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# Functional Dissection of sRNA Translational Regulators by Nonhomologous Random Recombination and In Vivo Selection

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## Summary

**Small nontranslated RNAs (sRNAs) regulate a variety of biological processes. DsrA and OxyS are two *E. coli* sRNAs that regulate the translation of *rpoS*, which encodes a protein sigma factor. Due to their structural complexity, the functional dissection of sRNAs solely by designing and assaying mutants can be challenging. Here, we present a complementary approach to the study of functional RNAs, in which highly diversified RNA libraries are generated by nonhomologous random recombination (NRR) and processed efficiently by in vivo selections that link RNA activities to cell survival. When applied to DsrA and OxyS, this approach rapidly identified essential and nonessential regions of both sRNAs. Resulting hypotheses about DsrA and OxyS structure-function relationships were tested and further refined experimentally. Our findings demonstrate an efficient, unbiased approach to the functional dissection of nucleic acids.**

## Introduction

Small, nontranslated RNAs (sRNAs) that regulate biological functions are abundant in nature [1–15]. Of the more than 50 sRNAs identified in *E. coli*, however, only a small subset has been well characterized [5, 6, 13, 14]. The sequence and structural diversity of sRNAs together with the relatively small number of well-understood examples can make their study difficult, creating the need for additional methods to dissect sRNA structure and function.

The central regulator of the general stress response in *E. coli* is the protein sigma factor RpoS. During normal growth conditions, RpoS protein levels remain low until stationary phase even though *rpoS* mRNA levels remain constant and abundant [16]. The *rpoS* mRNA contains a 567 nt 5′ untranslated region (UTR) proposed to fold into a structure in which the translation initiation region is base-paired, repressing translation in *cis* [16] (Figures 1A and 1B). Expression of *rpoS* is dependent on the RNA binding protein Hfq and translation of the *rpoS* message is regulated by many different factors, including several sRNAs [16, 17].

DsrA and OxyS are two sRNAs that regulate *rpoS* translation. DsrA, an 87 nt sRNA induced at low temperatures, activates translation by interacting with the *rpoS* 5′UTR through a proposed anti-antisense mechanism [18–23] (Figure 1B). DsrA is thought to fold into three stem-loops (SL1, SL2, and SL3) with an AU-rich SL1-SL2 linker [21] (Figure 1C). Based on studies by Gottesman, Lease, Belfort, and their coworkers, it has been proposed that SL1 and the SL1-SL2 linker form a duplex with the *rpoS* mRNA, freeing the translation initiation region for binding by the ribosome [20–23]. SL3 has been proposed to be a rho-independent transcriptional terminator [18, 20].

OxyS, a 109 nt sRNA transcribed in response to oxidative stress, represses *rpoS* translation through an unknown mechanism [24, 25]. Although unrelated in sequence to DsrA, OxyS is also predicted to fold into three stem-loops [26] (Figure 1D). The linker between SL2 and SL3 has been shown to be important for OxyS activity [25], and SL3 of OxyS also appears to act as a transcriptional terminator [27]. Regulation of *rpoS* by DsrA and OxyS is dependent upon Hfq, which likely mediates initial sRNA-mRNA interactions [25, 28–32].

The existence of two sRNAs that target the same message but induce opposite outcomes highlights the complexity and functional potential of these systems. To further our understanding of sRNA translational regulators, we chose to probe the requirements for sRNA regulation of *rpoS* expression in a broad and unbiased manner. We reasoned that selecting libraries of highly diversified DsrA or OxyS variants for *rpoS* activation or repression would efficiently identify essential and nonessential regions of both sRNAs in a manner that is independent of current assumptions. Nonhomologous random recombination (NRR), a diversification method that effects the rapid deletion, repetition, and reordering of subsequences with no sequence requirements, has previously been used to evolve DNA aptamers and protein enzymes with new functional or structural properties [33, 34].

Here, we report the first use of NRR to functionally dissect a natural nucleic acid. We coupled NRR with selections in *E. coli* cells to isolate highly diversified yet functional sRNA activators or repressors of *rpoS* translation starting from *dsrA* or *oxyS*. This approach rapidly identified essential and nonessential regions of both sRNAs, including some regions not previously implicated or excluded in their function. In addition to augmenting our current understanding of sRNA-mediated translational regulation of *rpoS*, our findings suggest that the use of NRR coupled with in vivo selection or high-throughput screening may prove valuable to the study of other RNAs.

## Results and Discussion

### Development of an In Vivo Selection for *rpoS* Translational Activation

Our approach requires a method for rapidly evaluating *rpoS* translational regulation. We began by developing an in vivo selection for translational activation based on the expression of *cat* (chloramphenicol acetyltransferase), which confers resistance to the antibiotic chloramphenicol. *E. coli* cells entering this selection

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Figure 1. Translational Regulation of *rpoS*

(A) The *rpoS* mRNA sequence including the 150 nt of the 5′UTR and the region coding for the first 73 amino acid residues. The base numbering of *rpoS* follows that of Brown and Elliott [35]. The Shine-Dalgarno sequence is underlined and the start codon is labeled. Based on our results and on previous studies, nucleotides proposed to pair with DsrA, OxyS SL1 (sense), and OxyS SL1 (antisense) are highlighted in yellow, red, and blue respectively.

(B) Model of *rpoS* 5′UTR secondary structure and proposed anti-antisense mechanism for translational activation by DsrA [20–22]. The Shine-Dalgarno sequence is boldfaced, the *rpoS* start codon is underlined, and *rpoS* bases 113–115 are highlighted in orange.

(C) DsrA and (D) OxyS secondary structure as predicted by nuclease footprinting and the mFOLD program, respectively [21, 26].

carry two plasmids. Plasmid pProt-Cat expresses Cat from an inducible *tac* promoter as a C-terminal fusion to the first 73 amino acids of RpoS [35]. The *rpoS* start codon in pProt-Cat is preceded by 150 nt of the *rpoS* 5′UTR (Figure 1A).

The second plasmid, pRNA, expresses a library of sRNAs. The sRNAs expressed from the *lpp* promoter of pRNA contain an additional 18 nt at their 5′ end from cloning sites that are not complementary to *rpoS* and are terminated by an *rrnB* terminator (see Experimental Procedures). Although this cloning scheme inserts additional nucleotides to the 5′ and 3′ ends of the sRNAs, the results of control selections (see below) indicate that DsrA and OxyS exhibit their expected effects on *rpoS* translation when expressed from pRNA. Moreover, northern blot analysis indicates that expression levels of DsrA from pRNA are similar, if not somewhat lower than, those from previously reported expression systems [20] (Figure S1 in the Supplemental Data available with this article online.).

