The ancestral SgrS RNA discriminates horizontally acquired *Salmonella* mRNAs through a single G-U wobble pair

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SgrS RNA is a model for the large class of Hfq-associated small RNAs that act to posttranscriptionally regulate bacterial mRNAs. The function of SgrS is well-characterized in nonpathogenic *Escherichia coli*, where it was originally shown to counteract glucose-phosphate stress by acting as a repressor of the *ptsG* mRNA, which encodes the major glucose transporter. We have discovered additional SgrS targets in *Salmonella* Typhimurium, a pathogen related to *E. coli* that recently acquired one-quarter of all genes by horizontal gene transfer. We show that the conserved short seed region of SgrS that recognizes *ptsG* was recruited to target the *Salmonella*-specific *sopD* mRNA of a secreted virulence protein. The SgrS–*sopD* interaction is exceptionally selective; we find that *sopD2* mRNA, whose gene arose from *sopD* duplication during *Salmonella* evolution, is deaf to SgrS because of a nonproductive G-U pair in the potential SgrS-*sopD2* RNA duplex vs. G-C in SgrS-*sopD*. In other words, SgrS discriminates the two virulence factor mRNAs at the level of a single hydrogen bond. Our study suggests that bacterial pathogens use their large suites of conserved Hfq-associated regulators to integrate horizontally acquired genes into existing posttranscriptional networks, just as conserved transcription factors are recruited to tame foreign genes at the DNA level. The results graphically illustrate the importance of the seed regions of bacterial small RNAs to select new targets with high fidelity and suggest that target predictions must consider all or none decisions by individual seed nucleotides.

seed pairing | small noncoding RNA | target discrimination

Horizontal gene transfer (HGT) is a driving force in general microbial evolution that allows bacterial pathogens to acquire new virulence factors from exogenous sources (1). The *Salmonella enterica* species is a group of enterobacterial pathogens that cause a range of diseases from gastroenteritis to typhoid fever, and it has horizontally acquired >25% of the total genetic material since the time *Salmonella* and *Escherichia coli* shared a common ancestor (2). The new genes include all virulence factors that *Salmonella* secrete into mammalian host cells through the two type 3 secretion systems (T3SSs) encoded on the *Salmonella* pathogenicity islands, SPI-1 and SPI-2 (3–5). The products of HGT genes generally have a fitness cost for recipient bacteria, and therefore, it is crucial that these genes are integrated into existing regulatory networks to prevent inappropriate expression (6, 7). Studies of bacterial regulators recruited to regulate HGT genes have identified signaling events that promote or suppress virulence, and they contributed to our understanding of the DNA recognition preferences of transcriptional regulators that mediate the repression or activation of newly acquired genes (8–10).

Small noncoding RNAs (sRNAs) are an emerging and abundant class of gene expression regulators that control many branches of cellular physiology. Most of the ~100 sRNAs known in *Salmonella* act on *trans*-encoded target mRNAs through short base-pairing interactions that are aided by the RNA chaperone, Hfq (11–16). It is not known whether and how these Hfq-dependent sRNAs exert control of HGT genes. Evidence has been circumstantial and based on observations that disruption of *hfq* alters expression of many *Salmonella* HGT loci (13, 17, 18, 19) and that Hfq binds many virulence factor mRNAs, suggesting that they might be targets of sRNAs (13).

The study of HGT targets could help better understand the building plan of sRNAs and how bona fide targets are discriminated from thousands of other cellular transcripts. For example, despite the great diversity in length (50–250 nt) and structure, increasing numbers of Hfq-dependent sRNAs are found to rely on a few highly conserved nucleotides—the seed—for binding to conserved targets. If new HGT targets were also recognized by the seed, this recognition would define the seed as the sRNA region that is generally responsible for mRNA binding.

In this paper, we show that the Hfq-associated SgrS RNA, present in both pathogenic and nonpathogenic enterobacteria (20, 21), was recruited to posttranscriptionally repress the synthesis of SopD, a recently acquired *Salmonella*-specific virulence protein that is secreted by both T3SSs (22). Pioneering work in *E. coli* had established SgrS as the centerpiece of a stress response to the accumulation of phosphorylated sugars, especially glucose (21, 23). This prior work also showed that the ~240-nt SgrS RNA is bifunctional (Fig. 1*A*): the 5′-located *sgrT* ORF encodes a 40-aa peptide that blocks glucose import by an unknown mechanism (24), whereas a 3′-located conserved region inhibits de novo synthesis of major sugar uptake proteins by direct base pairing with the *ptsG* and *manXYZ* mRNAs (21, 25). The SgrS–*ptsG* mRNA interaction has been exceptionally well-characterized and was shown to rely on only six nonredundant base pairs (26).

We show that, in *Salmonella*, similar regions of SgrS interact with both the *ptsG* and *sopD* mRNAs, suggesting that sRNAs preferably use their preestablished seed regions to sample incoming HGT mRNAs for potential regulation. Intriguingly, the closely related *sopD2* mRNA that contains an almost identical target site is refractory to SgrS regulation. Our analyses reveal that a single C to T transition in *sopD2*, resulting in a nonproductive G-U wobble base pair, enables SgrS to discriminate these similar mRNAs at the level of a single hydrogen bond. The results have ramifications for the prediction of sRNA target interactions.

Results

SgrS Targets in *Salmonella*. The *sgrS* gene (also known as *ryaA*) was originally identified in a global screen for Hfq-binding sRNAs in

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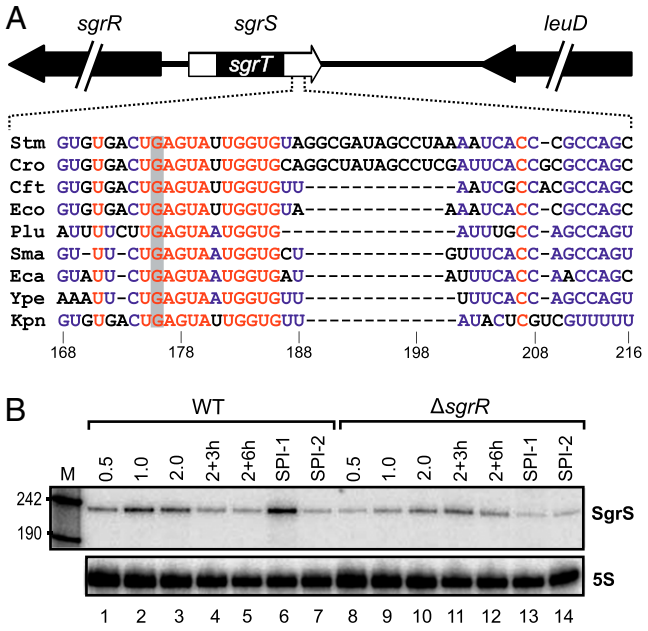

