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Physiological Consequences of Multiple-Target Regulation by the Small RNA SgrS in *Escherichia coli*

Yan Sun, Carin K. Vanderpool

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

Cells use complex mechanisms to regulate glucose transport and metabolism to achieve optimal energy and biomass production while avoiding accumulation of toxic metabolites. Glucose transport and glycolytic metabolism carry the risk of the buildup of phosphosugars, which can inhibit growth at high concentrations. Many enteric bacteria cope with phosphosugar accumulation and associated stress (i.e., sugar-phosphate stress) by producing a small RNA (sRNA) regulator, SgrS, which decreases phosphosugar accumulation in part by repressing translation of sugar transporter mRNAs (*ptsG* and *manXYZ*) and enhancing translation of a sugar phosphatase mRNA (*yigL*). Despite a molecular understanding of individual target regulation by SgrS, previously little was known about how coordinated regulation of these multiple targets contributes to the rescue of cell growth during sugar-phosphate stress. This study examines how SgrS regulation of different targets impacts growth under different nutritional conditions when sugar-phosphate stress is induced. The severity of stress-associated growth inhibition depended on nutrient availability. Stress in nutrient-rich media necessitated SgrS regulation of only sugar transporter mRNAs (*ptsG* or *manXYZ*). However, repression of transporter mRNAs was insufficient for growth rescue during stress in nutrient-poor media; here SgrS regulation of the phosphatase (*yigL*) and as-yet-undefined targets also contributed to growth rescue. The results of this study imply that regulation of only a subset of an sRNA's targets may be important in a given environment. Further, the results suggest that SgrS and perhaps other sRNAs are flexible regulators that modulate expression of multigene regulons to allow cells to adapt to an array of stress conditions.

All organisms must produce biomass and generate energy from external substrates in order to grow. Microbes have evolved complex regulatory mechanisms to optimize uptake and metabolism of glucose, which is a preferred carbon source for many species, while avoiding accumulation of unnecessary and potentially toxic metabolic intermediates. In many bacteria, glucose is transported into cells mainly by the phosphoenolpyruvate phosphotransferase system (PTS), which consists of two general sugar transport proteins enzyme I (EI) and histidine protein (HPr), as well as two glucose-specific proteins glucose-specific enzyme IIA ($EIIA^{Glc}$) and $EIICB^{Glc}$ (1). The expression of the *ptsG* gene encoding $EIICB^{Glc}$ is extensively regulated transcriptionally and posttranscriptionally. Transcription factor proteins responding to different environmental conditions regulate *ptsG*. For example, *ptsG* transcription is activated by the cyclic AMP (cAMP) receptor protein (CRP) (2), and negative control is exerted by the repressor Mlc, which inhibits *ptsG* transcription in the absence of glucose (3, 4). Posttranscriptional control of *ptsG* expression is mediated by the small RNA (sRNA) regulator SgrS (5). SgrS is produced in response to a metabolic stress known as sugar-phosphate or glucose-phosphate stress, which is characterized by cytoplasmic accumulation of certain phosphosugars and inhibition of cell growth. *Escherichia coli* cells require SgrS to resist stress—i.e., continue growing under stress conditions (6).

SgrS, like many other sRNA regulators, depends on the RNA chaperone Hfq for stability and to facilitate base pairing interactions with target mRNAs (7). SgrS forms base pairing interactions with *ptsG* mRNA that occlude the *ptsG* ribosome binding site (RBS), resulting in translation inhibition and subsequent RNase-E dependent degradation of the SgrS-*ptsG* duplex (5, 7, 8). We recently showed that SgrS represses translation of a second target mRNA, *manXYZ* (9, 10), which encodes the mannose (and auxiliary glucose) PTS transporter. SgrS-mediated inhibition of sugar transporter synthesis is believed to limit further uptake and accumulation of stressor phosphosugars during glucose-phosphate stress (Fig. 1) (10). SgrS was also recently demonstrated to act as a positive regulator of a novel target, *yigL*. SgrS-*yigL* mRNA base pairing selectively stabilizes a processed form of *yigL* mRNA by masking an RNase E cleavage site (38). SgrS-mediated stabilization of *yigL* mRNA allows for enhanced production of the encoded haloacid dehalogenase (HAD)-like phosphatase, which was previously shown to dephosphorylate glucose-6-phosphate and its analog 2-deoxyglucose-6-phosphate *in vitro* (11). This and other evidence suggests that SgrS enhances YigL production in order to promote dephosphorylation of sugar-phosphates so that the resulting uncharged sugars can be exported by an unknown efflux pump (Fig. 1) (38). In addition to regulating translation and stability of target mRNAs via base pairing interactions, we showed that *sgrS* encodes a functional protein, SgrT (12). Whereas SgrS base pairing activity affects new protein synthesis, SgrT inhibits the activity of extant sugar transporters (12). In a previous study, we showed that *E. coli* SgrS does not produce significant amounts of SgrT under typical glucose-phosphate stress conditions and that the base pairing function is sufficient for *E. coli* growth recovery (13).

While there are unanswered questions regarding the roles of SgrS and the stress response in natural environments, it is clear that the response is broadly conserved among enteric bacteria (14, 15). Glucose-phosphate stress occurs in several different circum-

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Address correspondence to Carin K. Vanderpool, cvanderp@life.illinois.edu.

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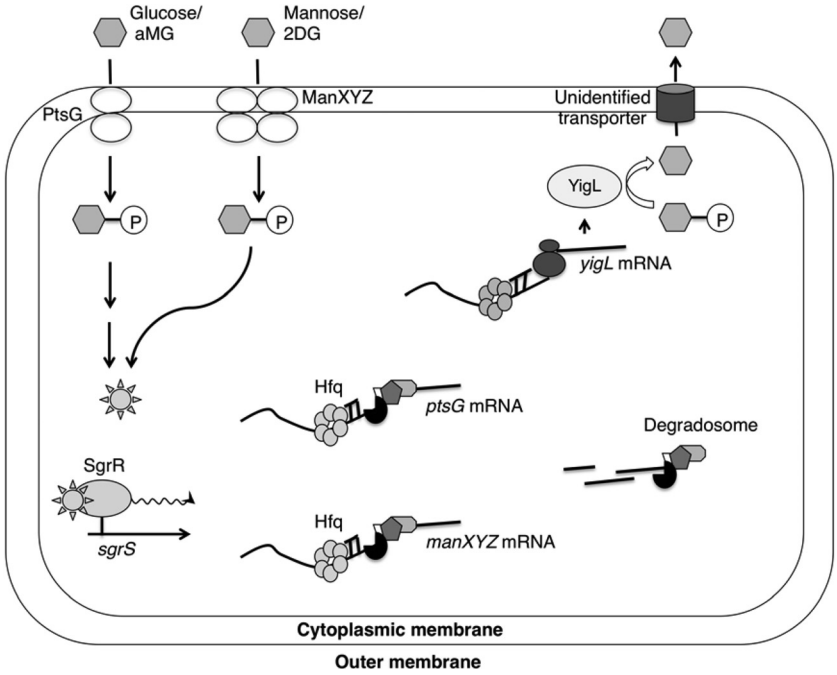

FIG 1 Model for the SgrS-mediated glucose-phosphate (GP) stress response. During glucose-phosphate stress, SgrR activates transcription of *sgrS*. SgrS associates with Hfq and negatively regulates the *ptsG* and *manXYZ* mRNAs, which encode major PTS sugar transporters. In addition, SgrS positively regulates the *yigL* mRNA, encoding a phosphatase. Dephosphorylation of sugars is a prerequisite for their efflux through unknown transporters. The regulation of these target mRNAs by SgrS, in turn, helps cellular recovery from GP stress. aMG, αMG; P, phosphate group.

stances, all having in common perturbed glycolytic metabolism resulting in accumulation of nonmetabolizable phosphosugars. Wild-type *E. coli* and *Salmonella enterica* cells induce the stress response when the glucose analogs α-methyl glucoside (αMG) or 2-deoxyglucose (2DG) are taken up through the glucose or mannose PTSs, respectively, causing accumulation of α-methyl glucoside-6-phosphate or 2-deoxyglucose-6-phosphate (5, 10). Interestingly, unlike *E. coli* and *Salmonella*, some microbes, such as *Klebsiella pneumoniae*, possess enzymes for αMG catabolism (16), while other organisms can utilize 2DG as a carbon source (17), raising the possibility that these (or similar) compounds may be present in some natural environments. These two glucose analogs, αMG and 2DG, are ideal model stress inducers for our studies because they enter *E. coli* cells through different PTS transporters that are regulated by SgrS. PtsG ($EIICB^{Glc}$) is the primary αMG transporter, whereas ManXYZ (mannose-specific enzyme IIABCD [$EIIABCD^{Man}$]) transports 2DG (18, 19).

Most studies so far have focused on the molecular mechanisms of individual target regulation by SgrS, and these have revealed novel and interesting aspects of sRNA-mediated regulation. However, it is unknown how coordinated regulation of these multiple targets contributes to the physiology of the glucose-phosphate stress response. Importantly, the issue of multiple target regulation by bacterial sRNAs is relevant for dozens of other Hfq-binding sRNAs that have also been shown to control expression of many genes (20–22), and little is known about how this property contributes to sRNA-mediated stress responses. In the present study, we sought to establish SgrS as a model sRNA for exploring the consequences of coordinated regulation of multiple targets with regard to stress resistance. We hypothesized that regulation of some targets would be more important than others in terms of rescuing cell growth during stress and that this arrangement might change to match fluctuations in the environment. To address this hypothesis, we began by testing how SgrS regulation of different targets impacts growth in response to different inducers of stress (αMG or 2DG) in different nutrient environments. Our results demonstrate for the first time that regulation of individual sRNA targets can contribute differentially to a stress response depending upon the particular source of stress and other environmental conditions. With regard to glucose-phosphate stress, our results highlight the importance of different carbon sources in modulating the severity of sugar-phosphate-associated metabolic stress. We show that under less severe stress conditions (in nutrient-rich media), SgrS needs only to repress synthesis of the relevant sugar transporter in order to ensure stress resistance. When stress becomes more severe (in nutrient-poor media), regulation of additional SgrS targets becomes crucial for growth recovery. These results show that regulation of only a subset of SgrS targets is important for responding to a given stressor in a particular environment, suggesting a broad role in nature for SgrS-mediated responses to metabolic stress. Our results imply that sRNAs may have evolved as flexible regulators that adjust their regulons in accordance with environmental changes, thus providing bacteria with efficient and adaptable responses to different stressors.

