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Regulation of Bacterial Virulence by Csr (Rsm) Systems

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SUMMARY

Most bacterial pathogens have the remarkable ability to flourish in the external environment and in specialized host niches. This ability requires their metabolism, physiology, and virulence factors to be responsive to changes in their surroundings. It is no surprise that the underlying genetic circuitry that supports this adaptability is multilayered and exceedingly complex. Studies over the past 2 decades have established that the CsrA/RsmA proteins, global regulators of posttranscriptional gene expression, play important roles in the expression of virulence factors of numerous proteobacterial pathogens. To accomplish these tasks, CsrA binds to the 5′ untranslated and/or early coding regions of mRNAs and alters translation, mRNA turnover, and/or transcript elongation. CsrA

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activity is regulated by noncoding small RNAs (sRNAs) that contain multiple CsrA binding sites, which permit them to sequester multiple CsrA homodimers away from mRNA targets. Environmental cues sensed by two-component signal transduction systems and other regulatory factors govern the expression of the CsrA-binding sRNAs and, ultimately, the effects of CsrA on secretion systems, surface molecules and biofilm formation, quorum sensing, motility, pigmentation, siderophore production, and phagocytic avoidance. This review presents the workings of the Csr system, the paradigm shift that it generated for understanding posttranscriptional regulation, and its roles in virulence networks of animal and plant pathogens.

INTRODUCTION

Prior to the discovery of CsrA in the early 1990s, bacterial gene expression was understood to be regulated at the level of transcription initiation and to a lesser extent by transcription termination. At that time, examples of posttranscriptional regulation included the role of Hfq (then called host factor 1) in bacteriophage Qβ replication and limited roles of ribosomal proteins in translation control (1, 2). Hfq is now recognized as an RNA chaperone that promotes the interaction of numerous base-pairing small RNAs (sRNAs) with their mRNA targets and CsrA as a sequence-specific RNA binding protein, both of which globally influence gene expression and virulence (3–8). Other posttranscriptional regulators also participate in bacterial virulence networks, including RNA helicases, ribonucleases, and the Crc protein of pseudomonads (9–15).

The *csrA* (carbon storage regulator A) gene was originally uncovered by a transposon mutagenesis screen that was designed to identify regulators of gene expression in the stationary phase of growth. The *csrA* mutation had pleiotropic effects on genes involved in carbon flux pathways and phenotypes, including cell morphology and surface adhesion (16, 17). A CsrA homolog (95% identity) called RsmA (repressor of secondary metabolites) was later identified in *Pectobacterium carotovorum* and shown to repress pathogenicity in plant hosts through effects on extracellular lytic enzymes and quorum sensing (18, 19). CsrA homologs have since been studied in a variety of animal and plant pathogens. In addition to controlling metabolism and fundamental physiological properties, CsrA is critical for the regulation of dedicated virulence systems required for host infection.

This review addresses the workings of the Csr system, including the structure and function of the CsrA protein, the Csr sRNAs that introduced the concept of molecular mimicry as a strategy for riboregulation, and the global role of this system in regulating gene expression. A discussion of the Csr regulatory circuits is provided for a number of important pathogens, including *Escherichia coli*, *Pseudomonas* spp., *Legionella pneumophila*, and *Vibrio cholerae*. These sections will highlight (i) important regulatory targets of CsrA/RsmA pertinent to virulence, (ii) environmental factors and regulatory circuits that control CsrA/RsmA activity, and (iii) features of CsrA/RsmA regulatory circuitry that are unique for each pathogen. The contributions of CsrA to virulence in *Salmonella* spp., *Yersinia* spp., pathogenic *E. coli* strains, and plant pathogens, as well as its regulatory role in beneficial biocontrol species, will also be discussed. Finally, we address recent studies on the regulation of CsrA by an anti-CsrA protein (FliW) in the Gram-positive bacterium *Bacillus subtilis* and the implications of these findings for bacterial pathogens. A summary of Csr (Rsm) systems is presented in Table 1.

CsrA/RsmA: Structure and Function

Because the amino acid sequence of CsrA was unrelated to known proteins, its regulatory mechanism was originally unclear (17, 20). Studies on its regulatory target *glgC*, which encodes the glycogen biosynthetic enzyme ADP-glucose pyrophosphorylase, allowed its mode of action to be determined. Surprisingly, CsrA regulation of a *glgC′-′lacZ* fusion did not depend on the presence of a native *glgC* promoter and required a region that overlapped the *glgC* ribosome binding site (RBS) (20). These results suggested that CsrA may control gene expression at the posttranscriptional level. Consistent with this hypothesis, *glgC* mRNA was destabilized by CsrA (20). It was later determined that CsrA binding to the *glgC* mRNA leader blocks ribosome access, suggesting that its effect on transcript stability in this case may be secondary to its effect on translation (21).

Subsequent to those seminal discoveries, the understanding of CsrA structure and function has advanced dramatically. Structural studies of *E. coli* CsrA and its homolog, *Yersinia enterocolitica* RsmA, revealed a dimer of identical subunits, each containing 5 tandem β-strands, a short α-helix, and a flexible C terminus (Fig. 1) (22, 23). The β-strands of the two polypeptide monomers are interwoven to form a hydrophobic core, and two C-terminal wing-like α-helices extend away from the protein. A comprehensive alanine-scanning mutagenesis study by Mercante et al. revealed that two identical surfaces of *E. coli* CsrA mediate RNA binding and regulation, which are formed primarily by the parallel β1 and β5 strands of opposite polypeptides (24). Structural analyses of CsrA-RNA complexes later confirmed that the amino acid residues that are critical for regulation interact directly with bound RNA (25). In addition, these structural studies revealed contacts between the polypeptide backbone and RNA that were not identified by alanine-scanning mutagenesis (24, 25).

The features of RNA that are recognized by CsrA were first suggested following the discovery of the CsrA-inhibitory sRNA CsrB. CsrB unexpectedly copurified in a large globular complex with CsrA and was found to compete with lower-affinity mRNA targets for CsrA occupancy (26, 27). Finding CsrB was fortuitous not only because of its importance as a regulator of the Csr system (discussed below) but also because it suggested the RNA sequence and structure elements of the CsrA binding site. A stochastic RNA folding algorithm predicted that CsrB contains 15 RNA hairpin structures, most of which possess a GGA motif located in a single-stranded loop with conserved flanking sequences, predominantly CAGGA(U/A/C)G (26). Single-stranded GGA sequence motifs also typify the CsrA binding sites of mRNA untranslated leader segments (21, 28–35). This conserved sequence resembles the Shine-Dalgarno (SD) sequence of ribosome binding sites, to which CsrA often binds in translation repression mechanisms.

To further define the CsrA binding site, systematic evolution of ligands by exponential enrichment (SELEX) experiments were performed to identify RNA ligands that contain high-affinity CsrA binding sites from a pool of random RNAs (36). A total of 55 selected RNA ligands were analyzed, 100% of which contained either one (50 RNAs) or two (5 RNAs) GGA motifs. A majority (92%) of the RNAs (51 RNAs) were predicted to contain GGA motifs within the loop of an RNA hairpin, and in fact mutations that disrupted the hairpin reduced CsrA binding affinity. The

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SELEX-derived consensus was determined to be RUACARGGA UGU, with the ACA and GGA motifs being 100% conserved. Recently, solution nuclear magnetic resonance (NMR) and other studies of the CsrA homolog RsmE binding to the sRNA RsmZ or short oligonucleotides derived from RsmZ have provided additional evidence that the sequence and structural context in which the GGA motif resides determine the binding affinity (37).

Since CsrA exists as a symmetrical dimer with two RNA binding surfaces, it was predicted to be able to bridge two sites on a single RNA molecule. Mutant CsrA dimers containing an alteration in one or both RNA binding surfaces were tested for binding to a series of model RNAs, revealing that the homodimer is able to bridge two binding sites separated by 10 to 63 nucleotides (nt) (38). This intersite bridging mechanism plays a regulatory role in *glgC* expression, where binding of a CsrA homodimer to a high-affinity site upstream of the RBS apparently allows CsrA to bridge to a lower-affinity site that overlaps the RBS, thus inhibiting translation (Fig. 2) (38). Other examples of CsrA function may not involve bridged CsrA binding sites (30). Recent structural studies confirmed dual-site binding and further revealed that CsrA/RsmA dimers assemble sequentially onto the binding sites of regulatory sRNAs, as opposed to binding to them randomly (37, 39).

Dynamic Effects of CsrA on Translation, RNA Turnover, and Transcription Termination

As discussed above, CsrA represses translation of *glgC* by directly occluding the RBS (Fig. 2), and similar translation repression mechanisms have been described for *sdiA*, *nhaR*, *cstA*, and other genes of *E. coli* and other organisms (25, 33, 34). Studies by Irie et al. revealed that CsrA can also repress translation by stabilizing the formation of RNA secondary structure that blocks translation (40) (Fig. 2). The following discussion addresses recently described mechanisms by which CsrA regulates translation, RNA stability, or Rho-mediated transcription termination.

Riboswitches are *cis*-acting RNA elements in which the binding of a small molecule ligand within a folded RNA aptamer regulates gene expression (41, 42). A class of riboswitches was recently proposed to bind the molybdenum cofactor (Moco), which serves as a redox center for many metabolic enzymes (43). *E. coli moaABCDE* mRNA is required for Moco biosynthesis and contains a prototypical riboswitch element from this family, which is negatively regulated by Moco. Studies by Patterson-Fortin et al. showed that CsrA binds to the 5′ untranslated leader of *moaA* at two sites within its proposed aptamer domain and activates translation, likely by increasing ribosome accessibility (Fig. 2) (35). These studies introduced a new model for riboswitch function in which two different factors interact with an RNA aptamer to regulate expression.

Pseudomonads synthesize phenazine compounds that serve as precursors of secreted factors that affect biocontrol and virulence pathways (44). *P. aeruginosa* contains two phenazine biosynthetic gene clusters referred to as *phz1* and *phz2*. Ren et al. proposed a model in which RsmA posttranscriptionally activates expression of *phz2* by binding to the leader transcript and disrupting an RNA structure that would otherwise block the RBS (Fig. 2) (45). Though this model has yet to be demonstrated *in vitro*, it is supported by RNA structure predictions and *in vivo* expression studies with wild-type and mutant *phz2* leaders. Similar to CsrA regulation of *moaA* expression in *E. coli*, this is an example of translational activation and is related to repression mechanisms

TABLE 1 Overview of Csr (Rsm) systems

Organism	Homolog	TCS(s)	Inhibitory RNA(s)	CsrD (MshH)	Direct target(s) (reference)[a]	Regulated phenotype(s)[c]
E. coli	CsrA	BarA/UvrY	CsrB, CsrC, McaS	+	*glgCAP* (21), *sdiA* (33), *pgaABCD* (29), *nhaR* (34), *cstA* (28), *csrA* (32), *flhDC* (46, 47), *LEE4 locus* (312), *grlRA* (312)	Motility, biofilm formation, glycogen metabolism, T3SS
S. Typhimurium	CsrA	BarA/SirA	CsrB, CsrC, *fimAICDHF*	+	*hilD* (269) *fimAICDHF* (285), *pefACDEF* (285), *STM1987*, *STM3375*, *STM3611*, *STM1344*, *STM1697* (84)	Motility, SPI1 T3SS, biofilm formation
P. aeruginosa	RsmA	GacS/GacA	RsmY, RsmZ	−	*psl* (40), *tssA1* (80), *fha1* (80), *phz2* (45), *rahU*, *algU*, *pqsR*, *PA1300* (56), *magA* (80)	T3SS, T6SS, quorum sensing, biofilm formation, type IV pili
	RsmF (RsmN)	?	?	−	*tssA1* (157)	T6SS, biofilm formation
P. fluorescens	RsmA	GacS/GacA	RsmX, RsmY, RsmZ	−	*hcnA* (167)	Motility, secreted biocontrol factors
	RsmE	GacS/GacA	RsmX, RsmY, RsmZ	−	*hcnA* (167)	Motility, secreted biocontrol factors
L. pneumophila	CsrA	LetS/LetA	RsmX, RsmY, RsmZ	−		Motility, T4SS, pigment production
V. cholerae	CsrA	VarS/VarA	CsrB, CsrC, CsrD	+		Quorum sensing, biofilm formation
Y. pseudotuberculosis	CsrA	BarA/UvrY, PhoQ/PhoP	CsrB, CsrC	+	*flhDC* (333)	Invasin production
P. carotovorum	RsmA	GacS (ExpS)/GacA (ExpA)	RsmB	+		Cell wall-degrading exoenzymes
Xanthomonas spp.	RsmA	?	?	−	*hrpG*, *hrpD* (100)	Motility and T3SS, exoenzymes, EPS
P. syringae	RsmA	GacS/GacA	RsmB, RsmX, RsmY, RsmZ	−		Motility, secreted virulence factors
B. subtilis	CsrA	?	?[b]	−	*hag* (381)	Motility

[a] Direct targets, discussed in this review, were demonstrated by *in vitro* binding assays.
[b] The CsrB gene was predicted via bioinformatics approaches, but it has not been tested (55).
[c] The list of regulated phenotypes is not all-inclusive and is meant to suggest the conservation as well as breadth of regulated processes.

