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Global regulation by CsrA and its RNA antagonists

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Abstract

The sequence-specific RNA binding protein CsrA is employed by diverse bacteria in the posttranscriptional regulation of gene expression. Its binding interactions with RNA have been documented at atomic resolution and shown to alter RNA secondary structure, RNA stability, translation and/or Rho-mediated transcription termination through a growing number of molecular mechanisms. In *Gammaproteobacteria*, small regulatory RNAs that contain multiple CsrA binding sites compete with mRNA for binding to CsrA, thereby sequestering and antagonizing this protein. Both the synthesis and turnover of these sRNAs are regulated, allowing CsrA activity to be rapidly and efficiently adjusted in response to nutritional conditions and stresses. Feedback loops between the Csr regulatory components improve the dynamics of signal response by the Csr system. The Csr system of *E. coli* is intimately interconnected with other global regulatory systems, permitting it to contribute to regulation by those systems. In some species, a protein antagonist of CsrA functions as part of a checkpoint for flagellum biosynthesis. In other species, a protein antagonist participates in a mechanism in which a type III secretion system is used for sensing interactions with host cells. Recent transcriptomics studies reveal vast effects of CsrA on gene expression through direct binding to hundreds of mRNAs, and indirectly through its effects on the expression of dozens of transcription factors. CsrA binding to basepairing sRNAs and novel mRNA segments, such as the 3′ UTR and deep within coding regions, predict its participation in yet to be discovered regulatory mechanisms.

INRODUCTION

The Csr (carbon storage regulator) or Rsm (repressor of stationary phase metabolites) system is among the most extensively studied bacterial RNA-based regulatory systems. Its central component, the RNA binding protein CsrA (RsmA), was uncovered by a transposon mutagenesis screen designed to identify regulators of gene expression in the stationary phase of growth, using glycogen biosynthesis and *glgC′-′lacZ* expression as reporters (1). Understanding of RNA binding proteins and their roles in regulation was limited at that

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time, but included Hfq and ribosomal proteins that mediate negative feedback by binding to their mRNAs (2–4). Soon after its discovery, the regulatory role of CsrA began to emerge, which included repression of other genes similar to *glgC*, which are expressed in stationary phase or under stress conditions (5), and evidence that CsrA activates gene expression that supports robust growth (6). Discoveries that CsrA (RsmA) regulates virulence genes of pathogens associated with plant disease (7) and mammalian cell invasion (8) offered early glimpses of the widespread roles played by CsrA proteins in microbe-host interactions (9). The role of CsrA in biofilm formation (10–14), quorum sensing (15), carbon metabolism (6, 16, 17), motility (18, 19), and stress responses (14, 20–23) is now well documented in *E. coli* and other species. New functions of CsrA are being uncovered at a rapid pace through the use of transcriptomics, proteomics, metabolomics and other systems approaches (12, 13, 20, 24–35).

Early evidence that CsrA regulates gene expression posttranscriptionally was that it activates *glgC* mRNA decay, which requires the *glgC* translation initiation region but not the promoter region (36), and that CsrA binds to *glgC* mRNA and blocks translation by occluding the SD sequence (37, 38). CsrA activity is regulated by noncoding RNAs that compete with mRNAs for CsrA binding. The first of these sRNAs, CsrB, was identified as a component of a ribonucleoprotein (RNP) complex, isolated by purification of a recombinant CsrA protein (39). The stoichiometry of the CsrA:CsrB RNP complex suggested that CsrA likely bound to CsrB RNA at highly repeated CAGGA(U/A/C)G sequences. These sequences resemble the SD sequence of mRNAs and were located in predicted stem-loops and single-stranded segments of this sRNA. Discovery of CsrC, an sRNA that functions similarly to CsrB, soon followed (40). While the *csrB* and *csrC* loci were both uncovered earlier in a genetic screen designed to identify regulators of *glgC* expression (41), understanding of the function of these regulatory RNAs awaited the discovery of CsrA. Altogether, these findings set the stage for the development of a new paradigm in genetic regulation, in which a sequence-specific RNA binding protein is sequestered and antagonized by a noncoding RNA containing binding sites that mimic its mRNA target sequences.

RNA SEQUENCE AND STRUCTURAL FEATURES OF CSRA BINDING SITES

Early studies with CsrB provided the first suggestion that a GGA motif in the loop of a short hairpin was an important component of a CsrA binding site (39). A consensus sequence for CsrA binding sites was determined using systematic evolution of ligands by exponential enrichment (SELEX). The SELEX-derived consensus sequence for a single high-affinity CsrA binding site was determined as RUACARGGAUGU, with the GGA motif being 100% conserved. The GGA motif was typically located in a hexaloop (ARGGAU) of an RNA hairpin, with the upstream AC and downstream GU residues always base-paired to one another (Fig. 1A). Mutagenesis of one SELEX-derived RNA target indicated that the GGA motif and the preceding A residue in the loop of the hairpin were critical for *E. coli* CsrA binding (42). This study established that the primary RNA sequence is most important for CsrA binding, and that the presentation of the GGA motif in a loop increases the affinity of CsrA-RNA interaction. More recently, a SELEX method that exploited deep sequencing of RNAs identified a consensus for *Pseudomonas aeruginosa* RsmA and RsmF as

CANGGAYG, with the GGA motif typically found in a hexaloop (43). This sequence and structural arrangement is remarkably similar to that determined for *E. coli* CsrA.