MATERIALS AND METHODS

Bacterial strain and plasmid construction. Most strains used in this study are derivatives of *E. coli* DJ480 (D. Jin, National Cancer Institute), and all bacterial strains are listed in Table 1. The sequences of all oligonucleotides (Integrated DNA Technologies) used in the construction of mutant strains and plasmids are listed in Table 2.

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description or relevant characteristic(s)	Source or reference
Bacterial strains		
MG1655	Wild-type *E. coli* K-12	D. Jin (NCI)
DJ480	MG1655 Δ*lacX74*	D. Jin (NCI)
CS104	DJ480 Δ*sgrS*	13
CS123	DJ480 *sgrS1*	13
CS168	DJ480 λ*attB*::*lacI*$^{q+}$ tetR Sp^r	23
CS194	DJ480 Δ*yigL*::FRT λ*attB*::*lacI*$^{q+}$ tetR Sp^r	This study
CS195	DJ480 Δ*yigL*::FRT Δ*sgrS* λ*attB*::*lacI*$^{q+}$ tetR Sp^r	This study
JH111	DJ480 Δ*sgrS* λ*attB*::*lacI*$^{q+}$ tetR Sp^r	10
JH116	DJ480 Δ*sgrS manX′-′lacZ lacI*$^{q+}$	10
JH171	DJ480 Δ*sgrS ptsG′-′lacZ lacI*$^{q+}$	10
YS185	DJ480 Δ*yigL*::FRT	This study
YS234	DJ480 Δ*sgrS yigL′-′lacZ* λ*attB*::*lacI*$^{q+}$ tetR Sp^r	This study
YS236	λ*attB*::tet	This study
YS237	DJ480 λ*attB*::tet	This study
YS238	DJ480 λ*attB*::tet *sgrS1*	This study
YS246	Δ*sgrS*::kan-*araC*-P$_{BAD}$-*ccdB*; mini λ	This study
YS247	DJ480 λ*attB*::tet Δ*sgrS*	This study
YS248	DJ480 *sgrS28*	This study
YS249	DJ480 λ*attB*::tet *sgrS28*	This study
YS258	DJ480 Δ*sgrS* cm-P$_{lac}$-*ptsG* λ*attB*::*lacI*$^{q+}$ tetR Sp^r	This study
YS259	DJ480 cm-P$_{lac}$-*ptsG* λ*attB*::*lacI*$^{q+}$ tetR Sp^r	This study
YS265	DJ480 cm-P$_{lac}$-*ptsG yigL*::FRT Δ*sgrS* λ*attB*::*lacI*$^{q+}$ tetR Sp^r	This study
YS269	DJ480 *sgrS26*	This study
YS270	DJ480 λ*attB*::tet *sgrS26*	This study
YS273	DJ480 λ*attB*::tet Δ*yigL*::FRT	This study
YS283	Δ*manXYZ*::kan Δ*sgrS* λ*attB*::tet	This study
YS284	Δ*ptsG*::cm Δ*sgrS* λ*attB*::tet	This study
YS285	Δ*manXYZ*::kan λ*attB*::tet	This study
YS286	Δ*ptsG*::cm λ*attB*::tet	This study
Plasmids		
pBRCS12	Vector control for pLCV1, pBRCS6, pBRJH19, pBR26 and pBRYS4	13
pLCV1	P$_{lac}$-*sgrS*	5
pBRCS6	P$_{lac}$-*sgrS1*	13
pBRJH26	P$_{lac}$-*sgrS26*	10
pBRYS4	P$_{lac}$-*sgrS28*	This study
pZE21	Vector control for pZEYS2	23
pZEYS2	P$_{LtetO-1}$-*yigL*	This study

Strains JH116 and JH171, which contain the *manX′-′lacZ* and *ptsG′-′lacZ* translational fusions, respectively, were described in a previous study (10). The *yigL′-′lacZ* translational fusion was created using a technique described previously (24). Briefly, a kanamycin cassette flanked by a FLP recombination target (FRT) site was amplified from template pKD13 using oligonucleotides O-YS206 and O-YS207 (Table 2) and integrated into the chromosome by λ Red recombination at the *yigL* locus. The kanamycin cassette was then removed using the helper plasmid pCP20 encoding the FLP recombinase, resulting in a strain carrying a single FRT site. Subsequently, translational fusion vector pCE40 (24) was integrated into the chromosome by FLP-dependent site-specific recombination, resulting in *′lacZ* fused to the 17th codon of *yigL* and linked to the kanamycin cassette. The fusion was then transduced by P1 phage into a previously described strain, JH111 (10) (which is *lacI*$^{q+}$ and Δ*sgrS* [Table 1]) to create strain YS234.

Strains CS104 and CS123, which carry the Δ*sgrS* and *sgrS1* mutations, respectively, were described previously (13). The Δ*manXYZ*::kan allele was moved into strain CS104 via P1 transduction to create strain CS184 by C. Wadler in our laboratory. The Δ*ptsG*::cm allele was transduced into strain CS104 to create strain CV106. Chromosomal *sgrS26* and *sgrS28* alleles (Fig. 2A) were constructed using a strategy modified from the one described in a previous study (25), and a strain that carries the kanamycin cassette fused to the *araC* gene and the toxin gene *ccdB* under the control of the P$_{BAD}$ promoter (a gift from N. Majdalani, National Cancer Institute). The kan-*araC*-P$_{BAD}$-*ccdB* region was PCR amplified by oligonucleotides O-YS226 and O-YS227 and inserted into the *sgrS* locus of strain NM300 (which carries a mini-λ encoding λ Red functions [5]), resulting in strain YS246. Mini-λ was maintained in strain YS246 by growth at 30°C. Subsequently, the *sgrS26* allele was PCR amplified from plasmid pBRJH26, using oligonucleotides O-YS261 and O-YS230. The *sgrS28* allele was amplified using genomic DNA from wild-type DJ480 as the template, and oligonucleotides O-YS228/O-YS230, which incorporated the desired point mutations. Following induction of λ Red functions in strain YS246, *sgrS26* and *sgrS28* PCR products were transformed by electroporation, yielding strains YS269 and YS248, respectively. Recombinants were obtained by counterselection against *ccdB* by plating cells on medium containing 1% L-arabinose.

To create the Δ*yigL*::FRT-kan-FRT allele, a kanamycin cassette flanked by FLP recombination target sites was amplified from template pKD13 (24) using oligonucleotides O-YS156/O-YS157. The Δ*yigL*::FRT-kan-FRT allele was then moved into the previously described strains

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
O-YS156	CCCAGCGGAAACCGCTCTACAGAGGTTTAAATTTCTTGTGTAGGCTGGAGCTGCTTCG
O-YS157	GCGAAGTATCAGGTTGACAACTGACCAAATAAAGAACGAATTCCGGGGATCCGTCGAC
O-YS206	CATACGTTATCCCCTTACGCCAAAGAACTCTGAAGCTGATTCCGGGGATCCGTCGA
O-YS207	GTATCCATTGTAGCGAAGTATCAGGTTGACAACTGACCAGTGTAGGCTGGAGCTGCT
O-YS212	TGAAAGTTGACTTGCCTGCATCATCACACACTGAGTATTGGTGTAAAATCACCCGC
O-YS213	ACCTTCCCGTTTCGCTCAAGTTAGTATAAAAAAGCACTAGACATCATTAATTCCTA
O-YS214	CCGGGCTATGAAATAGAAAAATGAATCCGTTGAAGCCGAAGCTAAATCTTCTTTATC
O-YS215	CCCAAGCTTATTAAAGAGGAGAAATTAACTATGTACCAGGTTGTTGCGTCTGAT
O-YS216	CCCGGATCCCCAAATAAAGAACGATTACGATAAATAGAGTTTACGCAGA
O-YS225	CCTGTGACGGAAGATCACTTCGCAGAATAA
O-YS226	CAGTGGGATGACCGCAATTCTGAAAGTTGACTTGCCTGCAATAGGAACTTCAAGATCC
O-YS227	TACGGCGAGCCATCGTCATTATCCAGATCATACGTTCCTTATATTCCCCAGAACATCAGG
O-YS228	CAGTGGGATGACCGCAATTCTGAAAGTTGACTTGCCTGCATCATCACACACTGAGTATT
O-YS230	TACGGCGAGCCATCGTCATTATCCAGATCATACGTTCCC
O-YS238	GTGCTCAGTATCTTGTTATCCGCTCACAATGTCAATGTTATCCGCTCACATTTATTTATCCGCTCACATTTATTTATCACTTAT
O-YS240	AGCTCGTAATTAATGGCTAAAACGAGTAAAGTTCACCCCTGTGACGGAAGATCACTT
O-YS241	CCTCGCCGTGTACAGGGCATCTAAGCGCCCTTTATTTATGTGCTCAGTATCTTGTTATC
O-YS261	CAGTGGGATGACCGCAATTCTGAAAGTTGACTTGCCTGCATCATCTGTGACTGAGTATT

