Characterization of homologs of the small RNA SgrS reveals diversity in function

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ABSTRACT

SgrS is a small RNA (sRNA) that requires the RNA chaperone Hfq for its function. SgrS is a unique dual-function sRNA with a base pairing function that regulates mRNA targets and an mRNA function that allows production of the 43-amino-acid protein SgrT. SgrS is expressed when non-metabolizable sugars accumulate intracellularly (glucose-phosphate stress) and is required to allow *Escherichia coli* cells to recover from stress. In this study, homologs of SgrS were used to complement an *E. coli* sgrS mutant in order elucidate the physiological relevance of differences among homologs. These analyses revealed that the base pairing function of *E. coli* and *Yersinia pestis* SgrS homologs is critical for rescue from glucose-phosphate stress. In contrast, base pairing-deficient SgrS homologs from *Salmonella typhimurium*, *Erwinia carotovora* and *Klebsiella pneumoniae* rescue *E. coli* cells from stress despite their failure to regulate target mRNAs. Compared with *E. coli* SgrS, *S. typhimurium* SgrS produces more SgrT and this rescues cell growth even when the base pairing function is inactivated. Genetic evidence suggests that a secondary structure in the *E. coli* SgrS 5' region inhibits sgrT translation. This structure is not present in *S. typhimurium* SgrS, which explains its higher level of SgrT production.

INTRODUCTION

Small RNAs have risen to prominence as post-transcriptional regulators of gene expression in virtually all organisms from bacteria to humans. The best-studied class of bacterial small RNAs (sRNAs) is characterized by a base pairing-dependent mechanism of action on target mRNAs and a requirement for the RNA chaperone Hfq (1). Most Hfq-dependent sRNAs are in the range of 60–100 nucleotides (nt) in length and do not encode proteins. Since the regulatory function of these molecules depends on RNA–RNA base pairing interactions, sRNAs are sometimes referred to as riboregulators. Hfq-dependent sRNAs are capable of both positive and negative regulation of mRNA translation and stability (2). To date, examples of negative regulation are more common: an sRNA base pairing with the 5' untranslated region (UTR) of its target mRNA prevents ribosome binding to that mRNA and either directly or indirectly stimulates mRNA degradation.

In the last decade, hundreds of sRNAs of unknown function have been identified in dozens of bacterial species. While the vast majority of these RNAs remain uncharacterized with regard to their roles in cell physiology, the few sRNAs that have been studied generally have roles in cellular stress responses. For example, the 227-nt sRNA SgrS is expressed under conditions of metabolic stress in *Escherichia coli* when cells are unable to appropriately metabolize phosphorylated sugars; SgrS is required for continued cell growth under these conditions (3,4). This metabolic stress is referred to as glucose-phosphate stress because the stress response is apparently induced by the accumulation of early glycolytic intermediates, such as glucose-6-phosphate (5,6). In the laboratory, the stress response is induced by exposing strains with mutations in early glycolytic genes (*pgi* or *pfk*) to glucose or by exposing wild-type cells to the non-metabolizable glucose analog α-methyl glucoside (α-MG). Both glucose and α-MG are transported into the cytoplasm and comitantly phosphorylated through the action of the phosphoenolpyruvate phosphotransferase system (PTS) (7). When it is expressed under stress conditions, SgrS base pairs with mRNAs encoding PTS proteins, which inhibits translation and promotes degradation of these messages (3,8). The proposed physiological significance of this regulation is that SgrS stops new sugar transporters from being produced under conditions where the accumulated sugar-phosphates have become toxic. Indeed, the growth of sgrS mutants is strongly and permanently inhibited under glucose-phosphate stress conditions, whereas sgrS+ cells are only slightly and transiently inhibited (3).
We recently reported that SgrS is the defining member of a novel class of Hfq-dependent RNAs with two distinct functions (4). The riboregulation function of SgrS on its target mRNAs is carried out by a mechanism that has been described for a number of other sRNAs. Base pairing interactions between SgrS and ptsG mRNA, which encodes the major glucose transporter (PtsG or EIICB Glc), occlude the ptsG ribosome-binding site and lead to translation inhibition and ptsG mRNA degradation (8). The second function of SgrS is encoding the 43-amino-acid ribosome-binding site and lead to translation inhibition and ptsG mRNA degradation (8). When SgrT is ectopically produced under glucose-phosphate stress conditions, it rescues the growth of cells by a mechanism that is distinct from riboregulation. Since SgrT can block glucose uptake without affecting levels of ptsG mRNA or PtsG protein, we have previously proposed that it acts by blocking PtsG activity by an as yet unknown mechanism (4).

SgrS is thus far unique in its dual functionality and we do not know how translation and riboregulation influence one another. Therefore, in this and a companion study (9) we sought to define the distribution of SgrS/SgrT systems and utilize SgrS homologs from other organisms as naturally occurring variants that might provide insight into the function of this sRNA. The current study describes our characterization of a subset of SgrS homologs in an E. coli sgrS mutant host. We have determined that while the riboregulation function is well conserved, there is diversity among homologs of closely related organisms, with respect to the role of SgrT in the glucose-phosphate stress response.

