

Regulation and Function of *Escherichia coli* Sugar Efflux Transporter A (SetA) during Glucose-Phosphate Stress[∇]

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Accumulation of certain nonmetabolizable sugar-phosphates (including α -methyl glucoside-6-phosphate) in *Escherichia coli* is growth inhibitory and elicits the glucose-phosphate stress response. The transcription factor SgrR activates transcription of the small RNA SgrS under stress conditions. SgrS represses translation of mRNAs encoding sugar transporters. The *sgrR* and *sgrS* genes are located directly upstream of *setA*, and this gene organization is conserved in numerous enteric species, prompting the hypothesis that SetA contributes to the glucose-phosphate stress response. SetA is a proton motive force-driven efflux pump capable of transporting various sugars and sugar analogs *in vitro*. This study demonstrates that *setA* expression is induced in response to glucose-phosphate stress, and this requires SgrR. Under stress conditions, *setA* is cotranscribed with *sgrS* from the *sgrS* promoter. A *setA* mutant exhibits a growth defect under stress conditions that can be complemented by *setA* *in trans*, suggesting that SetA contributes to the optimal cellular recovery from stress. Despite previous *in vitro* evidence that SetA can promote efflux of the stress-causing glucose analog α -methyl glucoside, *in vivo* data in this study indicate that SetA is not the major efflux pump responsible for removal of α -methyl glucoside under stress conditions.

The genome of *Escherichia coli* encodes three members of the SET (sugar efflux transporter) family, which was identified 10 years ago as a subfamily of the major facilitator superfamily (16). These are SetA, SetB, and SetC, which are also encoded in numerous other enterobacterial genomes (16). A fourth member, SotA, is encoded in the *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) genome (24, 30). SetA was originally identified in *E. coli* as a multicopy suppressor of the IPTG (isopropyl- β -D-thiogalactopyranoside)-induced toxicity associated with overproduction of a membrane protein, and this suppression was due to SetA pumping IPTG out of the cells (16). *In vitro* studies of SetA revealed that it is a proton motive force-driven efflux pump capable of transporting a wide range of substrates, including various sugars and sugar analogs (17). Despite this information regarding its biochemistry, prior to this study, nothing was known about the conditions that induce the expression of *setA* or its true physiological function *in vivo*. Previously, the only *in vivo* information regarding SET family members was limited to *sotA* of *D. dadantii*: the transcription of *sotA* is activated by the cyclic AMP (cAMP) receptor protein (CRP) and weakly repressed by the 2-keto-3-deoxygluconate repressor KdgR (4, 29). Nevertheless, based on the biochemical characterization (17), it was plausible to speculate that SetA and its homologs might function *in vivo* as sugar efflux pumps with roles in carbohydrate metabolism or metabolic stress.

One example of carbohydrate-related metabolic stress in *E. coli* is referred to as glucose-phosphate stress (7, 9–12, 15, 32, 40, 42), which can be induced by exposing wild-type (WT) *E.*

coli cells to the nonmetabolizable glucose analog α -methyl glucoside (α MG). Under glucose-phosphate stress conditions, *E. coli* cells initiate a unique cellular response mediated by the transcription factor SgrR, which activates transcription of a small RNA (sRNA), SgrS (Fig. 1A) (32–34). When expressed under stress, SgrS base pairs with the *ptsG* mRNA, which encodes EIICB^{Glc}, the phosphoenolpyruvate phosphotransferase system (PTS) protein responsible for transport of both glucose and α MG. The base-pairing interaction results in translation inhibition and degradation of the *ptsG* mRNA (13, 14, 21, 32, 33). The physiological significance of this regulation is that SgrS stops new synthesis of sugar transporters under conditions where sugar-phosphates are not being metabolized (32). SgrS also encodes the small protein SgrT, which inhibits uptake of glucose via an unknown mechanism (37).

Stress-responsive activation of *sgrS* requires the transcription factor SgrR, which is encoded by the *sgrR* gene divergently transcribed from *sgrS* (Fig. 1B) (34). The *sgrR* and *sgrS* genes are located upstream of *setA* on the chromosome, and this gene organization is conserved among numerous enteric species (Fig. 1B) (8), suggesting that SetA may play a role in the glucose-phosphate stress response. The very short intergenic region between *sgrS* and *setA* (27 nucleotides [nt] in the *E. coli* K-12 genome) also suggested the possibility that the two genes might form an operon.

In the present study, we sought to characterize the regulation and physiological function of SetA in the glucose-phosphate stress response. We determined that under stress conditions, *setA* is coexpressed with *sgrS* in an SgrR-dependent manner. The *setA* mutant has a growth defect under stress conditions that is medium dependent. The growth defect can be complemented by SetA *in trans*, confirming that SetA contributes to optimal cellular recovery from stress under some circumstances. The *setA* mutation also affects the stress-responsive activation of *sgrS* in a medium-specific manner, sug-

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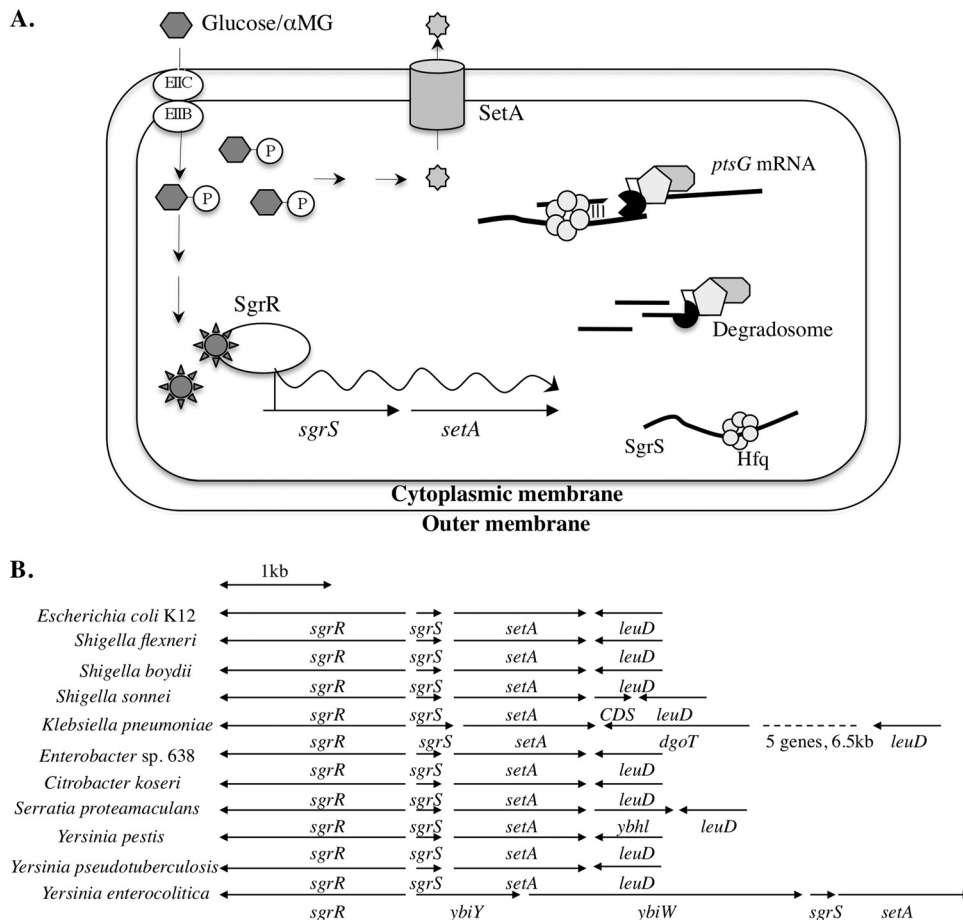


FIG. 1. Involvement of SgrS and SetA in the glucose-phosphate stress response. (A) Model of glucose-phosphate stress response in *E. coli*. (B) Organization of genes in the *sgrR-sgrS-setA* chromosomal region in organisms related to *E. coli* K-12. The directions of gene transcription are indicated by the arrows.

gesting that SetA activity affects signaling through SgrR. Efflux assays using radiolabeled αMG indicate that SetA does not play a significant role in directly pumping out this sugar analog. Together, these data suggest that SetA plays a complex role in the stress response.

