Translational control and target recognition by *Escherichia coli* small RNAs *in vivo*

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ABSTRACT

Small non-coding RNAs (sRNAs) are an emerging class of regulators of bacterial gene expression. Most of the regulatory *Escherichia coli* sRNAs known to date modulate translation of trans-encoded target mRNAs. We studied the specificity of sRNA target interactions using gene fusions to green fluorescent protein (GFP) as a novel reporter of translational control by bacterial sRNAs *in vivo*. Target sequences were selected from both monocistronic and polycistronic mRNAs. Upon expression of the cognate sRNA (*DsrA, GcvB, MicA, MicC, MicF, RprA, RyhB, SgrS and Spot42*), we observed highly specific translation repression/activation of target fusions under various growth conditions. Target regulation was also tested in mutants that lacked Hfq or RNase III, or which expressed a truncated RNase E (*rne*). We found that translational regulation by these sRNAs was largely independent of full-length RNase E, e.g. despite the fact that *ompA* fusion mRNA decay could no longer be promoted by MicA. This is the first study in which multiple well-defined *E.coli* sRNA target pairs have been studied in a uniform manner *in vivo*. We expect our GFP fusion approach to be applicable to sRNA targets of other bacteria, and also demonstrate that *Vibrio* RyhB sRNA represses a *Vibrio sodB* fusion when co-expressed in *E.coli*.

INTRODUCTION

Small non-coding RNAs (sRNAs) that act as regulators of gene expression are wide-spread in bacteria. Typically, these molecules are 50–200 nt in size, and do not contain expressed open reading frames (ORFs). Using a diverse array of approaches [reviewed in (1)], >70 *Escherichia coli* sRNAs have been identified in numerous screens [e.g. (2–7)] over the past five years, while hundreds of additional sRNA candidate genes still await experimental validation (8).

Two main modes of action have been established for the *E.coli* sRNAs. Some sRNAs modify the activity of proteins (9–11), while the majority act on *trans*-encoded target mRNAs to modulate their translation and/or stability. Several key features of antisense regulation by chromosomal sRNAs have emerged: (i) Unlike the *cis*-encoded antisense RNAs of plasmids and phages [reviewed in (12)], these *trans*-encoded antisense RNAs typically have only short and imperfect complementarity to their target(s). (ii) Base pairing most often occurs in the 5′-untranslated region (5′-UTR) of the target mRNA, and is aided by the bacterial Sm-like protein, Hfq. (iii) Binding may result in either the blockage of ribosome entry (translational repression), or the melting of inhibitory secondary structures, which sequester the ribosome binding site (RBS) of the mRNA (translational activation). (iv) Regulation is frequently coupled to nuclease-mediated cleavage of the mRNA, e.g. RNase E cleavage of *sodB* mRNA upon RyhB binding (13), and RNase III cleavage of *tisAB* mRNA upon IstR-1 binding (14).

Several *E.coli* sRNA target interactions have been well-defined. For example, the porin-regulating sRNAs, MicC and MicF, form an extended though imperfect RNA duplex with the 5′-UTRs of the *ompC* and *ompF* mRNAs, respectively (15,16), whereas MicA forms an almost perfect 16 bp duplex encompassing the RBS region of *ompA* mRNA (17,18). Shorter interactions underlie the repression of the *ptsG* message by SgrS (19), and of the *sodB* message by RyhB (20); in the latter case, 9 nt of either RNA are involved in duplex formation (21). Repression of the *fhlA* mRNA by OxyS is mediated by two short kissing complexes of 9 and 7 bp, respectively; the two target regions in OxyS and in *fhlA* mRNA are each separated by long spacers (22). DsrA was proposed to repress *hns* mRNA by binding it at both the start and the stop codon region; in other words, a bipartite interaction that would involve regions within the *hns* mRNA that are ~400 nt apart (23).

Some sRNAs are known to activate translation of mRNAs. The DsrA and RprA sRNAs pair with the *rpoS* mRNA leader, thereby preventing the formation of an inhibitory structure around the *rpoS* RBS that would otherwise repress *rpoS* translation (24–26). There are two other examples of *E.coli* sRNAs that function as mRNA activators, i.e. GadY and RydC (27,28).

In the early days of *E.coli* sRNA identification, these molecules were frequently recognized through their effect on a certain mRNA. For example, the *micF* gene was found...
within a multi-copy library insert that caused OmpF depletion, while the MicF::ompF mRNA interaction was shown in subsequent analysis (15,29). That is a (main) target was known before the regulator itself was identified. In contrast, the sheer numbers of new sRNAs recently identified in systematic genome-wide searches (1), which are \textit{a priori} of unknown function, require tools to efficiently predict and study interactions with target mRNAs. Besides, since sRNAs may also regulate multiple targets [\textit{e.g.} (26,30,31)], knowing a single target may not fully reflect the regulatory potential of a given sRNA.

Traditionally, genome-wide screens of randomly inserted reporter gene fusions as well as global protein pattern changes upon deletion or overexpression of an sRNA have played a major role in target identification [\textit{e.g.} (17,18,32)]. However, these approaches strictly require the target gene to be expressed at a measurable level under the assay condition, with the additional caveat that they provide little means to distinguish primary target effects from secondary pleiotropic changes upon deletion or overexpression of an sRNA have been the most common tool to study target gene regulation by sRNAs. However, since the fusion is typically driven by the target gene promoter, a specific effect on translation rather than on transcription has to be confirmed in independent experiments. Generally, \textit{lacZ} fusions represent a robust and well-established reporter system, however, come with the disadvantages of an enzymatic assay involving cell lysis to measure $\beta$-galactosidase activity.

Using the green fluorescent protein (GFP) from the jelly-fish \textit{Aequorea victoria} (37), which permits a non-invasive reporter assay, we have here studied a great number of \textit{E.coli} sRNAs and their targets in a uniform reporter system. This study has revealed novel aspects of regulation for several of these pairs. Furthermore, our GFP-based reporters will be helpful to rapidly validate bacterial sRNA targets of other prokaryotes.

**MATERIALS AND METHODS**

**DNA oligonucleotides**

The complete list of oligonucleotides used for cloning and as probes in hybridization is provided as Supplementary Table S2.

**Bacterial strains, media and growth conditions**

\textit{E.coli} strain Top10 (Invitrogen) was used to clone GFP fusions, and in all experiments that involved co-expression of GFP fusions and sRNAs. \textit{E.coli} strain Top10 F$^\prime$ (Invitrogen) was used to clone sRNA expression plasmids. All established mutant strains are derived from \textit{E.coli} Top10. Strains JVS-2001 (\textit{Δhfq::Km$^R$}) and JVS-2002 (\textit{rne701::Km$^R$}) were constructed by the one-step inactivation protocol (38) with PCR products obtained with primer pairs JVO-0515/-0516 or JVO-0856/-0857, respectively, using a modified Km$^R$ cassette of plasmid pKD4 as template (J. Vogel, unpublished data). Strain JVS-2003 (\textit{Δrnc14::Tet$^R$}) was constructed similarly, using primer pair JVO-0884/-0885 and chromosomal DNA of strain W3310 rnc14::Tn10 (39). Verification of the mutant strains was carried out by colony PCR using primer pairs JVO-0517/-0518 (for JVS-2001), JVO-0858/-0859 (for JVS-2002) and JVO-0886/-0887 (for JVS-2003). C-terminal truncation of RNase E in JVS-2002 was also verified by western blot using an RNase E antiserum (kindly provided by A. G. Carposis). Details of the aforementioned bacterial strains are given in Supplementary Table S1.

Growth in Luria–Bertani (LB) broth or on LB plates at 37$^\circ$C was used throughout this study. Antibiotics were applied at the following concentrations: 100 $\mu$g/ml ampicillin, 50 $\mu$g/ml kanamycin and 20 $\mu$g/ml chloramphenicol.

**Plasmids**

\textit{Fusion plasmids:} To construct plasmid pXG-0 (control plasmid to determine cellular autofluorescence), the p15A replicon of pZA31-luc was removed by SacI/AvrII restriction digest and replaced with a SacI/AvrII fragment containing the low-copy pSC101$^*$ replicon of pZS$^*$24-MCS1.

Plasmid pXG-10, the standard plasmid for \textit{gfp} fusion cloning was constructed as follows. A DNA fragment containing the pSC101$^*$ origin of replication, chloramphenicol resistance cassette and the \textit{P_{LtetO}} promoter was amplified from pXG-0 by PCR using primer pair JVO-0154/-0156, which adds BfrBI and NheI restriction sites right downstream of the promoter. The PCR product was digested with XbaI/NheI, and ligated to a \textit{gfp} encoding fragment amplified from plasmid pWH601 (40) with primer pair JVO-0517/0518, which introduces a point mutation at the promoter (changing the C codon precedes the NheI site, and thus results in expression of full-length GFP).

To construct plasmid pXG-1, the \textit{P_{LtetO}} promoter and RBS region of plasmid pZA31-luc was amplified with primers pZE-CAT/JVO-0330. Upon AatII/NheI digest, the fragment was inserted into plasmid pXG-10 digested with the same enzymes. Consequently, in plasmid pXG-1 an ATG start codon precedes the NheI site, and thus results in expression of full-length GFP.

