A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide

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SgrS is a 227-nt small RNA that is expressed in *Escherichia coli* during glucose-phosphate stress, a condition associated with intracellular accumulation of glucose-6-phosphate caused by disruption of glycolytic flux. Under stress conditions, SgrS negatively regulates translation and stability of the *ptsG* mRNA, encoding the major glucose transporter, by means of a base pairing-dependent mechanism requiring the RNA chaperone Hfq. SgrS activity mitigates the effects of glucose-phosphate stress, and the present study has elucidated a function of SgrS that is proposed to contribute to the stress response. The 5′ end of SgrS, upstream of the nucleotides involved in base pairing with the *ptsG* mRNA, contains a 43-aa ORF, *sgrT*, that is conserved in most species that contain SgrS-like small RNAs. The *sgrT* gene is translated in *E. coli* under conditions of glucose-phosphate stress. Analysis of alleles that separate the base pairing function of SgrS from the *sgrT* coding sequence revealed that either of these functions alone are sufficient for previously characterized SgrS phenotypes. SgrS-dependent down-regulation of *ptsG* mRNA stability does not require SgrT and SgrT by itself has no effect on *ptsG* mRNA stability. Cells expressing *sgrT* alone had a defect in glucose uptake even though they had nearly wild-type levels of PtsG ($IICB^{Glc}$). Together, these data suggest that SgrS represents a previously unrecognized paradigm for small RNA (sRNA) regulators as a bifunctional RNA that encodes physiologically redundant but mechanistically distinct functions contributing to the same stress response.

riboregulation | RNA stability | small proteins | phosphoenolpyruvate phosphotransferase system | glycolytic flux

Central metabolism is controlled by a complex regulatory network at many levels, including transcription, translation, and allosteric control of enzymes. Although it has been the subject of decades of research, knowledge of the mechanisms controlling glycolytic flux are still incomplete, even in the well studied model organism *Escherichia coli*. Numerous studies have noted that bacterial strains with an impaired capacity to metabolize phosphorylated sugars (including some of the substrates of glycolysis) often show strong phenotypes of growth inhibition or in some cases cell lysis (1–3). However, until recently, little was known about the mechanisms used by bacterial cells to deal with metabolic stress associated with intracellular phosphosugar accumulation. One form of phosphosugar stress occurs in *pgi* (phosphoglucose isomerase) mutant strains where stress is associated with accumulation of glucose-6-phosphate (G6P) when cells are exposed to glucose (4–6). An analogous condition occurs in wild-type strains exposed to the nonmetabolizable glucose analog α-methyl glucoside (αMG), resulting in accumulation of αMG-6-phosphate. In both situations, cell growth is inhibited and there is a specific destabilization of the *ptsG* mRNA, which encodes the major glucose transporter of the phosphoenolpyruvate phosphotransferase system (PTS) in *E. coli* (PtsG, $IICB^{Glc}$) (4, 5). This posttranscriptional regulation of the *ptsG* mRNA under conditions of G6P accumulation (glucose-phosphate stress) suggested the existence of at least one specific regulatory response to deal with such stresses.

We discovered a small RNA regulator, SgrS, that is induced under glucose-phosphate stress conditions and is responsible for destabilization of *ptsG* mRNA (6). Expression of SgrS during glucose-phosphate stress is clearly important for the adaptation to stress because *sgrS* mutant strains are strongly inhibited compared with wild-type strains under these conditions (6). The negative regulation of translation and stability of *ptsG* mRNA by SgrS stops synthesis of glucose transport proteins (7) and is hypothesized to limit further accumulation of G6P or αMG6P. SgrS is encoded divergently from *sgrR*, which encodes the transcriptional activator required for SgrS synthesis (6). SgrS is 227 nt in length and was originally identified on the basis of its binding to the RNA chaperone Hfq (8). Previously characterized small RNA (sRNA) regulators that require Hfq for function regulate mRNA targets via sRNA:mRNA base pairing interactions, a mechanism referred to as riboregulation. Hfq binding stabilizes most sRNAs and in some cases remodels secondary structure to facilitate base pairing (9–11) and increase the rate of sRNA:mRNA association (12). SgrS activity on *ptsG* mRNA requires an Hfq-mediated base pairing interaction between sequences at the 3′ end of SgrS (Fig. 1) and sequences in the 5′ untranslated region of *ptsG* mRNA. Although many sRNAs have multiple mRNA targets, our unpublished studies to identify other putative SgrS targets revealed only two additional candidates that might be down-regulated by SgrS at the level of mRNA stability. Again, sequences at the 3′ end of SgrS were predicted to base pair with these other targets, leaving open the question of the role of the SgrS 5′ end.

Although there are several mechanisms of regulation by sRNAs, most regulatory sRNAs in bacteria are ≈100 nt long, act by base pairing with mRNA targets, and are not predicted to encode protein products. One exception is the 500-nt RNA III in *Staphylococcus aureus* that functions by base pairing with several mRNA targets (13–15) and also encodes the small protein delta hemolysin. RNA III is somewhat unique in that it appears that the base pairing-dependent regulation performed by RNA III does not require Hfq (16). In this study, we report SgrS as an example of an Hfq-dependent sRNA regulator that also encodes a functional protein product. This opens up the possibility that there are other such bifunctional sRNAs that