**MATERIALS AND METHODS**

**Strain and plasmid construction**

Many strains constructed for this study are derivatives of DJ480 or DJ624 (D. Jin, National Cancer Institute) and are listed in Table S1. CV104 and CS136 were described in a previous study (4). CS104 contains a ΔsgrS allele that was constructed by homologous recombination using the λ Red system (10). To construct CS104, a cat-sacB cassette was amplified with primers O-CV178 and O-CV179 and the resultant PCR product was recombined with the chromosome after the fifth codon of sgrT to create strain CS100. A single-stranded oligonucleotide, O-CV211 (Table S2) was then transformed into CS100 to loop out the cat-sacB cassette and the resultant PCR product was recombined with the strain JH111 was created by J. B. Rice (in our laboratory) by P1 transduction of λatt::lacI, specR, tetR from donor DH5zZ1 (11) to recipient CS104. CS174 was created by moving ΔmanXYZ::kan into JH111 by P1 transduction. The ptsG::lacZ translational fusion in strain JH171 was created by J. B. Rice using λ Red and FLP-mediated site-specific recombination by a method described previously (12). A kanamycin cassette flanked by FRT sites was amplified from pKD13 (12) using primers O-JH159 and O-JH160 and was recombined at the ptsG locus after Pro141. After removal of the kanamycin resistance cassette and transformation of the strain with a plasmid carrying FRT-lacZ-lacY-kan-FRT, homologous recombination resulted in an in-frame fusion such that LacZ was fused to a cytoplasmic loop after the fourth transmembrane domain of PtsG.

DH5z (Invitrogen) and XL10 gold (Stratagene) were used for cloning procedures. Characteristics of all plasmids are summarized in Table S1. Plasmids pHDB3 (13), pLCV1 and pLCS1 were constructed as discussed previously (4). pBRCS6 was constructed by PCR amplifying sgrS from CS123 using primers PLlacO-sgrS1 and sgrSrev, followed by cloning of the PCR product into vector pHDB3. Plasmid pBRCS12 was constructed by annealing oligonucleotides O-CS138 and O-CS139 followed by ligation of the resulting double-stranded DNA product into pHDB3. Plasmids pBRCS22, pBRCS28, pBRCS30, pBRCS31, pBRCS32, pBRCS34 and pBRCS35 are all derivatives of pBRCS12. Primers used to obtain PCR product inserts for each of these plasmids are listed in Table S2. Plasmids pBRCS2, pBRCS27, pBRCS29, pBRCS33, pBRCS36, pBRCS37, pBRCS38, pBRCS39, pBRCS40, pBRCS41, pBRCS42 and pBRCS43 were all created by inverse PCR using primers listed in Table S2.

**β-Galactosidase assays**

Strains containing sgrS plasmids were grown overnight in TB medium supplemented with 100 μg/ml ampicillin and subcultured 1:200 to fresh medium. Cultures were grown to OD600 ~0.5 and induced with 0.1 mM IPTG. Samples were taken before induction and 1 h after induction and assayed for β-galactosidase activity as described previously (14).

**Phenotypic assays: αMG rescue**

The plasmids described in the text and Table S1 were transformed into CV104, which contains chromosomal lacF’ and ΔsgrS::kan alleles. For growth on plates, strains were streaked on LB with 100 μg/ml ampicillin, 0.1 mM IPTG and with or without 0.5% αMG and grown overnight at 37°C. Plates were imaged after ~18 h of incubation. For liquid cultures, strains were grown overnight in LB medium supplemented with 100 μg/ml ampicillin and 0.1 mM IPTG and sub-cultured 1:500 in fresh medium with the same amounts of ampicillin and IPTG. The cultures were then grown to an OD600 ~0.1 and stress was induced by the addition of 0.5% αMG to the medium. Growth was monitored by measuring the OD600 every 30 min before addition of αMG and every 20 min after addition.

**Phenotypic assays: glucose growth inhibition**

The lacF’+, ΔsgrS::kan host (CV104) carrying the plasmids described was grown for 2 days at 37°C on minimal 63 medium plates with 0.2% glucose, 100 μg/ml ampicillin and with or without 0.1 mM IPTG.
Phenotypic assays: inducer exclusion

The ΔsgrS::kan host (CS136) carrying the plasmids indicated was grown overnight in TB medium supplemented with 100 μg/ml ampicillin and subcultured 1:200 to fresh media with 100 μg/ml ampicillin, 0.2% glucose and 0.2% lactose. Samples were taken as indicated at mid-log phase (OD\textsubscript{600} ~0.5) and assayed for β-galactosidase activity as described previously (12).

