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Circuitry Linking the Csr and Stringent Response Global Regulatory Systems

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Summary

CsrA protein regulates important cellular processes by binding to target mRNAs and altering their translation and/or stability. In *Escherichia coli*, CsrA binds to sRNAs, CsrB and CsrC, which sequester CsrA and antagonize its activity. Here, mRNAs for *relA, spoT* and *dksA* of the stringent response system were found among 721 different transcripts that copurified with CsrA. Many of the transcripts that copurified with CsrA were previously determined to respond to ppGpp and/or DksA. We examined multiple regulatory interactions between the Csr and stringent response systems. Most importantly, DksA and ppGpp robustly activated *csrB/C* transcription (10-fold), while they modestly activated *csrA* expression. We propose that CsrA-mediated regulation is relieved during the stringent response. Gel shift assays confirmed high affinity binding of CsrA to *relA* mRNA leader and weaker interactions with *dksA* and *spoT*. Reporter fusions, qRT-PCR, and immunoblotting showed that CsrA repressed *relA* expression, and (p)ppGpp accumulation during stringent response was enhanced in a *csrA* mutant. CsrA had modest to negligible effects on *dksA* and *spoT* expression. Transcription of *dksA* was negatively autoregulated via a feedback loop that tended to mask CsrA effects. We propose that the Csr system fine-tunes the stringent response and discuss biological implications of the composite circuitry.

Keywords

CsrA; stringent response; ppGpp; global networks; regulatory systems

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Introduction

Bacteria utilize genetic regulatory mechanisms to adapt, compete and survive in response to changing environmental and physiological conditions. Moreover, global regulatory networks permit bacteria to coordinate expression of large sets of genes in multiple operons (Gottesman, 1984; Beisel and Storz, 2010). Two global regulatory networks, Csr (carbon storage regulator) and stringent response, provide mechanisms for sensing end products of carbon metabolism and nutrient availability, and regulating translation and transcription, respectively (Babitzke and Romeo, 2007; Chavez *et al.*, 2010; Potrykus and Cashel, 2008).

CsrA (and its orthologs, RsmA/E) is a small, dimeric RNA binding protein that post-transcriptionally coordinates expression of a diverse set of genes by positively or negatively regulating the translation and/or stability of target transcripts. In this way, CsrA activates exponential phase processes while repressing several stationary phase functions (Babitzke and Romeo, 2007). CsrA is widely distributed among eubacteria (White *et al.*, 1996; Mercante *et al.*, 2006) and regulates expression of genes for virulence factors (Fortune *et al.*, 2006; Bhatt *et al.*, 2009), quorum sensing (Cui *et al.*, 1995; Lenz *et al.*, 2005), motility (Wei *et al.*, 2001; Yakhnin *et al.*, 2007), carbon metabolism (Romeo *et al.*, 1993; Sabnis *et al.*, 1995; Yang *et al.*, 1996), biofilm formation (Jackson *et al.*, 2002; Wang *et al.*, 2005), cyclic di-GMP synthesis (Jonas *et al.*, 2008) and peptide uptake (Dubey *et al.*, 2003).

CsrA directly regulates gene expression by interacting with the 5' untranslated leaders of target mRNAs at sites characterized by a GGA sequence, which is often located within the loop of a short stem-loop structure (Liu *et al.*, 1997; Dubey *et al.*, 2005; Schubert *et al.*, 2007). CsrA typically represses translation initiation by binding to sites that overlap the Shine-Dalgarno sequence (SD), thus competing with the 30S ribosomal subunit and accelerating mRNA degradation (Liu and Romeo, 1997; Baker *et al.*, 2002; Dubey *et al.*, 2003; Babitzke *et al.*, 2009). CsrA can also activate gene expression by stabilizing a bound transcript, as exemplified by the *E. coli flhDC* mRNA (Wei *et al.*, 2001).

The *E. coli* Csr system includes other important regulatory components. CsrB and CsrC are noncoding RNAs that contain multiple CsrA binding sites, which permit them to sequester and antagonize CsrA (Liu *et al.*, 1997; Weilbacher *et al.*, 2003). In a negative feedback loop, CsrA is indirectly required for transcription of these sRNAs, which are directly activated by the two-component signal transduction system (TCS), BarA-UvrY (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003). Recently, acetate was identified as a physiological stimulus for BarA-dependent signaling (Chavez *et al.*, 2010). Because CsrA activates glycolysis (Sabnis *et al.*, 1995), a major source of acetate, this metabolic pathway was hypothesized to be a possible link for CsrA effects on *csrB/C* transcription. However, results from the present study do not support this hypothesis. CsrA represses *csrD*, encoding a GGDEF-EAL domain protein, which functions along with RNase E and PNPase to mediate CsrB/C turnover (Suzuki *et al.*, 2006).

The stringent response defines another global regulatory network of eubacteria. It is characterized by a rapid downshift in synthesis of stable RNAs, such as rRNA and tRNA, and the upregulation of a number of operons, such as those for amino acid biosynthesis, in response to starvation for amino acids or other nutrients (Potrykus and Cashel, 2008). The effector of this response is the nucleotide secondary messenger guanosine tetraphosphate (ppGpp) (Cashel and Gallant, 1969), which binds to RNA polymerase and positively or negatively affects transcription, depending upon promoter characteristics (Barker *et al.*, 2001). In *E. coli*, ppGpp levels are regulated by two enzymes: RelA and SpoT. In response to the presence of uncharged tRNA in the ribosomal A-site, RelA synthesizes pppGpp, which is converted to ppGpp, collectively referred to as (p)ppGpp (Wendrich *et al.*, 2002).

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SpoT is a bifunctional synthetase/hydrolase, which exhibits weak ppGpp synthetase activity and serves primarily to degrade ppGpp (Cashel *et al.*, 1996). However, SpoT synthesizes ppGpp in response to carbon starvation (Cashel *et al.*, 1996), fatty acid starvation (Seyfzadeh *et al.*, 1993; Gong *et al.*, 2002) and other stresses, such as iron limitation (Vinella *et al.*, 2005).

In most cases, regulation by ppGpp requires the transcription factor DksA (Paul *et al.*, 2004; Paul *et al.*, 2005), which interacts with the secondary channel of RNA polymerase (Paul *et al.*, 2004; Perederina *et al.*, 2004). Together, ppGpp and DksA regulate the expression or activity of a number of global regulators, including the stationary phase sigma factor RpoS (Brown *et al.*, 2002), the extracytoplasmic sigma factor RpoE (Costanzo *et al.*, 2008) and the master regulator of the motility cascade, $FlhD_4C_2$ (Lemke *et al.*, 2009).

Here, we used a high throughput sequencing approach to screen for novel, direct targets of CsrA regulation. Our results suggest a greatly expanded global regulatory role for CsrA. Among the transcripts identified were *relA, spoT* and *dksA*, which encode proteins involved in the stringent response, along with numerous RNAs for ppGpp-responsive genes. A reporter system designed to monitor post-transcriptional regulation and other approaches revealed complex regulatory interactions between the Csr and stringent response systems. The strong positive effects of ppGpp and DksA on *csrB/C* transcription and negative effects of CsrA on *relA* expression and (p)ppGpp accumulation during stringent response were notable. We present and discuss a model for this composite circuitry and its biological implications.

Results

Screening for novel targets of CsrA binding

Several studies suggest that CsrA orthologs regulate expression of a large number of transcripts (Lawhon *et al.*, 2003; Burrowes *et al.*, 2006; Brencic and Lory, 2009). Data from these studies were primarily derived from transcriptome analyses, which in large part, did not exclude effects of indirect regulation or examine expression from intergenic regions. To screen for putative direct targets of CsrA binding, recombinant His-tagged CsrA (CsrA-His_6) was ectopically expressed and purified from a *csrA csrB csrC* triple mutant. RNA that was noncovalently bound to CsrA-His_6 was isolated, converted to cDNA, and analyzed by 454 sequencing (Margulies *et al.*, 2005). The methods, results, and validation studies for this screen are provided in the online Supporting Information. While our screen was not saturated, this analysis suggested that CsrA binds to the RNAs of at least 721 genes (Tables S1, S2), representing many fundamental physiological and regulatory processes, including the stringent response. An additional screen for targets of CsrA regulation compared proteins from *csrA* mutant and wild-type strains, harvested from four growth conditions, and analyzed by two-dimensional PAGE and MALDI-ToF/ToF mass spectrometry (Table S3). Several of the abundant proteins that varied among the *csrA* mutant and wild type strains were expressed from mRNAs that copurified with CsrA (Table S4), suggesting the directionality of CsrA-mediated regulation for these genes.

Analysis of csrA binding to *relA* and *dksA* mRNA leaders

Because CsrA-His_6 copurified with *relA*, *spoT*, and *dksA* mRNAs, we began by testing these binding interactions in vitro. The *relA* leader contains six putative CsrA binding sites, while *dksA* contains two (Fig. 1A). Gel shift assays with CsrA protein and in vitro synthesized transcripts containing the 5' untranslated region and part of the coding region of *relA* (−178 nt to +22 relative to the start of translation) or *dksA* (−54 nt to +10 nt relative to the start of translation) revealed initial binding at 5 to 10 nM CsrA for *relA* and at 10 nM CsrA for *dksA*

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(Fig. 1C, E). As the CsrA concentration was increased, additional shifted species were observed for the CsrA-*relA* interaction, suggesting that multiple CsrA proteins were bound to each *relA* transcript. A nonlinear least-squares analysis of these data yielded apparent K_d values of 17 ± 1 nM CsrA for *relA* and 66 ± 4 nM CsrA for *dksA*.

Competition experiments with specific (*relA* and *dksA*) and nonspecific (*Bacillus subtilis trp* leader) unlabeled RNAs confirmed that CsrA binds to the *relA* mRNA leader with high affinity and specificity (Fig. 1D), while *dksA* RNA binding exhibited weak competition by the *B. subtilis trp* leader RNA (Fig. 1F). Thus, the binding affinity and specificity of the CsrA-*dksA* RNA interaction are not as strong.