We designed the selection such that only in the presence of *rpoS* translational activators would sufficient RpoS-Cat fusion protein be expressed to confer resistance to a specific concentration of chloramphenicol. This selection was validated by introducing pRNA-expressing wild-type DsrA, wild-type OxyS, or no sRNA into *E. coli* cells harboring pProt-Cat. We observed optimal growth differences when cells were plated on 40 μg/ml chloramphenicol; these conditions allowed 1% of cells expressing DsrA to survive, whereas only 1 in $10^4$ cells lacking an sRNA insert and 1 in $5 \times 10^5$ cells expressing OxyS survived. These results demonstrate that our system links *rpoS* translational activation with cell survival.

We used a secondary LacZ screen to verify the translational activation ability of sRNAs surviving this selection. *E. coli* strain NM22508 expresses a single copy of a chromosomal *rpoS-lacZ* translational fusion [20], enabling the quantitation of *rpoS* translation in a context different from that of the selection. RNA sequences that could both survive the Cat selection and pass the LacZ screen by inducing *lacZ* expression levels comparable to or greater than that of wild-type DsrA were considered positive activators. These sRNAs, like wild-type DsrA, are *rpoS* specific but are not dependent on a specific reporter gene.

### Development of an In Vivo Selection for *rpoS* Translational Repression

An analogous selection was developed for sRNAs that repress, rather than activate, *rpoS* translation. Plasmid pProt-CcdB was designed to express the toxic gyrase inhibitor CcdB [36] as a C-terminal fusion to the RpoS fragment described above. We hypothesized that the

toxicity of CcdB would prevent the growth of cells not expressing translational repressors of the *rpoS-ccdB* fusion.

The stringency of the selection was varied by titrating the concentration of IPTG used to induce *rpoS-ccdB* expression from its *tac* promoter. At an optimized concentration of 27.5 μM IPTG, the selection allowed 1 in 500 OxyS-expressing cells to survive, whereas control cells expressing no sRNA or DsrA survived at a rate of 1 in $1 \times 10^4$ or 1 in $3 \times 10^4$, respectively. To multiply this modest level of enrichment for authentic repressors, two iterated rounds of the selection were performed on the libraries described below. The activities of selected RNA repressors were also evaluated in the secondary LacZ screen described above; sRNAs that reduced LacZ expression to an extent comparable to or greater than that of wild-type OxyS were considered positives.

### Creation of RNA Libraries

The NRR method [33, 34] was used to diversify *dsrA* and *oxyS* separately into libraries of randomly and nonhomologously recombined fragments. Although the 5′ and 3′ sequences of the sRNA genes could play a role in sRNA activity, we diversified specifically those regions of *dsrA* and *oxyS* that are known to be transcribed and to allow full translational regulation of *rpoS* expression (Figures 1C and 1D).

The NRR-diversified *dsrA* (activator) library, A1, was constructed with randomly cleaved gene fragments ranging in size from 10–70 bp. The fragments were recombined to a target gene size of 80–150 bp. Two NRR-diversified *oxyS* (repressor) libraries, R1 and R2, were constructed. In R1, blunt-ended *oxyS* gene fragments 5–30 bp were recombined into 80–100 bp genes, while in R2, 20–70 bp fragments were joined into 100–150 bp recombinants. All three sRNA libraries were cloned into pRNA and the resulting plasmids were introduced into *E. coli* DH10B cells, generating libraries of $1 \times 10^6$ to $1 \times 10^8$ transformants. For comparison, we also prepared library N1, expressing 40 consecutive random RNA nucleotides ($8 \times 10^7$ transformants).

To assess the diversity introduced by NRR, 15 unselected library members from library A1 were characterized by DNA sequencing (Figures 2A and 2B). In Figures 2B and 2C, each fragment derived from the *dsrA* gene (Figure 2A, top line) is depicted as a single, colored arrow. The colors of the arrows indicate the arrangement of the fragments within a single transcript (5′-red-orange-green-blue-purple-3′). The position and orientation of the arrow indicates the origin of the fragment within the *dsrA* gene and whether the sequence is from the sense (right-pointing) or antisense (left-pointing) strand of *dsrA*. Unselected clone U15, for example, consists of two fragments derived from *dsrA* (Figure 2A). The first fragment (red) is from the 5′ end of the sense strand of *dsrA*. The second fragment (orange) is from the 5′ end of the antisense strand. Consistent with the library design goals, the diversified sequences of library A1 ranged in length from 29–174 bp and contained up to four crossovers between fragments ranging in size from 12–79 bp. As expected, the sense and antisense strands of the parental DNA before selection were similarly represented (48% sense strands).

### Translational Regulators Do Not Arise from Random or Unrelated Sequences

The high degree of diversification introduced by NRR raises the possibility that translational activators or repressors unrelated in structure or mechanism to that of DsrA or OxyS might arise by chance in our libraries. To determine the frequency with which sRNA translational regulators unrelated to DsrA or OxyS spontaneously arise from our libraries, we performed a series of control selections. *E. coli* expressing repressor libraries R1 and R2 ($5 \times 10^7$ transformants total), and random $N_{40}$ library N1 ($8 \times 10^7$ transformants) were separately selected for *rpoS* translational *activation* as described above. The RNA sequences surviving the selections (ten examples each picked from a survival rate of ~1 in $10^4$) were screened for their ability to activate the translation of *rpoS-lacZ* in *E. coli* strain NM22508. For all three libraries, none of the clones surviving selection expressed LacZ activity. Similarly, random library N1 ($1 \times 10^4$ transformants total) and activator library A1 ($1 \times 10^7$ transformants total) were selected for *rpoS* translational *repression* and screened using the RpoS-LacZ assay. Once again no active clones were observed.

Taken together, these results suggest that *rpoS* specificity and translational regulation activity are uncommon features of DsrA and OxyS that make the spontaneous formation of *rpoS* translational regulators from unrelated or random sequences highly unlikely (<1 in $10^7$ unrelated sequences of length similar to DsrA and OxyS). The inability of unrelated or random RNA sequences to give rise to translational activators and repressors supports our assumption that the active sRNAs emerging from the selections described below operate by DsrA-like and OxyS-like mechanisms.

### Selection of Translational Activators from a *dsrA*-Based Library

The A1 library was selected for *rpoS-cat* translational activation and screened for *rpoS-lacZ* translational activation as described above. In contrast with the inability of selected RNAs from the control selections to pass secondary screening, six sequences from the *dsrA*-based library activated *rpoS-lacZ* expression in NM22508 out of 38 clones that activated *rpoS-cat* expression in DH12S (pProt-Cat) (Figures 2C and 3A). During the course of our investigations, it became apparent that the presence of the genomic T-rich region 3′ of SL3 modestly increased the activity of all clones (Figure S2), likely due to the role of this region in rho-independent transcriptional termination (Figure S1). Therefore, each selected clone was further characterized with this T-rich region appended to its 3′ terminus.