Fig. 1. Expression of SgrS in *Salmonella*. (*A*) Genomic location of *Salmonella sgrS* and conservation of the antisense domain in enterobacteria. (Stm: *Salmonella typhimurium* LT2; Cro: *Citrobacter rodentium*; Cft: *E. coli* CFT 073; Eco: *Escherichia coli* K12; Plu: *Photorhabdus luminescens*; Sma: *Serratia marcescens*; Eca: *Erwinia carotovora*; Ype: *Yersinia pestis*; Kpn: *Klebsiella pneumoniae*). The SgrS 5′ end also encodes the SgrT ORF (24) and is flanked by the *sgrR* gene encoding its transcription factor. The antisense domain is located in the 3′ end of the molecule and characterized by a stretch of 6 + 5 conserved nucleotides. The gray bar indicates the G to C single-nucleotide exchange in SgrS* ($G_{176} \rightarrow C$). (*B*) Northern blot analysis of WT and *sgrR* mutant *Salmonella* collected from various stages of growth (OD_{600} of 0.5, 1.0, 2.0, 3, and 6 h after cells had reached OD_{600} = 2.0 as well as SPI-1 and SPI-2 induction conditions). 5S rRNA served as loading control.

E. coli and resides between *setA* and *sgrR* (27); the latter gene encodes a transcription factor that activates SgrS synthesis when high levels of phosphorylated sugars threaten to poison the cell (21, 28). We confirmed that SgrS is expressed in *Salmonella* Typhimurium strain SL1344 during exponential and early stationary phase (Fig. 1*B*, lanes 1–3), and as expected, it was greatly diminished in the absence of SgrR (Fig. 1*B*, lanes 8–10). In addition, we discovered that SPI-1–inducing conditions (high salt and low oxygen), known to activate *Salmonella* invasion genes, strongly induced SgrS expression, an effect that was abolished in the *sgrR* mutant strain (Fig. 1*B*, lane 6 vs. 13).

To identify the target suite of SgrS in *Salmonella*, we used a pulse expression approach (29, 30) and scored global changes in mRNA abundance with microarrays after transient (10 min) overexpression of the sRNA from a P_{BAD} promoter. Of the 4,716 ORFs represented on the arrays, just six mRNAs were altered more than or equal to threefold by SgrS (Table 1). In accordance with previous findings in *E. coli* (21, 31), overexpressed SgrS repressed the *ptsG* mRNA by 10-fold as well as the *manXYZ* operon, which encodes a mannose-specific uptake system (25). The *yigL* mRNA is an additional candidate target that was up-regulated by SgrS; it is present in both *Salmonella* and *E. coli*, and it encodes a potential haloacid dehalogenase (HAD)-like hydrolase with a predicted role in sugar metabolism (32). Most importantly, however, we discovered a significant down-regulation of the *Salmonella*-specific *sopD* gene. We followed up these global observations by repeating the SgrS pulse expression in a Δ*sgrS* strain and quantified transcript changes by quantitative RT-PCR, which showed a sevenfold reduction of both the *ptsG* and *sopD* mRNAs (Table 1). This finding identified the horizontally acquired *sopD* mRNA as a candidate target of SgrS.

SgrS Controls the Synthesis of Virulence Factor SopD. To address whether *sopD* is also regulated under physiologically relevant conditions, we treated *Salmonella* with the nonmetabolizable glucose analog α-methyl glucoside (αMG), a strong inducer of the chromosomal *sgrS* gene (21). Northern blots showed that addition of αMG to exponentially growing cells strongly up-regulated SgrS within 16 min and also reduced the levels of the monocistronic *sopD* mRNA more than fivefold (Fig. 2*A*, lanes 1–6). We identified SgrS as the silencer responsible for the reduction in *sopD* expression, because in the presence of αMG, *sopD* levels were only slightly reduced in a Δ*sgrS* strain (~1.3-fold) (Fig. 2*A*, lane 7 vs. 12). Carbon source availability can modulate SPI-1 gene expression (33) and might account for this basal reduction at the level of *sopD* transcription.

At the protein level, SgrS-dependent regulation was evident even under nonstress conditions, when higher levels of SopD protein were seen in Δ*sgrS* than WT bacteria throughout growth (Fig. 2*B*, lanes 1–5 vs. 7–11), as well as under the SPI-1–inducing conditions (Fig. 2*B*, lane 6 vs. 12). Collectively, these results identified SgrS as a repressor of SopD synthesis under both infection-relevant and standard in vitro conditions.

Regulation of SopD Requires the Seed Region of SgrS. Given that SgrS is a bifunctional RNA (Fig. 1*A*) (21, 24, 26), we needed to define whether the *sgrT* ORF, seed pairing domain, or both regulate *sopD* expression. To address this question, we complemented the Δ*sgrS* strain with plasmids that constitutively expressed WT SgrS or several rationally designed mutants, and we assessed impact on SopD levels (Fig. 2*C* and RNA expression

Table 1. Genes differentially regulated on SgrS pulse expression

Gene	ID	Fold regulation*	Fold regulation†	Description‡
manX	STM1830	−10.3	−34.9	Mannose-specific enzyme IIAB
manY	STM1831	−12.4	−20.2	Mannose-specific enzyme IIC
manZ	STM1832	−5.5	−13.1	Mannose-specific phosphotransferase system protein IID
ptsG	STM1203	−10.2	−7.1	Glucose-specific phosphotransferase system IIBC components
sopD	STM2945	−5.3	−7.0	Secreted effector protein
yigL	STM3962	+3.7	+4.0	Putative sugar phosphatase

*Fold regulation obtained by transcriptomic analysis of pBAD-driven SgrS expression on *Salmonella*-specific microarrays. Genes that were at least threefold differentially regulated and had a P value ≤ 0.01 are listed.
†Fold regulation obtained by quantitative RT-PCR.
‡Description is based on the annotation at *Colibase* (http://xbase.bham.ac.uk/colibase/).