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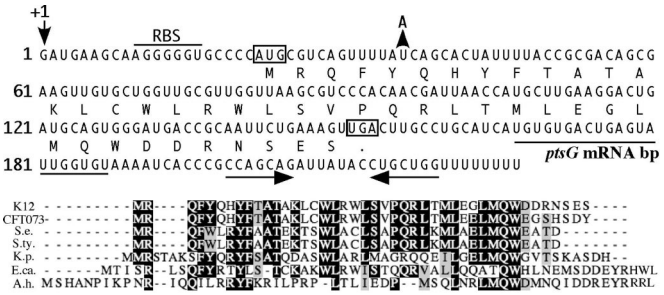


Fig. 1. The SgrS sRNA contains a conserved ORF, *sgrT*. (*Upper*) The SgrS sequence is shown with the translated product of *sgrT* below the nucleotide sequence. The putative ribosome binding site for *sgrT* is indicated by the horizontal bar labeled "RBS." The start and stop codons for *sgrT* are boxed. The fifth codon of *sgrT* was mutated to a "TAA" by a single base pair substitution indicated by the arrow and "A" (described in the text and in Fig. 2). The sequences involved in base pairing with the *ptsG* mRNA (7) are indicated by the horizontal line (labeled "*ptsG* mRNA bp") below the nucleotide sequences. The inverted repeat that forms the terminator at the 3′ end of SgrS is indicated by horizontal arrows below the nucleotide sequence. (*Lower*) The SgrT amino acid sequences from *E. coli* K12 (K12) and other bacterial species were aligned with ClustalW. Matches to the consensus are shaded. CFT073, *E. coli* CFT073; S.e., *Salmonella enterica paratyphi ATCC9150*; S.ty., *Salmonella enterica* serovar *Typhi Ty2*; K.p., *Klebsiella pneumoniae KP32*; E.ca., *Erwinia carotovora atroseptica SCRI1043*; A.h., *Aeromonas hydrophila ATCC7966*.

have not yet been discovered and expands the mechanistic repertoire of these versatile regulators.

Results

The Small Regulatory RNA SgrS Contains a Conserved ORF, *sgrT*. We have designated the ORF within the SgrS RNA sequence *sgrT*. This ORF is located at the 5′ end of SgrS and encodes a 43-aa polypeptide (Fig. 1); sequences that base pair with the *ptsG* mRNA are downstream from the stop codon of *sgrT*. SgrS-like sRNAs are fairly well conserved and can be found in the same genomic location in several bacterial species (6). Examination of SgrS orthologs from uropathogenic *E. coli* CFT073, *Salmonella* species, *Klebsiella pneumoniae*, and *Erwinia carotovora* revealed *sgrT* sequences that are quite well conserved with that of *E. coli* K12 (Fig. 1). Another putative *sgrT* ortholog was identified in *Aeromonas hydrophila*, a Gram-negative organism more distantly related to the aforementioned enteric species (Fig. 1). Interestingly, *E. coli* 0157:H7 and *Yersinia pestis* have SgrS orthologs; however, these RNAs do not appear to contain *sgrT*. The published sequence for *E. coli* 0157:H7 indicates that the *sgrT* start codon contains a substitution that alters the sequence to "ATT." The SgrS ortholog in *Y. pestis* is well conserved at the 3′ end (where base pairing sequences reside) but is truncated at the 5′ end relative to the other SgrS orthologs and does not appear to contain *sgrT*. Despite these exceptions, *sgrT* is conserved in the majority of organisms with an *sgrS* ortholog, suggesting that it encodes a functionally important protein.

Base Pairing and SgrT Functions both Play a Role in the Glucose-Phosphate Stress Response. Because the sequences involved in base pairing with *ptsG* mRNA are downstream from *sgrT*, several constructs were created (Fig. 2) to individually analyze these two putative functions of SgrS. All of the *sgrS* and *sgrT* derivatives were cloned on plasmids and were expressed under the control of a heterologous promoter (P_{lac}) that is IPTG-inducible. Wild-type SgrS possesses both base pairing and *sgrT* sequences [pP_{lac}-*sgrS* or pLCV1; supporting information (SI) Table 1 and Fig. 2]. [This construct was previously shown to complement a chromosomal Δ*sgrS*::kan mutation (6).] A single-base substitution in the fifth codon of *sgrT* created a "UAA" stop codon,

Plasmid name	Construct	Diagram
pHDB3	vector	
pLCV1	P_{lac}-*sgrS*	bp
pLCV5	P_{lac}-$sgrS_{UAA}$	bp
pBRCV7	P_{lac}-*sgrT*	
pBRCV8	P_{lac}-$sgrT_{UAA}$	

Fig. 2. Construction of alleles that separate base pairing and SgrT functions. Plasmids are described in more detail in *Methods* and SI Table 1. All constructs are under the control of the P_{lac} promoter. The SgrS molecule is depicted as an arrow, where the arrowhead is the 3′ end of SgrS. The location of *sgrT* is represented by the shaded rectangle at the 5′ end, and the location of the base pairing sequences at the 3′ end is indicated by the label "bp." $sgrS_{UAA}$ contains a stop codon that truncates the *sgrT* ORF. The *sgrT* construct lacks the base pairing region. The $sgrT_{UAA}$ construct lacks the base pairing region and also contains a truncated *sgrT* ORF.

resulting in plasmid pP_{lac}-$sgrS_{UAA}$ (pLCV5; SI Table 1 and Figs. 1 and 2). $SgrS_{UAA}$ should be able to perform the riboregulatory function of SgrS but not produce SgrT protein. To examine SgrT function in the absence of the base pairing sequences of SgrS, *sgrT* was cloned with a heterologous ribosome binding site (RBS), resulting in plasmid pP_{lac}-*sgrT* (pBRCV7; SI Table 1 and Fig. 2). As a negative control, a "UAA" stop codon was placed at the fifth codon of *sgrT*, resulting in plasmid pP_{lac}-$sgrT_{UAA}$ (pBRCV8; SI Table 1 and Fig. 2). This construct lacks the base pairing sequences and does not produce SgrT.