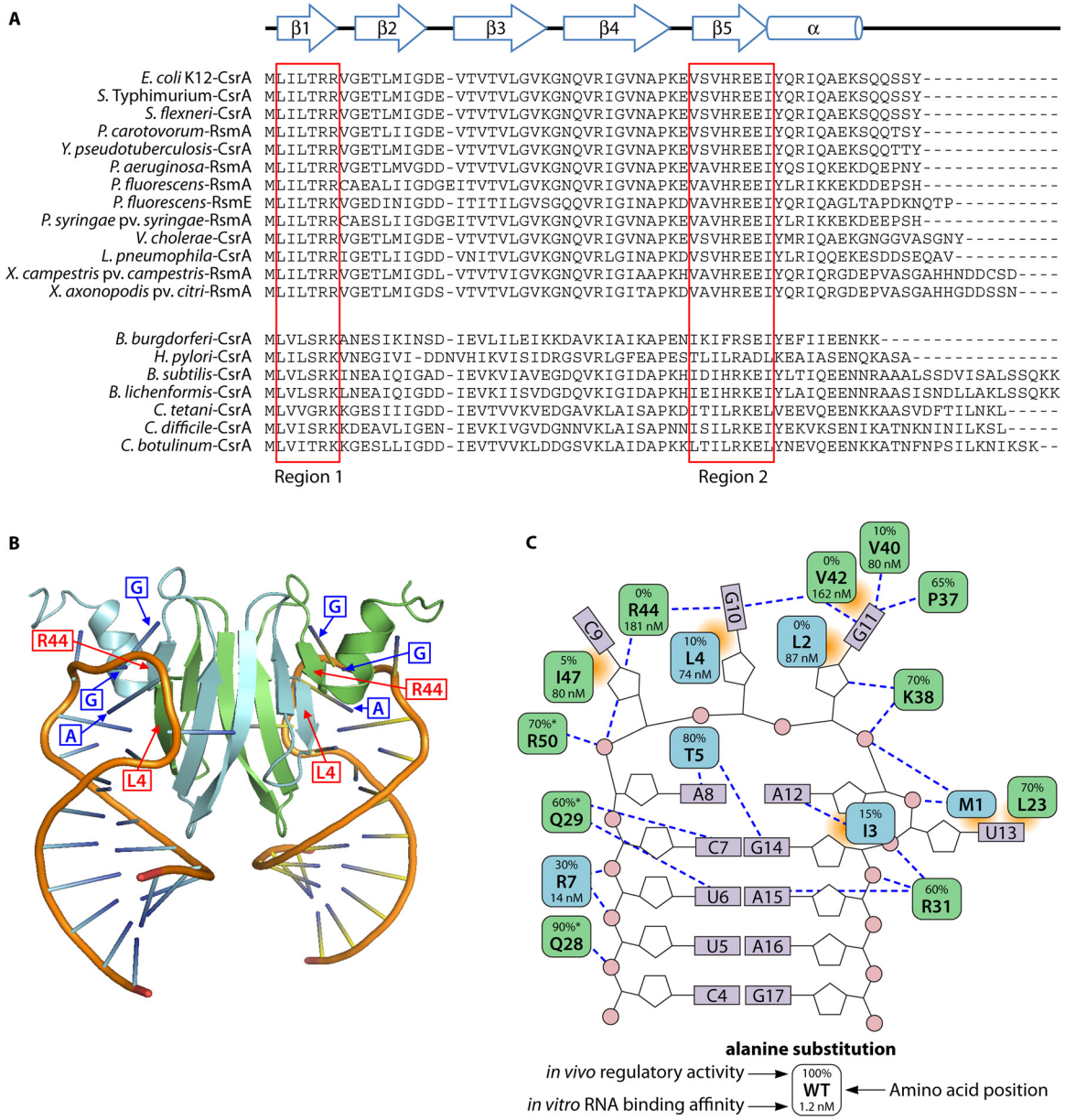


FIG 1 Structure of CsrA/RsmA/RsmE orthologs and the RsmE-*hcnA* RNA complex. (A) Protein secondary structure is shown at the top, with β-strands and α-helices depicted as arrows and cylinders, respectively, and amino acid sequence comparisons are shown immediately below. Sequence alignments from the *Gammaproteobacteria* (top) and from species containing the *csrA* gene in proximity to *fliW* (bottom) are depicted. Red boxes indicate the locations of conserved residues that are important for RNA binding and *in vivo* regulation in *E. coli* (24). (B) Ribbon diagram of the RsmE dimer (*P. fluorescens*) in a 1:2 complex with a 20-nucleotide segment of *hcnA* RNA. Individual RsmE polypeptides are colored with green or cyan, and the *hcnA* ribose-phosphate backbone is shown in orange. The critical R44 and L4 (red boxes) residues of RsmE and the GGA (blue boxes) recognition motif present on each *hcnA* molecule are indicated. The PDB structure file for RsmE-*hcnA* (2JPP) was downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/), and the protein structure was rendered using PyMOL. (Adapted from reference 25 by permission from Macmillan Publishers Ltd., copyright 2007.) (C) Two-dimensional interpretation of the interaction of RsmE with *hcnA* RNA. Amino acid residues contributed by an individual RsmE polypeptide are shown in green or cyan. Hydrogen bonds and hydrophobic interactions are indicated with dashed blue lines and orange highlights, respectively. The effects of alanine substitution on *in vivo* regulatory activities (% of wild type [WT], top) and *in vitro* binding affinities (K_d, bottom) for the *E. coli* CsrA protein were determined by Mercante et al. (24). Asterisks indicate amino acid positions that differ between *E. coli* CsrA and *P. fluorescens* RsmE. (Adapted from reference 25 by permission from Macmillan Publishers Ltd., copyright 2007.)

wherein CsrA mediates accessibility of the RBS by modifying RNA structure (40).

Several regulatory circuits converge on the master operon for flagellum biosynthesis, *flhDC*, to regulate motility in *E. coli*. Wei et al. demonstrated that CsrA activates expression of *flhDC* by binding to this mRNA and stabilizing it (46). Yakhnin et al. later identified the molecular mechanism of CsrA-mediated activation; CsrA binds to two sites within the *flhDC* RNA leader, which prevents 5′ end-dependent cleavage of this transcript by RNase E (Fig. 2) (47). While effects on translation often cause secondary

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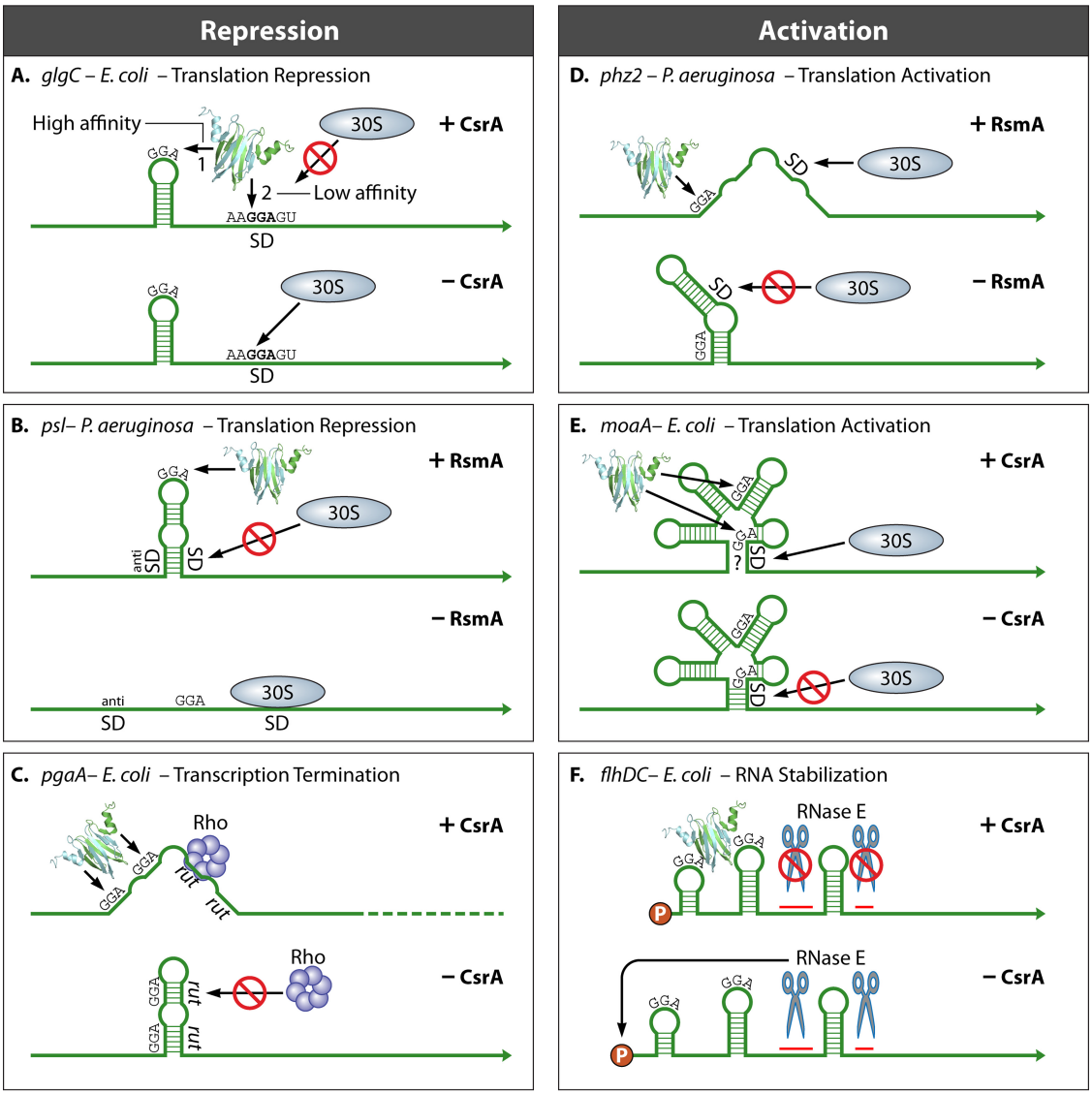


FIG 2 Models for repression and activation by CsrA/RsmA. (A) *E. coli* CsrA represses *glgC* translation by competing with ribosome (30S) binding. (Top) CsrA homodimer first binds to a high-affinity site present in the single-stranded region of an RNA hairpin, located in the 5′ untranslated leader of the *glgC* transcript (20, 21, 38). The tethered CsrA homodimer can then bind via its available RNA binding surface to a low-affinity site that overlaps the SD sequence, thus blocking ribosome binding. (Bottom) In the absence of free CsrA, the ribosome can bind to the SD sequence, and translation can proceed. (B) *P. aeruginosa* RsmA represses translation of *psl* by stabilizing a stem-loop structure that sequesters the RBS. (Top) RsmA can bind to a single site (GGA) present in the 5′ untranslated leader of the *psl* transcript (40). RsmA binding stabilizes an RNA hairpin formed between the SD and anti-SD sequences, thus blocking ribosome access. (Bottom) In the absence of RsmA, the predicted hairpin structure is unstable, and ribosome binding and translation can proceed. (C) *E. coli* CsrA binding promotes Rho-dependent transcription termination of *pgaA*. (Top) CsrA binds to six sites in the *pgaA* mRNA (29), two of which are located in a segment that forms a hairpin in the absence of CsrA (53). CsrA binding prevents hairpin formation and exposes *rut* sites for entry of Rho transcription termination protein. Rho binding leads to premature termination (dashed line) of *pgaA* transcription. (Bottom) In the absence of CsrA, *rut* sites are shielded by RNA base pairing, Rho is unable to bind *pgaA* mRNA, and transcription can proceed. (D) *P. aeruginosa* RsmA binding to the *phz2* untranslated leader prevents the formation of secondary structure that blocks translation (45). (E) *E. coli* activates translation of *moaA* by altering RNA structure. (Top) CsrA binds to two sites (GGA) present within the predicted *moaA* riboswitch aptamer that overlaps the SD (35). CsrA binding alters the aptamer structure, which reveals the SD for ribosome binding. (Bottom) In the absence of CsrA, the riboswitch aptamer sequesters the SD, thus blocking ribosome access. (F) *E. coli* CsrA stabilizes *flhDC* by preventing endonuclease cleavage by RNase E. (Top) CsrA binds to sites (GGA) present in the single-stranded region of two RNA hairpins located at the 5′ end of *flhDC* (47). CsrA binding prevents 5′ end-dependent cleavage by RNase E. (Bottom) In the absence of CsrA, RNase E binds to the 5′ monophosphorylated end of *flhDC* and performs several cleavages that initiate turnover of the transcript (47).

effects on transcript stability (48–50), in this case, regulation of *flhDC* stability is a primary consequence of CsrA binding (Fig. 2).

The *pgaABCD* operon of *E. coli* is required for the synthesis and secretion of a biofilm polysaccharide adhesin (51, 52), and CsrA binding to *pgaA* mRNA represses ribosome binding to and translation of this transcript (29). In addition to this translation repression mechanism, Figueroa-Bossi et al. recently demonstrated that CsrA binding to the 5′ untranslated region (5′ UTR) of the *pgaA* transcript remodels its structure, unveiling binding sites for Rho protein, which causes premature termination of the elongating

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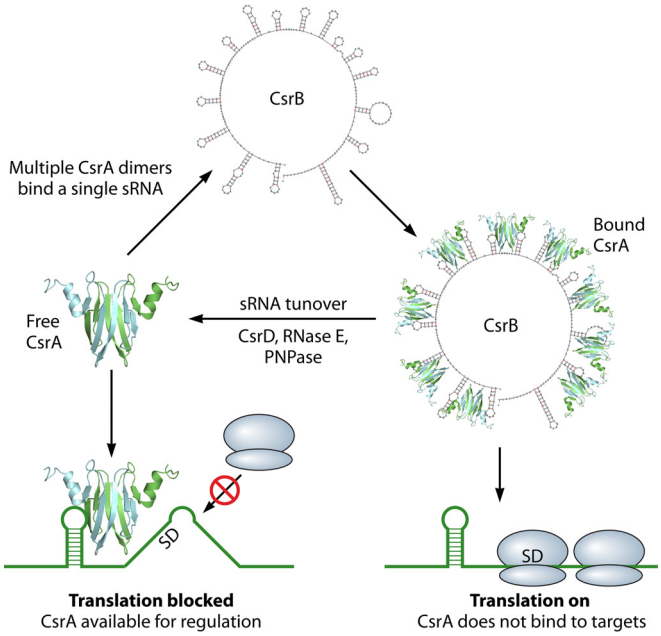


FIG 3 Outline of the Csr system in *E. coli*. CsrA binding to target mRNAs can have several regulatory outcomes: blocking translation initiation (as shown), stabilizing or destabilizing mRNA, or resulting in premature transcriptional termination. The concentration of free CsrA and therefore its regulatory activity depends on the levels of inhibitory small RNAs (CsrB is shown here). These sRNAs can bind to multiple CsrA dimers with high affinity and prevent them from binding target mRNAs. sRNA levels are regulated at the level of transcription (not shown) and turnover. Ribosomes are depicted in blue.

transcript (53, 54) (Fig. 2). This is the first example of CsrA/RsmA directly controlling transcription. How typical this mechanism is for CsrA function remains to be seen. Nevertheless, it is notable that the *pgaABCD* mRNA contains a long untranslated leader, with 6 CsrA binding sites, the largest number presently known for an mRNA and consistent with its complex regulation by CsrA (29).

Regulation of the Csr/Rsm System

sRNAs that sequester CsrA/RsmA proteins. The molecular mimicry paradigm for Csr (Rsm) sRNA function appears to be conserved throughout the *Gammaproteobacteria*, and many bacterial species have multiple Csr sRNAs (3, 55, 56). The inhibitory sRNAs likely represent the primary means of controlling CsrA activity in the species that produce them, and in a few cases, their synthesis and stability have been studied (57–59). Under the growth conditions that have been tested, CsrB appears to be the principle antagonist of CsrA activity in *E. coli* (26, 60). Mutation of *csrB* affects the expression of downstream CsrA targets, and its absence causes pleiotropic effects on *E. coli* physiology (Fig. 3). *E. coli* also produces additional sRNAs that control CsrA activity, CsrC and McaS, and these sRNAs substantially antagonize CsrA activity when overproduced, or in the case of CsrC, its effects are evident in a Δ*csrB* strain background (60, 61). Nevertheless, all three sRNAs bind to CsrA with high affinity *in vitro*. In principle, the presence of multiple negative regulators of CsrA might imply regulatory redundancy, designed to decrease intrinsic noise and increase the robustness of the Csr genetic circuitry (62), or it may also allow the cell to fine-tune CsrA activity in response to different environmental stresses and/or stimuli. Because growth conditions (carbon source) and regulatory factors (Crp, integration host factor [IHF], Hfq, etc.) differentially influence *csrB*, *csrC*, and *mcaS* expression (63–65), these sRNAs apparently have distinct regulatory roles.

The BarA/UvrY TCS activates transcription of CsrA-inhibitory sRNAs. The BarA/UvrY two-component system (TCS) (also referred to as GacS/GacA, VarS/VarA, ExpS/ExpA, LetS/LetA, and BarA/SirA in various species) is the primary regulator of *csrB* and *csrC* expression and is highly conserved throughout the *Gammaproteobacteria* (Table 1) (58, 66). BarA is a membrane-bound, tripartite histidine sensor kinase that uses an unusual phosphorelay mechanism to phosphorylate its cognate response regulator, UvrY (66). BarA activity is stimulated by short-chain fatty acids (SCFAs), the potency of which is negatively correlated with the aliphatic chain length (67, 68). The mechanistic basis of this stimulation remains to be determined. The SCFA acetate is abundant in the intestinal tracts of mammalian hosts, where it may serve as a potent activator of BarA during enteric colonization or infection (68). Alternatively, BarA-independent phosphorylation of UvrY has been observed to occur (both *in vitro* and *in vivo*) via acetyl-phosphate, a high-energy intermediate in the synthesis of acetate (68, 69). Though phosphorylation of UvrY and other response regulators by acetyl-phosphate has been observed in various bacterial species, the physiological relevance/significance of this observation is poorly understood (70–72).

UvrY is a member of the FixJ family of DNA binding proteins, which contain amino-terminal transceiver domains and carboxy-terminal DNA binding domains. UvrY directly binds to the *csrB* and *csrC* promoters *in vitro* (63, 73), yet the details of its transcription activation mechanism have not been determined. Though Csr/Rsm-inhibitory sRNAs of various species may bear little sequence identity outside the CsrA/RsmA binding sequences, their expression in most cases is highly dependent on the UvrY ortholog (73–79). Whether UvrY and its various orthologs have additional regulatory roles remains to be determined, although the GacA protein of *P. aeruginosa* was proposed only to regulate Rsm sRNA transcription (80).

