version of NarP retains biological activity, albeit at a reduced level compared to the wt protein (Supplementary Figure S2A).

The levels of NarP and NarP-P were then followed by western-blot using antibodies directed against the FLAG sequence upon overexpression of RprA and $SdsN_{137}$ sRNAs, either wt or variants that are defective in *narP* control. The western-blots were performed by separating total proteins on a polyacrylamide gel containing Phos-Tag to allow the separation of NarP-P from the non-phosphorylated form of NarP and directly assess the effect of the sRNAs on the two forms of the protein. As shown in Figure 4B, overexpression of wt RprA and $SdsN_{137}$ significantly decreased the level of both forms of the NarP

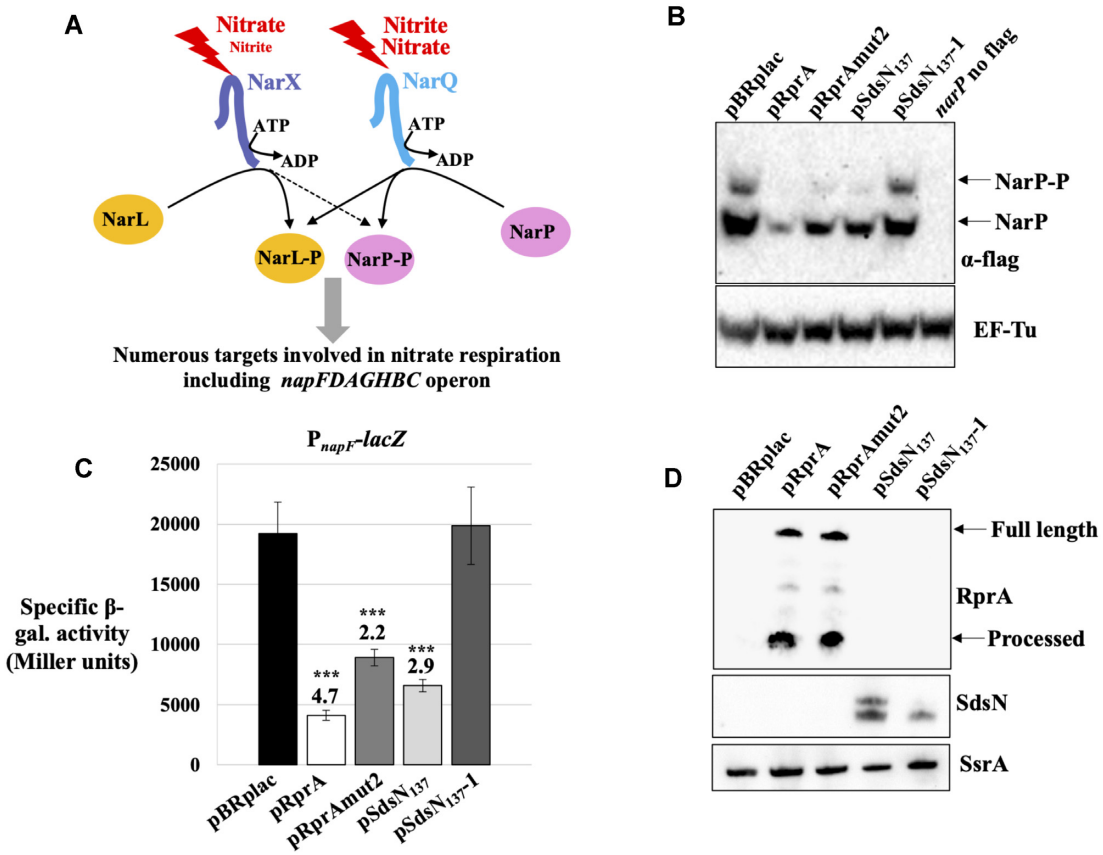

Figure 4. RprA and $SdsN_{137}$ decrease the levels of both the phosphorylated and the non-phosphorylated forms of NarP and indirectly affect transcription of a NarP target. (**A**) Schematic of the regulatory network involving the two-component system NarQ-NarP. See text for details. (**B**) Levels of the non-phosphorylated and phosphorylated forms of the NarP-3xFlag protein were followed by western-blot following protein separation on a Phos-Tag containing polyacrylamide gel. Protein samples were taken from cells grown in MMGly+Ni, supplemented with Amp_{100} and IPTG, under anaerobic condition. Strains used for this experiment are AB1082, transformed with the indicated plasmids, or AB1083, transformed with the vector control, to ensure specificity of the Flag signal (see no Flag lane). EF-Tu levels were determined from the same membrane and used as a loading control. An independent repeat of the same experiment is shown in Supplementary Figure S2C. (**C**) β-galactosidase activity of the P_{napF}-*lacZ* transcriptional fusion (in strain AB1042) was measured in the presence of plasmids overexpressing RprA and $SdsN_{137}$, wt or mutated in the pairing site with *narP* translation initiation region. Cells were grown in MMGly+Ni, supplemented with Amp_{100} and IPTG, under anaerobic condition. Data shown are the average from three independent experiments and, when statistically significant with a bilateral heteroscedastic Student's *t*-test (***P-value $< 5 \times 10^{-4}$, **P-value < 0.005, *P-value < 0.05), repression factors compared to the vector control are indicated above the bars. (**D**) RNA samples were extracted from the same cultures than used in panel (C) and the levels of RprA and SdsN sRNAs were analyzed by northern-blot, with detection of SsrA used as a loading control.