We showed previously that cells with wild-type SgrS have two prominent phenotypes related to glucose utilization and the glucose-phosphate stress response. SgrS activity is necessary for full recovery from glucose-phosphate stress caused by exposure of cells to αMG (6). Overexpression of SgrS also strongly inhibits growth when glucose is the sole carbon source (6). We originally hypothesized that both of these phenotypes were due to the base pairing function of SgrS and its activity on mRNA targets. Upon discovering *sgrT* within the SgrS sRNA, we set out to test whether SgrT also participates in glucose metabolism or stress physiology. A Δ*sgrS*::kan, $lacI^{q+}$ host (where expression of alleles is repressed in the absence of IPTG) carrying plasmids described in Fig. 2 was analyzed for αMG stress recovery and growth on glucose. For αMG stress recovery, cells were grown with inducer (IPTG) to early logarithmic phase, and αMG was added to induce stress. As shown previously (6), cells lacking *sgrS* (vector; Fig. 3*A*) were strongly inhibited and failed to recover from stress, whereas cells carrying pP_{lac}-*sgrS* (SgrS; Fig. 3*A*) recovered well. Cells expressing the $sgrS_{UAA}$ allele were able to recover from stress as well as cells expressing wild-type *sgrS* (Fig. 3*A*). This result suggested that *sgrT* is not absolutely essential for stress recovery under these conditions when the SgrS base pairing function is intact. Expression of the *sgrT* allele also rescued cell growth (Fig. 3*A*). The $sgrT_{UAA}$ allele failed to rescue growth (Fig. 3*A*), confirming that the SgrT phenotype was attributable to a functional SgrT polypeptide and not to nucleotide sequences within the 5′ region of SgrS. The same loss of rescue was observed with another *sgrT* allele where the ATG start codon was changed to a TAA stop codon (SI Fig. 7). Together, these results provided strong genetic evidence that *sgrT* encodes a functional protein that can participate in the glucose-phosphate stress response. The fact that alleles that possessed either base pairing ($sgrS_{UAA}$) or SgrT (*sgrT*) functions could rescue cells from stress suggests that these two properties of the SgrS sRNA may be physiologically redundant.

These same alleles were tested for the other known SgrS phenotype: growth inhibition when glucose is the sole carbon source. Cells expressing the constructs shown in Fig. 2 were grown in minimal glucose medium. Vector-containing cells grew

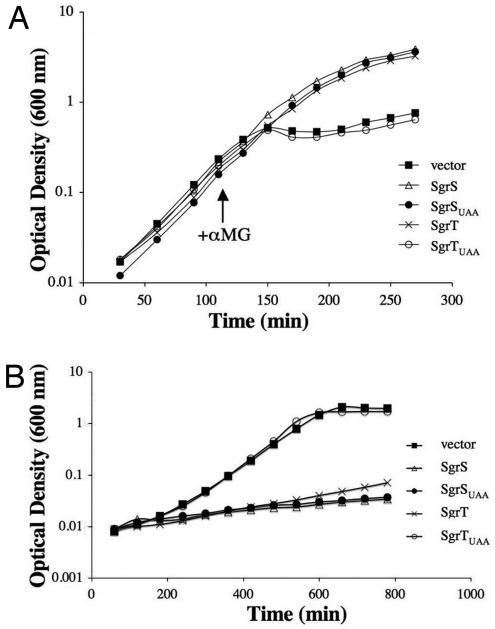


Fig. 3. Effect of SgrS base pairing and SgrT on recovery from glucose-phosphate stress and growth in glucose minimal medium. (*A*) A Δ*sgrS*::kan, *lacI*$^{q+}$ host strain (CV104) carrying plasmids (pHDB3, pLCV1, pLCV5, pBRCV7, and pBRCV8) with alleles described in Fig. 2 was grown in LB with ampicillin and IPTG. Cells were stressed by addition of 0.5% αMG at early log phase. The data shown are representative of at least three independent experiments. (*B*) The strains described in *A* were grown in minimal A medium with glucose in the presence of ampicillin and IPTG. The results shown are representative of at least three independent experiments.

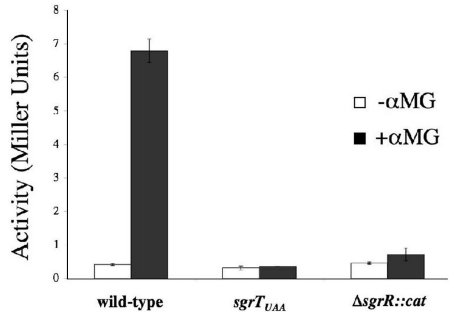


Fig. 4. Translation of *sgrT* is activated under stress conditions in an SgrR-dependent manner. All strains carry a translational *sgrT′-′lacZ* fusion at the native *sgrT* locus. Strains BH300 (wild-type), BH301 (*sgrT*$_{UAA}$), and BH302 (Δ*sgrR*::*cat*) were grown in rich medium to mid-log phase and split, and half of each culture was exposed to 0.005% αMG. β-galactosidase activity was measured at several time points thereafter. The data displayed are the average of three experimental trials at 3 h after exposure.

to a high final cell density (Fig. 3*B*). As expected, cells overexpressing wild-type SgrS were strongly inhibited and failed to grow significantly (Fig. 3*B*, compare vector and SgrS). Interestingly, both SgrS$_{UAA}$ and SgrT inhibited cell growth on glucose to nearly the same degree as wild-type SgrS (Fig. 3*B*). Cells expressing SgrT$_{UAA}$ were not inhibited and grew as well as vector-containing cells. These results suggested that either riboregulation or SgrT function is sufficient for this phenotype.