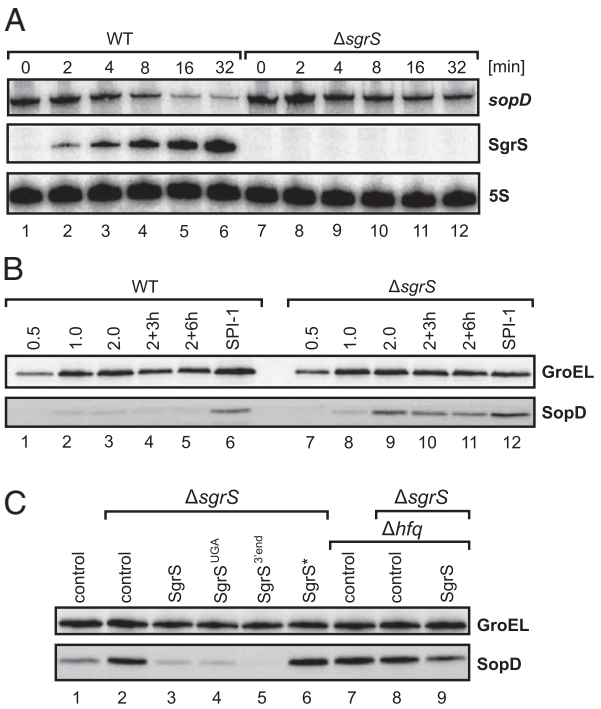

Fig. 2. The SgrS antisense domain is required for SopD repression. (*A*) Northern blot analysis of WT and *sgrS* mutant *Salmonella* challenged with the glucose analog αMG. Samples were withdrawn before and at the indicated time points posttreatment. Total RNA isolates were inspected for *sopD* and SgrS expression using gene-specific probes; 5S rRNA served as a loading control. (*B*) Western blot analysis of SopD::3×FLAG protein in WT and Δ*sgrS* strains at various conditions of *Salmonella* growth (OD_{600} of 0.5, 1.0, 2.0, 3, and 6 h after cells had reached OD_{600} = 2.0 as well as SPI-1–inducing conditions). GroEL served as an internal loading control. (*C*) Western blot analysis of SopD::3×FLAG protein in WT, Δ*sgrS*, Δ*hfq*, or Δ*sgrS*/Δ*hfq* bacteria. Strains were transformed with the indicated plasmids and grown under SPI-1–inducing conditions. Total protein was extracted, and SopD::3×FLAG proteins levels were inspected by Western blot.

data in *SI Appendix*, Fig. S1). In agreement with our previous results (Fig. 2*B*), under the SPI-1–inducing conditions, *sgrS* mutants displayed ∼2.5-fold higher SopD levels compared with WT (Fig. 2*C*, lane 2 vs. 1). In contrast, a plasmid overexpressing WT SgrS caused an approximately sevenfold reduction in SopD levels (Fig. 2*C*, lane 3 vs. 2), and similar repressions were mediated by SgrS with a severely truncated SgrT peptide (stop mutation at fifth codon) (Fig. 2*C*, lane 4) or just the 3′ region of the sRNA (Fig. 2*C*, lane 5). Thus, the 3′ end of the sRNA mediated *sopD* regulation. Furthermore, an SgrS* mutant RNA with a $G_{176} \rightarrow C$ point mutation in the 3′-located seed (Fig. 1*A*) fully abrogated regulation (Fig. 2*C*, lane 6). Importantly, G_{176} is essential for recognition of *ptsG* mRNA in *E. coli* (26), indicating that SgrS acted on *sopD* by direct base pairing.

To corroborate this finding, we tested the effect of SgrS in the absence of Hfq. A *Salmonella* Δ*hfq* strain showed the same elevated SopD levels as Δ*sgrS* (compared with WT) (Fig. 2*C*, lanes 1, 2, and 7) with no additional increase in a Δ*sgrS*/Δ*hfq* double mutant (Fig. 2*C*, lane 8), suggesting that both Hfq and SgrS are essential for *sopD* silencing. Overexpression of SgrS only caused a mild reduction in SopD expression (∼1.5-fold) in the absence of Hfq (Fig. 2*C*, lane 9). Collectively, these results suggested that SgrS uses its seed domain to regulate *sopD* posttranscriptionally by Hfq-dependent pairing.

***ptsG* and *sopD* mRNAs Are Regulated by Similar RNA Duplexes.** Most Hfq-associated sRNAs bind in the 5′ region of mRNAs, and similarly, SgrS down-regulated a translational *sopD::gfp* reporter containing only the 5′ UTR and first 20 codons of *sopD* (Fig. 3*A*, lanes 1 and 2). SgrS reduced the levels of SopD::GFP by approximately fivefold relative to sRNA control vector. We considered this regulation significant, particularly because it exceeded a previously observed 2.5-fold repression of a *ptsG::gfp* reporter by SgrS in *E. coli* (34).

Computer-aided analysis of antisense complementarity between the regions of the sRNA (3′ end) and target (5′ end) that were involved in regulation predicted an almost perfect 11-bp RNA duplex of SgrS with the early coding region of *sopD* (Fig. 3*B*). Importantly, this duplex involves all of the six SgrS nucleotides that form the critical core interaction with *ptsG* mRNA (Fig. 3 *B* and *C*), including G_{176}, which is required for the repression of both *ptsG* (26) and *sopD* (Fig. 2*C*).

To validate this RNA interaction through compensatory nucleotide changes in vivo, we confirmed that the SgrS* mutant did not repress the *sopD::gfp* reporter and constructed an *sopD**::gfp* fusion with a compensatory $C_{+5} \rightarrow G$ point mutation (Fig. 3*B*). This mutant reporter was refractory to WT SgrS but fully regulated by the compensatory SgrS* RNA (Fig. 3*A*, lanes 5 and 6).