To construct plasmid pXG-20, which is used for 5$'$-RACE product cloning, the \textit{P_{LtetO}} promoter from pZA31-luc was amplified with primers pZE-Cat/JVO-0339. The latter oligo introduces a point mutation at the promoter (changing the C at $\text{-1}$ to A) and adds a BfrBI restriction site to position +2. The fragment was digested with AatII/BfrBI and inserted into plasmid pXG-10 digested with the same enzymes. BfrBI/NheI cloning of a PCR product obtained on chromosomal \textit{E.coli} MC4100 DNA with primers JVO-0368/-0369 resulted in an insert that contains an internal fragment of the \textit{lacZ} coding region (651–976 amino acid) and a BsgI site; digest with BsgI will result in cleavage at the +1 site of \textit{P_{LtetO}}.
Plasmid pXG-30, the plasmid for operonic gfp fusion cloning, was constructed as follows. First, an E.coli DNA fragment spanning codons 2–59 of the lacZ gene was amplified with primers JVO-0642/-0685; oligo JVO-0685 added a FLAG epitope preceded by an ATG start codon to the N-terminus of the LacZ fragment. Upon KpnI/NheI digest, the fragment was inserted into plasmid pXG-1 digested with the same restriction enzymes, resulting in plasmid pJU-083. An internal fragment of the E.coli galETKM locus, from the last 58 codons of the galT C-terminal region to the 47th codon of galK, was PCR-amplified from E.coli MC4100 with primer pair JVO-0490/-0491, and inserted into pJU-083 by BfrBI/NheI cloning, which gave plasmid pJU-088. E.coli Top10 transformed with pJU-088 showed high fluorescence levels of GalK::GFP, but only low signals for the LacZ::GalT fusion protein. Therefore, a DNA fragment containing the RBS of plasmid pZA31-luc was PCR-amplified with primers JVO-1102/-1103, and fused in a subsequent PCR step to a DNA fragment containing a 3× FLAG epitope proceeded by an ATG start codon obtained by PCR on plasmid pSUB11 (41) with primers JVO-1100/-1101. The resulting DNA fragment was directly ligated to a PCR product obtained on pJU-088 with primers pZE-tet0/JVO-1104 to yield plasmid pXG-30.

For cloning of gfp fusions in pXG-10, chromosomal DNA fragments were amplified by PCR with a sense oligonucleotide which anneals to the transcriptional start-site (for many E.coli K12 genes annotated at http://ecocyc.org/). The gene of interest and a BfrBI restriction site and an antisense oligonucleotide which anneals in the N-terminal coding region of the gene and adds an in frame NheI restriction site. The corresponding primers for each gene are listed in Supplementary Table S2. Typically, the full-length 5′-UTR (from +1 of the most proximal promoter of a gene) and 30–150 bp (10–50 amino acid residues) of the N-terminal coding region were cloned. Inclusion of extracellular signal sequences (where known) were generally avoided to prevent traffic of the fusion proteins to the periplasm/membrane.

Intra-operonic fusions established in pXG-30 were cloned as above but the sense oligonucleotide annealed to the C-terminal coding region of the upstream gene and adds the BfrBI restriction site in frame.

sRNA plasmids: All sRNA plasmids constructed here are based on plasmid pZE12-luc. First, a DNA fragment of pZE12-luc was amplified by PCR using pjo-polymerase (Fermentas) and primers PLlacoB and PLlacoD, and subsequently digested with XbaI. This digest results in two DNA fragments of ~2.2 kb and ~1.7 kb, respectively. The ~2.2 kb fragment carries the PLlaco promoter (from the position –1), an ampicillin resistance cassette, a CoE1 replicon and a strong rrnB terminator followed by the sticky end created by XbaI digestion. After gel-purification, it served as the vector backbone for sRNA cloning. The E.coli micC sRNA gene was PCR-amplified using primers JVO-0486/-0489. The sense primer (JVO-0486) anneals to the +1 site of micC and carries a 5′ monophosphate for cloning. The antisense primer (JVO-0489) binds to the region downstream of the micC terminator and will add an XbaI site to the PCR product. Following XbaI digest, the product was ligated to the 2.2 kb XbaI fragment of pZE12-luc, to yield plasmid pSK-017 upon transformation. Plasmid pSK-019 expressing DicF sRNA was constructed similarly using primers JVO-0487/-0488. To construct sRNA plasmids pJVgcvB-6 (gcvB), pJV100IA-T4 (rprA) and pJV107-8 (ryhB), the sRNA genes were amplified with primer pairs gcvB6/gcvB7, jb-100-L/jb-100-1A, and jb-107-G/jb-107-H, respectively. Different to the micC cloning described above, however, these fragments were cloned by inserting them at the Km' site (pJV107-8, pJVgcvB-6), or the EcoRI site (pJV101A-T4) of pZE12-luc.

To lower the copy number, the ColE1 origin of pJV107-8 was swapped for the p15A origin of pZA31-luc by SpeI/AvrII cloning, resulting in plasmid pJU-002. Similarly, the ColE1 origin of pJVgcvB-6 was swapped for the p15A origin of pJU-002 by PacI/SpeI cloning, yielding plasmid pJU-014. To construct control plasmid pTP-011, the ColE1 origin of pJV300 was replaced by the p15A origin of pZA31-luc by SpeI/AvrII cloning.

5′ RACE and direct cloning of full-length gfp fusions

5′ RACE was carried out as described previously (4) but with modifications, the major being a new 5′ RNA adapter (A4: 5′-GACGAGCAGCGAGCAUGACAUUGGAGGAGGAGUGAAA-3′OH), which contains a BseRI recognition site (underlined) to facilitate cleavage of the obtained cDNA at the 5′ end of the ligated RNA. 5′ triphosphates were converted to 5′ monophosphates by treatment of μg total RNA (obtained on strain E.coli MC4100 grown to an OD600 of 2) with 10 μg of tobacco acid pyrophosphatase (TAP, Epicentre Technologies) at 37°C for 30 min. Control RNA was incubated in the absence of the TAP. Reactions were stopped by phenol chloroform extraction, followed by ethanol/sodium acetate precipitation. Pellets were dissolved in water, mixed with 300 pmol of 5′ RNA adapter A4, heat-denatured at 95°C for 5 min, followed by a 5 min quick-chill step on ice. The adapter was ligated at 17°C for 12 h with 40 U T4-RNA ligase (New England Biolabs) in the recommended buffer and 10% dimethyl sulfoxide (DMSO). Phenol chloroform-extracted, ethanol-precipitated RNA (2 μg) was then reverse-transcribed using 100 pmol random DNA hexamers and the SuperScriptIII RT system (Invitrogen) in a total volume of 20 μl. Reverse transcription was performed in four subsequent 15 min steps at 42°C, 50°C, 55°C and 60°C. The RT enzyme was inactivated at 85°C for 5 min, followed by RNase H (New England Biolabs, 1 U)-treatment for 20 min at 37°C.

For direct cloning of full-length gfp fusions, 1 μl cDNA served as template in a standard PCR using tag polymerase (New England Biolabs), and 25 pmol each of a gene-specific primer (antisense to the N-terminal coding region of the gene of interest and with a NheI site extension) and the adapter-specific primer JVO-0367. Products required a second PCR amplification step using the same primer combinations to increase DNA yields for cloning.
In vivo whole-cell colony plate fluorescence imaging

*E. coli* Top10 cells expressing plasmid-borne *gfp* fusions were streaked on standard LB plates supplemented with the appropriate antibiotics. After over night growth colonies were photographed in a FUJI LAS-3000 image analyzer using a CCD camera with a 510 nm emission filter and excitation at 460 nm.

**Liquid culture whole-cell fluorescence measurements and data processing**

To measure whole-cell fluorescence in liquid culture, *E. coli* strains harboring *gfp* fusion plasmids were inoculated 1/100 from overnight cultures into 20 ml fresh LB medium in erlenmeyer flasks. Three independent overnight cultures were used throughout the study for each strain. Cultures were incubated with aeration at 37°C through the study for each strain. Cultures were inoculated in 150 μl of each culture were transferred to a 96-well microtiter plate (Nunc, cat# 167008), and fluorescence measured at 37°C (optical excitation filter 480/31 nm, emission filter 520/10 nm, 0.2 s, CW lamp energy 21673, measurement height 8.0 mm) in a Victor® machine (1420 Multilable Counter, Perkin Elmer).

To calculate absolute fluorescence of a given strain, the mean fluorescence of the three aliquots from each of the three independently grown cultures was determined. Unless stated otherwise, cellular autofluorescence was subtracted to obtain the specific fluorescence of the *gfp* fusion. Herein, the fluorescence of strains harboring the same sRNA expression or control plasmid in combination with the negative control plasmid pXG-0 (expressing luciferase, i.e. no *gfp*) was measured as described above and subtracted from absolute fluorescence values obtained in presence of the *gfp* fusion plasmid of interest.