A previous study showed that overexpression of SgrS caused growth inhibition primarily on glucose and slightly on mannose but not on other carbon sources tested (6). The specificity of SgrT-mediated growth inhibition was tested by streaking SgrT-overproducing cells on minimal media with one of a number of different carbon sources. On plates, SgrT-dependent growth inhibition was most striking when glucose was the sole carbon source. SgrT-overproducing cells were slightly inhibited for growth on mannose and *N*-acetylglucosamine but not detectably inhibited on mannitol, fructose, or casamino acids (SI Fig. 8). One of the predicted targets for SgrS riboregulation that emerged from microarray studies (C.K.V. and S. Gottesman, unpublished data) is the polycistronic *manXYZ* message. These genes encode a PTS transporter of relatively broad sugar specificity, including mannose and *N*-acetylglucosamine. The fact that SgrT overproduction inhibits growth on mannose and *N*-acetylglucosamine is consistent with other data (Fig. 3) that suggest that SgrS riboregulation and SgrT functions are somehow redundant.

To obtain biochemical evidence that *sgrT* phenotypes were associated with a specific protein product, an epitope-tagged SgrT protein was constructed and its production monitored by Western blot analysis. A sequence specifying three tandem FLAG epitopes (3XFLAG) was inserted at the 3′ end of *sgrT* in the context of pP$_{lac}$-*sgrT*. The P$_{lac}$-*sgrT3XFLAG* allele rescued cells from αMG stress and caused growth inhibition on glucose (data not shown), indicating that the epitope at the C terminus of SgrT did not significantly interfere with the function of the protein. A Western blot revealed that these cells produced a protein that migrated at ≈8 kDa, as predicted for SgrT-3XFLAG (SI Fig. 9). When the UAA mutation was introduced in the SgrT-3XFLAG construct, the protein was no longer made (SI Fig. 9).

***sgrT* Is Translated Under Glucose-Phosphate Stress Conditions.** In the experiments described above, *sgrT* was ectopically expressed from a foreign promoter. To determine whether *sgrT* is translated under glucose-phosphate stress conditions in its native context, an *sgrT′-′lacZ* translational fusion was constructed. This fusion was placed in the natural chromosomal locus and joins *sgrT* coding sequence at the 38th codon with *′lacZ*. The fusion was constructed in three strain backgrounds: wild-type, *sgrT*$_{UAA}$, and Δ*sgrR*::*cat*. Because insertion of the fusion at the native locus effectively renders the fusion strains *sgrS* null mutants, a low concentration of αMG was used to avoid strong growth inhibitory effects. Strains were grown in rich (TB) medium and exposed to αMG in mid-log phase. β-galactosidase assays were performed on samples collected at several times after the addition of αMG. The basal level of activity in all strains in the absence of stress was very low (Fig. 4), but addition of αMG induced *sgrT′-′lacZ* activity in the wild-type background by ≈14-fold (Fig. 4). The data shown are from 3 h after induction; activity continued to increase as long as cells were cultured in the presence of αMG, reaching a level 25-fold greater than in uninduced cells after overnight culture (data not shown). When a stop codon was inserted upstream of the fusion junction (*sgrT*$_{UAA}$), αMG-inducible activation of the fusion was abrogated. This indicates that *sgrT* is indeed translated under glucose-phosphate stress conditions in its native context. In the Δ*sgrR*::*cat* background, activation of the fusion was also eliminated (Fig. 4). This result is not surprising because we have shown that synthesis of the sRNA SgrS (which encodes *sgrT*) under stress conditions depends on the transcription factor SgrR (6, 17).

SgrT Does Not Participate in Posttranscriptional Regulation of the *ptsG* mRNA. The hypothesis that SgrT may be redundant or cooperate with the base pairing function of SgrS in inhibiting translation and causing degradation of the *ptsG* mRNA was tested. In previous work, we showed that upon glucose phosphate stress, levels of SgrS increase, whereas levels of the *ptsG* mRNA diminish rapidly in an SgrS-dependent manner (6). Aiba

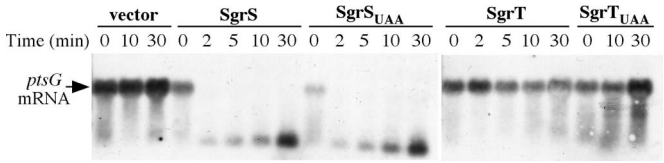
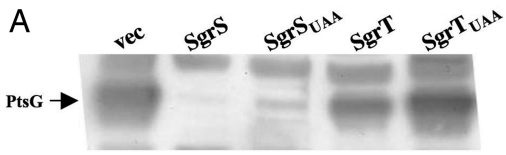


Fig. 5. SgrT does not affect levels of *ptsG* mRNA. The Δ*sgrS*::kan, *lacI*$^{q+}$ strain CV104 carrying constructs depicted in Fig. 2 was grown to mid-log phase in LB with ampicillin. Northern blot analysis was performed on total RNA extracts harvested at times indicated after cells were exposed to IPTG to induce expression of *sgrS* and *sgrT* constructs. The blot was probed for *ptsG* mRNA (indicated at the left).