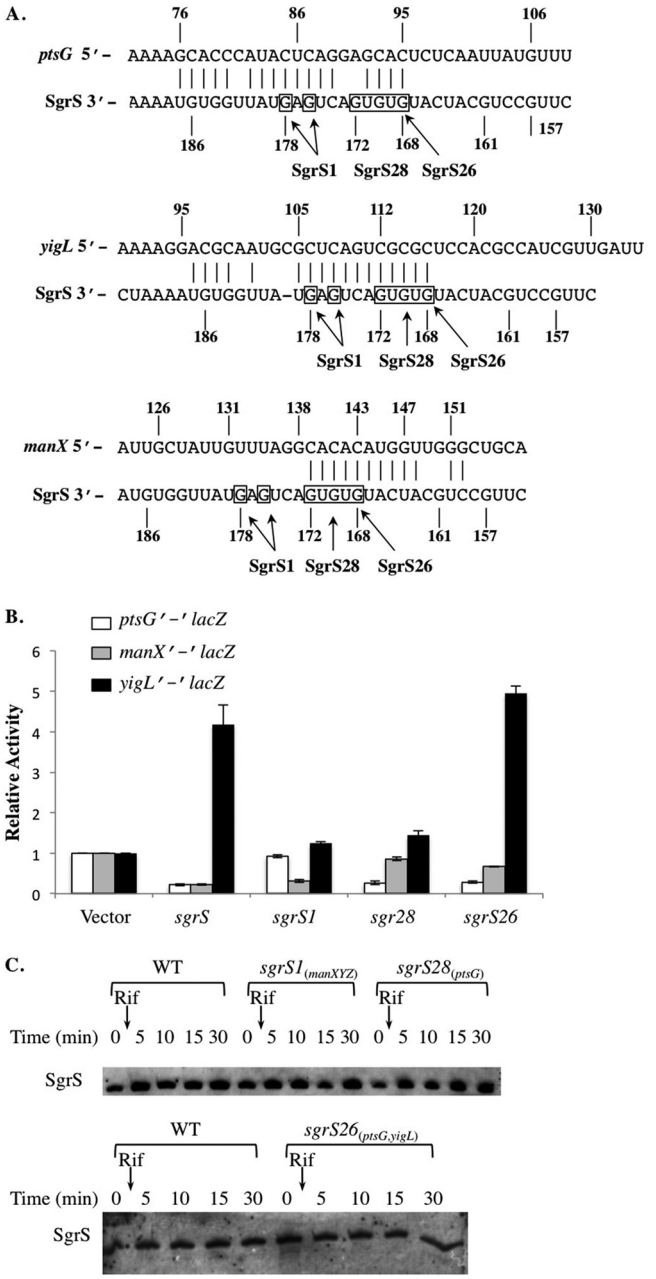

FIG 2 SgrS mutant alleles differentially regulate expression of *ptsG*, *manX*, and *yigL*. (A) Base pairing between SgrS and the three targets, the *ptsG*, *manX*, and *yigL* mRNAs, are indicated by vertical lines. The sequence directly above SgrS and allele names (SgrS1, SgrS26, and SgrS28) indicate the mutated bases and their positions in different SgrS mutants. (B) The Δ*sgrS* strains with *ptsG′-′lacZ*, *manX′-′lacZ*, or *yigL′-′lacZ* carrying an empty vector, P_{lac}-*sgrS*, P_{lac}-*sgrS1*, P_{lac}-*sgrS26*, or P_{lac}-*sgrS28* were grown to early log phase and exposed to 0.1 mM IPTG. Samples were collected 60 min after IPTG addition and assayed for β-galactosidase activity. Specific activities were normalized to the levels in the strains carrying the empty vector to yield relative activity (fold). Three independent experiments were performed; results reported are averages plus standard deviations (error bars). (C) Strains were grown to early log phase and exposed to 0.5% αMG for 10 min. Rifampin (Rif) (250 μg/ml) was then added to all cultures, and RNA samples were harvested at the indicated time points and subjected to Northern blot analysis. Blots shown are representative of three independent experiments. WT, wild type.

CS168 and JH111 (10, 13) by P1 transduction. Subsequently, the kanamycin resistance cassettes in these strains were eliminated using pCP20, a plasmid that expresses FLP recombinase (24); the resulting strains are YS184, CS194, and CS195.

The *attB*::tet allele in strain YS236 was created using primers O-YS213/O-YS214, with homology to the *attB* locus to amplify the tetracycline resistance cassette, followed by λ Red recombination (26). This mutant allele was then transduced into strains DJ480, CV106, CS104, CS123, CS184, YS185, YS208, YS248, and YS269 to yield strains YS237, YS284, YS247, YS238, YS83, YS273, YS285, YS249, and YS270, respectively. The Δ*ptsG*::cm allele was moved into strain YS237 by P1 transduction to produce strain YS286.

To insert the P_{lac} promoter on the chromosome upstream of *ptsG*, we first amplified a chloramphenicol cassette from strain CV700, using oligonucleotides O-YS225 and O-YS238 that contain sequences homologous to the *cat* gene and the P_{lac} promoter. The resulting PCR product, which has the chloramphenicol cassette linked to the P_{lac} promoter, then served as the template for the next round of PCR amplification. Using oligonucleotides O-YS240 and O-YS241, we obtained a new PCR product, which contains the *cat* gene-linked P_{lac} promoter that is flanked by the region from −177 to −140 relative to the *ptsG* start codon, as well as the first 30 nucleotides (nt) of *ptsG* coding sequence. Using this PCR product and the λ Red recombination system (26), we created the cm-P_{lac}-*ptsG* allele, which was then transduced into strains JH111, CS168, and CS195, resulting in strains YS258, YS259, and YS265, respectively.

Strains DH5α (Invitrogen) and XL10 (Stratagene) were used for cloning and QuikChange mutagenesis, respectively. To construct plasmid pZEYS2, the *yigL* gene was amplified by PCR using the forward primer O-YS215, which contains a HindIII site and a 24-nt fragment from the pQE80L vector (Qiagen) carrying the ribosome binding site, followed by *yigL* sequence. The reverse primer, O-YS216, contains a BamHI site and sequences homologous to the region downstream of the predicted *yigL* terminator. The HindIII- and BamHI-digested PCR product was then cloned into the previously described vector pZE21 (23). Plasmids pLCV1, pBRCS6, and pBRJH26 that carry wild-type *sgrS*, *sgrS1*, and *sgrS26*, respectively, were described in previous studies (10, 13). Plasmid pBRYS4, containing the *sgrS28* allele, was created using the QuikChange II site-directed mutagenesis kit (Agilent Technologies) with oligonucleotide O-YS212 and the previously described plasmid pBRJH19 (10) as the template.

β-Galactosidase assays. Strains containing translational fusions were grown overnight in TB (Bacto tryptone) medium (BD, Franklin Lakes, NJ) with 100 μg/ml ampicillin and subcultured 1:200 to fresh medium. Cultures were grown to an optical density at 600 nm (OD_{600}) of ~0.5 and exposed to 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Samples were taken 1 h later and assayed for β-galactosidase activity as described previously (23). The β-galactosidase activity (measured in Miller units) produced by cells carrying the vector control was set at 1.0. Activity values for other strains were normalized to the vector control to give relative activity for experimental samples.

RNA extraction and Northern blot analyses. To examine the stabilities of SgrS variants, strains were grown in LB medium to an OD_{600} of ~0.5 and exposed to 0.5% αMG (Sigma) for 10 min. Rifampin (250 μg/ml) was then added to the cultures, and RNA was extracted at the indicated time intervals by the previously described hot phenol method (5). Northern blot analysis with probe *sgrS*-1bio (5) was used to detect the SgrS RNA.

Growth experiments. For growth competition experiments, cells were grown overnight in LB or minimal MOPS (morpholinepropanesulfonic acid) medium (Teknova) supplemented with 0.4% glycerol or 0.2% fructose as indicated. Two competing strains were mixed at 1:1 ratios (based on OD_{600}), inoculated in fresh media, and grown to an OD_{600} of ~0.03 (minimal MOPS medium with glycerol) or to an OD_{600} of ~0.1 (LB and minimal MOPS medium with fructose). Cultures were then exposed to 0.5% αMG or 2DG (Sigma) or kept under nonstress conditions. Due to the overall lower growth rate in glycerol, cells grown with glycerol

were exposed to αMG until they reached a lower OD_{600} and incubated longer. Culture samples were collected right after the initial mixing, as well as 3 h (LB), 17 h (minimal MOPS medium with fructose), or 19 h (minimal MOPS medium with glycerol) after exposure of the cells to the stress inducer. Serial dilutions were made from the collected samples and plated on LB agar with or without 100 μg/ml tetracycline.

For the experiments involving removal of inducers, strains were grown overnight in LB or minimal MOPS medium supplemented with 0.4% glycerol (25 μg/ml kanamycin was added to the medium when working with strains carrying plasmids pZE21 and pZEYS2) in the presence of 0.1 mM IPTG and then subcultured 1:200 in fresh media. Anhydrotetracycline (aTc) (25 ng/ml) was added to the subcultures when working with strains carrying plasmids. Cells were harvested at an OD_{600} of ~0.1 by filtration, washed, and resuspended in fresh media with 0.5% αMG and in the presence or absence of 0.1 mM IPTG. aTc at 25 ng/ml was added back to these cultures when working with strains carrying plasmids pZE21 and pZEYS2. In other regular growth experiments, strains were grown in minimal MOPS medium supplemented with 0.4% glycerol, 25 μg/ml kanamycin, and 25 ng/ml aTc to an OD_{600} of ~0.1 and then exposed to 0.5% αMG.

For growth experiments involving the supplementation of Casamino Acids, strains were grown in minimal MOPS medium with 0.4% glycerol or 0.2% fructose overnight and subcultured to an OD_{600} of ~0.05 in fresh medium. Casamino Acids (final concentration of 0.1%) and/or 0.5% αMG were also added to the medium to the subcultures as indicated. The growth of strains was monitored using a FLUOstar Omega multimode microplate reader (BMG Labtech).