CSRA AND CSRA-RNA STRUCTURE

Crosslinking studies with purified CsrA established that *E. coli* CsrA functions as a homodimer (5, 43). Subsequent structural studies demonstrated that each subunit of the dimer contains five β-strands (β_1-β_5), an α-helix, and a flexible C terminus (44). Strands β_1 and β_5 of one monomer hydrogen bond to β_4 and β_2 of the other monomer, forming a mixed antiparallel β-sheet (44). Similar structures were later determined for RsmA of *Yersinia enterocolitica* (45), RsmE of *Pseudomonas fluorescens* (46), and CsrA of *Geobacillus thermodenitrificans* (47). Alanine scanning mutagenesis of *E. coli* CsrA identified amino acid residues in the β_1 and β_5 strands that are critical for RNA binding (48). The structure of a *P. fluorescens* RsmE-RNA complex confirmed and extended these findings (Fig. 1B). This structure revealed that the RsmE homodimer binds to two RNA molecules simultaneously (46). The RNAs were bound on positively charged surfaces formed by the β_1 and β_5 strands of opposite monomers, with each dimer interacting with all six bases in the hexaloop (ACGGAU). As expected, the GGA motif is specifically recognized by the protein; the Watson-Crick face of both G residues and the Hoogsteen face of the A residue form hydrogen bonds with the protein. In addition, the Watson-Crick face of the A residue preceding the GGA motif makes specific contacts with the protein (46). Interestingly, both of the SELEX studies described above identified an A residue at this position. Even though this upstream A is not as highly conserved as the GGA motif, mutagenesis of the *E. coli* consensus sequence indicated that altering this A residue caused a more severe binding defect than changing the A in the GGA motif itself (42).

Although the SELEX-derived consensus for *E. coli* CsrA and *P. aeruginosa* RsmA/RsmF is virtually identical, this method selected for high affinity sites. With the exception of the conserved GGA motif, CsrA binding sites within natural RNA targets exhibit considerable sequence variation. To identify the structural basis for recognition of varying RNA sequences, structures of *P. fluorescens* RsmE in complex with a variety of RNA sequences were determined. This study provided an explanation for how the variation of sequence and structural context of the GGA motif modulate the binding affinity (49). Recent structural studies also demonstrated that RsmE binding to multiple sites in its regulatory sRNAs is not random, but rather occurs sequentially in an ordered manner (50).

DIRECT CSRA-MEDIATED REGULATION

Repression of Translation Initiation

Repression of translation initiation was the first detailed regulatory mechanism identified for CsrA (38) (Fig. 2A). This common regulatory strategy has led to CsrA being called a “translational repressor.” However, this is an inadequate description of CsrA function, as this protein has been shown to participate in a wide variety of regulatory mechanisms, including mRNA stabilization and destabilization, transcription attenuation, and activation of translation.

The sequence of CsrB provided critical insight into possible modes of CsrA action. The fact that GGA is a common feature of the Shine-Dalgarno (SD) sequence, an important component of the ribosome binding site, led to the suggestion that CsrA might repress translation of mRNA targets. This prediction was first verified by the demonstration that CsrA represses translation initiation of *glgC,* which encodes a glycogen biosynthetic enzyme (38). CsrA binds to four sites in the 5′-UTR of *glgCAP* mRNA, one of which overlaps the *glgC* SD sequence, such that bound CsrA prevents 30S ribosomal subunit binding (38, 51). Since CsrA functions as a homodimer with two identical RNA binding surfaces, CsrA is capable of bridging a high-affinity binding site to the lower affinity site overlapping the *glgC* SD sequence (51). Structural studies confirmed dual-site binding (50). This general mechanism of translational repression has been substantiated for numerous *E. coli* mRNA targets, including *csrA* itself (5, 9, 11, 14, 20, 22, 23, 52–55). Similar translation repression mechanisms have also been identified in several other bacterial species (31, 56–59). In some cases, CsrA binding sites overlap the start codon (11, 22, 23, 55, 58, 59), or initially translated region (5, 15).

Several translational repression mechanisms differ substantially from the general mechanism just described. RsmA of *P. aeruginosa* represses translation of *psl*, a gene encoding a component of biofilm matrix. In this case, bound RsmA appears to stabilize a structure that sequesters the *psl* SD sequence in an RNA secondary structure (60). In another instance, *E. coli* CsrA indirectly represses translation of *iraD*, a gene encoding an antiadapter protein that inhibits RssB-mediated degradation of RpoS. CsrA represses translation of a leader peptide whose stop codon overlaps with the *iraD* start codon. Thus, translational repression of *iraD* occurs entirely via translational coupling (22). Lastly, unlike other known CsrA-mediated translational repression mechanisms in which CsrA binds to two or more sites, CsrA is capable of repressing *hfq* translation by binding to a single site that overlaps its SD sequence (61). The commonality in all of these examples is that bound CsrA inhibits ribosome binding. Although not always the case, reduced translation often leads to destabilization of the downstream mRNA (9, 53).

Transcription Attenuation

In addition to repressing translation initiation of *pgaA* (11), CsrA participates in a transcription attenuation mechanism in which bound CsrA prevents formation of an RNA secondary structure that would otherwise sequester a Rho binding site (Fig. 2B). Thus, bound CsrA mediates Rho-dependent termination of the nascent *pgaABCD* operon transcript (62). Since CsrA also represses translation of *nhaR*, which encodes an activator of *pgaABCD* operon transcription (14, 63), and the expression of GGDEF domain proteins that synthesize cyclic-di-GMP, an allosteric activator of poly-β-1,6-*N*-acetyl-D-glucosamine (PGA) synthesis (12, 13, 64), CsrA represses the biosynthesis and secretion of the PGA biofilm adhesin by at least four distinct mechanisms.

Activation Mechanisms

The *E. coli moaABCDE* operon is controlled by a MOCO-dependent riboswitch that represses its translation, whereas bound CsrA activates *moaA* translation by altering the RNA structure, thereby increasing the accessibility of the ribosome binding site (65). In

another interesting example, RsmA of *P. aeruginosa* activates translation of *phz2*, a phenazine biosynthetic gene cluster. In this case, bound RsmA appears to activate translation by destabilizing an SD-sequestering hairpin (66). Lastly, CsrA activates expression of the *E. coli flhDC* operon, which encodes a DNA binding activator of flagella biosynthesis and chemotaxis. CsrA binds to two sites that are >150 nucleotides (nt) upstream of the *flhD* SD sequence, one of which is positioned at the extreme 5′ end of the transcript. In this case, bound CsrA stabilizes the *flhDC* transcript by blocking 5′ end-dependent cleavage by RNase E (19) (Fig. 2C).