and coworkers (4) showed that declining levels of *ptsG* message were due to the RNase E-dependent destabilization of the *ptsG* mRNA. To determine whether SgrT is involved in destabilizing the *ptsG* mRNA, the alleles shown in Fig. 2 were expressed in a *lacI*$^{q+}$, *sgrS* mutant host, and the levels of *ptsG* mRNA were monitored at different time points after expression. As observed previously (6), in the absence of *sgrS*, the levels of *ptsG* mRNA remain steady, whereas when SgrS is expressed, the *ptsG* mRNA disappears (Fig. 5) because of RNase E-dependent degradation (4). The SgrS$_{UAA}$ variant also caused disappearance of the *ptsG* mRNA, suggesting that the base pairing function of SgrS does not require SgrT and is sufficient for posttranscriptional regulation of the *ptsG* mRNA. Northern blot analysis probing for SgrS and SgrS$_{UAA}$ showed that these two molecules accumulate after induction to approximately the same levels (SI Fig. 10), suggesting that the UAA mutation does not have a deleterious effect on the stability of SgrS$_{UAA}$. When SgrT alone was expressed, levels of the *ptsG* mRNA did not change significantly (Fig. 5). Likewise, the negative control SgrT$_{UAA}$ did not alter levels of the *ptsG* mRNA (Fig. 5). These results indicate that the role of SgrT is independent of the base pairing function of SgrS and that SgrT acts at a different level in the glucose-phosphate stress response.

Base Pairing and SgrT Functions both Inhibit Glucose Uptake but by Different Mechanisms. Wild-type cells recover from glucose-phosphate stress in a relatively short period and continue growing. Our current model for SgrS riboregulation of the *ptsG* message provides a mechanism for the cell to reduce influx of sugar phosphates indirectly by reducing the production of new sugar transport proteins. SgrT might contribute to recovery by a different mechanism; for example, by reducing the levels or activity of preexisting PtsG proteins. To examine the role of SgrS base pairing and SgrT on steady state levels of PtsG protein, the alleles described in Fig. 2 were expressed in a *lacI*$^{q+}$, *sgrS* mutant background, and Western blot analysis was used to detect PtsG (IICB^{Glc}). Levels of the PtsG protein were significantly lower in cells expressing SgrS and SgrS$_{UAA}$ compared with vector control cells (Fig. 6*A*). This result is consistent with the idea that SgrS molecules that can perform base pairing-dependent down-regulation of the *ptsG* mRNA stop new synthesis of PtsG protein, and preexisting PtsG is diluted as the cells continue to grow. PtsG levels in cells expressing SgrS$_{UAA}$ (containing the premature stop codon in *sgrT*) were approximately equivalent to levels in cells expressing the wild-type SgrS. This result again suggests that SgrT is not required and does not significantly contribute to the riboregulatory function of SgrS. Cells expressing SgrT alone had high levels of PtsG protein, as did cells expressing the negative control SgrT$_{UAA}$ (Fig. 6*A*). This result strongly suggests that SgrT does not function by promoting degradation of preexisting PtsG protein and led to the hypothesis that SgrT inhibits the transport activity of PtsG.

The data above show that SgrT does not affect levels of *ptsG* mRNA or PtsG protein during stress. If SgrT acts at a post-



B

Construct	% Glucose Remaining
Vector	25.2 ± 7.51
SgrS	93.5 ± 3.66
SgrS$_{UAA}$	100 ± 6.38
SgrT	92.3 ± 7.23
SgrT$_{UAA}$	16.8 ± 4.99

Fig. 6. SgrS base pairing and SgrT functions individually block glucose uptake by different mechanisms. (*A*) Strains carrying plasmid constructs (as in Fig. 2) were grown in MOPS defined medium with glucose and amino acids with IPTG. Western blot analysis was performed on total protein extracts from samples harvested after 5 h of growth. The blot was probed for PtsG by using an αIIB^{Glc} antibody. The position of the PtsG (IICB^{Glc}) protein is indicated at left. Bands above and below the PtsG band are cross-reacting proteins and served as loading controls. (*B*) Strains are as described in *A*. Cells were harvested after 5 h of growth, and the amount of glucose remaining in the medium was measured. The numbers reported represent the amount of glucose remaining at 5 h divided by the amount of glucose in media before inoculation (% glucose remaining). The average of three independent experiments is reported.

translational level on PtsG to inhibit its activity, this could account for the stress rescue and glucose growth inhibition phenotypes (Fig. 3). Alternatively, SgrT might interfere with metabolism of glucose at a step subsequent to transport. To test the hypothesis that SgrT interferes with glucose transport, the *lacI*$^{q+}$, *sgrS* mutant strain with induced *sgrS* and *sgrT* alleles (as shown in Fig. 2) was grown in defined medium (MOPS) with glucose and amino acids. The growth rate of cells expressing SgrS, SgrS$_{UAA}$, and SgrT was reduced compared with vector control and SgrT$_{UAA}$ cells (SI Fig. 11). (Growth of the former strains was not completely inhibited, as in Fig. 3*B*, because the medium used in this experiment contains amino acids that can be used as a carbon source.) The amount of glucose remaining in the supernatant was measured and normalized to the amount in the medium before cell inoculation (set at 100%). Cells carrying the vector control and negative control *sgrT$_{UAA}$* plasmids used a significant portion of the glucose present; only ≈20% of the initial amount remained (Fig. 6*B*). In contrast, culture supernatants from cells expressing *sgrS*, *sgrS$_{UAA}$*, or *sgrT* contained >90% of the initial amount of glucose (Fig. 6*B*), indicating that expression of each of these alleles prevented cells from taking up glucose. Together, these results support the model that the mechanism for inhibition of glucose uptake in cells expressing base pairing-proficient SgrS molecules is strong reduction of the amount of glucose transport protein. In contrast, SgrT-mediated inhibition of glucose uptake appears to occur at the level of PtsG activity.