protein. Furthermore, this effect was abolished when we tested the $SdsN_{137}$-1 mutant for which pairing to *narP* is eliminated (12), indicating that these changes are due to post-transcriptional control of *narP* by this sRNA. Strikingly however, RprAmut2, i.e. the RprA variant that no longer controls expression of the P_{tet}-*narP-lacZ* fusion (Figure 3B), was still very efficient at decreasing NarP and NarP-P levels (Figure 4B and Supplementary Figure S2, panels C and D). This surprising effect of RprAmut2 is further discussed below (see Figure 5). Changes in total NarP protein levels were also assessed in the same experiment by Western-Blot from a classical polyacrylamide gel where NarP and NarP-P are not separated; the results are in complete agreement with the Phos-Tag data and confirm the reduction in NarP levels in presence of pRprA, pRprAmut2, $pSdsN_{137}$, but not $pSdsN_{137}$-1 (Supplementary Figure S2B).

Overall, *narP* repression by RprA or $SdsN_{137}$ results in a clear decrease of both NarP and NarP-P levels.

Downstream effects of RprA and $SdsN_{137}$ on NarP targets

Previous work has shown that regulating the synthesis of TCSs may have unexpected outcomes on the expression of their targets (52). For instance, only one of the two sRNAs that repressed PhoP synthesis also repressed expression of PhoP-activated genes, while control of *ompR* by sRNAs affected only targets that were sensitive to the non-phosphorylated form of OmpR (17,18). It was thus of interest to determine how RprA and $SdsN_{137}$ control affected NarP-targets. For this purpose, we used the previously described P_{napF}-*lacZ* transcriptional fusion and measured its activity upon overproduction of wt or mutant RprA and $SdsN_{137}$ (Figure 4, panels C and D). Consistent with the observed reduction in NarP-P levels, RprA and $SdsN_{137}$ decreased expression of the P_{napF} fusion by 4.7- and 2.9-fold, respectively. In contrast, $SdsN_{137}$-1 had no effect, which was expected since this mutant no longer controls *narP*. The RprAmut2 variant still decreased expression of