The regulatory effect of a sRNA on a given *gfp* fusion was calculated as follows. Strains harboring the fusion of interest in combination with a specific negative control plasmid (i.e. without sRNA expression), were measured to obtain absolute fluorescence values and autofluorescence of strains harboring the same negative control plasmid in combination with pXG-0 was subtracted resulting in the ‘unregulated *gfp* fusion specific fluorescence’. Strains harboring the same *gfp* fusion of interest in combination with a specific sRNA expression plasmid were measured and the autofluorescence of strains harboring the same sRNA expression plasmid in combination with pXG-0 was subtracted to give the ‘regulated *gfp* fusion specific fluorescence’. Fold regulation mediated by expression of a sRNA was calculated by dividing the ‘unregulated *gfp* fusion specific fluorescence’ by the ‘regulated *gfp* fusion specific fluorescence’.

**Fluorescence measurements in microtiter plates and data processing**

Fluorescence measurements in 96-well microtiter plates was carried out as described in (42) with modifications. Single colonies (triplicate) of *E. coli* strains harboring *gfp* fusion and sRNA expression plasmids were inoculated in 150 μl LB in a 96-well microtiter plate and these cultures were overlaid with 50 μl mineral oil (Sigma) to prevent evaporation. Cultures were grown in a Victor® fluorimeter set at 37°C and assayed with an automatically repeating protocol of shaking (2 mm orbital, normal speed, 900 s), absorbance (OD) measurements (600 nm, P600 filter, 0.1 s) and fluorescence readings (optical excitation filter 480/31 nm, emission filter 520/10 nm, 0.2 s, CW lamp energy 21673). OD*600* and fluorescence were measured at 17 min intervals (60 in total).

To plot fluorescence over OD*600*, curves of all three cultures within a triplicate were independently established first. The linear range of increasing fluorescence during growth covered by all members within a triplicate was selected individually and a cut-off set at the OD*600* were at least one member showed non-linear increase of fluorescence. An average curve was calculated for each triplicate and the cellular autofluorescence curve of a strain harboring pXG-0 and pJV300 negative control plasmids subtracted. The OD*600* range in which all measured cultures showed near-linear fluorescence increase is shown in Figure 9A.

To calculate the regulatory effect of sRNA expression on the *ompC* fusion (Figure 9B), fluorescence of *E. coli* strains harboring the *ompC* fusion in combination with a sRNA expression plasmid was divided by the fluorescence of a strain harboring the *ompC* fusion in combination with the sRNA control plasmid, pJV300.

**Whole-cell protein fractions and western blot**

Culture samples were taken according to 1 OD*600* if not stated otherwise. Samples were spun 2 min at 16 100 g at 4°C. The cell pellet was resuspended in 1x sample loading buffer (Fermentas, #R0981) to a final concentration of 0.01 OD/μl. Samples were heated 5 min at 95°C.

A total of 0.01 or 0.05 OD of whole-cell protein fractions of strains expressing highly or weakly fluorescent GFP fusions, respectively, were separated by 15% SDS–PAGE. Gels were blotted for 60 min at 100 V at 4°C in a cable tank blotter (Pehlab) onto PVDF (PerkinElmer) membrane in transfer buffer (25 mM Tris base, 190 mM Glycin and 20% Methanol). After rinsing in TBST20 buffer (20 mM Tris base, 150 mM NaCl and 0.1% Tween 20), membranes were blocked for 1 h in 10% dry milk in TBTS*20*, followed by incubation with α-GFP monoclonal (Roche #11814460001) or α-FLAG monoclonal antibodies (Sigma #F1804; 1:1000 in 3% BSA, TBST*20*) for 1 h at RT, 5 × 6 min wash in TBST*20*, α-mouse-horseradish peroxidase (HRP) (Amersham Biosciences #NXA931; 1:5000 in 3% BSA in TBST*20*) for 1 h at RT, 6 × 10 min wash in TBST*20*. For simultaneous detection of GroEL (loading control), membranes were cut after the blocking step at the 47.5 kDa band indicated by the prestained protein marker (Fermentas), GroEL was detected using α-GroEL antiserum conjugated with HRP (Sigma #A8705; 1:1000 in 3% BSA, TBST*20*; 2 h incubation at RT, followed by 6×10 min washes in TBST*20*). Blots were developed using Western Lightning reagent (PerkinElmer), and signals detected with a Fuji LAS-3000 CCD camera.

**RNA isolation and northern detection**

TRIZOL reagent (Invitrogen) or the Promega SV total RNA purification kit were used according to the manufacturer’s protocol or as described at www.ifr.ac.uk/safety/microarrays/protocols.html, respectively, to isolate total RNA.
Unless stated otherwise, RNA was isolated from cells grown to an OD₆₀₀ of 1.

To detect gfp fusion mRNAs or sRNAs, RNA samples (corresponding to 0.7 OD culture volume) were denatured for 5 min at 95°C in loading buffer (containing 95% formamide), separated on 8.3 M urea – 5 or 6% polyacrylamide gels, and transferred to Hybond-XL membranes (GE Healthcare) by electro-blotting (1 h, 50 V, 4°C) in a tank blotter (Peqlab).

For detection of the chromosomal sdhCDAB polycistronic mRNA 20 µg total RNA was separated on a 1.5% Agarose gel containing 2.2 M formaldehyde and transferred to a Hybond-XL membrane by upward capillary transfer in 10× SSC overnight as described (43).

DsRr, GcvB, MicA, MicC, MicF, RyhB, SgrS and Spot42 were detected using 5' end-labeled oligodeoxynucleotides JVO-1367, JVO-0321, JVO-1371, JVO-1369, JVO-0909, JVO-0223, JVO-1366 and JVO-1368, respectively. 5S rRNA and gfp fusion mRNAs were detected with end-labeled oligodeoxynucleotide JVO-0322 and JVO-155, respectively. The sdhCD fusion mRNA was detected with a random-labeled ([³²P] dCTP; Readiprime II labeling kit, GE Healthcare) PCR fragment generated with primer pair JVO-0642/-1101. To detect the chromosomally expressed sdhCDAB polycistronic mRNA, a PCR fragment generated with primer pair JVO-1360/1361 was in vitro-transcribed from the T7 promoter (added by primer JVO-1361) in the presence of [α-³²P]UTP using Ambion’s T7 polymerase Maxiscript kit. Riboprobes were purified over a G50 column.

Prehybridization and hybridization of membranes with riboprobes, DNA probes, or oligonucleotides was carried out in Roti-Hybi-Quick buffer (Roth, #A981.1) at 70°C, 65°C, or 42°C, respectively, for 2 h. Membranes hybridized with riboprobes were washed at 65°C in three subsequent 15 min steps in SSC (2×, 1× and 0.5×)/0.1% SDS solutions, after rinsing the membrane first in 2× SSC/0.1% SDS. Membranes hybridized with PCR fragments were rinsed in 2× SSC/0.1% SDS, followed by 15 min washes in 2× (65°C), 1× and 0.5× (42°C) SSC/0.1% SDS. For end-labeled oligodeoxynucleotides hybridization membranes were rinsed in 5× SSC followed by three wash steps at 42°C in SSC (5×, 1× and 0.5×, respectively). Signals were visualized on a phosphorimager (FLA-3000 Series, Fuji), and band intensities quantified with AIDA software (Raytest, Germany).

RESULTS

General approach

To study sRNA-mediated translational control at the 5' region of a given target mRNA (from here on: target), we use two compatible plasmids derived from the pZ2 series of expression vectors (44) that can be stably maintained in an E.coli recA- strain (Figure 1A). The target plasmid is a low-copy vector that carries a pSC101 origin of replication (3–4 plasmid copies/cell), a cat chloramphenicol resistance marker, and the 5' sequence of the target as a translational fusion to the N-terminus of GFP. Transcription of the gfp fusion gene is driven by P_LtetO-1, a constitutive promoter that is derived from the native phase λ, P_L promoter (44). The sRNA plasmid is a high-copy vector carrying a CoIE1 origin of replication (~70 copies/cell) and a bla ampicillin resistance gene. The sRNA gene of interest is cloned under control of the constitutive P_LtetO-1 promoter [another modified version of λ P_L (44)] such that transcription will precisely start at the native +1 site of the sRNA.

The constant transcription rate of both the regulatory sRNA and the target fusion is a key feature of this system. It uncouples both players from the chromosomal transcriptional network, and diminishes the possible pleiotropic effects of sRNA expression on target fusion transcription. It also ensures high yields of the expressed RNAs, thus minimizing the contribution of any transcripts from the chromosomal copies of the respective sRNA or target genes. In case the high transcription rate of either promoter yields toxic RNA levels, it may be controlled in E.coli strains that encode the LacI or TetR repressor proteins [repressing P_LtetO-1 or P_LtetO-1, respectively; (44)] by addition of an appropriate inducer. Alternatively, sRNA genes are cloned on a plasmid carrying a p15A origin of replication, thus lowering the copy number to ~20 per cell (44).

E.coli cells carrying a target fusion plasmid of interest are transformed with plasmids expressing either the cognate regulatory sRNA or a nonsense (control) RNA. The GFP fluorescence of the resulting transformants is subsequently read out from colonies on LB agar plates or from cells grown in liquid culture, and corrected for the auto-fluorescence of E.coli. Fusions that exhibit higher GFP activity in the presence of a sRNA plasmid are considered activated, whereas lower GFP fluorescence indicates target repression. In cases where GFP activity is low, i.e. close to auto-fluorescence, western blotting with an anti-GFP antibody provides a more sensitive measure for quantification of fusion protein levels.