RNA methods

Strain CV104 carrying the plasmids described was grown overnight in LB supplemented with 100 μg/ml ampicillin and subcultured 1:500 in fresh media with antibiotic. When cultures reached an OD\textsubscript{600} ~0.5, they were exposed 0.1 mM IPTG, and samples were harvested for RNA extraction at the times indicated. RNA was extracted via the hot phenol method as described previously (13). The concentration of RNA samples was determined spectrophotometrically, and samples were prepared for electrophoresis using equal amounts of total RNA (3 μg for SgrS blots and 10 μg for ptsG blots). Samples to be probed for ptsG mRNA were run on a 1.2% agarose gel with a Millennium marker (Ambion) at 90 volts for approximately 1.5 h. Samples to be probed for SgrS were run on a 6% polyacrylamide gel with a Century marker (Ambion) at 100 V for approximately 1 h. The gels were prepared for transfer as described previously (16).

RNA was transferred from agarose gels to a 0.45 μm membrane (Whatman) for 4 h by capillary transfer. RNA was transferred from acrylamide gels to a 0.2-μm membrane (Whatman) by electrophoresis at 250 mA for 1 h. RNA was UV-crosslinked to the membrane. 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, SgrS-1bio, ptsG-1bio, or ssrA-bio for SgrS, ptsG and SsrA RNAs, respectively. Detection was performed according to Brightstar Biodetect kit (Ambion) specifications.

Protein methods

Protein harvesting and extraction. CV104 carrying the plasmids indicated was grown overnight in LB supplemented with 100 μg/ml ampicillin and subcultured 1:500 in fresh media with antibiotic. The cultures were grown to mid-log phase (OD\textsubscript{600} ~0.5) and then induced with 1 mM IPTG. Proteins were harvested immediately before induction (T = 0 min) and at 15 and 120 min after induction; the optical density of the cell cultures at 600 nm was measured when the samples were taken. To extract the protein, 1 ml of culture was added to a microcentrifuge tube on ice with a final concentration of 10% TCA. After 15 min, total cell protein was collected by centrifugation at 4°C and 12,000 rpm for 10 min. The protein pellet was washed in 0.5 ml 80% acetone and collected again by pelleting at 4°C and 12,000 rpm for 10 min. The acetone was removed from the microcentrifuge tube and the pellet briefly air dried. Proteins were resuspended in 1.5× sample buffer with DTT (New England Biolabs) at 0.05 OD\textsubscript{600} units/10 μl.

Protein gels. All protein gels and buffers were from Invitrogen. For detection of SgrT-3XFLAG, resuspended protein samples were run on a 4-12% Bis–Tris gel with MES-SDS running buffer at 170 V for the recommended time. The proteins were transferred to Immobilon-P\textsubscript{SO} membranes at 25 V for 70 min in NuPage transfer buffer. The membrane was blocked for at least 1 h in PBS-T with 5% milk. Remaining steps for western blots are as described previously (17). The monoclonal mouse anti-FLAG antibody was purchased from Sigma. The goat anti-mouse IgG horseradish peroxidase conjugate secondary antibody was purchased from Novagen.

RESULTS

SgrS homologs complement an E. coli K12 SgrS mutant

The homologs chosen for in vivo characterization in this study were identified in another study and are described in detail there (9). All sgrS homologs are found in genomes adjacent to and encoded divergently from sgrR homologs. All have a conserved 3' region predicted to be involved in riboregulation. The subset of homologs chosen for this study are described at length elsewhere (9) and briefly here. Homologs from E. coli K12 (sgrSK12) and Salmonella typhimurium (sgrSK12) both possess the sgrT coding sequence (CDS) and 3' base pairing region. The Y. pestis homolog (sgrSK12), is truncated at the 5' end compared to other homologs; it lacks the sgrT CDS but retains the 3' base pairing region. The K. pneumoniae homolog (sgrSK12) is predicted to be much longer at ~400 nt compared to other homologs that are ~200 nt. The E. carotovora homolog (sgrSEcar) is the most divergent at the nucleotide sequence level, but retains both the sgrT CDS and putative base pairing sequences. To assess the function of SgrS homologs, we used two phenotypic assays described in previous studies (3,4): (i) recovery from glucose-phosphate stress following exposure of cells to ζMG and (ii) growth inhibition on glucose minimal medium when SgrS is ectopically expressed. The genes encoding the homologs were placed under the control of the lac promoter on a medium-copy-number plasmid. The start of transcription chosen for each homolog is based on promoter predictions and alignment with E. coli K12 sgrS, for which the start site has been experimentally determined (3). The homolog-containing plasmids were transformed into a ΔsgrS, lacP\textsuperscript{+} (overexpresses the LacI repressor) E. coli host and the resulting strains were tested for stress recovery (Figure 1A) and growth on minimal glucose medium (Figure 1B and C). Northern blots showed that an SgrS RNA of the predicted size for each homolog was produced from the P\textsubscript{lac} constructs at levels comparable to (sgrSK12, sgrSK12, sgrSK12) or slightly less than (sgrSEcar, sgrSK12) that produced from the E. coli chromosome during glucose-phosphate stress (Figure S1). We showed previously (3,4) that the wild-type SgrSK12 expressed in trans from the P\textsubscript{lac} promoter rescues cells from stress and strongly inhibits growth on glucose. In the current experiments, expression of all the homologs tested promoted recovery of the E. coli host strain from stress (Figure 1A). However, only sgrSK12,
sgrSYp and sgrSEcar fully inhibited growth on minimal glucose medium. Expression of the sgrSEcar allele partially inhibited growth of the E. coli strain on minimal glucose medium and sgrSYp did not detectably inhibit (Figure 1B). All strains grew similarly in a rich medium without zMG and in glucose minimal medium when expression of alleles was not induced (data not shown).

