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The Novel Transcription Factor SgrR Coordinates the Response to Glucose-Phosphate Stress▽†

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SgrR is the first characterized member of a family of bacterial transcription factors containing an N-terminal DNA binding domain and a C-terminal solute binding domain. Previously, we reported genetic evidence that SgrR activates the divergently transcribed gene *sgrS*, which encodes a small RNA required for recovery from glucose-phosphate stress. In this study, we examined the regulation of *sgrR* expression and found that SgrR negatively autoregulates its own transcription in the presence and absence of stress. An SgrR binding site in the *sgrR-sgrS* intergenic region is required in vivo for both SgrR-dependent activation of *sgrS* and autorepression of *sgrR*. Purified SgrR binds specifically to *sgrS* promoter DNA in vitro; a mutation in the site required for in vivo activation and autorepression abrogates in vitro SgrR binding. A plasmid library screen identified clones that alter expression of a P_{sgrS}-*lacZ* fusion; some act by titrating endogenous SgrR. The *yfdZ* gene, encoding a putative aminotransferase, was identified in this screen; the *yfdZ* promoter contains an SgrR binding site, and transcriptional fusions indicate that *yfdZ* is activated by SgrR. Clones containing *mlc*, which encodes a glucose-specific repressor protein, also downregulate P_{sgrS}-*lacZ*. The *mlc* clones do not appear to titrate the SgrR protein, indicating that Mlc affects *sgrS* expression by an alternative mechanism.

The phenomenon of sugar-phosphate toxicity in bacterial cells is not new; however, the physiology underlying this stress remains uncharacterized. One type of sugar-phosphate stress occurs under conditions where glucose-6-phosphate (G6P) accumulates intracellularly and cannot be broken down through the glycolytic pathway, due to a mutation in one of the early steps. An analogous stress is induced in wild-type cells upon exposure to the nonmetabolizable glucose analog α-methylglucoside (αMG). Both glucose and αMG are transported and concomitantly phosphorylated by the phosphoenolpyruvate phosphotransferase system enzyme $IICB^{Glc}$, which is encoded by the *ptsG* gene. Studies from the Aiba laboratory reported that under glucose-phosphate stress conditions (accumulation of G6P or αMG6P), posttranscriptional regulation of the *ptsG* mRNA occurs such that the half-life of the *ptsG* mRNA is diminished ~10-fold (6). This regulated instability of the *ptsG* message was dependent upon the RNA chaperone Hfq and the endoribonuclease RNase E (6). These characteristics are hallmarks of regulatory processes mediated by small RNAs in bacteria (3).

Our previous work began from studies of a novel regulatory small RNA and demonstrated that this small RNA, SgrS, was responsible for the posttranscriptional regulation of *ptsG* (21). Furthermore, we found that a dedicated pathway to deal with glucose-phosphate stress involved, in addition to SgrS, a novel transcription factor protein, SgrR (21). Wild-type cells exposed to glucose-phosphate stress are transiently inhibited for growth but recover in a fairly short time. In contrast, cells that lack SgrS or SgrR are strongly inhibited under these conditions and fail to recover significantly (12, 21), indicating that this pathway is critical for the glucose-phosphate stress response.

The *sgrR* and *sgrS* genes are encoded on the *Escherichia coli* chromosome and are divergently transcribed (Fig. 1). Transcription of *sgrS* is induced under glucose-phosphate stress conditions, and induction is dependent upon the product of the *sgrR* gene (21). SgrS contains sequences that are complementary to the translation initiation region of the *ptsG* mRNA, and when expressed, SgrS forms base-pairing interactions with the *ptsG* mRNA (4, 21). The outcome of base-pairing between these RNAs is degradation of the *ptsG* message (4). SgrS-mediated degradation of *ptsG* mRNA under these conditions stops new synthesis of the $EIICB^{Glc}$ transport protein (12). We hypothesize that this function of SgrS mitigates the accumulation of toxic glucose-phosphate molecules.

SgrR is a member of a novel family of bacterial transcription factors (NCBI Clusters of Orthologous Groups designation COG4533). Genes encoding SgrR-like proteins can be found in the genomes of many *Enterobacteriaceae* as well as several species of gram-positive bacteria. SgrR and its family members are characterized by a two-domain structure with an N-terminal DNA binding domain of the winged helix family and a solute binding domain at the C terminus. Many SgrR family members are erroneously annotated in bacterial genomes as "putative periplasmic transport protein" due to the strong homology of the C-terminal domain with metal or sugar binding transport proteins found in the periplasm. The proteins of this family are almost certainly all cytoplasmic, as they do not contain signal sequences and would necessarily carry out their functions as transcription factors in the cytoplasm. As SgrR is the first member of this family to be characterized, little was known, prior to this work, about the mechanism of regulation of target genes or about the regulation of expression and activity of the SgrR protein itself. We hypothesize that SgrR is

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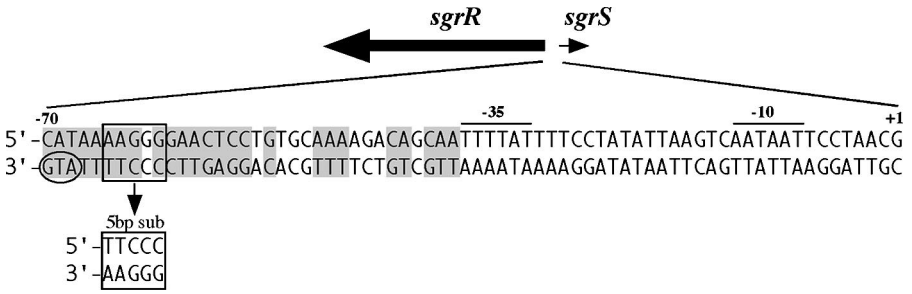

FIG. 1. Genetic organization of the *sgrR sgrS* region. The directions of transcription of the *sgrR* and *sgrS* genes are indicated at the top. Both strands of the *sgrR-sgrS* intergenic region are shown below the gene map. The +1 at right denotes the start of *sgrS* transcription, the −70 position at left is with respect to *sgrS* transcription. The −35 and −10 promoter elements are indicated by horizontal lines above the nucleotide sequence. The shaded base pairs represent conservation of putative regulatory sequences upstream of the −35 element (21). On the lower strand, the ATG start codon for *sgrR* is circled. The boxed nucleotides show the positions of conserved sequences where a 5-bp substitution was constructed. The substituted sequences marked "5 bp sub" are shown below the wild-type sequence.

the sensor for glucose-phosphate stress and that binding a small molecule (possibly G6P) under stress conditions modulates the activity of this protein.

The present study was undertaken in order to further understand the roles played by the small RNA SgrS and its transcriptional regulator SgrR in the response to glucose-phosphate stress. In vivo studies of *sgrR* regulation revealed that the SgrR protein functions as an activator of target genes such as *sgrS* and as a repressor of its own transcription. In vitro studies demonstrated that purified SgrR binds specifically to *sgrS* promoter DNA. A plasmid library screen identified at least one new member of the SgrR regulon with a potential role in altering metabolic flux during stress.

MATERIALS AND METHODS

Strain and plasmid construction. *E. coli* strains and plasmids that were used in this study are listed in Table 1. *E. coli* strain DH5α (Invitrogen) or XL-10 Gold (Stratagene) was used for routine cloning procedures. *E. coli* expression strain BL21(DE3) was used for overproduction of recombinant SgrR-His protein. The *E. coli* genomic library used in the screen was previously described (7). The medium-copy pHDB3 (19) or low-copy pWSK129 (22) plasmid was used in the construction of other recombinant plasmids. The vector pET24b (Novagen) was used for cloning and expression of recombinant SgrR-His protein. Transcriptional *lacZ* fusions were constructed as described previously (21), using plasmid pRS1553 (16).

Subclones of library plasmids (Table 1) were constructed by PCR and cloning. The sequences of oligonucleotides used in this study are given in Table S1 in the supplemental material. The 0.85-kb *sgrR′* fragment in pBRCV16 was amplified using primers ryaArev and O-CV142. The limits of this fragment are ~300 bp upstream and ~550 bp downstream of the *sgrR* ATG. The 0.65-kb fragment in pBRCV17 has the same upstream limit as pBRCV16 and a downstream limit ~350 bp within *sgrR* (primers used were ryaArev and O-CV143). The 0.43-kb *yfdZ-ypdA* intergenic region (amplified using primers LW-01 and LW-02) was cloned to create plasmid pBRCV11; the limits of this fragment extend 25 bp into the coding sequences for the flanking genes. The ~1.4-kb *mlc* fragment containing the native *mlc* promoter and protein-coding sequences was cloned to create plasmid pBRCV10 (PCR primers O-CV135 and O-CV136). Plasmid pBRCV21 carries an *mlc′* fragment with the same 5′ limit as for pBRCV10, but with a 0.6-kb deletion from the 3′ end (PCR primers O-CV135 and O-CV163).