Cloning and activity of translational gfp fusions

All gfp fusions described carry the gfp+ allele, which encodes a GFP variant that combines mutations for higher fluorescence yield and increased folding efficiency (45). Plasmid pXG-10 is the standard plasmid for directional cloning of a potential target mRNA sequence as N-terminal translational fusion to GFP (Figure 1B). Selected target regions are PCR-amplified using a sense primer that binds to the +1 site (if known) of the target gene and adds a BfrBI restriction site to this sequence, and an antisense primer that binds in the 5' coding region and adds a NheI restriction site in frame with the target gene. Plasmid pXG-10 has a single BfrBI site at the +1 position of the P_LtetO-1 promoter hence all fusion transcripts will have a uniform AUGCAU 5' end. The single NheI site (GCTAGC) of pXG-10 represents the 2nd and 3rd codon of the gfp reading frame, hence the translational target gene fusion will be to full-length GFP protein. Control plasmid pXG-0 does not contain a gfp gene but instead constitutively expresses luciferase, and is used to determine the auto-fluorescence background of E.coli cells. Control plasmid pXG-1 expresses full-length GFP and carries an artificial 5'-UTR containing a strong RBS that is derived from the pZ2 family of expression vectors (44). Two additional fusion vectors, pXG-20 and pXG-30, were constructed for cloning of 5'-RACE products and of intra-operonic target sequences, respectively (Figure 1B). The cloning strategies for these plasmids are described along with their applications further below.
Figure 1. Principle approach and gfp fusion cloning strategies. (A) Putative sRNA target sequences are cloned as translational fusions to gfp on a low-copy vector that carries a pSC101* origin of replication (3–4 plasmid copies/cell) and confers chloramphenicol resistance. The fusion is transcribed from a constitutive PLtetO-1 promoter (PL derivative). Regulatory sRNAs are cloned on a high-copy vector that carries a ColE1 origin of replication (70 copies/cell) and confers ampicillin resistance. The sRNA gene is cloned under control of the constitutive P LlacO-1 promoter (another derivative of PL) such that transcription will precisely start at the native +1 site of the sRNA. For E.coli cells that carry both plasmids, the effect of a given sRNA on a target fusion can be determined by monitoring GFP fluorescence of colonies grown on agar plates, of liquid cultures grown in standard laboratory flasks or in microtiter plates, or by flow cytometry.

Combinations of fusion and sRNA expression plasmids with control plasmids are used to determine (i) the basal fluorescence of E.coli cells and how it is affected by sRNA overexpression, (ii) the general effect of the plasmid-borne sRNA gene on GFP expression and (iii) the specific effect of an sRNA on a target fusion of interest. (B) Putative target sequences are PCR-amplified and cloned into specialized gfp fusion vectors. If the target sequence is derived from a monocistronic gene or the first gene of an operon, and its promoter is known (left panel), it is amplified with an upstream primer that binds at the +1 site of the target gene and adds a BfrBI site, and a downstream primer that binds in the N-terminal region of the target gene and adds an NheI site in frame with the target gene coding region. The resulting PCR product is inserted into the standard fusion vector, pXG-10, digested with BfrBI/NheI. If the promoter +1 site is unknown (middle panel), the target sequence is amplified from cDNA of total E.coli RNA that was ligated to a 5' RNA linker oligo upon treatment with TAP (this enzyme converts the 5' PPP group of primary transcripts to 5' P and thus allows the differential amplification of cDNAs that correspond to the native +1 site of an mRNA). The amplified cDNA will carry a 5' BseRI site (contained in the RNA linker sequence). Insertion of the NheI/BseRI-digested cDNA into NheI/BsgI-digested RACE fusion plasmid, pXG-20, ensures that transcription of the fusion mRNA starts at the native +1 site of the target gene. Target sequences that are derived from within polycistronic mRNAs are amplified and cloned into the operon fusion vector pXG-30 (right panel). The upstream primer adds a BfrBI site in frame with the C-terminus of the upstream ORF; cloning into pXG-30 will create a C-terminal fusion to a short artificial reading frame composed of a FLAG epitope and a truncated lacZ gene, thus mimicking operon miRNA expression. See text and Figure 6A for more details.
Using these vectors, we have thus far constructed >80 translational gfp fusions to diverse genes of E. coli, Salmonella typhimurium and Vibrio cholerae, which are listed in Table 1 and in Supplementary Table S3. These fusions include several known targets of E. coli sRNAs, which were the focus of this study, as well as various mRNAs that were predicted as sRNA targets in our laboratory (C. M. Sharma and J. Vogel, unpublished data). For simplicity, the fusions listed throughout this paper refer to E. coli genes unless stated otherwise.

A preliminary determination of fluorescence on standard LB agar plates by visual inspection revealed large variations of GFP activity among these reporter strains. Figure 2A shows images of five representative reporter strains that were used for a rough classification of GFP activity (Table 1). Fusions that show fluorescence similar to full-length GFP (control plasmid pXG-1), e.g. ompC, were classified ++. Interestingly, a sodB fusion exhibited a higher fluorescence than pXG-1, and was thus classified ++++. Fusions with intermediate yet readily detectable fluorescence, e.g. oppA were marked ++, whereas fusions, such as ptsG with levels just above the autofluorescence of pGX-0 cells were classified +. Altogether, >80% of the 68 E. coli fusions listed in Table 1 and Supplementary Table S3 had detectable fluorescence on agar plates, and all but the sodB fusion (smaller colonies) formed colonies of regular size.

We next determined the GFP activity of a broader set of fusions in liquid culture (Figure 2B). Overnight cultures were diluted into fresh LB media, and overall culture fluorescence was determined at five growth stages, i.e. at a cell density of OD_600 of 0.1, 0.3, 0.5, 1 and 2. We observed an almost linear correlation of cell number and culture fluorescence, as well as small standard deviations within triplicates, with fusions that had shown high GFP activity on plates (Figure 2B, right panel). Several fusions surpassed the full-length GFP expressed from pXG-1 in terms of fluorescence, i.e. ftsZ, hns, ompC, ompF, and sodB. In contrast, many fusions with low GFP activity required growth to an OD_600 > 0.5 for reliable detection (Figure 2B, left panel). Interestingly, some of the target genes that yielded low GFP activity had previously been fused to lacZ, e.g. fhlA or rpoS, and similarly small (<200) Miller unit numbers had been reported (22,25,26). However, we need to caution against a general comparison to previously published lacZ fusion results since these fusions greatly vary in their way of construction (chromosomal versus plasmid-borne fusions), the growth stage at which β-galactosidase activity was determined, as well as enzyme activity units.

We next sought to determine a correlation among reporter fluorescence, steady-state fusion mRNA levels and fusion protein accumulation. Northern blots of RNA samples taken at two growth stages were probed for the gfp portion of the fusion mRNAs, and likewise fusion protein levels were determined on western blots with a mixture of two monoclonal antibodies that recognize GFP. A cross-comparison of GFP fluorescence (Figure 2B) with the corresponding mRNA

### Table 1. Overview of relevant gfp fusion plasmids

| Target gene | Plasmid trivial name | Plasmid original name | Insert 5’ end | Fused codon | Fusion vector | Fluorescence on plate | Western blot detection | Comment |
|-------------|----------------------|-----------------------|----------------|-------------|--------------|-----------------------|------------------------|---------|
| E.coli      |                      |                       |                |             |              |                       |                        |         |
| dppA        | pDppA::gfp           | pSK-015               | −165           | 14          | pXG-10       | +                     | +                      | Predicted GevB target |
| galK        | pGalIK::gfp          | pUJ-147               | −180           | 47          | pXG-30       | +++                   | +                      | Repressed with Sp042  |
| hns         | pHns::gfp            | pSK-009               | −36            | 28          | pXG-10       | +++                   | +                      | Repressed with Sp042  |
| lacZ        | pLacZ22::gfp         | pJV-861-9             | −37            | 29          | pXG-10       | +                     | +                      | Repressed with DsrA   |
| ompC        | pOmpC::gfp           | pSK-003               | −81            | 12          | pXG-10       | +++                   | +                      | Repressed with MicC   |
| ompF        | pOmpF::gfp           | pSK-005               | −50            | 13          | pXG-10       | +++                   | +                      | Repressed with MicF   |
| ompA        | pOmpA::gfp           | pSK-008               | −133           | 16          | pXG-10       | +                     | +                      | Repressed with MicA   |
| rpoS        | pRpoS::gfp           | pSK-031               | −564           | 41          | pXG-10       | +                     | +                      | Activated by DsrA, RprA |
| sdhD        | pSdhD::gfp           | pSK-042               | −60            | 21          | pXG-10       | −                     | −                      | Repressed by RyhB     |
| sodB        | pSodBD::gfp          | pJV-162               | −59            | 21          | pXG-30       | ++                    | +                      | Repressed by RyhB     |
| Vibrio      |                       |                       |                |             |              |                       |                        |         |
| sodB        | pV.c.SodB::gfp       | pJU-066               | −82            | 69          | pXG-10       | +++                   | +                      | Repressed by RyhB     |

*Gene whose N-terminal coding sequence was fused to GFP. Gene names refer to the following genome annotations. E. coli K12 (NC_000913), V.cholerae O1 biovar eltor (NC_002505). Known sRNA targets are set in boldface.