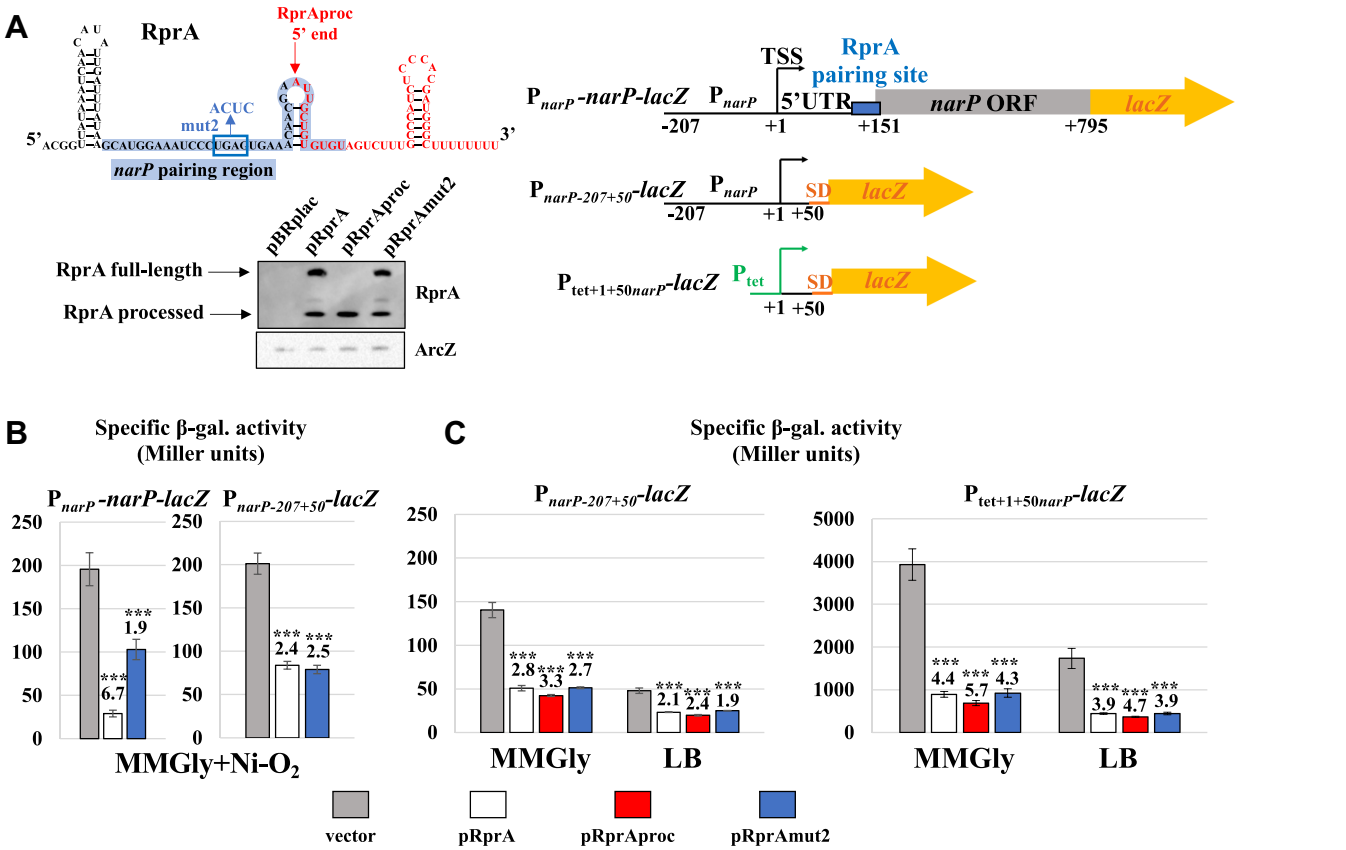

Figure 5. A second control of *narP* by RprA relies on the 5′ end of *narP* mRNA and the 3′ end of RprA. (**A**) Schematic of the different RprA versions and the three *narP-lacZ* fusions used in the next panels. The indicated RprA structure is based on (28); RprA processed is shown in red. The levels of the different RprA variants were analyzed by northern-blot after RNA was extracted from AB1184 strain transformed by the indicated plasmids and grown in LB-Amp$_{100}$-IPTG, using the same cultures than those used for the β-galactosidase assays of the right panel of Figure 5C. The detection of the ArcZ sRNA was used as a loading control. (**B**, **C**) The β-galactosidase activity of the *narP-lacZ* fusions was measured upon overproduction of wt RprA or of several RprA variants, in MMGly-Amp$_{100}$-IPTG medium (MMGly) or in LB-Amp$_{100}$-IPTG (LB). In panel B, cells were grown anaerobically in the presence of nitrate. Strains used in this experiment were AB1092 (P$_{narP}$-*narP-lacZ* fusion), AB1159 (P$_{narP-207+50}$-*lacZ*) and AB1184 (P$_{tet+1+50narP}$-lacZ). Data shown are the average of two independent experiments (panel B) or three independent experiments (panel C). Repression fold by the different plasmids compared to the vector control are given by the numbers above the bars after the statistical significance was assessed using a bilateral heteroscedastic student t-test (***: *P*-value $< 5 \times 10^{-4}$).

the P$_{napF}$ fusion by 2.2-fold, which is fully consistent with its intermediary effect on the NarP protein levels (Figure 4C). In other words, controlling NarP synthesis with RprA and SdsN$_{137}$ sRNAs can impact NarP-targets, as shown here for *napF*. However, whether this is systematically true for other NarP targets remains to be determined.

A second, independent, regulation of *narP* by RprA

The previous results clearly established the direct pairing between RprA and the *narP* TIR (Figure 3). However, mutating RprA in this pairing region (RprAmut2) strongly impaired control of a P$_{tet}$-*narP-lacZ* fusion (Figure 3B) but still allowed control of *narP* expression when looking at NarP protein levels and *napF* transcription (Figure 4B and C). This could be explained if the mut2 change does not completely prevent the RprA interaction with *narP* TIR under some conditions, but we also considered the possibility that another regulation of *narP* by RprA may explain the residual control by RprAmut2. This hypothesis was furthermore in agreement with two observations made with the processed form of RprA (RprAproc), corresponding to the last 47 nts of the sRNA and co-existing *in vivo* with the full-length primary transcript (57,28). First, even though RprAproc is mostly devoid of the region interacting with *narP* TIR (Figure 5A), it still repressed expression of *narP-lacZ* fusion (Figure 3B). Second, as full-length RprA, RprAproc was found to repress *narP* in a transcriptomic study performed in *Salmonella* (28). These data are consistent with RprA affecting *narP* not only *via* the canonical pairing shown in Figure 3A, but also *via* another mechanism involving a different region of RprA that is present in RprAproc.

To get further insight into this additional control, we used several *narP-lacZ* fusions that differed in their promoter or in the transcribed region of *narP* that they carry (Figure 5A); their control by RprA or RprAmut2 was measured in anaerobic conditions in the presence of nitrate (MMGly+Ni-O$_2$, i.e. the same experimental conditions used in Figure 4) or in aerobic growth in LB or in MMGly. In all tested media, RprAmut2 significantly repressed the

expression of all tested fusions (Figure 5, panels B and C). This repression was even similar to that obtained with wt RprA for fusions that lack the RprA pairing site identified in Figure 3. Importantly, fusions carrying only the first 50 nts of the transcribed region of *narP* are equally repressed by RprA, RprAmut2 and RprAproc, and this is true whether their transcription is driven by the *narP* promoter or the P_{tet} one. In other words, only the first 50 nts of the *narP* 5′ UTR are required for this second control by RprA.

It is not clear at this stage why this second regulation did not allow a stronger control of the P_{tet}-*narP-lacZ* fusion by RprAmut2 or of the P_{tet}-*narPmut2-lacZ* fusion by wt RprA (Figure 3B). It is certainly possible that the combination of the growth conditions used in this experiment (aerobic growth in rich medium) with the fusion used (allowing transcription of an artificial *narP-lacZ* mRNA from a strong P_{tet} promoter) is unfavorable for observing this regulation. Nevertheless, the weak residual control of the wt fusion/mutant sRNA or mutant fusion/wt sRNA combinations (Figure 3B) is most likely explained by this second mechanism of repression acting on the (+1+50) region of *narP* mRNA.