Stability of CsrA-inhibitory sRNAs is tightly controlled in *E. coli*. In a genetic screen for novel regulators of the Csr system, Suzuki et al. discovered CsrD, a protein that specifically targets CsrB and CsrC for RNase E-dependent turnover (57). CsrD-mediated turnover of CsrB/C indirectly regulates the expression of all CsrA target genes and processes that have been examined, including motility, glycogen biosynthesis, and biofilm production. The molecular mechanism by which CsrD facilitates RNase E-dependent cleavage of CsrB/C is presently unclear. CsrD is a membrane-bound protein (C. A. Vakulskas and T. Romeo, unpublished data) that contains GGDEF and EAL domains, which are normally associated with the synthesis and breakdown of the bacterial secondary messenger cyclic di-GMP (c-di-GMP), respectively. However, CsrD contains degenerate or, more aptly stated, evolved domains and does not synthesize or degrade this nucleotide. Other GGDEF or EAL domains of bacterial proteins have also evolved novel roles (81, 82). A CsrD homolog in *Vibrio cholerae*, MshH, was found to interact with the glucose-specific enzyme IIA ($EIIA^{Glc}$) of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), which is a central regulator of carbon metabolism (83). Whether carbon flux regulates CsrB/C RNA turnover through this interaction and which CsrD domain(s) is responsible for binding

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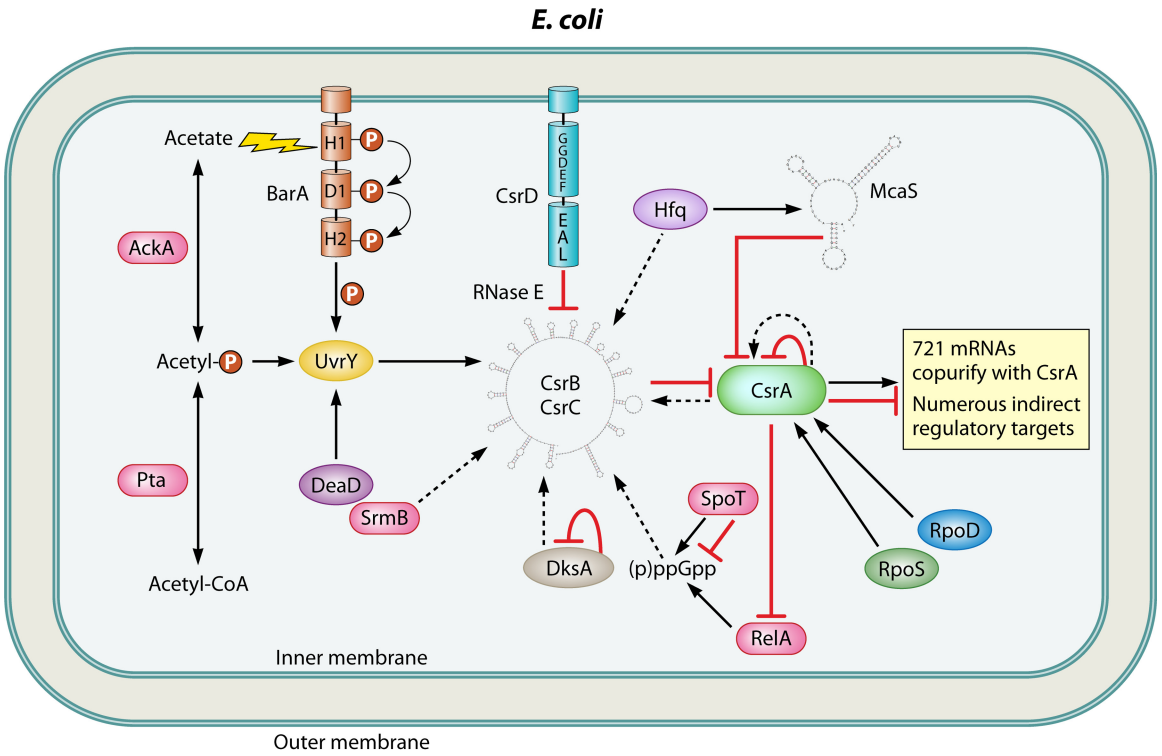


FIG 4 Csr regulatory circuitry in *E. coli*. The inner and outer membranes are indicated. Solid lines indicate regulatory connections with molecular mechanisms supported by experimental evidence, whereas dashed lines show regulatory effects for which a mechanism is lacking. Activation or repression is indicated by a black arrowhead or red T-bar, respectively. A phosphoryl group is indicated by "P." See the accompanying text for details.

to $EIIA^{Glc}$ remain to be determined. In *E. coli* and *Salmonella enterica* serovar Typhimurium, CsrA also represses the expression of *csrD*, suggestive of a negative feedback loop wherein CsrA indirectly stabilizes its sRNA antagonists, although the effect of CsrA on CsrB turnover seems to be negligible (57, 84).

CsrA apparently controls the stability of CsrA/RsmA-inhibitory sRNAs in *Pseudomonas fluorescens* through a mechanism that involves RsmA-dependent protection from RNase cleavage (59). Though the details of this mechanism are uncertain, a *csrA* mutation had little or no effect on the stability of CsrB/C sRNAs in *E. coli* (57). Perhaps this discrepancy is due to the presence of the decay specificity factor (CsrD) in *E. coli*, which is absent in the pseudomonads. A comparison of Csr/Rsm sRNA stability and the presence or absence of a *csrD* homolog in several bacterial genera is needed to further explore these relationships.

CsrA regulates its own expression. Autoregulation is common for global regulatory proteins, and CsrA is no exception. CsrA binds to the untranslated leader of its mRNA and represses translation by competing with ribosome binding (32). CsrA concomitantly activates its transcription indirectly through a mechanism that involves the σ^S-dependent P3 promoter, one of five *csrA* promoters that use either σ^{70} or σ^S for transcription. The finding that CsrA simultaneously activates its own transcription and represses its translation highlights the exquisite level of control that is maintained in this system. The fact that translational repression can be enacted more rapidly than transcriptional activation may suggest the former as a mechanism to rapidly shut off CsrA synthesis when its activity reaches a critical level (32). Thus, the autoregulation of CsrA activity is extremely complex, involving positive and negative feedback loops in CsrA synthesis and negative feedback loops that control the levels of its sRNA antagonists.

Multiple regulatory circuits feed into the Csr system. Other regulatory components besides BarA/UvrY control the levels of CsrB/C sRNAs, many of which function through or in conjunction with the BarA/UvrY TCS (Fig. 4). For example, CsrA indirectly activates the expression of its sRNA antagonists, CsrB/C, indicative of negative feedback regulation (32, 58, 60), and RsmA from *Pseudomonas* spp. similarly regulates expression of the sRNAs RsmX, RsmY, and RsmZ (85, 86). Epistasis analyses and other experiments indicate that these pathways involve UvrY (GacA). Though the available genetic data suggest that CsrA activates CsrB/C expression through the BarA/UvrY TCS, CsrA-dependent regulation of BarA/UvrY protein levels and/or phosphorylation state remains to be thoroughly examined. CsrA/RsmA activation of its antagonists may serve as a homeostatic mechanism that has evolved to minimize dramatic fluctuations in CsrA/RsmA activity under a given condition (3, 87).

Integration host factor (IHF) binds to the promoter of the *csrB* gene of *Salmonella enterica* serovar Typhimurium and the *rsmZ* promoter from *P. fluorescens* (63, 74). Deletion of *ihfA* in *S.* Typhimurium severely decreases *csrB* expression, similar to the case for a *uvrY* deletion, although it does not affect *csrC* expression. IHF typically modifies DNA architecture, affecting DNA supercoiling, duplex stabilization, and global gene expression (88). It commonly regulates transcription from promoters that are directly regulated by multiple transcription factors. Perhaps it should be no surprise that UvrY and IHF function in concert to activate *csrB* expression or that *csrB* transcription is tightly con-

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trolled by specific (UvrY) and global (IHF) regulatory factors. Whether IHF activates *csrB* expression by a typical DNA bending mechanism and the physiological role of this regulation are open questions.

Recently, Vakulskas et al. determined that the DEAD box RNA helicases DeaD and SrmB activate CsrB/C expression in the exponential phase of growth (10). These bacterial proteins traditionally have been associated with rRNA maturation activities, particularly at low temperatures (89). DeaD was found to activate *uvrY* translation by overcoming the effect of a long-range inhibitory RNA structure that forms between the *uvrY* RNA leader and proximal coding sequence. Though the mechanism of SrmB function is not understood, it did not affect the levels or phosphorylation state of UvrY (10). Despite their established roles as cold shock proteins, DeaD and SrmB activated CsrB expression over a broad temperature range, indicative of wider physiological roles for these proteins than was previously appreciated. DeaD also regulated *sirA* (*uvrY*) expression in *S.* Typhimurium, and bioinformatics analysis suggested that this regulatory mechanism is conserved in most of the *Enterobacteriaceae*. The physiological purpose of this kind of regulation remains to be determined, but it may help to maintain adequate levels of *uvrY* expression as the capacity for protein synthesis in the cell declines (10), similar to the biological role of (p)ppGpp in CsrB/C expression (31).

Components of the stringent response, including the alarmone (p)ppGpp and the (p)ppGpp-responsive transcription regulator DksA, strongly activate CsrB and CsrC expression (31). Interestingly, CsrA represses the *relA* gene, which encodes (p)ppGpp synthase I, and (p)ppGpp levels are elevated during the stringent response in a *csrA* mutant background. These findings depict a reciprocal regulatory circuit wherein amino acid starvation activates CsrB/C expression, and the resulting reduction in CsrA activity increases (p)ppGpp production, thus strengthening the stringent response. The Csr system also reinforces effects of the stringent response in another way: genes that are known to be posttranscriptionally repressed (*glgC*) or activated (*flhDC*) by CsrA are transcriptionally activated or repressed by (p)ppGpp, respectively. Thus, when the stringent response is invoked, the direct regulation of these genes by (p)ppGpp is heightened by the decrease in CsrA activity that is caused by (p)ppGpp-mediated induction of CsrB and CsrC expression (31).

The RNA binding protein Hfq positively affects CsrB levels under certain growth conditions (57). Hfq typically mediates pairing of sRNA and mRNA molecules that share weak complementarity, affecting the stability of one or both RNAs and/or translation of the mRNA (90). Because Hfq did not affect stability of the sRNA CsrB, it likely activates *csrB* gene expression indirectly (57).

The Csr system captures the outputs of stress response systems and converges them into global regulation. In various species, CsrA/RsmA regulates environmental stress responses and regulatory factors that mediate stress responses, including the stringent response (31), osmotic resistance (91), heat resistance (91), oxidative stress (92–94), the global stress sigma factors RpoS (94–96) and AlgU (97), and the RNA chaperone Hfq (30). Global studies of CsrA/RsmA target RNAs and transcriptome effects suggest that CsrA/RsmA regulates the expression of many additional stress response genes that have not been studied in detail (31, 56, 98–101). Furthermore, the levels of the CsrA/RsmA-inhibitory sRNAs are controlled by several regulators that mediate stress responses, including DeaD/SrmB (10), Hfq (57, 102), RpoS (32), AlgU (86), Crp (103), and (p)ppGpp/DksA (104–106). These diverse connections of the Csr system to stress responses, in which CsrA/RsmA activity is regulated by the effects of stress responses on Csr/Rsm sRNA levels and CsrA/RsmA regulates stress response genes, imply that Csr/Rsm systems are a centerpiece of global stress response circuitry. Apparently CsrA/RsmA, similar to RpoS, governs its regulon in response to diverse environmental stresses (3, 107). Just how extensively the Csr system serves to capture and channel the effects of stress responses into global posttranscriptional regulation is an important question that is worthy of additional study.

The Repertoire of Direct and Indirect CsrA Targets: the Csr Regulon

The effects of *csrA* deletion and overexpression have been studied in several species using microarray experiments, revealing a profound influence of CsrA/RsmA on the transcriptome. For example, Lawhon et al. demonstrated that in *S.* Typhimurium, a *csrA*::*kan* mutation causes ≥2-fold activation or repression of the steady-state levels of 365 different transcripts, including mRNAs critical for virulence factors such as motility and type III secretion systems (T3SS) (98). Microarray analysis of the *P. aeruginosa* transcriptome revealed that 9% of genes present on the array (506 of 5,570) were significantly affected by *rsmA* deletion, including those associated with virulence systems for iron acquisition, quorum-sensing, motility, and antibiotic resistance (99). Similar experiments have been performed in plant pathogens, *Xanthomonas campestris* pv. campestris, and *Pectobacterium wasabiae*, all of which demonstrated CsrA/RsmA-dependent effects on the expression of a large and diverse set of genes (100, 101). These studies greatly expanded the potential roles of the Csr/Rsm system but were unable to distinguish between direct and indirect regulatory targets.

In an attempt to identify the direct targets of CsrA, Jonas et al. pulse-induced CsrA expression in *E. coli* and monitored changes in mRNA levels as a function of time (108). The rationale was that the direct targets of CsrA would show changes in gene expression more rapidly than indirect targets, which was confirmed using known direct (*pgaA*) (29) and indirect (*csrB*) (58, 87) targets. Though this method was limited by the fact that CsrA is capable of regulating translation independently of effects on mRNA transcription and/or stability, this set of experiments revealed interesting new direct targets of CsrA, including the c-di-GMP signaling proteins YcdT and YdeH (108).

In an approach designed to identify binding targets of *P. aeruginosa* RsmA, Brencic and Lory coprecipitated RNA with RsmA, cloned cDNAs derived from the resulting RNA pool, and sequenced a number of these clones (80). Altogether, 40 likely direct targets of RsmA were identified, including genes involved in T6SS, fatty acid metabolism, cell division, and proteolysis. More recently, Edwards et al. introduced the use of high-throughput RNA sequencing (RNA-seq) to analyze RNA that copurified with *E. coli* CsrA (31). This study identified 721 transcripts that copurified with CsrA, representing genes with extremely diverse functions. These studies to identify the direct RNA targets of CsrA, in concert with observations that CsrA/RsmA serves as a regulator of many other regulators and has remarkable effects on bacterial physiology and virulence, confirm the critical role that the Csr system plays in bacterial gene expression. Current state-of-the-art approaches for studying RNA binding proteins can be used to iden-

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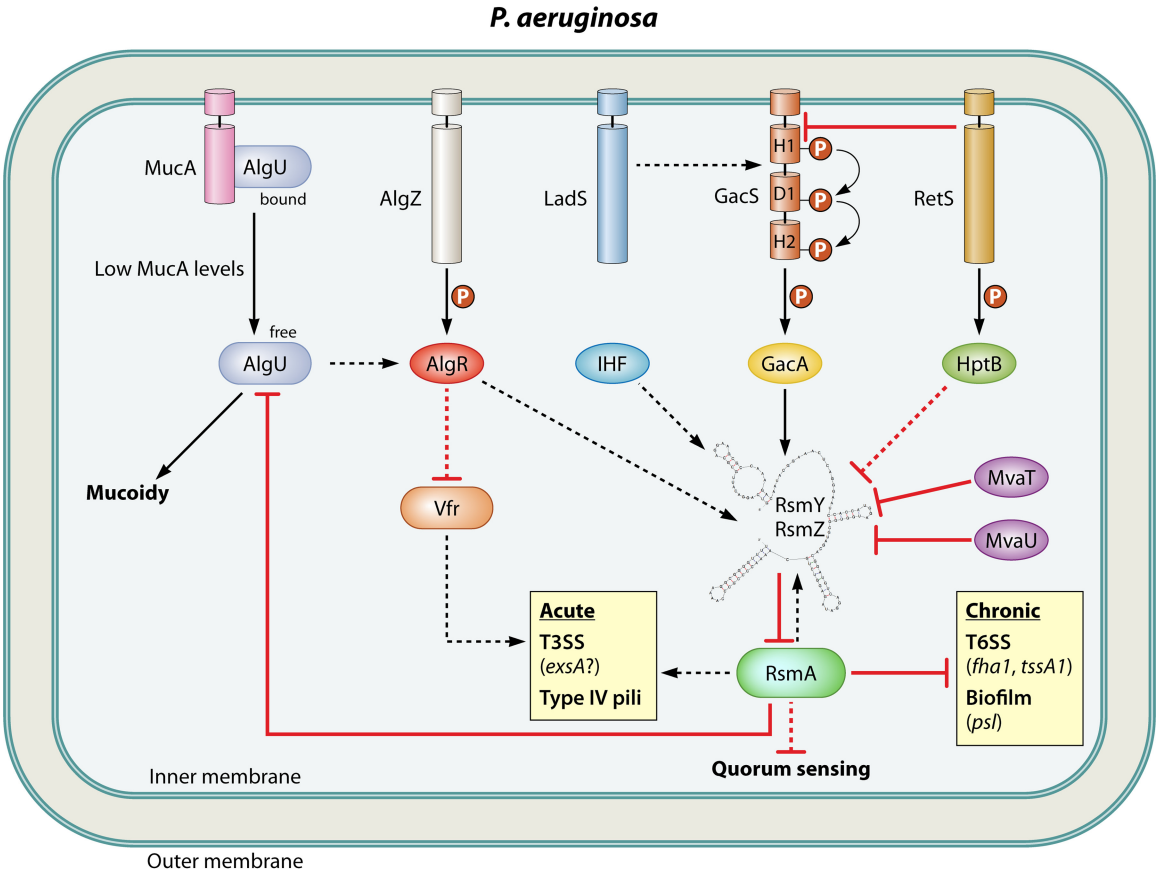


FIG 5 Rsm regulatory circuitry in *P. aeruginosa*, depicted as in Fig. 4.

tify the complete set of bound RNAs as well as the minimum region that is protected by a bound protein (10, 109–111).