RESULTS

Mutations in SgrS base pairing determinants have differential effects on regulation of three targets. Previous studies have demonstrated that duplex formation between SgrS and its target mRNAs is essential for regulation. For each of the targets, the residues of SgrS involved in pairing partially overlap and are partially distinct (10). For example, though two G-C base pairs formed by SgrS residues G176 and G178 (Fig. 2A) are critical for translational repression of *ptsG* by SgrS (7), they are not required for inhibition of *manX* translation (10). To begin to elucidate how SgrS coordinates regulation of multiple targets, we sought to identify additional mutations in *sgrS* that would result in differential target regulation. Regulation by wild-type and mutant SgrS was monitored using translational *lacZ* fusions to the three known SgrS targets. As shown in Fig. 2B, wild-type SgrS (expressed from a plasmid under the control of the P_{lac} promoter) repressed *ptsG′-′lacZ* (~3-fold repressed compared to the vector control) and *manX′-′lacZ* (~3-fold repressed) and activated *yigL′-′lacZ* (~4.4-fold increase compared to the vector control). These results were consistent with previous reports (10; Papenfort et al., submitted). The *sgrS* allele with G176C and G178C mutations (Fig. 2A), which we refer to as *sgrS1*, repressed *manX′-′lacZ* almost as efficiently as wild-type SgrS but no longer regulated the activities of *ptsG′-′lacZ* (as observed previously [7, 10]) or *yigL′-′lacZ* (Fig. 2B). Thus, with regard to these three targets, SgrS1 specifically regulated only *manXYZ* and is hereafter referred to as $SgrS1_{manXYZ}$. SgrS28 carries five point mutations (G172C, T171A, G170C, T169A, and G168C [Fig. 2A]). When expressed from the P_{lac} plasmid, SgrS28 repressed *ptsG′-′lacZ* to a degree similar to wild-type SgrS (Fig. 2B) but failed to regulate either *manX′-′lacZ* or *yigL′-′lacZ* (Fig. 2B). Since SgrS28 specifically regulated only *ptsG*, it is referred to as $SgrS28_{ptsG}$. A previous study in our laboratory identified the *sgrS26* allele (Fig. 2A) with a G168C mutation as being defective for regulation of *manX′-′lacZ* (10). We confirmed this finding and further showed that SgrS26 repressed *ptsG′-′lacZ* activity and enhanced *yigL′-′lacZ* activity to a degree similar to wild-type SgrS (Fig. 2B). Since SgrS26 regulated both *ptsG* and *yigL* but was deficient in regulation of *manXYZ*, it is now referred to as $SgrS26_{ptsG,yigL}$. Altogether, we defined three mutants with distinct target repertoires, two that each regulate only one of the three known SgrS targets ($SgrS1_{manXYZ}$ and $SgrS28_{ptsG}$) and the third, which regulates two of the three known targets ($SgrS26_{ptsG,yigL}$).

TABLE 3 Competition assays to measure the effects of *sgrS* mutations on growth with αMG in LB medium

E. coli strain A genotype	*E. coli* strain B genotype	Without αMG		With 0.5% αMG	
		CI[a]	*P* value[b]	CI	*P* value
λ*attB*::tet	WT	1.08 ± 0.06	NS	1.0 ± 0.3	NS
Δ*sgrS* λ*attB*::tet	WT	0.82 ± 0.01	NS	0.6 ± 0.1	0.024
$sgrS1_{manXYZ}$ λ*attB*::tet	WT	0.98 ± 0.21	NS	0.5 ± 0.04	0.01
$sgrS28_{ptsG}$ λ*attB*::tet	WT	0.93 ± 0.08	NS	0.8 ± 0.07	NS
$sgrS1_{manXYZ}$ λ*attB*::tet	Δ*sgrS*	1.02 ± 0.15	NS	1.0 ± 0.01	NS
$sgrS28_{ptsG}$ λ*attB*::tet	Δ*sgrS*	0.97 ± 0.12	NS	3.5 ± 0.3	0.03
$sgrS28_{ptsG}$ λ*attB*::tet	$sgrS1_{manXYZ}$	1.03 ± 0.08	NS	2.9 ± 0.1	0.013

[a] See Materials and Methods for detailed procedures. The competition index (CI) is calculated as follows: (log_{10} strain A output/log_{10} strain B output)/(log_{10} strain A input/log_{10} strain B input). The results presented here are the averages ± standard deviations of three independent experiments.

[b] Student's *t* test was used to compare the output and inoculum. NS, not significant ($P \geq 0.05$).

Regulation of *ptsG*, but not *manXYZ* or *yigL*, is crucial for recovery from αMG-induced stress in nutrient-rich medium. In order to verify that differences in regulation were not due to decreased stability of mutant sRNAs, we tested the induction of chromosomally encoded *sgrS* mutant alleles in response to αMG and monitored stability using a rifampin chase. After αMG treatment, all three mutant SgrS molecules were present at levels similar to that of the wild type and also showed similar stabilities (Fig. 2C). These results indicated that the observed differences in regulation of targets were not due to effects on SgrS stability.

To examine how strains expressing target-specific mutant alleles cope with stress in the presence of various levels of nutrients, we examined stress induced by αMG under nutrient-rich and nutrient-poor conditions. Growth competition experiments were used to monitor the relative fitness of strains grown under stress conditions; these experiments were conducted as described in Materials and Methods. Briefly, the two strains were mixed at a 1:1 ratio. The mixed culture was exposed to αMG at early log phase and then was grown to saturation. The numbers of viable cells at both the initial mixing and the end of the experiment were determined by plating for CFU, and a competition index (CI) (27) was calculated as follows: (log_{10} strain A recovered/log_{10} strain B recovered)/(log_{10} strain A inoculated/log_{10} strain B inoculated). A CI equal to 1 indicates that the two strains compete evenly for resources in mixed culture; a CI that is less than 1 suggests that strain B outcompetes strain A, whereas a CI that is greater than 1 shows that strain A outcompetes strain B.

For a control, we first compared the growth of a strain marked with tetracycline resistance at a neutral genomic location (*attB*::tet) with the wild-type parent. The CI for the *attB*::tet strain versus the wild-type parent was ~1.0 in both the absence and presence of αMG when cells were grown in nutrient-rich LB medium (Table 3). This result demonstrated that the tetracycline resistance gene does not cause a growth defect under these conditions. As shown

TABLE 4 Competition assays to measure the effects of mutations in the three *sgrS* targets on growth with αMG in LB medium

E. coli strain A genotype	*E. coli* strain B genotype	Without αMG		With 0.5% αMG	
		CI[a]	*P* value[b]	CI	*P* value
Δ*ptsG* λ*attB*::tet	WT	0.96 ± 0.05	NS	2.6 ± 0.1	2.0E−5
Δ*manXYZ* λ*attB*::tet	WT	1.01 ± 0.1	NS	1.05 ± 0.04	NS
Δ*sgrS* Δ*ptsG* λ*attB*::tet	Δ*sgrS*	1.02 ± 0.03	NS	2.26 ± 0.18	0.0025
Δ*sgrS* Δ*manXYZ* λ*attB*::tet	Δ*sgrS*	0.98 ± 0.04	NS	1.06 ± 0.11	NS
Δ*yigL* λ*attB*::tet	WT	0.98 ± 0.07	NS	1.02 ± 0.14	NS

[a] See Materials and Methods for detailed procedures. The competition index (CI) is calculated as follows: ($\log_{10}$ strain A output/$\log_{10}$ strain B output)/($\log_{10}$ strain A input/$\log_{10}$ strain B input). The results presented here are the averages ± standard deviations of three independent experiments.

[b] Student's *t* test was used to compare the output and inoculum. NS, not significant ($P \geq 0.05$).

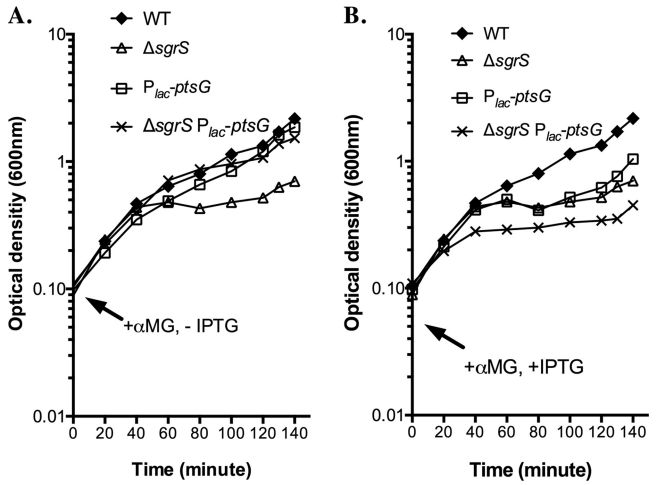

FIG 3 Regulation of *ptsG* by SgrS is crucial for recovery from αMG-induced stress. Strains were grown in LB medium overnight and then subcultured 1:200 in fresh medium, both in the presence of 0.1 mM IPTG. Cells were harvested at an optical density at 600 nm of ~0.1 by filtration, washed, and resuspended in fresh medium with 0.5% αMG and in the absence (A) or presence (B) of 0.1 mM IPTG. Growth of all cultures was monitored by OD_{600} throughout the whole procedure, but only the measurements following resuspension of cells are reported in the graphs. Results shown are representative of at least three independent trials.

in Table 3, in the absence of stress (LB medium without αMG), all strains competed equally, indicating that SgrS does not play a significant role in *E. coli* growth in rich medium. In contrast, under αMG-induced stress conditions, growth of the Δ*sgrS* mutant was attenuated when it competed with the wild-type strain (CI = 0.6), which is consistent with the important role of SgrS in the glucose-phosphate stress response, as reported previously (6). The $sgrS1_{manXYZ}$ mutant, which specifically regulates *manXYZ* but not *ptsG* or *yigL* (Fig. 2), also exhibited a growth defect in competition with the wild-type strain (CI = 0.5 [Table 3]). The CI for the $sgrS1_{manXYZ}$ mutant versus the Δ*sgrS* mutant was 1.0 (Table 3), indicating that SgrS regulation of *manXYZ* alone gives no growth advantage when the cells are stressed by αMG. In contrast, the strain with the $sgrS28_{ptsG}$ allele that allows regulation of *ptsG* but not *manXYZ* or *yigL*, performed well in growth competition with the wild-type strain (the CI, at 0.8, was not statistically different from 1.0 [Table 3]). Moreover, the $sgrS28_{ptsG}$ strain outcompeted the other two *sgrS* mutants with CIs of ~3 (Table 3). These results strongly suggested that during stress with αMG in rich medium, regulation of *ptsG*, which encodes the major transporter of αMG, is a crucial function of SgrS in the stress response, whereas regulation of *manXYZ* and *yigL* is not required.