Together, these data clearly show that, in addition to the direct pairing of nts 30–70 of RprA to *narP* TIR, this sRNA also represses *narP* via a second action involving only the first 50 nts of *narP* mRNA and the 47 last nts of the sRNA.

Down-regulation of *narP* upon physiological induction of *rprA* expression

To determine whether this effect of RprA or RprAproc on the *narP* (+1+50) region is specific, we measured expression of the $P_{tet+1+50narP}$-*lacZ* fusion upon overproduction of other Hfq-dependent sRNAs. We included in this set $SdsN_{137}$, the other known repressor of *narP*, and sRNAs that had a more modest effect on the full-length *narP-lacZ* fusion, such as OmrA, MicA, Spot42 (Figure 2A). The NarS and SdsR sRNAs were tested as well based on the NarS involvement in nitrate/nitrite metabolism (38) and the possible accumulation of SdsR mediated by the RpoS increase when RprA is produced (11,15),respectively. Importantly, these other plasmids had only a very modest or no effect on the $P_{tet+1+50narP}$-*lacZ* fusion (Figure 6A), showing that this effect is specific to RprA or RprAproc.

The physiological relevance of the control of *narP* by RprA was then investigated by comparing *narP* expression in wt and *ΔrprA* strains. To this end, we first used the P_{tet}-*narP-lacZ* fusion, but its expression was never significantly affected by the deletion of *rprA*, even under conditions known to allow RprA accumulation (data not shown). A likely hypothesis for this result is that RprA is not the only post-transcriptional regulator of this fusion, whose expression is also repressed by $SdsN_{137}$ sRNA and the Hfq protein, possibly independently of sRNAs (see below). These additional regulators could thus compensate and further repress *narP* in the absence of RprA. In contrast, the expression of the $P_{tet+1+50narP}$-*lacZ* fusion is not controlled by $SdsN_{137}$ (Figure 6A) or by Hfq (Figure 6B), and we compared its expression in wt and *ΔrprA* cells. This experiment was carried out in the presence of polymyxin B nonapeptide (PMBN), a known inducer of the Rcs phosphorelay that efficiently promotes RprA synthesis (58). In control conditions, i.e. without PMBN, RprA is poorly expressed and its deletion has no significant effect on the activity of the $P_{tet+1+50narP}$-*lacZ* fusion. When PMBN is present, however, RprA accumulates and expression of the fusion is increased by 50% in *ΔrprA* cells when compared to the wt background (Figure 6, panels C and D). Thus, physiological levels of RprA are sufficient to observe a modulation of *narP* expression.

The Hfq effect on *narP-lacZ* fusion may be only partially due to sRNA regulation

As described above, we initially focused on the regulation of *narP* by sRNAs because *narP* expression was derepressed by about 3-fold in an *hfq*$^-$ background (Figure 1). Interestingly however, a similar increase in *narP* expression was observed in the *hfq* mutant in the absence of either *rprA* or *sdsN* genes, or in the double mutant, clearly showing that the Hfq effect is largely independent of these two sRNAs (Figure 7A). This suggests that other Hfq-dependent sRNAs, not represented in the library used in Figure 2, could negatively regulate *narP* expression. Another possibility is that, in addition to being involved in RprA and SdsN action, Hfq could also directly modulate *narP* expression, this time independently of sRNAs, as shown for the *mutS* mRNA (7). To discriminate between these two (non-exclusive) possibilities, we made use of point mutants of Hfq that have been previously described.

Hfq forms a ring-shaped homohexamer that presents different surfaces involved in RNA binding: the proximal, distal, lateral faces and the C-terminal tail. In enterobacteria, several studies pointed to a major role of the proximal face in binding and stabilizing most sRNAs through interaction with the polyU stretch of the terminator, while the distal face displays a preference for (AAN) triplets, found in mRNAs and some sRNAs. The lateral face also participates to the binding of sRNAs or mRNAs via an arginine patch that can interact with UA-rich RNA sequences (31,32,59–62). The expression of the P_{tet}-*narP-lacZ* translational fusion was thus measured in point mutants affecting each of three surfaces: Q8A (proximal face), Y25D (distal face) and R16A (lateral face) (32). Q8A and R16A mutants had no effect on the expression of the fusion, while the Y25D change caused a 2.7-fold increase, i.e. similar to the effect of the *hfq* null allele (Figure 7B). Furthermore, several AAN motifs are present in the *narP* mRNA in the vicinity of the TIR and mutation of two of them to CCN abolished the repression by Hfq (Supplementary Figure S3), supporting binding of the distal face of Hfq to this site. Together, these data suggest that, as for *mutS* (7), *narP* expression is not only subject to sRNA control, but could also be directly controlled by Hfq. Since *phoP* expression was found to be up-regulated as well in the absence of Hfq, independently of the two known Hfq-dependent sRNAs that repress *phoP*, i.e. MicA and GcvB (18), the same set of mutants was tested with the P_{tet}-*phoP-lacZ* fusion. In this case, the Q8A mimicked the effect of the *Δhfq* mutation, while R16A and Y25D had no effect (Figure