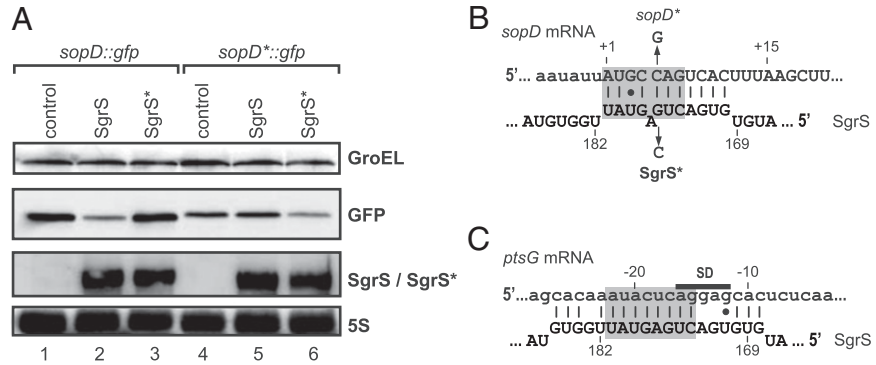

Fig. 3. SgrS binds *sopD* in the proximal coding sequence. (*A*) Western and Northern blot analyses of *Salmonella* harboring plasmid pP_L-SgrS or mutant plasmid pP_L-SgrS* in combination with either WT *sopD::gfp* or mutant *sopD**::gfp* fusion plasmids. Protein and RNA samples were collected at OD_{600} of 2.0, and SopD::GFP protein levels were determined by Western blot analysis. SgrS and SgrS* levels were detected by Northern blot using a gene-specific oligonucleotide. (*B*) Graphical presentation of the SgrS–*sopD* interaction. Numbering for *sopD* relative to the start-codon AUG (A is +1) and SgrS relative to +1 of the transcription start site. The *sopD* coding region and SgrS are shown in capital letters. Vertical arrows denote nucleotides introduced in *sopD::gfp* and SgrS, respectively. (*C*) Graphical presentation of the SgrS–*ptsG* interaction for comparison. The gray bars indicate SgrS nucleotides shared in the interactions *sopD* and *ptsG*.

These experiments show that the seed region of SgrS is used to target the laterally acquired *sopD* mRNA.

A single G-U Pair Prevents Regulation of the Nearly Identical *sopD2* mRNA by SgrS. *Salmonella* encodes an additional effector protein, SopD2, that is 42% identical to SopD (22). Only *S. enterica* species that are very closely related to the ancestral *S. bongori* lack this effector, indicating that *sopD2* arose from an early duplication of *sopD* and was followed by evolution to a divergent function (35). Intriguingly, the SgrS target site is almost identical within *sopD* and *sopD2* (*SI Appendix*, Fig. S2). We predicted an 11-bp RNA duplex for SgrS-*sopD2*, which only differs from SgrS-*sopD* by U instead of C opposite to G_{172} of SgrS (Fig. 4 *A* and *B*). However, our microarray experiment (Table 1) revealed no regulation of *sopD2* mRNA by SgrS. Similarly, the SgrS overexpression plasmid failed to down-regulate chromosomally expressed SopD2::FLAG protein under any growth condition tested (Fig. 4*C*), ruling out the possibility that regulation only occurred at the protein level. By contrast, SgrS depleted SopD::FLAG protein under all growth conditions (Fig. 4*C*), proving that the SgrS plasmid was functional.

Can a single noncanonical G-U instead of the canonical G-C pair suffice for discrimination and prevent *sopD2* from being targeted by SgrS? To address this question, an *sopD2::gfp* reporter was constructed that (as with *sopD::gfp*) included the entire 5′ UTR and the first 20 codons of *sopD2*. As expected, this reporter was not regulated by SgrS (Fig. 4*D*). Next, we introduced a T_{+9} to C point mutation within the *sopD2* region to generate essentially the same SgrS site as in the bona fide *sopD* target. Strikingly, this *sopD2*::gfp* reporter was fully regulated by SgrS, displaying approximately fivefold repression (Fig. 4*D*). In a reciprocal approach, we introduced a $C_{+9} \rightarrow T$ point mutation in *sopD::gfp* such that the duplex with SgrS would carry the *sopD2*-like G-U pair (Fig. 4*B*). The resulting *sopD**::gfp* fusion showed the same basal GFP activity as *sopD::gfp* but was not controlled by SgrS (Fig. 4*D*).

To prove that SgrS effectively discriminates between the native *sopD* and *sopD2* mRNAs, we mutated the chromosomal *sopD2* locus (*sopD2**; $T_{+9} \rightarrow C$). In contrast with the lack of regulation of the *sopD2* WT gene (Fig. 4*C*), SopD2 protein synthesis from *sopD2** was as strongly repressed by SgrS as the authentic SopD target (*SI Appendix*, Fig. S3). Thus, a single G-U pair in the seed duplex allows *sopD2* to escape regulation by SgrS.

Target Discrimination by the SgrS Seed at the Level of Translation. The target site location in the proximal coding sequence predicted that SgrS controlled the *sopD* mRNA at the level of translation (36, 37). We used a 70S ribosome translation assay (38–40) to determine the ability of SgrS to inhibit protein synthesis from in vitro synthesized *sopD::gfp* or *sopD2::gfp* mRNAs (Fig. 5*A*). In the absence of SgrS or Hfq, both mRNAs produced comparable amounts of protein with a linear increase over the course of a 30-min assay. Addition of Hfq protein or SgrS sRNA alone had a negligible effect on the translation, which was also reported for the *ptsG* mRNA in the work by Maki et al. (39). In contrast, the combined presence of SgrS and Hfq decreased SopD::GFP levels by ~3.5-fold at the 30-min time point (Fig. 5*A*, *Left*) but failed to repress *sopD2::gfp* (Fig. 5*A*, *Right*). The in vitro translation system offered additional evidence for the discrimination between *sopD* and *sopD2*, and it suggested that, as with SgrS-*ptsG* (39), translational interference is the primary event in SgrS-mediated *sopD* repression.

To narrow down a critical region of SgrS for *sopD* mRNA repression, we tested a seed-derived short SgrS RNA that the work by Maki et al. (38) had shown to suffice for *ptsG* inhibition in vitro. This 14-mer RNA (SgrS nucleotides 168–181) afforded the same fold regulation and selectivity as full-length SgrS with respect to *sopD::gfp* and *sopD2::gfp* translation, including the strict requirement for Hfq (Fig. 5*B* and *SI Appendix*, Fig. S4). The striking overlap with the previous *ptsG* data (38) lead us to conclude that the SgrS seed region, a critical RNA element that had already evolved in *Salmonella*, was acquired for the regulation of the horizontally acquired SopD virulence factor.