RNA-MEDIATED CSRA ANTAGONISM

Discovery of sRNA Antagonists Of CsrA

CsrB of *E. coli* was the first identified sRNA antagonist of CsrA (Table 1) (39). This 369 nt sRNA contains 22 potential CsrA binding sites and is capable of sequestering ~9 CsrA dimers (39). Soon after the discovery of *E. coli* CsrB, RsmB of *Pectobacterium carotovorum* was identified as an sRNA antagonist of its CsrA homolog, RsmA. This 479 nt sRNA contains 20 GGA motifs (67). A second sRNA, CsrC, was later identified as a redundant *E. coli* CsrA antagonist (40), although recent evidence indicates that these two sRNAs are differentially expressed (68). CsrC contains 13 GGA motifs, many of which were predicted to be in short RNA hairpins, as was previously observed for CsrB (40). Multiple sRNA antagonists have been identified in a variety of species, indicating that this is a common strategy for modulating CsrA/RsmA activity (69–73). However, the sRNAs in *P. aeruginosa* (RsmY and RsmZ) and *P. fluorescens* (RsmX, RsmY and RsmZ) are much shorter and contain only 5 to 8 GGA motifs (69, 71).

Additional RNAs may sequester CsrA

CsrB/C RNAs may be dedicated solely to CsrA sequestration; no additional roles for these sRNAs have been identified. Nevertheless, other RNAs appear to bind to and sequester CsrA as a “moonlighting” function, in addition to their other roles (Fig. 3). The 5′-UTR of *fimAICDHF* mRNA of *Salmonella enterica* contains two CAGGAUG sequences that sequester CsrA, along with CsrB/C, as part of a hierarchical control mechanism for fimbriae expression. In this mechanism, abundant expression of the mRNA for Type I fimbriae prevents expression of plasmid-encoded fimbriae from *pefACDEF* mRNA, which requires CsrA binding for its expression (74). This sequestration mechanism apparently helps *Salmonella* to avold the costly expression of plasmid-encoded fimbriae outside of the host. Because CsrA has little effect on *fimA* expression, it appears that high affinity binding of CsrA to *fimA* mRNA acts primarily in hierarchical control.

Two basepairing sRNAs have been reported to act by sequestering CsrA, McaS and GadY. These sRNAs contain GGA sequences that allow interaction with CsrA, and upon overexpression, these RNAs are able to activate *pgaA* expression by antagonizing CsrA activity (75, 76). Increased expression of the *pgaABCD* operon promotes both synthesis and secretion of the biofilm adhesin poly-β-1,6-*N*-acetyl-D-glucosamine, PGA, and biofilm formation (77). High-throughput sequencing of RNA isolated by crosslinking and immunoprecipitation (CLIP-seq) studies have indicated that CsrA interacts directly with

other sRNAs *in vivo* in *Salmonella* and *E. coli*, respectively (29, 35). High affinity binding of CsrA to *E. coli* sRNAs GadY, Spot 42, GcvB, and MicL, was confirmed *in vitro*, although biological functions for the three latter RNAs have not determined (35).

Ribosome pausing as a mechanism leading to CsrA sequestration?

Because there appears to be little RNA-free CsrA in the cell (53) any tight-binding RNA expressed at sufficient levels should compete for CsrA binding with lower affinity transcripts. Recent findings from transcriptomics studies have led to the discovery that CsrA binding occurs at the 3′ UTR of a few transcripts, but occurs predominantly deep within the coding regions of mRNAs (29, 31, 34, 35). Furthermore, CsrA binding within mRNA coding regions is significantly increased in frequency near ribosome pause sites (35). While CsrA was not found to cause ribosome pausing, it is possible that ribosome pausing facilitates CsrA binding near pause sites. On a transcriptome-wide scale, this might serve as an effective means of restricting CsrA availability when the capacity for translation is limited and pausing is increased. Described below, CsrA activity is inhibited during amino acid limitation by the positive effects of ppGpp and DksA on CsrB/C transcription (20). Conceivably, these two mechanisms might act together, thus increasing the expression of factors that deal with translational stress and other stresses, and decreasing gene expression required for rapid growth, under translational stress.

PROTEIN ANTAGONISTS OF CSRA

Although sRNA antagonism of CsrA/RsmA is a common feature of Csr/Rsm systems, some organisms use a protein to antagonize the activity of CsrA (Fig. 3). CsrA represses translation initiation of *Bacillus subtilis hag*, the gene encoding the flagellar filament protein (56). FliW was identified as the first protein antagonist of CsrA (78). FliW, CsrA and Hag participate in a protein partner switching mechanism to control Hag synthesis. Following completion of the flagellar hook, secretion of Hag releases FliW from a FliW-Hag complex. Once released, FliW can instead bind to CsrA, thereby relieving CsrA-mediated translational repression of *hag*, so that Hag synthesis is increased precisely when it is needed for synthesis of the flagella. Thus, Hag homeostatically restricts its own translation (78). FliW does not bind to the same residues of CsrA required for *hag* mRNA binding. Some *csrA* mutants abolished CsrA-FliW binding, but others did not, indicating that FliW and RNA interaction is not mutually exclusive (79). Structural analysis of the CsrA-FliW complex from *G. thermodenitrificans* indicates that each CsrA subunit binds to a FliW monomer (47). This structure also revealed that FliW interacts with a C-terminal extension of CsrA, and that FliW allosterically antagonizes CsrA in a noncompetitive manner by excluding RNA from the RNA binding surface of CsrA. An essentially identical FlaA-FliW-CsrA partner switching mechanism is responsible for controlling CsrA-mediated translational repression of *flaA*, which encodes the major flagellin in *Campylobacter jejuni*. Interestingly, *flaA* mRNA is expressed and localized at the cell poles, which depends on the FlaA-FliW-CsrA network (31). Notably, *B. subtilis* and *C. jejuni* lack an sRNA antagonist of CsrA, while *E. coli* lacks FliW and the C-terminal extension of CsrA.