The sequences of the active clones reveal significant structural rearrangements of *dsrA* (Figures 2C and S3). In contrast with the sequences prior to selection, 94% (15 out of 16) of the fragments from the selected sequences are from the sense strand of *dsrA*, consistent with the selection's enrichment for functional DsrA variants. Interestingly, the more active DsrA variants, such as A41 and A45, contain repetitions of subsequences suggesting that avidity effects may enhance translational activation.

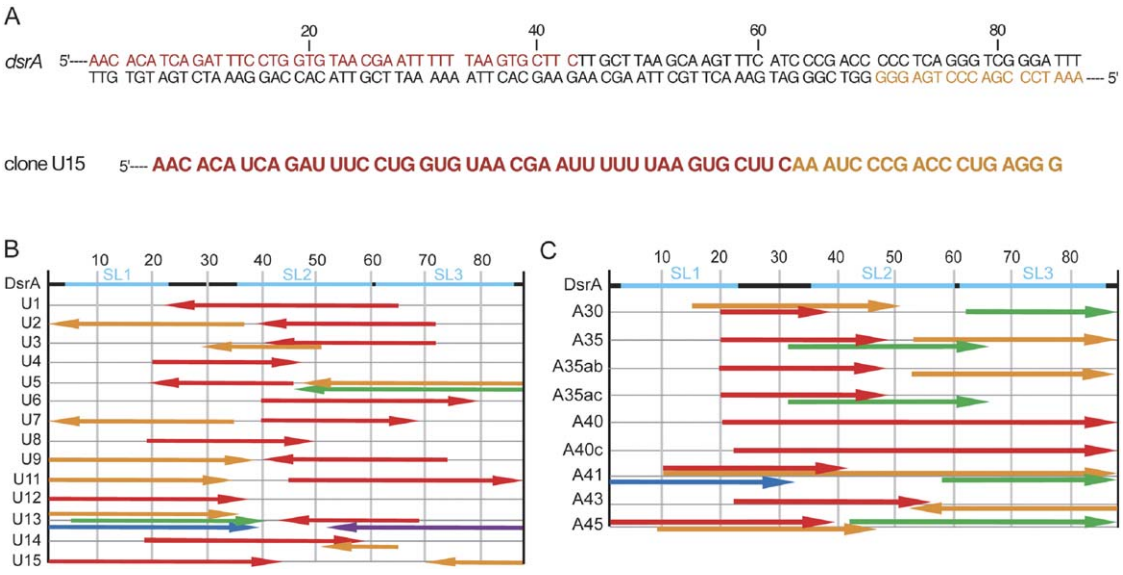


Figure 2. NRR-Mediated Diversification of DsrA

(A) Top line: sequence of *dsrA* with both the sense and antisense strands shown; numbering follows that of Figure 1C. Bottom line: sequence of clone U15, an example of an NRR-diversified DsrA variant. Color coding corresponds to that of *dsrA*, showing that sequences from both the sense and antisense strands of *dsrA* recombine to form variant U15.

(B) Composition of several NRR-diversified variants prior to selection. Numbering across the top corresponds to the nucleotide position in DsrA as shown in Figure 1C. Each arrow represents a *dsrA* fragment. Arrow positions indicate the origin of each fragment within the parental *dsrA* gene. Arrow colors indicate the order of the fragment reassembly (5′-red-orange-green-blue-purple-3′). The direction of each arrow identifies the sense (pointing right) or antisense (pointing left) strand of *dsrA*.

(C) Composition of active RNA activators of *rpoS* translation after selection and screening. The labeling scheme is as described in (B).

### Intracellular Abundance of Active DsrA Variants

As shown in Figure 3A, the activities of the selected translational activators vary widely. Differences in intracellular stability and abundance could account for differences in activity. In order to test this possibility, quantitative reverse transcriptase-PCR (qRT-PCR) was used to measure the intracellular levels of three representative groups of DsrA variants (Figures 3B and 3C). In all studies, controls lacking reverse transcriptase or template RNA showed no signal above background (data not shown).

Group 1 includes sRNAs with activities higher than that of DsrA (A45) or comparable to that of DsrA (A40 and A43), as well as wild-type DsrA. We observed no significant difference in RNA levels between A45 and DsrA that could account for the observed 3-fold greater activity of A45. In the case of A40 and A43, the selected sRNAs are 5- and 40-fold less abundant, respectively, than that of DsrA. The lack of SL1 in both sRNAs, together with the inversion of SL3 in A43, may explain their lower stability. Because both A40 and A43 are at least as active as wild-type DsrA, their low intracellular levels do not compromise our ability to interpret these clones as active translational activators.

Groups 2 and 3 represent the series of site-directed mutants of selected sRNAs described below. These results also show a similar lack of correlation between translational activation activity and intracellular abundance (Figures 3B and 3C). Specifically, A35ab and A35ac were comparably abundant, while A40, A40a, A40b, and A40c were also present at similar intracellular levels. Based on these observations, we conclude that while the selected DsrA variants differ in their intracellular abundance, these differences cannot account for their observed differences in activity within each group.

### Hfq Dependence of Active DsrA Variants

Each of the six active sequences contains at least one copy of the putative U-rich Hfq binding site (bases 23–35 of DsrA) [23], suggesting that Hfq mediates a required interaction between these sRNAs and the *rpoS* mRNA. To evaluate the dependence of translational activation on Hfq, we assayed a representative set of four active sequences in an Hfq-deficient strain. Sledjeski and coworkers have previously demonstrated that in this *hfq*$^{-}$ strain, DsrA stimulation of *rpoS* translation is significantly reduced though not abolished [28]. Similarly, we observed that the absence of Hfq significantly impairs, but does not eliminate, the activity of the selected sequences (Figure 3D). These results suggest that the selected sequences have inherited the features of DsrA that confer its Hfq-dependence and therefore likely activate *rpoS* translation through an analogous mechanism. Translational activation arising from the pRNA vector lacking an sRNA insert was higher in the presence of Hfq than in its absence (Figure 3D); this difference is presumably due to other Hfq-dependent sRNAs that activate *rpoS* translation. Additionally, in the absence of Hfq, the selected sRNAs all have comparable activity. The pleiotropic nature of Hfq and the