Analyses of csrA binding to *gmk* and *rpoZ* mRNA leaders

spoT is located within the five gene *spo* operon, *gmk-rpoZ-spoT-trmH-recG*, which is transcribed by three mapped promoters (Gentry *et al.*, 1993; Fig. 1B). A possible CsrA binding site, with modest sequence similarity to consensus, overlaps the *gmk* SD sequence, and two putative CsrA binding sites are present in *rpoZ*, one of which overlaps the SD sequence (Fig. 1A, B). RNA gel shift assays with 5' untranslated leader and part of the coding region of *gmk* (−42 nt to +25 nt relative to the start of translation) or *rpoZ* (−28 nt to +33 nt relative to the start of translation) revealed that CsrA did not interact with the *gmk* transcript (Fig. 1G) but bound to the *rpoZ* transcript, beginning at 5 to 10 nM CsrA and resulting in an apparent K_d value of 66 ± 4 nM (Fig. 1H). Unlabeled *rpoZ* RNA competed effectively for the CsrA-*rpoZ* interaction (Fig. 1I). *trp* leader RNA was also able to compete, although not as effectively as *rpoZ* RNA (Fig. 1I), implying that CsrA interacts with modest specificity to the *rpoZ* transcript.

Effects of csrA on *relA* expression

To examine *relA* expression, β-galactosidase specific activity from a chromosomal *relA*'-'*lacZ* translational fusion containing the upstream non-coding region through the first three codons (−880 nt to +11 nt relative to the start of translation) was monitored in wild-type and *csrA* mutant strains. Disruption of *csrA* increased expression ~40%, which was reversed by ectopic complementation (Fig. 2A). Two promoters driving *relA* transcription (P1 from −178 nt; P2 from −626 nt) have been mapped (Metzger *et al.*, 1988; Nakagawa *et al.*, 2006). In contrast to the translational fusion, expression from a transcriptional fusion containing the upstream non-coding region through the upstream transcriptional start of *relA* (−880 nt to −626 nt) was unaltered by the *csrA* mutation (Fig. 2B). We also constructed post-transcriptional reporter fusions for *relA*, in which the constitutive *lacUV5* promoter replaced the native promoters (P1, −178 nt to +11 nt; P2, −626 nt to +11 nt). Expression from both fusions, including the P1 replacement, which did not contain *relA* promoter DNA, was increased ~30% in the *csrA* mutant versus the wild-type strain as the cultures approached the stationary phase of growth (Fig. 2C). These data indicate that CsrA represses *relA* expression post-transcriptionally via the 5' untranslated segment of the *relA* mRNA.

Effects of csrA on *spoT* and *dksA* expression

In contrast, CsrA had little to no effect on the expression of a P*lacUV5*-*rpoZ*-spoT'-'*lacZ* leader fusion (Fig. 2D). Translational fusions for *gmk*, *rpoZ* or *spoT*, containing the native promoters and ribosome binding sites, produced extremely low levels of β-galactosidase and were not quantified. Finally, expression of the *dksA*'-'*lacZ* fusion was slightly decreased in the *csrA* mutant (up to ~30% at the transition to stationary phase), and was complemented by ectopic expression of *csrA* (Fig. 2E), suggesting that CsrA activates *dksA* expression. This effect is examined in more detail below.

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Effects of csrA on *relA, spoT* and *dksA* steady-state transcript levels

Because CsrA frequently affects the stability and steady-state levels of its target RNAs (Liu *et al.*, 1995; Wei *et al.*, 2001; Wang *et al.*, 2005), quantitative real-time reverse transcription polymerase chain reactions (rt-qRT-PCR) were performed on *relA*, *spoT* and *dksA* transcripts. In the *csrA* mutant, *relA* mRNA was increased 1.4-fold in exponential phase (OD_{600} = ~0.5), and 2.0-fold in stationary phase (Fig. 3A), suggesting that repression of *relA* involves an alteration in mRNA levels. While the *spoT* leader fusion showed no change (Fig. 2D), steady-state transcript levels of *spoT* RNA in the *csrA* mutant were increased 1.5-fold in exponential phase and 1.8-fold in stationary phase (Fig. 3A). Because CsrA did not affect expression of the *spoT* leader fusion, its effects on *spoT* transcript levels may be mediated indirectly. *dksA* transcript levels remained unchanged between the *csrA* mutant and wild type strains (Fig. 3A).

RelA protein accumulates in a csrA mutant while GMK and DksA remain unchanged

Western blotting (Fig. 3B) revealed that RelA was increased by the *csrA* mutation in mid-exponential (1.6-fold) and stationary phases (1.9-fold), similar to effects obtained with the reporter fusions (Fig. 2A, C) and the rt-qRT-PCR analysis (Fig. 3A). GMK, the product of the first gene of the *spoT* operon, and DksA were unchanged in the *csrA* mutant (Fig. 3C and D). A possible explanation for this result is that while CsrA binds to *dksA* mRNA and modestly activates *dksA* expression, other factor(s) might compensate for these effects. Western analyses of SpoT protein levels were inconclusive, due to technical problems caused by a cross reacting product (data not shown).

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csrA represses (p)ppGpp accumulation during stringent response

CsrA bound specifically to the *relA* mRNA leader and repressed *relA* expression (Figs. 1C, D, 2A–C, 3A, B). To assess the biological relevance of these effects, we monitored (p)ppGpp levels in *csrA* mutant and wild-type strains during induction of the stringent response by serine hydroxamate (SHX) treatment (Fig. 3E). The levels of (p)ppGpp upon SHX treatment were elevated ~1.5-fold in the *csrA* mutant relative to the wild-type strain, indicating that negative regulation of *relA* expression by CsrA downregulates the stringent response.

DksA regulates its own transcription

Because CsrA bound to *dksA* mRNA and a *dksA'-'lacZ* fusion responded modestly to CsrA, yet *dksA* transcript and protein levels were similar in the *csrA* mutant and wild-type strains, we sought to more carefully monitor the influence of CsrA on *dksA* expression. We reasoned that many global regulators are autoregulatory, which might have complicated these analyses. Expression of a *dksA*'-'*lacZ* translational fusion was increased ~2.5-fold in a *dksA* mutant, suggesting that DksA negatively regulates its own expression (Fig. 4A). This was confirmed by ectopic expression of *dksA* (Fig. 4A).

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To determine if DksA negative autoregulation influences the effect of CsrA on *dksA* expression, *dksA*'-'*lacZ* expression was compared in isogenic wild-type, *dksA, csrA* and *dksA csrA* strains. The *dksA csrA* double mutant expressed ~50% less β-galactosidase activity than the *dksA* single mutant (Fig. 4B), confirming that CsrA activates DksA expression and suggesting that DksA autoregulation tends to mask this effect. The *dksA csrA* double mutant exhibited an increase in β-galactosidase activity compared to the *csrA* single mutant (Fig. 4B), showing that DksA negative autoregulation occurs independently of CsrA regulation.

To assess the levels at which DksA and CsrA regulate *dksA* expression, a *dksA-lacZ* transcriptional fusion and a P*lacUV5-dksA'-'lacZ* leader fusion were examined. Expression

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from the transcriptional fusion was increased ~2.5-fold in the *dksA* mutant, similar to the translational fusion (compare Fig. 4C to 4B), and was restored by ectopic expression of dksA (Fig. S1A). This revealed that the promoter DNA present in the transcriptional fusion mediates transcriptional autoregulation. In contrast, CsrA did not regulate the *dksA-lacZ* transcriptional fusion, suggesting that CsrA regulates *dksA* post-transcriptionally (Fig. 4C). This was confirmed by the finding that β-galactosidase activity from the P*lacUV5-dksA'-'lacZ* was decreased (~30%) in a *csrA* mutant (Fig. 4D). A slight decrease in β-galactosidase activity from the P*lacUV5-dksA'-'lacZ* leader fusion in the *dksA* mutant (Fig. 4D) from 5 to 8 h, and no effect at 10 h, revealed that the 5' leader of *dksA* mRNA does not support negative autoregulation. Furthermore, ectopic expression of *dksA* did not substantially alter expression from the P*lacUV5-dksA'-'lacZ* fusion (Fig. S1B).

To further examine DksA autoregulation, primer extension analysis of *dksA* mRNA was conducted (Fig. 5). Transcription of *dksA* from the chromosome was below the level of detection in our hands, even after long exposures (Fig. 5B). Using a *dksA* mutant, we examined ectopic expression of *dksA* transcripts from strains containing plasmids that expressed either the wild-type *dksA* allele or a *dksA* allele that contained amino acid substitutions in the two aspartic acid residues (D71N D74N) that are critical for DksA activity (Perederina *et al.*, 2004). Both alleles were expressed from the native *dksA* promoters present in these plasmids. The wild-type *dksA* allele produced two transcripts, P1 and P2, in low abundance (Fig. 5B, Lane 3, see inset), which increased in the strain expressing the defective *dksA* D71N D74N allele (Fig. 5B, Lane 2). This finding revealed that a functional DksA protein is required for negative autoregulation and that the P1 and P2 transcripts are both subject to this regulation. The P1 promoter has been characterized previously (Kang and Craig, 1990), and in vitro transcription analysis resulted in two identically mapped transcripts (data not shown).

To confirm that DksA directly repressed transcription from the putative P1 and P2 promoters, we attempted in vitro transcription using a linear template and purified DksA and/or ppGpp, but were unable to demonstrate DksA-mediated inhibition (data not shown). This result may suggest that an additional factor or condition is required or that this effect is indirect.

Effects of dksA and ppGpp on *csrA* expression

The Csr and stringent response systems share a number of regulatory targets, e.g. *glgCAP* (Romeo and Preiss, 1989; Romeo *et al.*, 1990; Liu and Romeo, 1997) and *flhDC* (Wei *et al.*, 2001; Lemke *et al.*, 2009). Furthermore, the Csr system possesses feedback loops in which its components both control and are controlled by other factors. Thus, we reasoned that the stringent response components might regulate expression of the genes of the Csr system. Western blotting demonstrated that CsrA protein levels were modestly reduced ~50% in the *dksA* mutant as well as the *relA spoT* ($ppGpp^0$) mutant (Fig. 6A). CsrA protein levels in the *dksA* mutant were restored to wild-type or higher levels by ectopic expression of *dksA*. β-galactosidase activity from a *csrA'-'lacZ* translational fusion was also reduced in the *dksA* and $ppGpp^0$ mutants and complemented by ectopic expression of *dksA* (Fig. 6B). These observations indicate that DksA and ppGpp activate *csrA* expression.