Work in our laboratory has established that SgrSK12 targets the manXYZ message, which encodes an alternative glucose PTS (J. B. Rice and C. K. Vanderpool, unpublished data). We hypothesized that failure of SgrSYp and SgrSEcar to fully inhibit growth on glucose minimal medium might be due to failure to down-regulate the manXYZ mRNA. We therefore repeated the experiments with all alleles in an sgrS manXYZ mutant host. In this background, all homologs efficiently inhibited growth on glucose (Figure 1C), which implies that SgrSYp and SgrSEcar are unable to down-regulate the E. coli K12 manXYZ mRNA.

There can be subtle differences in rate or ability of cells to recover from glucose-phosphate stress that grow on solid media will not reveal. To further examine the complementation by SgrS homologs, growth in liquid media was monitored after cells were stressed with zMG at early logarithmic phase. All alleles tested promoted recovery from stress (data not shown) and the results were entirely consistent with those shown in Figure 1.

Homologs negatively regulate translation of E. coli ptsG mRNA

Previous work has shown that the base pairing interaction between SgrS and ptsG mRNA inhibits ptsG translation and promotes ptsG mRNA degradation (3,8,16). This regulation stops new synthesis of PtsG (EIICB(Glu)), which presumably limits further influx of non-metabolizable sugar-phosphates. Based on the rescue phenotypes demonstrated by all homologs (Figure 1A), we predicted that the ability to regulate E. coli ptsG mRNA by base pairing would be conserved. Alignment of each SgrS homolog with E. coli K12 ptsG mRNA revealed base pairing interactions that were predicted to encompass the same eight contiguous bases adjacent to and overlapping the ptsG ribosome-binding site (referred to hereafter as the ‘core’ base pairing region) (Figure 2A). It was previously shown (8) that mutations in two residues that disrupt G:C base pairs in this 8-bp region (SgrS G176C, G178C) prevent SgrSK12 from down-regulating ptsG mRNA stability. To test the validity of the base pairing predictions for E. coli K12 ptsG mRNA with SgrS homologs (Figure 2A), translational regulation of a ptsG-lacZ translational reporter fusion was examined (Figure 2B and C). The reporter fusion was efficiently down-regulated by >3-fold when expression of sgrSK12 was induced (Figure 2B, compare ‘K12’ to ‘vec’). Expression of all sgrS homologs resulted in reduced β-galactosidase levels compared with levels produced by cells carrying the vector control (Figure 2B). The fold-repression mediated by the homologs was similar to that of the native sgrSK12 allele. These results support the hypothesis that SgrS homologs base pair with E. coli K12 ptsG mRNA and inhibit its translation.

We next made G to C mutations expected to disrupt base pairing in the core pairing region (indicated in Figure 2A) in the context of P_tac-sgrS constructs. An sgrSK12 allele with the G176C, G178C point mutations was shown in another study to be defective in regulation of ptsG mRNA (8). Consistent with this finding, our sgrSI_k12 allele (SgrS G176C, G178C) (Table 1) fails to down-regulate the reporter fusion (Figure 2C, compare ‘vec’ to ‘K12’). The sgrSI_off, sgrSI_off, sgrSI_Ecar and sgrSI_Kp alleles were also expressed in the ptsG-lacZ reporter strain to determine if the analogous G:C base pairs (indicated in Figure 2A) were required for base pairing-dependent regulation of E. coli ptsG. Levels of reporter fusion activity were not significantly different between uninduced (-IPTG) and induced (+ IPTG) strains (data not shown) or in induced cells with vector compared with sgrSI alleles (Figure 2C). These data indicate that the point mutations in the sgrSI alleles abrogate the ability of the SgrSI molecules to repress ptsG translation. Furthermore, the data support the notion that the determinants for riboregulation are very similar among all SgrS homologs. This is not surprising given the conservation in this region at the nucleotide sequence level and the conservation of predicted base pairing interactions with cognate ptsG mRNAs (9).
SgrS homologs indicate positions of G176C and G178C mutations with horizontal lines and boxes, respectively. The boxed G residues of mRNA (8). The ptsG near the tarity are indicated with asterisks. The importance of the base pairs mRNA and SgrS homologs were aligned and regions of complementarity are indicated with asterisks. The numbers reported are an average of results from three separate experiments. Abbreviations are as described in the Figure 1 legend. The numbers carried by sgrS1 alleles. The sgrS (B) or sgrS1 (C) homologs were expressed (from plasmids, Table S1) by induction with IPTG (as described in Materials and Methods’ section) in an E. coli ΔsgrS strain containing a ptsG-lacZ translational fusion (JH171). Abbreviations are as described in the Figure 1 legend. The numbers reported are an average of results from three separate experiments.