SgrT Overexpression Prevents Inducer Exclusion. The glucose-specific PTS enzyme IIA^{Glc} phosphorylates IICB^{Glc} (PtsG) and also has important regulatory functions in carbon catabolite repression (18). The regulatory functions of IIA^{Glc} depend on its phosphorylation state, which in turn depends on IICB^{Glc} transport activity. If IICB^{Glc} is not actively transporting glucose, IIA^{Glc} is phosphorylated. If IICB^{Glc} is actively transporting glucose, IIA^{Glc} is mainly dephosphorylated and dephospho-IIA^{Glc} is responsible for inducer exclusion. Dephospho-IIA^{Glc} binds to transporters for other carbon sources, including the

lactose permease, and prevents their transport activity. This is one mechanism in wild-type cells that accounts for repression of lactose-inducible genes—e.g., *lacZ*—when cells are grown in the presence of glucose and lactose. We used this property of the PTS to indirectly measure IICB^{Glc} (PtsG) transport activity. We predicted that if SgrT inhibits glucose transport through PtsG, it should increase levels of phosphorylated IIA^{Glc} and therefore relieve repression of β-galactosidase (LacZ) activity in cells growing on glucose and lactose. A Δ*sgrS*::kan, *lac*$^+$ strain carrying vector control or P$_{lac}$-*sgrT* plasmids was grown in rich medium with glucose and lactose, and β-galactosidase activity was measured. In cells carrying the vector control, the activity was low (25 Miller units in mid-log phase), reflecting that glucose inhibited uptake of lactose, the inducer and substrate of LacZ (SI Fig. 12). In contrast, cells producing SgrT had dramatically higher levels of β-galactosidase activity (1557 Miller units in mid-log phase) (SI Fig. 12), indicating that SgrT somehow interferes with inducer exclusion. This result further supports our hypothesis that SgrT inhibits glucose transport at the level of PtsG transport activity.

Discussion

Given the relatively large size of SgrS (227 nt), we initially anticipated that SgrS might regulate a large set of mRNA targets similar to other characterized sRNAs; e.g., RyhB, which regulates at least 18 mRNAs by base pairing (19). Instead, the studies presented here suggest an alternative function for SgrS. We identified a conserved ORF in the 5′ region of SgrS that we designated *sgrT*. Genetic and biochemical data support the notion that the SgrT polypeptide itself has a distinct function that can promote recovery from stress and negatively affect glucose transport. Although the phenotypes for base pairing-only or *sgrT*-only alleles are the same—i.e., growth rescue (Fig. 3*A*), growth inhibition on glucose (Fig. 3*B*), and glucose uptake inhibition (Fig. 6*B*)—our data indicate that different mechanisms are used to effect these outcomes. The riboregulation function acts through promoting *ptsG* mRNA degradation (Fig. 5) (4, 6) and inhibiting synthesis of PtsG (Fig. 6*A*) (7). SgrT does not affect levels of *ptsG* mRNA or PtsG protein (Figs. 5 and 6*A*) yet still inhibits glucose uptake (Fig. 6*B*). Together, these data suggest that the two functions encoded on SgrS have some physiological redundancy.

We propose that the small RNA SgrS encodes two distinct functions that participate in the glucose phosphate stress response. Our previous studies (6), as well as those of Aiba and coworkers (12), have shown that SgrS base pairing activity is required for down-regulation of *ptsG* mRNA stability and for preventing new synthesis of IICB^{Glc} proteins under stress conditions. Stopping new synthesis of glucose transporters may not be sufficient for relief from stress under conditions where preexisting glucose transporters remain competent for glucose uptake because phosphosugars would continue to accumulate. The identification of SgrT and the evidence for its role in inhibiting glucose transporter activity suggest a mechanism to overcome this problem. It is remarkable that the 227-nt SgrS molecule apparently carries out dual strategies, riboregulation and coding for a protein inhibitor of glucose transport, that reduce uptake of glucose-phosphate under conditions where it is not appropriately metabolized. Inhibition of glucose transport at the level of IICB^{Glc} (PtsG) activity by SgrT might be mediated by protein–protein interactions that result in "plugging" the transport channel or inhibiting IICB^{Glc} phosphorylation. A prediction of this model is that SgrT mediates a rapid response to stress that reduces influx of sugar phosphates without affecting levels or stability of the transport protein. The riboregulation activity of SgrS might normally be an important adaptation to prolonged stress, because it stops synthesis of new transporters, and extant transporters are diluted out as cells continue to grow (Fig. 6*A*).