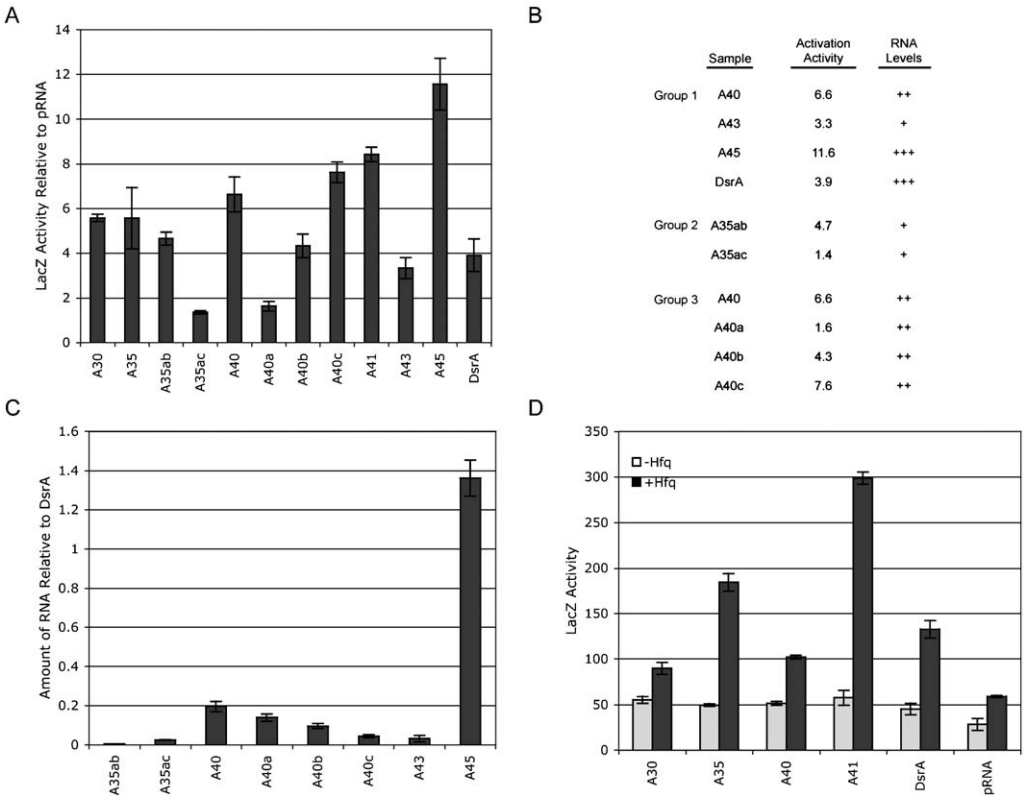


Figure 3. Activation of *rpoS* Translation by Selected DsrA Variants

(A) Level of *rpoS-lacZ* translational activation by DsrA variants. Miller units are used to quantitate expression of β-galactosidase from an RpoS-LacZ fusion, and activities are expressed in Miller units relative to a pRNA control lacking a sRNA insert.

(B) Comparison of translational activation activity (listed as RpoS-LacZ activity relative to pRNA) and intracellular abundance (see Figure 3C) of selected RNA sequences.

(C) Intracellular abundance of sRNA activators, relative to DsrA, as measured by quantitative reverse transcriptase PCR (see Experimental Procedures).

(D) Activities (in Miller units) of selected RNA sequences in the presence and absence of Hfq.

Error bars represent standard deviations of three or more independent trials.

presence of other sRNAs that activate *rpoS* translation may account for these observations.

### The Role of DsrA Stem-Loop 3

A comparison of the active and inactive DsrA variants emerging from this work identifies regions essential for DsrA activity (Figures 2B and 2C). For example, all DsrA variants surviving selection contained SL3. RNAs lacking a complete SL3 yet containing other regions found to be essential (e.g., clones U4, U8, U12, U14, and U15) were inactive, strongly suggesting that an intact SL3 is required for activity. The order in which the essential fragments occur is also important. For example, the sequence of inactive clone U11 contains a complete SL3 followed by a nearly complete SL1 and Hfq binding site, but these components occur in the opposite order compared with active clones A35, A41, and A45. Interestingly, A43 contains the antisense strand of SL3, yet is still active. This result suggests that both the sense and antisense strand of SL3 may function, a hypothesis that is consistent with SL3's putative role as a hairpin-forming factor-independent terminator [18, 20]. The antisense version of SL3, however, renders A43 a weaker translational activator than its sense-strand counterpart (*e.g.* A40).

Highly active clone A35 consists of three fragments containing: (a) the end of SL1 + half of SL2; (b) SL3; and (c) SL2 (Figure 2C). To study the role of SL3 in detail, mutants of A35 missing either (b) or (c) were constructed and assayed. While deletion of the fragment after SL3 (mutant A35ab) did not affect *rpoS* translational activation, deletion of the fragment containing SL3 (A35ac) completely abolished activity (Figures 2C and 3A), confirming the importance of SL3 in this clone. These results are in agreement with Northern blot analysis which indicates that only the first two fragments of A35 are transcribed (Figure S1). Collectively, these results indicate that SL3 retains its role as a transcriptional terminator in the selected DsrA variants.

### The Role of DsrA Stem-Loop 1

Previous studies have suggested that SL1 is essential to DsrA activity and participates in base pairing with the *rpoS* mRNA [20–23]. While all six active clones contain the SL1-SL2 linker, four of the clones lack large portions of SL1 (Figure 2C). Notably, clone A40 begins

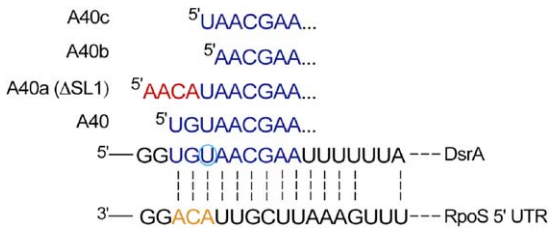


Figure 4. Mutational Analysis of Selected *dsrA* Variant A40

Potential base pairs between A40 variants and the *rpoS* leader. Complementary bases are shown in blue, while mismatches are red. DsrA U22 is circled; *rpoS* nt 113–115 are highlighted in orange (see text).

with only the last three bases of SL1 (UGU) followed by the remainder of DsrA, yet is at least as active as wild-type DsrA (Figures 3A and 4). These findings indicate that the majority of SL1 is not necessary for translational activation. In apparent contradiction of our findings, Gottesman and coworkers reported that deletion of SL1 (ΔSL1; 5′-AACAU followed by the SL1-SL2 linker) resulted in the complete loss of *rpoS* translation [20]. To investigate this inconsistency, we generated a series of A40 mutants differing in their 5′ termini (Figure 4). A40a is identical to the previously characterized ΔSL1 sequence and begins with 5′-AACAU. A40b contains a deletion of all the nucleotides before the linker and therefore starts with 5′-AACGAA, whereas A40c begins with the last U of SL1 (5′-UAACGAA).