Since translational repression is coupled with mRNA degradation in many cases of sRNA-mediated regulation, we used Northern blots to determine the levels of SgrS RNA and ptsG mRNA in cells expressing wild-type or sgrS1K12 alleles (Figure 3A and B). Wild-type SgrS K12 and mutant SgrS1K12 were expressed at similar levels (Figure 3A). Wild-type SgrS K12 promoted degradation of ptsG mRNA and full-length ptsG mRNA became undetectable (Figure 3B); this was concomitant with the appearance of a stable degradation product as we and others have shown previously (3,19). Northern blots showed that wild-type SgrS K12 and SgrSYP similarly promoted degradation of ptsG mRNA (data not shown). In contrast, when sgrS1K12 was expressed, both full-length ptsG mRNA and the degradation product were detected (Figure 3B). This result confirms that the two G:C base pairs in the core pairing region (Figure 2A) are necessary for full downregulation of ptsG mRNA stability.

Variations in SgrT production and function among SgrS homologs

SgrS is a dual-function sRNA, and we are interested in determining the relative contributions of riboregulation and SgrT to recovery from glucose-phosphate stress. We showed previously (4) that SgrT itself has no effect on either ptsG mRNA or PtsG protein levels, but when ectopically expressed (using heterologous promoter and ribosome binding sequences), SgrT can rescue cells from glucose-phosphate stress. However, the contribution of SgrT to growth rescue when it is produced from its native ribosome-binding site has not been analyzed. Therefore, all sgrS1 alleles were expressed in a ΔsgrS background and growth of cells in the presence of ZMG was followed. The strain carrying the vector control was strongly inhibited, as were strains expressing the native sgrS1K12 allele and the sgrS1YP allele (Figure 4). In contrast, growth of strains expressing the sgrS1St, sgrS1Ecar and sgrS1Kp alleles was rescued (Figure 4).

Table 1. Alleles

<table>
<thead>
<tr>
<th>Name</th>
<th>Allele description</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgrS</td>
<td>Wild-type sgrS</td>
</tr>
<tr>
<td>sgrS1</td>
<td>sgrS with two point mutations that correspond to sgrS1K12 G176C,G178C and abolish base pairing with ptsG mRNA</td>
</tr>
<tr>
<td>sgrS2</td>
<td>sgrS with ATG start codon of sgrT mutated to a TAA stop codon</td>
</tr>
<tr>
<td>sgrS3</td>
<td>sgrS with both mutations from sgrS1 and sgrS2 alleles</td>
</tr>
<tr>
<td>sgrS4</td>
<td>sgrS1K12 C19T,C21T, weakens 5’ hairpin formation, G:U base pairs</td>
</tr>
<tr>
<td>sgrS5</td>
<td>sgrS1K12 C19A,C21A, weakens 5’ hairpin formation, no base pairing</td>
</tr>
</tbody>
</table>

Figure 2. Base pairing interactions between SgrS homologs and E. coli ptsG mRNA mediate riboregulation. (A) Sequences of E. coli ptsG mRNA and SgrS homologs were aligned and regions of complementarity are indicated with asterisks. The importance of the base pairs near the ptsG RBS has been verified for the native E. coli SgrS and ptsG mRNA (8). The ptsG RBS and start codon are indicated with horizontal lines and boxes, respectively. The boxed G residues of SgrS homologs indicate positions of G176C and G178C mutations.
It was unsurprising that the base pairing-deficient \textit{Y. pestis} allele could not rescue cells from stress, since our bioinformatic analysis indicated that SgrS 

\textit{Y. pestis} lacks the 5' region that contains \textit{sgrT} and should therefore only be capable of riboregulation (9). In contrast, \textit{E. coli} SgrS\textsubscript{K12} does possess \textit{sgrT}, but this is apparently not sufficient to rescue growth in the context of a base pairing-deficient SgrS molecule. Since \textit{E. coli} K12 SgrT is capable of rescue when produced ectopically from a heterologous promoter and strong ribosome-binding site (4), the current data suggest that in its normal context, \textit{E. coli} SgrT is either not produced at high-enough levels or is less effective than the SgrT produced by other organisms.