MATERIALS AND METHODS

Strain and plasmid construction. Most strains used for this study are derivatives of DJ480 (D. Jin, National Cancer Institute) and 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.

The *sgrS-lacZ* transcriptional reporter fusion was constructed by B. Hussain in our laboratory via previously described methods (33). Briefly, the *sgrS* promoter fragment was amplified by primers O-BAH102 and O-CV142 (Table 2) containing EcoRI or BamHI sites, respectively, and cloned into plasmid pRS1553. The fusion was crossed into the *λattB* site on the host chromosome (33), resulting in BAH100. The *setA-lacZ* transcriptional fusion in YS131 was constructed using the λ-Red and FLP-mediated site-specific recombination method described previously (6). Briefly, a kanamycin cassette flanked by FLP recombination target (FRT) sites was amplified from template pKD13 (6) using primers O-YS125 and O-YS126 (Table 2) and integrated into the chromosome by λ-Red recombination at the *setA* locus. The remaining steps were as described in reference 6.

BAH101 was created by B. Hussain (in our laboratory) by moving a $\Delta sgrR::cm$ allele into the BAH100 host via P1 transduction. CS168 was created by C. S. Wadler in our laboratory by P1 transduction of $\lambda attB::lacI^q$ Sp^r tetR from donor DH5αZ1 (18) into recipient DJ480. The $\Delta setA::cm$ deletion-insertion mutation in ST101 was constructed by S. Tsang in our laboratory using the λ-Red recom-

bination system (43) and primers O-CV170 and O-CV171 (Table 2). YS133 is derived from YS131 and contains a $\Delta sgrR::cm$ allele that was constructed by homologous recombination (43) with a PCR product obtained using primers $\Delta yabNcm1$ and $\Delta yabNcm2$ (Table 2). To construct YS135, a single-stranded oligonucleotide, O-YS135 (Table 2), was transformed into CV1300 to replace the *cat-sacB* cassette carried by the latter in the SgrR-binding site. The *setA-lacZ* fusion was then introduced into YS135 by the same method used for YS131 construction, resulting in strain YS140. YS137 was derived from CS100 (38) by looping out the *cat-sacB* cassette in the -10 region of the *sgrS* promoter using the oligonucleotide O-YS134 (Table 2). YS142 was subsequently made by constructing the *setA-lacZ* fusion in the YS137 background. YS143 was created by moving the $\Delta setA::cm$ allele into CS168 via P1 transduction. YS144 is derived from CS123, which contains the *sgrS1* mutation (38), and was constructed by transducing the $\lambda attB::lacI^q$ Sp^r tetR allele into CS123. YS214 was created by transducing the $\Delta setA::cm$ allele into BAH100. YS215 is derived from CS104 (38), which contains a $\Delta sgrS$ allele, and was constructed by inserting the *sgrS-lacZ* transcriptional fusion in single copy into the *λattB* site as described previously (33). YS219 was made first by P1 transduction of a $\Delta kdgr::FRT$ -kan-FRT allele from donor JW1816 (1) to recipient DJ480; the kanamycin cassette was then removed by a previously described method (6), and the *setA-lacZ* fusion was moved in by P1 transduction. YS224 is derived from CS123 and was created by inserting the *sgrS-lacZ* transcriptional fusion in single copy into the *λattB* sites of the respective strains as described previously (33). Using the same method, the *sgrS-lacZ* fusion was inserted into YS219, resulting in strain YS225. YS228 was constructed by moving a $\Delta crp::cm$ allele into BAH100 through P1 transduction. YS231 was created by moving a $\Delta crp::cm$ allele to YS131 by P1 transduction.

DH5α (Invitrogen) was used for routine cloning procedures. Plasmid pZE21 was described in a previous study (18) and was kindly provided by J. Cronan. To

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
MG1655	Wild-type <i>E. coli</i> K-12	D. Jin (NCI)
DJ480	MG1655 $\Delta lac X74$	D. Jin (NCI)
BAH100	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i>	This study
BAH101	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i> , $\Delta sgrR::cm$	This study
CS168	DJ480 $\lambda attB::lacI^q tetR Sp^f$	This study
CV107	DJ480 $\Delta ptsG \Delta manXYZ \Delta sgrS$	C. K. Vanderpool (NIH)
CV700	DJ480 $\Delta sgrR::cm$	33
ST101	DJ480 $\Delta setA::cm$	This study
YS131	DJ480 <i>setA'</i> - <i>lacZ</i>	This study
YS133	DJ480 <i>setA'</i> - <i>lacZ</i> $\Delta sgrR::cm$	This study
YS135	DJ480 sub1	This study
YS137	DJ480 sub2	This study
YS140	DJ480 <i>setA'</i> - <i>lacZ</i> sub1	This study
YS142	DJ480 <i>setA'</i> - <i>lacZ</i> sub2	This study
YS143	DJ480 $\lambda attB::lacI^q tetR Sp^f \Delta setA::cm$	This study
YS144	DJ480 $\lambda attB::lacI^q tetR Sp^f sgrS1$	This study
YS214	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i> $\Delta setA::cm$	This study
YS215	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i> $\Delta sgrS$	This study
YS219	DJ480 <i>setA'</i> - <i>lacZ</i> $\Delta kdgR::FRT$	This study
YS224	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i> <i>sgrS1</i>	This study
YS225	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i> $\Delta kdgR::FRT$	This study
YS228	DJ480 <i>imm</i> ²¹ <i>sgrS'</i> - <i>lacZ</i> $\Delta crp::cm$	This study
YS231	DJ480 <i>setA'</i> - <i>lacZ</i> $\Delta crp::cm$	This study
Plasmids		
pZE21	Vector control for plasmid pZEYS1	18
pZEYS1	pZE21 with <i>setA</i> under the control of the P _{LtetO-1} promoter	This study
pHDB3	Vector control for plasmid pBRBAH3	31
pBRBAH3	pHDB3 with <i>sgrR1</i>	This study

construct plasmid pZEYS1, the *setA* gene was amplified by PCR using primer pairs O-YS130 and O-CV193 (Table 2), which contain BamHI and KpnI sites, and subsequently cloned into the BamHI- and KpnI-cut vector pZE21. Plasmid pBRBAH3 was created by B. Hussain in our laboratory using the XL1-Red system (Stratagene). Briefly, plasmid pLCV3 carrying the wild-type *sgrR* gene (33) was transformed into the *E. coli* mutator strain XL1-Red, and the transformants were grown over many generations using serial passages. Pools of mutagenized plasmids generated by this method were then transformed into host strain CV5230, which contains an *sgrS'*-*lacZ* fusion and a $\Delta sgrR$ mutation on the

chromosome (33). The transformants were screened on lactose MacConkey indicator plates for the Lac⁺ phenotype, and plasmid pBRBAH3 obtained from the screening was then sequenced to identify the locations of mutations in the *sgrR* gene.