Seed Composition Affects Target Discrimination by the G-U Pair. Successful seed pairing of small RNAs with targets has been proposed to largely depend on the thermodynamic stability of the RNA duplex that is formed (41, 42). Accordingly, most target search algorithms, including the popular *RNAhybrid* (43), calculate minimum free energy (MFE) to determine an energy optimum of intermolecular RNA hybridization. Using this al-

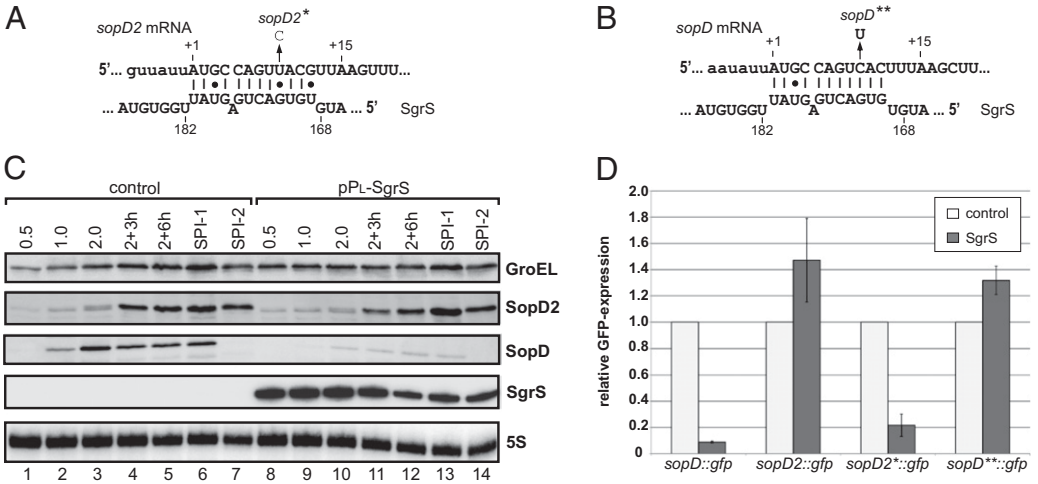

Fig. 4. A single nucleotide exchange protects *sopD2* from targeting by SgrS. (*A*) Graphical presentation of predicted SgrS–*sopD2* interaction. The vertical arrow denotes the U to C change in the *sopD2** allele. (*B*) Graphical presentation of the SgrS–*ptsG* interaction. The vertical arrow denotes the C to U change in the *sopD*** allele. (*C*) Western and Northern blot analyses of strains harboring a chromosomal *sopD2*::3×FLAG allele transformed with a control plasmid (lanes 1–7) or the pP_L-SgrS plasmid (lanes 8–14). Samples were collected at the indicated time points of growth in rich media or under SPI-1– and SPI-2–inducing conditions, and were subjected to Western and Northern blot analyses. Regulation of the SopD::3×FLAG is shown for comparison. (*D*) Quantitative Western blot analysis of *sopD::gfp*, *sopD2::gfp*, *sopD2*::gfp*, and *sopD**::gfp* reporters combined with a control vector or pP_L-SgrS. *Salmonella* (Δ*sgrS*) double transformants were grown in rich media to OD_{600} of 2.0, and total protein samples were subjected to Western blot analysis of GFP protein. Relative GFP expression was calculated from the average of three independent experiments. The standard deviation is indicated by error bars.

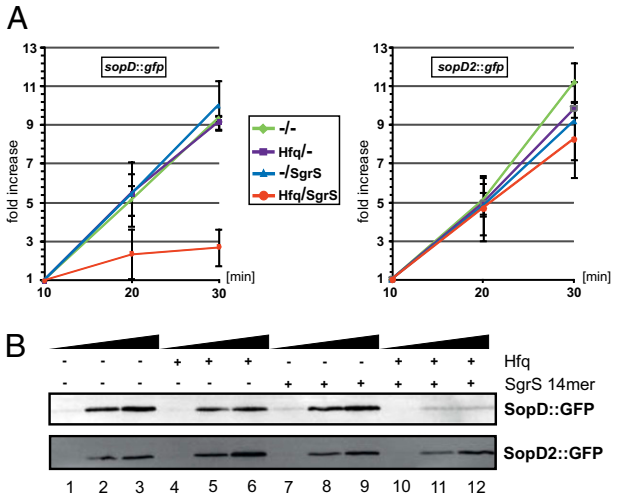

Fig. 5. In vitro translation assay of *sopD*::*gfp* and *sopD2*::*gfp* translation. (*A*) *sopD*::*gfp* (*Left*) and *sopD2*::*gfp* (*Right*) mRNAs were supplemented with mRNAs only, equimolar Hfq, 10-fold excess of SgrS, or a combination of Hfq and SgrS, and they were subjected to in vitro translation assays. Samples were collected at 10, 20, and 30 min postaddition of 70S ribosome, and GFP expression was monitored by Western blot. Experiments were carried out in triplicates, and translation efficiency was normalized to the levels of GFP observed after 10 min of translation. The error bars indicate the standard deviation. (*B*) Analogous to *A*; however, SgrS was exchanged by an RNA-oligo covering the SgrS antisense domain (SgrS residues 168–181) (Fig. 2*A*). Levels of SopD::GFP (*Upper*) and SopD2::GFP (*Lower*) were determined by Western blot, and triplicate results of these experiments are shown in *SI Appendix*, Fig. S4.

gorithm, a typical MFE for bacterial seed pairing ranges from −24.2 to −18.8 kcal/mol^{-1}, which was established with 10 targets of RybB sRNA (44).

The ability of the 14-mer seed to substitute for full-length SgrS in target regulation (see above) allows the 14-mer to be used as a proxy to calculate RNA duplex stability of SgrS and *sopD*/*sopD2* (*SI Appendix*, Table S1). With an MFE of −17.4 kcal/mol, the nonproductive SgrS–*sopD2* interaction was indeed slightly weaker than the productive SgrS–*sopD* pairing (−18.6 kcal/mol), and both were much weaker than SgrS–*ptsG* (−22.7 kcal/mol^{-1}). However, the marginal difference (−1.2 kcal/mol) between SgrS-*sopD* and SgrS-*sopD2* sheds doubt on the assertion that RNA duplex strength is solely responsible for the observed discrimination.