One of the regulatory functions of the PTS is inducer exclusion, which prevents uptake of substrates like lactose when glucose is present (7). Inducer exclusion is mediated by dephosphorylated EII\textsubscript{AGlc} (the form that accumulates when glucose is transported) binding and inhibiting activity of proteins like Lac permease. Thus, when wild-type cells are growing in the presence of glucose and lactose, lactose is excluded and expression of \textit{lac} genes is very low. The activity of the \textit{lacZ} gene product (\(\beta\)-galactosidase) in cells growing in media with glucose and lactose serves as an \textit{in vivo} measure of PTS function. Inducer exclusion assays demonstrated that ectopic expression of the wild-type SgrS or SgrT inhibits glucose transport (4). In the present study, we monitored \(\beta\)-galactosidase activity in a \textit{lac}\^+ \textit{E. coli} host expressing the \textit{sgrS} alleles of \textit{E. coli}, \textit{Y. pestis}, \textit{S. typhimurium}, \textit{E. carotovora} or \textit{K. pneumoniae} (Figure 5). \(\beta\)-Galactosidase levels in the strain expressing the \textit{sgrS\textsubscript{K12}} or \textit{sgrS\textsubscript{YP}} alleles remained low, similar to the vector control. This indicates that when base pairing is disrupted, these alleles (\textit{sgrS\textsubscript{K12}} and \textit{sgrS\textsubscript{YP}}) are unable to down-regulate PtsG production or activity and therefore glucose transport and inducer exclusion are unaffected. This is consistent with previous data (Figure 4) suggesting that the base pairing activity of SgrS\textsubscript{K12} and SgrS\textsubscript{YP} is the most important function for these homologs (and the only function for SgrS\textsubscript{YP}).

In contrast, strains expressing the \textit{sgrS\textsubscript{St}}, \textit{sgrS\textsubscript{Ecar}} or \textit{sgrS\textsubscript{Kp}} alleles had high levels of \(\beta\)-galactosidase activity (Figure 5), indicating that inducer exclusion was disrupted (reflecting a block of glucose uptake). These data support the idea that SgrS\textsubscript{St}, SgrS\textsubscript{Ecar} and SgrS\textsubscript{Kp} utilize SgrT to block glucose (or \(\alpha\)MG as in Figure 4) uptake when the base pairing function is inactivated.

Comparison of \textit{Salmonella} and \textit{E. coli} alleles reveals differences in production of SgrT

The data presented in Figures 4 and 5 suggest that there are differences among homologs with respect to the roles of base pairing and SgrT in rescue from stress. To further examine these differences, we compared the activities of a series of alleles derived from \textit{sgrS\textsubscript{K12}} and \textit{sgrS\textsubscript{St}} (Table 1). The mutation in \textit{sgrS2} alleles changed the \textit{sgrT} start codon to a TAA stop codon, eliminating production of SgrT. The TAA stop codon was combined with the base pairing mutations to yield \textit{sgrS3} alleles, which we expected to be functionally null as they cannot produce SgrT and cannot perform the base pairing function. These constructs were tested in an \textit{sgrS} mutant host for their ability to rescue cells from growth inhibition by \(\alpha\)MG (Figure 6). For the \textit{E. coli} alleles, \textit{sgrS}\textsuperscript{2} and \textit{sgrS}\textsuperscript{2} (lacking \textit{sgrT}) rescued cells from growth inhibition by \(\alpha\)MG while \textit{sgrS1} and \textit{sgrS2} failed to rescue. In contrast, \textit{S. typhimurium} \textit{sgrS}\textsuperscript{2}, \textit{sgrS1} and \textit{sgrS2} all rescued cells from stress while only \textit{sgrS3} behaved as a null allele. These results support our interpretation of the data in Figures 4 and 5; i.e. that the base pairing function of \textit{E. coli} SgrS is critical (and SgrT contributes very little) whereas either base pairing or SgrT of the \textit{S. typhimurium} homolog is sufficient for rescue.

The data described above indicate that differences in either the amount of SgrT produced or the activity of SgrT between \textit{E. coli} and \textit{S. typhimurium} homologs...
The results reported are an average of three independent experiments.

SgrT was undetectable in samples from cells expressing the amount of SgrT produced. SgrT was tagged with a 3X-FLAG epitope was inserted upstream of the ribosome-binding site and start codon. The circled cytosines (C) upstream of sgrT (Figure 8A) were changed to either adenines (A) or thymines (T). We reasoned that the A residues would disrupt the hairpin as they would not base pair with the guanine (G) residues in the other half of the inverted repeat, whereas the T residues [uracils (U) in the RNA sequence] could form weaker G:U base pairs and possibly preserve the hairpin. These mutations were made in the context of the sgrS1K12 allele; the C to T mutant allele is designated sgrS4K12 and the C to A mutant allele is sgrS5K12. In contrast with the parent allele, sgrS1K12, which is not able to rescue cells from stress (Figure 4), both sgrS4K12 and sgrS5K12 partially rescued the sgrS mutant host from growth inhibition imposed by zMG (Figure S2A). To examine whether these mutations also increased production of SgrT from sgrS4K12 and sgrS5K12, a sequence specifying the 3X-FLAG epitope was inserted upstream of the sgrT stop codon in these alleles. Western blots were performed on protein extracts from cells expressing sgrS1K12 (positive control), sgrS1K12, sgrS4K12 and sgrS5K12. As observed in Figure 7, SgrT was detected in extracts from cells expressing the sgrS1K12 allele, but not the sgrS1K12 allele. SgrT was also detected in cells expressing sgrS4K12 and sgrS5K12 (Figure 8B). Taken together, these data indicate that the mutations in the translation initiation region of