Consistent with previous findings, A40a (ΔSL1) was 6-fold less active than A40 (Figure 3A). In contrast, A40c activates translation at least as potently as wild-type DsrA, while A40b (differing from A40c only in the loss of a single 5′-U) was 2-fold less active than A40c. The current model for DsrA activation of *rpoS* translation invokes an anti-antisense mechanism that must precisely balance intramolecular DsrA hairpin formation with intermolecular DsrA-*rpoS* duplex formation [20–23] (Figures 1B and 1C). If base pairing between DsrA and *rpoS* mRNA is too weak, translational activation cannot take place, whereas if hybridization is robust, the level of *rpoS* translation is high. In this model, DsrA U22 (directly preceding the SL1-SL2 linker) pairs with A113 of *rpoS* mRNA (Figures 1B and 4B). Clone A40 contains U22 as well as the two preceding nucleotides (U20 and G21); these two bases can further pair with A115 and C114 of *rpoS* mRNA, favoring intermolecular hybridization. A40a, however, replaces U20 and G21 with AACA, bases that cannot pair with the *rpoS* leader; duplex formation is less favorable as a result and *rpoS* activation decreases 6-fold (Figure 3A). Removal of the mismatching AACA (clone A40c) fully restores LacZ activity.

Comparing the activities of A40b and A40c reveals that a single DsrA-*rpoS* base pair can significantly affect translational activation. Reducing the number of possible base pairs between DsrA and *rpoS* mRNA from 12 (A40c) to 11 (A40b) reduces translational activation 2-fold (Figures 3A and 4). Collectively, these results show that pairing interactions between the SL1-SL2 linker of DsrA and the complementary region of the *rpoS* UTR can precisely tune translational activation.

Our findings indicate that beyond the small number of bases that pair with the RpoS mRNA, the substantial majority of SL1 is not required for translational activation. When present, however, SL1 can contribute to pairing as well, as demonstrated by previous mutagenesis studies [20]; this extended pairing could be particularly important when DsrA levels are low. Because there is little thermodynamic incentive for SL1 to unfold and swap intramolecular base pairs for intermolecular ones, it is not surprising that most of SL1 is not required for DsrA activity when sRNA concentrations are higher. The evolutionary conservation of SL1, however, suggests that this region plays a significant role in *rpoS* translational activation. The intracellular abundance assays described earlier suggest that one possible role for SL1 consistent with all of the above observations is to stabilize the sRNA, rather than to form necessary base pairs with the *rpoS* mRNA.

### Selection of Translational Repressors from an *oxyS*-Based Library

NRR-diversified repressor libraries R1 and R2 were separately introduced into *E. coli* cells harboring pProt-CcdB, each resulting in $\sim 10^6$ transformants. Following selection, RNA-encoding inserts from surviving colonies (1 in $8 \times 10^3$) were pooled, recloned into pRNA, and reselected. One in 600 transformants survived reselection, suggesting that repressors were enriched 13-fold by reselection. Two highly represented sequences, R1-1 (12 out of 64 clones) and R2-1 (6 out of 64 clones), were confirmed to repress the translation of both *rpoS-ccdB* and *rpoS-lacZ* (Figures 5A, 5B, and S3).

Sequence analysis of R1-1 and R2-1 revealed an unexpected 3′ T-rich fragment (5′-TTTTTTTTGCC) in both selected clones that entered the NRR process through the use of pOxyS as a PCR template. The presence of this fragment in both active sequences strongly suggested the importance of rho-independent transcriptional termination after SL3 of *oxyS* (Figure 5B, compare OxyS and OxyST). Therefore, we constructed two additional NRR-diversified *oxyS* libraries, R3 and R4, which either allowed the T-rich region to be recombined throughout the library (R3) or which appended this T-rich region to the 3′ end of all library members (R4). R3 and R4 were selected for translational repression as described above ($5 \times 10^6$ and $1 \times 10^6$ transformants, respectively). After two rounds of selection, four unique sequences from R3 and five from R4 repressed *rpoS-lacZ* expression (Figures 5A and 5B). Including R1-1 and R2-1, 10 of the 11 selected sequences contain two small regions of OxyS (Figure 5A), suggesting that these regions are required for efficient translational repression.

All of the selected sequences, moreover, contain the U-rich putative Hfq binding region, found in the linker between OxyS SL2 and SL3 [25]. When a representative set was assayed in an Hfq-deficient strain, all five of the selected sequences were inactive, mirroring the Hfq dependence of wild-type OxyS (Figure 5C) [25, 30]. Together with our control selections described above, these results strongly suggest that the selected sequences repress *rpoS* translation in a manner similar to that of wild-type OxyS.

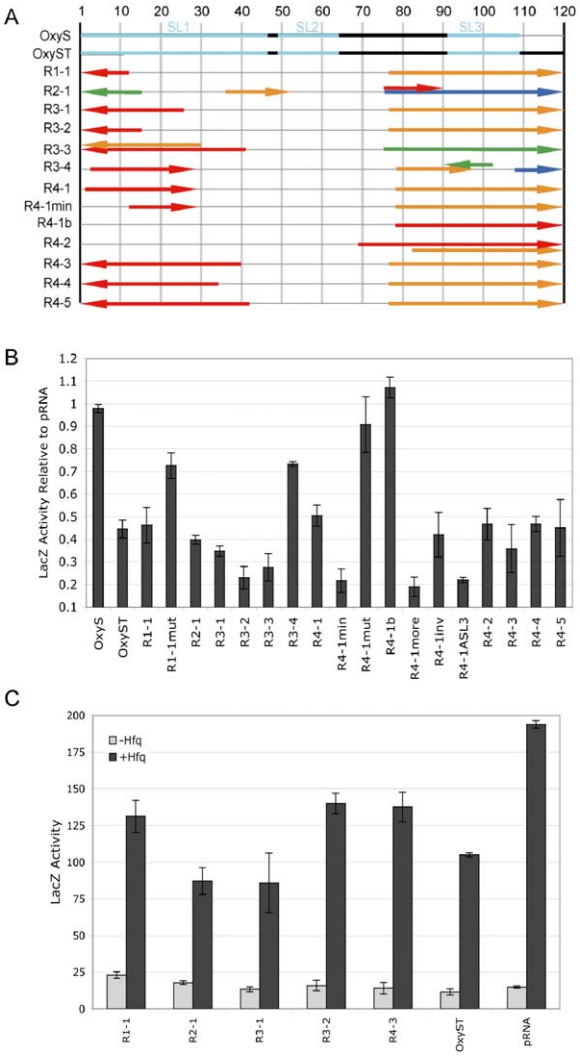


Figure 5. Translational Repression of *rpoS* by Selected OxyS Variants

(A) Composition of selected OxyS variants that repress *rpoS* translation. The labeling scheme is described in Figure 2B.