β -Galactosidase assays. Strains were grown overnight in LB (100 μ g/ml ampicillin was added to the medium when working with strains carrying plasmids pHDB3 and pBRBAH3) or minimal MOPS (morpholinepropanesulfonic acid) medium (Teknova) supplemented with 0.4% glycerol or 0.2% fructose and subcultured 1:200 in fresh medium. The cultures were grown to an optical density

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5'-3')
O-BAH102.....	CCCGGATCCCATCGTTAGGAATTATTGACTTAATATAGG
O-CV142.....	CCCGAATTCAAAAGAAACGCCAGTGAAGCGGTG
O-CV170.....	TTTTATTCTCGCCGCGCTAAAAAGGGAACGTATGATCTGGATACCTGTGACGGAAGATCACTTCGC
O-CV171.....	TTTAACCTTTGCGGTTAAAAATAATGCGACAACAGAAATAACCTTATCACTTATTCAGGCGTAGCACC
O-CV193.....	CCCGGATCCGTATTGGGCTTACCTTGCAGCACGAC
O-YS101.....	ACCGCAATTCTGAAAGTTGACTTGCC
O-YS102.....	GAGCCATCGTCATTATCCAGATCATAACG
O-YS107.....	CGCCCCGGCCACCCCATCATAAAA
O-YS109.....	ATAGCATTCACCGTATAAAAAGA
O-YS110.....	CCTGACTGTCAGAACGTTTTGC
O-YS125.....	CATTTATGCTGGTCTGCTTTTATGATGGGGGTGGCCGGGGCGTGTAGGCTGGAGCTGCTTC
O-YS126.....	ACCGCAATTACCCAGTAGACAGCAAAGTGCCCCCAACTTTATTCCGGGGATCCGTCGACC
O-YS130.....	CCGGTACCGTGTAAAATCACCCGCCAGCAGATT
O-YS134.....	AACCAGCACAACCTTCGCTGTCGCGGTTAAAATAGTGCTGATAAAACTGACGCATGGGGCACCCCTTG CTTCATCGTTAGGAGTTGTTGACTTAATATAGGGAAAAAT
O-YS135.....	GAAAATAAAATTGCTGTCTTTTGCACAGGAGTTCGGGAATTATGCCATCTGCTCGTCTGCAACAACA GTTTCATCCGCTGTGGCAA
O-YS138.....	GCCGCGTAAACACCGTTCATACGGC
For PCR-RACE.....	GATATGCGCGAATTCCTGTAGAACGAAC
$\Delta yabNcm1$	GCTCGTCTGCAACAACAGTTATCCGCTGTGGCAATGCTGCCTGTGACGGAAGATCACTTCGC
$\Delta yabNcm2$	GGGTATTCATGCGCAGGCCGCGCATACTGCGTTGCCCTGTTATCACTTATTCAGGCGTAGCACC

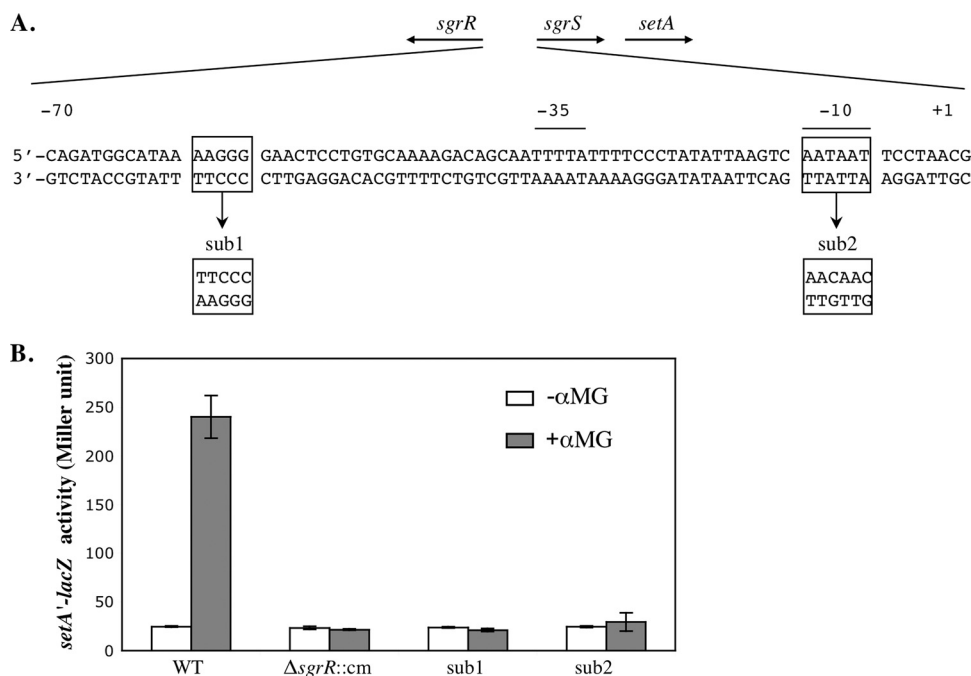


FIG. 2. SgrR-dependent activation of *setA* expression. (A) Genetic organization of the *sgrR-sgrS* region. The directions of transcription of the *sgrR* and *sgrS* genes are depicted by the arrows at the top. The -35 and -10 promoter elements are indicated by horizontal lines above the nucleotide sequence. The boxed nucleotides show the positions where mutations sub1 and sub2 were constructed. The substituted sequences are shown below the wild-type sequence. (B) Cells were grown in LB, and 0.1% αMG was added to half of the cultures. β-Galactosidase activity was assayed 45 min after the addition of αMG. The error bars indicate the standard deviations of three independent experiments.

at 600 nm (OD_{600}) of ~0.5 and split into two separate cultures. αMG (Sigma) was added to one of the cultures to the indicated final concentrations. For testing differences in growth under stress conditions, αMG was used at a final concentration of 0.5%. We had shown previously that wild-type and Δ*sgrS* strains have distinctly different growth characteristics at this αMG concentration (37, 38). For experiments testing signaling leading to transcriptional activation of *sgrS-lacZ*, a lower concentration of αMG was used (0.001%) because the *sgrS* promoter is very sensitive to induction under stress conditions and higher concentrations saturate the activation. For testing signaling through activation of *setA-lacZ*, a final concentration of 0.1% αMG was used because this fusion is less sensitive to induction than the *sgrS-lacZ* fusion. Samples were taken 45 min after the addition of αMG to the appropriate cultures and assayed for β-galactosidase activity as described previously (3).

RT-PCR. Strains were grown overnight in LB medium and subcultured 1:200 in fresh medium. Total RNA was extracted by the hot-phenol method (33) both before and 10 min after exposure of the cells to 0.5% αMG. Reverse transcription (RT) was performed with 5 μg of Turbo DNase (Ambion)-treated RNA using the Superscript III reverse transcriptase (Invitrogen) and oligonucleotide O-YS110 (Table 2). The PCR products were amplified from cDNA using the Go Taq (Promega) PCR system with primers O-YS101 and O-YS102 (Table 2).

5' RACE. 5' Rapid amplification of cDNA ends (RACE) analysis of the *setA* transcript was performed as described previously with and without tobacco acid pyrophosphatase to detect transcription initiation sites versus processed ends (2) with RNA isolated from mid-log-phase wild-type (DJ480) cells grown in minimal MOPS medium supplemented with 0.4% glycerol 10 min following exposure of the cells to 0.5% αMG. Oligonucleotide O-YS109 was used for reverse transcription, primers O-YS107 and ForPCR-RACE were used for PCR, and O-YS138 was used for sequencing (Table 2).

Growth experiments. Strains were grown overnight in minimal MOPS medium supplemented with the indicated carbon source (0.4% glycerol or 0.2% fructose) and 25 μg/ml kanamycin and subcultured (OD_{600} , ~0.01) into fresh medium containing 1 ng/ml anhydrotetracycline (aTc) (Sigma). The cultures were then grown at 37°C with shaking to an OD_{600} of ~0.1, and αMG was added to the medium at a concentration of 0.5%. Growth was monitored hourly by measuring the OD_{600} .

Efflux assays. Strains were grown overnight in minimal MOPS medium supplemented with 0.4% glycerol and subcultured 1:200 into fresh medium. The

cultures were grown at 37°C to an OD_{600} of ~0.1. The cells were incubated with [¹⁴C]αMG (ARC; 3.3 μM; 1 μCi/ml) at room temperature for 20 min and then diluted 200-fold with fresh medium. At the indicated times, 20 ml of the culture was filtered (Fisher; 25 mm; filter pore size, 0.45 μm), and the radioactivity of the filtered cells was determined with a scintillation counter.