Putative secondary structure at the 5’ end of SgrS1K12 inhibits sgrT translation

The 5’ ends of SgrS homologs were examined to identify sequences in the sgrT translation initiation region that might explain differences in SgrT production among homologs. This analysis revealed an inverted repeat in SgrS3K12 (Figure 8A) that could form a hairpin which occludes ribosome binding and inhibits sgrT translation. This GC-rich inverted repeat (5’-GGGGGTGCCCC-3’ in E. coli) was not conserved in Salmonella, Klebsiella or Erwinia homologs (Figure 8A), which could explain why more SgrT is produced from these SgrS homologs compared with E. coli SgrS. Site-directed mutagenesis of SgrS1K12 was utilized to alter the region between the sgrT ribosome-binding site and start codon. The circled cytosines (C) upstream of sgrT (Figure 8A) were changed to either adenines (A) or thymines (T). We reasoned that the A residues would disrupt the hairpin as they would not base pair with the guanine (G) residues in the other half of the inverted repeat, whereas the T residues [uracils (U) in the RNA sequence] could form weaker G:U base pairs and possibly preserve the hairpin. These mutations were made in the context of the sgrS1K12 allele; the C to T mutant allele is designated sgrS4K12 and the C to A mutant allele is sgrS5K12. In contrast with the parent allele, sgrS1K12, which is not able to rescue cells from stress (Figure 4), both sgrS4K12 and sgrS5K12 partially rescued the sgrS mutant host from growth inhibition imposed by zMG (Figure S2A). To examine whether these mutations also increased production of SgrT from sgrS4K12 and sgrS5K12, a sequence specifying the 3X-FLAG epitope was inserted upstream of the sgrT stop codon in these alleles. Western blots were performed on protein extracts from cells expressing sgrS1K12 (positive control), sgrS1K12, sgrS4K12 and sgrS5K12. As observed in Figure 7, SgrT was detected in extracts from cells expressing the sgrS1K12 allele, but not the sgrS1K12 allele. SgrT was also detected in cells expressing sgrS4K12 and sgrS5K12 (Figure 8B). Taken together, these data indicate that the mutations in the translation initiation region of

Figure 7. Salmonella typhimurium SgrS produces more SgrT than E. coli SgrS. Strains carrying plasmids with sgrS and sgrS1 alleles with sgrT-3X-FLAG from E. coli (K12) or S. typhimurium (St) were grown in rich media to an OD600 ~0.5 and induced with 1 mM IPTG. Protein samples were harvested 15 and 120 min after induction and a western blot was performed to detect SgrT-3X-FLAG.

SgrT production from the S. typhimurium homolog compared with the E. coli homolog. This result is consistent with our predictions based on the phenotypic results (Figures 4, 5 and 6).
sequences. The inverted repeat is indicated by arrows above the K12 ribosome-binding site is indicated by a bracket below the aligned region. In low SgrT production. (A) sgrT ribosome-binding region of the homologs were examined and a potential hairpin was identified in the Methods' section. Alleles are described in Table 1 and briefly as follows: sgrS, wild-type; sgrS4, C residues indicated in (A) changed to T; sgrS5, C residues indicated in (A) changed to A.

Figure 8. Putative secondary structure in the 5' end of SgrS5K12 results in low SgrT production. (A) Aligned sequences of the 5' end of SgrS homologs were examined and a potential hairpin was identified in the sgrT ribosome-binding region of the sgrS5K12 allele. The putative sgrT ribosome-binding site is indicated by a bracket below the aligned sequences. The inverted repeat is indicated by arrows above the K12 sequence. Two C residues in this inverted repeat that were mutated to T (sgrS4) or A (sgrS5) are indicated with circles. The sgrT start codon is boxed. (B) Strains carrying plasmids with sgrS, sgrS4 and sgrS5 alleles with sgrT-3X-FLAG from E. coli (K12) or S. typhimurium (St) were grown as described in the legend for Figure 7 and a western blot using anti-FLAG antibody was performed as described in ‘Materials and Methods’ section. Alleles are described in Table 1 and briefly as follows: sgrS, wild-type; sgrS4, C residues indicated in (A) changed to T; sgrS5, C residues indicated in (A) changed to A.

sgrT increase production of SgrT by the E. coli sgrS4 and sgrS5 alleles. There is slightly more SgrT produced from the sgrS5K12 allele compared with the sgrS4K12 allele, which supports the argument that the proposed secondary structure (Figure 8A) modulates SgrT production because the sgrS4K12 allele contains mutations that would preserve a weakened form of the structure (with G:U base pairs instead of G:C). Further evidence that this structure inhibits SgrT production came from inducer exclusion experiments. Cells expressing sgrS4K12 and sgrS5K12 had ~12-fold higher levels of β-galactosidase activity compared with control cells (Figure S2B), strongly suggesting that these alleles produce sufficient SgrT to block glucose uptake through the PTS. Taken together, all of these data support our hypothesis that a hairpin formed in the translation initiation region of sgrT in the SgrSK12 sRNA is responsible for repressing production of SgrT. Unfortunately, it is not possible to construct mutations in the other half of the inverted repeat that would compensate and restore the predicted hairpin as these would alter the Shine–Dalgarno sequence of sgrT and would therefore themselves have a negative effect on sgrT translation.