We recently found that the stationary phase and general stress response sigma factor, RpoS (σ^S), directly regulates *csrA* transcription (H. Yakhnin and P. Babitzke, unpublished results). Furthermore, ppGpp and DksA are required for full expression of RpoS (Brown *et al.*, 2002; Hirsch and Elliott, 2002). We therefore asked whether DksA activation of *csrA* expression requires RpoS. Expression of the *csrA*'-'*lacZ* fusion was reduced ~60% in the *rpoS* and *dksA* single mutant strains, while the *rpoS dksA* double mutant exhibited a further reduction in β-galactosidase activity (Fig. 6C). Ectopic expression of *dksA* from an IPTG-inducible plasmid

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partially restored β-galactosidase activity in the double mutant, but not to wild-type levels. These findings indicate that activation of *csrA* expression by DksA depends in part on RpoS.

Transcription of *csrA* is driven by at least three promoters (H. Yakhnin and P. Babitzke, unpublished results). RpoS directs *csrA* transcription from P3, while P1 and P5 are transcribed by the housekeeping sigma factor, σ^{70}. Using transcriptional *lacZ* fusions for each promoter, we found that the P1-*csrA*-*lacZ* and P5-*csrA*-*lacZ* fusions were unaffected by the *dksA* mutation, whereas the P3-*csrA*-*lacZ* fusion was decreased (3-fold) in the *dksA* mutant (Fig. 6D). These data further suggest that DksA activates *csrA* expression primarily through RpoS-driven transcription of P3. Interestingly, ppGpp was required for full activity of the P1-*csrA*-*lacZ* and P3-*csrA*-*lacZ* fusions (Fig. 6D), suggesting that ppGpp likely influences *csrA* expression through additional RpoS-independent pathway(s). Furthermore, expression from the minor promoter P1 was not affected by DksA, but was partly dependent on ppGpp. This exemplifies the finding that DksA and ppGpp independently regulate certain genes (see Discussion).

DksA and ppGpp activate *csrB* and *csrC* expression

Further examination of DksA and ppGpp effects on the Csr system by Northern blotting revealed that CsrB and CsrC RNA levels were reduced 10-fold in *dksA* and ppGpp^0 mutant strains (Fig. 7A). Additionally, *csrB* and *csrC* transcripts were reduced in the *csrA* mutant, as previously observed (Fig. 7A; Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003). Ectopic expression of *dksA* partly or fully complemented the *dksA* mutation. Expression of *csrB-lacZ* and *csrC-lacZ* transcriptional fusions was decreased substantially in the *dksA* mutant and ppGpp^0 strains (Fig. 7B). Furthermore, activity was restored in the *dksA* mutant strain by ectopic expression of *dksA* (Fig. 7B). These findings indicate that DksA and ppGpp activate *csrB* and *csrC* transcription.

CsrA indirectly activates transcription of CsrB and CsrC sRNAs, through the BarA-UvrY TCS (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003). BarA is a membrane bound tripartite sensor kinase, while UvrY functions as its cognate response regulator (Pernestig *et al.*, 2001). Epistasis studies were conducted to determine whether the effects of DksA on *csrB* and *csrC* expression were dependent upon UvrY. Ectopic expression of *uvrY* restored *csrB-lacZ* and *csrC-lacZ* expression in a *dksA uvrY* double mutant while ectopic expression of *dksA* did not (Fig. 7C, D). This suggested that *dksA* might lie upstream of *uvrY* in this signaling pathway. Furthermore, the promoter sequences of *csrB*, *csrC* and *uvrY* do not contain AT-rich discriminator sequences, as observed for genes that are subject to direct positive regulation by ppGpp (Da Costa and Artz, 1997; Barker *et al.*, 2001). The *barA* promoter has not been determined. Expression from a *uvrY*'-'*lacZ* translational fusion was unaffected in the *dksA* mutant strain (Fig. S2), indicating that DksA does not affect *uvrY* expression, and raising the possibility that it may affect UvrY activity. CsrA is required for normal signaling through the BarA-UvrY TCS (Suzuki *et al.*, 2002), and since DksA activates CsrA, epistasis studies were performed to determine if DksA activated *csrB* and *csrC* expression through CsrA. However, neither ectopic expression of *csrA* nor *dksA* restored *csrB-lacZ* or *csrC-lacZ* expression in a *dksA csrA* double mutant (Fig. 7E, F). These findings revealed that CsrA and DksA are both necessary for full expression of *csrB* and *csrC*, and suggest that their effects in this circuitry are not mediated in series, i.e., sequentially.

DksA, ppGpp and csrA affect acetate production

Because acetate acts as a stimulus for BarA-UvrY signaling (Chavez *et al*. 2010), we asked whether CsrA, DksA or ppGpp affect *csrB* and *csrC* expression via effects on acetate accumulation. Acetate levels were monitored in isogenic wild-type, *csrA*, *dksA*, *csrA dksA*,

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and ppGpp^0 strains. No substantial differences in acetate accumulation were observed in LB medium (Fig. S3A). However, in a medium requiring glycolytic carbon metabolism (Kornberg medium; Fig. S3B), all of the mutant strains accumulated less acetate than the parent strain. Nevertheless, these effects were likely insufficient to account for strong regulation of *csrB* and *csrC* expression by these genes. A *barA'*-*'lacZ* translational fusion was constructed to monitor effects of ppGpp, DksA and CsrA on *barA* expression, but its expression was too low to measure.

Discussion

The motivation for these studies was our observation that the mRNAs for the three stringent response genes, *relA*, *spoT* and *dksA*, copurified with a recombinant CsrA protein. While the Csr and stringent response systems were known to govern a number of the same genes and processes, the present study has revealed novel regulatory interactions within and among the components of these global regulatory systems (summarized in Fig. 8).

The strongest effects (10-fold) observed in this study were the positive transcriptional effects of ppGpp and DksA on *csrB* and *csrC* expression and CsrB/C RNA levels (Fig. 7). The positive effects of DksA and ppGpp on CsrA levels were relatively modest in comparison (Fig. 6). Furthermore, one molecule of CsrB or CsrC RNA is able to sequester ~10 or 5 CsrA dimers, respectively (Babitzke and Romeo, 2007), which further magnifies the inhibitory influence of ppGpp on CsrA activity. The increased ppGpp levels that are generated in response to the stringent response or other metabolic stresses should antagonize CsrA activity during such conditions. This is in keeping with the regulatory roles of CsrA in repressing metabolic pathways and processes that are activated upon the entry into stationary phase growth, while activating central metabolic pathways (Babitzke and Romeo, 2007). Previous studies demonstrated that CsrB and CsrC RNA levels are elevated in minimal media and decline drastically upon amino acid supplementation, while CsrA levels were only modestly affected under these conditions (Jonas and Melefors, 2009). Thus, our present observations point to the genetic circuitry responsible for the effects of amino acids on the Csr system, and the prior physiological studies strengthen our model for the composite system (Fig. 8).

In reciprocal regulatory interactions, CsrA bound with high affinity to *relA* mRNA and negatively regulated *relA* expression, *relA* transcript levels, RelA protein levels, and (p)ppGpp accumulation during the induction of stringent response. Thus, the Csr system plays a negative regulatory role in the stringent response. The effects of CsrA on other genes of the stringent response system were more subtle, and their implications less certain. Despite the strong binding interactions of CsrA with the *relA* transcript (Fig. 1C, D), its effects on *relA* expression and RelA protein levels were relatively modest (Figs. 2, 3). These results differed from the much stronger effects of CsrA on various structural genes, whose mRNAs were bound by CsrA with affinities that do not differ greatly from that of *relA* (K_d = 17 nM CsrA), e.g., *glgCAP* at 39 nM (Baker *et al.*, 2002), *cstA* at 40 nM (Dubey *et al.*, 2003), *pgaABCD* at 22 nM (Wang *et al.*, 2005). While the molecular basis of the distinct effect of CsrA on *relA* expression remains to be determined, high affinity binding in the context of modest regulation implies that CsrA fine-tunes *relA* expression, as opposed to functioning as a course-control mechanism or an on-off switch.

As a consequence of CsrA-mediated repression of RelA protein levels, CsrA repressed (p)ppGpp accumulation ~1.5-fold during SHX-induced stringent response (Fig. 3E). Furthermore, CsrA affected the relative accumulation of pppGpp versus ppGpp; the *csrA* mutant accumulated substantially greater amounts of pppGpp (Fig. 3E). The enzyme responsible for dephosphorylation of pppGpp to form ppGpp is encoded by *gpp* mRNA

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(Keasling *et al.*, 1993), which copurified with CsrA protein (Table S2). While the biological role for differential effects on pppGpp and ppGpp remains to be determined, our observations raise the possibility that *gpp* mRNA may be a target of positive regulation by CsrA.

Our studies also suggest that there is substantial overlap between the stringent response and Csr regulons. Altogether, 40% of the genes whose transcripts copurified with CsrA (Table S1) were found previously to respond to ppGpp and/or DksA (Aberg *et al.*, 2009). Even more striking, 68% of such genes in COG C, encoding proteins involved in energy production and conversion, were found to be common between these two studies (Table S1). These values represent minimum estimates of the regulatory overlap, as only the exact gene matches between the two data sets were tabulated.

Our working model for the composite regulatory network has interesting implications for genes that respond to both regulatory systems (Fig. 8). Presently, two genetic systems have been demonstrated to respond directly to both CsrA and ppGpp: the glycogen biosynthetic operon, *glgCAP* (Romeo and Preiss, 1989; Romeo *et al.*, 1990; Liu *et al.*, 1997; Baker *et al.*, 2002), and the *flhDC* operon, which encodes a transcription factor that activates the motility and chemotaxis cascade (Wei *et al.*, 2001;Lemke *et al.*, 2009). In these examples, CsrA and ppGpp have opposite effects on expression, i.e. *glgCAP* is repressed by CsrA and activated by ppGpp, while *flhDC* is activated by CsrA and repressed by ppGpp. Because the major effect of ppGpp and DksA on the Csr system is to increase CsrB/C RNA levels and thereby decrease CsrA activity, the stringent response should reinforce its direct effects on *glgCAP* and *flhDC* by down-regulating the opposing post-transcriptional effects of CsrA. We caution that although this attractive model is based on established regulatory nodes, additional studies are required to demonstrate the role of cross regulation from ppGpp to CsrA in the full system. In addition, it is not clear that CsrA and ppGpp function antagonistically with respect to other genes of their overlapping regulons.