In the past few years, sRNA regulators have garnered a great deal of attention and study, yet we are still discovering novel physiological functions and mechanisms of action. By far the most well studied class of sRNAs are those that act by base pairing with target messages, like the microRNAs in eukaryotes. The analogous bacterial sRNAs also act as base pairing-dependent riboregulators that require the action of protein cofactors, most notably Hfq. The majority of characterized bacterial sRNAs base pair with sequences in the 5′ UTRs of their target messages and down-regulate their translation and/or stability. However, new variations on this theme are emerging. Some riboregulators positively regulate translation of their target messages, and there are now examples of riboregulators that act negatively on some targets and positively on others. Our work has now provided evidence for yet another functional class of sRNAs. SgrS and RNA III of *S. aureus* are bifunctional sRNAs that can act through a base pairing mechanism on mRNA targets and also serve as mRNAs themselves. Although SgrS shares the basic feature of bifunctionality with RNA III, there are some important and intriguing differences. For example, Hfq is required for riboregulation by SgrS but apparently is not required for RNA III. Furthermore, SgrS is thus far unique in that the function of the encoded protein product (SgrT) appears to be redundant physiologically (although not mechanistically) with the riboregulation function. One of the lessons that may be taken from this study is that small ORFs encoded by sRNAs should be examined carefully for potential function. Undoubtedly there are other bifunctional sRNAs that await characterization.

Methods

Strain and Plasmid Construction. The strains used in this study are listed in SI Table 1, and oligonucleotides are listed in SI Table 2. Alleles were moved between strains by P1 transduction or inserted via λ Red recombination (20). Strains DH5α (Invitrogen) and XL10 gold (Stratagene) were used for cloning procedures. Plasmid vectors were pBR322 derivatives, pHDB3 (21) or pBRP$_{lac}$ (22).

Strains BH300 and BH301 were created by using a modified λ Red and FLP-mediated recombination protocol (23). Briefly, a kanamycin cassette flanked by FRT sites was amplified from template pKD13 (23) by using primers O-CV247 and O-CV248 and integrated into the chromosome by λ Red recombination at the 3′ end of *sgrT*. The remaining steps were as described in ref. 23. The resulting strains, BH300 (wild-type *sgrT*) and BH301 (*sgrT$_{UAA}$*), carry in-frame translational fusions between *sgrT* and *lacZ*. Strain BH302 was created by insertion of the Δ*sgrR*::*cat* allele into strain BH300 by λ Red recombination.

Plasmid pLCV1 carries P$_{lac}$-*sgrS* and is described in ref. 6. Plasmid pLCV5 was derived from pLCV1 by whole-plasmid PCR mutagenesis using primers O-CV111 and O-CV112. pLCV5 (P$_{lac}$-*sgrS$_{UAA}$*) contains a single base pair substitution that changed the fifth codon of *sgrT* from "TAT" to "TAA" to create a stop codon. The *sgrT* ORF was amplified with forward primer O-CV115, containing an AatII restriction site and heterologous 5′ leader and ribosome binding site, and reverse primer O-CV116, which had an EcoRI restriction site. This PCR product was cloned into the vector pBRP*lac* (22), resulting in plasmid pBRCV7 (P$_{lac}$-*sgrT*). Plasmid pBRCV8 (P$_{lac}$-*sgrT$_{UAA}$*) was derived from pBRCV7 and was constructed by PCR mutagenesis using primers O-CV118 and O-CV119 to incorporate the stop codon mutation as described for pLCV5. Plasmids pBRCS1 and pBRCS4 were created by PCR mutagenesis on pBRCV7 and pBRCV8, respectively, using primers O-CV207 and O-CV208. pBRCS1 and pBRCS4 carry additional sequences that encode a 3XFLAG tag fused to the C terminus of SgrT.

Media and Reagents. LB medium was used for all liquid cultures and plates unless otherwise noted. Media were supplemented with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, or 25 μg/ml tetracycline where indicated. IPTG was used at a concentration of 0.1 mM for induction of P$_{lac}$-*sgrS* or P$_{lac}$-*sgrT* alleles and at a concentration of 1 mM for induction of pBRCS1 and pBRCS4 for protein extraction. MOPS EZ Rich defined medium (Teknova) or minimal A medium with 0.2% glucose or 0.4% glycerol was used for some experiments.

Phenotypic Assays. **αMG rescue.** Strains were grown overnight in LB with ampicillin and 0.1 mM IPTG and then subcultured 1:500 in fresh medium. Cultures were grown to early-log phase ($OD_{600} \sim 0.1$), and stress was induced by the addition of 0.5% αMG to the medium. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) every 30 min before addition of αMG and every 20 min thereafter until cells reached stationary phase.

Glucose growth inhibition. The cultures were grown overnight in minimal A medium with ampicillin, IPTG, and glycerol. Strains were subcultured 1:200 in fresh medium with ampicillin, IPTG, and glucose. Growth was monitored by measuring OD_{600} every hour until cells reached stationary phase.

Glucose uptake assays. Cultures were grown overnight in rich defined medium (MOPS EZ Rich) with ampicillin, IPTG, and glycerol. Strains were subcultured 1:500 in fresh medium with ampicillin, IPTG, and glucose. Samples were taken immediately after subculture and again after 5 h of growth. Cells were pelleted by centrifugation, and a 10-fold dilution of supernatant was used with the glucose (HK) assay kit (Sigma), according to the manufacturer's instructions, to determine the amount of glucose in supernatant samples. Protein samples were harvested by precipitation with trichloroacetic acid (TCA) at the 5-h time point. Processing of protein samples and Western blotting is described below.

β-Galactosidase assays. Strains containing a translational *sgrT'-'lacZ* fusion were grown overnight in TB medium and subcultured 1:200 to fresh medium. Cultures were grown to $OD_{600} \sim 0.5$ and then split into two cultures, one of which was induced with 0.005% αMG. Samples were taken as indicated after induction and assayed for β-galactosidase activity as described in ref. 24.