Substitution mutations were constructed by whole-plasmid PCR mutagenesis using the QuikChange system (Stratagene) according to the manufacturer's instructions. Plasmid pBRCV18 was derived from pBRCV16 (Table 1) using primers 5ntmut-S plus 5ntmut-AS. Plasmid pBRCV20 was derived from pBRCV11 (Table 1) using primers O-CV144 plus O-CV145. Plasmids pBRCV24 and pBRCV25 were derived from pBRCV10 (Table 1) using primers O-CV190 plus O-CV191 and O-CV188 plus O-CV189, respectively.

Plasmid pWSKCV1 (pP_{lac}-*sgrR*) was constructed by cloning the 1.7-kb *sgrR* open reading frame as an EcoRI-PstI fragment (PCR amplified using primers O-CV102 plus O-CV111) into low-copy plasmid pWSK129 (22). The vector P_{lac} promoter drives transcription of *sgrR*.

The Δ*sgrR*::cat allele was constructed and analyzed in a previous study (21). This allele was moved to various strain backgrounds by P1 transduction and selection for chloramphenicol resistance. The P_{bla}-*ptsG* construct linked to a chloramphenicol resistance cassette (11) was kindly provided by Hiroji Aiba; this construct was moved to the P_{sgrS}-*lacZ* fusion strain by P1 transduction.

Media and reagents. Bacteria were routinely cultured on LB (Lenox) agar plates or in liquid LB medium at 37°C. For SgrR overexpression, cells were cultured in MOPS (morpholinepropanesulfonic acid) rich defined medium (Teknova) with 0.4% glycerol as the carbon source. MacConkey agar plates containing 1% lactose and the appropriate antibiotic (50 μg/ml ampicillin or 25 μg/ml kanamycin) were used to analyze activity of *lacZ* reporter fusions.

Plasmid library screen. A pBR322-based plasmid DNA library (19) was screened as described previously (7). Plasmid DNA was delivered to strain CV5200 by electroporation, and transformants were plated on lactose MacConkey agar containing ampicillin (50 μg/ml) and α-methylglucoside (0.05%). Plasmids were recovered from white colonies and retransformed to strain CV5200 to confirm that the Lac^- phenotype was associated with the plasmid. Plasmid inserts were sequenced using pBRlibfor and pBRlibrev primers as described previously (2).

Construction and analysis of *lacZ* transcriptional fusions. Transcriptional fusions were constructed by cloning the promoter of interest into the vector pRS1553 (16; http://www.mimg.ucla.edu/bobs/vectors/Alpha-lac/alphaLac.htm). The 0.22-kb P_{sgrR1} fragment was PCR amplified (primers SgrR1-lacZEco and SgrR2-lacZBam) and cloned into pRS1553 as an EcoRI-BamHI fragment. This plasmid is pRSCV11; the corresponding lambda clone is λRSCV11. The 5′ limit of this P_{sgrR1} clone is ~200 bp upstream of the *sgrR* ATG; the 3′ limit is 20 bp downstream. For the mutant P_{sgrR1}-*lacZ* fusion, the same 0.22-kb fragment was PCR amplified using primers SgrR1-lacZEco and SgrR-5ntmut-Bam and cloned into pRS1553. The reverse primer, SgrR-5ntmut-Bam, incorporated the 5-nucleotide substitution shown in Fig. 1. The resulting mutant P_{sgrR1}-*lacZ* plasmid is pRSCV13; the corresponding lambda clone is λRSCV13. The P_{sgrR2}-*lacZ* fusion was constructed by amplifying a 0.75-kb fragment by using primers SgrR1-lacZEco and O-CV206. This fragment was cloned into pRS1553 as an EcoRI-BamHI fragment, resulting in plasmid pRSBH1. The corresponding lambda clone is λRSBH1. The P_{sgrR2} fragment has the same 5′ limit as P_{sgrR1}, that is, ~200 bp upstream of the *sgrR* ATG; the 3′ limit of P_{sgrR2} is 550 bp within the *sgrR* gene. The mutant P_{sgrR2}-*lacZ* plasmid (pRSBH2) was derived from the wild type by PCR mutagenesis using primers 5ntmut-S and 5ntmut-AS. The corresponding lambda clone is λRSBH2.

The 0.43-kb P_{ypdA} and P_{yfdZ} fragments were amplified using primers LW-07 plus LW-08 and LW-01 plus LW-09, respectively (the limits of these fragments are the same as for clone pBRCV11 [see above]). These fragments were cloned into pRS1553 as EcoRI-BamHI fragments. The P_{ypdA}-*lacZ* plasmid and corresponding lambda clone are pRSLW1 and λRSLW1, respectively. The P_{yfdZ}-*lacZ* plasmid and corresponding lambda clone are pRSLW2 and λRSLW2, respectively. Transcriptional fusions were delivered in single copy to the λ *att* site as described previously (16).

Kinetic assays for β-galactosidase activity were performed using a SpectraMax 250 microtiter plate reader (Molecular Devices) as described previously (8).

TABLE 1. Strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Description	Reference or source
Strains		
MG1655	Wild-type *E. coli* K-12	
DJ480	MG1655 Δ*lac*X74	D. Jin (NCI)
DJ624	DJ480 *mal*::*lacI*^{q}	D. Jin (NCI)
CV105	DJ624 *mal*::*lacI*^{q} Δ*sgrS*::kan Δ*ptsG*::cat	This study
CV5200	DJ480 *imm*^{21} P$_{sgrS}$-*lacZ*	21
CV5282	CV5200 *imm*^{21} P$_{sgrS}$-*lacZ*, cat-P$_{bla}$-*ptsG*	This study
CV5500	DJ480 *imm*^{21} mutant P$_{sgrS}$-*lacZ*	This study
CV9000	DJ480 *imm*^{21} P$_{sgrR1}$-*lacZ*	This study
CV9030	CV9000 Δ*sgrR*::cat	This study
CV9100	DJ480 *imm*^{21} mutant P$_{sgrR1}$-*lacZ*	This study
CV9200	DJ480 *imm*^{21} P$_{sgrR2}$-*lacZ*	This study
CV9201	DJ480 *imm*^{21} P$_{sgrR2}$-*lacZ* Δ*sgrR*::cat	This study
CV9300	DJ480 *imm*^{21} mutant P$_{sgrR2}$-*lacZ*	This study
LW1000	DJ480 *imm*^{21} P$_{ypdA}$-*lacZ*	This study
LW1010	LW1000 *imm*^{21} P$_{ypdA}$-*lacZ* Δ*sgrR*::cat	This study
LW2000	DJ480 *imm*^{21} P$_{yfdZ}$-*lacZ*	This study
LW2010	LW1000 *imm*^{21} P$_{yfdZ}$-*lacZ* Δ*sgrR*::cat	This study
Plasmids		
pWSK129	Low-copy plasmid vector control	22
pWSKCV1	pWSK129 with 1.7-kb *sgrR* open reading frame; *sgrR* expression controlled by P$_{lac}$	This study
pHDB3	pBR322 derivative; vector control	19
pBRlib15	Clone identified in library screen; contains 1.5-kb *sgrR′ sgrS setA′* region	This study
pBRCV16	pHDB3 with 0.85-kb *sgrRS* DNA region; minimal in vivo titrating fragment	This study
pBRCV17	pHDB3 with 0.65-kb *sgrRS* DNA region; does not titrate	This study
pBRCV18	pBRCV16 with 5-bp mutation in P$_{sgrS}$ promoter (Fig. 1)	This study
pBRCV2	pHDB3 with 0.08-kb *sgrR-sgrS* intergenic region	This study
pBRCV3	pBRCV2 with 5-bp mutation in SgrR binding site	This study
pBRlib1	Clone identified in library screen; contains 1.2-kb *yfdZ′-ypdA′* region	This study
pBRCV11	pHDB3 with ~0.43-kb *yfdZ-ypdA* intergenic region and 25 bp of each open reading frame	This study
pBRCV20	pBRCV11 with 3-bp substitution mutation in predicted SgrR binding site	This study
pBRlib8	Clone identified in library screen; contains 3.0-kb *′clcB ynfK mlc ′ynfL* region	This study
pBRCV10	pHDB3 with ~1.4-kb *mlc* gene under control of native promoter	This study
pBRCV21	pHDB3 with ~1.1-kb *mlc′* fragment	This study
pBRCV24	pBRCV10 with 3-bp substitution "sub1" in P$_{mlc}$	This study
pBRCV25	pBRCV10 with 3-bp substitution "sub2" in P$_{mlc}$	This study
pRS1553	Vector for construction of transcriptional *lacZ* reporter fusions	16
pRSCV11	pRS1553 with 0.22-kb wild-type P$_{sgrR1}$ fragment	This study
pRSCV13	pRS1553 with 0.22-kb mutant P$_{sgrR1}$ fragment	This study
pRSBH1	pRS1553 with 0.75-kb wild-type P$_{sgrR2}$ fragment	This study
pRSBH2	pRS1553 with 0.75-kb mutant P$_{sgrR2}$ fragment	This study
pRSLW1	pRS1553 with 0.43-kb P$_{ypdA}$ fragment	This study
pRSLW2	pRS1553 with 0.43-kb P$_{yfdZ}$ fragment	This study
Bacteriophages		
λRSCV11	Lambda vector carrying wild-type P$_{sgrR1}$-*lacZ* transcriptional fusion	This study
λRSCV13	Lambda vector carrying mutant P$_{sgrR1}$-*lacZ* transcriptional fusion	This study
λRSBH1	Lambda vector carrying wild-type P$_{sgrR2}$-*lacZ* transcriptional fusion	This study
λRSBH2	Lambda vector carrying mutant P$_{sgrR2}$-*lacZ* transcriptional fusion	This study
λRSLW1	Lambda vector carrying P$_{ypdA}$-*lacZ* transcriptional fusion	This study
λRSLW2	Lambda vector carrying P$_{yfdZ}$-*lacZ* transcriptional fusion	This study