Whereas FliW appears to be the only CsrA antagonist in *B. subtilis* and *C. jejuni*, enteropathogenic *E. coli* (EPEC), whose genome contains the *csrB* and *csrC* genes (80), uses a recently identified protein, CesT, to antagonize CsrA (55). CesT functions as a chaperone for effectors that are injected into host epithelial cells by a type III secretion system (T3SS). Following effector injection, CesT binds to and antagonizes CsrA. The CesT-CsrA interaction leads to changes in virulence and metabolic gene expression, which is likely required for EPEC's adaptation to life on the epithelial surface (55).

REGULATION OF CSRB/C TRANSCRIPTION

BarA-UvrY TCS and its orthologs

The recognition that CsrB and CsrC antagonize CsrA activity in *E. coli* (39, 40) made it crucial to understand how the levels of these RNAs are regulated (Fig. 4). CsrB/C transcription requires the two component signal transduction system (TCS) BarA-UvrY (40, 81, 82), referred to as GacS-GacA or other names in various species (9, 68). This TCS likely activates transcription of most, though not necessarily all of the Csr/Rsm sRNAs in the *Gammaproteobacteria,* a notable exception being CsrC of *Yersinia pseudotuberculosis* (83). The membrane-bound sensor-kinase, BarA, is a protein with tripartite architecture, which appears to use a His -> Asp -> His phosphorelay prior to phosphorylation of its cognate response regulator, UvrY, a FixJ-family DNA binding protein (81, 84). In *E. coli*, BarA-UvrY signaling is activated by short chain carboxylate compounds such as formate and acetate, which are sensed by BarA (85). In addition, acetyl-phosphate can directly phosphorylate and activate UvrY (85, 86). Abundant acetate and other short chain carboxylates in the mammalian intestinal tract suggests that CsrA activity may be decreased in this environment. TCA cycle intermediates and citrate appear to serve as signals for orthologous TCS in *Pseudomonas fluorescens* and *Vibrio fischeri*, respectively (87, 88). The precise signaling mechanisms in all of these cases remain mysterious.

Genomic crosslinking experiments (ChIP-exo) in *E. coli* and *Salmonella* showed that P-UvrY or P-SirA crosslinks to *csrB* and *csrC* DNA at two locations; one far upstream of and another overlapping the promoter (68). The *csrB* upstream site contains an inverted repeat sequence (TGTGAGAGATCTCTTACA) followed by a partial repeat (TGTAGGAGA) in both species; binding to *csrC* occurred at similar sequences. Binding at the promoter apparently represents indirect formaldehyde crosslinking of P-UvrY to DNA, e.g. via RNA polymerase, as only the upstream location is bound by the purified P-UvrY protein *in vitro*. ChIP-exo and ChIP-seq experiments in *E. coli*, *Salmonella* and *P. aeruginosa* have demonstrated that the Csr/Rsm sRNA genes represent the major or sole binding targets of the response regulator UvrY, SirA, or GacA, respectively, suggesting that the global regulatory effects of these TCS are mediated largely or entirely via the Csr/Rsm system (26, 68).

Factors that regulate BarA and UvrY expression affect CsrB/C levels. These include two DEAD-box RNA helicases, DeaD (CsdA), which activates translation of *uvrY* by counteracting long-range inhibitory basepairing interactions between the 5′-UTR and coding regions of this transcript (89), and SrmB, which somehow stimulates the binding of P-UvrY to *csrB* DNA without affecting the levels of this protein (68, 89). Regulation by

DeaD helicase may help to support *uvrY* translation under conditions of reduced translation capacity (89). The CsrA protein itself activates *uvrY* transcription and translation indirectly, and is required to switch BarA protein from acting as a UvrY phosphatase to a kinase (90). The autoregulatory circuitry that includes these interactions is discussed below.

Regulation of CsrB/C transcription by starvation and stress conditions

Several factors in addition to BarA-UvrY regulate CsrB/C sRNA transcription in response to nutrient starvation, extracytoplasmic stress and other stresses. Furthermore, CsrA mediates reciprocal effects on the genes for a number of the corresponding stress response factors, apparently to fine tune regulation of stress responses (discussed below). The stringent response system detects amino acid starvation and other stresses and responds by activating the synthesis of the alarmone (p)ppGpp, which in turn activates *csrB/C* transcription (20, 68). Effects of (p)ppGpp are often potentiated by the protein DksA; both of these molecules bind to RNA polymerase and modify its activity (91, 92). Because ppGpp and DksA stimulated expression from a *csrB* promoter reporter *in vitro*, these effects appear to be mediated directly (68). Recent studies revealed that *csrB/C* transcription is also activated by the extracytoplasmic stress response system, which is centered on the sigma factor RpoE or σ^E (23). However, in this case, RpoE indirectly activates transcription of CsrB/C sRNAs from σ^{70} promoters. Physiological implications of this regulation are discussed below. The catabolite repression system also affects CsrB/C expression (21). In contrast to the effects of stringent response and extracytoplasmic response systems, CsrB and CsrC transcription is repressed by the mediator of this carbon starvation stress response, cAMP-CRP. As discussed below, CsrB/C turnover is also stimulated by active glucose transport, causing complex interplay of the Csr circuitry with cAMP-CRP and other carbon regulatory systems. Finally, we should note that the regulation of Csr/Rsm sRNA transcription can vary greatly in other species. For example, in *P. aeruginosa* three sensor kinases in addition to GacS (BarA) govern the phosphosporylation state of GacA (UvrY), none of which is present in *E. coli* (93).