Csr/Rsm REGULATE VIRULENCE LIFESTYLES OF GAMMAPROTEOBACTERIAL PATHOGENS

The Pseudomonads

Opportunistic pathogens of the *Pseudomonadaceae* reside primarily in soil and aquatic environments and are known to infect a broad range of hosts using a vast arsenal of secreted factors, including protein toxins and secondary metabolites with antihost properties. *P. aeruginosa* is the most prevalent human pathogen of this group and a major cause of morbidity and mortality in immunocompromised, burned, or wounded patients, as well as debilitating and difficult-to-treat chronic lung infections of cystic fibrosis (CF) patients (112–114). Although *P. fluorescens* is a biocontrol agent that protects plants against fungal pathogens, it is included here because it has served as an important model for studies of *Pseudomonas* Csr/Rsm systems (115–117). Studies have focused on regulation of the virulence and biocontrol systems of each organism. In *P. fluorescens*, this includes genes involved in the production and secretion of the antimicrobial factors alkaline metalloprotease A, hydrogen cyanide, and 2,4-diacetylphloroglucinol (59, 118, 119). In *P. aeruginosa*, RsmA studies have focused on genes involved in T3SS, T6SS, motility, quorum sensing, and biofilm formation (80, 85, 112, 120, 121). *Pseudomonas syringae* strains cause many important diseases of higher plants and will be discussed below along with the other plant pathogens.

***P. aeruginosa*: RsmA regulates the switch between acute and chronic infection.** *P. aeruginosa* causes acute respiratory infections that frequently become chronic in patients with the genetic disorder cystic fibrosis (CF) (122, 123). Patients suffer recurring episodes of acute *P. aeruginosa* infection that can become septic and are nearly impossible to eradicate with antibiotic therapy (124). The switch between acute and chronic infection is characterized by major changes in virulence factor gene expression, which RsmA mediates by positively regulating factors associated with acute virulence, including T3SS and type IV pili, and negatively regulating factors associated with chronic virulence, including biofilm formation and a T6SS (Fig. 5) (113, 125). In a mouse model of acute *P. aeruginosa* infection, *rsmA* mutation significantly reduced colonization during the initial stages of infection, but the loss of *rsmA* ultimately favored persistent infection (112). RsmA also represses the expression of the acyl homoserine lactone (AHL)-producing quorum-sensing systems *las* and *rhl* in *P. aeruginosa*; however, quorum-sensing signals have complex and seemingly conflicting roles in the regulation of acute and chronic virulence phenotypes (126).

Expression of T3SS genes of *P. aeruginosa* is tightly controlled by a partner-switching mechanism involving a cascade of regulatory proteins called ExsA, ExsC, ExsD, and ExsE (127). Transcription of T3SS-associated genes is intimately coupled to the state of the T3SS secretory apparatus by the small, T3SS-secreted regulatory factor ExsE (128). In the absence of inducing conditions (high Ca^{2+} or host cell contact) the secretion channel is closed,

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and ExsE remains cytoplasmic and bound to its chaperone, ExsC. Cytoplasmic ExsE sequesters ExsC away from its other binding partner, ExsD. ExsD is then free to bind ExsA and inhibit its DNA binding ability. ExsA is the master transcriptional activator of all T3SS genes. In the presence of induction, the secretory apparatus is open and ExsE is secreted or translocated into host cells, ExsC binds to and sequesters ExsD, and ExsA is then free to bind T3SS-associated promoters. ExsA activates transcription by recruiting RNA polymerase and promoting open complex formation through a direct interaction with σ^{70} (129–134).

Microarray experiments by Brencic et al. determined that *rsmA* mutation reduces the expression of 22 T3SS-associated genes, including representatives from genes that facilitate secretion, translocation, regulation, and every exotoxin secreted by *P. aeruginosa* strain PAK (80, 133). These results implied that a T3SS-associated transcription factor was likely a target for RsmA regulation. Consistent with this hypothesis, RsmA regulated the T3SS by activating *exsA* expression (80, 86). Whether RsmA directly binds to *exsA* mRNA and how it controls ExsA protein levels is presently unknown. While this RsmA activation pathway serves as a regulatory output for the GacA/GacS TCS to control the T3SS, in the absence of a known environmental stimulus for GacS, the physiological importance of this regulation remains unclear. Other studies by Intile et al. (discussed below) suggested that *P. aeruginosa* isolates from chronic CF patients may lack a functional T3SS in part due to disruption of RsmA-ExsA regulation (86).

Type IV pili are fiber-like protein structures present on the surfaces of a wide variety of bacteria and archaea that are utilized for activities ranging from the uptake of DNA to electron transport (134). In *P. aeruginosa*, type IV pili have known roles in adhesion to host cells, biofilm formation, twitching motility, host immune system evasion, and phage transduction and are critical for establishing acute infection (80, 125, 135, 136). Studies by Brencic and Lory showed that RsmA activates expression of 9 genes involved in type IV pilus biogenesis (80). RsmA-dependent activation of the *pilMNOPQ* operon was observed using transcriptional fusions to *lacZ*, but no effect was observed using translational fusions to *lacZ* driven by a constitutive promoter (also called a leader fusion) (80). These results indicated that most of these effects likely occur indirectly through other factors. AlgR is a known regulator of type IV pilus gene expression; however, it was not identified in the microarray studies (80, 137). The magnitude of RsmA's effect on type IV pilus gene expression (~2 to 4-fold) pales in comparison to its effects on T3SS gene expression (~3- to 25-fold), suggesting that RsmA plays a more nuanced role in controlling type IV pilus production. Additional experiments to determine the effects of RsmA on type IV pilus-dependent phenotypes, such as twitching motility, adhesion, biofilm formation, and pathogenicity, are still needed.

Patients with chronic *P. aeruginosa* infections preferentially express virulence factors that increase persistence and immune system evasion (138–142). Examples of RsmA-regulated virulence factors associated with chronic disease include the ability to form biofilm and the type VI secretion system (T6SS), described below (80, 143, 144). *P. aeruginosa* strains secrete one or more polysaccharides that contribute to the biofilm extracellular matrix, namely, alginate, Pel, and/or Psl (145). Whereas alginate is a uronic acid polymer that is produced by mucoid strains commonly found in patients with late-stage CF infections (146, 147), Pel and Psl are complex biofilm polysaccharides produced by non-mucoid strains from early- to mid-stage CF infections (148, 149). The Psl polysaccharide is a repeating pentamer of D-mannose, L-rhamnose, and D-glucose. Psl surrounds the cell in a helical distribution and is thought to play a role in facilitating cell-cell and cell-surface interactions during biofilm formation (145, 150). Irie et al. demonstrated that deletion of *rsmA* resulted in high biofilm formation and increased levels of *psl* mRNA (40). RsmA binds to the 5′ UTR of *psl* mRNA and facilitates the formation of a stem-loop structure that blocks the ribosome binding site. The Pel polysaccharide forms a fabric-like matrix that connects cells at the air-liquid interface (145, 148). Microarray experiments indicate that RsmA represses the expression of genes involved in Pel polysaccharide biosynthesis (80), though it is presently unclear whether this regulation occurs directly or indirectly.

The T6SS is a specialized secretion system and was originally thought to secrete/translocate only toxic products into host eukaryotic host cells (151–155). However, it is now known to also mediate interbacterial interactions by directly injecting proteins into the cytoplasm of neighboring bacterial cells (156). RsmA-dependent control of the T6SS in *P. aeruginosa* was suggested by results from microarray studies comparing gene expression in wild-type and *rsmA* mutant strains (80). Furthermore, RsmA repressed the expression of at least 12 T6SS-associated genes and bound directly to *tssA1* and *fha1* mRNAs (80, 157). Interestingly, both RsmA and its paralog RsmF (described below) bind the *tssA1* mRNA *in vitro*, and both proteins repress *tssA1* expression *in vivo* (157). RsmF binding to *fha1* was not examined in these studies. Both *tssA1* and *fha1* encode structural components of the T6SS (158). Further studies are needed to better define the mechanism(s) by which RsmA and RsmF repress T6SS and the role(s) played by T6SS in chronic infection.

***P. fluorescens*: RsmA regulates the expression of secreted biocontrol factors.** *P. fluorescens* is a soilborne bacterium that protects plant roots from fungal and nematode diseases. *P. fluorescens* induces systemic resistance phenotypes in plants and secretes lytic enzymes (phospholipase C, tryptophan side chain oxidase, and exoprotease) and antimicrobial compounds (pyoluteorin and 2,4-diacetylphloroglucinol) (118, 159–164). The GacS/GacA (BarA/UvrY) TCS controls the expression of genes involved in the production/secretion of these factors, including the genes for 2,4-diacetylphloroglucinol biosynthesis, *phlACBDE*, the major exoprotease, *aprA*, and hydrogen cyanide synthase, *hcnABC* (119, 160, 163–165). In the closely related pathogen *P. aeruginosa*, GacA activates the expression of RsmY and RsmZ sRNAs, and GacA from *P. fluorescens* follows a similar trend via RsmX, RsmY, and RsmZ (74, 76). Therefore, some or all of the GacA effects on secreted biocontrol factors probably occur through activation of RsmX/Y/Z expression and, hence, inhibition of RsmA activity. Consistent with this hypothesis, deletion of either *gacA* or the *rsmXYZ* sRNA genes reduces hydrogen cyanide secretion, 2,4-diacetylphloroglucinol secretion, exoprotease activity, and swarming motility to a similar extent (166). Lapouge et al. demonstrated that RsmA directly bound to the *hcnA* mRNA at three sites within the untranslated leader using RNA footprinting and toeprinting techniques (167).

RsmA negatively regulates the production of quorum-sensing AHLs in *Pseudomonas* spp. *P. aeruginosa* encodes at least two different AHL pathways, the *rhl* and *las* pathways, which collectively affect the expression of approximately 10% of the genome (168). The production of AHL-dependent exoproducts in *P.*

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aeruginosa, including exoenzymes, pyocyanin, and hydrogen cyanide, is repressed by RsmA (169). Consistent with this finding, RsmA negatively affected the production of both *las* and *rhl* AHLs. Nevertheless, the addition of exogenous AHLs did not fully restore exoproduct production in a strain that overproduces RsmA, which suggested that RsmA may affect exoproduct production at multiple levels. For example, RsmA regulates the *P. aeruginosa* hydrogen cyanide synthesis transcript *hcnABC* directly (169). It was later confirmed that RsmY and RsmZ positively affect the production of quorum-sensing AHLs and exoproducts, consistent with their roles as negative regulators of RsmA (85). The generally accepted role for RsmA in *P. aeruginosa* pathogenesis is that it activates gene expression of virulence factors associated with acute infection (T3SS and motility) and that it represses the expression of virulence factors associated with chronic infection (T6SS and biofilm). Quorum-sensing systems are important for both acute and chronic infections, where they regulate secreted bacterial virulence factors and the host immune response (170). Additional studies are needed to fully understand how RsmA-mediated repression of quorum-sensing systems fits into the chronic/acute paradigm for RsmA regulation or if this model is an oversimplification of its regulatory responsibilities.

In *P. fluorescens* strain CHA0, which lacks quorum-sensing mechanisms controlled by AHLs and other known autoinducers (85, 171), a solvent-extractable, cell population density-dependent signal activates RsmX/Y/Z expression. The chemical nature of this signal is not known; however, it did not appear to be related to AHLs or other known signaling molecules (172). Carboxylate compounds with short aliphatic tails stimulate BarA activity in *E. coli*, and Takeuchi et al. recently demonstrated that increased cellular pools of the carboxylate compounds succinate, malate, and 2-oxoglutarate correlated with an increase in RsmXYZ sRNA levels in *P. fluorescens* (67, 173). While an uncharacterized quorum-sensing chemical could certainly be influencing GacS/GacA function, these findings might also be explained by changes in carboxylate-containing metabolites that fluctuate throughout growth (174).

CsrA paralogs of *Pseudomonas* spp.: RsmA, RsmE, and RsmF. Pseudomonads contain RsmA paralogs that appear to have redundant regulatory roles in *P. fluorescens* (RsmA and RsmE) and both redundant and unique regulatory roles in *P. aeruginosa* (RsmA and RsmF). The RsmA and RsmE proteins from *P. fluorescens* share 71% amino acid identity, and mutations in the *rsmA* and *rsmE* genes have similar effects on repression of the target mRNAs *hcnA*, *aprA*, and *phlA* and activation of the inhibitory sRNAs *rsmY* and *rsmZ* (59). Both RsmA and RsmE bind to the RsmY/RsmZ sRNAs *in vitro* with similar affinity/specificity and antagonize the regulatory effects of these proteins similarly *in vivo*. Though the functions of these two proteins are remarkably similar, their regulation appears to be different. RsmA levels modestly increase throughout growth, while RsmE levels peak sharply in the stationary phase. Regulation of *rsmE* expression appears to occur at least in part due to repression by both RsmA and RsmE (59). At present, regulators that control RsmA levels in *P. fluorescens* have yet to be reported. While future experiments might reveal regulatory targets exclusive to RsmA or RsmE, the available data suggest that the role of RsmE is to reinforce RsmA function in the stationary phase of growth.

In contrast, RsmA and RsmF (RsmN) in *P. aeruginosa* share only 31% amino acid identity and have both shared and exclusive

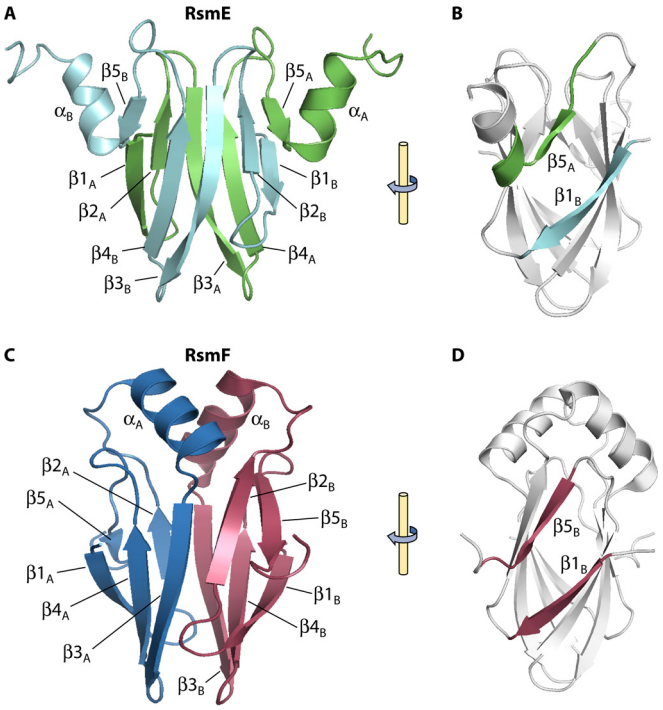


FIG 6 Structural comparison between RsmE of *P. fluorescens* and RsmF (RsmN) of *P. aeruginosa*. (A and C) Cartoons depicting the NMR solution structure of RsmE and crystal structure of RsmF (157, 175). Individual polypeptides of the RsmE homodimer are shown in green or cyan, RsmF polypeptides are shown in blue or maroon, and secondary structural elements are labeled with subscript "A" or "B," as shown. (B and D) Structures of RsmE (top) and RsmF (RsmN) (bottom) rotated 90°. The β-strands that form the RNA interaction surfaces are highlighted using the coloring scheme from panels A and C. Protein structures were rendered using PyMOL. The PDB structure files for RsmE-*hcnA* (2JPP) and RsmF (4K59) were downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/).