*Fusion plasmid name used throughout the manuscript.

*Original plasmid name used for construction and storage (to be cited when requesting plasmids).

*5’ End of the target gene insert relative to annotated ATG.

*Target gene codon that is fused to the NheI site preceding the reading frame in the cloning vectors.

*Vector type used for cloning.

*Fluorescence on LB agar plates of E. coli strains carrying a fusion plasmid as shown in Figure 2A. (−) denotes background fluorescence, (+) weak but detectable fluorescence, (+++) intermediate fluorescence, (++++) fluorescence similar to full-length GFP (control plasmid pXG-1), and (++++) stronger fluorescence than that of a pXG-1 strain.

*Western blot detection of the fusion protein in cells grown to OD_600 of 1. (−) = no detection, (+) = protein detected.
and protein levels (Figure 2C) indicates a good correlation in five cases, i.e. the sodB, ptsG, rpoS and lacZ29 fusions, and wild-type GFP. For example, both the mRNA and fusion protein levels of the bright sodB fusion far exceed those of wild-type GFP. In contrast, the ptsG and rpoS fusions, both being in the lower fluorescence range, are hardly detectable at the mRNA and protein level. However, the case of the two different lacZ::gfp fusions included here merits further
description. Fusion lacZ186 differs from lacZ29 by the additional inclusion of residues 30–186 of LacZ (Table 1). Even though there were drastic differences between the mRNA levels and processing patterns of the two fusions, comparable amounts of fusion protein were detected (Figure 2C). Fluorescence still differed by a factor of 3 (Figure 2B), indicating that the larger LacZ portion of lacZ186 may affect proper folding or solubility of the GFP fusion protein. It may thus be advisable to keep the fused target sequence as short as possible, thereby also avoiding the inclusion of intact signal peptides of extracytoplasmic target proteins.



Repression of target fusions by sRNAs

We cloned several regulatory sRNAs previously reported by us and others (see Table 2) into a ColE1-based vector that is compatible with the aforementioned gfp fusion plasmids. Our strategy ensures transcription from the plasmid-borne constitutive P_{lacO} Promoter to start precisely at the native +1 site of the sRNA (see Materials and Methods). Plasmid pJV300, which expresses a ~50 nt nonsense RNA derived from the rrrB terminator region (46), is the standard control vector for these P_{lacO}-driven sRNA expression plasmids.

The RyhB and the GcvB plasmids gave aberrantly small colonies after transformation of E.coli; we thus lowered their copy number by replacing the ColE1 origin with p15A. We also note that on three sRNA expression plasmids (DsrA, RyhB and Spot42) were observed to affect bacterial growth, either causing a longer lag phase or earlier entry into stationary phase (data not shown), this does not seem to influence GFP expression.

Subsequently, we combined 10 sRNA plasmids with eight target fusions. We expected to see repression with the sRNA/target pairs, DsrA/RyhB and Spot42 positively changed fluorescence up to 1.5-fold. This unspecific effect will have to be taken into account when calculating the regulation of target mRNA fusions. Although some of these plasmids (DsrA, RyhB and Spot42) were observed to affect bacterial growth, either causing a longer lag phase or earlier entry into stationary phase (data not shown), this does not seem to influence GFP expression.

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Figure 3. Specific regulation of target fusions by co-expression of cognate sRNAs. (A) E.coli strains carrying the full-length GFP control plasmid pXG-1 (upper and lower left panel) or target gene fusions (as indicated above the individual panels) were co-transformed with the sRNA expression plasmids given below each panel. Fluorescence of liquid cultures was determined at an OD600 of 1. Fold regulation was calculated from the relative fluorescence by dividing the fluorescence obtained in the presence of the sRNA plasmid by that obtained with a control plasmid that does not express a regulatory RNA. Gray and black bars indicate positive regulation (target activation) and negative regulation (target repression), respectively. The cognate regulatory sRNA for each fusion is boxed. The ompC fusion was repressed >20-fold in the presence of the MicC expression plasmid. (B) Specific regulation of the low-activity ptsG fusion by SgrS as determined by western blot analysis. Total cell protein samples of the same strains as in (A), samples were taken at an OD600 of 1. The PtsG::GFP fusion protein was detected with α-GFP antibodies (upper panel). Detection of GroEL protein with a specific antibody was used to confirm equal loading (lower panel). (C) Fluorescence of strains that carry the ompC, ompF, hns or sodB fusion in combination with either a control plasmid (−), or MicC, MicF, DsrA or RyhB expression plasmids, respectively (denoted by +). Strains were grown on agar plates and images were taken in the visible light or fluorescence mode as in Figure 2A. (D) As in (C) but showing dppA, ptsG, ompA or galK fusion regulation by GcvB, SgrS, MicA or Spot42 co-expression, respectively.
Figure 4. Regulation of various ompA fusions by MicA. (A) Structure of the E.coli ompA leader adapted from (17). Nucleotides that pair with MicA are indicated by black boxes. The 5′ ends of the two truncated ompA fusion, ompA-30 and ompA-95, are circled. (B) Introduction of compensatory base pair changes into MicA sRNA, yielding MicA_M6, restored regulation of the mutated ompAM6 fusion mRNA, thus confirming specific pairing of the two RNAs in vivo. The M6 mutation in ompA or MicA simultaneously disrupts base pairing between ompA and MicA at six positions, as described previously (17). E.coli strains carrying the ompA wild-type or ompAM6 mutant fusion plasmid were combined with a control plasmid (no sRNA expression), or the MicA or MicAM6 expression plasmids. Samples were taken at an OD 600 of 1, and subjected to western (upper panel) and northern (lower two panels) blot analysis as in Figure 2C. (C) Effects of 5′ truncations on ompA* fusion mRNA stability and regulation by MicA. In the ompA* wild-type fusion (constructed by 5′ RACE cloning, see text), transcription from the constitutive P0 promoter starts at the native ompA +1 site. Destruction of the terminal stem–loop of the ompA leader neither affects stability or translation of the mutant fusion ompA-95, nor its repression by MicA. However, further shortening of the ompA leader as in mutant ompA-30, which is deprived of both stem–loops, results in partial degradation of the fusion mRNA and loss of fusion protein translation. Samples were taken and probed as in (B).

from the constitutive P LtetO promoter starts at the native regulation factors difficult. To measure ptsG fusions were introduced in ompA::gfp the MicA/sRNA and its mRNA target. The so-called M6 mutation in the introduction of compensatory base pairs in the regulatory ptsG strength of the SgrS/sRNA, shows that SgrS reduced the PtsG::GFP signal to background clear-cut regulation in liquid culture measurements. ((Figure 4. Regulation of various ompA fusions by MicA. (A) Structure of the E.coli ompA leader adapted from (17). Nucleotides that pair with MicA are indicated by black boxes. The 5′ ends of the two truncated ompA fusion, ompA-30 and ompA-95, are circled. (B) Introduction of compensatory base pair changes into MicA sRNA, yielding MicA_M6, restored regulation of the mutated ompAM6 fusion mRNA, thus confirming specific pairing of the two RNAs in vivo. The M6 mutation in ompA or MicA simultaneously disrupts base pairing between ompA and MicA at six positions, as described previously (17). E.coli strains carrying the ompA wild-type or ompAM6 mutant fusion plasmid were combined with a control plasmid (no sRNA expression), or the MicA or MicAM6 expression plasmids. Samples were taken at an OD 600 of 1, and subjected to western (upper panel) and northern (lower two panels) blot analysis as in Figure 2C. (C) Effects of 5′ truncations on ompA* fusion mRNA stability and regulation by MicA. In the ompA* wild-type fusion (constructed by 5′ RACE cloning, see text), transcription from the constitutive P0 promoter starts at the native ompA +1 site. Destruction of the terminal stem–loop of the ompA leader neither affects stability or translation of the mutant fusion ompA-95, nor its repression by MicA. However, further shortening of the ompA leader as in mutant ompA-30, which is deprived of both stem–loops, results in partial degradation of the fusion mRNA and loss of fusion protein translation. Samples were taken and probed as in (B).)

(~38 000 to ~40 000) and renders reliable calculations of regulation factors difficult. To measure ptsG regulation more precisely, we determined PtsG::GFP protein expression (by western blot) in the presence of all sRNAs. Figure 3B shows that SgrS reduced the PtsG::GFP signal to background levels, whereas all other tested sRNA plasmids had negligible effects. This confirms the high specificity and regulatory strength of the SgrS/ptsG interaction, while it also indicates the requirement for a minimal fusion activity to observe clear-cut regulation in liquid culture measurements.

Ultimate proof for in vivo interaction is typically obtained by the introduction of compensatory base pairs in the regulatory sRNA and its mRNA target. The so-called M6 mutation in the MicAlompA pair refers to simultaneous disruption of 6 bp in this interaction site, either obtained by mutation of MicA or ompA. Previously, introduction of six compensatory mutations in ompA to restore base pairing with the MicA_M6 mutant RNA (and vice versa) successfully restored regulation of MicA/ompA as measured by the activity of plasmid-borne ompA::lacZ fusions (17). Since this provided a means for direct comparison between a lacZ and a gfp reporter, the same mutations were introduced in ompA::gfp. Similar to the data reported by (17), wild-type ompA::gfp was hardly regulated by MicA_M6 at the fusion mRNA or protein level, whereas ompAM6::gfp was regulated by MicA_M6 but hardly responded to wild-type MicA (Figure 4B). This finding proves that gfp is as reliable a reporter of ompA regulation as lacZ.