To further test the roles of *manXYZ* and *yigL* in growth during stress, strains with deletions in each locus were tested in growth competition assays in rich LB medium with αMG (Table 4). The Δ*manXYZ* mutant (CI = 1.05) competed evenly with the wild-type strain under these conditions, and the Δ*manXYZ* Δ*sgrS* double mutant competed evenly with its Δ*sgrS* parent (CI = 1.06 [Table 4]), indicating that the absence of the ManXYZ sugar transporter provides no protection from αMG stress. Likewise, the Δ*yigL* mutant performed well in competition with the wild-type strain (CI = 1.02 [Table 4]), consistent with the results above and demonstrating that regulation of *yigL* is dispensable for growth recovery when cells are stressed with αMG in rich medium. In contrast, a Δ*ptsG* mutant outcompeted the wild-type strain (CI = 2.6), and a Δ*ptsG* Δ*sgrS* double mutant outcompeted its Δ*sgrS* parent strain (CI = 2.26) during growth in LB with αMG (Table 4). These results support the conclusions from analyses of *sgrS* mutant alleles (Table 3) and are consistent with the notion that PtsG is the major αMG transporter and that repression of PtsG synthesis is important for growth recovery in the presence of αMG.

Repression of *ptsG* by an SgrS-independent mechanism promotes recovery from αMG-induced stress in nutrient-rich medium. The *sgrS* base pairing mutations may affect the ability of SgrS to regulate other as-yet-unknown mRNA targets. Transcriptome analyses conducted in our laboratory have suggested that in addition to *ptsG*, *manXYZ*, and *yigL*, there are other mRNAs whose levels are altered upon SgrS induction (M. Bobrovskyy, G. Richards, D. Balasubramanian, and C. K. Vanderpool, unpublished data). While we do not yet know which (if any) of these are direct targets of SgrS, we wondered whether the *sgrS* mutations we constructed might affect recovery from stress by altering regulation of other targets. To address this issue, we tested whether cell growth could be rescued (in LB with αMG) by specific downregulation only of *ptsG* by a mechanism not dependent on SgrS. To this end, a P_{lac} promoter was inserted upstream of *ptsG* on the chromosome to control its expression at the level of transcription. By removing the inducer IPTG, new synthesis of PtsG ($EIICB^{Glc}$) protein could be stopped via turning off *ptsG* transcription in a manner that is independent of SgrS function. If stopping new synthesis of PtsG were sufficient for recovery from stress, we expected that a Δ*sgrS* mutant in which *ptsG* transcription had been turned off (Δ*sgrS* P_{lac}-*ptsG* mutant [Fig. 3A]) would show better growth than its Δ*sgrS* parent with *ptsG* under the control of the native promoter (Δ*sgrS* mutant [Fig. 3A]). Cells that were wild type for *sgrS* grew well under stress conditions, regardless of the transcriptional control of *ptsG* (Fig. 3A, compare the wild type to P_{lac}-*ptsG* mutant). This indicated that in the presence of SgrS, growth recovery under αMG stress conditions was not significantly affected by turning off transcription of P_{lac}-*ptsG* (by removal of IPTG). In contrast, in the Δ*sgrS* mutant background, cells with P_{lac}-*ptsG* recovered significantly better than cells expressing *ptsG* from its native promoter (Fig. 3A, compare Δ*sgrS* strain to Δ*sgrS* P_{lac}-*ptsG* strain). This result suggested that in the absence of SgrS, cell growth in the presence of αMG can be rescued by turning off new PtsG synthesis by an SgrS-independent

TABLE 5 Competition assays to measure the effects of *sgrS* mutations on growth with αMG in minimal MOPS medium with glycerol

E. coli strain A genotype	*E. coli* strain B genotype	Without αMG		With 0.5% αMG	
		CI[a]	*P* value[b]	CI	*P* value
λ*attB*::tet	WT	0.94 ± 0.04	NS	1.04 ± 0.06	NS
Δ*sgrS* λ*attB*::tet	WT	0.98 ± 0.1	NS	0.38 ± 0.11	<0.0001
$sgrS1_{manXYZ}$ λ*attB*::tet	WT	1.03 ± 0.12	NS	0.42 ± 0.06	0.014
$sgrS28_{ptsG}$ λ*attB*::tet	WT	0.96 ± 0.3	NS	0.34 ± 0.02	0.022
$sgrS26_{ptsG,yigL}$ λ*attB*::tet	WT	0.92 ± 0.07	NS	0.5 ± 0.03	0.015
$sgrS1_{manXYZ}$ λ*attB*::tet	Δ*sgrS*	0.96 ± 0.13	NS	1.01 ± 0.19	NS
$sgrS26_{ptsG,yigL}$ λ*attB*::tet	Δ*sgrS*	1.1 ± 0.51	NS	1.86 ± 0.64	0.023
$sgrS28_{ptsG}$ λ*attB*::tet	Δ*sgrS*	0.93 ± 0.21	NS	1.11 ± 0.16	NS
$sgrS26_{ptsG,yigL}$ λ*attB*::tet	$sgrS1_{manXYZ}$	1.03 ± 0.06	NS	1.75 ± 0.13	0.019
$sgrS28_{ptsG}$ λ*attB*::tet	$sgrS1_{manXYZ}$	0.91 ± 0.12	NS	1.02 ± 0.1	NS
$sgrS26_{ptsG,yigL}$ λ*attB*::tet	$sgrS28_{ptsG}$	1.03 ± 0.11	NS	2.09 ± 0.25	0.02

[a] See Materials and Methods for detailed procedures. The competition index (CI) is calculated as follows: ($\log_{10}$ strain A output/$\log_{10}$ strain B output)/($\log_{10}$ strain A input/$\log_{10}$ strain B input). The data presented are the averages ± standard deviations of three independent experiments.

[b] Student's *t* test was used to compare the output and inoculum. NS, not significant ($P \geq 0.05$).

TABLE 6 Competition assays to measure the effects of mutations in the three *sgrS* targets on growth with αMG in minimal MOPS medium with glycerol

E. coli strain A genotype	*E. coli* strain B genotype	Without αMG		With 0.5% αMG	
		CI[a]	*P* value[b]	CI	*P* value
Δ*ptsG* λ*attB*::tet	WT	1.02 ± 0.15	NS	2.87 ± 0.12	0.0008
Δ*manXYZ* λ*attB*::tet	WT	0.98 ± 0.11	NS	1.02 ± 0.008	NS
Δ*sgrS* Δ*ptsG* λ*attB*::tet	Δ*sgrS*	0.97 ± 0.02	NS	2.23 ± 0.46	0.04
Δ*sgrS* Δ*manXYZ* λ*attB*::tet	Δ*sgrS*	0.97 ± 0.12	NS	0.97 ± 0.07	NS
Δ*yigL* λ*attB*::tet	WT	1.01 ± 0.02	NS	0.26 ± 0.06	0.031
Δ*yigL* λ*attB*::tet	Δ*sgrS*	0.98 ± 0.04	NS	1.0 ± 0.11	NS

[a] See Materials and Methods for detailed procedures. The competition index (CI) is calculated as follows: ($\log_{10}$ strain A output/$\log_{10}$ strain B output)/($\log_{10}$ strain A input/$\log_{10}$ strain B input). The results presented here are the averages ± standard deviations of three independent experiments.

[b] Student's *t* test was used to compare the output and inoculum. NS, not significant ($P \geq 0.05$).

mechanism, i.e., stopping *ptsG* transcription. On the other hand, when IPTG was persistently present in the culture media, strains harboring P_{lac}-*ptsG* strains grow worse under stress than their corresponding parental strains with *ptsG* expressed from its native promoter (Fig. 3B), presumably because the P_{lac} promoter drives *ptsG* overexpression, resulting in higher αMG-6-phosphate accumulation. Collectively, these results are consistent with the idea that inhibition of PtsG synthesis is one of the primary adaptive effects mediated by SgrS under αMG stress conditions in rich medium.