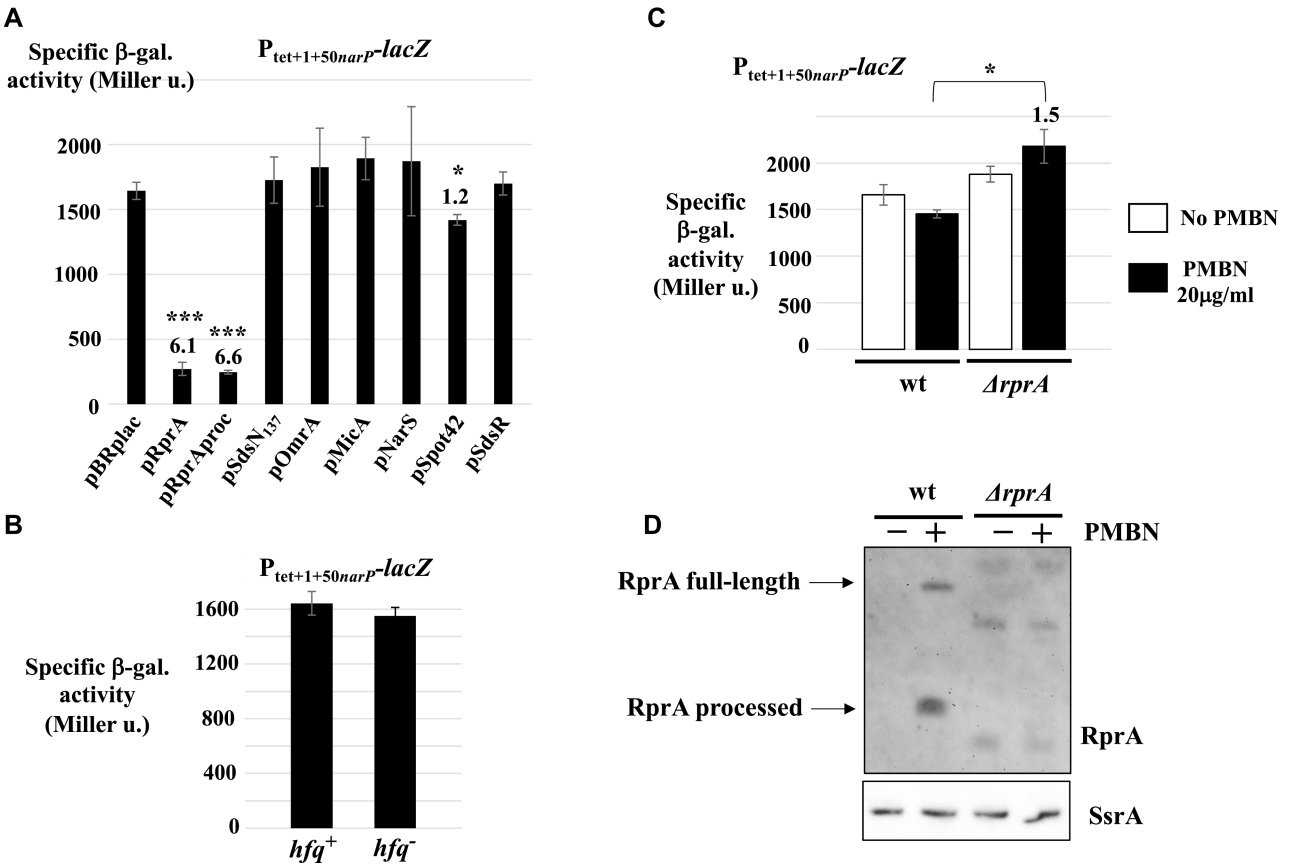

Figure 6. The endogenous copy of RprA specifically represses *narP* expression. (**A**) The β-galactosidase activity of the $P_{tet+1+50narP}$-*lacZ* fusion was measured upon overproduction of different Hfq-binding sRNAs. For the significant changes, the repression factors compared to the vector control are given by the numbers above the bars. For panels A, B and C, the data shown correspond to the average of three independent experiments and statistical significance was determined with a bilateral heteroscedastic Student's *t*-test; **P*-value < 0.05; ***P*-value < 0.005; ****P*-value < 5×10^{-4}. (**B**) The activity of the same $P_{tet+1+50narP}$-*lacZ* fusion was measured in LB in wt and *hfq*$^-$ strains. Strains are AB1184 (wt) and AB1194 (*hfq*$^-$). (**C**) The activity of the same fusion was measured in wt or *ΔrprA* cells, grown in LB or LB supplemented with 20 µg/ml polymyxin B nonapeptide (PMBN). Strains used in this experiment are AB1184 (wt) and PB70 (*ΔrprA*). When significant, the fold-difference in the β-galactosidase activity in Δ*rprA* and wt cells is indicated. (**D**) Northern-blot analysis of RprA using total RNA samples extracted from the same cultures than in (C). Detection of SsrA is used as a loading control. The bands that are detected with the RprA probe in the Δ*rprA* strain most likely correspond to RNA fragments carrying the RprA terminator, still present in the *ΔrprA::tet* allele that was used (see Materials and Methods for details).

7C), consistent with the hypothesis that *phoP* expression is regulated by Hfq-dependent sRNAs that remain to be identified.