The length of the fused target mRNA sequence could be another determinant of sRNA regulation. Ideally, the cloned region would encompass the entire 5′-UTR and include a short stretch of the coding region. We reasoned that fusion cloning should be based on the native 5′-UTR to ensure a comparable stability of the fusion transcript to the parental mRNA. However, the +1 site or promoter is only known for a subset of the E.coli genes, and even less information on transcription start sites is available for other bacteria. To solve this problem, we developed vector pXG-20 as part of a cloning strategy that combines +1 site mapping and rapid fusion cloning (Figure 1B). Briefly, this includes a 5′ RACE protocol that distinguishes primary 5′ mRNA ends (carrying a triphosphate) group from processed mRNA species (2,4,53,54), followed by the direct insertion of a target mRNA 5′ RACE fragment into vector pXG-20 such that transcription from P0 will precisely start at the mapped +1 site. The full protocol and an example of 5′ RACE fusion cloning are provided in the Supplementary Data. Following this approach we were able to directly clone an ompA::gfp fusion using E.coli total cDNA as PCR template. The obtained plasmid, pOmpA*::gfp, contains the same full-length fusion as ompA::gfp, but without the 5′-ATGCAT extension added in the standard cloning procedure. To investigate how varying the length of a target 5′-UTR would affect regulation, two shorter derivatives of the ompA*::gfp fusion were constructed. The 133 nt ompA 5′-untranslated leader (Figure 4A), as contained in ompA*::gfp, is well-characterized in terms of both its 5′ end structure (55) and the MicA interaction site (17,18). Moreover, the first 115 nt containing two stem–loop structures were shown to act as a stabilizer of ompA mRNA in vivo (56,57). In ompA-95, the first stem–loop was destroyed, thus creating a 5′ end that should be single-stranded. In ompA-30, transcription was expected to start only 30 nt upstream of the ompA start codon; this mutant retained the MicA target site but not the ompA Hfq binding site (58). Figure 4C shows that destruction of the stabilization stem–loop I (ompA-95) had no effect on fusion mRNA or protein abundance, and that repression by MicA was unaffected. However, ompA-30 yielded a much less abundant and partially degraded fusion mRNA, and no detectable fusion protein, which rendered it difficult to determine regulation by MicA.

Activation of an rpoS fusion by sRNAs

While repression of target translation is the predominant mode of sRNA action, the rpoS mRNA provides an excellent example to study activation by sRNAs [reviewed in (59)].
We tested activation of an \textit{rpoS} fusion with a set of sRNA plasmids, expecting higher fluorescence exclusively with DsrA and RprA, two sRNAs that act to melt the inhibitory structure that sequesters the \textit{rpoS} RBS. Although \textit{rpoS}::\textit{gfp} fluorescence is in the lower activity range (Table 1), its activation in the presence of DsrA or RprA plasmids is already visible on agar plates (Figure 5A). Such activation was also observed in liquid culture (Figure 5B), and generally the two sRNAs elevated fluorescence stronger than any other sRNA or the control plasmid. However, in these measurements the poor fluorescence of the \textit{rpoS} fusion rendered calculation of activation factors difficult. Thus, the regulation factor in this case is given as the ratio of \textit{rpoS} fusion fluorescence to \textit{E.coli} autofluorescence, each obtained in the presence of the same control or sRNA expression plasmid. As seen before with other low fluorescence fusions (e.g. \textit{ptsG}; Figure 3A and B), direct detection of the fusion protein on western blots provided a much clearer picture of regulation (Figure 5C).

\textbf{Intra-operonic sRNA target sites}

The target genes investigated so far were either monocistronic or first in an operon. Some sRNAs, however, target...
UTRs of downstream reading frames within a polycistronic mRNA. In the case of the polycistronic galETKM mRNA, Spot42 binds to the galK RBS region, which leads to translational repression of galK without affecting expression of the two upstream genes, galE and galT; consequently, this type of regulation was termed discoordinate operon expression (50). RyhB, which is predicted to block the sdhD RBS, may regulate the sdhCDAB operon mRNA in a similar fashion (20). Such intronic targets could pose a challenge for our approach since transcription of the fusion mRNA would not start at its native +1 site, thus creating an arbitrary 5’ end that could destabilize the fusion mRNA. To solve this problem, we developed vector pXG-30, in which intronic target genes are expressed as part of an artificial dicistronic mRNA (Figure 1B). Putative target genes are cloned on pXG-30 as dual fusions: the upstream coding sequence is fused to the C-terminus of a FLAG epitope-tagged, truncated lacZ gene (FlacZ), whereas the actual target gene is fused to gfp as described above. In addition, the FlacZ ORF is preceded by a strong RBS derived from protein expression vector pZE12-luc (44) to ensure efficient translation initiation of the dicistronic operon mRNA. Next we cloned the sdhCD and galTK target sequences of RyhB and Spot42, respectively, in plasmids pXG10 and pXG30, and compared the GFP activity of these fusions (Figure 6A). Following the GFP activity over growth, we observed striking differences for the two vector types in the case of either fusion (Figure 6B). Specifically, when the sdhCD target site is cloned on standard vector pXG-10, the fluorescence of this fusion is close to background levels. However, if the same sequence is cloned into operon plasmid pXG30, it yields a fusion with well-detectable GFP activity. Moreover, activity of the galTK fusion was also enhanced 2-fold by cloning into pXG30 as compared to pXG10. In the pXG30-based galTK and sdhCD constructs, the upstream and downstream fusion proteins can be specifically detected with anti-FLAG and anti-GFP antibodies, respectively (Figure 6C). According to the concept of discoordinate gal operon expression (50), we expected a reduction of GalK::GFP levels upon Spot42 co-expression but no change of FlacZ::GalT levels. Quantification of the western blot signals shown in Figure 6C revealed an 8-fold decrease of GalK::GFP in the presence of the Spot42 plasmid, but also a 10-fold reduction of FlacZ::GalT (Figure 6C, compare...
lanes 5 and 6). While the first was in keeping with the previously published model, the strong reduction of the GalT fusion protein seemed to contradict it. However, Spot42 has been consistently observed to have a ~3-fold negative effect on the FlacZ moiety in other non-target constructs, e.g. FlacZ::SdhC (Figure 6C, lanes 1 and 2, and data not shown). Since no other sRNA tested by us has shown a similar effect, we believe that this is a currently unexplained peculiarity of Spot42. If corrected for this target-independent FlacZ-dependent effect, Spot42 regulates the galT part only 3.3-fold as opposed to 8-fold galK regulation. Notably, this is in very good agreement with (50), who reported 1.4-fold and 4.9-fold regulation of galT and galK expression, respectively.

Whereas this data confirmed discordant gal operon expression by Spot42, we were unable to mimic RyhB-mediated regulation of the sdhCDAB operon. As shown in Figure 6C, RyhB co-expression did not regulate the sdhCD fusion whereas it had a drastic effect on the sodB fusion (Figures 3A and 8B). However, in contrast to the sdhCD fusion, we did see a RyhB effect on the native sdhCDAB operon mRNA (Figure 6D). As outlined in the discussion section, this result does not call into question sdhCDAB as a RyhB target, or the use of pXG30 to study intra-operonic sRNA targets.

**Regulation is independent of major RNA processing factors**

Bacterial RNA metabolism involves a large number of ribonucleases and other RNA-binding proteins, three of which—Hfq, RNase E and RNase III—are known to play prominent roles for the activity of *trans*-encoded antisense RNAs. In principle, our GFP system is well-suited to test the contribution of such factors by determining sRNA/target regulation in the respective *hfq* and *RNase E* deletion strains. For RNase E is encoded by an essential gene (*rne*), we resorted to a viable *rne* deletion mutant. This mutant expresses a C-terminally truncated RNase E that is defective both in interaction with Hfq and in assembly of a functional degradosome, and was recently shown to impair RyhB and SgrS action on their targets mRNAs (13,60). The *rne* deletion reduced the steady-state levels of most sRNAs, which would also contribute to the observed loss of regulation. However, the data also indicates that Hfq contributes to regulation independent of sRNA stabilization. For example, the *hfq* deletion reduced RyhB levels to ~30%, yet *sodB* regulation to 1.6-fold as compared to ~12-fold in wild-type cells. In addition, *DsrA* levels in the *Δhfq* strain were indistinguishable from the wild-type background; in the absence of Hfq, DsrA could still promote *rpoS* activation whereas it entirely failed to repress *hns* translation. Interestingly, this is in keeping with a previous observation that multi-copy *DsrA* could partially bypass the *hfq* requirement for *rpoS* but not for *Hfq* regulation (61).