ACTIVATION OF CSRB/C DECAY BY PREFERRED CARBON SOURCES

The intracellular level of an RNA molecule reflects the rates of its synthesis, decay and dilution by growth. Decay of CsrB/C sRNAs of *E. coli* is tightly regulated, and requires the endonuclease RNase E and the 3′-to-5′ exonuclease polynucleotide phosphorylase (PNPase), housekeeping enzymes widely involved in RNA turnover (94). In contrast to basepairing sRNAs, turnover of CsrB/C is unaffected by Hfq, but requires a specificity factor referred to as CsrD. The *csrD* gene was uncovered in a transposon screen for mutations that decreased transcription of a *csrB-lacZ* fusion; yet this mutation increased the levels of CsrB (94). This initially puzzling result occurs because the Csr system operates via negative feedback loops. Disruption of *csrD* stabilizes CsrB/C, causing sequestration of CsrA. Because CsrA activates *csrB/C* transcription (82, 90, 95), this causes *csrB-lacZ* transcription to decrease. Unlike CsrB/C RNA, *lacZ* mRNA stability is not affected by CsrD, thus in the *csrD* mutant *csrB-lacZ* expression is decreased. This feedback loop also prevents CsrC RNA from accumulating in the *csrD* mutant, although it is greatly stabilized in this strain, and attenuates the effect of *csrD* disruption on CsrB levels.

The CsrD protein has features suggestive of a signaling protein. It contains two N-terminal membrane-spanning domains, a HAMP-like domain, and degenerate GGDEF and EAL domains (94). All domains are required for its activity, with the exception of the membrane-spanning domains, which are dispensable if the protein is overexpressed. Unlike the classical GGDEF and EAL domain proteins, the domains of CsrD are not involved in synthesis, turnover or recognition of the signaling molecule c-di-GMP, presumably because of the degeneracy of these domains. In addition, CsrD itself is not a nuclease (94, 96). Recent studies demonstrate that its EAL domain mediates a binding interaction that activates CsrD and triggers CsrB/C decay in response to the availability of a preferred carbon substrate, such as glucose (97). The CsrD EAL domain binds only to the unphosphorylated form of $EIIA^{Glc}$, which predominates when glucose is being transported via the phosphotransferase system (PTS). In this way, glucose activates CsrB/C decay through its effect on the phosphorylation state of $EIIA^{Glc}$. Because $EIIA^{Glc}$ does not affect CsrD levels, this binding interaction appears to allosterically activate CsrD (Fig. 4). The CsrD-$EIIA^{Glc}$ decay pathway appears to also operate in *Vibrio cholerae* (97) and perhaps throughout *Enterobacteriaceae, Vibrionaceae*, and *Shewanellaceae* families (94). The absence of CsrD in other *Gammaproteobacteria*, which express Csr/Rsm sRNAs, raises questions about its evolution.

While *Gammaproteobacteria* extensively use sRNAs to sequester CsrA, most families of this bacterial class lack a CsrD ortholog (68, 94, 96). Thus, their Csr-family sRNAs apparently decay differently than CsrB/C of *E. coli*. Turnover of RsmY sRNA is relatively slow in *Pseudomonas fluorescens,* 26 min vs. ~2 – 4 min for CsrB/C in *E. coli* (94, 98). Furthermore, while *csrA* disruption in *E. coli* has little or no effect on CsrB/C decay in a *csrD* wild type strain (95, 96), *rsmA (csrA*) disruption destabilizes RsmY. Examination of the CsrB decay mechanism helps to explain the distinct turnover patterns in these species, and the reason that RNase E is unable to trigger CsrB decay in the absence of CsrD in *E. coli* (94, 96). RNase E initially cleaves CsrB within an unstructured RNA segment located immediately upstream of the intrinsic terminator (96). CsrA binding to two sites adjacent to this cleavage site blocks RNase E cleavage in the absence of CsrD. In a *csrD* mutant and *in vitro*, CsrA binding to CsrB protects it against turnover and RNase E cleavage, respectively, similar to the effect of RsmA (CsrA) on RsmY sRNA in *P. fluorescens* (96). Therefore, the evolution of CsrD has provided *E. coli* with a means of bypassing CsrA-mediated protection of CsrB/C sRNAs; even in the presence of CsrA their turnover is activated by the availability of glucose or other preferred carbon sources. Therefore, end products of metabolism, such as formate and acetate, activate CsrB/C transcription (85) while preferred carbon activates their turnover (97). Thus, carbon nutritional cues favor enhanced CsrA activity when glycolytic metabolism and other growth promoting pathways are required and decreased CsrA activity when carbon nutrition is limited. Additional carbon nutrition effects on the behavior of Csr circuitry are discussed below.

FEEDBACK AND AUTOREGULATORY CIRCUITS PERMIT RAPID CSR RESPONSES

Following the discovery that CsrB and CsrC sRNAs act as antagonists of CsrA, CsrA itself was found to indirectly activate CsrB/C transcription in *E. coli* (40, 82, 95). This indirect

activation occurs via positive effects of CsrA on *uvrY* expression and the ability to cause BarA to switch from its phosphatase to kinase activity (90). The negative feedback loop that this creates in the Csr system allows CsrB and CsrC to mediate compensatory effects on each other's expression (40, 94) (Fig. 4). A separate negative feedback loop is created in which CsrA represses CsrD, which is needed for turnover of CsrB/C RNAs (13, 94). Negative feedback can cause a number of regulatory outcomes, including acceleration of response times and decreased cell-cell variability (99, 100). In the Csr system, negative feedback has been shown to reduce response times (101). Sequestration of CsrA by CsrB/C allows rapid reduction of CsrA activity without the need for its dilution via growth, while CsrD-mediated decay of CsrB/C rapidly releases CsrA for interaction with other RNAs. Compensatory regulatory interactions between CsrB and CsrC should reduce cell-cell variability in CsrA activity, although this remains to be demonstrated. Following its description in *E. coli*, compensatory sRNA regulation has been demonstrated in other Csr systems (102) and in circuitry involving redundant basepairing sRNAs (103).