Inducer exclusion. Strains were grown overnight in TB with ampicillin and subcultured 1:200 in fresh media with antibiotic, IPTG, 0.2% glucose, and 0.2% lactose. Samples were taken as indicated, roughly to OD_{600} 0.1, 0.5, and 2, respectively, and were used for Miller assay (24).

RNA Methods. RNA was extracted by the hot phenol method as described in ref. 25. The concentration of RNA samples was determined spectrophotometrically, and samples were prepared for electrophoresis by using equal amounts of total RNA (15 μg for *ptsG* Northern blots). Samples were run on 1.2% agarose gels alongside the Millennium size marker (Ambion) at 90 V for ≈2 h. The gel was prepared and RNA transferred as described in ref. 26. Prehybridization was performed in ULTRAhyb (Ambion) solution at 42°C for at least 30 min; the membrane was probed overnight with a 5′-biotinylated probe, RyaA-1bio or ptsG-1bio, for SgrS and *ptsG* mRNA, respectively. Detection was performed according to BrightStar BioDetect kit (Ambion) specifications.

Protein Methods. For Western blot analysis, strains were grown overnight in LB with ampicillin and subcultured 1:500 in fresh media. The cultures were grown to mid-log phase ($OD_{600} \sim 0.5$) and induced with IPTG. Proteins were harvested by precipitation with TCA, as described in ref. 17, at the time points indicated; the OD_{600} was measured when the samples were harvested. All proteins were resuspended in sample buffer with DTT (New England Biolabs).

All protein gels and buffers were from Invitrogen. Protein samples were run on 10% Bis-Tris gels with MOPS-SDS buffer for PtsG or 4–12% Bis-Tris gels with Mes-SDS buffer for SgrT-3XFLAG at the recommended running times and voltages. The proteins were transferred to Immobilon membranes (Millipore): Immobilon-P or Immobilon-P_{SQ} for PtsG ($IICB^{Glc}$) and SgrT-3XFLAG, respectively. Membranes were blocked and Western blots performed as described in ref. 17. The rabbit primary antibody against PtsG was a gift from Hiroji Aiba (Nagoya University, Nagoya, Japan) and was used at a dilution of 1:5,000, and goat anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (used at 1:5,000) was from Calbiochem. The ECL Plus reagent (GE Healthcare) was used for detection. The autoradiography film used was Kodak BioMax light film.

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1. Englesberg E, Anderson RL, Weinberg R, Lee N, Hoffee P, Huttenhauer G, Boyer H (1962) *J Bacteriol* 84:137–146.
2. Kadner RJ, Murphy GP, Stephens CM (1992) *J Gen Microbiol* 138:2007–2014.
3. Yarmolinsky MB, Wiesmeyer H, Kalckar HM, Jordon E (1959) *Proc Natl Acad Sci USA* 45:1786–1791.
4. Kimata K, Tanaka Y, Inada T, Aiba H (2001) *EMBO J* 20:3587–3595.
5. Morita T, El-Kazzaz W, Tanaka Y, Inada T, Aiba H (2003) *J Biol Chem* 278:15608–15614.
6. Vanderpool CK, Gottesman S (2004) *Mol Microbiol* 54:1076–1089.
7. Morita T, Mochizuki Y, Aiba H (2006) *Proc Natl Acad Sci USA* 103:4858–4863.
8. Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, Gottesman S (2003) *Mol Microbiol* 50:1111–1124.
9. Massé E, Majdalani N, Gottesman S (2003) *Curr Opin Microbiol* 6:120–124.
10. Møller T, Franch T, Hojrup P, Keene DR, Bachinger HP, Brennan R, Valentin-Hansen P (2002) *Mol Cell* 9:23–30.
11. Sledjeski DD, Whitman C, Zhang A (2001) *J Bacteriol* 183:1997–2005.
12. Kawamoto H, Koide Y, Morita T, Aiba H (2006) *Mol Microbiol* 61:1013–1022.
13. Boisset S, Geissmann T, Huntzinger E, Fechter P, Bendridi N, Possedko M, Chevalier C, Helfer AC, Benito Y, Jacquier A, *et al.* (2007) *Genes Dev* 21:1353–1366.
14. Huntzinger E, Boisset S, Saveanu C, Benito Y, Geissmann T, Namane A, Lina G, Etienne J, Ehresmann B, Ehresmann C, *et al.* (2005) *EMBO J* 24:824–835.
15. Morfeldt E, Taylor D, von Gabain A, Arvidson S (1995) *EMBO J* 14:4569–4577.
16. Bohn C, Rigoulay C, Bouloc P (2007) *BMC Microbiol* 7:10.
17. Vanderpool CK, Gottesman S (2007) *J Bacteriol* 189:2238–2248.
18. Deutscher J, Francke C, Postma PW (2006) *Microbiol Mol Biol Rev* 70:939–1031.
19. Massé E, Vanderpool CK, Gottesman S (2005) *J Bacteriol* 187:6962–6971.
20. Yu DG, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL (2000) *Proc Natl Acad Sci USA* 97:5978–5983.
21. Ulbrandt ND, Newitt JA, Bernstein HD (1997) *Cell* 88:187–196.
22. Guillier M, Gottesman S (2006) *Mol Microbiol* 59:231–247.
23. Ellermeier CD, Janakiraman A, Slauch JM (2002) *Gene* 290:153–161.
24. Miller JH (1972) *Experiments in Bacterial Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
25. Aiba H, Adhya S, de Crombrugghe B (1981) *J Biol Chem* 256:11905–11910.
26. Majdalani N, Chen S, Murrow J, St. John K, Gottesman S (2001) *Mol Microbiol* 39:1382–1394.