RNA targets (157, 175). The *rsmF* deletion strain appears phenotypically virtually indistinguishable from wild-type cells when comparing the expression of known RsmA targets, including genes involved in biofilm, T3SS, and T6SS. However, deletion of both *rsmA* and *rsmF* greatly exacerbated these effects of an *rsmA* deletion, implying a supportive role for the RsmF protein. Unlike RsmA/RsmE from *P. fluorescens*, however, RsmF binds to the RsmY and RsmZ sRNAs with 245- and 58-fold-lower affinity (K_{eq}) than RsmA, and predictably, neither sRNA appeared to regulate RsmF activity *in vivo*. Furthermore, RsmA but not RsmF binds to the *pslA* mRNA with high affinity and specificity. How these differences in the Csr/Rsm paralogs favor the unique lifestyles of these two species remains to be seen. Studies are needed to determine whether RsmF and RsmA are expressed at different phases of growth or under different growth conditions.

The structure of RsmF has been solved by X-ray crystallography, and although the RNA binding surfaces are similar to those of CsrA and RsmE, the overall dimer structure is distinctly different (Fig. 6). For example, RsmF is a homodimer that contains antiparallel β-sheets formed by β1, β3, and β4 from one polypeptide and β2 and β5 from the opposite polypeptide (157, 175). This is in contrast to the case for CsrA/RsmA/RsmE, wherein β-sheets are formed by β2, β3, and β4 from one polypeptide and β1 and β5 from the opposite polypeptide. Furthermore, whereas the C-ter-

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minal α-helices of CsrA/RsmA/RsmE form wing-like structures that extend away from the body of the protein, the α-helices encoded by RsmF are located between β-strands β2 and β3 and form a solvent-exposed, unique interaction surface.

The crystal structure of RsmF bound to a hairpin from the RsmZ sRNA has been solved, revealing that, in contrast to the case for CsrA and RsmE (25), where each RNA binding surface of the dimer is comprised of two β-strands contributed by opposite polypeptides, the RsmF RNA binding surface is composed of two β-strands originating from the same polypeptide (Fig. 6) (175). How do these topological differences in the RsmE and RsmF proteins explain how both proteins bind to a single RsmZ hairpin with similar affinities (175), yet RsmA binds the complete RsmZ RNA with 58-fold-higher affinity than does RsmF (157)? Perhaps the difference in the proximity of RNA binding surfaces in RsmF constrains the ability of this protein to bridge two binding sites within an RNA target relative to CsrA/RsmE (38), especially a target such as RsmZ that contains multiple closely spaced binding sites (39).

The RsmA/RsmE-inhibitory sRNAs. *P. aeruginosa* and *P. fluorescens* have different pairs of CsrA-like proteins, as well as a different set of inhibitory sRNAs. Both organisms produce the sRNAs RsmY and RsmZ, and *P. fluorescens* also produces a third sRNA called RsmX. All of these sRNAs antagonize the respective RsmA protein *in vivo* (59, 85, 166). It is important to note that RsmA-inhibitory sRNAs from pseudomonads differ greatly from those of *E. coli* and *Salmonella* spp. in length, number of predicted CsrA/RsmA binding sites, and stability (57, 59, 176). The CsrB RNA from *E. coli*, for example, is 369 nt in length, possesses 18 to 22 predicted CsrA binding sites, and has a half-life of 1.7 min (26, 57). In contrast, the Rsm sRNAs of the pseudomonads are on average half as long, possess one-half to one-third as many RsmA binding sites, and are much more stable (20 to 60 min) (59, 177). The greater stability of the latter sRNAs may be related to the absence of an apparent CsrD homolog in *Pseudomonas* spp., which is essential for turnover of *E. coli* CsrB/C sRNAs (57). Rapid synthesis and turnover of CsrB/C suggest that CsrA activity can be rapidly altered in response to changes in environmental conditions in *E. coli*, an idea that is supported by mathematical modeling studies (178). The kinetic response of RsmA activity to conditions that affect the Rsm sRNAs remains to be investigated.

Complex regulatory networks of *Pseudomonas* Rsm systems. The genetic circuitry that controls the expression of Rsm sRNAs in *Pseudomonas* spp. is complex, involving several regulatory proteins and presumably multiple environmental stimuli. Regulation is mediated through the BarA/UvrY homologs GacS/GacA. GacS is a membrane-bound sensor kinase that phosphorylates GacA, which binds to the *rsmX*, *rsmY*, and *rsmZ* promoters and activates transcription (59). Though a specific stimulus has yet to be conclusively demonstrated for any BarA homolog, mutations in Krebs cycle genes affect GacA-dependent activation of RsmZ expression, suggesting the involvement of Krebs cycle intermediate(s) or derivatives thereof in GacS activation (173). The Krebs cycle involves 9 intermediates that possess one or more carboxylate groups, and carboxylate compounds with short aliphatic tails stimulate BarA activity in *E. coli* (67).

Two unusual hybrid sensor kinase-response regulator proteins called RetS (LemA) and LadS regulate GacS through repression and activation mechanisms, respectively (Fig. 5). RetS is a membrane-bound protein that binds to GacS and prevents autophosphorylation, which reduces GacA phosphorylation and ultimately Rsm sRNA expression (179). LadS is a membrane-bound protein that binds to GacS and stimulates its activity through an unknown mechanism (180, 181). Though the stimuli for RetS and LadS regulation are unclear, these proteins act antagonistically to control T3SS, motility, T6SS, quorum sensing, and biofilm formation through GacS/GacA and RsmA (180). Recent studies demonstrated that a novel phosphotransfer protein called HptB interacts with and is phosphorylated by RetS and that HptB, similar to RetS, represses RsmY expression (182, 183). Nevertheless, RetS and HptB regulate RsmY expression through separate pathways. Interestingly, whereas RetS repressed both RsmY and RsmZ expression, HptB appeared to control only RsmY expression. As in *E. coli*, these findings imply that the presence of multiple Csr/Rsm-inhibitory sRNAs may serve to provide multiple regulatory inputs to control the Csr/Rsm regulon(s) in response to different environmental stimuli.

Strains of *P. aeruginosa* isolated from chronic CF patients typically lack a functional T3SS. One reason for this is the frequent occurrence of mutations affecting the MucA/AlgU and AlgZ/AlgR signal transduction systems (141, 142). MucA is a membrane-bound anti-sigma factor that sequesters AlgU (sigma 22), preventing it from activating the expression of ~300 genes, including genes for the T3SS (184–187). AlgU activates transcription of the response regulator AlgR, which inhibits T3SS gene expression via two independent pathways (86). The first involves AlgR activation of RsmY and RsmZ expression through an unknown mechanism. The second pathway involves AlgR-dependent repression of Vfr, a cyclic AMP (cAMP)-activated global transcription factor that is homologous to *E. coli* cAMP receptor protein (188). Though the mechanism has yet to be established, it is believed that Vfr activates transcription of T3SS genes through activation of the *exsCEBA* operon (188). Given the shear number of TCSs in *P. aeruginosa* (approximately 64 response regulators and 63 sensor histidine kinases) and the diverse repertoire of potential hosts/environments it can inhabit, it is perhaps no surprise that multiple signal transduction networks should control the Rsm system.

A number of cytoplasmic global regulators also control the levels of RsmA-inhibitory sRNAs in *Pseudomonas* species. For example, IHF (discussed above) binds to and activates transcription from the *rsmZ* promoter in *P. fluorescens*. IHF serves to maintain DNA architecture (supercoiling and duplex stabilization). The mechanism for IHF effects on *rsmZ* expression is presently unknown. Interestingly, the H-NS (histone-like nucleoid structuring protein) orthologs MvaT and MvaU bound to the *rsmZ* promoter region *in vivo* and repressed its expression (76). Similar to IHF, H-NS also affects DNA architecture, and it is known to repress gene expression under conditions of low temperature or low osmolarity (189). While the conditions that control MvaT/MvaU activity are unknown, IHF antagonizes H-NS-dependent silencing of gene expression in bacterial pathogens, including *E. coli*, *V. cholerae*, and *S. enterica* (190–192). Further studies are needed to determine whether IHF antagonizes MvaT/MvaU-dependent repression of RsmZ.

Posttranscriptional regulators of gene expression also influence RsmA activity in *Pseudomonas* species. For example, Hfq appears to modestly stabilize RsmY in *P. aeruginosa* through a direct interaction that prevents RNase E-dependent cleavage (102, 177). Deletions of *hfq* and *rsmY* similarly reduced expression of the quorum-sensing AHL synthetase *rhlI*. It has been proposed

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that Hfq activates *rhlI* expression indirectly via RsmA, though this hypothesis has not been experimentally tested. Hfq also enhances CsrB expression in *E. coli*, apparently through indirect effects on its synthesis (57).

Legionella pneumophila

Legionella pneumophila is the causative agent of a respiratory pneumonia called Legionnaires' disease. This bacterium is found primarily in aquatic environments, where it infects and replicates within numerous species of protozoa (193). Humans are infected with *L. pneumophila* by inhaling contaminated water droplets, which are present in both natural and man-made sources (194). The infectious life cycle of *L. pneumophila* can be broken down into two phases: replication and transmission. In the replicative phase, abundant nutrients cause intracellular replication to proceed rapidly and transmission traits to be repressed. When nutrients become limiting, transmission traits are expressed, including motility, resistance to heat and osmotic pressure, sodium sensitivity, and the ability to avoid phagosome-lysosome fusion (195, 196). The postexponential growth phase of *L. pneumophila* is strongly correlated with virulence phenotypes, and bacteria from the exponential or stationary phase of growth are used to study the replicative or transmission phase, respectively. The leading hypothesis to explain these findings is that nutrient depletion perturbs fatty acid biosynthesis, which in turn stimulates production of ppGpp by SpoT. When ppGpp levels are high, both the stationary-phase sigma factor RpoS and the LetS/LetA TCS cooperate to induce the expression of transmission genes (197–200).

CsrA controls the switch from replicative to transmissive virulence phase of infection. CsrA in *L. pneumophila* was originally studied in plasmid overexpression experiments, where it was determined that CsrA represses transmission traits, including pigmentation and motility, and promotes morphological changes leading to filamentation (201). Molofsky and Swanson demonstrated that CsrA-dependent repression of transmission traits is alleviated by the LetS/LetA (BarA/UvrY) TCS (91). The RsmY and RsmZ sRNAs were identified by bioinformatics analyses (55), and Sahr et al. demonstrated that RsmY/Z link the LetS/LetA TCS and CsrA to the control of replication versus transmission phases (202). The function of RsmY/Z in *L. pneumophila* is consistent with the *Gammaproteobacteria* model in which LetS/LetA (BarA/UvrY) modulates CsrA activity by activating transcription of the RsmY/Z sRNAs (3).

Though the majority of LetS/LetA-regulatory effects depended on RsmY/Z, regulation of several motility genes did not (202). This may suggest that *L. pneumophila* encodes additional CsrA-inhibitory sRNAs or that LetA directly controls the expression of these genes. A third Csr sRNA, RsmX, is present in many (but not all) *L. pneumophila* strains. Its expression is also dependent on LetS/LetA (203). The regulatory contributions of this sRNA, however, did not account for LetA-dependent control of motility gene expression. Further studies are needed to determine how the LetS/LetA TCS affects motility gene expression and whether this regulatory circuit involves CsrA. Whether RsmX, RsmY, and RsmZ of *L. pneumophila* function in a redundant fashion or whether they are independently expressed in response to different environmental parameters is an open question.

The Dot/Icm type IV secretion system. When humans inhale aerosolized *L. pneumophila*, the bacterium either immediately triggers contact-dependent apoptosis or rapidly multiplies within the engulfing alveolar macrophage by establishing the *Legionella*-containing vacuole (LCV) (204). Either fate depends on effectors that are secreted by the Dot/Icm type IV secretion system (T4SS), which is important for transporting *L. pneumophila* virulence components into the host. The T4SS structural genes are homologous to the T-DNA transfer system of *Agrobacterium tumefaciens* and the Tra transfer genes of the IncI ColIb-P9 plasmid of *Shigella flexneri* (205). The Dot/Icm complex includes approximately 27 protein components that are assembled into one of three subcomplexes: the inner membrane substrate receptor, the transmembrane bridge connecting the inner and outer core subcomplexes, and the outer membrane core containing the secretory components for host cell penetration (206). This T4SS secretes approximately 300 effectors (207–210) and is important for intracellular replication, organelle trafficking, and effector-dependent egress from the host cell (204, 211–213). CsrA regulates the expression of at least 26 of 44 tested Dot/Icm effector genes and at least three genes encoding regulatory proteins that influence Dot/Icm expression (78, 214). Though many of these putative CsrA targets appear to contain multiple CsrA binding sites, *in vitro* CsrA binding experiments in *L. pneumophila* have yet to be reported.

Complex regulation of CsrA and the *L. pneumophila* life cycle. Three TCSs in *L. pneumophila* apparently regulate CsrA activity: LetS/LetA, PmrB/PmrA, and LqsS/LqsT/LqsR (Fig. 7). Although it is known that *rsmY/Z* expression is stimulated as growth approaches stationary phase, the exact nature of the inducing signal is unknown. In other Gram-negative bacteria, the signal for the homologous sensor kinase (BarA, GacA, etc.) seems to be tied to carbon metabolism intermediates, including short-chain fatty acids such as acetate or intermediates of the tricarboxylic acid cycle (67, 173). Likewise, *L. pneumophila* requires its LetS/LetA TCS to induce transmission traits in response to SCFAs, as well as to other metabolic stresses and ppGpp (199, 200). However, a direct stimulatory factor has never been definitively demonstrated in this or any other bacterium.

The PmrB/PmrA TCS modulates the composition of lipopolysaccharide (LPS) in response to signals in host and nonhost environments (215) in a variety of pathogens, (216–222). The PmrB sensor kinase is activated by high Fe^{3+} (~100 μm), high Al^{3+} (~100 μM), and mildly acidic pH (pH 5.8) (215). Surprisingly, *L. pneumophila* PmrA did not regulate LPS composition (223) and instead activated transcription of several Dot/Icm T4SS genes (224). PmrA also activated transcription of the *csrA* gene in both exponential and stationary growth phases (78). The net result of these effects was PmrA-dependent activation of Dot/Icm gene expression at the transcriptional level and CsrA-dependent repression of Dot/Icm genes at the posttranscriptional level. An explanation for this somewhat paradoxical finding may be that PmrA and CsrA work in concert to fine-tune the levels of Dot/Icm products as the infection cycle progresses.