CsrA mediates complex regulation of its own expression, including direct negative and indirect positive effects, which occur simultaneously (Fig. 4) (52). CsrA binds to 4 sites in its own mRNA leader, preventing ribosome binding to the SD sequence and allowing for rapid inhibition of its translation (52). Transcription of *csrA* involves 5 promoters, recognized by σ^{70} and/or σ^{S} RNA polymerase. CsrA indirectly activates transcription from the strong P3 promoter. This promoter responds to σ^{S} as well as σ^{70} and permits the CsrA protein to accumulate approaching the stationary phase of growth. CsrB/C levels also accumulate at this stage of growth (40, 89, 95). The ability of CsrB/C RNAs to sequester a large number of CsrA dimers allows them to cause a net decrease in CsrA activity upon entry to stationary phase growth, even as CsrA protein levels are increased. Perhaps the latter increase in CsrA levels positions the Csr system for a robust response when conditions favorable for growth are restored.

INTERACTION OF CSR WITH OTHER GLOBAL REGULATORY SYSTEMS

In the wake of early evidence that CsrA (RsmA) influences the expression of other regulators (18, 104), transcriptomics and other studies have uncovered vast potential for bacterial Csr systems to interact with other regulators and global regulatory systems (e.g. 12, 13, 20, 24–35). Most of these interactions have not been further investigated, but in a few cases the integration of transcriptional and Csr posttranscriptional regulatory circuitry have been studied. For example, RNA-seq analysis indicated that CsrA binds to mRNAs for stringent response components (RelA, DksA, SpoT), leading to the discovery of reciprocal circuitry connecting these two regulatory networks (20) (Fig. 5A). Amino acid starvation or other stresses trigger the synthesis of (p)ppGpp by the RelA or SpoT proteins (91, 105). In conjunction with DksA, (p)ppGpp activates transcription of the CsrA antagonists CsrB/C (20, 68). In addition, CsrA represses both the *relA* and *dksA* genes, although the latter effect can be partly masked by DksA autoregulation (20, 105). The positive feedback loop: (p)ppGpp –> CsrB/C –| CsrA –| RelA enhances expression of RelA and perhaps DksA during the stringent response and should allow *relA* expression to be repressed after stress has been eliminated. For the many genes that respond oppositely to CsrA and ppGpp, this

circuitry should allow Csr to reinforce the transcriptional effects of (p)ppGpp at the posttranscriptional level (20).

Reciprocal interactions also occur between the Csr and extracytoplasmic function (ECF) systems (23) (Fig. 5B). The *E. coli* ECF system responds to cell envelope stress via the proteolytic inactivation of a cytoplasmic membrane protein, RseA, which acts as an anti-sigma factor of the ECF sigma factor, RpoE or σ^E (106). Release of σ^E from its membrane association upon RseA cleavage allows it to access core RNA polymerase and activate transcription of a large set of genes (107, 108). RseA and RpoE mRNAs were identified by copurification with CsrA as well as CLIP-seq analysis (20, 35). Further studies showed that CsrA binds to *rpoE* mRNA at 4 sites and inhibits *rpoE* translation by directly blocking ribosome access to the translation initiation region of *rpoE* mRNA (23). Reciprocally, Eσ^E activates CsrB/C transcription indirectly (23). The resulting positive feedback loop: RpoE –> CsrB/C –| CsrA –| *rpoE*, presumably allows CsrA to reinforce regulation by the ECF system and to assist in restoring basal σ^E levels when membrane damage has been repaired. Full appreciation of the role of Csr in extracytoplasmic stress will require further investigation of CsrA inhibitory effects on RseA (35). Interestingly, ppGpp activates *rpoE* expression in the stationary phase of growth, independently of envelope stress (109), which should create additional positive feedback between the Csr and ECF systems (Fig. 5B).

Multiple regulatory connections are apparent between CsrA and genes for other carbon metabolism regulators, most of which are not well understood (20, 35). Interactions with the classical catabolite repression system have been investigated, with unexpected results (21) (Fig. 5C). In addition to regulating CsrB/C sRNA decay (described above), the phosphorylation state of $EIIA^{Glc}$ has other vast effects on physiology and metabolism. When glucose is being transported by the PTS system, unphosphorylated $EIIA^{Glc}$ binds to and inhibits transporters of alternative carbon sources, while under carbon limitation P-$EIIA^{Glc}$ activates adenylate cyclase (110). Thus synthesized, cAMP binds to cAMP receptor protein (CRP) and globally reprograms *E. coli* transcription, the basis of “catabolite repression” (111–113). RNA-seq analysis of transcripts that copurified with CsrA identified the *crp* and *cyaA* transcripts, a finding that was confirmed by *in vitro* binding studies (20, 21). While CsrA exhibited modest, conditional inhibitory effects on *crp* expression, cAMP and CRP exhibited substantial negative effects on transcription of *csrB* and *csrC*. cAMP-Crp repressed *csrC* transcription by directly competing with UvrY-P for binding to the *csrC* promoter region, while it repressed *csrB* transcription indirectly. These findings imply that $EIIA^{Glc}$-dependent regulatory pathways permit glucose to stimulate both the turnover (97) and synthesis (21) pathways of CsrB/C. In this way, it appears that the presence of glucose has the potential to create a futile cycle that should poise Csr sRNAs for rapid response to changing conditions, while carbon starvation may reduce both arms of this cycle, thereby conserving resources.