RESULTS

***setA* expression is induced in an SgrR-dependent manner under glucose-phosphate stress conditions.** In order to determine the involvement of *setA* in the glucose-phosphate stress response, we first examined the expression of *setA* under stress conditions. A *setA-lacZ* fusion was constructed on the chromosome at the *setA* locus in a Δ*lac* host strain as described in Materials and Methods. In the presence of αMG, the *setA-lacZ* fusion was activated ~10 fold over the nonstress level (Fig. 2B, WT), demonstrating that *setA* is indeed induced in response to the stress.

The gene located 27 nt upstream of *setA* on the *E. coli* K-12 chromosome and transcribed in the same orientation is *sgrS*, which encodes the sRNA required for recovery from glucose-phosphate stress. In many organisms that contain SgrS homologs, *setA* is present in this position relative to *sgrS* (Fig. 1B). Our previous work (33) and data in Fig. 2B (WT) demonstrated that expression of *sgrS* and *setA* is induced under the same stress conditions. Based on what is known about *sgrS* regulation, we predicted that SgrR (34), the transcriptional activator of *sgrS*, would also regulate *setA*. To elucidate the role of SgrR in regulation of *setA*, a Δ*sgrR*::cm allele was introduced into the strain carrying the *setA-lacZ* fusion. As shown in Fig. 2B (Δ*sgrR*::cm), the absence of SgrR did not affect the basal

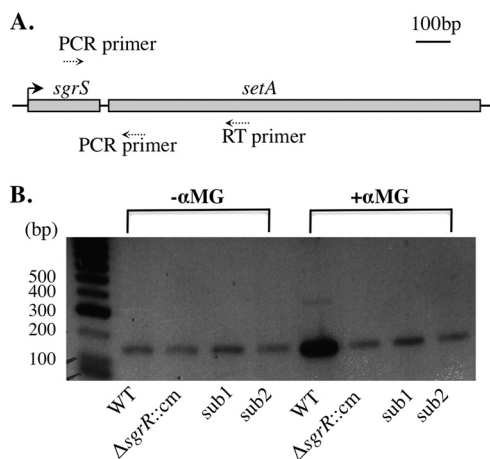


FIG. 3. RT-PCR analysis of *sgrS* and *setA*. (A) Schematic diagram showing the positions of the RT primer and PCR primers within the *sgrS-setA* region. (B) Wild-type (DJ480), $\Delta sgrR::cm$ mutant (CV700), and *sgrS* promoter mutant sub1 (YS135) and sub2 (YS137) cells were grown in LB medium at 37°C to an OD_{600} of ~ 0.5 . Total RNA was prepared from cells before and 10 min after exposure to 0.5% α MG, reverse transcribed, and amplified by PCR as described in Materials and Methods. Control experiments were also performed on each sample without addition of the reverse transcriptase, and no PCR product was detected in any samples (data not shown).

level of *setA'-lacZ* activity in the absence of stress but abolished activation following the exposure of cells to α MG. This result demonstrated that SgrR is required for stress-dependent induction of *setA* transcription.

Previous studies defined a conserved region between positions -70 and -55 relative to the start of *sgrS* transcription as required for the SgrR-dependent activation of *sgrS* (32, 33). To determine whether the same region is needed for the SgrR-mediated activation of *setA*, a 5-bp substitution in this region on the chromosome was constructed in the context of the *setA'-lacZ* chromosomal fusion. This 5-bp substitution (Fig. 2A, sub1) was shown previously (32) to disrupt the interaction between SgrR and the *sgrS* promoter and, consequently, to eliminate the SgrR-dependent activation of *sgrS*. Compared with the wild-type fusion, the mutant fusion had a similar basal activity in the absence of the stress. The sub1 mutant fusion was not activated upon exposure of cells to α MG (Fig. 2B, sub1), indicating that this SgrR-binding site is crucial for stress-dependent induction of *setA*. Similarly, point mutations in the -10 region of the *sgrS* promoter (Fig. 2A, sub2) that abrogate *sgrS* activation in response to glucose-phosphate stress (data not shown) also prevented the activation of the *setA'-lacZ* fusion under stress conditions (Fig. 2B, sub2). Together, these results suggested that the stress-responsive induction of *setA* was due to SgrR-dependent activation at the *sgrS* promoter.

***setA* is coexpressed with *sgrS* under glucose-phosphate stress conditions.** Based on the *setA'-lacZ* fusion data (Fig. 2), we predicted that *sgrS* and *setA* are cotranscribed under glucose-phosphate stress. To confirm this, RT-PCR was performed on total RNA from wild-type cells in the presence and absence of α MG. Using an *sgrS*-specific primer and a *setA*-specific primer (Fig. 3A), an amplicon of the expected size of ~ 150 bp, encompassing the *sgrS-setA* intergenic region, was produced (Fig. 3B). Levels of this product were reproducibly

higher in samples from wild-type stressed cells than in nonstressed cells (Fig. 3B, WT, compare $-\alpha$ MG and $+\alpha$ MG). Though this is semiquantitative RT-PCR, the fact that an *sgrS-setA* product was detected at high levels in stressed wild-type cells was consistent with the *setA'-lacZ* fusion results (Fig. 2). This indicated that *sgrS* and *setA* are cotranscribed and both are upregulated under glucose-phosphate stress conditions. To further support the results of *setA'-lacZ* fusions, RT-PCR was performed on total RNA from the *sgrR* mutant (Fig. 3B, $\Delta sgrR::cm$), as well as the two *sgrS* promoter mutants (Fig. 3B, sub1 and sub2). The results showed lower abundance of the *sgrS-setA* product in stressed cells carrying the *sgrR* sub1 or sub2 mutation compared with stressed wild-type cells, supporting the notion that the stress-responsive activation of *setA* requires SgrR and *sgrS* promoter determinants.

Transcriptional fusions (Fig. 2) and RT-PCR (Fig. 3) demonstrate that when transcription at the *sgrS* promoter is activated, some transcripts initiating at P_{sgrS} read through the *sgrS* terminator into *setA*. However, it was also possible that an additional *setA*-specific promoter was responsible for the basal transcription of *setA'-lacZ*. To identify any additional *setA* transcription initiation sites, 5' RACE (2) was performed on RNA harvested from stressed ($+\alpha$ MG) and unstressed ($-\alpha$ MG) wild-type cells. The only *setA*-specific product obtained in this experiment was one that initiated at the previously mapped SgrS 5' end (data not shown and reference 33). Therefore, if another *setA*-specific promoter exists, these transcripts must be present at levels too low to detect by 5' RACE. Together, these analyses indicate that under glucose-phosphate stress, *setA* is cotranscribed with *sgrS* from the *sgrS* promoter, and these two genes form an operon. The basal level of fusion activity (Fig. 2B) and the product observed in RT-PCR (Fig. 3B) in samples from the nonstressed cells may originate from a transcription start site that could not be detected in our experiments or may represent the basal level of expression from the *sgrS* promoter.