SgrS-mediated regulation of *ptsG* and *yigL*, but not *manXYZ*, is required for recovery from αMG stress in certain minimal media. We reported previously that *sgrS* mutant strains have more pronounced growth defects when glucose-phosphate stress is induced in minimal medium compared with rich medium (23). Therefore, we were interested in determining how regulation of different SgrS targets contributed to growth recovery in the more stringent stress induced in minimal medium. To address this, we tested growth competition between wild-type and *sgrS* mutant strains grown with αMG as the stressor in minimal MOPS medium with glycerol (Table 5). (We also performed competition assays in minimal MOPS medium with fructose and αMG, and found that, as for competitions in LB with αMG, regulation of *ptsG* mRNA was crucial, whereas regulation of *manXYZ* and *yigL* appeared dispensable [Table 3; data not shown].) In minimal MOPS medium with glycerol, the *attB*::tet marker had no effect on growth without or with αMG (Table 5), and all strains competed evenly in the absence of the stressor (Table 5, without αMG). With αMG, both the Δ*sgrS* mutant (CI = 0.38 [Table 5]) and the $sgrS1_{manXYZ}$ mutant (CI = 0.42 [Table 5]) were at a significant growth disadvantage compared to the wild-type cells, whereas they competed evenly with one another (CI = 1.01 [Table 5]). In addition, the Δ*manXYZ* mutant (CI = 1.02 [Table 6]) and the Δ*manXYZ* Δ*sgrS* mutant (CI = 0.97 [Table 6]) competed equally with their respective parent strains during growth in minimal medium with αMG. These results (together with those in Tables 3 and 4) are consistent with the notion that the regulatory action of SgrS on *manXYZ* does not play a significant role in the response to αMG regardless of the nutrient content of the growth medium. In contrast, the competitiveness of strains with $sgrS28_{ptsG}$ was very different between rich and minimal media containing glycerol and αMG. The $sgrS28_{ptsG}$ mutant was severely attenuated in competition with the wild-type strain in minimal medium containing glycerol and αMG (CI = 0.34 [Table 5]). In fact, under these severe stress conditions, the $sgrS28_{ptsG}$ mutant competed evenly with the Δ*sgrS* mutant (CI = 1.11 [Table 5]) and the $sgrS1_{manXYZ}$ mutant (CI = 1.02 [Table 5]). On the other hand, under the same conditions, the Δ*ptsG* mutant outcompeted the wild-type strain (CI = 2.87 [Table 6]), and the Δ*ptsG* Δ*sgrS* mutant grew much better than its Δ*sgrS* parent strain (CI = 2.23 [Table 6]), suggesting that blocking αMG uptake by eliminating its major transporter PtsG can protect cells from αMG stress in minimal medium with glycerol. We interpret these results to mean that when cells are stressed while growing in minimal medium containing glycerol and αMG, regulating *ptsG* is not the only crucial contribution of SgrS to the stress response, and regulation of other targets is also required.

It was recently reported that *yigL* also plays a critical role in the glucose-phosphate stress response (38). Consistently, we found that a Δ*yigL* mutant growing in competition with its wild-type parent in minimal MOPS medium containing glycerol and αMG was at a significant disadvantage (CI = 0.26 [Table 6]). Under these conditions, the Δ*yigL* mutant competed evenly with the Δ*sgrS* mutant (CI = 1.0 [Table 6]), implying that *yigL* plays an essential role in recovery from αMG stress in minimal medium with glycerol. This result led us to hypothesize that cells expressing $sgrS28_{ptsG}$ fail to grow well in minimal medium containing glycerol and αMG because $SgrS28_{ptsG}$ cannot regulate *yigL*. We tested this hypothesis—that SgrS regulation of both *ptsG* and *yigL* is required for growth recovery during αMG stress in minimal medium—by competing a strain expressing $sgrS26_{ptsG,yigL}$, which expresses SgrS capable of regulating *ptsG* and *yigL* (Fig. 2A and B) with *sgrS* mutant strains. Consistent with our hypothesis, the $sgrS26_{ptsG,yigL}$ strain outcompeted the Δ*sgrS* (CI = 1.86 [Table 5]), $sgrS28_{ptsG}$ (CI = 2.09 [Table 5]), and $sgrS1_{manXYZ}$ (CI = 1.75 [Table 5]) mutants. These results strongly suggest that SgrS regulation of *ptsG* and *yigL* together protects cells from αMG stress under nutrient-poor conditions. However, while necessary, regulation of these two targets alone was not sufficient to promote full growth recovery, since the $sgrS26_{ptsG,yigL}$ mutant still displayed a growth deficit in competition with the wild-type strain (CI = 0.5 [Table 5]). More broadly, these results suggest that the importance of regulating different subsets of mRNA targets varies depending on specific stress conditions.

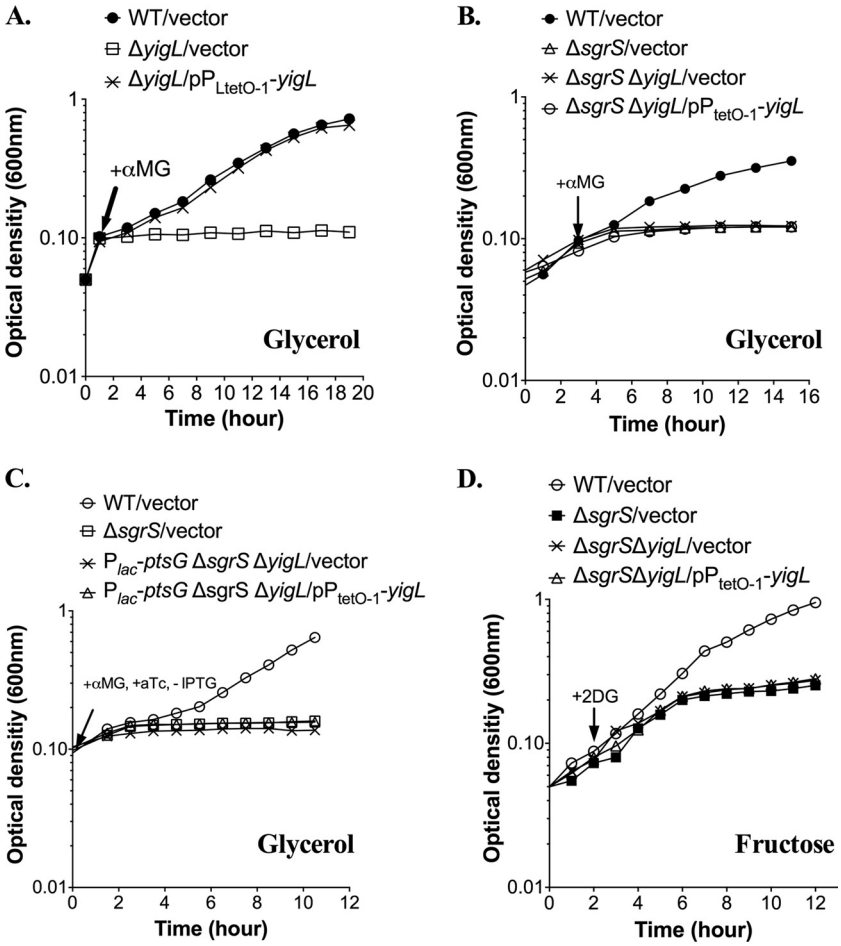

FIG 4 SgrS-mediated regulation of multiple targets, including *ptsG*, *yigL*, and additional targets, is required for recovery from αMG-induced stress. (A and B) Strains were grown in minimal MOPS medium supplemented with 0.4% glycerol in the presence of 25 ng/ml aTc to an OD_{600} of ~0.1 and then exposed to 0.5% αMG. (C) Strains were grown overnight in minimal MOPS medium supplemented with 0.4% glycerol and 25 μg/ml kanamycin and then subcultured 1:200 in fresh medium, both in the presence of 0.1 mM IPTG. aTc (25 ng/ml) was also present in all the subcultures. Cells were harvested at an OD_{600} of ~0.1 by filtration, washed, and resuspended in fresh medium with 0.5% αMG and 25 ng/ml aTc. Growth of all cultures was monitored by OD_{600} throughout the whole procedure, but only the measurements following resuspension of cells were reported in the graphs. (D) Strains were grown in minimal MOPS medium supplemented with 0.2% fructose in the presence of 25 ng/ml aTc to an OD_{600} of ~0.1 and then exposed to 0.5% αMG. All results are representative of at least three independent experimental trials.

To further test the contributions of *ptsG* and *yigL* regulation to recovery from αMG-induced stress, we again employed the Δ*sgrS* P_{lac}-*ptsG* strain, where the expression of *ptsG* can be manipulated in an SgrS-independent fashion. In this strain background, *yigL* was deleted from the chromosome and expressed in *trans* from the inducible $P_{LtetO-1}$ promoter. Strains were grown and stressed in minimal MOPS medium supplemented with glycerol and αMG. The Δ*yigL* strain carrying a vector control or the $P_{LtetO-1}$-*yigL* plasmid behaved as expected: the *yigL* mutant was immediately and strongly inhibited by the addition of αMG to cultures grown on minimal MOPS medium with glycerol (Fig. 4A), and induction of the plasmid-borne copy of *yigL* restored a wild-type pattern of growth to the Δ*yigL* mutant, confirming that the plasmid complements the *yigL* growth defect in an *sgrS*$^+$ host (Fig. 4A). The Δ*sgrS* Δ*yigL* double mutant experienced immediate growth inhibition similar to the Δ*sgrS* and Δ*yigL* parent strains (Fig. 4B). However, *yigL* carried on a plasmid failed to restore growth during stress in the Δ*sgrS* Δ*yigL* double mutant background (Fig. 4B), confirming the importance of SgrS-mediated regulation of targets in addition to *yigL* under these conditions.

By controlling *ptsG* transcriptional repression and *yigL* induction, independent of SgrS, we further validated the results of competition assays (*sgrS26*$_{ptsG,yigL}$ [Table 5]), suggesting that regulation of these two targets is necessary but not sufficient for growth rescue during αMG stress in minimal medium. Wild-type cells recovered from αMG stress, whereas Δ*sgrS* cells were severely growth inhibited (Fig. 4C). The growth of Δ*sgrS* Δ*yigL* P_{lac}-*ptsG* cells carrying the vector control was similarly inhibited, even though *ptsG* transcription was turned off (by removal of IPTG), validating the results of growth competition experiments (*sgrS28*$_{ptsG}$ [Table 5]) that showed that repression of *ptsG* alone did not provide a growth advantage in minimal medium with αMG. Introduction of the $P_{LtetO-1}$-*yigL* plasmid in the Δ*sgrS* Δ*yigL* P_{lac}-*ptsG* strain with simultaneous regulation of *yigL* and *ptsG* (by addition or removal of the appropriate inducers) did not rescue cells from stress caused by αMG (Fig. 4C).