DksA and ppGpp robustly activated *csrB/C* transcription in a UvrY-dependent fashion, and in parallel with CsrA (Fig. 7). Because DksA did not affect *uvrY* expression (Fig. S2), and ectopic expression of *uvrY* restored *csrB* and *csrC* expression in a *dksA uvrY* mutant background (Fig.7C, D), DksA may affect UvrY activity. In *Pseudomonas aeruginosa*, the BarA sensor-kinase ortholog (GacS), as well as two other sensors that do not have orthologs in *E. coli*, RetS (Goodman *et al.*, 2004; Goodman *et al.*, 2009) and LadS (Ventre *et al.*, 2006), have been proposed, based on genetic experiments, to regulate the activity of the UvrY ortholog (GacA). The absence of an AT-rich discriminator sequence (Da Costa and Artz, 1997; Barker *et al.*, 2001) in the *csrB* and *csrC* promoters is inconsistent with direct activation of their transcription by ppGpp, although the limited information on transcriptional activation by ppGpp makes such predictions uncertain. Whether BarA alone or other factors in *E. coli* mediate DksA and ppGpp effects on *csrB* and *csrC* expression will require additional investigation.

Previous studies revealed that DksA protein levels remain relatively constant throughout growth (Brown *et al.*, 2002; Paul *et al.*, 2004; Rutherford *et al.*, 2007). A recent stringent response model suggests that since DksA levels are constant, ppGpp levels mediate stringent control upon nutritional stress (Dalebroux *et al.*, 2010). Our results demonstrated that DksA levels are regulated by a negative feedback loop, whereby DksA represses its own transcription. Negative feedback loops tend to promote homeostasis (Becskei and Serrano, 2000), and are not unexpected for a regulatory gene whose product is maintained within a relatively narrow range. Presumably, negative autoregulation permits transcription of *dksA* to be adjusted in response to DksA protein levels. Autoregulation of *dksA* tended to mask the opposing and relatively weak effect of CsrA, as observed in epistasis analyses (Fig. 5).

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Our data do not resolve the apparent discrepancy between the weak positive effects of CsrA on *dksA* reporter expression (Figs. 2E, 5) versus the absence of effects of CsrA on DksA mRNA and protein levels (Fig. 3A, D). However, CsrA copurified with RNAs representing various factors (e.g., ribonucleases and proteases) capable of differentially influencing DksA mRNA or protein levels versus reporter expression.

We previously demonstrated that the Csr system of *E. coli* contains multiple negative feedback loops (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003; Suzuki *et al.*, 2006; Fig. 8), which may provide distinct advantages for this regulatory network. Negative feedback loops produce graded responses, thus reducing cell-cell variability (Nevozhay *et al.*, 2009) and stochastic events and noise (Becskei and Serrano, 2000), and they can also accelerate regulatory responses (Rosenfeld *et al.*, 2002). A central feedback loop of the Csr system involves a multistep pathway from CsrA to *csrB* and *csrC* expression via the BarA-UvrY TCS (Fig. 8). The recent finding that acetate and other short-chain fatty acids act as a stimulus for BarA signaling (Chavez *et al.*, 2010), coupled with previous studies showing that CsrA activates glycolysis (Sabnis *et al.*, 1995), suggested that glycolysis might provide the link between CsrA and BarA activity. However, acetate accumulation (Fig. S3) cannot explain the effects of CsrA, DksA or ppGpp on *csrB* and *csrC* transcription. Thus, acetate signaling to BarA in *E. coli* likely allows the Csr system to respond to conditions of the large intestinal lumen (discussed in Chavez *et al.*, 2010), although this remains to be experimentally demonstrated.

An unexpected observation was that CsrA had opposite effects on *relA* and *dksA* expression (Fig. 2). Although ppGpp and DksA often potentiate each other's effects, there have been several reports of independent and even antagonistic effects of ppGpp and DksA (Magnusson *et al.*, 2007; Aberg *et al.*, 2008; Aberg *et al.*, 2009; Lyzen *et al.*, 2009; Merrikh *et al.*, 2009). Indeed, ppGpp^0 and *dksA* mutant strains exhibit multiple, but not identical, amino acid auxotrophies (Brown *et al.*, 2002; Potrykus *et al.*, 2010b), perhaps signifying alternative gene expression roles for ppGpp and DksA. Presently, it is not clear how the modest effect of CsrA on *dksA* expression might impact differential genetic regulation or whether negative feedback by DksA tends to overshadow the effect of CsrA under most or all physiological conditions.

Given that the stringent response was only one of many transcriptional regulatory systems whose mRNAs copurified with CsrA (Tables S1, S2), the complexity of the regulatory circuitry surrounding the Csr system is undoubtedly vast. Supporting this hypothesis, transcripts for regulatory factors necessary for critical cellular processes were identified, including alternative sigma factors (*rpoE* and *rpoH*), universal stress proteins (*uspA, uspB* and *uspD*) and the proteins that mediate catabolite repression (*crp* and *cyaA*). We expect that the high-throughput sequencing of CsrA-bound transcripts from the present study will spur additional research on the complex circuitry and global regulatory role of Csr system.

Experimental Procedures

Bacterial strains, phage, plasmids and growth conditions

All *E. coli* K-12 strains, plasmids and bacteriophage used in this study are listed in Table S5. Unless otherwise indicated, bacteria were grown at 37°C, shaking at 250 rpm, in Luria-Bertani (LB) medium (Miller, 1972). Media were supplemented with antibiotics, as needed, at the following concentrations: kanamycin, 100 μg ml^{-1}; ampicillin, 25 μg ml^{-1}; spectinomycin, 25 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}, except that ampicillin was used at 100 μg ml^{-1} during the construction of *lacZ* fusion plasmids. P1*vir* transduction was performed as previously described (Miller, 1972).

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Construction of transcriptional, translational and leader fusions and integration into the chromosome

Plasmids pRELZ and pDKSZ were constructed by PCR amplification of an 888 bp fragment containing the upstream regulatory region through the first three codons of *relA* and 628 bp fragment containing the upstream regulatory region through the first three codons of *dksA* using the primer pairs relA-F/relA-R and dksA-F/dksA-R. Table S6 lists primer sequences. The products were digested with *Eco*RI and *Bam*HI and cloned into the same sites of pMLB1034 to create *relA*'-'*lacZ* and *dksA*'-'*lacZ* translational fusions. Both fusions were moved into the *E. coli* CF7789 chromosome using λInCh1 and confirmed by PCR analysis, as previously described (Boyd *et al.*, 2000).

A series of plasmids using the pAH125 plasmid backbone (Haldimann and Wanner, 2001) were created for the generation of various *lacZ* fusions. pLFX was generated to replace the kanamycin resistance marker of pAH125 with ampicillin resistance. The *bla* PCR product was generated using the primer pair AmpRF/AmpRR and plasmid pUC19 DNA as template. Purified PCR product was digested with *Cla*I and *Not*I. *Cla*I/*Not*I digestion of pAH125 generated three fragments; the 1.2 kb and 3.5 kb DNA fragments were purified from 1X TAE agarose gels and ligated with the *bla* PCR product to generate the *lacZ* transcriptional fusion vector, pLFX. pLFX was used to generate a *lacZ* translational fusion vector. *BssH*II/*Eco*RI digestion of pLFX and pMLB1034 liberated 4.3 kb and 1.5 kb fragments, respectively. These fragments were gel purified and ligated to generate the *lacZ* translation fusion vector, pLFT. pLFT was also used to create a post-transcriptional (or leader) fusion vector containing the constitutive *lacUV5* promoter. The *lacUV5* promoter was amplified using the primer pair LPF-19/LPF-20 and pUV5moaA (L. M. Patterson-Fortin and T. Romeo, unpublished data) plasmid DNA as template, and the purified PCR product was digested with *Pst*I and *Eco*RI and cloned into *Pst*I/*Eco*RI digested pLFT plasmid DNA. The resulting plasmid pUV5 allows construction of *lacZ* translational fusions expressed from the constitutive *lacUV5* promoter.

Plasmid pPFINT was constructed to replace the ampicillin resistance marker of the parent plasmid, pINT-ts (Haldimann and Wanner, 2001), with tetracycline resistance. The *tet* PCR product was generated using the primer pair LPF-21/LPF-22 and pBR322 plasmid DNA as template, and the purified PCR product was digested with *Cla*I. pINT-ts was digested with *Bgl*I, blunt-ended using DNA polymerase I, and digested with *Cla*I. The resulting 4 kb fragment was gel purified and ligated with the *Cla*I-digested *tet* PCR product to generate pPFINT.

The plasmids pRELZtxn and pDKSZtxn were constructed by PCR amplification of a 255 bp fragment containing the upstream regulatory region of *relA* from −880 to −625 relative to the translational start and a 66 bp fragment containing the upstream regulatory region of *dksA* from −118 to −52 relative to the translational start using the primer pairs relA-F-txn/relA-R-txn and dksA-F-txn/dksA-R-txn, respectively. The products were digested with *Pst*I and *Bam*HI and cloned into the same sites of pLFX to create *relA-lacZ* and *dksA-lacZ* transcriptional fusions. Plasmids pRELZplacP1, pRELZplacP2, pDKSZplac, pGMKZplac, and pSPOZplac were constructed by PCR amplification of the 5' untranslated mRNA leaders of *relA*, *dksA*, *gmk*, and *rpoZ-spoT* using the primer pairs relAP1-F-plac/relA-R, relAP2-F-plac/relA-R, dksA-F-plac/dksA-R, gmk-F-plac/gmk-R-plac, and spoT-F-plac/spoT-R-plac, respectively, to create leader fusions under the control of the *lacUV5* promoter. These products were digested with *Eco*RI and *Bam*HI and cloned into the same sites of pUV5. All fusions were integrated into the CF7789 chromosome as previously described (Haldimann and Wanner, 2001). All oligonucleotide primers used in this study (Table S6) were synthesized by Integrated DNA Technologies Inc., Coralville, Iowa, and all cloned DNA inserts were sequenced to confirm the absence of mutations.