L. pneumophila produces the quorum-sensing autoinducer molecule 3-hydroxypentadecane-4-one (LAI-1 or *Legionella* autoinducer 1). LAI-1 is produced by the LAI-1 synthase LqsA and is sensed by sensor kinase proteins LqsS/LqsT that stimulates phosphorylation of the response regulator LqsR (225, 226). LqsA and LqsS are homologs of *V. cholerae* CqsA and CqsS, respectively (227). *L. pneumophila lqsR* mutants are poorly phagocytosed by amoeba and macrophage hosts and are defective in intracellular

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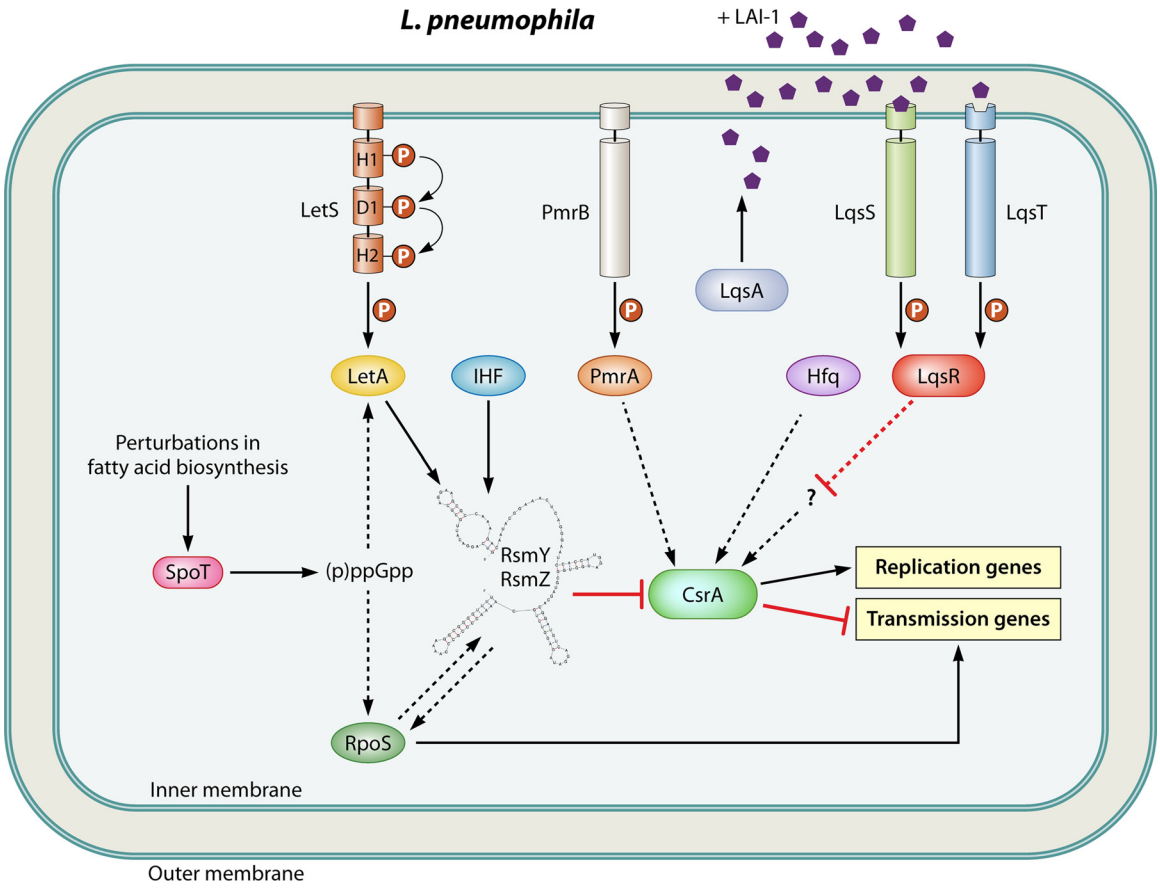


FIG 7 Csr regulatory circuitry in in *L. pneumophila*, depicted as in Fig. 4. Pentagons depict the autoinducer LAI-1.

replication (226). Mutations in *letA* lead to reduced LqsR levels, suggesting that LqsR is repressed by CsrA. Perhaps the Lqs quorum-sensing system facilitates the transition from transmission-phase gene expression to the replication phase, a role complementary to that of Csr, which facilitates the switch from replication to transmission phase.

In many bacterial species, the regulatory activities of CsrA/RsmA and RpoS appear to oppose one another (3), yet in *L. pneumophila*, the LetS/LetA TCS destabilizes the *rpoS* transcript in stationary phase (228). Furthermore, while RpoS in other bacterial species promotes stationary-phase resistance to various environmental stresses (229), RpoS in *L. pneumophila* does not appear to perform this function (197, 230). Clearly, RpoS has evolved a unique role to accommodate the life cycle of *L. pneumophila*.

IHF in *L. pneumophila* is expressed in stationary phase and is required for full virulence in amoebae (231). IHF directly binds to and activates transcription of the *rsmY* and *rsmZ* promoters (232). Expression of the genes encoding IHF, *ihfA* and *ihfB*, is negatively regulated by LetA. This regulatory circuit, wherein LetA directly activates *rsmY/Z* and indirectly represses *rsmY/Z* through *ihfA/B*, appears to comprise an incoherent feedforward loop. Incoherent feedforward loops are known to have diverse regulatory effects, including the ability to alter the dynamics or the dynamic range of target regulation, to facilitate the generation of transient pulses in regulation, or to permit the integration of multiple signals and accelerate response times (233).

Salmonella Species

The top three food-borne infections causing hospitalization in the United States are *Salmonella*, norovirus, and *Campylobacter*, with *Salmonella* being the leading cause of death (234). *Salmonella enterica* can infect a remarkably broad range of host species, including a large number of different animals and even plants (235–238). In humans, the serovar *S.* Typhimurium causes an acute gastroenteritis characterized by an inflammatory diarrhea and fever (239–242). In rare cases this is followed by reactive arthritis (243, 244). Besides being a significant cause of human morbidity and mortality, *S.* Typhimurium was one of the first and most powerful models for bacterial genetics.

The primary virulence systems of *S.* Typhimurium are two distinct type III secretion systems (T3SS) both of which are regulated by CsrA. T3SS1 is encoded within SPI1 (*Salmonella* pathogenicity island 1) and is involved primarily in the intestinal phase of disease. T3SS1 effectors are directly injected into intestinal epithelial cells (245–247), which causes uptake of bacterial cells via macropinocytosis and an inflammatory response (247–263). T3SS2 (encoded within SPI2) is induced after *Salmonella* has invaded or is phagocytized by eukaryotic cells and is required for survival in macrophages and systemic disease (264–268). These observations led to a simple model in which T3SS1 is needed to invade intestinal cells but is not required during the subsequent phases of *Salmonella* pathogenesis, while T3SS2 is expressed only when the bacte-

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ria reside within eukaryotic cells. However, the regulation and function of the *Salmonella* T3SSs may be more complicated. SPI1 and SPI2, which contribute to inflammation, and the anaerobic respiration pathways that take advantage of inflammation are all regulated by CsrA, as detailed below (98, 269).

SPI1 is regulated by SirA/CsrA. The *uvrY* ortholog in *Salmonella*, *sirA* (*Salmonella* invasion regulator A), was first discovered by screening for mutations that confer a defect in SPI1 gene regulation (270). Another study identified *barA* and *csrB* as regulators of SPI1, which led to further studies on the effects of *csrA* (271, 272). CsrC was identified by its homology with *E. coli csrC* (77, 273). Mutations in *barA*, *sirA*, *csrB*, and *csrC* all cause decreases in SPI1 gene expression. Interestingly, either mutation of *csrA* or overexpression of *csrA* causes a decrease in SPI1 gene expression (271).

CsrA regulates the dedicated regulators of SPI1. There are three large operons in SPI1 that encode regulatory, structural, and effector proteins. There are also at least five regulatory genes encoded within SPI1: *hilA*, *hilC*, *hilD*, *sprB*, and *invF*. HilC, HilD, and RtsA (not encoded within SPI1) are AraC/XylS family regulators which comprise a feedforward loop that regulates *hilA* (274–277). HilA is an OmpR/ToxR family protein that regulates a number of SPI1 genes by activating transcription from the *invF* and *prgH* promoters (278, 279). InvF is another AraC/XylS family regulator that, in association with a coactivating chaperone SicA, activates the transcription of numerous genes encoding T3SS effector proteins, encoded inside and outside SPI1, including *sip/sspABCD*, *sopA*, *sopB/sigD*, *sopE*, *sopE2*, *sspH1*, *sptP*, and *slrP* (280–283).

The effects of *barA*, *sirA*, *csrB*, and *csrC* on SPI1 are mediated through the binding of CsrA to the *hilD* transcript (269). BarA phosphorylates SirA (73, 269), which then binds as a dimer to an 18-bp inverted repeat sequence between positions −190 and −173 of *csrB* and −168 and −151 of *csrC* relative to their transcription start sites (63). CsrB and CsrC antagonize CsrA, preventing it from binding the *hilD* transcript. The net result of BarA/SirA activity is increased HilD expression, which has downstream effects on the remainder of SPI1 (269). HilD also regulates SPI2 under certain conditions (269, 284). CsrA affects *invF* and *sipC*, but not *prgH*, in the absence of *hilA*, suggesting that CsrA may also directly regulate other genes within SPI1, which lie above and below the *hilA* regulatory level (271).

Other genes regulated by CsrA. SirA and CsrA also regulate the expression of type 1 and Pef fimbriae (285, 286). Under laboratory conditions, only type 1 fimbriae are expressed, unless the 5′ UTR of the *fimAICDHF* mRNA is deleted, which then allows expression of Pef fimbriae. The mechanism of this regulation is that CsrA binds the 5′ UTR of the *fimAICDHF* transcript, which has weak regulatory effects on the expression of type 1 fimbriae but has surprisingly large effects on the expression of Pef fimbriae (285). The 5′ UTR of the *fimAICDHF* mRNA is so abundant that it titrates CsrA from other transcripts, including the *pefA* transcript, for which CsrA is a positive regulator. Thus, the 5′ UTR of *fimAICDHF* acts as a third regulatory RNA in addition to *csrB* and *csrC* (285).

In a microarray study, mRNAs whose levels are affected by the *csrA* gene included those of SPI1, flagellum synthesis and chemotaxis, maltose, propanediol and ethanolamine utilization, tetrathionate reductase and hydrogen sulfide production, and cobalamin biosynthesis (98). As in *E. coli*, CsrA represses biofilm formation, and *sirA* or *csrB csrC* double mutants are defective in biofilm development (286). CsrA has been shown to bind to the mRNAs of 5 of the 20 GGDEF/EAL domain proteins in *Salmonella*, which are involved in switching between biofilm and motile phases of growth (84).

Csr and the response to short-chain carboxylate compounds. The intestinal environment contains high concentrations of short-chain fatty acids (SCFAs), which regulate *Salmonella* invasion through Csr-dependent and -independent pathways. Acetate appears to activate *Salmonella* invasion independently of *barA*, but dependent upon *sirA*, through acetyl-phosphate (68). Formate activates *Salmonella* invasion, apparently independently of *barA/sirA and csrA* (287). Propionate represses invasion gene expression via the posttranslational control of HilD, independently of the Csr system (288). Because formate and acetate levels are elevated in the small intestine, while propionate is elevated in the colon, these SCFAs may serve as cues to prepare *Salmonella* for invasion of or exit from the host, respectively. In contrast to these findings, all of these SCFAs activated *csrB* expression via BarA signaling in *E. coli* (67).

Vibrio cholerae

V. cholerae primarily inhabits aquatic environments and causes the human disease known as cholera (290). Virulent serogroups of *V. cholerae* form robust biofilms and express the virulence factors cholera toxin (CT) and the toxin-coregulated pilus (TCP) (290, 291). The maturation and dispersal of *V. cholerae* biofilms, as well as the production of CT and TCP, are controlled by multiple independent quorum-sensing systems, whose effects converge on a single regulatory circuit (Fig. 8) (227, 292, 293). CsrA exerts its influence on virulence factor expression and quorum sensing in *V. cholerae* through this circuitry.

There are two well-studied quorum-sensing circuits in *V. cholerae* that regulate gene expression in response to autoinducers that are referred to as CAI-1 [cholera autoinducer-1; (*S*)-3-hyroxytridecan-4-one] and AI-2 [autoinducer 2; (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate] (227, 289, 294, 295). These quorum-sensing systems coordinate gene expression so that virulence factors are produced at low cell population density. At low population density the concentration of CAI-1 and AI-2 is low, and the sensor kinases CqsS (CAI-1) and LuxQ (AI-2) facilitate phosphorylation of LuxU and ultimately the LuxO response regulator protein (296–298). LuxO-P, Fis, and σ^{54}-RNA polymerase activate transcription of the Qrr1 to -4 sRNAs, which bind to and destabilize the *hapR* mRNA in an Hfq-dependent manner (299–301). HapR represses transcription of *vpsR* and *vpsT*, which encode transcription factors that activate biofilm formation genes. HapR also regulates the expression of 14 genes involved in the synthesis and degradation of c-di-GMP, whose net effect is to reduce c-di-GMP levels (302). The Qrr1 to -4 sRNAs also activate translation of the transcription factor AphA (303, 304). AphA indirectly activates *toxT* expression, and ToxT activates transcription of genes encoding the CT and TCP (227, 305, 306). At high cell population density, the autoinducers CAI-1 and AI-2 are produced in sufficient quantities to switch CqsS and LuxQ from kinases to phosphatases. This system is reinforced by mutual repression wherein HapR represses *aphA* transcription and AphA represses *hapR* transcription. High cell population density therefore triggers quorum-sensing systems to reprogram gene expres-

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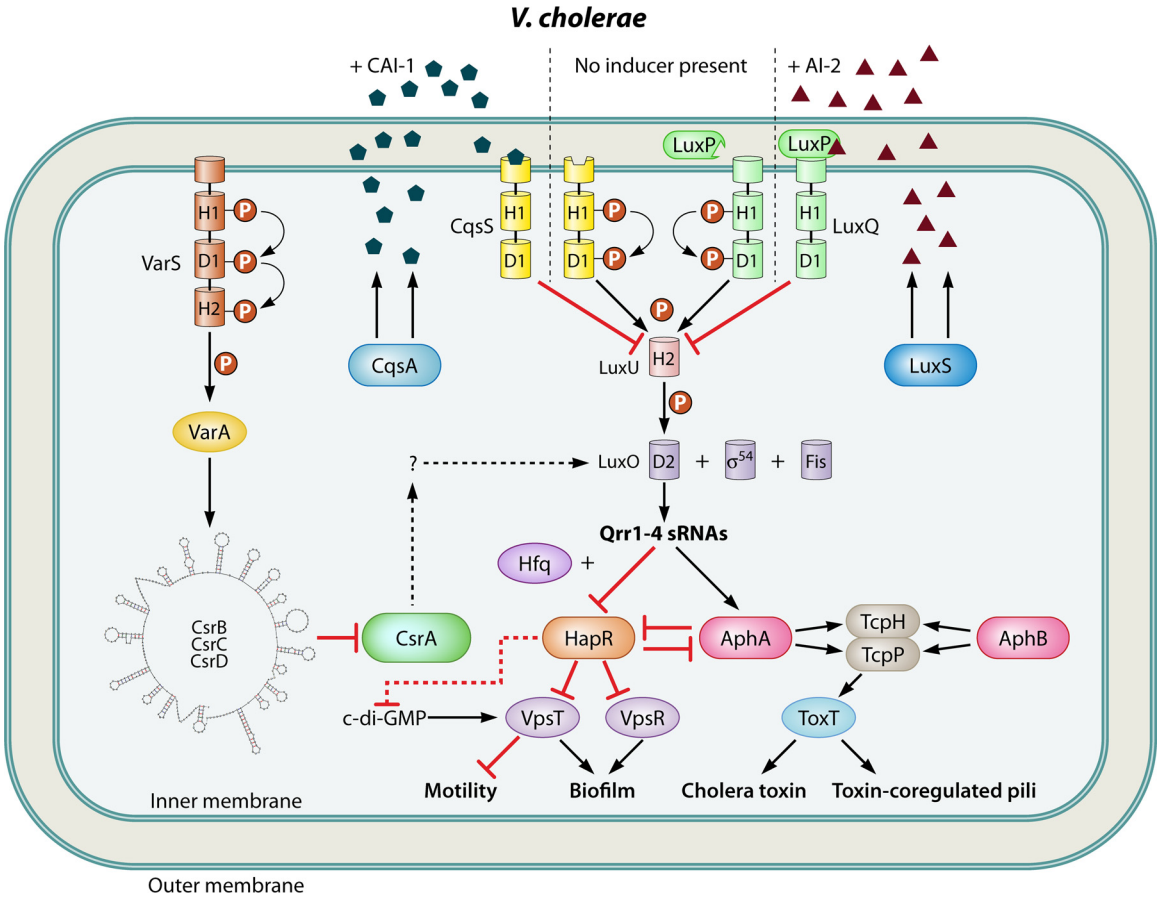


FIG 8 Csr regulatory circuitry in *V. cholerae*, depicted as in Fig. 4. Autoinducers CAI-1 and AI-2 are shown as pentagons and triangles, respectively. (Adapted from reference 75 with permission of the publisher.)

sion, which halts production of virulence factors and increases production of transmission factors such as motility.