***crp* and *kdgR* mutations affect signaling through the glucose-phosphate stress response.** The experiments described above established that transcription of the *sgrS-setA* bicistronic transcript initiates at the *sgrS* promoter under glucose-phosphate stress conditions. In studies of *sotA*, a SET family member in *D. dadantii*, the global regulators CRP and KdgR were implicated in transcriptional regulation. CRP activated expression of *sotA* (4), while KdgR, which represses transcription of many genes involved in transport and catabolism of 2-keto-3-deoxygluconate in *E. coli* (22, 27), was shown to weakly repress the expression of *sotA* in *D. dadantii* (29). We therefore tested the effects of CRP and KdgR on regulation of *sgrS* and *setA* in *E. coli*. Given the evidence presented above regarding the coregulation of *sgrS* and *setA* (Fig. 2 and 3), we reasoned that CRP and KdgR might regulate *setA* through one of two mechanisms. First, these regulators might directly bind upstream of the *sgrS* promoter and directly activate or repress *sgrS-setA* expression. Second, these regulators could alter *sgrS-setA* expression indirectly by modulating the signal that results in SgrR-mediated activation at the *sgrS* promoter. To determine whether CRP and KdgR were involved in one of these modes of regulation, activities of *setA'-lacZ* and *sgrS'-lacZ* fusions were monitored in two different host strains: host 1, an *sgrR*⁺ host in which stress was induced by addition of α MG to cul-

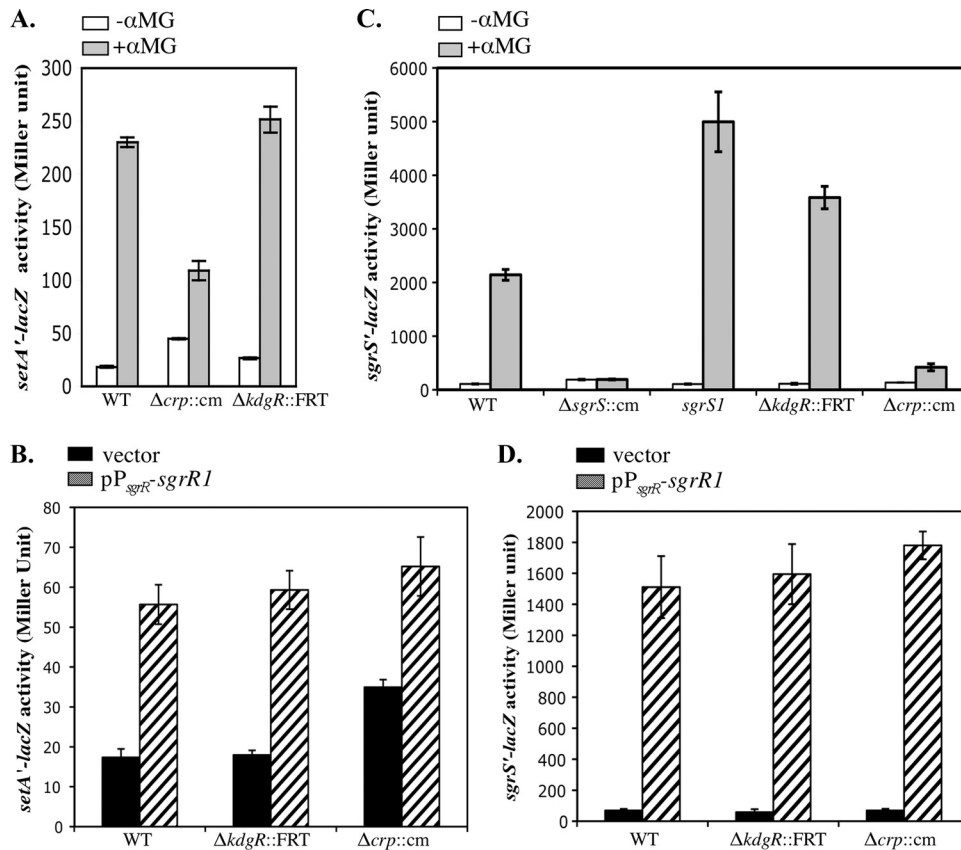


FIG. 4. Effects of CRP and KdgR on *setA* and *sgrS* expression. (A) Cells were grown in LB to an OD_{600} of ~ 0.5 , and 0.1% α MG was added to half of the cultures. β -Galactosidase activity was assayed 45 min after the addition of α MG. (B) Cells were grown in LB supplemented with 100 μ g/ml ampicillin to an OD_{600} of ~ 0.5 and assayed for β -galactosidase activity. (C) Cells were grown in LB to an OD_{600} of ~ 0.5 , and 0.001% α MG was added to half of the cultures. β -Galactosidase activity was assayed 45 min after the addition of α MG. (D) Cells were grown in LB supplemented with 100 μ g/ml ampicillin to an OD_{600} of ~ 0.5 and assayed for β -galactosidase activity. The error bars indicate standard deviations from three independent experiments.

tures, and host 2, a $\Delta sgrR$ host complemented with a stress signal-independent *sgrR* allele (i.e., an *sgrR* mutant that does not require stress to activate the *sgrS* promoter). This *sgrRI* allele encodes a glycine-to-glutamate substitution at position 118 of SgrR; the *sgrRI* plasmid promotes *sgrS* transcription independent of glucose-phosphate stress conditions (Fig. 4D, WT). If *crp* or *kdgR* mutations have an effect on regulation of fusions in the context of both host 1 and host 2, it would suggest direct regulation. On the other hand, if *crp* or *kdgR* mutations have an effect on regulation of fusions in host 1 but not host 2, it would indicate an indirect mechanism of regulation via effects on signaling through SgrR.

$\Delta crp::cm$ and $\Delta kdgR::FRT$ alleles were introduced into the hosts described above carrying the *setA'*-*lacZ* fusion. In the *sgrR*⁺ host, the *kdgR* mutation did not cause a significant change in the basal level of *setA* expression (Fig. 4A, - α MG). The level of *setA'*-*lacZ* activity in stressed cells (Fig. 4A, + α MG) was reproducibly slightly higher in the $\Delta kdgR$ mutant than in the *kdgR*⁺ strain, suggesting that KdgR may play a very modest role in regulating *setA* expression. The *crp* mutation resulted in a basal level of *setA'*-*lacZ* activity that was 2.4-fold higher than in the wild-type strain (Fig. 4A, - α MG), suggesting that CRP might repress *setA* expression under nonstress

conditions. Upon exposure of the Δcrp mutant cells to α MG, *setA'*-*lacZ* expression increased ~ 2.4 -fold compared to nonstressed Δcrp mutant cells, though the induced level of *setA'*-*lacZ* activity in the Δcrp mutant was lower than in the wild-type strain (Fig. 4A, + α MG). These results suggested that during glucose-phosphate stress, CRP might exert a slight effect on stress-responsive activation of *setA* expression.

To address whether the slight effects of the *crp* and *kdgR* mutations on *setA'*-*lacZ* expression were direct or through effects on signaling through SgrR, we repeated the experiments described above in the $\Delta sgrR$ host carrying the stress signal-independent *sgrRI* plasmid. In the wild-type background, the *sgrRI* plasmid caused a 3.2-fold increase in the level of *setA'*-*lacZ* activity over the vector control levels. *sgrRI* plasmid-containing cells had ~ 3.3 -fold-increased *setA'*-*lacZ* activity compared to vector control-containing cells in the $\Delta kdgR$ mutant background (Fig. 4B, $\Delta kdgR::FRT$ and pP_{*sgrR*}-*sgrRI*). Consistent with our previous observation (Fig. 4A, - α MG), *setA'*-*lacZ* activity was ~ 2.2 -fold higher in the Δcrp mutant than in the wild type when both strains carried the vector control, i.e., the basal level of activity was higher in the absence of CRP (Fig. 4B, $\Delta crp::cm$ /vector). *sgrRI* plasmid-containing cells had ~ 2 -fold-increased *setA'*-*lacZ* activity compared to vector con-

tol-containing cells in the Δcrp mutant background (Fig. 4B, $\Delta crp::cm$ and $pP_{sgrR-sgrR1}$). Importantly, cells carrying the $sgrR1$ plasmid had similar levels of $setA'-lacZ$ expression in wild-type and $\Delta kdgR$ and Δcrp mutant backgrounds independent of glucose-phosphate stress. Together, these results suggest that while CRP may repress basal levels of $setA$ transcription, neither CRP nor KdgR is likely to exert any direct effect on $setA$ transcription under stress conditions.