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Cloning of the *csrA* gene

The plasmid pCsrA, encoding the *csrA* gene including 400 bp upstream from the start of *csrA* translation through the coding region, was constructed by PCR amplifying the *csrA* gene with primers csrA-F-pGB2 and csrA-R-pGB2. The PCR product was digested with *Hind*III and *Eco*RI and cloned into the same sites of pGB2 (Churchward *et al.*, 1984).

RNA Gel Shift Assays

E. coli CsrA-His^6 protein for gel shifts was purified from pCsrH6-19 as described previously (Mercante *et al.*, 2006). This plasmid was also the source of CsrA-His$_6$ in the RNA pull-down experiments (Tables S1, S2). Quantitative gel mobility shift assays followed a previously published procedure (Yakhnin *et al.*, 2000). DNA templates for *relA* and *gmk* transcripts were PCR-amplified from MG1655 genomic DNA using the primer pairs relA-F-T7/relA-R-T7 and gmk-F-T7/gmk-R-T7. DNA templates for *rpoZ* and all *dksA* transcripts were produced by annealing primer pairs rpoZ-T7/GC-rpoZ-T7, dksA-T7/GC-dksA-T7, dksA-BS1/GC-dksA-BS1, dksA-BS2/GC-dksA-BS2, and dksA-BS1-2/GC-dksA-BS1-2. RNA was synthesized in vitro using the MEGAshortscript kit (Ambion, Austin, TX) and purified PCR products (for *relA* and *rpoZ*), annealed DNA primers (*gmk* and all *dksA* transcripts) or linearized plasmid pPB77 (for nonspecific *trp* leader RNA from *B. subtilis*) (Babitzke *et al.*, 1994) as templates, and RNA was gel purified. Transcripts were 5' end-labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. Radiolabeled RNA was gel purified and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), heated to 85°C and chilled on ice. Increasing concentrations of purified CsrA-His$_6$ recombinant protein were combined with 80 pM radiolabeled RNA in 10μl binding reactions [10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 3.25 ng total yeast RNA, 20 mM DTT, 7.5% glycerol, 4U SUPERasin (Ambion, Austin, TX)] for 30 min at 37°C to allow for CsrA-RNA complex formation. Competition assays were performed in the absence or presence of unlabelled RNA specific and non-specific competitors, and used CsrA concentrations that reflected the direct binding interactions (minimal concentrations required for full shift). Binding reactions were separated using 10% native TBE polyacrylamide gels, and radioactive bands were visualized with a Molecular Dynamics phosphorimager. Free and bound RNA species were quantified with Quantity One (Bio-Rad, Hercules, CA), and an apparent equilibrium binding constant (K_d) was calculated for CsrA-RNA complex formation according to a previously described cooperative binding equation (Mercante *et al.*, 2006).

β-galactosidase and total protein assays

β-galactosidase activity was determined as described previously (Romeo *et al.*, 1990), except that 100 μl chloroform and 50 μl 0.01% SDS were used for cell membrane permeabilization. Total cellular protein was measured by the bicinchoninic acid (BCA) assay with bovine serum albumin as the protein standard (Pierce Biotechnology, Rockford, IL). All analyses compared isogenic strains, derived from CF7789.

rt-qRT-PCR

To measure steady-state levels of *relA, spoT* and *dksA* transcripts, wild-type (MG1655) and *csrA* mutant strains were grown at 37°C in LB medium, and cells were harvested in exponential ($OD_{600} \approx 0.5$) and early stationary phases. Total RNA was isolated using the Ribo-pure Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was quantified by its absorbance at 260 and 280 nM, and rRNA integrity was analyzed on formaldehyde agarose gels. Real-time quantitive reverse-transcription PCR (rt-qRT-PCR) was performed using the iScript one-step RT-PCR Kit (Bio-Rad, Hercules, CA) along with primer pairs for the coding regions (~70 nt) of the mRNAs, specifically, relA-F-taqman and relA-R-taqman, spoT-F-taqman and spoT-R-taqman, and dksA-F-taqman and dksA-R-

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taqman, and the probes relA-6FAM-BHQ1, spoT-6FAM-BHQ1, and dksA-6FAM-BHQ1, which were 5'-end labeled with 6-carboxyfluorescein (6FAM) and 3'-end labeled with Black Hole Quencher 1(BHQ1). Reactions were conducted using the Lightcyler 480 (Roche Diagnostics) under the following conditions: 50°C for 10 min, 95°C for 5 min, and 40 cycles of 95°C for 15 sec and 60°C for 30 seconds with real time measurements taken at the 60°C step. Each reaction was performed in triplicate in two independent experiments, each time with 100 ng and 10 ng template RNA, and the mean values of the two experiments were determined. A reaction lacking reverse transcriptase was included for each sample, which served as a control for DNA contamination. For normalization of *relA, spoT* and *dksA* transcript levels, rt-qRT-PCRs were performed with each sample for 16S rRNA quantitation using the primer pair 16S-Fw and 16S-Rv and the probe 16S-6FAM-BHQ1 (Baker *et al.*, 2007). The reaction conditions for rt-qRT-PCR of 16S rRNA were identical to the other transcripts except that 1 ng and 0.1 ng of RNA were used for each example. The identities of all of the PCR products were confirmed by electrophoresis on 1% agarose gels with ethidium bromide staining. The $2^{-\Delta\Delta CT}$ method was used to calculate relative transcript levels (Livak and Schmittgen, 2001).

Western blotting

Cultures for western blot analyses were grown at 37°C with shaking, and at indicated time points, cells from 1 ml of culture were concentrated and resuspended in Lysis Buffer A (90 mM Tris-HCl, 2% SDS, pH 6.8). Samples were boiled for 3 min, cell debris was removed by centrifugation, and the supernatant saved and assayed for total protein using the BCA assay with bovine serum albumin as the protein standard (Pierce Biotechnology, Rockford, IL). 10 μg total protein was applied to 8–15% SDS-polyacrylamide gels and transferred to Immunoblot PVDF membrane (Bio-Rad, Hercules, CA) after separation. RelA, DksA and CsrA proteins were detected as previously described (Brown *et al.*, 2002; Gudapaty *et al.*, 2001). GMK antibody was obtained from the Cashel lab and was raised against GMK protein purified as previously described (Gentry *et al.*, 1993). Protein bands were quantified with Quantity One (Bio-Rad, Hercules, CA).

Primer Extension

Primer extension was carried out as previously described (Potrykus *et al.*, 2010a) except that 10 ug of total RNA was used. RNA was isolated from the following strains: CF9239 (MG1655 dksA::kan), CF9239/pJK537 and CF9239/pHM1684.

Northern Blotting

Bacterial cells were grown in LB at 37°C with shaking, and cellular RNA was stabilized by the addition of 2 vol of the RNAprotect Bacteria Reagent (Qiagen). Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen), quantitated by absorbance (260 nm). The resulting RNA (5 μg) was separated on 5% polyacrylamide gels containing 7 M urea, rRNAs were visually examined by ethidium bromide staining, and the RNA was transferred to positively charged nylon membranes (Roche Diagnostics) by electroblotting using the Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. RNA was cross-linked to nylon membranes by exposure to UV light (120,000 μJ) followed by baking at 80°C for 30 min. Membranes were then blotted with DIG-labeled anti-sense RNA probes using the DIG Northern Starter Kit (Roche Diagnostics) according to the manufacturer's instructions. Blots were developed using the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA) and densitometry was performed using Quantity One image analysis software (Bio-Rad, Hercules, CA).

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Thin-layer chromatography of (p)ppGpp levels

MG1655 (WT) and *csrA::kan* strains were grown in MOPS minimal media containing 0.2 % glucose, 0.1 mM serine, and the remaining amino acids and vitamins as described previously (Wanner *et al.*, 1977). Nucleotides were labeled in vivo by the addition of 0.33 mM K_2HPO_4 and 20 μCi ml^{-1} of $^{32}P_i$ (Perkin-Elmer) to the growth medium. Cells were grown to an OD_{600} of 1.0 at which time 200 μg ml^{-1} serine hydroxamate (SHX) was added and aliquots were sampled every four minutes up to 16 minutes. Nucleotide extraction was performed by mixing 200 μl of culture with 40 μl of cold formic acid and incubating on ice for approximately 20 minutes. Following centrifugation, 10 μl of supernatant solution was spotted onto PEI-F cellulose TLC plates, and nucleotides were separated using 1.0 M KH_2PO_4 (pH 3.4) as a solvent. Labeled nucleotides were detected by phosphorimaging and densitometry was performed using Quantity One software (Bio-Rad, Hercules, CA). Cold GTP was detected by UV shadowing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Gel shift analyses of CsrA binding to mRNA leaders of *relA, dksA, gmk* and *rpoZ*. (A) The nucleotide sequences of *relA, gmk, rpoZ* and *dksA* mRNA leaders are shown with putative CsrA binding sites underlined. Positions of the experimentally determined transcriptional starts are depicted as capital letters, and positions of the Shine-Dalgarno (SD) sequences and initiation codons (Met) are shown. (B) The structure of the *spo* operon; arrows denote promoters while asterisks indicate potential CsrA binding sites. (C-I) RNA gel shifts: 5'-end-labeled transcripts (80 pM) were incubated with CsrA at the concentration indicated below each lane. Reactions were performed in the absence (C, E, G and H) or presence (D, F and I) of specific or nonspecific (*trp* from *B. subtilis*) unlabeled RNA competitors, at the concentrations shown. The positions of free (F) and bound (B) RNA are shown. (C, D) CsrA-*relA* interactions. (E, F) CsrA-*dksA* interactions. (G) CsrA-*gmk* interaction. (H, I) CsrA-*rpoZ* interaction.