DISCUSSION

A new connection between sRNAs and nitrate metabolism

Enterobacteria such as *E. coli* and *Salmonella* are facultative anaerobes and often encounter low oxygen conditions, e.g. in the gut of mammalian hosts. Their ability to respire on nitrate or nitrite, which are efficient alternative electron acceptors to oxygen, is certainly an advantage under anaerobic conditions and this has been linked to efficient colonization (63) and competitive growth in host (64,65). Several transcriptional regulators ensure control of gene expression in response to the availability of different electron acceptors. In particular, FNR and ArcB-ArcA both respond to anaerobiosis, with the oxidation status of quinones being involved in signaling to ArcB-ArcA. The NarX-NarL and NarQ-NarP TCSs are involved in the response to nitrate and nitrite. While the NarQ sensor phosphorylates both the NarP and NarL RR in response to nitrate and nitrite, NarX preferentially phosphorylates NarL in response to nitrate mostly (66,67). NarL and NarP regulons partially overlap and include genes for enzymes required for nitrate/nitrite respiration, among them the nitrate reductases NarGHJI (membrane-bound) and NapFDAGHBC (periplasmic), and the nitrite reductases NirBDC and NrfABCDEFG. Other members of the NarP and the NarL regulons, e.g. the *hcp-hcr* operon, are involved in the response to nitrogen stress and NO detoxification following nitrate respiration (68–71). The identification of *narP* as one of the direct targets of RprA suggests that it may be advantageous for the cell to limit NarP levels under conditions where RprA is expressed, such as cell surface stress that would signal to the Rcs phosphorelay (72). In line with this, transcriptomic analyses of strains lacking the RcsB regulator identified several genes related to nitrogen metabolism, e.g. *napAB*, *nirBDC*, *narK* or