Specific activities were calculated using the formula V_{max}/optical density at 600 nm (OD_{600}); these are approximately 25-fold lower than standard Miller units. The results reported represent data typical of at least three experimental trials.

Overexpression and purification of SgrR-His. The 1.7-kb *sgrR* open reading frame was amplified by PCR using the primers pET-sgrRfor and pET-sgrRrev and cloned into the expression vector pET24b (Novagen) as an EcoRI-HindIII restriction fragment. The resulting construct, pET-*sgrR*, encodes a recombinant SgrR protein containing the T7 tag epitope at the N terminus and the His_6 epitope at the C terminus. The pET-*sgrR* plasmid was used to transform *E. coli* strain BL21(DE3) for overexpression. Cultures were grown in MOPS EZ rich defined medium (Teknova) with 0.4% glycerol as a carbon source at 18°C with shaking until the culture reached an OD_{600} of ~0.2. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added at a final concentration of 100 μM, and cultures were grown for an additional 8 to 12 h. Cells were pelleted at 4°C and pellets stored at −80°C.

Cell pellets were resuspended in lysis buffer (pH 8.0) composed of 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 10% glycerol. Throughout purification, cell fractions were maintained at 4°C. Bacteria were lysed using a French pressure cell, and soluble and insoluble fractions were separated by centrifugation. The presence of SgrR-His in the soluble fraction of the lysate was confirmed by running protein samples from uninduced and induced cells on sodium dodecyl sulfate-polyacrylamide gels and staining with Coomassie blue. Soluble fractions containing SgrR-His were incubated in batch with Ni^{2+}-nitrilotriacetic acid agarose (Invitrogen) for 1 h at 4°C. The matrix with bound protein was then applied to a column for four washes (each wash was 5 times the bed volume) with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole,

10% glycerol, pH 8.0). SgrR-His was eluted in four fractions (each 0.5 times the bed volume) in wash buffer containing 200 mM imidazole. Eluates were exchanged into storage buffer containing 20 mM HEPES, 300 mM NaCl, and 10% glycerol by using PD-10 desalting columns (GE Biosciences) and stored at −80°C.

Electrophoretic mobility shift assays. A 74-bp DNA probe containing the P_{sgrS} region from position −70 to +4 (with respect to the *sgrS* +1) was constructed by annealing complementary oligonucleotides PryaA1 and PryaA2. The double-stranded DNA molecule was run on a 2.5% agarose gel, and the 74-bp band was cut and purified. The mutant P_{sgrS} probe was similarly constructed with oligonucleotides PsgrS1-mut1-S and PsgrS1-mut1-AS. DNA probes were end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Invitrogen) according to the manufacturer's recommendations.

Binding reactions were set up using labeled probe at a final concentration of 0.1 nM in a volume of 20 μl. The binding buffer contained 20 mM HEPES, 10% glycerol, 1 mM dithiothreitol, 3 mM $MgCl_2$, 50 mM NaCl, 0.1 μg/μl bovine serum albumin, and 0.05 μg/μl poly(dI-dC). SgrR-His was freshly diluted from concentrated stock solutions into binding reaction mixtures over a range of concentrations and incubated at room temperature for 20 min. Reaction mixtures were loaded on 6% DNA retardation gels (Invitrogen) and allowed to run at 100 V for ~40 min. Gels were dried and exposed to film with intensifying screens at −80°C.

Western blots. Strains were grown to mid-log phase in LB medium, and total proteins were harvested by precipitation with trichloroacetic acid. Ice-cold trichloroacetic acid was added to 1 ml of culture at a final concentration of 10% and incubated on ice for 10 min. Proteins were spun down and pellets washed with 0.5 ml of ice-cold 80% acetone. Dried pellets were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer at volumes normalized to the OD_{600} of cultures. The equivalent volume for 0.05 OD unit of culture was loaded on a 10% bis-Tris gel and run in MOPS buffer, and proteins were transferred to a polyvinylidene difluoride membrane according to the manufacturer's instructions (Invitrogen). The anti-$EIIB^{Glc}$ primary antibody (provided by Hiroji Aiba) was used at a dilution of 1:5,000; the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Calbiochem) was used at a dilution of 1:10,000. The blot was developed using Immobilon Western horseradish peroxidase substrate (Millipore) according to the manufacturer's instructions.

RESULTS

Regulation of divergent *sgrR* and *sgrS* genes: SgrR is an activator and a repressor. The 227-nucleotide SgrS small RNA is encoded at ~1.65 min on the *E. coli* chromosome (21). Transcription of *sgrS* is activated under glucose-phosphate stress conditions and is dependent upon the product of the divergently transcribed gene *sgrR* (21). The SgrR protein contains two domains, an N-terminal DNA binding domain of the winged-helix family and a C-terminal solute binding domain. The divergent *sgrR* and *sgrS* coding sequences are separated by only 67 bp (Fig. 1). In a previous study, we found that sequences required for SgrR-mediated activation of *sgrS* were localized to a conserved region between positions −70 and −55 relative to the start of *sgrS* transcription (21). This corresponds to the region that would contain the translation initiation sequences for *sgrR*, suggesting that SgrR binding at this site for activation of *sgrS* transcription may result in simultaneous negative autoregulation of *sgrR* transcription.

Two P_{sgrR}-*lacZ* transcriptional fusions were constructed to evaluate possible autoregulation by SgrR. The 5′ limit of the first fusion, P_{sgrR1}-*lacZ*, was located ~200 bp upstream of the *sgrR* AUG start codon (within the *sgrS* coding sequence), and the 3′ junction with the promoterless *lacZ* gene occurred 26 bp into the *sgrR* coding sequence. This fusion was delivered in single copy to the λ *att* site in wild-type and Δ*sgrR*::cat hosts. β-Galactosidase activity was measured when strains were grown in the presence and absence of αMG to induce glucose-phosphate stress (Fig. 2A). Analyses of the P_{sgrR1}-*lacZ* fusion showed that in the absence of stress, transcription of the *sgrR* promoter was consistently at least 1.5-fold higher in an *sgrR* mutant background than in $sgrR^+$ cells, demonstrating that SgrR at least modestly negatively autoregulates its own transcription. Experiments that will be described below provided evidence that sequences within the *sgrR* coding sequence, not contained within P_{sgrR1}, may be involved in regulation of *sgrR* transcription. To analyze this possibility, a second fusion (P_{sgrR2}-*lacZ*) (Fig. 2A), which contained an additional 550 bp

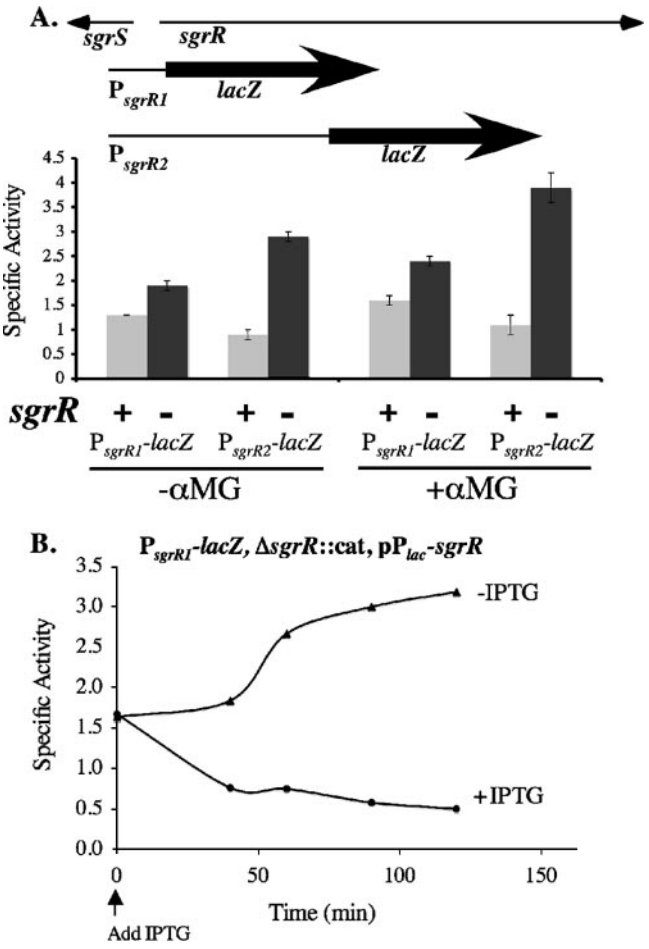

FIG. 2. Negative autoregulation by SgrR. A. The relative limits of the short (P_{sgrR1}) and long (P_{sgrR2}) *sgrR-lacZ* transcriptional fusions are represented at the top. Wild-type (+; CV9000 and CV9200) (Table 1) and Δ*sgrR*::cat mutant (−; CV9030 and CV9201) (Table 1) host strains carrying the P_{sgrR1}-*lacZ* or P_{sgrR2}-*lacZ* transcriptional fusion (as indicated below the graph) were grown to mid-log phase, split, and cultured with αMG (+αMG) or without αMG (−αMG). After an additional 45 min, samples were harvested and assayed for β-galactosidase activity. Gray bars represent activity of wild-type strains; black bars represent activity of *sgrR* mutant strains. The presence or absence of αMG is indicated below the graph. B. The Δ*sgrR*::cat host strain (CV9030) carrying the P_{sgrR1}-*lacZ* transcriptional fusion and a low-copy plasmid with *sgrR* under control of the heterologous P_{lac} promoter (pWSKCV1) was grown to mid-log phase, split, and cultured with or without IPTG to induce overexpression of *sgrR*. The graph of β-galactosidase activity over time begins at the point where the culture was split. Circles represent activity of the culture that was exposed to IPTG; triangles show activity of cells cultured in the absence of IPTG. For both panels A and B, the results reported are representative of at least three experimental trials. Error bars indicate standard deviations.

of the *sgrR* gene, was constructed and analyzed. Without stress, the activity of the longer P_{sgrR2}-*lacZ* fusion was ~3-fold-higher in the *sgrR* mutant host than in the wild type (Fig. 2B), again suggesting that SgrR negatively autoregulates its own transcription. These results support the notion that sequences within the *sgrR* coding sequence are important for negative autoregulation, as will be discussed further below. Under stress conditions (with αMG), the activities of both fusions showed the same pattern as under nonstress conditions; transcription of *sgrR* in both cases was increased in the *sgrR* mutant host. This result suggested that, unlike the activation activity of SgrR at the *sgrS* promoter, the autorepression activity of the SgrR protein is not significantly affected by the stress.

To further investigate negative autoregulation of the *sgrR* promoter, activity of the P_{sgrR1}-*lacZ* fusion was examined in strains containing the chromosomal Δ*sgrR*::cat allele and carrying either a plasmid vector control or pP_{lac}-*sgrR* (pWSKCV1) (Table 1). After growth to mid-log phase, cultures of cells carrying the pP_{lac}-*sgrR* plasmid were split; one of the cultures after the split was exposed to IPTG to induce the expression of *sgrR*. The β-galactosidase activities of induced and uninduced cultures were assayed at several time points after IPTG addition. Uninduced cells showed increasing levels of β-galactosidase activity over the course of the experiment, while cells induced for *sgrR* expression showed decreasing activity. At the final time point, activity of the P_{sgrR1}-*lacZ* fusion was ~6-fold higher in uninduced cells than in cells where *sgrR* expression was induced (Fig. 2B). A similar result was seen when the activity of the fusion in IPTG-induced cells carrying the plasmid vector control was compared with that of the pP_{lac}-*sgrR* construct (data not shown). Addition of αMG had no significant effect on the extent of negative autoregulation by SgrR when *sgrR* expression was induced by IPTG from P_{lac}-*sgrR* (data not shown). These results confirm that SgrR negatively autoregulates its own transcription. The greater level of autorepression of P_{sgrR1}-*lacZ* seen when SgrR was produced from a heterologous promoter (compare the fold repressions in Fig. 2A and B) suggested that endogenous SgrR levels may be limiting.

We had hypothesized that the activity of SgrR might be regulated at the level of DNA binding depending on the presence of the stress signal, that is, that SgrR binding to its target sequences may only occur in the presence of αMG (21). However, the negative autoregulation phenotype in the absence of glucose-phosphate stress (Fig. 2) strongly suggests that SgrR can bind DNA in a repression-competent form in the absence of the stress signal.

A site required for SgrR-mediated activation and repression. We showed previously that deletion of sequences from −70 to −55 with respect to the start of *sgrS* transcription abrogated the SgrR-dependent activation of P_{sgrS} in response to glucose-phosphate stress. Alignment of P_{sgrS} sequences from closely related organisms showed that the sequences in this region were highly conserved (21), supporting the notion that a conserved SgrR binding site may occur here. A 5-bp substitution mutation that altered conserved nucleotides in this region (Fig. 1) was constructed in the context of P_{sgrS}-*lacZ* and both P_{sgrR}-*lacZ* chromosomal fusions, and the activities of wild-type and mutant fusions were compared (Fig. 3). We predicted that if these sequences were important for SgrR binding, the

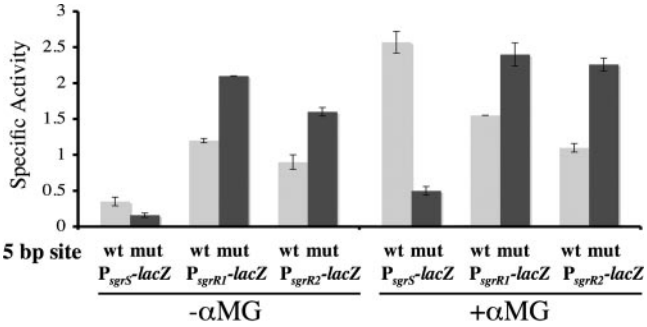

FIG. 3. The same SgrR binding site is required for activation of *sgrS* and autorepression of *sgrR*. β-Galactosidase assays were performed as described for Fig. 2A. Host strains were wild type (*sgrR*$^+$); *lacZ* transcriptional fusions had either a wild-type (wt) sequence in the *sgrR-sgrS* promoter region (shown in Fig. 1) or the 5 bp substitution mutation (mut) (also as shown in Fig. 1) in the P_{sgrS}, P_{sgrR1}, or P_{sgrR2} fragments. Strains are as follows (see Table 1): CV5200, P_{sgrS} (wt); CV5500, P_{sgrS} (mut); CV9000, P_{sgrR1} (wt); CV9100, P_{sgrR1} (mut); CV9200, P_{sgrR2} (wt); and CV9201, P_{sgrR2} (mut). Gray and black bars represent activities of fusions with the wild-type or mutant 5-bp site, respectively. The presence or absence of the stress signal αMG is indicated below the graph. Error bars indicate standard deviations.

mutation would result in a loss of activation of P_{sgrS} under stress conditions and a relief of autorepression of P_{sgrR}. In the absence of the stress signal αMG (Fig. 3), activities of both the wild-type and mutant P_{sgrS}-*lacZ* fusions were very low. Under nonstress conditions, the mutant derivatives of both the short (P_{sgrR1}) and the long (P_{sgrR2}) P_{sgrR}-*lacZ* fusions showed ~1.5-fold-higher activity than the corresponding wild-type fusions (Fig. 3). This result suggests that this putative SgrR binding site in the *sgrR-sgrS* intergenic region (Fig. 1) contributes approximately equally to SgrR autorepression regardless of the presence of any additional regulatory sites within *sgrR* (contained only in P_{sgrR2}). In the presence of the stress signal αMG (Fig. 3), the wild-type P_{sgrS}-*lacZ* fusion was activated ~6-fold over the nonstress levels, consistent with our previous observations (21). In contrast, mutation of the 5-bp site in the context of P_{sgrS}-*lacZ* abrogated this activity, demonstrating that this site is crucial for SgrR-dependent *sgrS* activation during stress. For both P_{sgrR}-*lacZ* fusions under stress conditions, the 5-bp mutation again resulted in ~1.5-fold-higher activity compared with the wild-type fusions. Taken together, these in vivo results suggest that the same SgrR binding site is required for activation of *sgrS* transcription under glucose-phosphate stress conditions and negative autoregulation of *sgrR* transcription in the presence and absence of stress.

Purified SgrR-His binds *sgrS* promoter DNA in vitro. Genetic analyses of the regulation of *sgrR* (Fig. 2) and *sgrS* (21) (Fig. 3) showed that SgrR is required for activation of *sgrS* transcription under glucose-phosphate stress conditions and autorepression of *sgrR*, prompting the hypothesis that SgrR directly binds DNA to mediate these effects. To examine the DNA binding characteristics of SgrR in vitro, an epitope-tagged SgrR-His protein was overexpressed and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography. Purified SgrR-His was then incubated with radiolabeled wild-type or mutant P_{sgrS} fragments (Fig. 1), and electrophoretic mobility shift assays were performed. SgrR bound to the wild-type P_{sgrS}

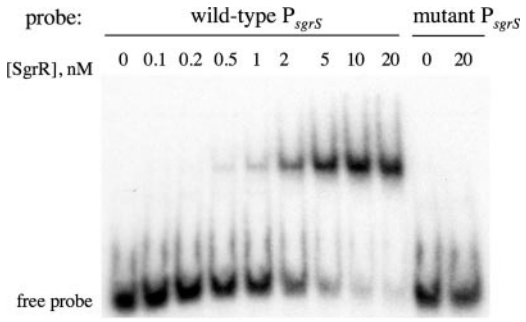

FIG. 4. SgrR binds *sgrS* promoter DNA in a site-specific manner. Gel mobility shift assays were carried out using purified SgrR-His protein as described in Materials and Methods. Wild-type P_{sgrS} is a 74-bp fragment encompassing nucleotides −70 to +4 with respect to *sgrS*. Mutant P_{sgrS} contains the 5-bp substitution as pictured in Fig. 1.

fragment to produce a shifted band over a range of protein concentrations (Fig. 4); the apparent K_D for this interaction was ≈5 nM. In contrast, even at the highest concentration of SgrR protein tested, no band shift was detected (Fig. 4) with the mutant P_{sgrS} fragment that carries the 5-bp substitution (Fig. 1). This result is entirely consistent with genetic experiments indicating that these sequences are required for SgrR-dependent activation of *sgrS* and autorepression of *sgrR*.

Given the domain structure of SgrR and the evidence showing that SgrR requires a stress signal in order to activate *sgrS* expression, we hypothesize that binding of a small-molecule inducer may alter the function of the SgrR protein. One candidate small molecule is G6P (or αMG6P), since it has been shown that intracellular accumulation of this molecule is correlated with the stress response (10). Gel shifts were repeated where purified SgrR protein was preincubated with or without G6P and then mixed with P_{sgrS} DNA. The results of these experiments showed that exogenously added G6P had no significant effect on either the apparent affinity or the band pattern of SgrR binding to the *sgrS* promoter fragment (data not shown).

In vivo titration identifies additional SgrR binding sites. During glucose-phosphate stress, SgrR is required for activation of *sgrS* transcription (21). There may also be other components of the stress signaling or recovery pathways that have not yet been discovered. In order to identify such putative factors, a genomic library was screened for clones that altered the expression of a P_{sgrS}-*lacZ* fusion in the presence of αMG. Three classes of clones containing distinct genomic regions were identified in this screen; each type of clone resulted in reduced activity of the P_{sgrS}-*lacZ* fusion under glucose-phosphate stress conditions. The clones chosen for further analysis contained the shortest insert fragment of their representative class.

(i) The *sgrRS* genomic region. Members of the first class of clones were isolated several times in the screen and comprised different DNA fragments within the *sgrR sgrS* genomic region (one such isolate is pBRlib15 [Fig. 5]). Since this screen was carried out under glucose-phosphate stress conditions (in the presence of αMG), cells carrying the plasmid vector showed a high level of P_{sgrS}-*lacZ* activity, which was set at 100% for purposes of comparison with library clones (Fig. 5). Clones

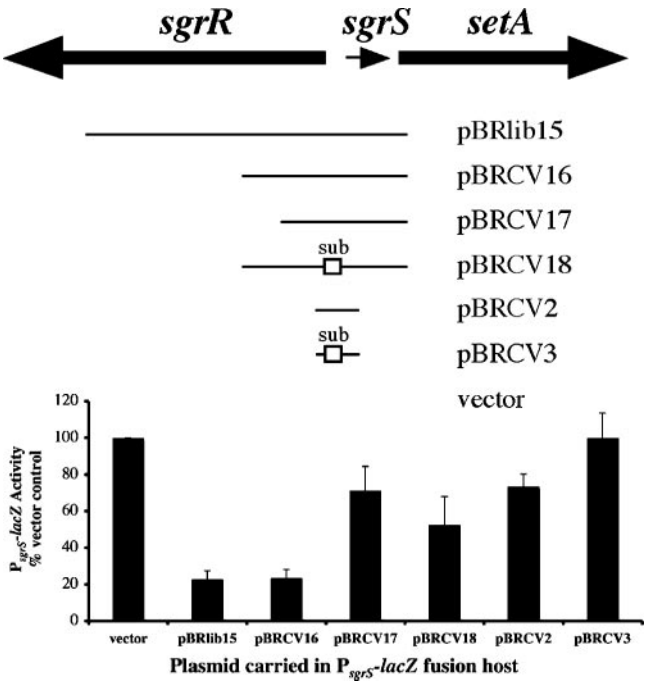

FIG. 5. Titration of endogenous SgrR by DNA fragments from the *sgrR-sgrS-setA* region. The relative sizes and orientations of transcription of the *sgrR*, *sgrS*, and *setA* genes are represented by the gene map at top. The limits of the original titrating clone pBRlib15 and all subclones are represented by horizontal lines below the gene map. The relative position of the mutation in clones pBRCV18 and pBRCV3 is denoted by the box over the horizontal line. For these two plasmids, the "sub" mutation is the 5-bp substitution described in the text and shown in detail in Fig. 1. All plasmids were transformed to a wild-type host strain carrying the P_{sgrS}-*lacZ* fusion (strain CV5200 [Table 1]) (21); activity of the fusion in each strain was measured after 45 min of exposure to αMG. β-Galactosidase activity was quantitated and normalized to the activity of cells carrying the vector control, which was set at 100%. Error bars indicate standard deviations.

exemplified by pBRlib15, which contained a fragment from the *sgrR sgrS setA* region, reduced the activity of P_{sgrS}-*lacZ* in the presence of αMG to ~20% of vector levels (Fig. 5). We theorized that clones like pBRlib15 negatively affected P_{sgrS}-*lacZ* activity because they bound free SgrR protein and titrated it away from the chromosomal fusion. Titration of a regulator can be observed when concentrations of the regulatory protein are limiting, and in vivo titration assays have been used to identify novel binding sites for known regulators (17). To determine the limits of the minimally active titrating *sgrRS* fragment, sequential deletions were made, starting from the 5′ limit of pBRlib15. Deletions of up to 0.8 kb from the 5′ end of the pBRlib15 insert produced active clones, such as pBRCV16, that diminished activity of P_{sgrS}-*lacZ* in the presence of αMG also to ~20% of vector levels (Fig. 5). Deletion of an additional 200 bp significantly relieved titration and restored activity of P_{sgrS}-*lacZ* to ~70% of vector levels (Fig. 5, compare activities of strains carrying pBRCV16 and pBRCV17). This result suggested that there were sequences between the limits of clones pBRCV16 and pBRCV17, within the coding region for SgrR, that were important for SgrR binding. To define the contribution of the known SgrR binding site in the *sgrR-sgrS* intergenic region (Fig. 1) to the titration phenotype, a mutant

derivative of the active clone pBRCV16 was constructed. The mutant plasmid, pBRCV18 (Fig. 5), contained the same 5-bp substitution mutation in the SgrR binding site shown in Fig. 1 that has been described in other experiments in this study (Fig. 3 and 4). Plasmid pBRCV18 downregulated P_{sgrS}-*lacZ* slightly; the activity of cells carrying pBRCV18 was ~50% of that of cells carrying the vector (Fig. 5). This result is consistent with the idea that the site located in the *sgrR-sgrS* intergenic region is necessary but not sufficient for strong SgrR binding that leads to titration in this in vivo system. To examine this further, a subclone carrying only the *sgrR-sgrS* intergenic region, pBRCV2, was tested in the in vivo titration assay. Consistent with our prediction, this fragment only slightly reduced activity of P_{sgrS}-*lacZ* (Fig. 5) (pBRCV2 has ~70% activity compared with the vector control). Mutation of the known SgrR binding site in this short region (clone pBRCV3 carries the 5-bp substitution, as in Fig. 1) completely abrogated titration. Taken together, these results suggest that two regions are necessary for strong binding and full titration of SgrR: the region defined by the 5-bp substitution mutation (Fig. 1 and 5) and the region in the *sgrR* coding sequence located between the limits of plasmid clones pBRCV16 and pBRCV17 (Fig. 5).