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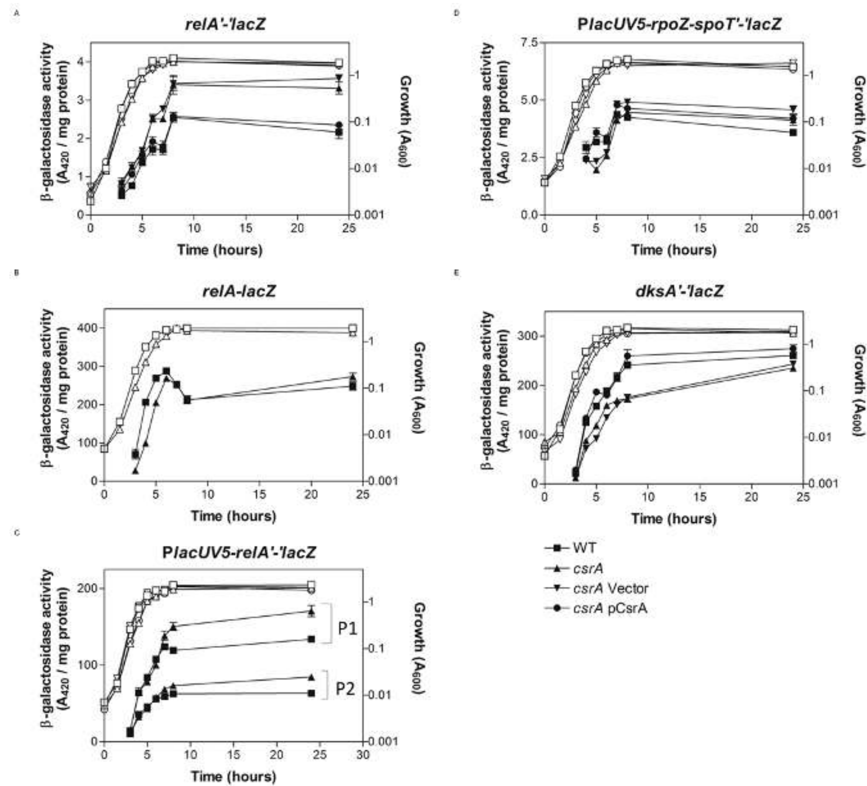

Fig. 2.

Effects of *csrA* on expression of *relA, spoT* and *dksA* reporter fusions. Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). The values represent the average of two independent experiments. Error bars depict standard error of the mean. (A–E) Activity of indicated fusions in wild-type, ■; *csrA*, ▲; *csrA* pGB2 (empty vector), ▼; and *csrA* pCsrA (*csrA*+), ●. (E) Strain identities were identical, except pBR322 (empty vector, ▼) and pCRA16 (*csrA*+, ●) were used for complementation. Growth curves are represented by corresponding open symbols.

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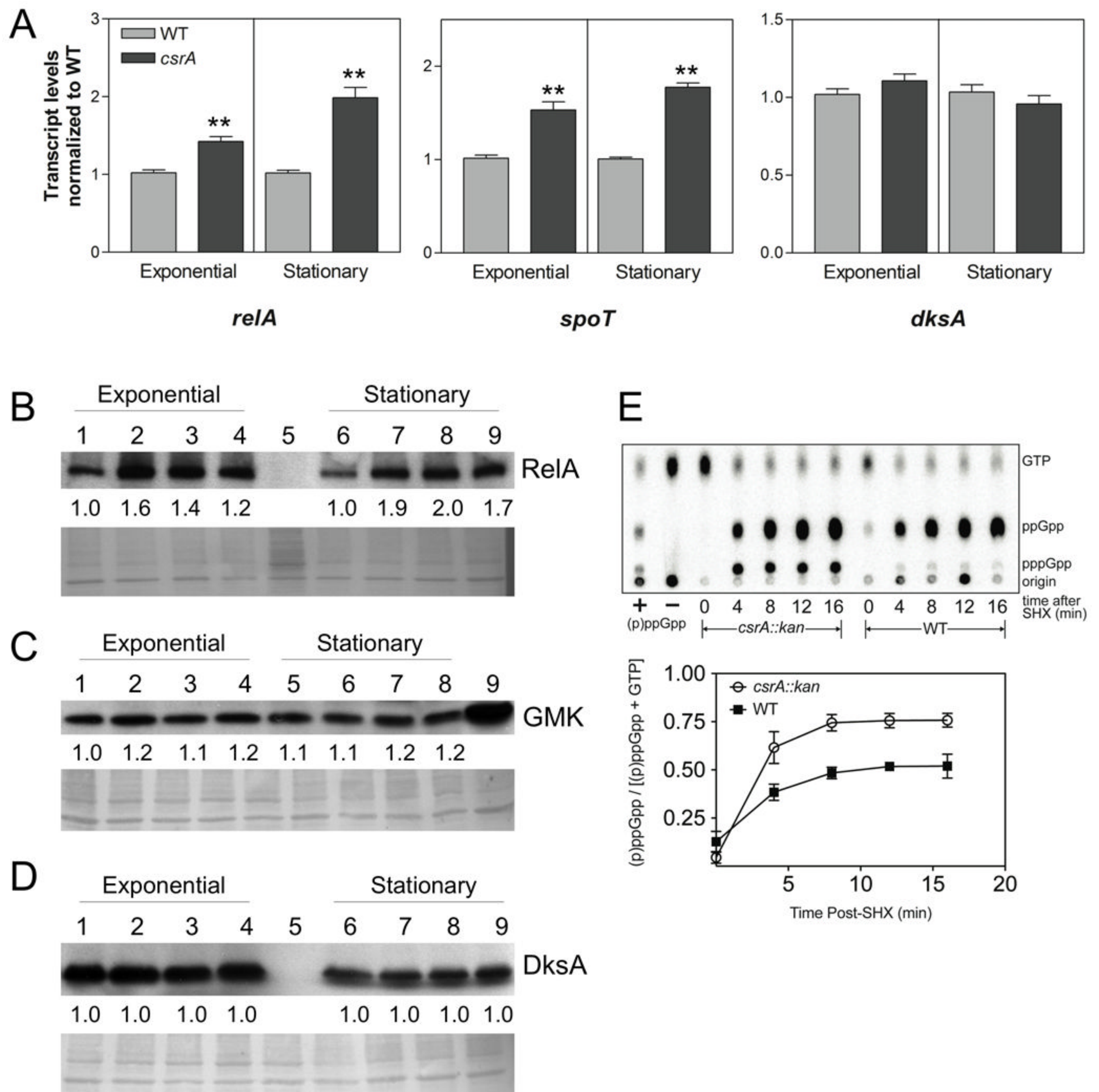

Fig. 3.

Effects of *csrA* on *relA, spoT* and *dksA* transcripts, gene products, and (p)ppGpp levels during stringent response. (A) Samples of MG1655 (WT) and TRMG1655 (*csrA::kan*) were taken for RNA isolation at exponential phase (OD_{600} = 0.5) and stationary phase (at 8 hours of growth), and RNA samples were analyzed by rt-qRT-PCR. The values represent the average of two independent experiments. Error bars depict standard error of the mean (** *P* < 0.001). (B–D) Effects of *csrA* disruption and complementation on RelA, GMK and DksA protein levels by Western blotting. Shown below each representative blot, the PVDF membrane was stained using the MemCode™ Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, IL) and imaged as a loading control. Protein was harvested at

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exponential (OD_{600} = 0.5) and stationary phases (after 8 hours of growth). (B) RelA; Lane 1,6 MG1655; Lane 2,7 *csrA::kan*; Lane 3,8 *csrA::kan* pGB2; Lane 4,9 *csrA::kan* pCsrA; Lane 5, *relA::kan spoT::cat*. (C) GMK; Lane 1,5 MG1655; Lane 2,6 *csrA::kan*; Lane 3,7 *csrA::kan* pGB2; Lane 4,8 *csrA::kan* pCsrA; and Lane 9, CF80005 (pGMK). (D) DksA; Lane 1,6 MG1655; Lane 2,7 *csrA::kan*; Lane 3,8 *csrA::kan* pGB2; Lane 4,9 *csrA::kan* pCsrA; Lane 5, *dksA::kan*. Fold differences in protein levels (relative to wild-type) represent the average of three independent experiments. The standard deviation for all values was less than 10% from the mean. (E) Determination of CsrA effects on (p)ppGpp accumulation during stringent response. MG1655 (wild-type) and *csrA::kan* strains were grown to mid-logarithmic phase, treated with serine hydroxamate (SHX) (200 µg ml^{-1}) to induce the stringent response, and nucleotides were labeled and analyzed by thin-layer chromatography (TLC) as described in Experimental Procedures. The upper panel depicts phosphorimaging of the TLC plate, the lower panel depicts the ratio of (p)ppGpp to guanine nucleotides [(p)ppGpp and GTP)] for the wild-type and *csrA* mutant strains. These values represent the averages of three independent experiments. Error bars depict the standard errors of the means.