To address this issue, we looked for loss or gain of regulation of the *sopD* and *sopD2* reporters after mutation of the SgrS seed at three selected positions (Fig. 6*A*). First, we changed G_{172} to A (SgrS^{A}), which dramatically increases the MFE (to −13.4 kcal/mol) of the SgrS-*sopD* duplex and also changes its continuity; as expected, *sopD* was no longer regulated (Fig. 6*B*). The same mutation would endow SgrS-*sopD2* with an A-U Watson–Crick pair at the critical G-U pair position. However, because no additional hydrogen bond forms (unlike the three-hydrogen bond G-C pair, both G-U and A-U make only two hydrogen bonds), this mutation does little to MFE and unsurprisingly, failed to bring about regulation of *sopD2* (Fig. 6*B*). These data show that a mere discrimination of a wobble vs. Watson–Crick pair cannot be responsible for the target selectivity of SgrS.

Second, we sought to increase overall duplex strength with a U_{179} → C mutation in SgrS (SgrS^{C}), which changes an unrelated G-U pair to G-C in both SgrS-*sopD* and SgrS-*sopD2*. This change lowered the predicted MFE of the SgrS-*sopD* duplex by −2 kcal/mol^{-1}, and it doubled the regulation of the *sopD* reporter. This SgrS mutation also strengthened the interaction with *sopD2* beyond the stability of the WT SgrS-*sopD* duplex (−19.4 vs. −18.6 kcal/mol) but again, it failed to bring about *sopD2* regulation.

Third, we discovered how to regulate *sopD2* with SgrS, by deleting A_{177} (SgrS$^{\Delta A}$), a nucleotide that bulges out from the predicted SgrS-*sopD* and SgrS-*sopD2* duplexes and weakens them by disrupting helix continuity. SgrS$^{\Delta A}$ has a predicted favorable change of MFE with *sopD2* by −2.8 kcal/mol^{-1}. This single-nucleotide deletion in SgrS brought *sopD2* regulation from zero to eightfold (Fig. 6*B*) (in other words, similar to the level of repression achieved with WT SgrS on the *sopD* reporter). We note that, for *sopD* itself, despite a −1.8 kcal/mol difference in MFE, regulation by the SgrS^{A} and SgrS^{C} mutants of SgrS was the same. Both mutants afforded ∼18-fold repression, indicating potential saturation of regulation.

These data show that, in SgrS seed pairing, positional constraints rather than a continuum of MFE values are responsible for the all or none discrimination of individual seed nucleotides between cognate and near-cognate targets.

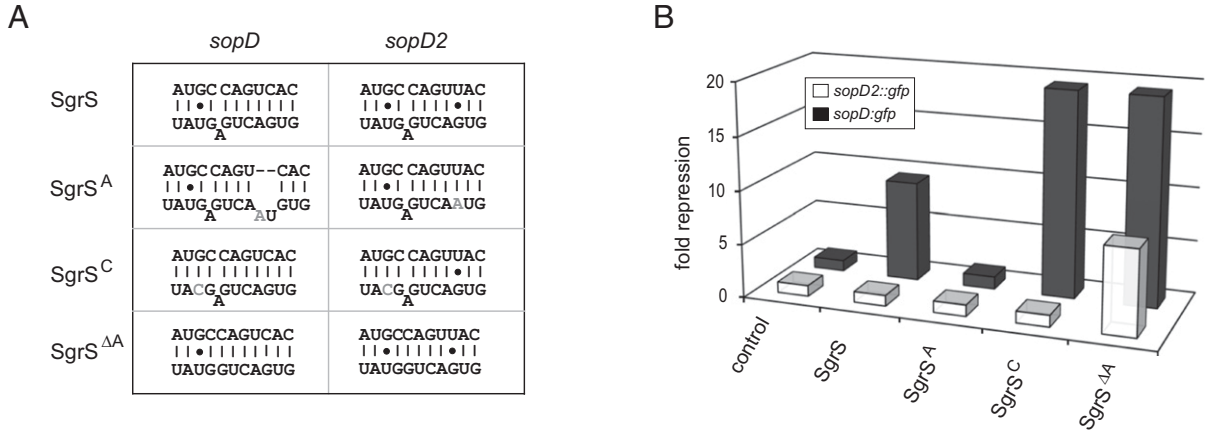

Fig. 6. Systematic analysis of the SgrS–*sopD*/*sopD2* interaction. (*A*) Graphical presentation of the SgrS-*sopD*/*sopD2* duplexes and SgrS mutants used in *B*. Nucleotides exchanged in the SgrS mutants have been marked in gray. (*B*) Quantitative Western blot analysis of *sopD*::*gfp* and *sopD2*::*gfp* reporters combined with a control vector, pP$_L$-SgrS, pP$_L$-SgrS^{A}, pP$_L$-SgrS^{C}, or pP$_L$-SgrS$^{\Delta A}$. *Salmonella* (Δ*sgrS*) double transformants were grown in rich media to OD$_{600}$ of 2.0, and total protein samples were subjected to Western blot analysis of GFP protein. Data are shown as fold repression relative to the control samples (average of three independent experiments). All SgrS variants are expressed equally (*SI Appendix*, Fig. S1).

Discussion

The identification of the recently acquired *sopD* mRNA as an SgrS target in *Salmonella* combined with unexpected non-regulation of the almost identical *sopD2* mRNA reveal that bacterial seed pairing can distinguish between mRNAs at the level of a single hydrogen bond. A critical wobble G-U pair, forming only two of three hydrogen bonds of Watson–Crick G-C, accounts for the deafness of *sopD2* to SgrS. This unprecedented discrimination by a single hydrogen bond seems to be independent of base topology, which can allow noncanonical base pairs to impact the formation and strength of RNA duplexes (45). First, flipping the G-C pair in the SgrS-*sopD* duplex (*SI Appendix*, Fig. S5) maintains target regulation, despite a predicted spatial change of the seed helix. Second, an A-U pair at this position, which restores the glycosidic angle from 54° in G-U wobble to the general 65° of Watson–Crick pairs, does not rescue SgrS-*sopD2* regulation (Fig. 6*B*). By contrast, amending the SgrS-*sopD2* duplex with a single G-C pair allows SgrS to fully repress *sopD2*.