DISCUSSION
Numerous Hfq-dependent sRNAs have been identified in recent years. To date, all of these appear to function solely as non-coding riboregulators. Some of these [e.g. the E. coli RyhB sRNA (20)] target dozens of mRNAs for translational regulation or degradation and thus have far-reaching effects on cell physiology. It appears as though the E. coli SgrS sRNA has a more limited scope for its riboregulation function. We know of only two E. coli mRNAs that are directly targeted for negative regulation by SgrS; these both encode PTS carbohydrate transporters. However, SgrS has the additional unusual property of encoding a small protein with its own individual function in the glucose-phosphate stress response. In the present study, we used a set of homologs of SgrS (identified in a separate study, 9) as natural ‘mutants’ that would provide insight into the individual roles of riboregulation and SgrT in the glucose-phosphate stress response. To our knowledge, this is the first study to report a side-by-side functional comparison of homologous sRNAs.

The 3' region of SgrS contains sequences involved in base pairing with target mRNAs and is the most conserved at the primary sequence level (9), suggesting that the riboregulation function is also well-conserved. Among the homologs tested, only SgrSYp and SgrSEcar failed to inhibit growth on glucose minimal medium (Figure 1B). We showed that the failure to inhibit growth on glucose was not due to failure to regulate ptsG translation (Figure 2B). Since we know that SgrSK12 targets the manXYZ mRNA (J. B. Rice and C. K. Vanderpool, unpublished data), which encodes a PTS system of broad substrate specificity, we hypothesized that the sgrSYp and sgrSEcar alleles' failure to down-regulate this message may account for their inability to fully inhibit growth on glucose. Indeed, we found that both of these homologs could fully inhibit growth on glucose in a ΔmanXYZ host (Figure 1C). These observations highlight one aspect of heterogeneity among SgrS homologs. We have not yet determined whether these SgrS homologs also fail to regulate their cognate manXYZ mRNAs or whether the base pairing determinants for SgrS and manXYZ mRNA have evolved compensatory mutations that preserve the regulation.

Phenotypic differences in rescue from stress and glucose growth inhibition among sgrS homologs indicate that of the two functions of sgrS (riboregulation and SgrT production) that SgrT production is more variable. The major functional difference among SgrS homologs was revealed by analyses of mutants with defective base pairing functions (Figures 2C, 4 and 5). The sgrSLK12 and sgrS1Yp alleles failed to rescue cells from stress, indicating that for these homologs, the base pairing function is primarily responsible for the rescue phenotype. This was not surprising for sgrSLYp, since it lacks the sgrT CDS altogether (9). However, for the sgrSLK12 allele, it was an unexpected result as we had previously shown that ectopic expression of sgrT alone was sufficient for rescue (4). Furthermore, other homologs that encode sgrT (sgrS1St, sgrS1Ecar and sgrS1Kp) were fully capable of rescue even with defective base pairing functions. The disparate requirements for base pairing among these homologs likely can be explained by the occurrence of a translation-inhibitory secondary structure in the sgrSK12 5' region. Mutations that disrupt the putative secondary structure allow higher-level production of SgrT (Figure 8B). It is unclear whether this structure serves a
regulatory role. There may be a condition or additional regulatory factor that we have not identified that modulates sgrT translation in E. coli K12. On the other hand, it is possible that some organisms, e.g. E. coli K12 and Y. pestis, have lost the need for SgrT and cope with stress using the base pairing function alone, while other organisms still require both base pairing and production of SgrT to adequately respond to stress.

The exact nature of the signal that initiates the glucose-phosphate stress response remains unclear, though the activities of SgrS appear to be aimed at limiting sugar transport or the glycolytic pathway, these other species should also have a mechanism for responding to stress. Beyond the identity of the stress signal, the natural conditions that induce this stress response are also unknown. The distribution of SgrRST homologs is limited to γ-Proteobacteria (9). However, many species outside this group of organisms also utilize the PTS for glucose transport and glycolysis for its catabolism. If this metabolic stress is inherently generated by either PTS-mediated transport and glycolysis for its catabolism. If this metabolic stress is inherently generated by either PTS-mediated transport or the glycolytic pathway, these other species should also have a mechanism for responding to stress. It seems likely that we will not understand this issue until we have further elucidated the nature of the stress signal and the effects of the stress on cell physiology.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCES