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Stringent Response Regulators Contribute to Recovery from Glucose Phosphate Stress in *Escherichia coli*

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ABSTRACT In enteric bacteria such as *Escherichia coli*, the transcription factor SgrR and the small RNA SgrS regulate the response to glucose phosphate stress, a metabolic dysfunction that results in growth inhibition and stems from the intracellular accumulation of sugar phosphates. SgrR activates the transcription of *sgrS*, and SgrS helps to rescue cells from stress in part by inhibiting the uptake of stressor sugar phosphates. While the regulatory targets of this stress response are well described, less is known about how the SgrR-SgrS response itself is regulated. To further characterize the regulation of the glucose phosphate stress response, we screened global regulator gene mutants for growth changes during glucose phosphate stress. We found that deleting *dksA*, which encodes a regulator of the stringent response to nutrient starvation, decreases growth under glucose phosphate stress conditions. The stringent response alarmone regulator ppGpp (synthesized by RelA and SpoT) also contributes to recovery from glucose phosphate stress: as with *dksA*, mutating *relA* and *spoT* worsens the growth defect of an *sgrS* mutant during stress, although the *sgrS relA spoT* mutant defect was only detectable under lower stress levels. In addition, mutating *dksA* or *relA* and *spoT* lowers *sgrS* expression (as measured with a P_{sgrS}-*lacZ* fusion), suggesting that the observed growth defects may be due to decreased induction of the glucose phosphate stress response or related targets. This regulatory effect could occur through altered *sgrR* transcription, as *dksA* and *relA spoT* mutants also exhibit decreased expression of a P_{sgrR}-*lacZ* fusion. Taken together, this work supports a role for stringent response regulators in aiding the recovery from glucose phosphate stress.

IMPORTANCE Glucose phosphate stress leads to growth inhibition in bacteria such as *Escherichia coli* when certain sugar phosphates accumulate in the cell. The transcription factor SgrR and the small RNA SgrS alleviate this stress in part by preventing further sugar phosphate transport. While the regulatory mechanisms of this response have been characterized, the regulation of the SgrR-SgrS response itself is not as well understood. Here, we describe a role for stringent response regulators DksA and ppGpp in the response to glucose phosphate stress. *sgrS dksA* and *sgrS relA spoT* mutants exhibit growth defects under glucose phosphate stress conditions. These defects may be due to a decrease in stress response induction, as deleting *dksA* or *relA* and *spoT* also results in decreased expression of *sgrS* and *sgrR*. This research presents one of the first regulatory effects on the glucose phosphate stress response outside SgrR and SgrS and depicts a novel connection between these two metabolic stress responses.

KEYWORDS DksA, RelA, SgrS, SpoT, glucose phosphate stress, ppGpp, stringent response

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All organisms must coordinate the regulation of metabolic functions in response to myriad fluctuations in nutrient availability, and disruption of this balance can lead to stress. For example, enteric bacteria such as *Escherichia coli* experience stress in the

form of growth inhibition when sugar phosphates such as glucose-6-phosphate accumulate in the cell due to a block in glycolysis (1–5). This metabolic imbalance, termed glucose phosphate stress, can be induced either by glucose analogs such as α-methyl glucoside (αMG) (which is transported into the cell but cannot be metabolized) (5–7) or by mutations in glycolytic genes such as *pgi*, encoding phosphoglucose isomerase (which results in the accumulation of glucose-6-phosphate) (8, 9). While the cause of glucose phosphate stress is not entirely understood, current evidence supports the notion that the depletion of metabolic intermediates resulting from these blocks in glycolysis contributes to stress (4, 8–11). For example, the introduction of glycolytic intermediates such as glucose-6-phosphate can rescue the αMG-induced growth defect (4).

In *E. coli*, the transcription factor SgrR and the small RNA (sRNA) SgrS regulate the response to glucose phosphate stress (5, 12–15). Both regulators are required for the ability to recover from stress, as *sgrR* and *sgrS* mutants exhibit severe stress-related growth defects (5, 12). Under stress conditions, SgrR rapidly (within 2 min of αMG exposure [5]) activates the transcription of *sgrS*, which encodes the primary effector of the glucose phosphate stress response (5, 15). SgrS is an Hfq chaperone-binding sRNA (5, 16) that regulates the translation of multiple mRNA targets through specific base-pairing interactions (3, 5, 10, 12, 17, 18). On the basis of the encoded functions of characterized targets, SgrS alleviates stress and helps restore growth in two main ways. First, it stops the transport and intracellular accumulation of stressor sugar phosphates in part by inhibiting the translation of *ptsG* and *manXYZ* mRNAs, which encode phosphoenolpyruvate (PEP) phosphotransferase system (PTS) transporters of glucose and related sugars (3, 5, 12, 18, 19). SgrS exerts a positive stabilizing effect on the translation of a third transcript, *yigL*, which encodes a sugar phosphatase that is thought to help dispatch accumulated sugar phosphates (20). Second, four recently characterized targets suggest an additional role of SgrS in bypassing the stress-induced glycolytic block and related depletion of metabolic intermediates (10). These targets encode diverse metabolism-related enzymatic and regulatory functions and include *adiY* (encoding an arginine decarboxylase gene activator), *asd* (encoding aspartate semialdehyde dehydrogenase), *folE* (encoding GTP cyclohydrolase I), and *purR* (encoding a repressor of purine synthesis). SgrS represses the translation of all four transcripts through varied mechanisms, and the overexpression of these targets adversely affects growth during glucose phosphate stress, particularly in mutants defective in producing the glycolytic intermediate PEP (10). PEP depletion is thought to contribute to glucose phosphate stress (4, 11, 21); therefore, it has been posited that SgrS regulation of these targets prevents the depletion of PEP or related intermediates and/or helps reroute metabolism around the glycolytic block (10).

While the regulatory mechanisms of the glucose phosphate stress response are thus well characterized, little is known about how the SgrR-SgrS stress response itself is regulated or whether it relates to other types of metabolic stress (13, 14, 17). To begin to identify other regulatory connections to the glucose phosphate stress response, we screened strains with insertion-deletion mutations in genes encoding global regulators of metabolism and/or related stress responses for changes in growth during glucose phosphate stress. Here, we describe a novel role for the transcription factor DksA and the nucleotide "alarmones" guanosine tetraphosphate and guanosine pentaphosphate (collectively abbreviated as ppGpp), global regulators of the stringent response to nutrient starvation (22–26), in aiding the recovery from glucose phosphate stress. The stringent response involves changes in the expression of hundreds of genes (27–29) and is induced under a variety of nutrient-limiting conditions, including amino acid and carbon starvation as well as phosphate and iron limitation (22, 23, 30, 31). ppGpp is synthesized by the enzymes RelA (22, 32, 33) and SpoT, the latter of which also has hydrolase activity (22, 34). ppGpp is produced by RelA in response to amino acid starvation and by SpoT in response to carbon starvation and other nutrient limitations (22, 23). Both DksA and ppGpp affect transcription through direct interactions with RNA polymerase (22, 24, 25, 35, 36). DksA and ppGpp have distinct and overlapping regulons

(27–29), but a major function of the stringent response is to decrease protein synthesis and increase biosynthesis and therefore conserve energy during nutrient starvation (22).

Previous observations also imply a connection between the stringent and glucose phosphate stress responses. The stringent response to carbon starvation has similarities to the metabolic block that induces glucose phosphate stress; for example, both stress responses can be induced by αMG (5, 22, 37, 38). In addition, glucose phosphate stress can be induced by mutations in glycolytic genes, including *fda* (which encodes fructose-1,6-diphosphate aldolase) (9), and mutating *fda* also increases ppGpp production (39). In this study, we present genetic evidence implicating the stringent response regulators DksA and ppGpp in the recovery from glucose phosphate stress. We demonstrate that deleting *dksA* or *relA* and *spoT* worsens the growth defect of an *sgrS* mutant under glucose phosphate stress conditions. *dksA* and *relA spoT* mutants also exhibit decreased expression of *sgrS* and *sgrR*, indicating that the stringent regulators could contribute to the induction of the glucose phosphate stress response as well as independent regulation of related downstream targets. These findings represent one of the first regulatory connections to the glucose phosphate stress response beyond SgrR and SgrS, and we also discuss the physiological implications of the interaction between these two metabolic stress responses.

RESULTS

Screen of global regulator mutants for changes in growth during glucose phosphate stress. To identify potential regulatory connections to the glucose phosphate stress response, we screened mutants lacking global regulator genes for changes in growth during glucose phosphate stress. We prioritized the regulators of functions related to glucose phosphate stress, such as metabolic stress responses and carbon, amino acid, and phosphate metabolism. These regulators include the stringent response regulators DksA and ppGpp; CreB, a two-component response regulator of carbon source usage (40, 41); cyclic AMP receptor protein (CRP), a transcriptional regulator of catabolite repression and catabolism of diverse carbon sources (42); the nucleoid-associated protein H-NS, which regulates genes involved in transcription, translation, and adaptation to a variety of stresses (43, 44); leucine-responsive protein (Lrp), a regulator of amino acid metabolism, nutrient transport and limitation, and pilus synthesis (45–47); and RpoS (σ^S), the general stress response and stationary-phase sigma factor (48). To determine if these regulators affect the recovery from glucose phosphate stress, insertion-deletion gene mutations were introduced into wild-type and Δ*sgrS* mutant *E. coli* backgrounds that were assessed for growth changes (based on colony size compared to that of the parent strains) during αMG-induced stress on rich and minimal media (Table 1). The Δ*sgrS* mutant background was examined because the absence of this major effector of the glucose phosphate stress response has previously been shown to reveal subtler phenotypes of genes involved in the recovery from glucose phosphate stress (4, 49–51). A Δ*relA* Δ*spoT* mutant (also termed ppGpp^0) was included because it completely lacks the ability to synthesize ppGpp (34, 52, 53). (Because Δ*spoT* mutations are lethal in the presence of *relA* due to unchecked ppGpp accumulation [22], a Δ*spoT* mutant was not examined.) Of the regulator mutants tested, only the deletion of *dksA* resulted in a stress-specific decrease in growth compared with that of the *sgrS* parent on the rich medium (Table 1; Fig. 1, left plate).

Deleting *dksA* worsens the growth defect of an *sgrS* mutant during glucose phosphate stress. To further characterize the effect of deleting *dksA* on glucose phosphate stress, the growth of the *dksA* and *sgrS dksA* mutants (as well as their respective wild-type and *sgrS* parent strains) was monitored during αMG-induced stress in liquid culture. Consistent with the defect on solid medium, the *sgrS dksA* mutant exhibited decreased growth compared with that of the parent *sgrS* strain under stress conditions in liquid medium (Fig. 2A). An *sgrR dksA* deletion mutant exhibited a similar defect during stress (Fig. 2B), which is consistent with the role of SgrR in activating *sgrS* transcription during glucose phosphate stress (15). There was also a slight but repro-

TABLE 1 Growth of regulator mutants during glucose phosphate stress

Strain[a]	Growth on[b]:			
	LB[c]	LB+αMG[c]	M63[d]	M63+αMG[d]
Wild type	+	+	+	+
Δ*creB*	+	+	+	+
Δ*crp*	+	+	+	+/−
Δ*dksA*	+	+	−	−
Δ*hns*	+/−	+/−	−	−
Δ*lrp*	+	+	+	+/−
Δ*relA*	+	+	+	+
Δ*relA* Δ*spoT*	+	+	−	−
Δ*rpoS*	+	+	+	+
Δ*sgrS*	+	+/−	+	−
Δ*sgrS* Δ*creB*	+	+/−	+	−
Δ*sgrS* Δ*crp*	+	+/−	+	−
Δ*sgrS* Δ*dksA*	+	−	−	−
Δ*sgrS* Δ*hns*	+/−	+/−	−	−
Δ*sgrS* Δ*lrp*	+	+/−	+	−
Δ*sgrS* Δ*relA*	+	+/−	+	−
Δ*sgrS* Δ*relA* Δ*spoT*	+	+/−	−	−
Δ*sgrS* Δ*rpoS*	+	+/−	+	−

[a]Strains with indicated mutations in the wild-type (DJ480) and Δ*sgrS* (CS104) backgrounds were examined.
[b]The growth of the indicated strains on described media was determined by qualitative evaluation of bacterial colony size compared with that of parent strains as follows: +, size was indistinguishable from that of the wild-type control; +/−, size was reduced compared to that of wild type; −, growth was strongly inhibited.
[c]Colony size 24 h after inoculation on solid LB medium with or without 0.5% αMG to induce glucose phosphate stress.
[d]Colony size 48 h after inoculation on solid M63 plus 0.2% fructose minimal medium with or without 0.5% αMG to induce glucose phosphate stress.

ducible stress-specific growth defect in the *dksA* mutant compared to its wild-type parent strain (Fig. 2) that was not detectable on solid medium (Table 1).

To confirm the causality of the *dksA* mutation for the observed growth defect, a wild-type copy of *dksA* ectopically expressed from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{lac} promoter in plasmid pCA24N (54) was introduced into the wild-type and *sgrS*, *dksA*, and *sgrS dksA* mutant strains. During growth with αMG to induce stress, expression of P_{lac}-*dksA* restored the growth of the *sgrS dksA* mutant to *sgrS* parent levels (Fig. 1, right plate), verifying that the *dksA* mutation is responsible for the growth defect of the *sgrS dksA* mutant during glucose phosphate stress. As a control, the *sgrS dksA* mutant carrying the pCA24N lacking *dksA* exhibited the same αMG-induced growth defect observed previously (Fig. 1, left plate). Taken together, these results indicate that *dksA* contributes to the recovery from glucose phosphate stress.

Deleting *relA* and *spoT* worsens the growth defect of an *sgrS* mutant under low-stress conditions. Since DksA and ppGpp both regulate the stringent response,

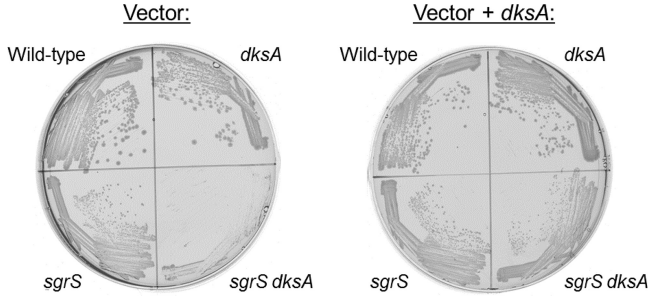

FIG 1 Complementation of the *sgrS dksA* mutant glucose phosphate stress growth defect. Growth of wild-type (DJ480), Δ*sgrS* (CS104), Δ*dksA* (GR128), and Δ*sgrS* Δ*dksA* (GR200) strains carrying either vector pCA24N (left plate, negative control) or pCA24N with a wild-type copy of *dksA* under the control of the P_{lac} promoter (right plate). Strains were grown for 24 h on solid LB medium containing 0.2 mM IPTG to induce P_{lac} expression and 0.5% αMG to induce stress.

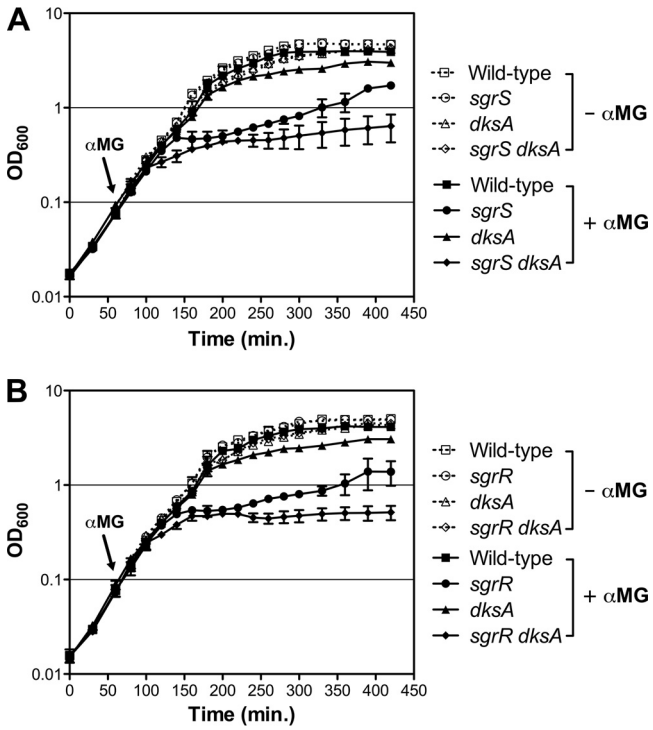

FIG 2 Deleting *dksA* worsens the glucose phosphate growth defect of *sgr* mutants. (A) Wild-type (DJ480), Δ*sgrS* (CS104), Δ*dksA* (GR128), and Δ*sgrS* Δ*dksA* (GR200) strains or (B) wild-type (DJ480), Δ*sgrR* (CV700), Δ*dksA* (GR128), and Δ*sgrR* Δ*dksA* (GR129) strains were grown in liquid LB medium in the absence or presence of 0.5% αMG to induce glucose phosphate stress. αMG was added to the indicated cultures at an OD_{600} of approximately 0.1 (arrows), and OD_{600} was monitored over time. Error bars indicate standard deviations ($n = 3$).

we further investigated the effect of deleting *relA* and *spoT* on growth during glucose phosphate stress. Although they did not exhibit decreased colony size on solid medium (Table 1), it is possible the *relA spoT* ($ppGpp^0$) mutants have a subtler growth defect during glucose phosphate stress. We thus assessed the growth in liquid culture of *relA spoT* and *sgrS relA spoT* mutants compared with that of the wild-type and *sgrS* parent strains in the presence and absence of 0.5% αMG to induce stress. Consistent with their growth on solid medium (Table 1), the *relA spoT* mutants did not exhibit growth defects during stress compared to the parent strains (Fig. 3A). Similar results were observed in an *sgrR* mutant background (Fig. 3B).

It is possible that the already considerable growth defect of the *sgrS* mutant under conditions of high (0.5% αMG) glucose phosphate stress could obfuscate any effect of the *relA* and *spoT* mutations. To more clearly ascertain whether there is an observable growth difference between the *sgrS relA spoT* mutant and its parent, we examined growth under low-stress (0.01% αMG) conditions, which allows for improved but still defective growth of the *sgrS* parent. At low-stress levels, the *sgrS relA spoT* mutant did display decreased growth compared with that of the *sgrS* parent (Fig. 4A). This growth defect was also observed in an *sgrR* mutant background (Fig. 4B) and on solid medium under low-stress conditions (Fig. 5, left plate). To verify that the inability to synthesize ppGpp is responsible for this growth defect, a wild-type copy of *spoT* was ectopically expressed from an IPTG-inducible P_{lac} promoter in plasmid pCA24N (54) and introduced into wild-type and *sgrS*, *relA spoT*, and *sgrS relA spoT* mutant strains. During growth under low-glucose-phosphate-stress conditions, the expression of P_{lac}-*spoT* restored the growth of the *sgrS relA spoT* mutant to *sgrS* parent levels (Fig. 5, right plate), demonstrating that the *spoT* mutation is responsible for the observed growth defect. Overall, these results suggest that ppGpp (Fig. 4 and 5) affects the recovery from glucose phosphate stress in a manner similar to yet distinct from that of DksA (Fig. 1 and 2).

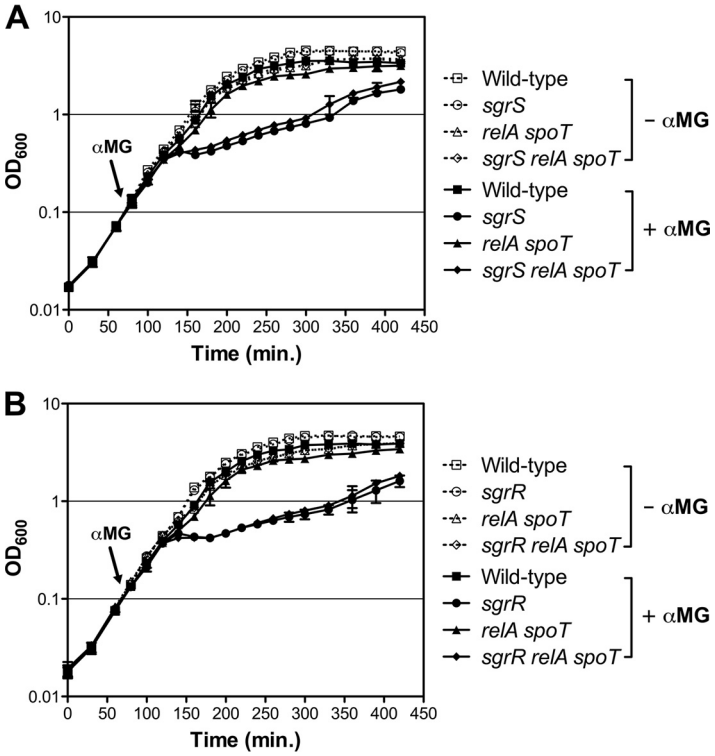

FIG 3 Growth of *relA spoT* mutants under high levels of glucose phosphate stress. (A) Wild-type (DJ480), Δ*sgrS* (CS104), Δ*relA* Δ*spoT* (GR184), and Δ*sgrS* Δ*relA* Δ*spoT* (GR202) strains or (B) wild-type (DJ480), Δ*sgrR* (JK120), Δ*relA* Δ*spoT* (GR247), and Δ*sgrR* Δ*relA* Δ*spoT* (GR249) strains were grown in liquid LB medium in the absence or presence of 0.5% αMG to induce glucose phosphate stress. αMG was added to the indicated cultures at an OD_{600} of approximately 0.1 (arrows), and OD_{600} was monitored over time. Error bars indicate standard deviations ($n = 3$).

Deleting *dksA* or *relA* and *spoT* decreases expression of the glucose phosphate stress response. Deletion of *dksA* (Fig. 1 and 2) or *relA* and *spoT* (Fig. 4 and 5) results in growth defects during glucose phosphate stress. Since both DksA and ppGpp primarily exert regulatory effects at the level of transcription (22, 24, 55), we hypothesized that the observed growth defects could be due to a decrease in transcriptional activation of the glucose phosphate stress response. To examine the effect of the stringent response regulators on the induction of the stress response, we monitored the expression of a chromosomal P_{sgrS}-*lacZ* transcriptional fusion (18, 49) introduced into wild-type and *dksA*, *sgrS*, and *sgrS dksA* mutant strains grown under αMG-induced stress. *sgrS* was chosen as a reporter because it is the major glucose phosphate stress response effector and is induced specifically in response to glucose phosphate stress (5). The expression of P_{sgrS}-*lacZ* was decreased in the *sgrS dksA* mutant compared with that in its *sgrS* parent strain (Fig. 6A). While P_{sgrS}-*lacZ* activity was slightly though reproducibly lower in the *dksA* mutant than in the wild-type parent in each individual experimental replicate, this result was not significantly different due to the variation between replicates (Fig. 6A). The P_{sgrS}-*lacZ* fusion also was introduced into *relA spoT* and *sgrS relA spoT* mutants, which exhibited decreased P_{sgrS}-*lacZ* expression compared with that of their respective parent strains (Fig. 6B). In both cases, the *sgrS* mutant strains exhibited higher levels of P_{sgrS}-*lacZ* induction than wild-type strains due to the absence of a functional glucose phosphate stress response, as observed previously (49, 50).

Because SgrR is known to directly activate *sgrS* expression (5, 15), the decreased *sgrS* expression observed in the stringent regulator mutants could occur via direct effects of DksA and ppGpp and/or via altered *sgrR* expression. To test this notion, we introduced a chromosomal P_{sgrR}-*lacZ* transcriptional fusion (15) into *dksA* and *relA spoT* mutants in both wild-type and *sgrR* mutant backgrounds and measured its activity during αMG-

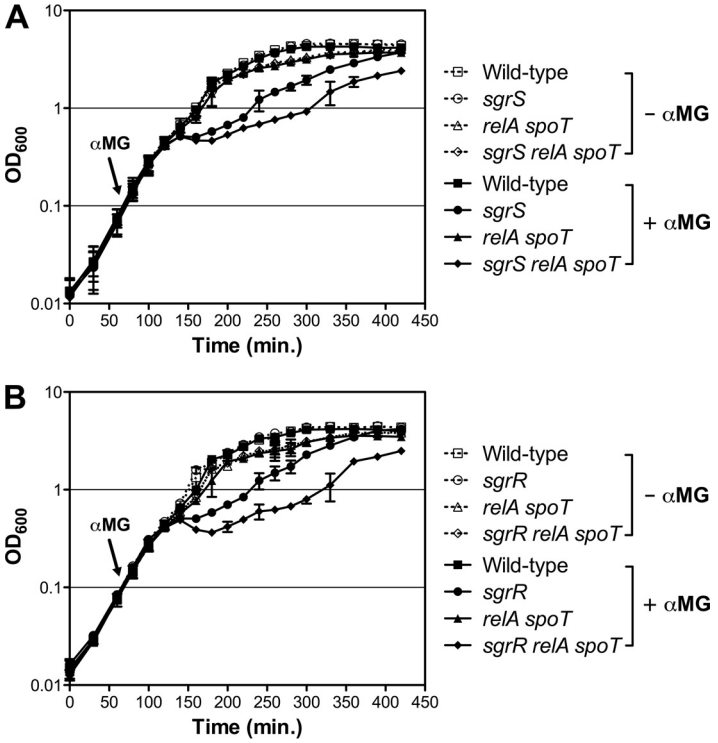

FIG 4 Deleting *relA* and *spoT* worsens the growth defect of *sgr* mutants under low levels of glucose phosphate stress. (A) Wild-type (DJ480), Δ*sgrS* (CS104), Δ*relA* Δ*spoT* (GR184), and Δ*sgrS* Δ*relA* Δ*spoT* (GR202) strains or (B) wild-type (DJ480), Δ*sgrR* (JK120), Δ*relA* Δ*spoT* (GR247), and Δ*sgrR* Δ*relA* Δ*spoT* (GR249) strains were grown in liquid LB medium in the absence or presence of 0.01% αMG to induce glucose phosphate stress. αMG was added to the indicated cultures at an OD_{600} of approximately 0.1 (arrows), and OD_{600} was monitored over time. Error bars indicate standard deviations ($n = 3$).

induced stress. In both the *dksA* and *sgrR dksA* mutants, P_{sgrR}-*lacZ* expression was decreased compared with that in their respective parent strains (Fig. 7A). The *relA spoT* and *sgrR relA spoT* mutants also exhibited similar decreases in P_{sgrR}-*lacZ* activity (Fig. 7B), indicating that the decreased *sgrS* expression observed in the stringent mutants (Fig. 6) is likely due at least in part to decreased *sgrR* expression (Fig. 7). The increased P_{sgrR}-*lacZ* expression in the *sgrR* strains compared with that in their wild-type counterparts has been observed previously and is due to the absence of SgrR autorepression (15). Altogether, these results indicate that the absence of either *dksA* or ppGpp decreases the expression of the glucose phosphate stress response, which could

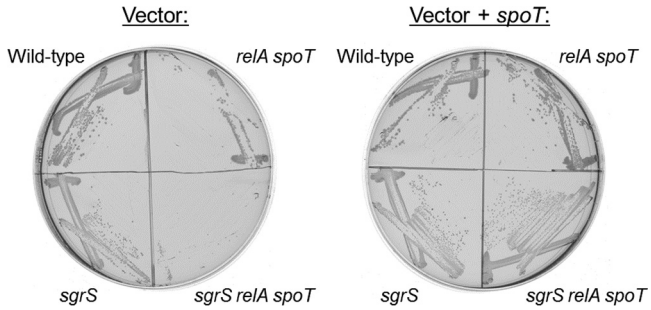

FIG 5 Complementation of the *sgrS relA spoT* mutant glucose phosphate stress growth defect. Growth of wild-type (DJ480), Δ*sgrS* (CS104), Δ*relA* $spoT_{E319Q}$ (GR259), and Δ*sgrS* Δ*relA* $spoT_{E319Q}$ (GR260) strains carrying either vector pCA24N (left plate; negative control) or pCA24N with a wild-type copy of *spoT* under the control of the P_{lac} promoter (right plate). Strains were grown for 24 h on solid LB medium containing 0.2 mM IPTG to induce P_{lac} expression and 0.01% αMG to induce stress.

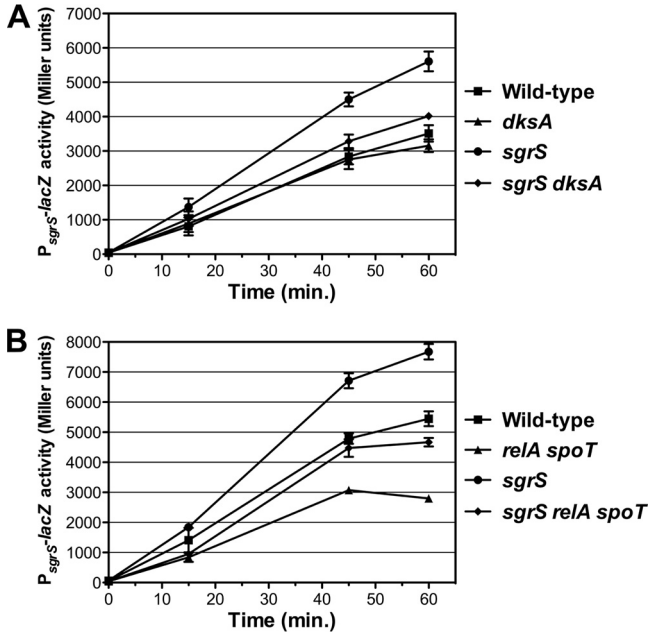

FIG 6 Expression of P_{sgrS}-*lacZ* in stringent regulator mutants during glucose phosphate stress. (A) Wild-type (BAH100), Δ*dksA* (GR206), Δ*sgrS* (GR195), and Δ*sgrS* Δ*dksA* (GR231) strains or (B) wild-type (BAH100), Δ*relA* Δ*spoT* (GR196), Δ*sgrS* (GR195), and Δ*sgrS* Δ*relA* Δ*spoT* (GR243) strains with chromosomal P_{sgrS}-*lacZ* fusions were grown in LB medium to an OD_{600} of approximately 0.1, at which point 0.01% αMG was added to induce glucose phosphate stress. β-Galactosidase activity was monitored at indicated times after the addition of αMG. Error bars indicate standard deviations ($n = 3$).

potentially contribute to the *dksA* and *relA spoT* mutant growth defects during glucose phosphate stress.

DISCUSSION

While the molecular mechanisms by which SgrR and SgrS regulate the response of *E. coli* to glucose phosphate stress have been extensively characterized, the connections of this stress response to other regulatory networks are poorly understood. In this study, we describe a novel role for stringent response regulators DksA and ppGpp in contributing to the recovery from glucose phosphate stress. We demonstrate that mutating *dksA* (Fig. 2) or *relA* and *spoT* (Fig. 4) worsens the growth defect of *sgrS* and *sgrR* mutants during glucose phosphate stress. To our knowledge, with the exception of *sgrR* and *sgrS* mutants, the stringent regulator mutants display the most pronounced glucose phosphate stress growth defects observed to date (5, 10, 20, 49, 51). *dksA* and *relA spoT* mutants also exhibit a decrease in the induction of the glucose phosphate stress response, as measured by lower *sgrS* (Fig. 6) and *sgrR* (Fig. 7) expression. These findings depict one of the first regulatory connections to glucose phosphate stress outside SgrR and SgrS themselves. To our knowledge, this is also the only reported regulatory effect on *sgrR* expression apart from SgrR autorepression (15). Overall, the implication of the stringent response regulators in the recovery from glucose phosphate stress broadens the known regulatory reach of the glucose phosphate stress response.

These results are consistent with previous observations implying a link between the stringent and glucose phosphate stress responses. Stringent response carbon starvation and glucose phosphate stress are both induced by the nonmetabolizable sugar analog αMG (5, 22, 37, 38). Moreover, stringent carbon starvation bears similarity to the glycolytic block that contributes to glucose phosphate stress through the depletion of downstream metabolic intermediates (4, 8–11). Indeed, mutating glycolytic genes such as *fda* induces glucose phosphate stress (9), and *fda* mutants also exhibit increased ppGpp production, which leads to stringent regulatory effects, including decreased

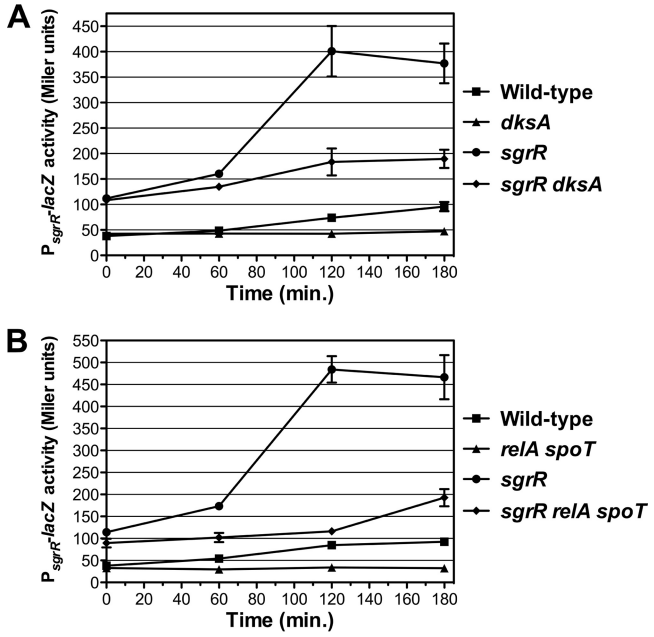

FIG 7 Expression of P_{sgrR}-*lacZ* in stringent regulator mutants during glucose phosphate stress. (A) Wild-type (CV9200), Δ*dksA* (GR233), Δ*sgrR* (CV9201), and Δ*sgrR* Δ*dksA* (GR234) strains or (B) wild-type (CV9200), Δ*relA* Δ*spoT* (GR248), Δ*sgrR* (GR251), and Δ*sgrR* Δ*relA* Δ*spoT* (GR250) strains with chromosomal P_{sgrR}-*lacZ* fusions were grown in LB medium to an OD_{600} of approximately 0.1, at which point 0.01% αMG was added to induce glucose phosphate stress. β-Galactosidase activity was monitored at indicated times after the addition of αMG. Error bars indicate standard deviations ($n = 3$).

rRNA transcription (39). Further supporting an interaction between the two, genomic studies (27–29, 56) suggest that the stringent and glucose phosphate stress response regulons have at least some targets in common. For example, results from a microarray analysis indicate that a Δ*dksA* ppGpp^0 mutant exhibits at least 2-fold statistically significant differences in expression levels of confirmed SgrS targets *adiY*, *asd*, *manXYZ*, and *ptsG* compared with those in the wild type (27). While not all expression effects of the stringent response are direct, the fact that SgrS and DksA/ppGpp likely share at least some regulatory targets is also consistent with our data showing an effect of the stringent regulators on recovery from glucose phosphate stress.

Since DksA and ppGpp exert regulatory effects transcriptionally (22, 24, 55), the *dksA* and *relA spoT* mutant growth defects could be due at least in part to the observed decrease in transcriptional activation of the glucose phosphate stress response (as measured by *sgrS* expression) (Fig. 6) and/or independent effects on related downstream targets. The defect in *sgrS* expression is most pronounced at 60 min after the addition of αMG (Fig. 6) and correlates with the onset of the observed growth defects (Fig. 2 and 4). Furthermore, this decrease in *sgrS* stress response induction could occur through a regulatory effect of DksA and ppGpp on *sgrR* transcription; SgrR is known to be the major activator of *sgrS* transcription (5, 15), and mutating *dksA* or *relA* and *spoT* also lowers *sgrR* expression (Fig. 7). Previous research suggests that the timing of induction for the stringent and glucose phosphate stress responses is consistent with these potential regulatory interactions; both ppGpp synthesis (22, 23) and *sgrS* expression (5) can be detected rapidly (within minutes) in response to their respective stressors. Whether DksA and ppGpp have direct or indirect effects on the expression of the glucose phosphate stress response is not yet clear; indeed, the stringent regulators have many direct and indirect effects on transcription (27–29). Independent of their effect on *sgrR* or *sgrS* expression, the fact that the *dksA* and *relA spoT* mutant growth defects are most pronounced in the absence of *sgrS* or *sgrR* (Fig. 2 and 4) suggests that DksA and ppGpp likely also affect the expression of one or more downstream targets involved in the recovery from glucose phosphate stress. Indeed, since genomic analyses

(27–29, 56) show that at least some SgrS targets are also regulated by DksA and/or ppGpp, the *sgrS dksA* and *sgrS relA spoT* mutant growth defects could be due to a cumulative effect on the expression of multiple glucose phosphate stress-related targets. Alternatively, given that the stringent response regulators impact the expression of hundreds of genes (27–29, 56), it is possible that the effects of DksA and ppGpp on glucose phosphate stress also are due to other as-yet-uncharacterized targets. Regardless of the specific regulatory mechanism, as a whole, these results strongly suggest that the stringent regulator mutant growth defects are due to the effects on expression of genes involved in the glucose phosphate stress response, and future research will aim to identify other stringent-regulated gene(s) that are important under glucose phosphate stress conditions.

While the *sgrS dksA* and *sgrS relA spoT* mutants both exhibit growth defects during glucose phosphate stress, the specific conditions vary. In contrast to the *sgrS dksA* mutant, which exhibits a growth defect under high-stress (0.5% αMG) conditions (Fig. 2), the *sgrS relA spoT* mutant growth defect is only apparent under low-stress (0.01% αMG) conditions (Fig. 4). This growth difference is in keeping with other studies that have reported both similar (24, 57–59) and distinct (57, 58, 60, 61) phenotypes for *dksA* and *relA spoT* mutants. Both DksA and ppGpp are required for the negative regulation of rRNA gene promoters and the activation of amino acid biosynthesis gene promoters (24, 59), and both mutants display similar though not identical amino acid auxotrophies and defects in stationary-phase induction of *rpoS* (57, 58). DksA and ppGpp tend to exert similar expression effects (24, 59), but in some cases they have opposite effects on the expression of the same target; *in vivo* expression of type 1 fimbria genes are increased in a *dksA* mutant but decreased in a ppGpp^0 mutant (61). Reflecting these regulatory complexities, the regulons of DksA and ppGpp contain both overlapping (e.g., genes encoding rRNAs and amino acid biosynthesis enzymes [24, 27, 59]) and unique (e.g., genes involved in motility, chemotaxis, and carbon metabolism and transport [27, 56, 62, 63]) targets. This regulatory diversity is likely rooted in the molecular mechanisms of DksA and ppGpp, including, for example, the way in which they interact with RNA polymerase. ppGpp has two binding sites, one at the interface of β' and ω subunits (36) and the other at the site of the RNA polymerase-DksA interaction (35). It has been posited that alterations in ppGpp binding at the two sites in response to various cellular levels of ppGpp could help account for the wide range of expression differences that ppGpp and DksA affect under various nutritional and environmental conditions (35). Therefore, while both stringent regulators affect the recovery from glucose phosphate stress, they may do so through discrete (if related) mechanisms.

To date, the *dksA* and *relA spoT* mutants are the only regulators besides *sgrR* and *sgrS* mutant strains to display both glucose phosphate stress-related growth defects (Fig. 2 and 4) and decreased induction of the associated stress response (Fig. 6 and 7). This and other studies also support the likelihood of additional regulatory interactions with the glucose phosphate stress response. CRP (the global regulator of catabolite repression and alternate carbon source metabolism) and KdgR (a regulator of carbon transport and catabolism) (64) also appear to affect the expression of *sgrS* under certain conditions, possibly via effects on SgrR (49). Consistent with this, a *crp* mutant exhibits a slight decrease in growth during glucose phosphate stress on minimal medium (Table 1). An *lrp* mutant exhibits a similar defect (Table 1), implying a role for one or more Lrp regulon members in the glucose-phosphate stress response. The induction of the phosphate starvation (Pho) regulon partially rescues the glucose phosphate growth defect of an *sgrS* mutant through an unknown mechanism, perhaps by improving phosphate (and by extension, PEP) availability and therefore helping to relieve the glycolytic depletion associated with stress (50).

The need to coordinate regulatory inputs from multiple metabolic pathways during glucose phosphate stress is also underscored by a recent study showing that regulation of certain SgrS targets is important under different nutritional and environmental conditions (51). SgrS inhibition of *ptsG* or *manXYZ* translation is sufficient to enable

stress recovery in rich media (51), while the regulation of additional SgrS targets such as *yigL* is required to rescue cells from stress in minimal media (51). There is likely a comparable need for regulatory coordination between the stringent and glucose phosphate stress responses, as highlighted by the similar yet distinct nutritional contexts in which these two stress responses operate. While both responses are induced by αMG, stringent carbon starvation is typically induced with a combination of glucose and αMG (38). These particular conditions do not adversely affect the growth of an *sgrS* mutant, presumably because sufficient glucose is able to enter the cell to enable carbon catabolism. In addition, glucose phosphate stress can be induced by the addition of αMG to rich or minimal media, whereas the stringent response typically is induced under minimal medium conditions. Moreover, the stringent regulators respond to a broader array of nutrient limitations (e.g., amino acid and iron starvation) than do SgrR and SgrS. Given the overlapping but different nutritional conditions under which they act, the involvement of regulators such as DksA and ppGpp during glucose phosphate stress could be a way for the cell to adjust the stress response in a manner appropriate to the particular nutritional environment. In conjunction with other regulatory connections from the studies described above, the work presented here represents a recent shift in our understanding of glucose phosphate stress from an initial mechanistic focus on regulation by SgrR and SgrS to a more holistic view in which the cell must coordinate multiple metabolic and regulatory inputs to fine-tune the response to stress. Future research emphasizing interactions of related regulatory and metabolic pathways could uncover additional pieces that enhance our knowledge of the regulatory puzzle surrounding the response to glucose phosphate stress.

MATERIALS AND METHODS

Bacterial strain construction. *E. coli* strains and plasmids used in these experiments are listed in Table 2. Strains are derived from the K-12 wild-type strain DJ480 (D. Jin, National Cancer Institute), a Δ*lac* derivative of MG1655. Deletion-insertion alleles of the *dksA*, *relA*, *sgrR*, *creB*, *crp*, *hns*, *lrp*, and *rpoS* loci containing kanamycin (kan) cassettes flanked by FLP recombination target (FRT) sites were obtained from the Keio collection of single-gene mutations in the wild-type background strain BW25113 (65). Δ*spoT*::cm is a deletion-insertion allele containing a chloramphenicol resistance cassette, and the $spoT_{E19Q}$ allele abolishes ppGpp synthesis activity but retains ppGpp hydrolase activity (like a Δ*relA* Δ*spoT* mutant, a Δ*relA* $spoT_{E19Q}$ mutant is unable to synthesize ppGpp) (66). Allele mutations were transferred into the indicated parent strains (Table 2) by P1 phage transduction with the exceptions noted here. Mutations were verified by PCR using Go*Taq* polymerase (Promega, Madison, WI) according to the manufacturer's instructions. The kanamycin resistance cassettes were removed from GR184 and GR246 strains using FLP-mediated site-specific recombination (67), resulting in strains GR247 and GR248. The FRT-kan-FRT allele of *sgrR* was then transduced into strains GR247 and GR248 to construct, respectively, GR249 and GR250. To construct strains GR195 and GR196, P_{sgrS}-*lacZ* transcriptional reporter fusions (18, 49) were inserted into the λ*attB* chromosomal loci of strains CS104 and GR184, respectively, as described previously (5). For ectopic expression of the *dksA* and *spoT* genes, wild-type *dksA* and *spoT* cloned into the vector pCA24N under the control of the IPTG-inducible $P_{T5\text{-}lac}$ promoter were obtained from the ASKA library of *E. coli* open reading frame (ORF) clones in host background strain AG1 (ME5305) (54). pCA24N, pCA24N/*dksA*, and pCA24N/*spoT* were transformed by electroporation into the indicated strains.

Media and growth conditions. Bacteria were cultured in Luria-Bertani (LB) rich medium (68) at 37°C unless stated otherwise. M63 minimal medium (68) supplemented with 0.2% fructose as a carbon source was used to assess growth under minimal conditions. For experiments examining growth during glucose phosphate stress, either 0.5% αMG (high-stress conditions) or 0.01% (low-stress conditions) was added to induce stress. To maintain pCA24N plasmids, 25 $\mu g \cdot ml^{-1}$ chloramphenicol was added to the medium, and IPTG (Sigma-Aldrich, St. Louis, MO) was added at a concentration of 0.2 mM to induce expression of the $P_{T5\text{-}lac}$ promoter.

Growth curve experiments were performed as described previously (50). Briefly, overnight cultures of strains were subcultured into new LB medium and normalized to an optical density at 600 nm (OD_{600}) of approximately 0.02. Once they reached an approximate OD_{600} of 0.1, cultures were split in two and αMG was added to one of the two flasks to induce stress. Growth was monitored for 7 h via OD_{600} measurements. To screen for stress-related growth differences of global regulator gene mutants, the colony sizes in comparison to those in wild-type (DJ480) or Δ*sgrS* (CS104) parental controls were assessed on solid agar LB medium after 24 h at 30°C and M63 medium after 48 h at 37°C in the presence or absence of 0.5% αMG to induce stress. To examine the effects of ectopic *dksA* or *spoT* expression, the colony sizes of strains containing pCA24N (as a control), pCA24N/*dksA*, or pCA24N/*spoT* were likewise measured during growth on solid LB agar medium with chloramphenicol (to maintain plasmids), αMG (to induce stress), and IPTG (to induce gene expression from the $P_{T5\text{-}lac}$ promoter).

β-Galactosidase assays. Strains containing either the P_{sgrS}-*lacZ* (18, 49) or P_{sgrR}-*lacZ* (15) transcriptional fusion were grown overnight and subcultured in fresh LB medium as described above for growth

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Description or relevant characteristics	Source or reference(s)
E. coli strains		
DJ480	MG1655 Δ*lac X74*	D. Jin (NCI)
CS104	DJ480 Δ*sgrS*	69
CV700	DJ480 Δ*sgrR*::cm	5
BAH100	DJ480 λ*attB*::P_{sgrS}-*lacZ*	18, 49
CV9200	DJ480 λ*attB*::P_{sgrR}-*lacZ*	15
CV9201	CV9200 Δ*sgrR*::cm	15
JK120	DJ480 Δ*sgrR*::FRT-kan-FRT	This study
GR121	DJ480 Δ*relA*::FRT-kan-FRT	This study
GR128	DJ480 Δ*dksA*::FRT-kan-FRT	This study
GR129	CV700 Δ*dksA*::FRT-kan-FRT	This study
GR138	DJ480 Δ*lrp*::FRT-kan-FRT	This study
GR140	DJ480 Δ*crp*::FRT-kan-FRT	This study
GR142	DJ480 Δ*hns*::FRT-kan-FRT	This study
GR143	CS104 Δ*hns*::FRT-kan-FRT	This study
GR184	GR121 Δ*spoT*::cm	This study
GR186	DJ480 Δ*rpoS*::FRT-kan-FRT	This study
GR195	CS104 λ*attB*::P_{sgrS}-*lacZ*	This study
GR196	GR184 λ*attB*::P_{sgrS}-*lacZ*	This study
GR198	CS104 Δ*crp*::FRT-kan-FRT	This study
GR199	CS104 Δ*lrp*::FRT-kan-FRT	This study
GR200	CS104 Δ*dksA*::FRT-kan-FRT	This study
GR201	CS104 Δ*relA*::FRT-kan-FRT	This study
GR202	GR201 Δ*spoT*::cm	This study
GR206	BAH100 Δ*dksA*::FRT-kan-FRT	This study
GR230	GR195 Δ*relA*::FRT-kan-FRT	This study
GR231	GR195 Δ*dksA*::FRT-kan-FRT	This study
GR233	CV9200 Δ*dksA*::FRT-kan-FRT	This study
GR234	CV9201 Δ*dksA*::FRT-kan-FRT	This study
GR243	GR230 Δ*spoT*::cm	This study
GR245	CV9200 Δ*relA*::FRT-kan-FRT	This study
GR246	GR245 Δ*spoT*::cm	This study
GR247	GR184 Δ*relA*::FRT	This study
GR248	GR246 Δ*relA*::FRT	This study
GR249	GR247 Δ*sgrR*::FRT-kan-FRT	This study
GR250	GR248 Δ*sgrR*::FRT-kan-FRT	This study
GR251	CV9200 Δ*sgrR*::FRT-kan-FRT	This study
GR259	GR184 $spoT_{E319Q}$ *zib563*::Tn*10*	This study
GR260	GR202 $spoT_{E319Q}$ *zib563*::Tn*10*	This study
GR263	CS104 Δ*rpoS*::FRT-kan-FRT	This study
GR264	DJ480 Δ*creB*::FRT-kan-FRT	This study
GR265	CS104 Δ*creB*::FRT-kan-FRT	This study
Plasmids		
pCA24N	Cm^r; $lacI^q$; IPTG-inducible promoter $P_{T5\text{-}lac}$	54
pCA24N/*dksA*	pCA24N plus *dksA*	54
pCA24N/*spoT*	pCA24N plus *spoT*	54

experiments. At an OD_{600} of approximately 0.1, 0.01% αMG was added to induce stress; this concentration of αMG was used because the expression of these fusions is known to be very sensitive and rapidly saturated at higher αMG concentrations (15, 49). Samples were taken at the indicated times and subjected to Miller assays as described previously (68). Briefly, samples were suspended in Z buffer and incubated at 28°C. Reactions were performed using 4 mg/ml 2-nitrophenyl β-D-galactopyranoside as a substrate, and 1 M Na_2CO_3 was used to stop the reactions (68).

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