(ii) The *yfdZ ypdA* genomic region. Another class of clones that diminished activity of P_{sgrS}-*lacZ* encompassed portions of the divergent *yfdZ* and *ypdA* genes and their 376-bp intergenic region; this class is exemplified by clone pBRlib1 (Fig. 6A). The pBRlib1 plasmid diminished P_{sgrS}-*lacZ* levels by ~50% compared with vector-containing cells. *yfdZ* has been annotated as encoding a hypothetical aminotransferase that is likely involved in amino acid biosynthesis. *ypdA* and the downstream *ypdB* gene encode a putative two-component signal transduction system, where YpdA is the predicted inner membrane sensor kinase. The fact that active clones such as pBRlib1 did not contain the complete coding sequence of either of the flanking genes (Fig. 6A) suggested that the YfdZ and YpdA proteins were not themselves responsible for the diminished activation of the *sgrS* promoter. If *yfdZ* and/or *ypdA* was a member of the SgrR regulon, these clones may be acting through a titration mechanism as discussed above. To examine this possibility, a smaller fragment containing only the 376-bp *yfdZ-ypdA* intergenic region and a short piece of each flanking gene was cloned and tested for its effect on P_{sgrS}-*lacZ* activity (pBRCV11) (Fig. 6A). This smaller region diminished activity to the same levels (~50% of vector) as the larger parental clone, indicating that the sequences active for SgrR titration were fully contained in this DNA sequence.

Alignment of the *yfdZ-ypdA* intergenic region with the *sgrS* promoter (Fig. 6B) identified a short conserved sequence with similarity to the −70 to −55 region of P_{sgrS} (Fig. 6), which we showed previously is required for SgrR-dependent activation of this promoter (21) (Fig. 3). This conservation was oriented 5′ to 3′ in the same direction as *yfdZ* and located ~250 bp upstream from the *yfdZ* coding region. Aside from this short stretch of conservation, the P_{sgrS} and P_{yfdZ} regions bore little similarity. However, an alignment of the *yfdZ* upstream regions from a number of *Enterobacteriaceae* (see Fig. S1 in the supplemental material) revealed strong conservation of the sequence shown in Fig. 6B, as well as the flanking sequences, suggesting that this region is important for regulation of *yfdZ*. Since the transcription start site for *yfdZ* is not known, it cannot yet be determined if the spacing between the putative SgrR binding site and the *yfdZ* promoter is similar to the spacing in P_{sgrS}. Nevertheless, to determine whether these sequences were indeed required for the in vivo titration of SgrR, a 3-bp substitution mutation was constructed in the context of plasmid pBRCV11 to yield mutant plasmid pBRCV20. This mutation in pBRCV20 abrogated the titration effect and restored P_{sgrS}-*lacZ* activity to 100% (Fig. 6A), strongly suggesting that these sequences constitute an SgrR binding site and that the wild-type *yfdZ-ypdA* intergenic region titrates SgrR.

To determine which of the genes flanking this region was regulated by SgrR, transcriptional *lacZ* fusions were created with both P_{yfdZ} and P_{ypdA} and the activity of the fusions tested in wild-type and *sgrR* mutant backgrounds (Fig. 6C). The activity of the *ypdA* promoter was low and was similar in wild-type and *sgrR* mutant hosts in the absence and presence of αMG, suggesting that *ypdA* is not regulated by SgrR. In contrast, in the wild-type strain background, the basal level of activity of P_{yfdZ}-*lacZ* was high, and it was further increased by approximately twofold in the presence of αMG. In the *sgrR* mutant strain, basal levels of P_{yfdZ} activity were slightly lower, and αMG addition did not result in a significant activation (Fig. 6C). These data suggest that *yfdZ* is a member of the SgrR regulon and imply that the YfdZ protein may play a role in glucose-phosphate stress.

(iii) The *mlc* genomic region. A single isolate of the third type of clone identified in this screen contained the *mlc* genomic region (plasmid pBRlib8) (Fig. 7A). For this class of clones, the effect on P_{sgrS}-*lacZ* was analyzed using MacConkey lactose indicator plates; the strain carrying the vector control was red (Lac^+), while the strain carrying pBRlib8 was white (Lac^-). *mlc* encodes a regulator that represses expression of *ptsG* (and several other genes encoding sugar transport proteins) when cells are grown in the absence of glucose (14). In the presence of glucose, Mlc derepression at the *ptsG* promoter results in higher levels of *ptsG* transcription (5, 13). A portion of pBRlib8 containing only the *mlc* coding sequences was subcloned to determine if the *mlc* gene itself was active for repression of P_{sgrS}-*lacZ* activity. Plasmid pBRCV10 (containing *mlc* alone) repressed activity of P_{sgrS}-*lacZ* as well as the parental clone pBRlib8 (Fig. 7A), indicating that *mlc* sequences were responsible for the effect. Since Mlc acts as a repressor of *ptsG* transcription, overproduction of Mlc might repress *ptsG* expression to the extent that there is insufficient $EIICB^{Glc}$ protein for transport of the inducer αMG. To test this hypothesis, levels of $EIICB^{Glc}$ (PtsG) were measured by Western blotting in strains carrying the vector control or *mlc* (pBRCV10) plasmid. This analysis showed that cells carrying the *mlc* plasmid had reduced levels of $EIICB^{Glc}$ compared with cells carrying the vector control (Fig. 7B, P_{ptsG}-*ptsG*). This result was consistent with our hypothesis that Mlc overproduction downregulated P_{sgrS}-*lacZ* expression by reducing the amount of $EIICB^{Glc}$ and thus presumably the amount of αMG brought into the cells. To further test this hypothesis, an allele of *ptsG* under the control of the constitutive P_{bla} promoter (not susceptible to repression by Mlc) was moved to the P_{sgrS}-*lacZ* strain, and the *mlc* clone pBRCV10 was tested in this background. As expected, Western blots showed that in the P_{bla}-*ptsG* background, the *mlc* plasmid pBRCV10 no longer affected levels of $EIICB^{Glc}$ (Fig. 7B), since the P_{bla} promoter