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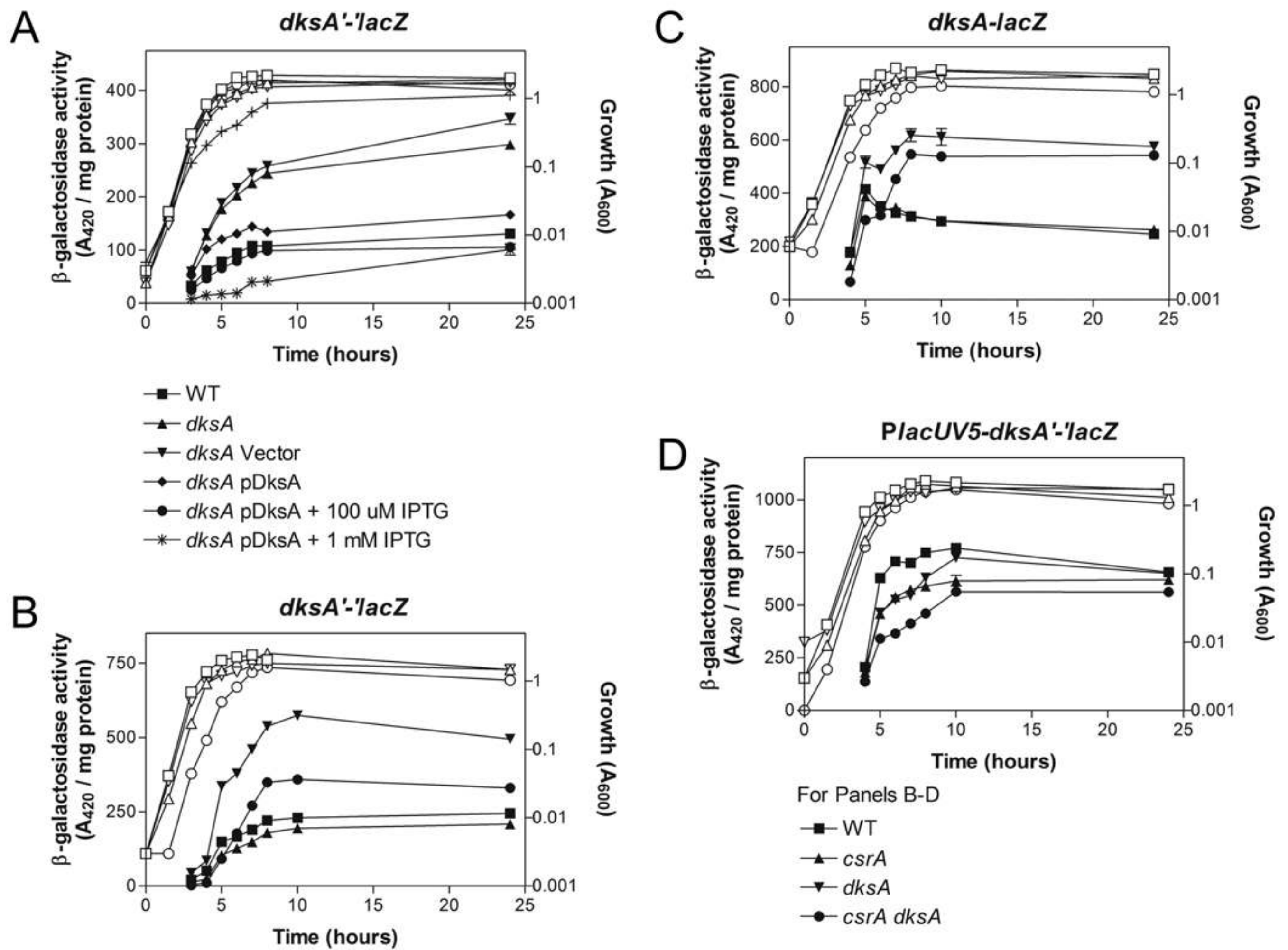

Fig. 4.

Effects of *dksA* and *csrA* on expression of chromosomally-encoded *dksA* translational, transcriptional, and leader fusions. (A–D) Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). Values represent the average of two independent experiments. Error bars depict standard error of the mean. Error bars smaller than the symbols are not shown. (A) Activity from a chromosomal *dksA'-'lacZ* translational fusion. Growth curves are represented by corresponding open symbols except for *dksA* pHM1506 + 1 mM IPTG (+). (B–D) Activity from chromosomal *dksA'-'lacZ*, *dksA-lacZ*, and P*lacUV5dksA'-'lacZ* fusions. Growth curves are represented by corresponding open symbols. Where error bars are not visible, they are obscured by the symbols.

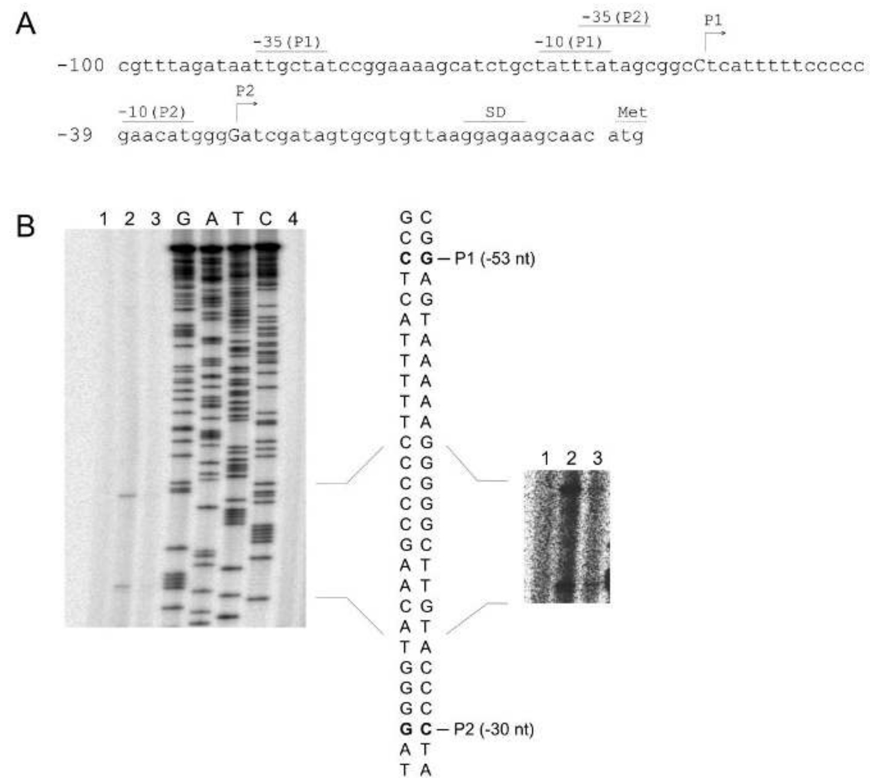

Fig. 5.

Effect of DksA on *dksA* transcription. (A) Nucleotide sequence of the *dksA* promoters. Positions of the mapped P1 and putative P2 transcriptional starts are depicted as capital letters, and predicted −35 and −10 promoter elements are shown. Numbering is with respect to the start of *dksA* translation. (B) Primer extension analysis of the *dksA* 5' end. Lane 1, *dksA* (disrupted with *kan*); Lane 2, *dksA* pHM1684 (plasmid containing *dksA* with the D71N and D74N mutations); Lane 3, *dksA* pJK537 (wild-type *dksA*); Lane 4, wild-type. The dideoxynucleotide sequencing ladder (G, A, T and C) was generated with the same primer (pdksA2) used for the primer extension analysis. The image to the right shows an enhanced exposure to accentuate the extension products present in lane 3.

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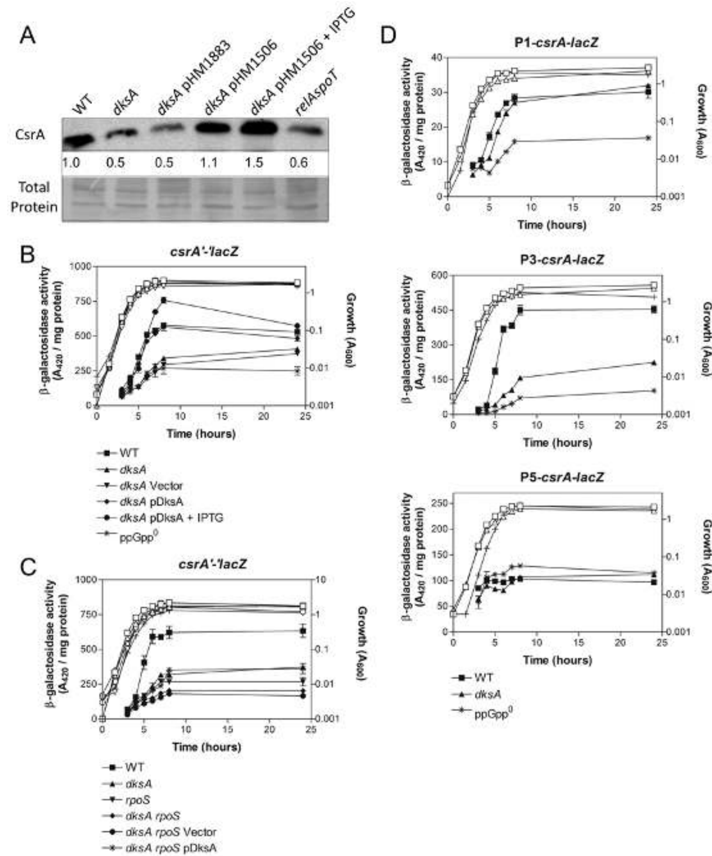

Fig. 6.

Effects of *dksA*, ppGpp and *rpoS* on *csrA* expression. (A) Western blot of CsrA protein levels in MG1655 (wild-type) and *dksA* mutant strains (upper panel) and PVDF membrane stained with the MemCode™ Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, IL) and imaged as an internal loading control (lower panel). Cells were harvested at exponential phase ($OD^{600} = 0.5$) and stationary phase (at 8 hours of growth). Lane 1, MG1655; Lane 2, *dksA::kan*; Lane 3, *dksA::kan* pHM1883 (empty vector); Lane 4, *dksA::kan* pHM1506 ($dksA^+$); Lane 5, *dksA::kan* pHM1506 + 0.1 mM IPTG; Lane 6, *relA spoT* ($ppGpp^0$). Fold differences in protein levels (relative to wild-type) are indicated and represent an average of three independent experiments. The standard deviation for these values was < 10% from the mean. (B–F) Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). The values represent the average of two independent experiments. Error bars depict standard error of the mean. (B, C) Activity from a chromosomal *csrA'-'lacZ* translational fusion. (D) Activity from chromosomal P1-*csrA-lacZ*, P3-*csrA-lacZ* and P5-*csrA-lacZ* transcriptional fusions. Growth curves are represented by open symbols, except for *dksA rpoS* pHM1506 + 1 mM IPTG (+) in panel C.