Since expression of $setA$ under stress conditions depends on the $sgrS$ promoter, we also tested the effects of KdgR and CRP on an $sgrS'-lacZ$ promoter fusion harbored at the $\lambda attB$ site. In addition to wild-type and $kdgR$ and crp mutant strains, the activity of the $sgrS'-lacZ$ fusion was also monitored in $\Delta sgrR$ and $sgrS1$ (38) mutant hosts. The SgrS1 molecule is defective for regulation of $ptsG$ under glucose-phosphate stress (13) and is more sensitive to αMG than the wild-type strain. Upon exposure to αMG , the $sgrS'-lacZ$ fusion in the wild-type strain was induced ~ 20 -fold (Fig. 4C, WT), whereas the $\Delta sgrR::cm$ mutation completely abolished induction, as observed previously (33). As expected, fusion activity in the $sgrS1$ mutant was significantly higher than that of the wild-type strain (induced ~ 47 -fold) (Fig. 4C), because this mutant cannot reduce uptake of αMG by downregulating the glucose transporter $ptsG$ mRNA. Interestingly, the induced level of $sgrS'-lacZ$ activity in the $\Delta kdgR$ mutant was also higher than in the wild-type strain (induced ~ 32 -fold) (Fig. 4C), although the basal expression levels were similar in the two strains, suggesting that KdgR functions as a repressor for the stress-dependent induction of $sgrS$ expression. For the crp mutant, basal levels of expression of $sgrS'-lacZ$ were similar to those in the wild-type strain. When stress was induced with αMG , $sgrS$ transcription was induced by 3-fold in the crp mutant compared to 20-fold in the wild-type strain (Fig. 4C). This result suggested that CRP is required for optimal induction of $sgrS$ under stress conditions.

To investigate whether KdgR and CRP directly or indirectly regulate $sgrS$ promoter activity, we again utilized the plasmid carrying the $sgrR1$ mutant allele. In the wild-type strain carrying the $sgrR1$ plasmid, $sgrS'-lacZ$ activity was increased ~ 22 -fold compared to the vector control strain (Fig. 4D, WT). Both basal (vector control) and induced ($sgrR1$) levels of $sgrS'-lacZ$ activity in the $kdgR$ and crp mutants were very similar to those in the wild-type cells (Fig. 4D). Collectively, these results suggested that under glucose-phosphate stress conditions, KdgR represses the activation of $sgrS$ (and very slightly of $setA$) indirectly by altering signaling through the stress response system and therefore activity of SgrR. Similarly, the effect of CRP on the induced level of $setA$ and $sgrS$ transcription was mediated through SgrR-dependent signaling. Interestingly, CRP had an SgrR-independent effect on the basal level of $setA$ transcription, but not on $sgrS$. This suggested that there might be a small direct effect of CRP on regulation of an unidentified promoter present in the $setA'-lacZ$ but not the $sgrS'-lacZ$ fusion.

A $setA$ mutation causes a defect in recovery from growth inhibition by αMG . After establishing that $setA$ is activated during glucose-phosphate stress, its role in the stress response was investigated. The growth of wild-type (CS168) and $\Delta setA::cm$ mutant (YS143) strains was monitored before and after they were exposed to αMG . No growth differences between the two strains were apparent in LB medium with or

without αMG (data not shown). We have noticed in the course of other studies that wild-type and $sgrS$ mutant strains have more pronounced growth defects when stress is induced in minimal medium compared with LB (C. S. Wadler and C. K. Vanderpool, unpublished data). Therefore, the stress phenotypes of wild-type and $\Delta setA::cm$ cells were also tested in minimal MOPS medium with fructose or glycerol as a carbon source. In minimal MOPS-fructose medium, the $\Delta setA::cm$ mutant strain carrying the vector control (Fig. 5A, $\Delta setA::cm/vector$) had a subtle but reproducible phenotype: a longer lag following addition of αMG to the culture than the wild-type strain (Fig. 5A, WT/vector). The $setA^+$ plasmid ($pZEYS1$, where expression of $setA$ was controlled by a P_{tet} promoter) eliminated the lag and restored the growth of the $\Delta setA::cm$ strain to a pattern very similar to that of wild-type cells (Fig. 5A, $\Delta setA::cm/pP_{LtetO-1-setA}$). In minimal MOPS medium with glycerol as the carbon source, the growth defect of the $\Delta setA::cm$ mutant compared with the wild type was more pronounced (Fig. 5B) and was similar to the $sgrS1$ mutant (Fig. 5C), which is defective in base pairing and subsequent regulation of $ptsG$ during glucose-phosphate stress (13). After stress was induced, the $setA$ mutant strain failed to recover to the wild-type level even after being cultured overnight. The effect of the $setA$ mutation under these conditions was bacteriostatic rather than bactericidal (data not shown). Again, the plasmid carrying $P_{LtetO-1-setA}$ complemented the stress-related growth defect of the $setA$ mutant (Fig. 5B). These data supported our hypothesis that, at least under some growth conditions, SetA plays a role in allowing cells to recover from glucose-phosphate stress. On the other hand, in both media, the $setA^+$ plasmid failed to complement the stress phenotypes of the $sgrS1$ mutant (Fig. 5C). This indicates that the $sgrS1$ phenotype is not due to polarity on $setA$ and, further, that the role of SgrS in the stress response cannot be substituted for by the activity of SetA.

The $setA$ mutation affects signaling through SgrR. Our original hypothesis was that under stress conditions, SetA facilitates removal of nonmetabolizable sugars, for example, αMG -6-phosphate, by pumping them out. If this were true, we would expect to see differences in signaling through SgrR in a $setA$ mutant compared to the wild-type strain. Since $sgrS$ transcription is controlled by SgrR (33, 34) (Fig. 4C), we measured signaling through SgrR using the $sgrS'-lacZ$ fusion. The activation of the fusion in wild-type and $\Delta setA::cm$ backgrounds was compared to activation in a $\Delta sgrS$ strain, where stress and $sgrS'-lacZ$ transcription are increased because the stress response is defective. This experiment was performed in minimal MOPS medium with fructose or glycerol as a carbon source. In minimal MOPS-glycerol medium, basal expression levels ($-\alpha MG$) of the $sgrS'-lacZ$ fusion were similar in the wild type and the $\Delta setA$ mutant. In wild-type cells, $sgrS'-lacZ$ was induced by ~ 2.8 -fold upon exposure to αMG , while induction in the $\Delta setA$ mutant was ~ 5.6 -fold (Fig. 6A). The activation of $sgrS'-lacZ$ was induced by ~ 8.8 -fold in the $\Delta sgrS$ mutant. This suggested that the $\Delta setA$ mutation impairs the stress response and therefore increases stress signaling through SgrR, though to a lesser degree than the $\Delta sgrS$ mutation. In minimal MOPS-fructose medium, however, both the basal and induced levels of $sgrS'-lacZ$ expression in the $\Delta setA::cm$ mutant were very