In stark contrast, the *hfq* deletion abrogated regulation of almost all sRNA/target pairs (Table 3). Since numerous sRNAs were previously observed to be unstable in the absence of Hfq, we compared the amounts of overexpressed sRNAs between wild-type and *Δhfq* cells. As shown in Table 3 and Figure 7B, the *Δhfq* deletion reduced the steady-state levels of most sRNAs, which would also contribute to the observed loss of regulation. However, the data also indicates that Hfq contributes to regulation independent of sRNA stabilization. For example, the *hfq* deletion reduced RyhB levels to ~30%, yet *sodB* regulation to 1.6-fold as compared to ~12-fold in wild-type cells. In addition, *DsrA* levels in the *Δhfq* strain were indistinguishable from the wild-type background; in the absence of Hfq, *DsrA* could still promote *rpoS* activation whereas it entirely failed to repress *hns* translation. Interestingly, this is in keeping with a previous observation that multi-copy *DsrA* could partially bypass the *Hfq* requirement for *rpoS* but not for *Hfq* regulation (61).

**Table 3. Regulation in the absence of full-length RNase E or Hfq**

| sRNA, fusion | Wild-type | *rne*701 | *Δhfq* | sRNA levels in *Δhfq* versus wild-type |
|--------------|-----------|---------|--------|----------------------------------------|
| *DsrrA, has* | -7.1      | -4.5    | -1.2   | 100                                    |
| *DsrrA, rpoS* | +7.0      | +4.6    | +3.8   | 100                                    |
| *GcvB, dppA* | -3.2      | -4.9    | -1.6   | 19                                     |
| *MicA, ompA* | -6.0      | -2.4    | -1.5   | 63                                     |
| *MicC, ompC* | >20       | >20     | -1.2   | 9                                      |
| *MicF, ompF* | -14.5     | -8.1    | -1.3   | 10                                     |
| *RprA, rpoS* | +3.4      | +3.6    | +1.3   | 69                                     |
| *RyhB, sodB* | -11.7     | -11.2   | -1.6   | 30                                     |
| *SgrS, ptsG* | -2.6      | -5.4    | 1      | 36                                     |
| *Spot42, galK* | -2.4      | -2.6    | -1.4   | 71                                     |

*aCombination of sRNA expression and target gfp fusion plasmid.

*bFold-regulation observed in wild-type background.

*cFold-regulation observed in a strain that expresses a truncated RNase E.

*dFold-regulation observed in an *hfq* deletion strain.

*eSRNA signals in the *hfq* deletion strain in % of the signal obtained in the wild-type. Signals were quantified on northern blots (Figure 7B) in strains carrying the indicated sRNA expression plasmid co-transformed with pKG-1. RNA was prepared from cells grown to an OD600 of 1. Signals were normalized to 5S rRNA detected on the same blot.

*fRegulation was generally determined by measurement of fluorescence of liquid cultures grown to an OD600 of 1 but for the *rpoS* fusion, which was assayed on western blots.
Assaying heterologous sRNA–target interactions

Few other bacteria in which sRNAs have been identified offer as excellent genetic tools as *E.coli* to study regulation of putative sRNA targets *in vivo*. However, in some cases sRNAs of even distantly related species were shown to regulate their target upon co-expression in *E.coli*, e.g. lhtA RNA of *Chlamydia trachomatis* (62). To test if we could use *E.coli* as a host to assay heterologous sRNA/target pairs, we co-expressed *V.cholerae* RyhB along with a *sodB* fusion derived from this bacterium. RyhB/sodB regulation in *V.cholerae* was previously suggested by (63). Interestingly, *V.cholerae* RyhB (∼225 nt) is more than twice as long as *E.coli* RyhB (∼90 nt), and the two homologues show little similarity except for the *sodB* interaction site (Figure 8A). Similarly, the *sodB* 5'-UTR differs substantially between the two bacteria. Nonetheless, we found that *Vibrio* RyhB effectively represses translation of both the *Vibrio* and the *E.coli* *sodB* fusion (Figure 8B). Reciprocally, *E.coli* RyhB effectively represses both the *Vibrio* and the *E.coli* *sodB* fusion.

Growth in microtiter plates

The experiments thus far described were carried out under standard laboratory growth conditions, i.e. growth in culture flasks with aeration. To test if specific regulation could also be obtained in a set-up that is more suited for high-throughput screening, we grew the *ompA* fusion strain co-transformed with various sRNA plasmids in small culture volumes (150 μl) overlayed with mineral oil in microtiter plates. Following inoculation from single colonies, cell density and fluorescence were monitored in 15 min intervals over a course of 16 h (Figure 9A). Fluorescence increased almost linear after an initial phase for all strains but MicC/ompC; fluorescence of the latter remained almost constant throughout and only increased slightly towards the end of the assay. Specific *ompC* repression by MicC is observed early in growth, and increases to 7-fold at the end of the assay (Figure 9B). Taken together, although the degree of *ompC* regulation is about one third as compared to culture in laboratory flasks (Figure 3A), this microtiter plate-based assay provides the same specificity.

DISCUSSION

We have studied sRNA-mediated control of mRNA targets by using translational fusions to *gfp*, encoding a non-invasive reporter of bacterial gene expression (64,65). We have observed faithful regulation of target fusions with all sRNA/target pairs whose interactions had previously been dissected at the molecular level. Of these, several had been tested by fusions to other reporter genes. Our results show that the GFP fusions constructed here perform at least equally well in terms of sRNA regulation. For example, ∼8-fold activation of an *rpoS::lacZ* fusion was observed upon DsrA overexpression (25,26), whereas overexpressed MicA caused a ∼6-fold decrease in the activity of an *ompA::lacZ* reporter gene (17). The corresponding regulation of *rpoS::gfp* and *ompA::gfp* (Figures 3A and 5B) perfectly match these previous results. Interestingly, MicC repressed a translational
Figure 8. E.coli as a host to assay heterologous sRNA/target interactions. (A) Alignments of E.coli and V.cholerae RyhB RNA and sodB fusion mRNA (the cloned sodB DNA fragment is shown). Sequence information is based on (20,21,63). Note that the native +1 site of sodB mRNA in V.cholerae is unknown (B. Davis and M. K. Waldor, personal communication). The nucleotides of the RyhB/sodB pairing regions, as experimentally determined for E.coli (21), are boxed. Note that a different interaction has been proposed for the Vibrio RyhB/sodB pairing regions, as experimentally determined for E.coli (21), are boxed. The sodB coding region is set in boldface. (B) Western blot detection of GFP and SodB::GFP fusion proteins from E.coli strains that expressed GFP (control plasmid pXG-1), a V.cholerae sodB fusion, or the E.coli sodB fusion, each in combination with the control plasmid pJV300 (C0), the V.cholerae RyhB (V) or the E.coli RyhB (E) expression plasmid. Samples were taken at an OD 600 of 1.

Figure 9. Microtiter plate-based assay of MicC-mediated ompC fusion repression. (A) E.coli strains carrying the ompC fusion in combination with one of the 10 sRNA expression plasmids or the pIV300 control plasmid (specified by the color code) were inoculated in 150 µL LB medium in a 96-well microtiter plate, overlaid with mineral oil and grown with agitation at 37°C in a Victor3 plate reader for 16 h. Cell density and fluorescence were determined in 15 min intervals. Plotting of fluorescence values over growth (OD 600*) shows specific repression of the ompC fusion by the MicC expression plasmid. Cell density is given as OD600* values of 600 nm light absorption in the microtiter plate well (0.2 OD600* units corresponds to 1 OD600 standard units). Fluorescence values were corrected for the autofluorescence of an E.coli strain carrying control plasmids pXG-0 and pJV300. (B) Factor of ompC fusion regulation by sRNAs (relative to the control plasmid pIV300, calculated as in Figure 3A) at five selected growth stages (OD600* of 0.1, 0.15, 0.2, 0.25 and 0.3). Black bars indicate ompC repression, whereas gray bars ompC activation.
ompC fusion to luciferase ~2.5-fold (16), whereas >20-fold repression of ompC::gfp was observed here (Figure 3A). While other inhibitory sRNA/target interactions, i.e. RyhB/sodB, DsrA/hns, MicF/ompF, Spot42/salK and SgrS/pstS, were previously validated by *in vitro* complex formation, the fusions used here independently confirm that sRNA-mediated mRNA repression occurs in the 5'-UTR.

Several cases merit further discussion. First, repression of *hns* mRNA by DsrA was proposed to involve two RNA duplexes with 13 nt at the *hns* RBS and with 11 nt upstream of the *hns* stop codon, in effect leading to a circularization of *hns* mRNA (23). Although our *hns::gfp* fusion included the 13 nt RBS target region only, it was still subject to ~7-fold regulation by DsrA (Figure 3A). Thus, our system may report regulation even if only partial target sequences are included in a fusion. Second, a chromosomal *gcvB* deletion was previously shown to elevate expression of a dppA::lacZ fusion but it remained unclear if GcvB acted on the cloned dppA mRNA fragment or regulated dppA transcription (51). Our results obtained with a dppA::gfp fusion strongly suggest that GcvB post-transcriptionally regulates dppA by targeting its mRNA in the 5'-UTR (Figure 3A). This has also been confirmed by biochemical analyses of the GcvB interaction site on the dppA mRNA (C. M. Sharma, F. Darfeuille and J. Vogel, manuscript in preparation). Similarly, a GcvB target site was predicted in the dppA RBS region in biocomputational analyses (30). Third, Wagner and Darfeuille (66) evaluated the free energy values (ΔG°) of several confirmed sRNA/target duplexes as well as near-cognate and non-cognate combinations, and found that ΔG° values are rarely good predictors of unknown target interactions. For example, some cognate combinations, such as RyhB/sodB and RprA/ropO80 were predicted to have ΔG° values of −17 and −24 kcal/mol, respectively, but the values for near-cognate combinations such as MicG/ompF and MicF/ompC were similarly low (−19.3 and −20.9 kcal/mol, respectively). Although the latter may indicate cross-regulation of ompF and ompC by MicC and MicF, respectively, no such regulation is seen with our GFP fusions (Figure 3A). This is of particular interest since many sRNAs target RBS regions which by default have lower sequence complexity than other mRNA parts (because of interaction with 16S rRNA). However, our data indicate that this lower complexity does not seem to compromise specificity.