(B) Translational repression activities of selected OxyS variants. Miller units are used to quantitatively represent β-galactosidase expression from an RpoS-LacZ fusion, which are shown relative to pRNA lacking an sRNA insert. OxyS represents the 109 nt wild-type OxyS sequence; OxyST represents the wild-type OxyS sequence with the T-rich region appended to the 3′ end (see text).

(C) Translational repression activities of OxyS variants in the presence and absence of Hfq.

Error bars represent standard deviations of three or more independent trials.

## Intracellular Abundance of Selected sRNA Repressors

As with the translational activators described above, qRT-PCR was used to analyze the intracellular abundance of a representative set of selected translational repressors and their mutants (Figures 6A and 6B). Control assays performed without reverse transcriptase or template RNA yielded no signal above background (data not shown). All OxyS variants analyzed in this way were comparably abundant (within 2-fold of wild-type OxyS containing the 3′ T-rich region), indicating that differences in activities among the OxyS mutants described cannot be explained simply by changes in their intracellular abundance. For example, although R4-1min was 5-fold more active than R4-1b, there was no significant difference in their intracellular abundance.

## The Role of OxyS Stem-Loop 1

The first region conserved among selected OxyS variants is the 5′ end of SL1. Ten of the 11 selected OxyS variants contain either a short antisense portion of the 5′ end of the OxyS parent (e.g., the reverse complement of OxyS bases 3–12 as in clone R1-1), or a longer sense portion (e.g., OxyS bases 2–29 as in clone R4-1) (Figure 6C). Both of these conserved SL1 fragments are partially complementary to the coding region of the *rpoS* mRNA (Figures 6D and 6E). Additionally, the sense fragment of OxyS SL1 recovered from our selections and the complementary region of *rpoS* are both evolutionarily highly conserved. These results suggest that the 5′ end of SL1 may interact with the *rpoS* mRNA through base pairing. The absence of the majority of SL1 among active clones establishes that the SL1 stem-loop structure is not required for OxyS translational repressor activity.

To further isolate the putative *rpoS*-pairing region in R4-1, the first 11 bases of R4-1 were deleted to generate mutant R4-1min. An alignment predicts that these eliminated bases do not pair with the *rpoS* mRNA (Figure 6D). This “minimal OxyS” construct was indeed fully active as a translational repressor (Figure 5B), consistent with the dispensability of the first third of SL1.

Four additional mutants were constructed to further test the hypothesis that a portion of SL1 pairs with the *rpoS* mRNA (Figure 6). The SL1 fragment complementary to *rpoS* was mutated in clones R1-1 and R4-1 such that the resulting mutants (R1-1mut and R4-1mut) cannot base pair with the *rpoS* mRNA (Figure 6C). The entire SL1 fragment of R4-1 was also deleted to create R4-1b. All three of these mutants possess no significant translational repression activity (Figures 5B and 6A). In contrast, when the SL1 fragment of R4-1min was altered to increase, rather than remove, possible base pairing with the *rpoS* mRNA (resulting in clone R4-1more), the resulting mutant remained a highly active translational repressor of *rpoS* expression (Figure 5B). Taken together, these results are consistent with a regulatory mechanism in which the central third of OxyS SL1 pairs with the *rpoS* mRNA to repress translation.

## The Role of OxyS Stem-Loop 3

The ten most active OxyS variants all contain a second conserved region: the last 40 bases of OxyS (comprising a portion of the SL2-SL3 linker, SL3, and the 3′ T-rich region). While this region appears necessary for translational repression, the inactivity of mutant R4-1b demonstrates that this region is not sufficient for activity (Figure 5B). This region includes a stretch of 13 nucleotides containing 11 bases complementary to the *rpoS* mRNA (Figure 6F), raising the possibility that it may interact with the *rpoS* mRNA through base pairing. To test this possibility, base pairs in SL3 of R4-1min

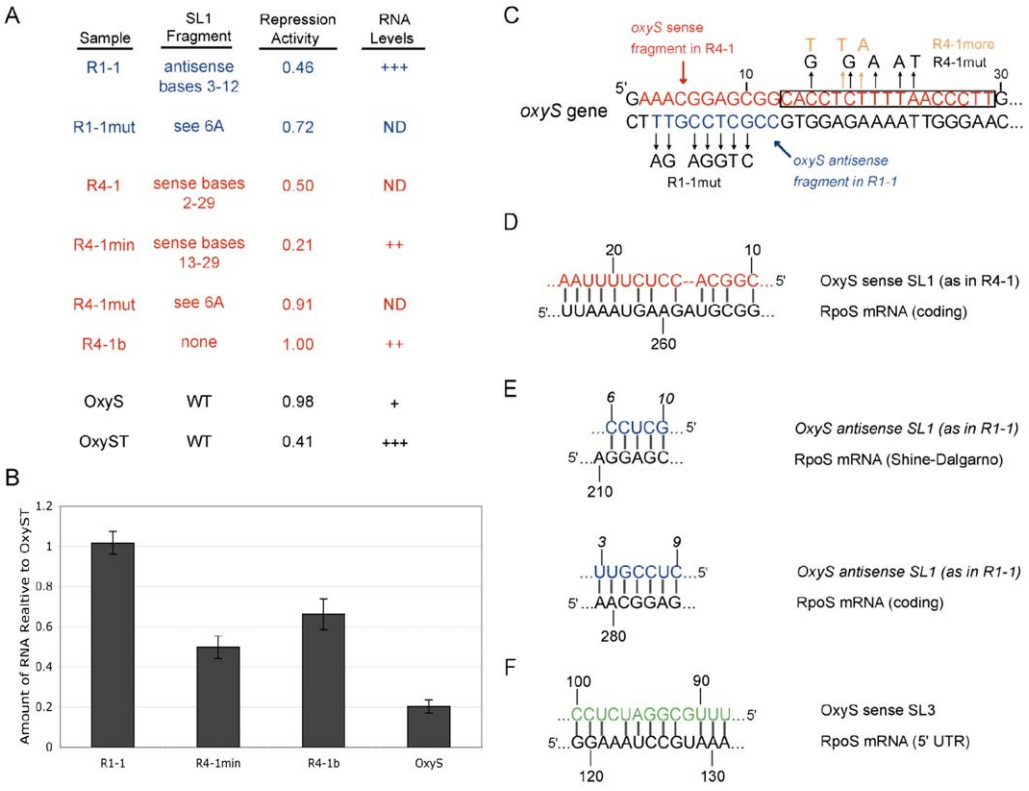


Figure 6. Sequence Analysis of Selected Translational Repressors

(A) Comparison of SL1 fragment, translational repression activity (see Figure 5A) and intracellular abundance (see Figure 6B); ND = not determined. Sequences containing the antisense OxyS SL1 sequence are in blue. Sequences containing the sense OxyS SL1 sequence are in red.