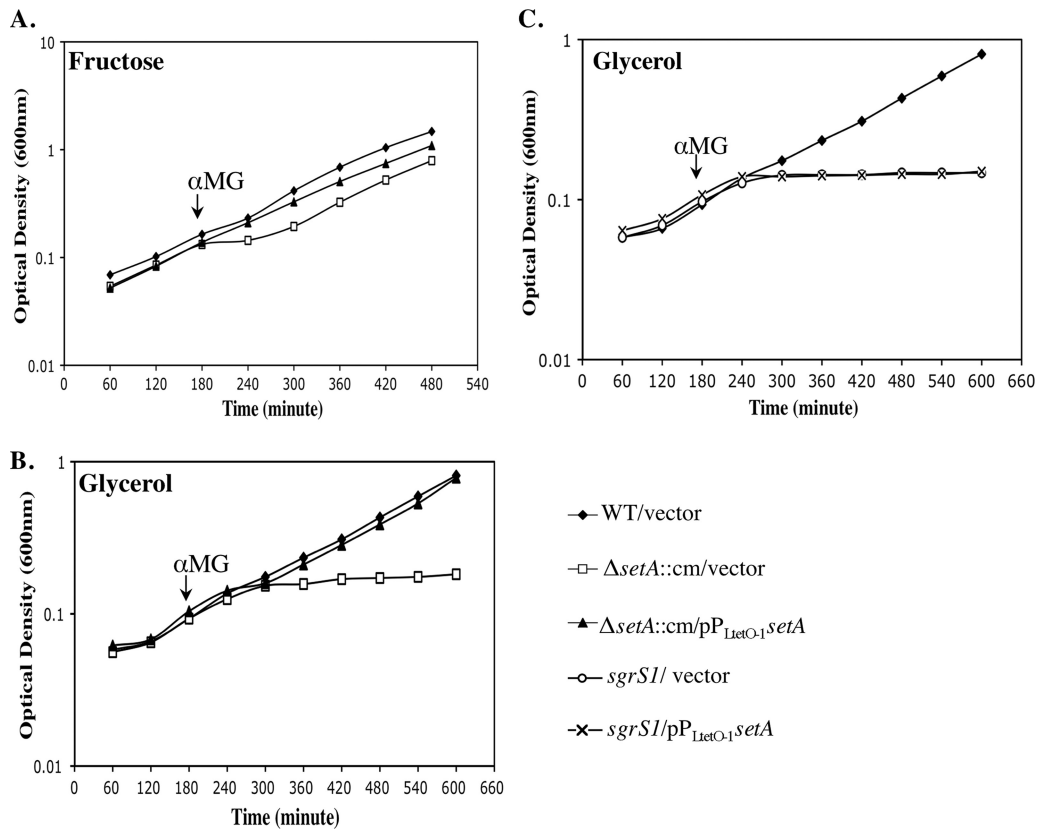


FIG. 5. Growth of wild-type and $\Delta setA$ strains in the presence of α MG. Cells were grown at 37°C in minimal MOPS medium supplemented with 1 ng/ml aTc, 25 μ g/ml kanamycin, and 0.2% fructose (A) or 0.4% glycerol (B and C) to an OD₆₀₀ of \sim 0.1, and α MG was added to the cultures to a final concentration of 0.5%. Each graph is representative of three independent trials.

similar to those of the wild type, while the *sgrS* mutant showed higher induction (Fig. 6B). The differential phenotypes in the two media were reminiscent of the growth experiments (Fig. 5) in that the effect of the *setA* mutation was more dramatic in minimal glycerol medium than in minimal fructose medium. Together, these results suggested that the *setA* mutation affects signaling through SgrR in a medium-specific manner.

Efflux of α MG by the cells is independent of SetA. To directly test the hypothesis that SetA promotes efflux of α MG under stress conditions, the efflux of 14 C-labeled α MG from wild-type and $\Delta setA::cm$ mutant cells was monitored using an assay described previously (41). α MG was implicated as an *in vitro* substrate for SetA in a previous study (17). We reasoned that if SetA is the major transporter responsible for removing the nonmetabolizable sugar from the cells during stress, the *setA* mutant would exhibit an elevated steady-state intracellular α MG level compared to that of the wild-type strain and would also show slowed kinetics of α MG efflux. Since ManXYZ and PtsG are the two main transporters responsible for α MG uptake in *E. coli* (26), a $\Delta manXYZ \Delta ptsG$ mutant was also included in the experiment as a negative control. All strains were grown in minimal MOPS-glycerol medium and stressed with [14 C] α MG. These are the conditions under which the *setA* mutant had the strongest growth phenotype (Fig. 5B). After 20 min of incubation, the cells were diluted into fresh medium lacking [14 C] α MG to promote its efflux. As shown in Fig. 7, the steady-state level of cell-associated radioactivity in

wild-type cells at time zero (immediately upon dilution) was \sim 10-fold higher than in the α MG uptake mutant ($\Delta ptsG \Delta manXYZ$). In wild-type cells, cell-associated counts decreased rapidly in the first 10 min following dilution and leveled out at a new, lower steady state. This result resembles the pattern reported in other, similar SetA efflux studies (5). The $\Delta setA::cm$ mutant showed a steady-state level of cell-associated radioactivity that was nearly identical to the level in wild-type cells. Moreover, the pattern of [14 C] α MG efflux for the $\Delta setA::cm$ mutant was also very similar to that of the wild-type cells, suggesting that SetA does not function as the major α MG efflux pump under these stress conditions.

DISCUSSION

In the current study, we have demonstrated that the sugar efflux transporter SetA contributes to the cellular response to nonmetabolizable sugar-phosphates. Under glucose-phosphate stress conditions, *setA* is coexpressed with *sgrS* (Fig. 2 and 3), which encodes the small RNA effector of the stress response (32–34). Our data are consistent with an operonic arrangement for *sgrS* and *setA*, with transcription initiating upstream of *sgrS* at a promoter that is activated under stress conditions by the transcription factor SgrR. Most well-characterized sRNA genes to date are transcribed independently (39), but a few cases have been documented where sRNA expression seems to be related to expression of flanking pro-

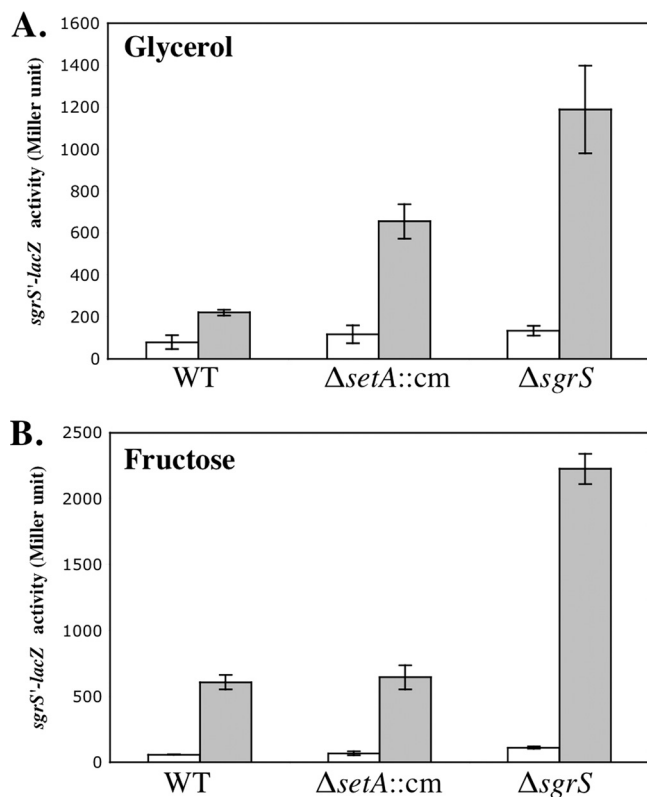


FIG. 6. Effects of *setA* mutation on *sgrS'*-*lacZ* activity in response to α MG. Cells were grown in minimal MOPS medium supplemented with 0.4% glycerol (A) or 0.2% fructose (B), and 0.001% α MG was added to half of the cultures. β -Galactosidase activity was assayed after 45 min of α MG addition. White bars represent the absence of α MG, while gray bars represent the presence of α MG. The error bars indicate the standard deviations of three independent experiments.

tein-encoding genes. For example, the recently identified *spd-sr37* and *ccnA* in *Streptococcus pneumoniae* (31) and *pel/sagA* in group A streptococcus (19, 23) are cotranscribed with downstream protein-coding genes. In *E. coli*, a previous study identified several sRNAs, e.g., SroC, that may be products of transcriptional attenuation of downstream genes and other sRNAs, e.g., SroD and SroE, that are likely processed from longer mRNAs (36). 6S RNA in *E. coli* is also processed from a bicistronic RNA, but it is a protein-binding sRNA (39) rather than a base-pairing sRNA like SgrS. The promoter of *ryhB*, which encodes an Hfq-dependent sRNA in *E. coli* that regulates various genes involved in iron metabolism (20), was implied to control the expression of the downstream gene *yhhX* (35). This gene organization is perhaps most analogous to the arrangement between *sgrS* and *setA*. However, we have clearly demonstrated here that, unlike YhhX, which encodes a putative D-galactose-1-dehydrogenase (28) whose functional link to RyhB is unclear, SetA contributes to the same stress response system as SgrS (Fig. 5).