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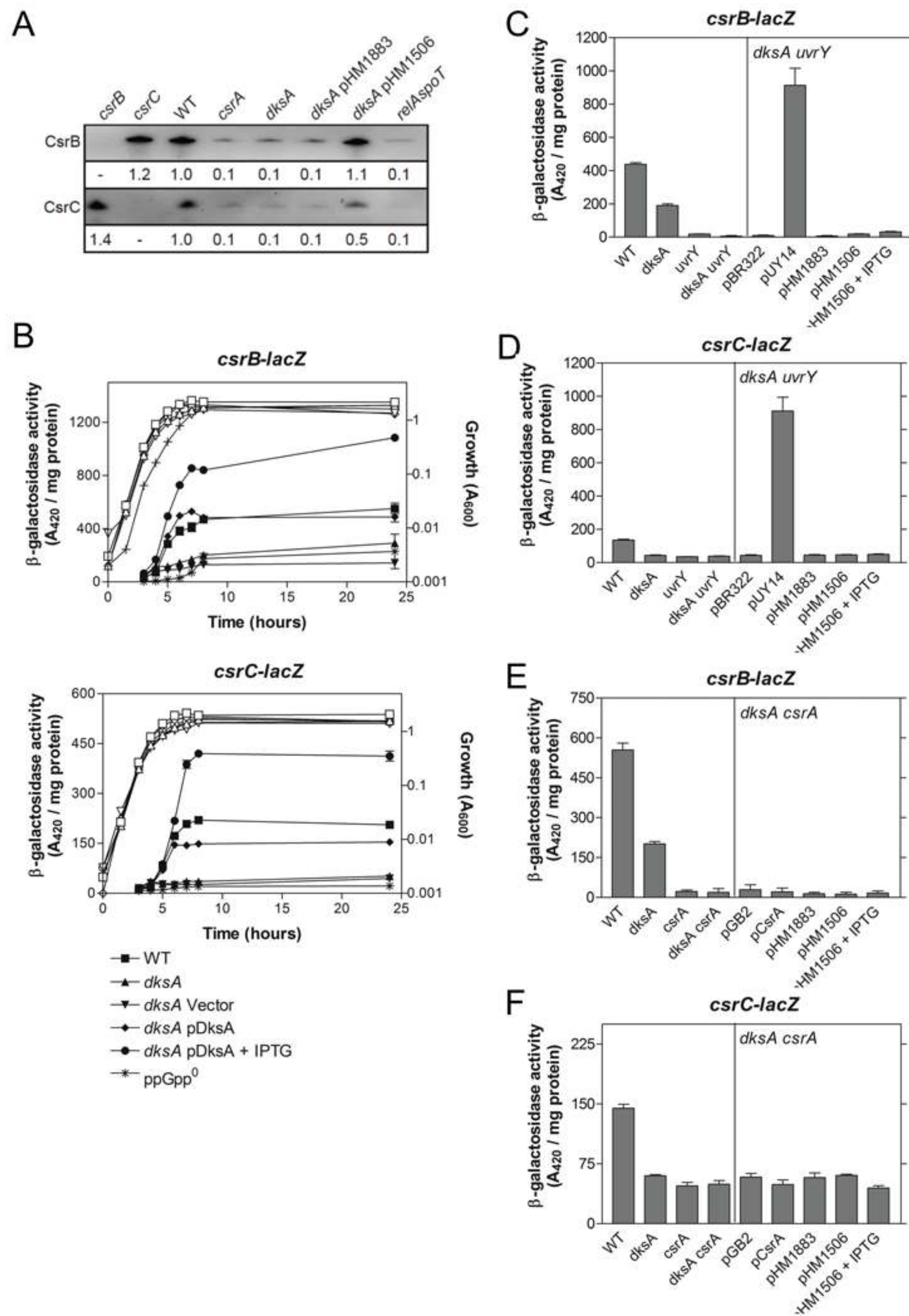

Fig. 7.

Effects of *dksA* and ppGpp on CsrB and CsrC RNA levels and gene expression. (A) A representative Northern blot of RNA from MG1655 (WT) and isogenic mutants is shown. A plasmid vector (pHM1883) and a *dksA* expression plasmid (pHM1506) were used for *dksA* complementation analysis. Fold differences in RNA levels (relative to wild-type) are indicated and represent an average of three independent experiments. The standard deviation for all values was <10% from the mean. (B) Activity from chromosomal *csrB-lacZ* and *csrC-lacZ* transcriptional fusions. Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). The values represent the average of two independent experiments, and error bars depict the standard error of the

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mean. Growth curves are represented by open symbols except for *relA spoT* (ppGpp^0) (+). (C, D) Epistasis studies with *csrB-lacZ* (C) and *csrC-lacZ* transcriptional fusions (D) in *dksA, uvrY* and *dksA uvrY* backgrounds with ectopic expression of *uvrY* (pUY14) or *dksA* (pHM1506) at 8 hours of growth. The vector controls were pBR322 and pHM1883, respectively. (E, F) Epistasis studies with *csrB-lacZ* (E) and *csrC-lacZ* transcriptional fusions (F) in *dksA, csrA* and *dksA csrA* backgrounds with ectopic expression of *csrA* (pCsrA) or *dksA* (pHM1506) at 8 hours of growth. The vector controls were pGB2 and pHM1883, respectively.

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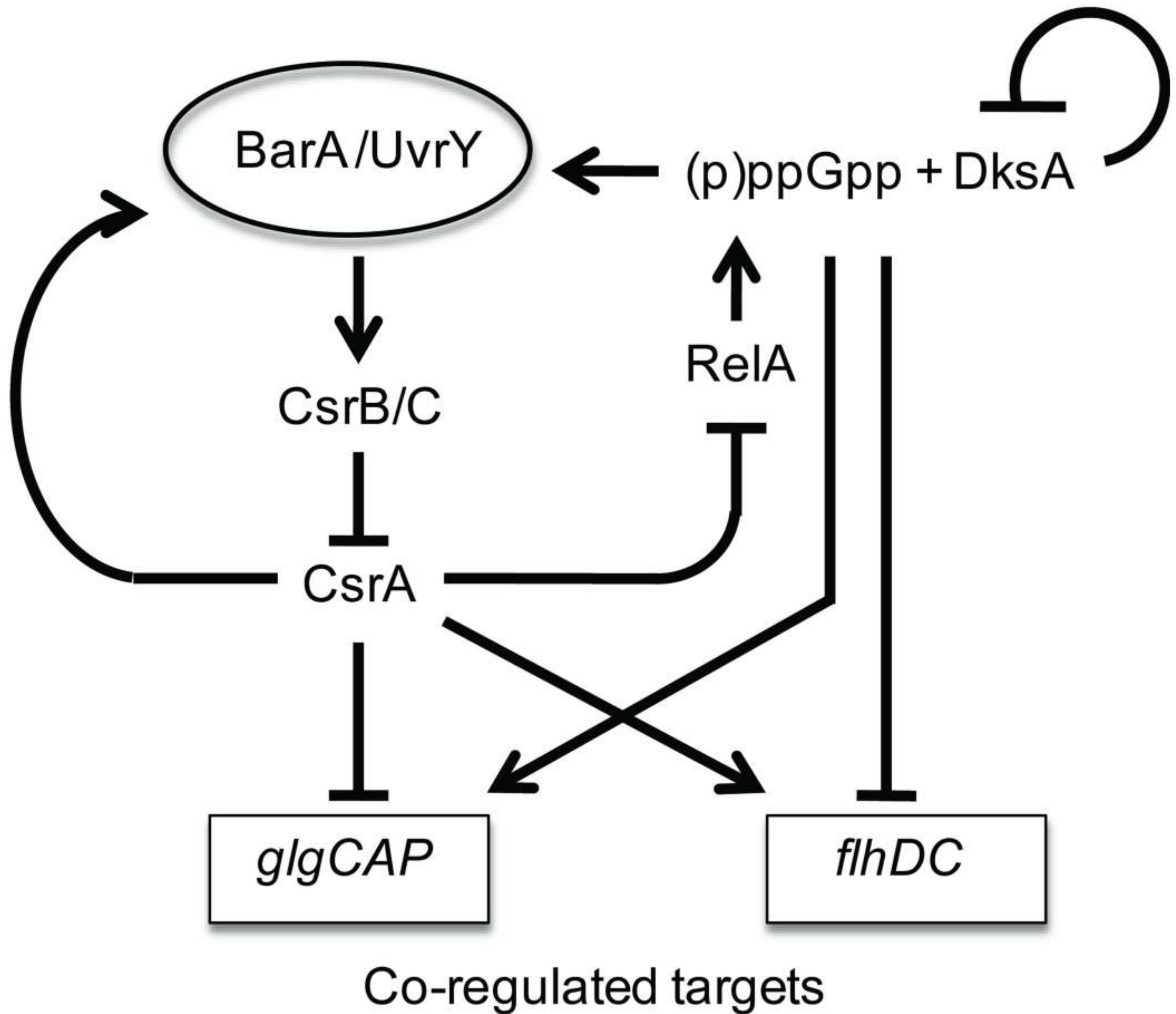

Fig. 8.

Model for the regulatory circuitry of the Csr and stringent response systems. Composite circuitry depicting feedback loops of the system. CsrA activates *csrB* and *csrC* expression via the BarA-UvrY TCS (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003). In turn, CsrB and CsrC RNAs sequester and antagonize CsrA (Liu *et al.*, 1997; Weilbacher *et al*, 2003). DksA and ppGpp activate (10-fold) transcription of CsrB/C RNAs. In turn, this should down-regulate CsrA activity during the stringent response. A prediction of this circuitry is that during stringent response, the direct effects of ppGpp on target genes that respond oppositely to ppGpp and CsrA, e.g., glycogen synthesis (*glgCAP*) genes (Romeo and Preiss, 1989; Romeo *et al.*, 1993; Baker *et al.*, 2002), and motility (*flhDC*) genes (Wei *et al.*, 2001; Lemke *et al.*, 2009) will be reinforced by the downregulation of CsrA activity. The modest effects of DksA and ppGpp on CsrA are likely overshadowed by their strong effects on CsrB/C, and are not shown in the diagram. Similarly, the modest effect of *csrA* on *dksA'-'lacZ* expression, which is masked by DksA negative autoregulation, is not shown. In

another feedback loop (not shown), CsrA represses *csrD* expression, which is needed for RNase E-dependent turnover of CsrB/C RNAs (Suzuki *et al.*, 2006).

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