TABLE 7 Competition assays to measure the effects of *sgrS* mutations on growth with 2DG in minimal MOPS medium with fructose

E. coli strain A genotype	*E. coli* strain B genotype	Without 2DG		With 0.5% 2DG	
		CI[a]	*P* value[b]	CI	*P* value
λ*attB*::tet	WT	0.84 ± 0.11	NS	0.84 ± 0.3	NS
Δ*sgrS* λ*attB*::tet	WT	1.09 ± 0.14	NS	0.46 ± 0.1	0.023
$sgrS1_{manXYZ}$ λ*attB*::tet	WT	0.97 ± 0.16	NS	1.03 ± 0.13	NS
$sgrS28_{ptsG}$ λ*attB*::tet	WT	1.00 ± 0.15	NS	0.68 ± 0.04	0.01
$sgrS1_{manXYZ}$ λ*attB*::tet	Δ*sgrS*	0.98 ± 0.22	NS	2.25 ± 0.59	0.015
$sgrS28_{ptsG}$ λ*attB*::tet	Δ*sgrS*	0.96 ± 0.11	NS	1.12 ± 0.34	NS
$sgrS28_{ptsG}$ λ*attB*::tet	$sgrS1_{manXYZ}$	1.01 ± 0.06	NS	0.59 ± 0.08	0.019

[a] See Materials and Methods for detailed procedures. The competition index (CI) is calculated as follows: ($\log_{10}$ strain A output/$\log_{10}$ strain B output)/($\log_{10}$ strain A input/$\log_{10}$ strain B input). The results presented here are the averages ± standard deviations of three independent experiments.

[b] Student's *t* test was used to compare the output and inoculum. NS, not significant ($P \geq 0.05$).

TABLE 8 Competition assays to measure the effects of mutations in the three *sgrS* targets on growth with 2DG in minimal MOPS medium with fructose

E. coli strain A genotype	*E. coli* strain B genotype	Without 2DG		With 0.5% 2DG	
		CI[a]	*P* value[b]	CI	*P* value
Δ*ptsG* λ*attB*::tet	WT	1.03 ± 0.15	NS	0.98 ± 0.008	NS
Δ*manXYZ* λ*attB*::tet	WT	0.99 ± 0.06	NS	2.04 ± 0.12	0.003
Δ*sgrS* Δ*ptsG* λ*attB*::tet	Δ*sgrS*	0.96 ± 0.14	NS	1.08 ± 0.07	NS
Δ*sgrS* Δ*manXYZ* λ*attB*::tet	Δ*sgrS*	1.02 ± 0.11	NS	1.90 ± 0.27	0.04
Δ*yigL* λ*attB*::tet	WT	0.97 ± 0.10	NS	0.92 ± 0.08	NS

[a] See Materials and Methods for detailed procedures. The competition index (CI) is calculated as follows: ($\log_{10}$ strain A output/$\log_{10}$ strain B output)/($\log_{10}$ strain A input/$\log_{10}$ strain B input). The results presented here are the averages ± standard deviations of three independent experiments.

[b] Student's *t* test was used to compare the output and inoculum. NS, not significant ($P \geq 0.05$).

SgrS-mediated regulation of *manXYZ* mRNA becomes crucial under different stress conditions. The experiments described so far establish that when glucose-phosphate stress is induced by αMG, the regulatory action of SgrS on *ptsG* plays a prominent role in stress recovery, whereas regulation of *manXYZ* does not contribute to growth (Tables 3, 4, and 5 and Fig. 3). These results are consistent with the substrate preferences of these two PTS transporters: $EIICB^{Glc}$ (PtsG) is more specific for αMG, and $EIIABCD^{Man}$ has higher specificity for the glucose analog 2-deoxyglucose (2DG) (28, 29). We previously demonstrated that PtsG plays a bigger role in induction of the stress response, as measured by increased *sgrS* transcription, when cells are exposed to αMG, whereas ManXYZ is required for induction in response to 2DG (5, 10, 19). We therefore theorized that regulation of *manXYZ* by SgrS would be important for growth recovery during 2DG-induced stress. To test this hypothesis, growth competition was performed with wild-type and *sgrS* mutant strains stressed in minimal MOPS medium with fructose (Table 7), because stress and growth inhibition of *E. coli* cells were previously observed under this condition (30). With 2DG, the Δ*sgrS* mutant was at a significant growth disadvantage in competition with the wild-type strain (CI = 0.46 [Table 7]), highlighting the crucial role of SgrS in mitigating 2DG-induced stress. Interestingly, the $sgrS1_{manXYZ}$ mutant, which specifically regulates *manXYZ*, but not *ptsG* or *yigL* (Fig. 2), competed equally with the wild-type strain (CI = 1.03 [Table 7]) and outcompeted the Δ*sgrS* mutant (CI = 2.25 [Table 7]). In contrast, the $sgrS28_{ptsG}$ strain (regulation of *ptsG* but not *manXYZ* or *yigL*) was at a growth disadvantage compared with both the wild type (CI = 0.68 [Table 7]) and the $sgrS1_{manXYZ}$ strain (CI = 0.59 [Table 7]). (All strains competed evenly in the absence of stress, and the selective marker [*attB*::tet] did not affect growth with or without 2DG [Table 7].) Similar results were observed when glycerol was used as the sole carbon source (data not shown). Collectively, these data indicate that, as predicted, regulation of *manXYZ* by SgrS becomes essential when the stressor is 2DG, a ManXYZ substrate, whereas regulation of *ptsG* and *yigL* does not contribute to growth recovery under these conditions.

Consistent with the observations described above, the Δ*manXYZ* mutant outcompeted the wild-type strain (CI = 2.04 [Table 8]), and the Δ*manXYZ* Δ*sgrS* mutant had a growth advantage over its Δ*sgrS* parent (CI = 1.90 [Table 8]) during growth with 2DG. Both the Δ*ptsG* (CI = 0.98) and Δ*ptsG* Δ*sgrS* (CI = 1.08) mutant strains competed evenly with their respective parent strains under the same conditions (Table 8), which is consistent with the notion that PtsG does not contribute significantly to 2DG uptake. In addition, the Δ*yigL* mutant competed well against the wild-type strain (CI = 0.92 [Table 8]), indicating that YigL is unlikely to play a significant role in the cellular response to 2DG under these conditions. We further tested the effect of YigL on growth with 2DG in minimal media, using the $P_{tetO\text{-}1}$-$yigL^+$ plasmid. While the wild-type strain recovered from 2DG stress, the plasmid-borne copy of YigL failed to improve the growth of the Δ*sgrS* Δ*yigL* mutant in the presence of 2DG (Fig. 4D). Together, these results strongly suggested that recovery from stress induced by 2DG requires SgrS-mediated regulation of *manXYZ*, whereas regulation of *ptsG* and *yigL* is dispensable for the response to 2DG.

Nutrient supplementation in minimal media improves growth during GP stress. Our results so far have demonstrated that variations in nutrient content of the growth medium can influence glucose-phosphate (GP) stress-associated growth phenotypes, as well as the requirements for the regulatory activities of SgrS. When growing in LB medium, *E. coli* cells use amino acids as the carbon source (31, 32), whereas under our minimal medium growth conditions, cells were given glycerol or fructose as a carbon source and had to synthesize their own amino acids. Another study from our laboratory (an accompanying article [33]) revealed that one underlying cause of glucose-phosphate stress is depletion of central glycolytic metabolites. Given this, we reasoned that *E. coli* cells may be more stressed during growth in minimal media compared to rich media, because in minimal media, already low pools of central metabolites would be further reduced because of the need to draw on these metabolites for precursors of amino acid biosynthesis. To investigate whether supplementation of amino acids reduced the severity of glucose-phosphate stress-associated growth inhibition, we compared growth of wild-type and *sgrS* mutant strains in minimal media with fructose or glycerol in the presence and absence of Casamino Acids (CAA) (Fig. 5). As expected, CAA supplementation enhanced the growth rates of wild-type and mutant strains in these minimal media in the absence of stress (Fig. 5A and C). Similarly, in both media, the presence of CAA improved the growth of wild-type cells stressed with αMG (Fig. 5B and D, compare the wild type [WT] plus αMG to the WT plus αMG plus CAA). The growth improvement conferred by CAA on the stressed wild-type strain resembled that observed for nonstressed cells, suggesting that the growth potential of wild-type cells stressed in minimal medium is not drastically limited by depleted pools of central metabolites.