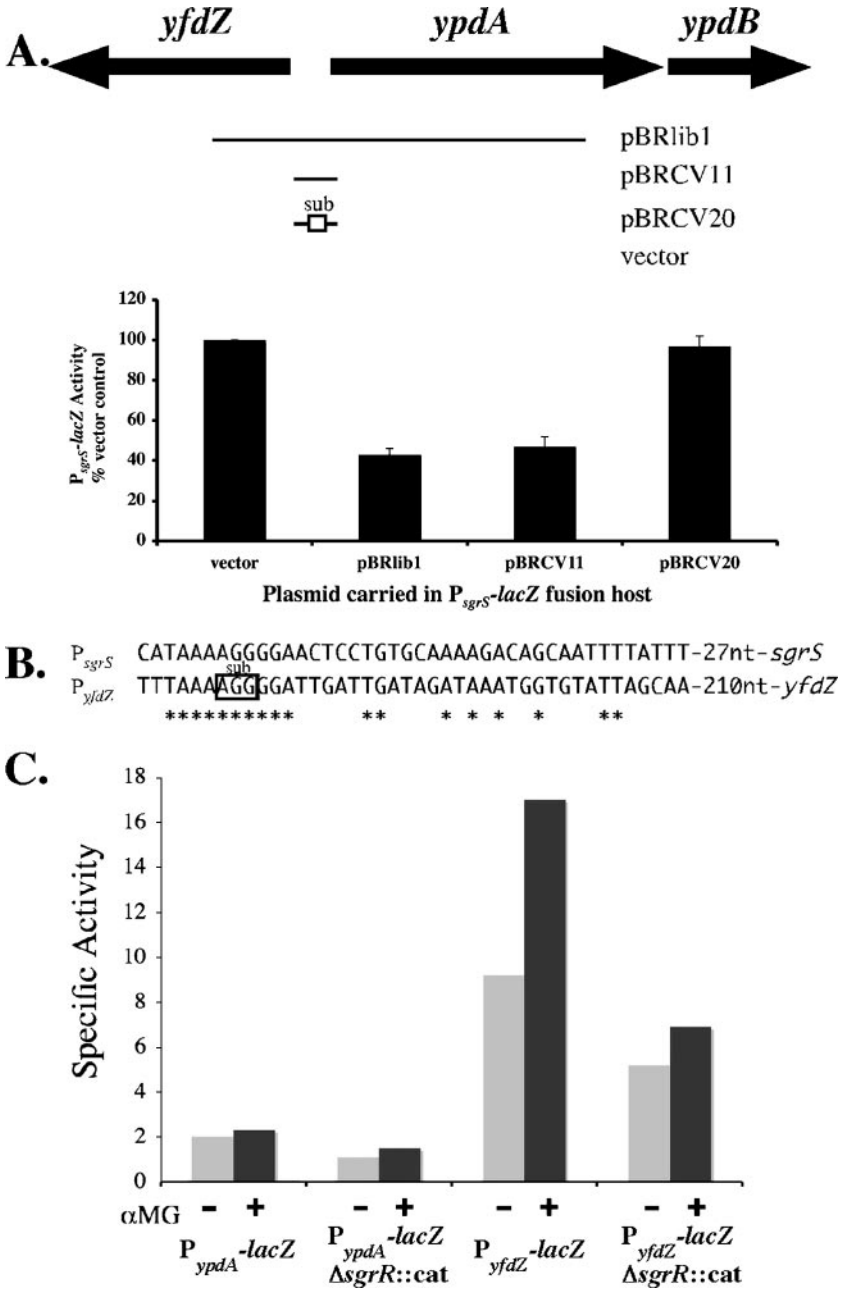

FIG. 6. Titration of endogenous SgrR by DNA fragments from the *yfdZ-ypdA* region. A. The relative sizes and directions of transcription of the *yfdZ* and *ypdA* genes are indicated by the gene map at top. The limits of titrating clones and location of a 3-bp substitution mutation are indicated as described for Fig. 5. The host strain carrying plasmid clones and relative levels of β-galactosidase activity are as described for Fig. 5. B. Alignment of the P_{sgrS} and P_{yfdZ} sequences. Conserved residues are indicated by asterisks below the sequence alignment. The position of the 3-bp substitution mutation constructed in plasmid pBRCV20 is indicated by the box. "5′-AGG-3′" was changed to "5′-TCC-3′" by QuikChange mutagenesis as described in Materials and Methods. C. Cells were cultured and β-galactosidase assays were performed as described for Fig. 2A. Host strains carried P_{ypdA}-*lacZ* (LW1000 and LW1010 [Table 1]) or P_{yfdZ}-*lacZ* (LW2000 and LW2010 [Table 1]) transcriptional fusions in the wild-type or Δ*sgrR*::cat backgrounds, as indicated. Gray and black bars represent activities of cultures without and with αMG, respectively.

cannot be repressed by Mlc. However, surprisingly, in the P_{bla}-*ptsG* strain, the *mlc* plasmid pBRCV10 still downregulated the P_{sgrS}-*lacZ* fusion (Fig. 7B). These data suggest that even though Mlc overproduction does reduce levels of $EIICB^{Glc}$ protein, this effect does not account for the phenotype of reduction of P_{sgrS}-*lacZ* activity.

These results prompted us to test one additional hypothesis. If *mlc* were a member of the SgrR regulon, *mlc* clones might affect P_{sgrS}-*lacZ* by an SgrR titration mechanism. However, deletion analyses showed that removal of segments from the 3′ end of *mlc* abrogated the negative effect on P_{sgrS}-*lacZ* (Fig. 7A, plasmid pBRCV21), suggesting that the whole *mlc* coding sequence was required for downregulation of P_{sgrS}-*lacZ*. While an alignment of P_{mlc} with P_{sgrS} showed some conservation (Fig.

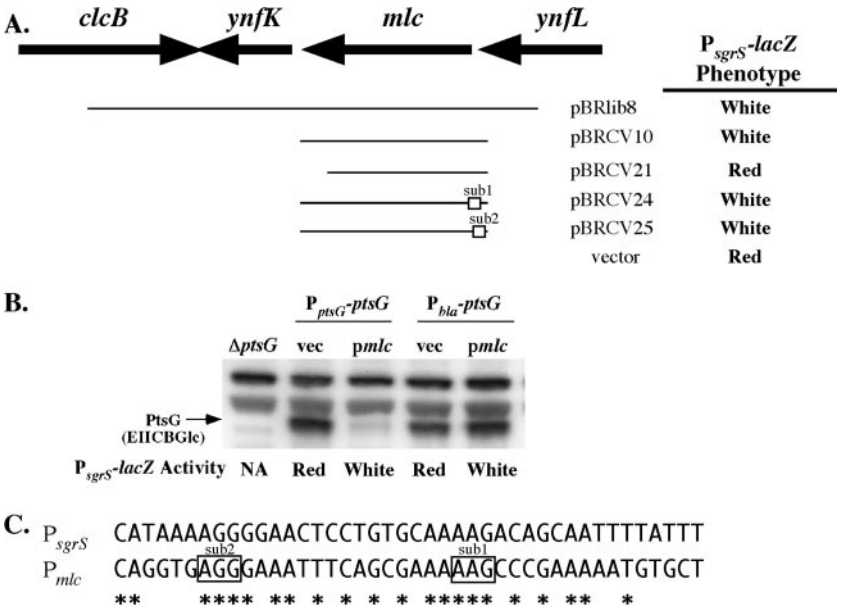

FIG. 7. Mlc affects activation of *sgrS* by an undefined mechanism. A. The relative sizes and directions of transcription of the *clcB*, *ynfK*, *mlc*, and *ynfL* genes are indicated by the gene map at top. The limits of clones and the location of 3-bp substitution mutations are indicated as described for Fig. 5. The host strain carrying the clones is CV5200 (Table 1), which contains the P_{sgrS}-*lacZ* fusion. Cells were streaked on MacConkey lactose indicator plates containing αMG at 0.05%; cells were grown for 18 h and the Lac phenotype observed. The vector control-containing cells were red, or Lac^+, since αMG strongly induces P_{sgrS}-*lacZ* expression. Strains carrying plasmids that downregulated the fusion had a Lac^- phenotype (white). B. Western blotting for $EIICB^{Glc}$ (PtsG) protein was performed as described in Materials and Methods. The Δ*ptsG*::cat strain (CV105) lacks $EIICB^{Glc}$ and is included for comparison. The two bands above the PtsG band are proteins that cross-react with the αIIB^{Glc} antibody and were used as loading controls. The wild-type host carries P_{sgrS}-*lacZ* and *ptsG* under the control of the native promoter elements (CV5200). Strain CV5282 also has P_{sgrS}-*lacZ*, but *ptsG* is under the control of the constitutive P_{bla} promoter. The p*mlc* plasmid is pBRCV10 as described in panel A. Relative levels of activity from P_{sgrS}-*lacZ* for each strain are indicated below the Western blot. C. Alignment of the P_{sgrS} and P_{mlc} sequences. Conserved residues are indicated by asterisks below the sequence alignment. The positions of the 3-bp substitution mutations constructed in plasmids pBRCV24 and pBRCV25 are indicated by boxes. For sub1, "5′-AAG-3′" was changed to "5′-TTC-3′"; for sub2, "5′-AGG-3′" was changed to "5′-TCC-3′" by QuikChange mutagenesis as described in Materials and Methods.

7C), the pattern of conservation was different than that between P_{sgrS} and P_{yfdZ}. Substitution mutations in these conserved sequences in P_{mlc} did not alter the repressive effect of the *mlc* clones (plasmids pBRCV24 and pBRCV25 in Fig. 7A). Taken together, these data suggest that *mlc* affects activity of P_{sgrS}-*lacZ* by a mechanism independent of repression of *ptsG* and probably not involving titration of the SgrR protein.

DISCUSSION

The small RNA SgrS and the transcription factor that controls *sgrS* expression, SgrR, are both required for recovery from glucose-phosphate stress (21). In this study, we focused on further characterization of SgrR, which we propose is the sensor and regulator of the glucose-phosphate stress response. SgrR is the first characterized member of a previously unstudied family of proteins that have been annotated primarily as solute binding proteins or periplasmic transport proteins due to their conserved C-terminal solute binding domains. However, as we observed previously (21), SgrR family members also contain N-terminal DNA binding domains, suggesting that these proteins are instead transcription factors. As predicted by the presence of this domain, we find that SgrR binds to a specific DNA sequence required for transcriptional regulation of *sgrS* (Fig. 4). Regulation of *sgrS* transcription is coupled to the regulation of the divergent *sgrR* gene. Based on the arrangement of the *sgrR* and *sgrS* coding sequences, we proposed that SgrR binding to P_{sgrS} sequences for activation may have a negative effect on *sgrR* expression (21). Consistent with this prediction, we found that SgrR negatively autoregulates its own transcription (Fig. 2A and B) under both stress and nonstress conditions. The SgrR binding site in the *sgrR-sgrS* intergenic region (Fig. 1) is required for both activation of *sgrS* and autorepression of *sgrR* (Fig. 3). An additional target of SgrR activation, *yfdZ*, was also identified; the *yfdZ* promoter region contains a regulatory sequence similar to the one in the *sgrR-sgrS* intergenic region (Fig. 6B).