The presence of a putative Rho-independent *sgrS* terminator strongly suggests that bicistronic *sgrS-setA* transcripts are generated by readthrough of the *sgrS* terminator. This is reminiscent of the case reported in group A streptococcus where a Rho-independent terminator between the sRNA coding gene

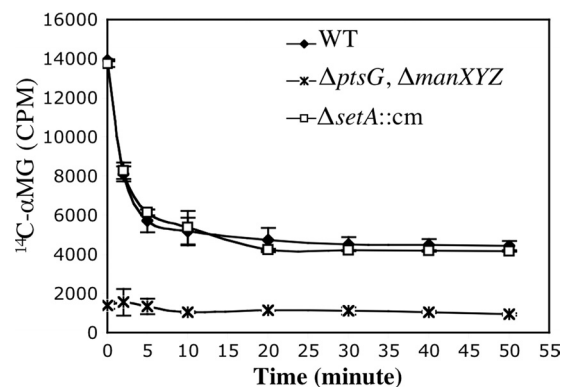


FIG. 7. Effects of *setA* mutation on the efflux of [14 C] α MG. Wild-type (DJ480), $\Delta setA::cm$ mutant (ST101), and $\Delta manXYZ::kan \Delta ptsG::cm$ mutant (CV107) strains were grown at 37°C in minimal MOPS medium supplemented with 0.4% glycerol to an OD₆₀₀ of ~0.1. The cells were incubated with [14 C] α MG (3.3 μ M; 1 μ Ci/ml) at room temperature for 20 min and then diluted 200-fold with fresh minimal MOPS-glycerol medium. Radioactivity was examined at the indicated times as described in Materials and Methods. The error bars indicate the standard deviations of three independent experiments.

pel/sagA and the downstream gene allowed and regulated the readthrough (23). Northern blots using a *setA*-specific probe did not yield a signal at the size predicted for the *sgrS-setA* transcript even under stress conditions (data not shown), whereas a strong ~200-nt signal was seen for SgrS using an SgrS-specific probe under stress conditions (31). Real-time PCR experiments revealed that under glucose-phosphate stress, steady-state levels of the *sgrS* transcript are ~600-fold higher than those of the *sgrS-setA* bicistronic transcript (data not shown), highlighting the significantly greater abundance of the SgrS RNA than of the bicistronic transcript. Collectively, these results suggest that termination usually occurs at the 3' end of SgrS and rather infrequently transcriptional readthrough produces the bicistronic transcript. Alternatively, the *sgrS-setA* RNA may be very unstable relative to SgrS.

We cannot rule out an additional *setA* promoter either within *sgrS* or further upstream. A basal level of activity for the *setA'-lacZ* fusion independent of the known *sgrS* promoter was observed (Fig. 2). Two transcriptional regulators, CRP and KdgR, which influence the expression of *sotA*, encoding a SetA paralog in *D. dadantii* (4, 29), were tested for their roles in regulating *setA* expression. A *crp* mutation increases the basal level of *setA'-lacZ* activity (Fig. 4A and B), but not the activity of *sgrS'-lacZ*, suggesting there may be another promoter controlling *setA* expression that is repressed by CRP. Further analysis of transcriptional regulation of *setA* may provide additional clues to its role in cell physiology.

Both KdgR and CRP had modest effects on the activation of *setA'-lacZ* and more significant effects on *sgrS'-lacZ* under stress conditions (Fig. 4A and C). The results of experiments using the plasmid-encoded signal-independent SgrR (Fig. 4B and D) implied that the *crp* and *kdgR* mutations alter the ability of wild-type SgrR to sense and respond to the stress signal. In the case of *kdgR*, its deletion is known to cause derepression of *eda*, which encodes 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase that cleaves KDPG to pyruvate and triose-3-phosphate (22). Interestingly, pyruvate was re-

ported previously to abrogate the degradation of *ptsG* in a Δ *pgi* mutant (14), usually indicative of altered SgrS levels or function. Furthermore, addition of pyruvate to the growth medium increases *sgrS'-lacZ* fusion activity in stressed wild-type cells (M. Patel, G. Richards, and C. K. Vanderpool, unpublished data). It is therefore possible that increased endogenous pyruvate concentrations caused by the *kdgR* deletion alter signaling through SgrR and, subsequently, stress-dependent activation of *sgrS*.

This study showed that SetA is required for optimal recovery from glucose-phosphate stress under certain growth conditions (Fig. 5). The hypothesis that SetA directly pumps out nonmetabolizable sugars (at least α MG) was not supported by our efflux experiments (Fig. 7). Consistent with this result, the stress phenotype exhibited by the *sgrS* mutant could not be bypassed by ectopic expression of *setA* (from $P_{\text{LetO-1}}\text{-setA}$) (Fig. 5C). This demonstrates two important points: (i) that the growth defect of a *sgrS* mutant is not due to polarity on *setA* and (ii) that the role of SgrS in the stress response cannot be replaced by the activity of SetA, suggesting that SgrS and SetA affect stress recovery by fundamentally different mechanisms. SgrS inhibits production and activity of the α MG transporter PtsG (28, 29) and therefore inhibits intracellular α MG6P accumulation. Since SetA cannot compensate for the lack of SgrS activity, it follows that SetA acts at a level in the stress response that does not directly impact intracellular α MG6P levels. This line of reasoning is consistent with our efflux data (Fig. 7).

SetA is not fully conserved in enterobacterial genomes that encode SgrS (8); for example, it is absent in some pathogenic *E. coli* and *Salmonella* strains. While we do not currently understand why *setA* is absent in these organisms, it may be due to potentially redundant functions of the other two SET proteins, namely, SetB and SetC, at least one of which is encoded in these genomes that lack *setA*. SetA shares a high degree of amino acid sequence similarity with SetB and SetC (16). Similar to SetA, SetB was also capable of transporting glucose and lactose *in vitro*, although it had a narrower substrate range (17). The substrate of SetC remains unidentified. Like *setA*, the genomic locations of these other SET family members suggest involvement in carbohydrate metabolism. In the *E. coli* K-12 genome, *setB* is located adjacent to the *fruBKA* operon encoding the major fructose transporter (26), while *setC* is in close proximity to genes encoding a putative galactose-pentose-hexuronide transporter (25). While transcription of *setB* and *setC* in *E. coli* K-12 was not induced by glucose-phosphate stress (data not shown), it is possible that in organisms that lack *setA*, SetB or SetC functionally replaces SetA and contributes in a similar fashion to the glucose-phosphate stress response.

Future studies are required to more fully elucidate the role of SetA in the glucose-phosphate stress response in *E. coli*. The growth defect and differences in signaling through SgrR (for activation of *sgrS* transcription) in the *setA* mutant under certain conditions confirm its connection to the stress response. The conditional nature of the SetA requirement, as well as medium-dependent differences in the severity of the stress, may provide important clues to two major unresolved questions: (i) what is the actual inducing signal and (ii) what is the nature of the metabolic problem experienced by stressed cells? Experiments are under way to determine the bona fide *in vivo*

substrate of SetA, and its identification will no doubt shed new light on the physiology of the glucose-phosphate stress response.

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