Target regulation by seed pairing is an emerging concept in bacteria. It was inspired by the ability of 22-nt microRNAs of eukaryotes to use their conserved seed region (nucleotides 2–7) to recognize mRNAs (46) and observations that Hfq-associated sRNAs carry conserved short regions that mediate target recognition (40, 44, 47, 48). The latter includes pioneering work on SgrS, in which the work of Vanderpool and Gottesman (21) predicted targeting of *ptsG* mRNA by highly conserved nucleotides of SgrS, which are now referred to as the seed. Subsequently, the work by Kawamoto et al. (26) showed that only six nucleotides of this region acted nonredundantly in *ptsG* repression. Our results with *sopD*/*sopD2* shed light on the SgrS seed, showing that a single nucleotide can make an all or none decision through a critical Watson–Crick pair. This natural example of high-fidelity target selection illustrates how cells may reduce the potential regulatory noise from off-target interactions of sRNAs with near-cognate cellular transcripts. Moreover, our results suggest that bacterial sRNA target predictions, which have chiefly relied on thermodynamic stability of RNA duplexes, must pay attention to G-U pairs in seeds to reduce the number of false positives.

The SgrS–*sopD* interaction is a 7 + 4-bp RNA helix interrupted by a bulged adenosine (Fig. 3*B*); with respect to MFE, it is weaker than most Hfq-dependent sRNA-mRNA duplexes (44, 49) including the well-studied SgrS–*ptsG* interaction (*SI Appendix*, Table S1). In fact, weaker duplexes have been found to allow posttranscriptional control [e.g., FnrS-*folE* (50) or ArcZ-*sdaC* (37)]. Specifically, the MFE of ArcZ-*sdaC* (−17.4 kcal/mol) matches the predicted −17.5 kcal/mol of SgrS-*sopD2*, but SgrS cannot repress *sopD2* (Fig. 4). A minor increase in MFE achieved by fortification of a distal base pair to G-C cannot compensate for the local weakness of the critical G-U pair, which was evident from failure to regulate *sopD2* with the SgrS U_{179} → C variant (Fig. 6*B*). Increasing duplex stability closer to the critical G-U pair by removing the bulged A, however, did bring about regulation of *sopD2* (Fig. 6*B*). Although a recent study of *E. coli* RyhB sRNA concluded that target selection reflected a thermodynamic continuum of general RNA duplex strength (42), our results with SgrS favor a model where the strength of critically positioned base pairs is pivotal to target selection. To test these results further, we monitored SgrS-mediated *sopD* vs. *sopD2* discrimination at lower (20 °C) or elevated (44 °C) temperature. SgrS-mediated down-regulation of *sopD2::gfp* did not occur at either temperature, whereas repression of *sopD::gfp* was observed at both temperatures (*SI Appendix*, Fig. S6), supporting our conclusion that overall changes in RNA duplex stability play a lesser role in target selection. We caution, however, that changes in temperature might also affect RNA duplex formation at a different level, including sRNA-related activity of Hfq (51).

There is an intriguing commonality between bacterial and eukaryotic seed pairing. Animal microRNAs are most potent in gene silencing when fully composed of Watson–Crick base pairs (46, 52). Similarly, a recent survey of target interactions of Hfq-dependent sRNAs suggested that G-U pairs within the first three duplex positions (excluding terminal G-U pairs) are extremely rare (i.e., found in 2 of 49 validated interactions) (49). Nonetheless, judging from the failure to generate *sopD2* regulation by simply changing the critical G-U pair to A-U (Fig. 6), we conclude that local duplex stability within the seed region is more relevant than a simple Watson–Crick vs. non-Watson–Crick discrimination. Additional experiments will be needed to fully understand the biochemical basis of target selection. The successful restoration of selective *sopD::gfp* vs. *sopD2*::*gfp* repression in an in vitro translation assay (Fig. 5) provides an important experimental framework to understand how intrinsic RNA elements, together with Hfq, determine productive regulation.

With a length of ∼240 nt, SgrS has much greater potential sequence space to bind mRNAs, but it uses only the short seed region to select the *sopD* target. Thus, one may liken the sRNA seed sequence to the DNA contact binding face of a transcription factor. Just as the binding of transcription factors is inherently constrained to a fixed pattern in DNA, Hfq-dependent sRNAs with a single seed region such as SgrS are inherently limited in their capability to incorporate new genes into their target suites. Importantly, this constrained capacity is crucially determined by a previously established lead target(s); in the case of SgrS, the ancestral regulation of the *ptsG* and/or *manXYZ* mRNAs in the context of phosphosugar stress (21, 25) has likely shaped the boundaries of flexibility for the evolution of new SgrS–mRNA interactions.

The biological reason for the regulation of *sopD* and not *sopD2* by SgrS remains to be understood. Why is *sopD* regulated in this way? Intuitively, a repression of SopD would suggest a role of SgrS in *Salmonella* virulence, and a global screen in mice did show that *sgrS* played a minor role in virulence (53). We have yet to account for this mild attenuation by phenotypes caused by SgrS deficiency; the Δ*sgrS* strain did not show any attenuation during host cell invasion or intracellular replication assays. Interestingly, a comparative Northern blot analysis indicates that expression of SgrS is differentially controlled between *Salmonella* and nonpathogenic *E. coli* strain MG1655 (*SI Appendix*, Fig. S7). This finding would be consistent with a virulence-associated function of SgrS specific to enteric pathogens with lifestyles that involved colonization and invasion of the inflamed gut epithelium. SopD is known as a general virulence factor, with multiple roles in the development of gastroenteritis, replication in mouse macrophages, and systemic virulence of *Salmonella* (35, 54). SopD also acts as a dual effector delivered by both the SPI-1 and SPI-2 T3SSs that is expressed at later stages of infection when other SPI-1 effectors are no longer produced (22). Considering the broad expression and functions of SopD, it may be necessary to regulate this effector protein at multiple levels, including the posttranscriptional level at which SgrS and Hfq come into play. Intriguingly, although SgrS is primarily viewed as responding to sugar stress, its sugar-dependent expression may be exploited by *Salmonella* to recognize different host cell types and subcellular environments through carbon source availability and accordingly adjust the levels of SopD.