SgrR activities: activation versus repression. While the sequences in the intergenic region are sufficient for SgrR-dependent activation of *sgrS* (21) (Fig. 3), other experiments suggest that additional sequences are necessary for the tight binding that allows titration of SgrR in vivo (Fig. 5). Full titration required additional sequences located within the *sgrR* open reading frame (Fig. 5). The analyses of P_{sgrR}- and P_{sgrS}-*lacZ* fusions indicate that while SgrR binding determinants located in the *sgrR* coding sequence are not necessary for activation of *sgrS*, they are important for full autoregulation of *sgrR* (Fig. 2A and 3). The strong titration of SgrR by a plasmid containing the full *sgrR* region may explain why plasmid-borne *sgrR* under the control of its own promoter (pP_{sgrR}-*sgrR*) fails to complement an *sgrR* mutation, as we observed previously (21). Indeed, when the 5-bp substitution mutation was moved to the pP_{sgrR}-

sgrR construct, these plasmids fully complemented the *sgrR* mutant for activation of P_{sgrS}-*lacZ* and rescued from glucose-phosphate stress (data not shown). These observations suggest that negative autoregulation is an important mechanism for maintaining SgrR at limiting concentrations.

SgrR synthesis appears to be controlled at several levels. Endogenous SgrR could not be detected by Western blotting using a polyclonal antibody raised against SgrR-His (data not shown), indicating that cellular levels of SgrR are quite low. The use of in vivo titration of SgrR to identify new SgrR binding sites in the plasmid DNA library implies that SgrR concentrations are normally limiting (Fig. 5 and 6). Negative autoregulation of *sgrR* at the transcriptional level was demonstrated in this study (Fig. 2 and 3) and likely provides one measure of control over SgrR at the level of synthesis. However, this is almost certainly not the only mechanism for control of SgrR amounts, since mutation of the sequences required for negative autoregulation (5-bp substitution as shown in Fig. 1) did not allow accumulation of SgrR to detectable levels (data not shown). SgrR levels may also be modulated by translational regulation or regulation of stability by mechanisms that have not yet been discovered. Why the regulation of this protein is so critical is not clear, but we have been unable to clone *sgrR* except on a low-copy plasmid under repressing conditions, suggesting that overproduction of SgrR may be toxic.

The SgrR regulon. The function thus far defined for SgrR is relief of the stress associated with accumulation of sugar-phosphates. *sgrR* mutants are more sensitive to growth inhibition during stress (21). With respect to the stress response, *sgrS* is likely to be the most important target for SgrR, since ectopic production of SgrS in an *sgrR* mutant mostly suppresses the sensitivity to sugar-phosphate stress (21). A genomic library screen for plasmids that diminished activity of P_{sgrS}-*lacZ* under inducing conditions identified five independent plasmids carrying the *sgrR-sgrS* region. These were found to interfere with P_{sgrS} activation by an SgrR titration mechanism. Two additional classes of interfering plasmids were identified. One class (comprised of four independent isolates) had in common the intergenic region between the *ypdA* and *yfdZ* genes. These clones have now also been shown to function through a titration mechanism. A putative SgrR binding site was identified by sequence comparison with P_{sgrS} (Fig. 6B), and a substitution mutation in these sequences abrogated the titration (Fig. 6A). Analysis of fusions indicated that the *yfdZ* gene is positively regulated by SgrR (Fig. 6C). Unlike *sgrS*, the other known target for positive regulation by SgrR, transcription of the *yfdZ* promoter has a significant SgrR-independent component (Fig. 3 and 6C). This suggests that *yfdZ* expression may be controlled by two promoters, one SgrR dependent and one SgrR independent. YfdZ is a protein of unknown function but is predicted to belong to the class I pyridoxal phosphate-dependent aminotransferase family, alanine aminotransferase subfamily (UniProtKB/Swiss-Prot family/domain classification). Enzymes of this family are involved in alanine biosynthesis and catalyze the following reaction: pyruvate + glutamate ↔ L-alanine + α-ketoglutarate. It is currently unclear how alanine biosynthesis may be tied to glucose-phosphate stress. However, pyruvate, a putative substrate for YfdZ, is a glycolytic intermediate downstream of the steps that seem to be involved in generation of the stress. Kimata et al. found that addition of pyruvate to stressed cells that had accumulated intracellular G6P restored the stability of the *ptsG* mRNA (6). Perhaps SgrR-dependent induction of *yfdZ* under glucose-phosphate stress conditions works to alter flux in the lower part of the glycolytic pathway by affecting pyruvate levels and this helps in the adaptation to stress. Minimally, these results demonstrate that, in addition to *sgrS*, at least one unlinked gene is activated by SgrR in response to sugar-phosphate stress.

The third class of clones that affected P_{sgrS}-*lacZ* activity contained the *mlc* gene. The mechanism by which *mlc* clones affect induction of *sgrS* is currently unclear. Deletion analyses and site-directed mutagenesis of putative regulatory sequences suggest that the Mlc protein itself is required and that these clones are not functioning through an SgrR titration mechanism. Mlc is a DNA binding regulatory protein that represses transcription of its target genes, including *ptsG*, in the absence of glucose (14). While we found that overproduction of Mlc reduced levels of the glucose transporter $EIICB^{Glc}$, this did not fully account for the Mlc-dependent downregulation of *sgrS*. In future studies we will investigate whether Mlc overproduction negatively affects the function of $EIICB^{Glc}$ or whether it might affect the *sgrS* promoter by direct repression. Another possibility is that Mlc may act through another, uncharacterized target that affects metabolic flux or stress signaling.

The mRNA targets of SgrS, including *ptsG*, can also be considered part of the SgrR regulon, albeit indirectly (via SgrS regulation). We conducted preliminary microarray studies that suggested that SgrS has a limited set of target mRNAs. In these studies, only three candidates were identified as potential SgrS targets. In addition to the *ptsG* mRNA, we found that two other messages encoding phosphotransferase system sugar transporters, *manXYZ* and *fruBKA* mRNAs, were downregulated by SgrS (C. K. Vanderpool and S. Gottesman, unpublished data). These targets fit with the hypothesized physiological role of SgrS in stopping synthesis of sugar transport proteins whose substrates might contribute to sugar-phosphate stress. It is possible that other SgrS targets could not be identified by microarray analysis, since this method requires that the target message be expressed at a detectable level under the culture conditions used and that there is a difference in the abundance of target messages in the absence versus presence of SgrS. If SgrS base-pairs with other targets and inhibits their translation without affecting their stability, as is the case for Spot 42, another small RNA that controls expression of genes involved in sugar metabolism (9), such targets would not have been detected by this approach.

While *sgrS* transcription is activated by SgrR only in the presence of a stress signal (21), repression of *sgrR* by SgrR occurs independently of stress (Fig. 2 and 3). These observations, coupled with our finding that SgrR binds in vitro to a specific DNA fragment without the addition of exogenous small molecules, suggests that SgrR DNA binding is independent of signal. It may be instead that the specific characteristics of the binding change depending upon the presence of the signal. If this was the case, SgrR would be similar in some respects to the LysR family of proteins, which autoregulate their own expression and often the expression of divergent target genes and bind to DNA sites with and without signal (15). We currently do not know the identity of the putative

small-molecule signal that is bound by SgrR and modulates its activity. The regulated degradation of *ptsG* mRNA, which we now know to be caused by SgrS, is correlated with intracellular accumulation of G6P or fructose-6-phosphate (F6P) (10), suggesting that SgrR might directly bind G6P or F6P when levels increase past a certain threshold. SgrR with bound sugar-phosphate would then activate transcription of *sgrS*. We found in this study that addition of G6P to binding reactions did not alter the affinity or pattern of binding of SgrR to *sgrS* promoter DNA; however, this does not rule out G6P as the signaling molecule. One line of evidence suggesting that the signal may be something other than G6P or F6P is provided by experiments performed by Kimata et al., who showed that even in *pgi* mutant cells growing on glucose (where intracellular levels of G6P are high), the *ptsG* mRNA can be stabilized by addition of glycolytic intermediates downstream of the block (6). It therefore remains possible that SgrR senses another small molecule that accumulates or is depleted under glucose-phosphate stress conditions.

SgrR defines a new family of proteins, named COG4533 by NCBI (18), that may all be controlled by small-molecule metabolic signals. This family includes one other member in *E. coli*, YbaE, and members in both gram-positive bacteria (bacilli and listeriae) and other gram-negative bacteria. In some genomes, the genes encoding SgrR family members are divergent from genes predicted to be involved in transport processes. In these cases, we predict that the divergent gene is a target of regulation and that a small molecule is the regulatory effector. SgrR may be somewhat unique in that its primary regulatory target is a small RNA. It joins only two other examples, OxyR/OxyS (1) and GcvA/GcvB (20), where a transcriptional regulator is divergently transcribed from the small RNA it regulates. However, as more small RNAs are characterized, we may find additional cases of coupled regulation of divergently encoded small RNAs and their transcriptional regulators.

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