Using a genetic screen to isolate genes that affect quorum-sensing gene expression, Lenz et al. determined that transposon insertion mutations in the *barA*/*uvrY* orthologs *varS*/*varA* exhibited reduced *luxCDABE* expression (75). *luxCDABE* is a bioluminescent reporter for quorum-sensing gene expression in *Vibrio harveyi*, and it was determined that this locus also faithfully reports effects on quorum-sensing gene expression in *V. cholerae* (75). These effects did not depend on the CAI-1 or AI-2 quorum-sensing system, and epistasis experiments subsequently demonstrated that the VarS/VarA TCS most likely functions through the response regulator LuxO. Genetic screens and epistasis experiments determined that VarS/VarA regulates quorum sensing indirectly through CsrA and three CsrA-inhibitory sRNAs called CsrB, CsrC, and CsrD. As is true for the other *Gammaproteobacteria*, transcription of the CsrA-inhibitory sRNAs depends on VarS/VarA and likely is activated directly by VarA.

Studies are needed to determine the pathway(s) by which CsrA (and VarS/VarA/CsrB/CsrC/CsrD) affects LuxO/HapR/AhpA and/or other components of the quorum-sensing circuitry. Because CsrA/RsmA is known to regulate the expression of virulence factors in other pathogens by both direct and indirect mechanisms, these pathways are likely to be complex. Perhaps intermediates in carbon pathways, such as acetate, other SCFAs, or tricarboxylic acid (TCA) cycle intermediates, stimulate VarS activity and therefore tie the metabolic status of *V. cholerae* to virulence factor gene expression. Toward this end, it is also of high interest to understand the effects of CsrA on the *V. cholerae* transcriptome.

Pathogenic *Escherichia coli* and Related Species

A variety of *E. coli* strains have gained and/or lost genetic information relative to commensal isolates and in so doing have become important sources of intestinal or extraintestinal infections (307–309). Pathotypes causing infections are classified according to the traits and genes that distinguish them. Nevertheless, sharing of genetic information among strains is common, and new hybrid forms can arise that complicate the classification schemes. A common core of roughly 1,700 genes is found in all strains. Along with the genes that vary among strains, they constitute a pangenome of over 16,000 genes, highlighting the remarkable plasticity of the *E. coli* genome (310, 311). Genes contributing specifically to virulence vary among the pathotypes and are often clustered together in horizontally transferred loci, present in the chromosome or in mobile elements such as plasmids or phage and referred to as pathogenicity islands. Although not yet demonstrated, it seems likely that the central role of the Csr system in controlling the expression of common genes for metabolism, biofilm formation, motility, and stress responses, such as the stringent response, broadly impacts the host interactions of all *E. coli* strains (3, 27,

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31). In addition, however, the Csr system is integrated directly into virulence gene networks of *E. coli* pathogens.

EPEC. A relatively detailed analysis of CsrA regulation in the enteropathogenic *E. coli* (EPEC) virulence gene network was conducted (312, 313). EPEC causes noninvasive diarrhea, characterized histopathologically by the formation of attaching and effacing (A/E) lesions in the small intestine, and is responsible for thousands of infant deaths each year in developing countries (307, 314, 315). The A/E lesions involve destruction of the intestinal microvilli and the formation of actin rearrangements in EPEC-attached host cells to form pathognomonic protrusions, referred to as pedestals (316). The main virulence factor of EPEC is a T3SS, encoded within the locus of enterocyte effacement (LEE) of the bacterial chromosome (317). This system transfers protein effectors into host cells, which interact with host signaling proteins and lead to pedestal formation (318). The EPEC LEE locus consists of five multigene operons, LEE1 to -5, along with *grlRA* and several monocistronic operons (313). Expression of LEE genes is coordinated by a large number of regulators that converge to govern expression of a DNA binding protein, referred to as the LEE expression regulator or Ler. Ler is encoded in LEE1 and acts as the master activator of the LEE gene expression, in large part by relieving repression caused by its paralog H-NS (319). CsrA does not directly regulate *ler* expression but directly activates expression of the Lee4 locus by binding to its mRNA leader and indirectly activates *escD* (312). The *escD* and LEE4 genes encode translocators that must be produced and assembled prior to the secretion of the effectors, suggesting that CsrA specifically activates this step in the secretion pathway. CsrA also appears to regulate LEE at a higher tier in the circuitry, based on ectopic expression studies showing that elevated CsrA levels repress *grlRA* expression by binding directly to *grlRA* mRNA (312). Because GrlA activates Ler expression (320), this causes extensive repression of LEE operons and genes. The ability of CsrA to switch from being an activator to a repressor of virulence properties as its levels vary was first observed in *Salmonella* (271). While the biological relevance of this kind of "seesaw" regulation needs to be established (3), its features hint that CsrA activity may be governed according to the particular niche environment within the host and in turn coordinates the workings of the T3SS and other processes. Interestingly, CsrA also activates expression of tryptophanase in EPEC, which produces indole and derivatives of indole that can be can be toxic to *Caenorhabditis elegans* (321) or can affect LEE expression and attenuate disease in a mouse model of infection (322).

UPEC. Uropathogenic *E. coli* (UPEC) strains commonly cause urinary tract infections, where they replicate in intracellular and extracellular locations with the help of a battery of virulence factors that include fimbriae, toxins, and iron siderophores (323). The BarA/UvrY TCS and CsrA have been implicated in regulating UPEC virulence in animal models and virulence factors expressed *in vitro*, although the mechanisms for these effects have not been determined (324–326).

***Shigella* species.** *Shigella* species are closely related to *E. coli* and cause invasive, localized diarrheal disease. Infection by *Shigella* involves entry of the bacterium into host M cells of the colon and rectum, destruction of the phagosomal membrane and release into the cytoplasm, intracellular motility via the host actin system, and lysis of host cells and infection of neighboring cells during repeated cycles of reinfection (327). Pathogenicity is dependent upon the presence of a 220-kb virulence plasmid and the T3SS that it encodes (328). In *S. flexneri*, CsrA was found to regulate metabolic genes of the chromosome, which are important for causing infection, e.g., *pfkA* for phosphofructokinase, as well as the *virF* and *virB* genes of the virulence plasmid, which activate expression of secreted T3SS effectors (329). However, the mechanisms for these effects remain to be determined.

Yersinia species

The Csr system of *Yersinia pseudotuberculosis* is the best studied of this important genus. This species, along with the related *Yersinia enterocolitica*, is the causative agent of a variety of gut-associated diseases, including colitis, diarrhea, and mesenterial lymphadenitis, collectively referred to as yersiniosis (330). These pathogens live both in environmental reservoirs and in a variety of animal hosts and are spread by fecal-oral contamination. Infections in humans result mainly from consumption of contaminated food, most commonly pork (4).

Infection begins when the bacteria tightly associate with the mucosal surface of the intestine. This initial interaction occurs at M cells of Peyer's patches and is mediated primarily via the cell surface-associated virulence factor invasin (331). The bacteria are rapidly translocated across the intestinal barrier into underlying lymphoid tissue, where they replicate. Infection can then spread to mesenteric lymph nodes and, in later stages of infection, to the liver and spleen. Expression of invasin and other early-stage virulence factors of *Y. pseudotuberculosis* pathogenesis is regulated by RovA, a member of the MarR/SlyA family of transcription regulators (332, 333). A *rovA* mutant is deficient in its ability to invade and colonize Peyer's patches, in part due to reduced expression of invasin and ability to induce inflammation through interleukin-1 (IL-1) production. After the initial stages of infection, virulence factors important for immune evasion and dissemination of the infection are induced, while expression of early virulence factors is reduced. These virulence factors include the adhesins Ail and YadA as well as T3SSs and their associated effectors, including Yops (*Yersinia* outer proteins) (331).

RovA expression varies in response to nutrient availability, which is in part due to effects of another transcriptional regulator, RovM. This LysR family protein is induced during growth in minimal media and it acts in concert with H-NS to repress the expression of RovA (334). Studies to determine how expression of RovM and RovA is linked to nutrient availability led to the identification of a Csr system in *Y. pseudotuberculosis* (333). In minimal media, CsrA was found to indirectly activate RovM expression, leading to repression of RovA. As in many other species, two inhibitory small RNAs, CsrB and CsrC, regulate CsrA activity. Of these, CsrC was found to provide the most dramatic regulatory effect, likely as a result of the very low level of CsrB expression under the conditions tested. It appears that the nutrient-sensitive expression of RovM and thus RovA is due primarily to high expression of CsrC in rich media. Subsequent work has shown that Crp is also involved in linking nutrient availability to CsrA activity via indirect activation of CsrC and repression of CsrB (103). The relevance of these opposing effects of Crp is uncertain. Regulation of CsrA activity in response to nutrient availability may allow *Y. pseudotuberculosis* to express early virulence factors only in the appropriate host compartment and under growth conditions that might lead to a successful infection.

Further studies identified an interesting distinction in the *Y. pseudotuberculosis* Csr regulatory circuitry: the BarA/UvrY TCS

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directly activates only the expression of CsrB, and not that of CsrC (333), while another TCS, the PhoQ/PhoP system, directly activates CsrC but not CsrB expression (335). The latter TCS regulates a number of virulence determinates in response to low magnesium, acidic pH, and antimicrobial peptides and is important for replication in macrophages (336). Such differential expression of Csr sRNAs by distinct TCSs has yet to be identified in the other characterized Csr systems, and its physiological role remains uncertain.

Plant Pathogens

Pectobacterium carotovorum. *Pectobacterium carotovorum* (previously known as *Erwinia carotovora*) is an important plant pathogen and is the etiological agent of soft-rot diseases in a variety of commercially important plant species (337, 338). As a close relative of *E. coli* (family *Enterobacteriaceae*), *P. carotovorum* is a genetically tractable organism in which some of the first studies on the structure and function of plant cell wall-degrading enzymes were conducted. These studies revealed that secreted proteases, cellulases, and pectinases play critical roles in *P. carotovorum* virulence (338–340). Further investigation of *P. carotovorum* virulence pathways led to the discovery of a quorum-sensing signal used to alter gene expression in response to changes in cell population density (338, 341–345). The quorum-sensing signal *N*-(3-oxohexanoyl)-L-homoserine lactone (acyl homoserine lactone [AHL]) is required for production of *P. carotovorum* virulence factors and related products, including the previously mentioned cell wall-degrading enzymes, antibiotics, hypersensitive responses, and the T3SS (346, 347).

Shortly after the discovery of CsrA in *E. coli*, its homolog RsmA in *P. carotovorum* was found to repress tissue maceration, the production of cell wall-degrading enzymes, and the AHL quorum-sensing signal (18, 19). Furthermore, Chatterjee et al. determined that AHL-dependent activation of exoenzyme production is regulated by *rsmA* (19). As found in many other species, *P. carotovorum* produces an RsmA-inhibitory sRNA called RsmB, which depends on the GacS/GacA TCS for its expression (348, 349). It was later shown that AHL activates the production of cell wall-degrading enzymes by repressing *rsmA* expression (350, 351). More specifically, AHL production inhibits the activity of the AHL receptor proteins ExpR1 and ExpR2, both of which activate *rsmA* expression in the absence of AHL (352–354). *P. carotovorum* subspecies may contain one or both ExpR receptor proteins.

RsmA and RsmB have been studied in other soft-rot disease-causing *Pectobacterium* species, including *P. atrosepticum* and *P. wasabiae.* RsmA also represses the expression of cell wall-degrading enzymes in these bacteria (101, 355). It is interesting to note that induction of the alarmone (p)ppGpp activates *rsmB* expression in *P. atrosepticum*, which is reminiscent of links between the Csr and the stringent response systems in *E. coli* and *L. pneumophila* (31, 198, 355, 356). Whether nutrient starvation through the stringent response system generally serves as a trigger for inhibiting CsrA activity in other bacteria remains to be seen.

***Xanthomonas* spp.** The genus *Xanthomonas* includes a diverse set of pathogens that infect over 200 different families of plants (357). Two *Xanthomonas* pathovars of great concern to agriculture are (i) *X. campestris* pathovar *campestris*, the causal agent of black rot, a disease of cruciferous plants, and (ii) *X. axonopodis* pathovar *citri*, the causal agent of citrus canker, a disease that reduces the vitality of susceptible citrus hosts. RsmA has been studied in the *X. campestris* pv. campestris and *X. axonopodis* pv. citri pathovars (100, 358). In both cases, RsmA promoted virulence through the activation of genes associated with a T3SS. Comparative genome analyses indicate that species of *Xanthomonas* encode every known type of bacterial secretion system (type I to type VI) and suggest that the T3SS is essential for pathogenicity of most *Xanthomonas* pathovars (359). *Xanthomonas* species typically translocate as many as 40 T3SS effectors that suppress host defenses and alter host gene expression to promote dissemination and tissue destruction (359).

Studies by Chao et al. revealed that *rsmA* is required for infection of host plants by *X. campestris* pv. campestris and significantly reduces the hypersensitive response in nonsusceptible hosts (358). Deletion of *rsmA* significantly reduced motility and the extracellular levels of virulence factors, including endoglucanase, amylase, and extracellular polysaccharide (EPS) (also called "xanthan gum"). Microarray analysis of wild-type cells versus *rsmA* deletion mutants revealed that *rsmA* affects the expression of at least 435 transcripts whose gene products are associated with a diverse variety of cellular processes. These studies did not differentiate between direct and indirect RsmA targets, which is needed to define the RsmA regulon.

Work by Andrade et al. similarly demonstrated that *rsmA* deletion in *X. axonopodis* pv. citri significantly affects its pathogenicity and ability to elicit a hypersensitive response in nonsusceptible plant hosts (100). Microarray experiments comparing an *rsmA* deletion mutant to the wild type revealed effects of *rsmA* on the levels of 170 transcripts. As seen in *X. campestris* pv. campestris, several T3SS regulatory and effector genes were affected by *rsmA* deletion. RsmA bound to the 5′ UTR of the *hrpG* and *hrpD* transcripts *in vitro* and stabilized these messages against degradation *in vivo*. HrpG is the master regulator of T3SS effector gene expression, and RsmA may regulate T3SS gene expression through HrpG. Consistent with this hypothesis, ectopic expression of *hrpG* from a plasmid restored pathogenicity and the hypersensitive response in an *rsmA* deletion strain. RsmA-dependent control of T3SS gene expression via master transcription regulatory factors, as seen in both animal and plant pathogens, is a conserved feature of virulence-associated secretion systems.