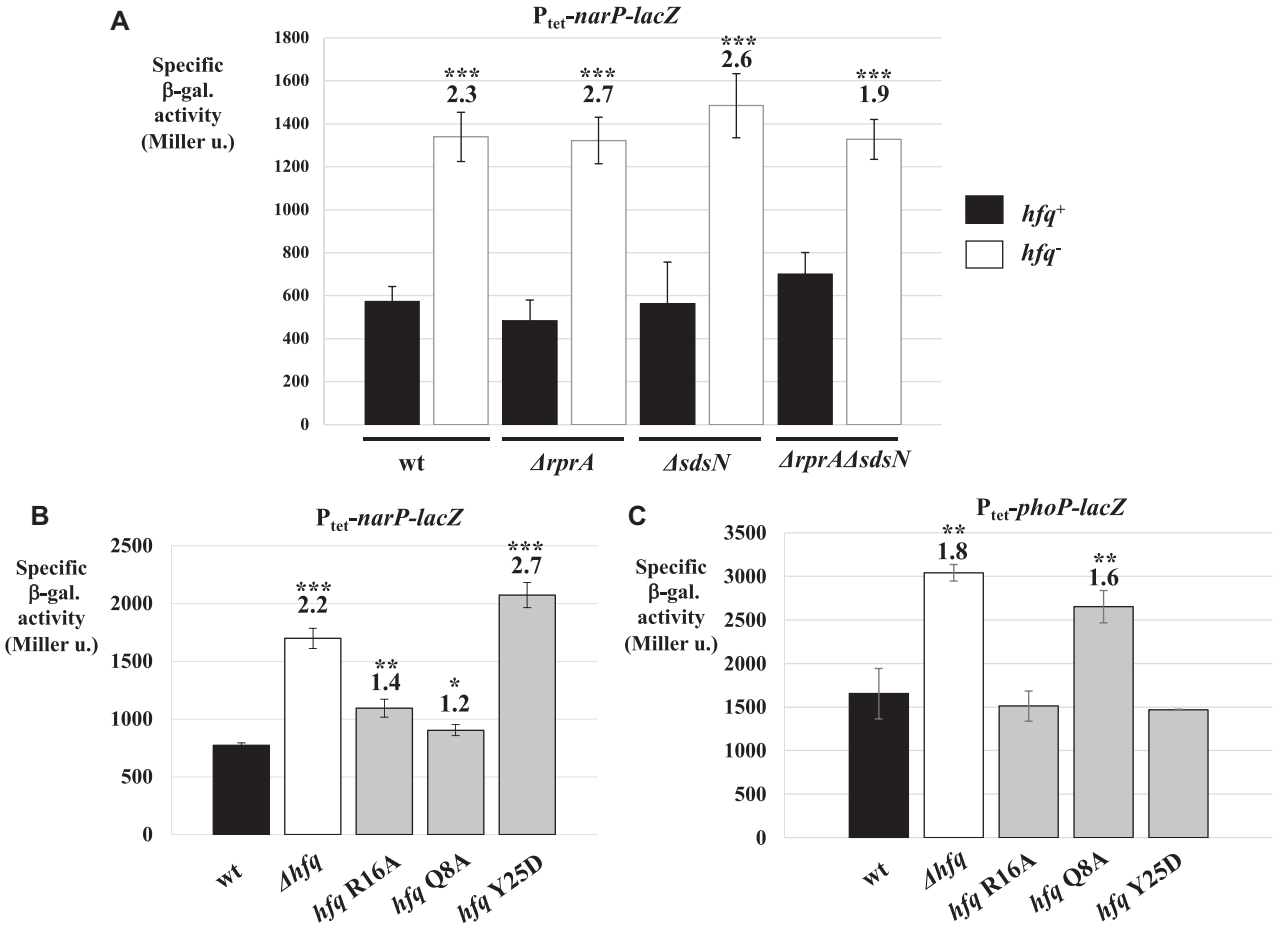

Figure 7. The Hfq effect on *narP* is largely independent of RprA and $SdsN_{137}$ sRNAs. (**A**) The β-galactosidase activity of the P_{tet}-*narP-lacZ* fusion was measured in LB medium in hfq^+ and hfq^- background, in the presence and absence of the chromosomal copies of *rprA* and *sdsN* genes. Strains used here were AB1004, AB1013, AB1018, AB1109, AB1028, AB1110, AB1029 and AB1111. The effect of an *hfq* deletion or of mutations in the lateral face (R16A), proximal face (Q8A) or distal face (Y25D) of the Hfq protein was assessed on the activity of the same fusion (**B**) or that of the P_{tet}-*phoP-lacZ* translational fusion (**C**). Strains used were AB1141, AB1142, AB1143, AB1144, AB1145, AB1151, AB1152, AB1153, AB1154 and AB1155, and were grown in LB. Shown are the average of three independent experiments (panel A) or two independent experiments (panels B and C). Numbers above the bars give the activation fold in the different *hfq* mutants compared to the hfq^+ background when considered statistically significant with a bilateral heteroscedastic Student's *t*-test (**P*-value < 0.05; ***P*-value < 0.005; ****P*-value < 5×10^{-4}).

narGH, as repressed by the Rcs system in several bacteria (73,74).

Reducing NarP levels could facilitate the activation of NarL by NarX or NarQ; this would be true for control by either RprA or $SdsN_{137}$. Such a precise balance between NarP and NarL functions may be important for a proper response to different nitrate concentrations. For example, NarX-NarL has been found to be more important for intestine colonization than NarQ-NarP, possibly because it corresponds to relatively high nitrate conditions where the NarGHI reductase, whose expression is activated by NarL, plays a key role (63). In this regard, it is interesting to note that $SdsN_{137}$ levels vary in response to some nitrogen sources (12). Even though RprA and $SdsN_{137}$ production may not peak under the same conditions, the two sRNAs can be co-expressed, for example in stationary phase (12,57,75), which could contribute to stronger reduction of *narP* expression under such conditions.

Interestingly, *narL* can also be targeted by sRNAs as its mRNA levels were shown to be decreased upon overexpression of DicF, an sRNA that accumulates under micro-aerobic conditions (76), and that was identified here as a possible regulator of *narP* as well (Figure 2A). Furthermore, NarL also promotes synthesis of the NarS sRNA, which is processed from the NarL-activated *narK* mRNA, encoding a nitrate/nitrite antiporter (38). In turn, NarS represses the expression of the gene encoding the nitrite transporter NirC. Although this NarS-control does not affect the expression of the other genes of the *nirBDC-cysG* operon, the synthesis of the NirB subunit of the NADH-dependent nitrite reductase is also subject to sRNA control, in this case by the RyhB sRNA (77). The link between sRNAs and nitrogen metabolism is further illustrated by the fact that $SdsN_{137}$ represses the synthesis of the NfsA nitroreductase and the HmpA nitric oxide dioxygenase (12). Additionally, several global approaches looking at ProQ or Hfq targets, or at RNA–

RNA interactions mediated by these chaperones, indicate that yet other genes related to nitrate/nitrite metabolism are likely to be controlled by sRNAs in enterobacteria (40,42,43).

Lastly, connections between sRNAs and nitrogen are not restricted to enterobacteria and many other examples have been reported in extremely diverse bacteria as well (see 78 for a review). They include the sRNAs related to denitrification in *Pseudomonas aeruginosa* or *Paracoccus denitrificans* (79,80), the RoxS/RsaE sRNA that responds to nitric oxide in firmicutes (81) and the sRNAs involved in the control of carbon-nitrogen balance in cyanobacteria (82).

New connections between Hfq and two-component systems

This study adds RprA to the list of sRNAs that include genes for TCSs in their regulons. Previous work has shown that altering the levels of transcriptional regulators with sRNAs may not always lead to a change in their activity, especially in the case of TCS regulators that must be activated by phosphorylation. In particular, the EnvZ-OmpR TCS was found to be robust, i.e. the level of the phosphorylated form of OmpR is insensitive to large changes in total EnvZ or OmpR levels (52). Consistent with this, repressing *ompR* expression with OmrA and OmrB sRNAs decreased only the amount of the non-phosphorylated form of OmpR. This allows these sRNAs to indirectly limit their own synthesis as their transcription responds to both the phosphorylated and the non-phosphorylated forms of OmpR (17). Although different, the outcomes of controlling PhoP synthesis with MicA or GcvB sRNA were also surprising: of these two sRNAs that repressed *phoP* via competition with ribosome binding, only MicA decreased the expression of positive PhoP-targets (18).

In the case of *narP*, our results show that repression by RprA or $SdsN_{137}$ sRNAs decreased the level of both the phosphorylated and the non-phosphorylated form of this RR and, consistently, repressed transcription from the NarP-dependent promoter P_{napF}. This sRNA effect on the phosphorylated form of the RR differs from what has been observed for *ompR* and indicates that robustness is not true for all TCS. However, for most of the previously reported cases, genes for RRs whose expression is repressed by sRNAs are in an operon with their cognate sensor kinase genes, leading to the prediction that expression of the kinase would also be repressed by the sRNA. This is different for *narP* since this is one of the few *E. coli* RR genes that is not part of an operon. Because RprA does not appear to control *narQ* (our preliminary data), its control of *narP* should thus change the RR/sensor kinase ratio. It would be interesting to determine whether this explains the observed difference in the levels of phosphorylated forms of NarP and OmpR in response to repression by sRNAs, which are decreased or unaffected, respectively (this study and (17)).