CONCLUDING REMARKS

The Csr system is among the best known RNA-based regulatory systems, with a wide-reaching influence on gene expression and physiology in diverse bacterial species. The structural biology of RNA binding by CsrA is now firmly understood and is contributing to

the unraveling of new RNA regulatory mechanisms. Transcriptomics and other systems-based investigations are uncovering the details of its vast influence on bacterial life-style decisions, which involve major shifts in physiology. They have also revealed that CsrA binding is not restricted to the 5′ segment of mRNAs, but occurs extensively within mRNA coding regions and is enriched near ribosome pause sites. In addition, CsrA binds not only to sRNAs that appear to serve only to sequester this protein, such as CsrB and CsrC, but also to sRNAs that function in RNA-RNA basepairing. Understanding the functions of these newly discovered RNA binding activities of CsrA will be crucial for a full appreciation of its regulatory capacity. Regulatory circuitry of the Csr system is well suited for rapid homeostatic responses. Csr interacts in complex ways with other global regulatory systems, in some cases it reinforces transcriptional effects of stress response systems at a posttranscriptional level. While much progress has been made in understanding the regulatory roles of CsrA and its sRNA antagonists, it is clear that much more remains to be learned.

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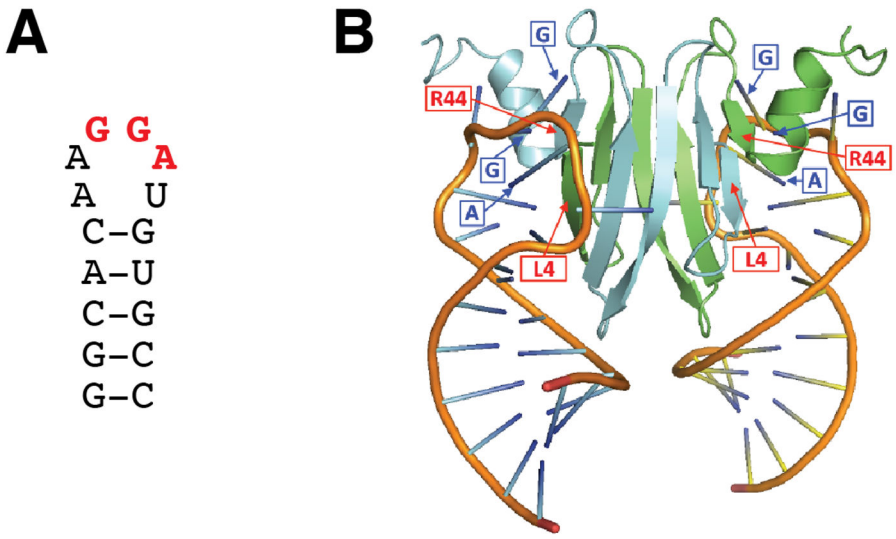

Fig. 1.

(A) Example of a high affinity CsrA binding site. The conserved GGA motif is in red. (B) structure of the CsrA-RNA complex. The GGA motifs are indicated by blue boxes and the critical L4 and R44 residues are indicated in red. Adapted from (9) with permission.

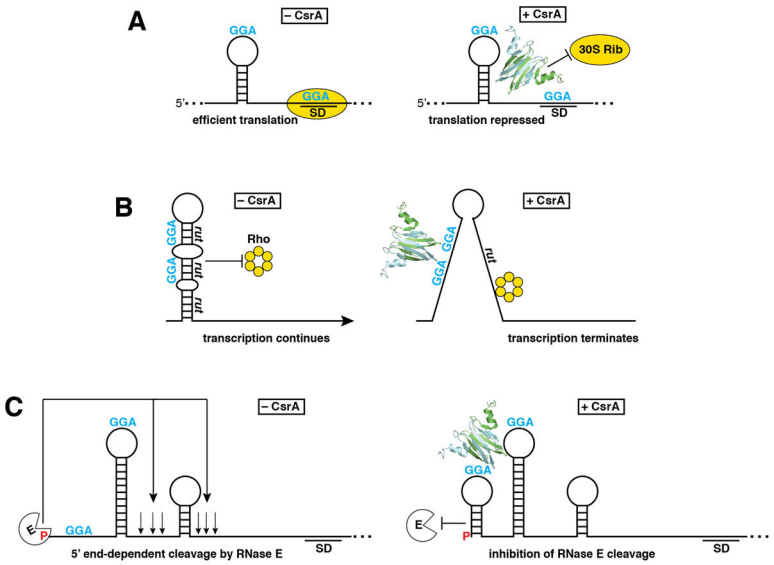

Fig. 2.

Mechanisms for CsrA-mediated translational repression (A), transcription termination (B) and protection of mRNA from nuclease cleavage. Adapted from (9) with permission.

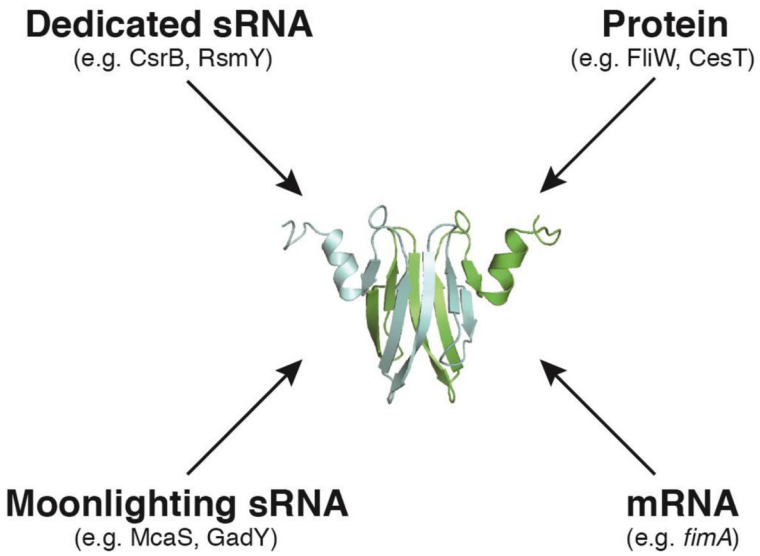

Fig. 3.