(B) Intracellular abundance of sRNA repressors, relative to OxyS, as measured by quantitative reverse transcriptase PCR (see Experimental Procedures). Error bars represent standard deviations of three or more independent trials.

(C) The first third of the *oxyS* gene showing both the sense and antisense strands. The SL1 sense fragment found in R4-1 is highlighted in red. The SL1 antisense fragment found in R1-1 is highlighted in blue. The boxed sequence represents the minimal SL1 found in R4-1min. Mutations introduced to generate R1-1mut, R4-1more, and R4-1mut are indicated by the arrows.

(D–F) Proposed pairing between the *rpoS* mRNA and (D) the OxyS SL1 sense fragment; (E) SL1 antisense fragments; and (F) SL3 fragments. Base numbering is the same as that used in Figure 1; for reference, the *rpoS* start codon begins at nt 223. Nucleotides from the antisense strand are numbered in italics according to their sense strand base-pairing partners.

were inverted (R4-1inv) to abolish the possibility of pairing between SL3 and the *rpoS* mRNA while preserving the ability of the modified SL3 to form a stem-loop. This mutant was only 2-fold less active than R4-1min, suggesting that SL3 base pairing with the *rpoS* mRNA is not required for translational repression. Similarly, when SL3 of R4-1min was replaced with SL3 of DsrA, the resulting mutant R4-1ΔSL3 was still a strong repressor of *rpoS* translation (Figure 5B).

As OxyS SL3 has been described as a transcriptional terminator [27], its essential role is most likely to stabilize the *oxyS* transcript in vivo. The discovery of two regions of OxyS that participate in translational repression but are not contiguous in primary sequence illustrates a strength of NRR in revealing multiple functional components of nucleic acids. A traditional truncation analysis in which the termini of a gene are progressively shortened might not reveal these regions as distinct essential elements.

## Significance

**We applied NRR diversification and in vivo selection to functionally dissect sRNA regulators of *rpoS* translation in a manner independent of prior hypotheses. This approach requires no prior knowledge of sRNA function beyond that necessary to establish a selection or an efficient screen. Our results suggest two essential components for DsrA activity: the SL1-SL2 linker and SL3. The above findings also suggest that the primary role of SL1 lies in stabilizing DsrA, rather than playing a direct role in translational regulation, and that the majority of SL1 is not needed for activity. In the case of OxyS, we identified two regions that are necessary and collectively sufficient for activity: (1) a small fragment of the 5′ half of SL1 that may function by base pairing with the *rpoS* mRNA, and (2) a region including the transcriptional terminator SL3. We anticipate that the application of this approach to addi-**

**tional biological pathways will continue to prove useful for the functional dissection of novel natural nucleic acids.**

## Experimental Procedures

### Strains

*E. coli* strains DH10B and DH12S were purchased from Invitrogen. *E. coli* strain NM22508 (*dsrA*$^-$, expressing a single-copy, chromosomal *rpoS-lacZ* fusion) and plasmid pNM13 were generous gifts of S. Gottesman [20]. Plasmid pOxyS [25] and *E. coli* strain DDS1631 (*hfq::kan*) [28] were kindly provided by G. Storz and D. Sledjeski, respectively.

### Oligonucleotides for Library Construction

HPA (5′-P-CATACACGTCATCCGAATTC*AGGCCT*CCGGGCGCGCCCGG*AGGCCT*GAATTCCGGATGACGTGTATG-3′) contains an *Afl*III site (underlined) and HPB (5′-P-CATGGTCACCCATCCGAATTC*AGCTG*GCGGCGGCCGCCGC*CAGCTG*AATTCGGATGGGTGACCATG-3′) contains a *Bst*EII site (underlined) for ligation into the selection plasmid pRNA. HPA and HPB also contain *Stu*I and *Pvu*II sites (italicized) for removal of hairpin ends, and both end with *Nsi*I half-sites (ATG/CAT) for digesting hairpin dimers. Primers P1 (CCTGAATTCGGATGACGTGTATG) and P2 (CTGAATTCGGATGGGTGACCATG) were used for PCR amplification (see below).

For construction of the random RNA library, P3 (5′-GGCGGCGGCGGTGACC$(N)_{40}$CTAGCCATGACACACGTGGCGGC-3′) contains a *Bst*EII site (underlined) and P4 (5′-GCCGCCACGTGTGTCATGGACTAG-3′) contains an *Afl*III site (underlined) for ligation into pRNA.

### Plasmid Construction

The *rpoS-cat* fusion used for the selection of *rpoS* translational activators contains the following components: (1) the last 150 nt of the *E. coli rpoS* 5′UTR, followed by (2) the first 73 codons of *rpoS*, and (3) the chloramphenicol acetyltransferase gene (*cat*) from pACYC184 for expression as a C-terminal protein fusion (lacking its natural start codon). Selection plasmid pProt-Cat contains the above construct together with the p15A replication origin from pACYC184, the *kanR* gene from pACYC177, and a *tac* promoter upstream of the *rpoS-cat* cloning site. An analogous plasmid, pProt-CcdB, was constructed for selection of translational repressors in which the *cat* gene of pProt-Cat was replaced by the cytotoxic gyrase inhibitor gene *ccdB* from pZero-1 (Invitrogen).

Plasmid pRNA was used for the expression of all sRNA variants and is a derivative of high-copy (colE1 origin) plasmid pBAD24 [37] in which the arabinose promoter ($P_{BAD}$) was replaced by the *lpp* promoter ($P_{LPP}$) using an upstream *Cla*I site and a downstream *Nhe*I site. The library insertion site, flanked by synthetic *Bst*EII and *Afl*III sites, is downstream of the transcription start site and upstream of the *rrnB* terminator. This cloning scheme appended 18 nt (5′AACGCGCTAGCGGTGACC) to the 5′ end of each sRNA construct; this sequence is not complementary to the *rpoS* leader.

PCR was used to generate DNA encoding the 87 bp DsrA and 109 bp OxyS RNAs, each flanked by a 5′ *Bst*EII and a 3′ *Afl*III site, using pNM13 [20] and pOxyS [25] as templates, respectively. These products were ligated into the same sites on pRNA to generate pRNA-DsrA and pRNA-OxyS. Mutants of selected sRNA regulators were cloned in a similar manner.