Another conclusion of this study is that, in addition to allowing sRNAs function, Hfq could also play a direct role in controlling TCSs expression. More direct experiments are required to definitively show that the effect of the Hfq Y25D mutant on *narP* (Figure 7), or of the mutation of the (AAN) motifs in *narP* TIR (Supplementary Figure S3), is due to a defect in Hfq binding to *narP* mRNA, and that sRNAs are not involved. Nevertheless, these results already indicate that *narP* expression could vary in response to signals that affect Hfq synthesis, stability and/or activity. While a similar direct Hfq effect on *phoP* seems unlikely at this stage, these data also raise the question of whether other TCSs can be controlled by Hfq independently of sRNAs, as previously shown for *mutS* (7).

In general, it will be important to determine how these post-transcriptional control mechanisms involving Hfq and/or sRNAs affect the various TCS signaling properties and, especially in the case of NarQ-NarP, the crosstalk with other systems.

Two independent mechanisms participate in the control of *narP* by RprA

This study is also interesting from a mechanistic standpoint, with the finding that an sRNA can repress a single target via two mechanisms, involving different regions of both the sRNA and the mRNA. The first, canonical, control relies on the pairing of RprA to the *narP* TIR, which presumably blocks translation initiation. The second control only requires the first 50 nts of *narP* mRNA, i.e. a region that is distant from the TIR since *narP* 5′UTR is 150 nt-long. These two pathways are most likely independent as the 5′end region of *narP* mRNA is sufficient to observe regulation by RprA in the absence of the TIR (Figure 5) and, similarly, RprA efficiently represses expression of the P_{BAD}-*narP-lacZ* fusion used in (12) that carries a 78-nt 5′ UTR and is thus devoid of the 5′ end of the *narP* 5′ UTR used here (our unpublished results).

The second control can be performed with similar efficiencies by either the full-length or the processed form of RprA carrying only the last 47 nts of the sRNA. Because we could not predict a convincing base-pairing between the 5′end of *narP* messenger (*narP*(+1+50) region) and RprAproc, this second control is likely not due to a direct sRNA-mRNA interaction. Instead, one can envision that RprA, or RprAproc, would control the level or the activity of a factor that would act on the *narP*(+1+50) region. A first possibility is that this factor mediating the observed control of RprA on *narP*(+1+50) is another sRNA. Such a scenario would be similar to other regulatory circuits involving sponge RNAs (here, RprA) that can titrate and/or destabilize an sRNA and thereby prevent its action (83,84). We have already tested a possible role of a few candidates sRNAs in the *narP* control by RprA. These candidates were sRNAs whose levels vary according to aerobic/anaerobic conditions (FnrS, ArcZ or NarS), sRNAs predicted to interact with RprA based on RIL-seq data (sRNA from the *ariR-ymgC* intergenic region), or sRNAs whose expression could be related to that of *narP* (narP 3′UTR). However, our results so far do not support the involvement of any of these sRNAs in this control (our unpublished data). Although possible factor(s) intervening in the effect of RprA on *narP*(+1+50) remain to be precisely identified, the dual-control mechanism analyzed here is most likely different from the reported examples of RprA

pairing to two distinct sites on a single target, such as *csgD* (27) or *hdeD* (29).

Another question raised by our results is related to the step at which this second control takes place. Given that the promoter and the TIR of *narP* are dispensable for this regulation, it is very unlikely to occur at the level of initiation of *narP* transcription or translation. However, an 8-aa upstream ORF is predicted in the first 50 nts of *narP* and its translation could be the target of this second control. Alternatively, the action on *narP*(+1+50) could primarily affect the stability of *narP* mRNA, as previously reported for other bacterial sRNAs, possibly by targeting the very 5′ end of mRNAs (85–87). The effect could also be transcriptional, for instance if RprA indirectly induced a premature termination of *narP* transcription.

There are several examples of feed-forward regulatory motifs where sRNAs regulate the expression of a target both directly and indirectly; the indirect effect is often mediated by a transcriptional regulator acting on the target promoter. One such example is the activation of the synthesis of the conjugation-inhibiting protein RicI by RprA that relies both on the pairing of RprA to *ricI* mRNA and on RprA promoting translation of the RpoS sigma factor that activates *ricI* transcription (28). It is likely that the dual control of *narP* by RprA reported here will also result in a feed-forward regulatory circuit, although with different details. Identifying the missing clues of this circuit, and in particular the nature of the indirect factor(s) involved and the way it acts, will be crucial to assess how this dual control can precisely impact *narP* expression.

Regardless of the precise mechanism of this second control, it is clear that the processed version of the sRNA is sufficient to promote it, even though this does not necessarily tell us whether the full-length or the processed form of RprA is preferentially responsible for this regulation when they are both present in the cell. RprA is not the only sRNA that exists under different isoforms, either because of processing, leading for instance to short forms of ArcZ, SdsR, MicL, RbsZ just to name a few (16,35,43,88,89), or of multiple TSS, which explains the different SdsN species (12). In the future, it will be interesting to decipher whether full-length and short forms of other sRNAs can also complement each other in the regulation of some targets, as reported here for RprA and *narP*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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