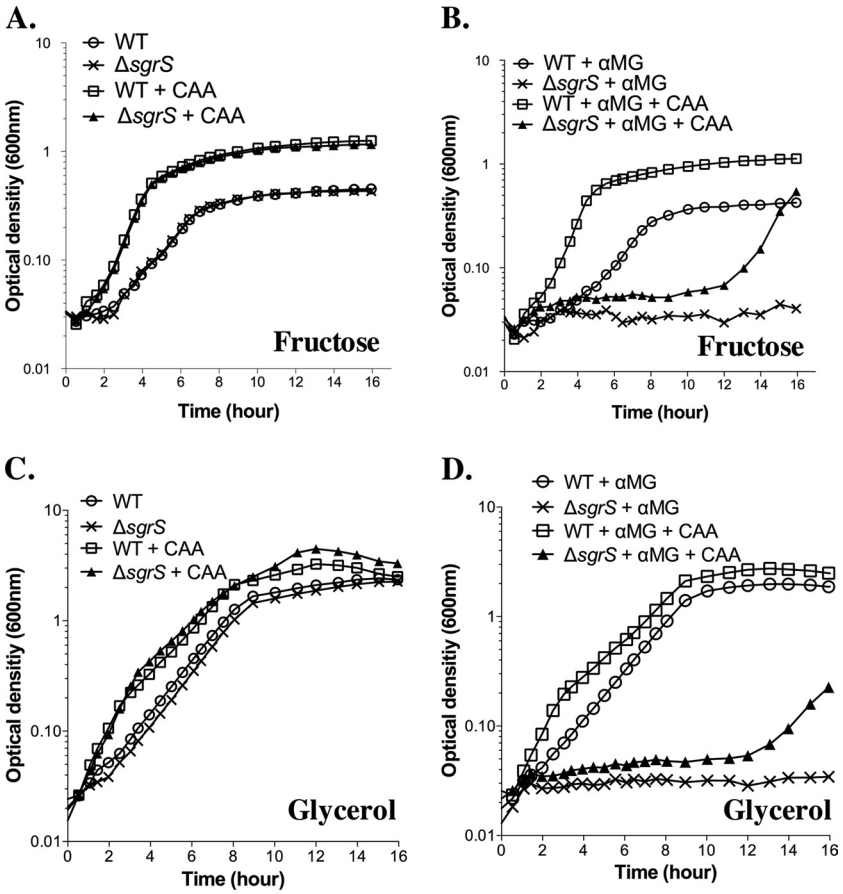

FIG 5 Supplementation with Casamino Acids improves growth during glucose-phosphate stress. Strains were grown in minimal MOPS medium supplemented with 0.2% fructose (A and B) or 0.4% glycerol (C and D). In addition, 0.5% αMG and/or 0.1% Casamino Acids (CAA) were present in the media as indicated. Results shown are representative of at least three independent experimental trials.

We expected this because wild-type cells induce SgrS to reduce αMG uptake and subsequent metabolite depletion. The *sgrS* mutant strain, on the other hand, is much more severely inhibited by αMG, yet added CAA also improved growth of this strain (Fig. 5B and D, compare the Δ*sgrS* mutant plus αMG to the Δ*sgrS* mutant plus αMG plus CAA), albeit after a much longer lag (approximately 12 h following exposure of the cells to αMG). These results are consistent with the idea that at least one factor accounting for lack of growth of *sgrS* mutant cells stressed in nutrient-poor conditions is a lack of central metabolites available to divert to amino acid biosynthesis. These results provide a rationale for the less severe growth inhibition experienced by cells growing in rich media containing amino acids compared with cells growing in minimal media.

DISCUSSION

In recent years, hundreds of novel sRNAs have been identified in *E. coli*, *Salmonella*, and many other bacterial species. However, SgrS is one of only a few base pairing sRNA regulators for which we have detailed knowledge concerning its regulation, targets, and perhaps most importantly, a clearly associated growth phenotype. These features make SgrS an excellent model for unraveling molecular mechanisms of sRNA-mediated coordinate regulation of multiple targets and unifying these mechanisms with their physiological relevance. While many studies have demonstrated that Hfq-dependent sRNAs regulate multiple mRNA targets (34–36), how regulation of individual targets or target subsets contributes specifically to growth physiology under different conditions has not been well studied. Here, we began to investigate this issue by studying the physiological impact of SgrS regulation of its multitarget regulon.

We report some of the first evidence supporting the idea that coordinated regulation of multiple genes in an sRNA's regulon directly contributes to cell growth potential during stress. Identification of SgrS mutants with altered target specificities allowed us to assess the importance of SgrS-mediated regulation of different targets under a variety of conditions. One stress variable that we manipulated was the stress-inducing phosphosugar. The two sugars we used, αMG and 2DG, are both glucose analogs, but are taken up via distinct PTS transporters, PtsG and ManXYZ, respectively (30, 37). We found that when αMG was the stressor, SgrS regulation of *ptsG* was crucial for continued growth, both in the context of growth competition (Table 3) and when strains were growing in pure culture (Fig. 3). In contrast, regulation of *manXYZ* by SgrS conferred no growth advantage during αMG-induced stress (Table 3). On the other hand, when cells were stressed by uptake of 2DG, regulation of *manXYZ* by SgrS was crucial, whereas regulation of *ptsG* was dispensable (Table 7). These specific results track with the known substrate specificities

of the two PTS transporters and make perfect biological sense based on what we know about the glucose-phosphate stress response. However, prior to our study, it had not been demonstrated that regulation of different subsets of sRNA target genes could allow cells to respond effectively to changing stress conditions. Thus, our experimental approaches and results have helped to shed light on a broader issue: how sRNA-mediated regulation of multiple mRNA targets can provide a flexible stress response that promotes optimal cell physiology under fluctuating environmental conditions.

Changing the nutrients available to cells by culturing in rich or minimal media allowed us to discern that factors other than the sugar stressor can modulate glucose-phosphate stress-associated growth phenotypes. Cells stressed with αMG show different patterns of growth depending on the nutrient content of the medium. In rich (LB) medium, wild-type and *sgrS* mutant cells continue growing for ~2 generations after αMG exposure. After that, growth of wild-type cells is unaffected, while *sgrS* mutant growth slows dramatically (5) (Fig. 3). With plentiful nutrients available in LB, restoring regulation of a single target, *ptsG* mRNA, was sufficient to rescue growth of *sgrS* mutant cells (Table 3 and Fig. 3), suggesting that simply reducing αMG uptake relieves stress under these conditions. Results from another study in our laboratory showed that one important cause of glucose-phosphate stress-associated growth inhibition of *sgrS* mutant strains is depletion of glycolytic metabolites, including phosphoenolpyruvate (PEP) (33).We propose that in wild-type cells growing in nutrient-rich media, SgrS-mediated repression of PtsG synthesis reduces uptake of the nonmetabolizable sugar and consequently reduces PEP consumption by the PTS. This activity of SgrS allows cells to continue growing using the amino acids available in LB as a carbon source.

In contrast, when growing in minimal medium, both wild-type and *sgrS* mutant cells experience almost immediate inhibition after exposure to αMG (23) (Fig. 4B). Wild-type cells subsequently recover, but *sgrS* mutant growth remains inhibited (Fig. 4B). We postulate that reduced levels of central metabolites is more growth limiting in minimal medium (compared to rich medium), because cells have to draw on central metabolite pools for amino acid biosynthetic precursors. Restoring regulation of *ptsG* alone failed to rescue growth of *sgrS* mutant cells in minimal medium containing glycerol and αMG (Table 5 and Fig. 4). Regulation of both *ptsG* and *yigL* provided some relief of growth inhibition but failed to fully restore *sgrS* mutant growth to wild-type levels (Table 5 and Fig. 4). These observations indicate that both reducing αMG uptake (via repression of *ptsG*) and enhancing αMG efflux (via activation of the sugar phosphatase *yigL* mRNA, a prerequisite for efflux [38]) are necessary but not sufficient for the stress response in nutrient-poor conditions. Our results imply that other as-yet-uncharacterized SgrS target mRNAs must also be regulated for full recovery under these conditions. We speculate that these other SgrS target mRNAs may encode metabolic enzymes or regulators that help reroute metabolism in order to replenish these metabolites. Ongoing studies in our laboratory are testing this hypothesis.

Consistent with the idea that reduced levels of central metabolites (including amino acid biosynthetic precursors) are limiting for *sgrS* mutant growth (33), we found that supplementation of minimal media with amino acids mitigates growth inhibition associated with αMG stress (Fig. 5). We postulate that when stressed cells growing in minimal media are provided with exogenous amino acids, they are spared from utilizing the already limited central metabolites for amino acid biosynthesis. In addition, the amino acids may help rescue the growth of *sgrS* mutant cells by serving as the substrates for gluconeogenesis that allow cells to make more PEP and other upstream metabolites. The lag we observe in growth recovery of the αMG-stressed *sgrS* mutant provided with amino acids may reflect the time it takes for cells to replenish the limiting metabolites through gluconeogenesis.

This study provides insight into two separate aspects of the glucose-phosphate stress response. First, our demonstration that cells require SgrS regulation of different target subsets depending on the nature of the environmental conditions when stress is induced suggests that SgrS and perhaps other sRNAs have evolved to be flexible regulators that modulate expression of multigene regulons in order to allow cells to adapt to an array of related stress conditions. Second, our analysis here of growth and competitiveness of wild-type and mutant strains stressed under different nutritional conditions, combined with our other study (33) is fully consistent with our model that glucose-phosphate stress is caused by an imbalance of central metabolites. In sum, our work shows that the SgrS-mediated response to stress has three main components that vary in importance depending upon the nutrients available. The first arm of the stress response, repression of sugar transport protein synthesis, is all that is required if stress occurs in a nutrient-rich environment with available amino acids, perhaps because this reduces the drain on central metabolites for biosynthesis and provides cells a route to replenish these limiting metabolites. Under nutrient-poor conditions, the cell needs at least two additional SgrS-dependent functions in order to recover from stress—activation of sugar efflux (through activation of *yigL* and subsequent dephosphorylation and efflux of accumulated sugars [38]) and another unknown activity. These studies set the stage for future work aimed at answering two important questions. (i) What is the exact nature of metabolic defects of cells experiencing stress in different nutritional environments? (ii) What are the other SgrS-mediated cellular responses that are important for overcoming stress under nutrient-limiting conditions? Answering the first question will require detailed analyses of intracellular metabolite levels and changes in metabolic fluxes in response to glucose-phosphate stress. Resolving the second will involve identification of other members of the SgrS regulon and assessing how their regulation contributes to stress-associated growth phenotypes. Both of these are active areas of investigation in this laboratory, and we anticipate they will lead to new insights into the regulation of central metabolism as well as physiology of glucose-phosphate stress.

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