By developing a specialized vector to clone fusions to intra-operonic target sites, we were able to mimic discordinate expression of the *galETKM* operon as mediated by Spot42. In addition, the inclusion of an upstream fusion to the artificial *FlacZ* gene appeared to greatly enhance fusion mRNA translation or stability, resulting in a detectable activity of its *sdhD* fusion. Using this vector, we have meanwhile identified more polycistronic mRNAs that are subject to discordinate regulation by *E. coli* sRNAs (J. H. Urban and J. Vogel, unpublished data). Although we failed to detect RyhB regulation of an *sdhD* fusion (Figure 6C) this does not call the *sdhCDAB* operon as a RyhB target into question. Parallel probing of the chromosomal *sdhCDAB* mRNA confirmed downregulation of this target mRNA by RyhB (Figure 6D) as previously shown by (20). Moreover, other results from our lab obtained for *Salmonella* RyhB regulation strongly support *sdhD* as a RyhB target (unpublished data).

Hence, the lack of *sdhCD* fusion repression hints at additional determinants of this regulation. For example, RyhB targeting may require additional residues or an Hfq binding site of the *sdhCDAB* mRNA that are located outside of the cloned 122 bp fragment, or an unknown protein factor that associates with the native *sdhCDAB* transcript but not with the *sdhCD* fusion mRNA.

While sRNA-target complexes have been extensively studied *in vitro*, less is known about the factors that contribute to regulation *in vivo*. Most of the sRNAs studied here require the bacterial RNA chaperone, Hfq, for target interaction *in vitro*. However, since many sRNAs also fail to accumulate in Δhfq mutant strains because of largely reduced stability, the contribution of Hfq to sRNA-target annealing *in vivo* is hard to assess. In contrast, sRNA overexpression as shown here (Figure 7 and Table 3) may provide a better means to evaluate an involvement of Hfq in sRNA function *in vivo*. For example, we found DsrA/hns regulation to be abrogated in Δhfq cells although plasmid-borne *dsrA* and the *hns* fusion were expressed normally. Thus this system could be used to study possible defects of Hfq-dependent ribonucleoprotein complex (RNP) formation or ribosome association of the two RNAs.

RNase E-based RNPs, either containing other degradosome components or Hfq, were recently implicated in translational repression and decay of the *ptsG* and *sodB* mRNAs *in vivo* (13,60). We have studied the regulation of *ptsG*, *sodB* and other target gene fusions in an *rne701* mutant strain that cannot assemble either of these RNPs because of the C-terminal RNase E truncation (Figure 7A and Supplementary Figure S2). Surprisingly, fusion regulation was found to be almost as effective as in the wild-type background. Although overexpression likely results in a stoichiometry that is different from that of chromosomally expressed sRNA and targets, we still expected to see some effect of the *rne* mutation. Nonetheless, our results were in better keeping with a more recent report showing that sRNA-mediated repression of the *ptsG* and *sodB* mRNAs in *rne701* cells does occur at the level of translation inspite of the defect in mRNA degradation (67). However, there are important differences between the experiments described by Morita et al. (67) and our experimental set-up. In the former case, the *ptsG* and *sodB* mRNA decay defects in *rne701* cells were most apparent upon short-term induction of the regulatory sRNA genes, which may be closer to studying the natural kinetics of sRNA-mediated gene silencing in the absence of native RNase E (67). In contrast, the experiments described here report on steady-state levels of target (fusion) mRNAs and proteins. Similar to (67) we find that upon extended SgrS expression, the *ptsG* target mRNA is effectively degraded in the *rne701* cells, too, and we observe the same effect on fusion mRNA decay for four other target mRNAs, i.e. *hns*, *sodB*, *ompC* and *ompF*. However, while the *ompF* fusion mRNA is fully degraded upon MicA overexpression in wild-type cells, it fails to get depleted when MicA is expressed in *rne701* cells. That this can be seen upon long-term sRNA expression makes the MicA-*ompF* pair an attractive model to study the contribution of RNase E to sRNA-mediated control of target mRNAs.

Although an RNase E-homologue is found in many bacteria (68), neither its RNA recognition sequences nor its ability to form RNPs are known to be conserved. Since
overexpressed sRNAs regulate their targets largely independent of RNase E-based RNP pairs, we expect that *E. coli* will be a suitable host for the validation of putative sRNA/target pairs from remotely related bacteria. Here we demonstrated the regulatory capacity of such a heterologous sRNA/target pair, i.e. *Vibrio* RyhB/sodB, while others recently used *E. coli* as a host to show translational control of a *C. trachomatis* sRNA/target pair (62).

The double-strand specific RNase III was shown to act on *E. coli* sRNAs (2,69), and to cleave IstR RNA and its target upon interaction (14). The ideal RNase III substrate is a >20 bp full duplex (equivalent to about two turns of A-form dsRNA). However, considerably shorter and/or imperfect duplexes were also shown to be substrates [e.g. (70,71)]. Since MicC and MicF form extended duplexes with their targets *ompC* and *ompF*, respectively, we chose to study the regulation of *ompC/F* fusions in an RNase III-deficient strain. No difference in *ompC/F* repression was found in this strain as compared to wild-type *E. coli*, suggesting that the formed duplexes are no substrates of RNase III, or that inhibition of translation initiation is sufficient for regulation.

Taken together, our translational GFP fusion approach offers a rapid and reliable tool to study sRNA control of targets that are derived from both monocistronic and poly-cistronic mRNAs. GFP was previously used by others as a reporter of translational control to engineer artificial ribo-regulators (72); the major difference to our approach being that RNA regulator and its target were expressed from the same plasmid. However, we believe that our two-plasmid system is better suited to meet the requirements of larger screens because of the ease with which already existing system is better suited to meet the requirements of larger screens because of the ease with which already existing sRNA and fusion plasmids can be combined. Similar two-plasmid systems with *lacZ* reporter genes were successfully used to study interactions of *cis*-encoded antisense RNAs with their targets [e.g. (73)].

The results presented here encourage further work to improve and refine our methodology. First, although fusions with low GFP activity can be easily assayed on Western blots, the use of *gfp* alleles with increased fusion fluorescence will facilitate screening approaches. While this work was in progress, a new GFP variant, superfolder GFP, with brighter fluorescence and higher tolerance of fusion partners was described (74). Preliminary results from our lab suggest that this variant enhances the activity of some of the fusions described here (unpublished data). Second, the vast majority of the sRNA targets sites known to date are located in mRNA 5′ regions. In contrast, *E. coli* GadY sRNA overlaps in antisense orientation with the 3′ end of its target mRNA (27).

Since GFP tolerates fusion to its carboxy terminus, it should be possible to adapt our approach to studying sRNA interactions with the 3′ end of target mRNAs. Third, many target mRNAs encode proteins that are exported to the periplasm or which integrate into membranes. We have thus far avoided inclusion of signal sequences to ensure cytoplasmic localization of the *gfp* fusions. Recent work from the Aiba lab, however, showed that membrane localization of the native *ptsG* mRNAs is required for its repression by SgrS (49). It will thus be interesting to determine how the inclusion of extracytoplasmic signal sequences affects regulation of a fusion by its cognate sRNA. Fourth, GFP as a reporter that does not require a chromogenic substrate allows studying gene regulation at the single-cell level. Several recent studies have shown considerable heterogeneity of transcriptional responses within bacterial populations [e.g. (75,76)]. Whether this also holds true for post-transcriptional processes could be determined using the reporter system described here. On this line, preliminary results with our *rpoS::gfp* fusion indicate that co-expression of DsrA or RprA results in bacterial populations that can be separated from control strains by high-speed flow cytometry (unpublished data).

Small non-coding RNAs have been discovered at a staggering rate in *E. coli* and many other eubacteria (1,77–83). Given the hundreds of sRNAs of unknown function, target identification has become a pressing issue but has been lagging behind, mainly due to an incomplete understanding of molecular rules for sRNA/target pairing. Although a first algorithm for target prediction has been implemented and even suggested additional targets for hitherto well-studied sRNAs (30), it has created rather than obviated the need for rapid and independent methods to validate the increasing numbers of predicted targets by independent methods. We believe that GFP-based reporters as the ones constructed here will be particularly useful when having to test larger numbers of predicted sRNA targets.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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