Modes of CsrA antagonism. In various species, dedicated sRNAs, moonlighting sRNAs, mRNA, and/or proteins have been found to bind to CsrA and inhibit is activity.

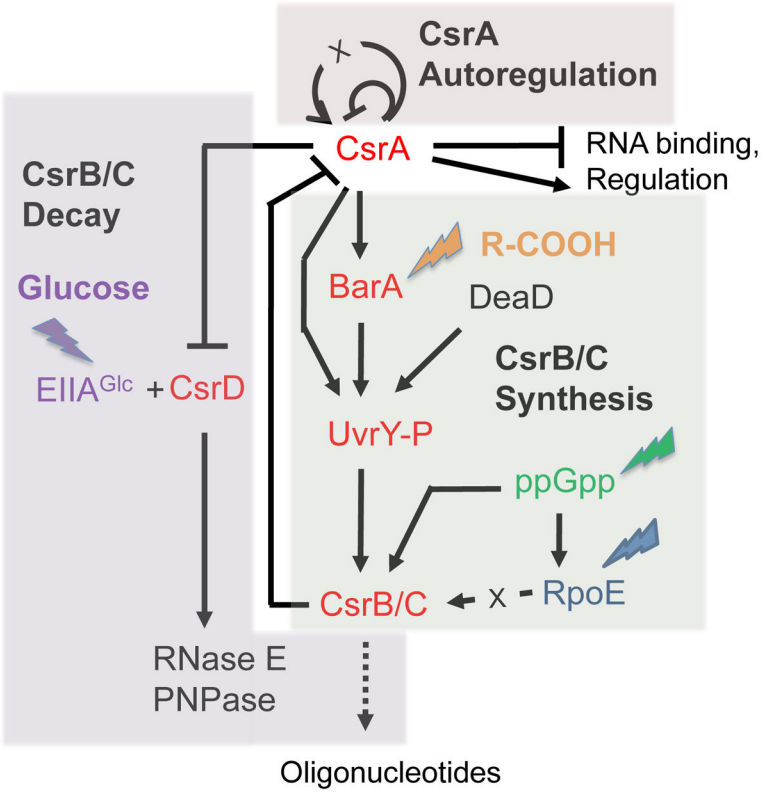

Fig. 4.

Central regulatory circuitry of the Csr system. Dedicated components of the Csr system are highlighted in red.

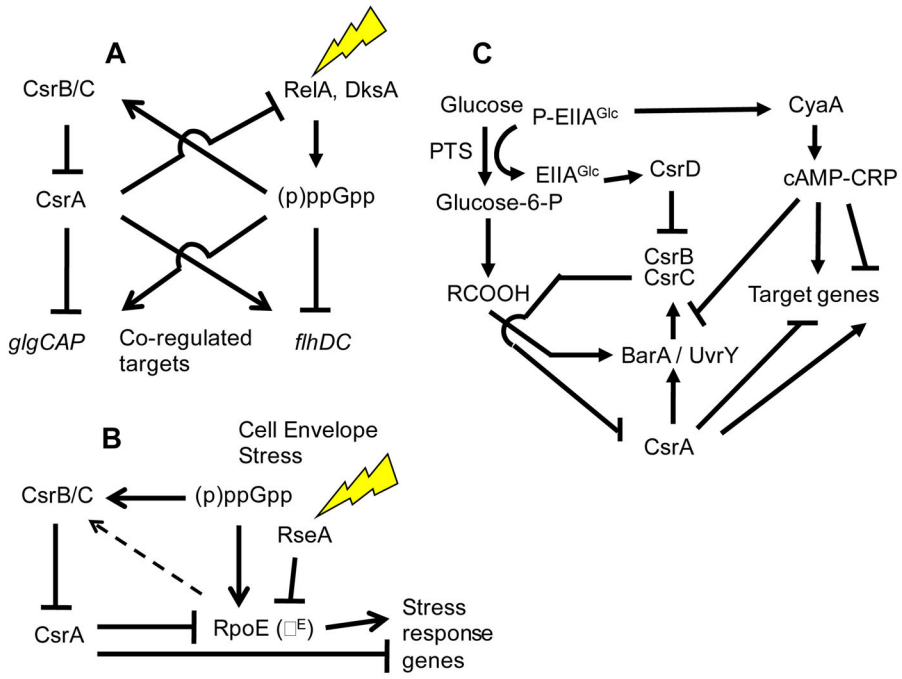

Fig. 5.

Regulatory interactions of the Csr system with stringent response (A), extracytoplasmic stress (B), and carbon catabolite repression (C) global regulatory systems. Adapted from (20), (23), (21) with permission.

TABLE 1

CsrA and its antagonists

Organism	CsrA homolog	Antagonist[a]
E. coli	CsrA	CsrB, CsrC
E. coli (EPEC)	CsrA	CesT
P. carotovorum	RsmA	RsmB
S. Typhimurium	CsrA	CsrB, CsrC, *fimA*
P. aeruginosa	RsmA, RsmF (RsmN)	RsmY, RsmZ
P. fluorescens	RsmA, RsmE	RsmX, RsmY, RsmZ
B. subtilis	CsrA	FliW
G. thermodenitrificans	CsrA	FliW
C. jejuni	CsrA	FliW

[a]All of these antagonists are RNAs, with exception of the CesT and FliW proteins.