Pseudomonas syringae. *P. syringae* is a remarkable species that includes over 50 pathovars that infect a wide variety of plant hosts. Economically important diseases caused by *P. syringae* pathovars include bacterial speck of the tomato (360), bleeding canker of horse chestnut (361), halo blight of bean (362), and various cankers and distortions of trees (338). As is the case for many other plant and animal pathogens, *P. syringae* secretes a variety of products, including quorum-sensing compounds, phytotoxins, antibiotics, exoproteases, fluorescent pigments, and alginate, which influence infection and disease processes (363). These secreted products as well as biofilm formation, host colonization, and lesion formation are all regulated by the GacS/GacA TCS (364–379). Furthermore, genetic analyses of various *P. syringae* pathovars have identified homologs of the RsmA-inhibitory sRNAs RsmX, RsmY, and RsmZ of *P. fluorescens*, as well as RsmB of *P. carotovorum* (55). Studies by Kong et al. demonstrated that *rsmA* overexpression from a plasmid negatively affects pathogenicity of several *P. syringae* pathovars and appears to do so by repressing the production of numerous secreted virulence factors, including phytotoxin, exoprotease, and pyoverdin (363). All tested *P. syringae* pathovars also showed reduced swarming motility in response to

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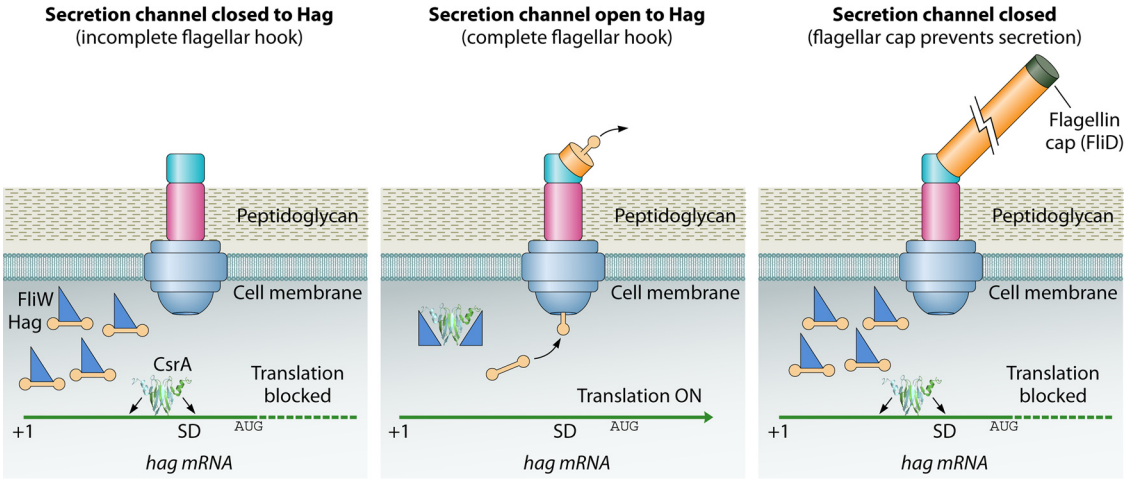


FIG 9 Model for CsrA-FliW-Hag control of flagellin homeostasis in *B. subtilis*. Flagellin homeostasis is controlled by a partner-switching mechanism involving FliW (dark blue triangles), CsrA, and Hag (orange barbells). The basal body (blue and pink), flagellar hook (cyan), filament (orange), cytoplasmic membrane, and peptidoglycan layer are indicated. (Adapted from reference 381 with permission of the publisher.)

rsmA overexpression, while its impact on biofilm formation and alginate production varied by pathovar. An analysis of the phenotypic effects of deleting the *rsmA* and/or *rsmXYZ* genes has yet to be performed in *P. syringae*.

A PARTNER-SWITCHING MECHANISM REGULATES CsrA ACTIVITY IN *BACILLUS SUBTILIS*: IMPLICATIONS FOR BACTERIAL PATHOGENS

Although the Csr system has been extensively studied in a variety of Gram-negative bacteria, *Bacillus subtilis* is the only Gram-positive organism in which this system has been examined. In *B. subtilis*, *csrA* is the terminal gene of a large flagellum-biosynthetic operon. In addition to a σ^D-dependent promoter that controls expression of the entire operon, a σ^A-dependent promoter drives expression of the last two genes (*fliW* and *csrA*) (380). The gene encoding flagellin, *hag*, is located in a separate σ^D-dependent operon, immediately downstream from *csrA*. CsrA represses translation initiation of *hag* by binding to two sites in the *hag* leader transcript, one of which overlaps the *hag* SD sequence (380). This translation repression mechanism is similar to those controlling gene expression in Gram-negative species. Although a *csrB* gene has been predicted in the *B. subtilis* genome, experimental evidence of its role as an sRNA antagonist of CsrA is lacking (55).

A recent and unexpected discovery from studies of *B. subtilis* CsrA regulation was that a protein (FliW) functions as an antagonist of CsrA, and together these proteins constitute part of a morphogenic checkpoint in flagellum assembly (Fig. 9) (381). Prior to flagellum hook assembly, FliW is largely bound in a complex with cytoplasmic Hag (flagellin) protein, leaving CsrA free to repress *hag* translation. Once assembly of the flagellar hook structure is completed, Hag can be secreted and assembled to form the flagellum filament. The resulting unbound FliW protein in the cytoplasm is then capable of binding to CsrA, thereby relieving CsrA-mediated translational repression of *hag*. This leads to derepression of Hag synthesis at the precise time in which Hag is needed in large quantities (381). Thus, flagellin homeostatically restricts its own translation as a consequence of a partner-switching mechanism involving alternative FliW-Hag, FliW-CsrA, and CsrA-*hag* mRNA complexes. The finding of this regulatory mechanism suggests that CsrA in *B. subtilis* might play a more limited role than it does in the *Gammaproteobacteria*, in which it controls gene expression in coordination with flagellum morphogenesis (381).

Bioinformatics analysis of the phylogenetic occurrence of *fliW* and *csrA* together in a flagellum gene cluster further suggests that CsrA may have had an ancestral role in controlling flagellar assembly and motility, which subsequently evolved to include additional cellular processes in other bacteria (381). The *csrA* and *fliW* genes are encoded adjacent to each other in many *Firmicutes*, *Spirochaetales*, *Thermotogales*, and *Delta-* and *Epsilonproteobacteria*, including human pathogens (Fig. 10). Although largely absent from the *Alpha-* and *Betaproteobacteria*, *csrA*, but not *fliW*, reappears in the *Gammaproteobacteria*, along with its associated sRNA antagonists. These observations raise the possibility that early in proteobacterial evolution, the *Gammaproteobacteria* may have obtained *csrA* via horizontal gene transfer, followed by the emergence of a large number of CsrA-controlled genes and the sRNA antagonists that replace FliW (381). In view of the fact that motility is often required for pathogenesis, the CsrA-FliW partner-switching mechanism seems worthy of investigation in additional species.

CONCLUDING REMARKS

The Csr system is extensively integrated into gene expression networks that govern bacterial physiology, metabolism, and virulence. Inhibitory sRNAs integrate signaling from the BarA/UvrY TCS and pathogen-specific regulatory factors into this system by sequestering the CsrA/RsmA protein away from its lower-affinity mRNA targets. Studies in *E. coli* K-12 revealed that CsrA often has opposite effects on opposing metabolic pathways, e.g., gluconeogenesis versus glycolysis, or physiological processes such as motility versus biofilm formation (3). Pathogens appear to use the Csr system to coordinate the expression of virulence factors according to alternate stages of infection or shifts in the host microenvironment. For example, CsrA activates *L. pneumophila* virulence factors associated with intracellular replication and represses genes for transmission traits (91, 201). RsmA activates *P. aeruginosa* genes associated with acute infection and represses genes associated with

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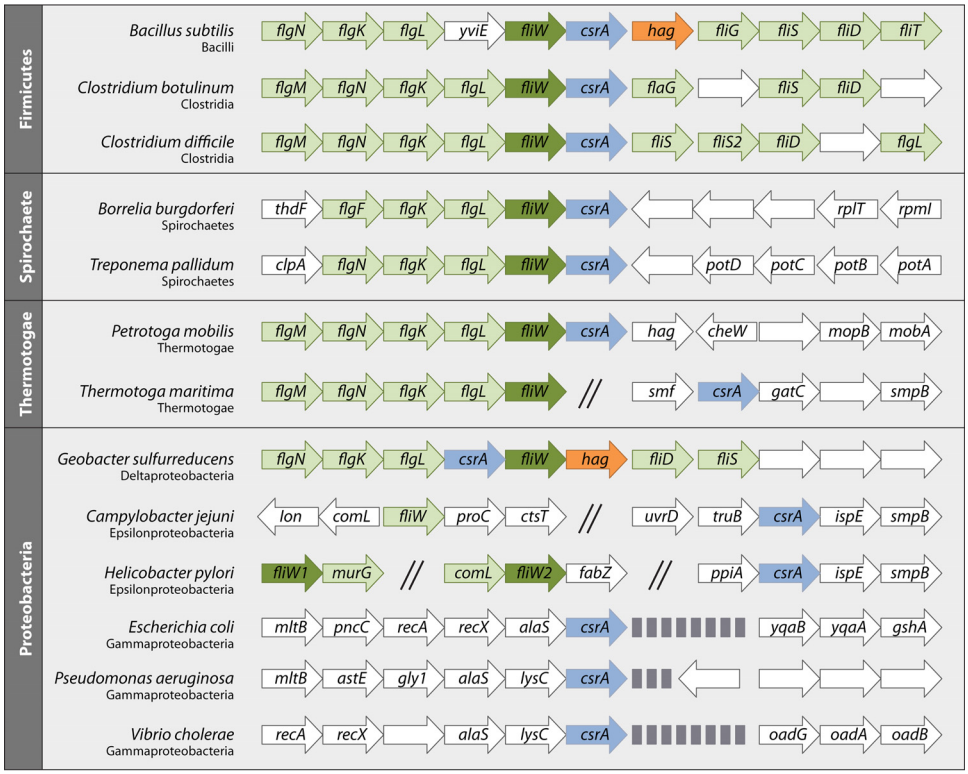


FIG 10 The *csrA* and *fliW* genes tend to cluster in the genomes of many taxonomically diverse species, with the notable exception of the *Gammaproteobacteria*, which lack FliW. Gene neighborhoods around *csrA* (blue) and/or *fliW* (dark green) in *Clostridium botulinum* A strain ATCC 19397, *Clostridium difficile* 630, *Borrelia burgdorferi* B31, *Treponema pallidum* subsp. *pallidum* strain Nichols, *Petrotoga mobilis* SJ95, *Thermotoga maritima* MSB8, *Geobacter sulfurreducens* PCA, *Campylobacter jejuni* NCTC 11168, *Helicobacter pylori* J99, *Escherichia coli* MG1655, *Pseudomonas aeruginosa* PAO1 and *Vibrio cholerae* MZO-2 are shown. The associated phylum and class taxonomic information is shown to the left of and immediately beneath the species names. Protein-coding genes are represented as arrows, and tRNA genes are represented with gray bars. Flagellin genes are colored orange, and other motility-associated genes are colored pale green. Slashes indicate that *csrA* and *fliW* are not within the same genomic neighborhood of a species. Annotation and gene position information was determined using MicrobesOnline (http://www.microbesonline.org/) and reference 381.

chronic infection (80). CsrA represses *V. cholerae* motility genes and activates genes for biofilm formation and cholera toxin (75). CsrA regulates genes necessary for the switch between early colonization and persistent infection in *Y. pseudotuberculosis* (4). Thus, the Csr system governs pivotal lifestyle decisions of *Gammaproteobacteria*, including the pathogens of this bacterial class.

Our understanding of the role of CsrA/RsmA in the physiology and virulence of pathogens is heavily influenced by the known virulence mechanisms, which tend to attract the greatest research attention. However, CsrA/RsmA protein is a global regulator of gene expression in every pathogen examined thus far, and its functions other than regulation of virulence factor expression are likely to be required for successful infection. High-throughput RNA sequencing and next-generation protein-RNA interaction studies that utilize the HITS-CLIP (high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation) (10, 109), PAR-CLIP (photoactivatable-ribonucleoside-enhanced CLIP) (110), or iCLIP-seq (individual-nucleotide-resolution CLIP) (111) method would be helpful for assessing the overall influence of CsrA/RsmA on gene expression in pathogens. Yet even with this information, it will be important to determine how global gene expression varies during the life cycle of a pathogen and the role played by CsrA/RsmA in this dynamic process. This is a goal that has not yet been achieved, even for a model pathogen.

Some questions arising from studies of proteobacterial pathogens are how and why the Csr system has evolved to become integrated into so many diverse virulence factor regulatory circuits. The virulence factors and their dedicated regulators typically are encoded within pathogenicity islands that are acquired by horizontal gene transfer (382). Immediately following acquisition, transcription of foreign genetic material tends to be silenced by the action of nucleoid-associated proteins, e.g., H-NS and Stp (383). If the newly acquired virulence genes are to confer a selective advantage for a pathogen, then evolution must occur to permit them to be expressed and appropriately regulated. While the details remain obscure, there must be strong selection for regulating virulence circuitry by CsrA. Perhaps the broad use of the Csr system in controlling metabolic and physiological functions makes it well suited for regulating virulence genes in response to metabolic shifts that occur during infection. Without a doubt, determining the environmental cues or physiological signals that control the BarA/UvrY-orthologous TCSs of various species will be necessary for addressing this hypothesis. It is worth noting that CsrA regulates gene expression by recognizing a relatively small and adaptable sequence motif, which should evolve rapidly. The striking similarity of the CsrA binding sequence to the Shine-Dalgarno sequence was recognized early (26) and no doubt allows translational regulation by CsrA to evolve with minimal mRNA sequence alteration. The Csr sRNAs may have evolved by duplication of a CsrA

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binding mRNA target, which subsequently accumulated additional CsrA binding sequences. The *Salmonella fimAICDHF* mRNA exemplifies how this might have occurred, as this abundant mRNA contains an untranslated leader that permits its own regulation by CsrA and competes with *pefACDEF* mRNA for CsrA binding (285). Finally, spontaneous mutations in the Csr system have been observed as colony morphotypes, although the significance of such mutations for pathogen evolution remains to be determined (40).

Given the wide distribution of the BarA/UvrY TCS, its strong effects on Csr/Rsm sRNA expression, and its critical roles in physiology and virulence pathways, it is remarkable that a direct stimulus for this TCS has yet to be identified. Some of the most compelling findings toward this goal originate from Chavez et al., who demonstrated that the addition of carboxylate compounds containing short aliphatic tails (such as acetate and formate) stimulates BarA/UvrY-dependent expression of *E. coli csrB in vivo* (67). Consistent with these observations, *P. fluorescens* strains lacking enzymes in the Krebs cycle accumulated carboxylate-containing compounds and stimulated RsmXYZ expression (173). These results suggest that the BarA/UvrY TCS and its homologs may serve as a barometer for the metabolic state of the bacterial cell. BarA autophosphorylation may occur when products of metabolism are abundant, for example, in the mammalian intestinal tract or in a localized nidus of infection. Furthermore, LetS/LetA-dependent differentiation of *L. pneumophila* occurs in response to perturbations in fatty acid biosynthesis, a stress that also activates SpoT to generate ppGpp (199, 200). Additional studies are needed to determine (i) whether carboxylate compounds bind directly to and stimulate BarA or GacS phosphorylation in *E. coli* or *P. fluorescens*, respectively, (ii) whether similar compounds serve as activators for other species of bacteria, and (iii) the way in which the metabolic environment of the host niche influences signaling through this TCS during an infection.

Recent studies have uncovered novel mechanisms of regulation by CsrA and new ways of regulating CsrA activity in the cell. The first detailed molecular mechanism of activation by CsrA was described for the *E. coli flhDC* operon, in which bound CsrA inhibits 5′ end-dependent cleavage of the transcript by RNase E (47). In addition, CsrA was recently shown to regulate termination of the *E. coli* biofilm transcript *pgaABCD* by altering its structure and making it accessible to Rho protein (53). This is the fourth mechanism by which CsrA regulates the expression of this transcript, along with effects on transcript stability, translation initiation, and transcription initiation (29, 34). An mRNA (*fimA*) that is naturally expressed at high levels and contains two CsrA binding sites in its untranslated leader antagonizes CsrA activity in *S.* Typhimurium, in a mechanism resembling regulation by sRNAs (285). This mechanism contributes to the hierarchical synthesis of *Salmonella* fimbriae. Finally, Mukherjee et al. demonstrated a new model for regulation in the *B. subtilis* Csr system (381), a partner-switching mechanism in which the FliW protein binds to CsrA and prevents it from repressing translation of the flagellin (*hag*) mRNA. Whether CsrA of *B. subtilis* is also regulated by sRNAs or regulates other genes besides *hag* is unclear. Bioinformatics analyses suggest that this partner-switching mechanism might operate in diverse Gram-positive and negative pathogens (Fig. 10). Without a doubt, many such surprises must be unearthed before the workings of these complex regulatory systems can be fully appreciated.

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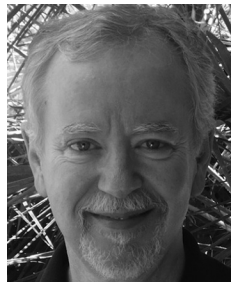
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