### Construction of NRR-Diversified *dsrA* and *oxyS* Libraries

Using pNM13 and pOxyS as templates, *dsrA* and *oxyS*, respectively, were amplified by PCR (*dsrA* primers: 5′-pAACACATCAGATTTCCTGGTGTAACGAATTTTTTAAGTGC-3′ and 5′-pAATCCCGACCCCTGAGGGGGTCGGGATGAACTTGC-3′), (*oxyS* primers: 5′-pGAAACGGAGCGGCACCTC-3′ and 5′-pGCGGATCCTGGAGATCCGC-3′). NRR was performed on the resulting PCR products as previously described [34]. Recombined genes were amplified by PCR using primers P1 and P2, and the product was digested with *Afl*III and *Bst*EII. The desired size range of recombined DNA was purified by gel electrophoresis, then ligated into pRNA.

### Construction of the Random 40 nt Library

Primers P3 and P4 (500 pmol each) were annealed and extended with *Taq* DNA Polymerase. The resulting random DNA library was digested with *Afl*III and *Bst*EII, then purified by gel electrophoresis and ligated into pRNA.

### In Vivo Selection for Translational Activation

An RNA library cloned into pRNA was transformed into 320 μl of electrocompetent DH12S cells harboring pProt-Cat and recovered in 2× yeast/tryptone (2×YT) medium at 37°C for 30 min. A fraction of the culture was plated on 2×YT plus carbenicillin (Cb) to determine the size of the library. The remaining cells were washed and plated on glycerol minimal media containing 20 μg/ml thiamine, 0.1 mg/ml casamino acids, 25 μM IPTG, 100 μg/ml Cb, and 40 μg/ml chloramphenicol (Cm) at 37°C. After 36 hr colonies were picked, cultured, and screened by PCR to confirm the presence of sRNA inserts. Putative active inserts were religated into pRNA and retransformed into DH12S (pProt-Cat) to confirm activity.

### In Vivo Selection for Translational Repression

Using the above protocol, a pRNA library was transformed into DH12S (pProt-CcdB) and recovered at 37°C for 60 min. A fraction of culture was plated on 2×YT + Cb to determine the size of the library. The remaining cells were washed and plated onto glycerol minimal media containing 5 μg/ml thiamine, 1 mg/ml casamino acids, 27.5 μM IPTG, 100 μg/ml Cb, and 40 μg/ml kanamycin (Kan) at 37°C. After 36 hr, cells were harvested and the plasmids from the collected cells were isolated. Inserts from these plasmids were religated into pRNA and subjected to reselection as above. The resulting sRNA-encoding inserts were religated into pRNA and retransformed into DH12S (pProt-CcdB) to confirm activity.

### β-Galactosidase Secondary Screen

Plasmids with putative active sRNAs were transformed into *E. coli* strain NM22508, which links *rpoS* translation with LacZ activity. Cells were grown at 37°C to an $OD_{600}$ of 0.7–0.8. Assays were performed as previously described [38]. LacZ activities were normalized to the cell density ($OD_{600}$) of each sample. Each assay was independently repeated three or more times.

### Construction of Second-Generation *oxyS* Libraries

Once the importance of the 11 nt (5′-TTTTTTTTGCC-3′) following *oxyS* became apparent (see above), two new libraries were constructed. Library R3 used an extended (120 bp) *oxyS* PCR product (generated from the original 5′ primer for *oxyS* and a new 3′ primer, 5′-GGCAAAAAAAAGCGGATCCTGGAGATCCGC-3′) as the starting material for NRR. Library R4 used the 109 bp *oxyS* PCR product for NRR, but used an alternate hairpin sequence containing the 11 nt region above (5′-CTTTTTTTTGCCACACGTGAATTCGGCCCGCGGGCCGAATTCACGTGTGGCAAAAAAAAG-3′). Primers P2 and P5 (5′-GCGAATTCACGTGTGGCAAAAAAAAG-3′) were used to amplify this library after NRR.

### Assay for Hfq Dependence

*E. coli* strain DDS1631 (*hfq::kan*) [28] was supplemented with a plasmid, pBadHfq, that contained the *hfq* gene under control of a pBad promoter. This plasmid contains the following key components: (1) the p15A origin of replication and the Cm resistance gene from pACYC184; (2) the $P_{BAD}$ promoter from plasmid pBAD24; and (3) the *hfq* gene, obtained by PCR from *E. coli* genomic DNA introducing *Nhe*I and *Pst*I restriction sites for cloning. To evaluate Hfq dependence, pRNA plasmids were introduced into DDS1631 (pBadHfq), grown in 2×YT with 0.2% glucose (Hfq repressed) or 0.2% arabinose (Hfq expressed), and assayed for LacZ activity as above.

### Quantitative Reverse Transcriptase PCR

NM22508 was transformed with the plasmid encoding the sRNA of interest and grown at 37°C to an $OD_{600}$ of 0.6. From 700 μl of the resulting culture, total RNA was isolated by hot-phenol extraction [39]. Total RNA was treated with 10 U RNase-free DNase I (New England Biolabs) in the presence of 2.5 mM $MgCl_2$ for 30 min at

37°C. DNase cleavage was terminated by phenol-chloroform extraction followed by ethanol precipitation. From the resulting material, 1 μg total RNA was treated with reverse transcriptase from the Moloney Murine Leukemia Virus (New England Biolabs) at 42°C as described by the manufacturer in the presence of 50 pmol primer A (for the activators, 5′CAAGAAGCACTTAAAAAATTC) or primer B (for the repressors, 5′GATCCGCAAAAGTTCACG). These primers anneal to regions common to all activators (or repressors) assayed. Reverse transcriptase activity was terminated by heating at 95°C for 10 min.

Serial dilutions of purified and quantified plasmid DNA were used as reference templates to facilitate the accuracy of comparisons between RNA samples during quantitative (real-time) PCR. The reference DNA or 1 μl of the reverse transcriptase reaction was mixed with 25 pmol primer A or B, sense primer (5′AACGCGCTAGC GGTGACC), 2× QuantiTect SYBR Green PCR Master Mix (Qiagen) and sterile water to a final volume of 50 μl. Quantitative PCR reactions were performed using a DNA Engine Opticon 2 (MJ Research) with an initial denaturation step of 15 min at 95°C followed by 40 cycles of 30 s at 94°C, 45 s at 50°C, and 45 s at 72°C. The fluorescence was measured at the end of each extension step. Finally, a melting curve was recorded between 48°C and 99°C with a hold every 2 s. Relative RNA concentrations were calculated by comparison to the standard curves. Control reactions were performed for each run and included samples not treated with RT or samples lacking template RNA. In all cases, no signal was observed in these control cases.

## Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/7/757/DC1/.

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