Why then is *sopD2* not regulated by SgrS? The *sopD2* gene is not present in the ancestral *S. bongori* strain (22), and the duplication event at which *sopD* gave rise to *sopD2* likely occurred after or concomitant with the acquisition of major pathogenicity island SPI-2 (55); in other words, it happened very recently on the timescale of enterobacterial evolution. Nonetheless, the *sopD* and *sopD2* genes have already diverged sufficiently to serve distinct functions in host–pathogen interplay (54, 56) as well as differ with respect to their own transcriptional control (57, 58). Perhaps the selective SgrS-mediated repression of *sopD* fostered the func-

tional diversification of the two genes. Sequence comparison of the two genes strongly argues that the regulation of *sopD* vs. nonregulation of *sopD2* has been under selective pressure (*SI Appendix*, Fig. S2). That is, although mutations have accumulated throughout both genes, the first six codons containing the SgrS site have been fully conserved (*SI Appendix*, Fig. S2) (22). This N-terminal domain is crucial for delivery of SopD through the T3SS and essential for virulence (59). All base changes in this region of *sopD* or *sopD2* were silent in terms of amino acid sequence (*SI Appendix*, Fig. S2), and this finding includes the conserved crucial C/U discrepancy at position +9 of *sopD* and *sopD2* (Fig. 4). Therefore, the development of this point mutation has enabled selective posttranscriptional control of the two mRNAs by SgrS that maintains the ability of the encoded proteins to be delivered by the T3SS. Generally, gene duplication events play important roles in the evolution of new biological functions (60), and HGT genes show a higher propensity than indigenous genes to undergo duplication (61). In *Salmonella*, additional effector protein pairs, such as SifA and SifB, resulted from gene duplication (35), and it will be interesting to see whether these pairs are also under selective sRNA-mediated control.

In conclusion, previous global phenotypic and gene expression analyses (18) as well as RNA–protein interaction studies (13) raised the possibility that Hfq-associated sRNAs could directly engage in the regulation of secreted effectors of *Salmonella*. Our discovery that SgrS regulates SopD synthesis provides proof of such a scenario; it suggests that conserved sRNAs with seemingly unrelated physiological functions constitute a reservoir of regulators that act like conserved transcription factors to tame foreign genes and integrate them into existing regulons. In addition to the HGT genes of the major SPI-1 and SPI-2 regions, *Salmonella* possesses another 12 pathogenicity islands and many single virulence genes (62); most transcripts from these regions are targets of Hfq. We predict that Hfq governs a large network of post-transcriptional control of HGT genes by either conserved or *Salmonella*-specific sRNAs that have evolved since *Salmonella* and *E. coli* diverged from each other 100–160 million years ago (63).

Experimental Procedures

Bacterial Strains and Growth. Bacterial strains and their construction details are listed in *SI Appendix*, Table S2. Strains were grown at 37 °C in LB or on LB plates. Ampicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (20 μg/mL), and L-arabinose (0.2%) were added where appropriate. *Salmonella* WT (SL1344) or mutant strains were transformed with plasmids by electroporation. SPI-1– and SPI-2–inducing conditions were as described (13, 18). Briefly, for SPI-1 induction, cultures were inoculated in 5 mL LB containing 0.3 M NaCl in 15-mL Falcon tubes with tightly closed lids. Cultures were incubated vertically with shaking for 12 h at 37 °C.

Oligonucleotides and Plasmids. Oligonucleotides (DNA and RNA) and plasmids are listed in *SI Appendix*, Tables S3 and S4. Details on plasmids construction are given in *SI Appendix*, *SI Methods*. Target fusions to *gfp* were constructed as described (34).

Western Blot Analysis and Plate Fluorescence. Culture samples were taken according to 1 OD_{600} and centrifuged for 4 min at 16,100 × *g* at 4 °C, and pellets resuspended in sample loading buffer to a final concentration of 0.01 OD/μL. After denaturation for 5 min at 95 °C, 0.1-OD equivalents of sample were separated on SDS gels. Western blot analyses of GFP and FLAG fusion proteins followed previously published protocols (34).

Northern Blot and Microarray Experiments. Total RNA was prepared and separated in 5% or 6% (vol/vol) polyacrylamide–8.3 M urea gels (5–10 μg RNA per lane) and blotted as described (18). Membranes were hybridized at 42 °C with gene-specific (32P) end-labeled DNA oligonucleotides in Rapid-hyb buffer (GE Healthcare). Microarray experiments were carried out as described before (30), and SgrS pulse expression was achieved using the pBAD-based pKP12-2 plasmid. Microarray data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession code GSE34851).

In Vitro Translation Assays. These assays were carried out with modifications as previously described in ref. 40. Briefly, DNA templates carrying a T7 promoter sequence for in vitro transcription were generated by PCR. Primers and sequences of the T7 transcripts are given in *SI Appendix*, Table S4. T7 templates of *gfp* fusion mRNAs were amplified from plasmids using a sense primer that adds a T7 promoter to the +1 site of the 5′ UTR and an antisense oligo pZE-T1 122 nt downstream from the *gfp* stop codon. These transcripts end with the *rrnB* terminator of the fusion plasmids. RNA was in vitro-transcribed and quality-checked as described in ref. 18. Translation reactions were carried out using PureSystem (PGM-PURE2048C; Cosmo Bio Co., Ltd) according to the manufacturer's instructions. Reactions (10 μL) contained, in addition to 70S ribosomes, mRNA template (40 nM), Hfq (40 nM), and where applicable, full-length SgrS RNA or the 14-mer RNA oligonucleotide. Before addition of PureSystem mix, RNA was denatured for 1 min at 90 °C and chilled on ice for 5 min. Hfq was mixed with mRNA (and sRNA/oligonucleotide) and preincubated for 10 min at 37 °C. PureSystem mix was added, and incubation continued at 37 °C for the time indicated in the figures. Reactions were stopped with 4 volumes ice-cold acetone and kept on ice for 15 min, and proteins were collected by centrifugation (10,000 × *g* for 10 min at 4 °C). Proteins were quantified by Western blot analysis using a monoclonal GFP antibody.

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