The RNA-Binding Protein CsrA Controls Virulence in *Erwinia amylovora* by Regulating RelA, RcsB, and FlhD at the Posttranscriptional Level

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CsrA, an RNA-binding protein, binds to target transcripts and alters their translation or stability. In *Erwinia amylovora*, CsrA positively regulates the expression of type III secretion system (T3SS), exopolysaccharide amylovan, and motility. In this study, the global effect of CsrA and its noncoding small RNA (ncsRNA) *csrB* in *E. amylovora* was determined by RNA-seq, and potential molecular mechanisms of CsrA-dependent virulence regulation were examined. Transcriptomic analyses under the T3SS-inducing condition revealed that mutation in the *csrA* gene led to differential expression of more than 20% of genes in the genome. Among them, T3SS genes and those required for cell growth and viability were significantly downregulated. On the other hand, the *csrB* mutant exhibited significant upregulation of most major virulence genes, suggesting an antagonistic effect of *csrB* on CsrA targets. Direct interaction between CsrA protein and *csrB* was further confirmed through the RNA electrophoretic mobility shift assay (REMSA). However, no direct interaction between CsrA and *hrpL* and *hrpS* transcripts was detected, suggesting that HrpL and HrpS are not targets of CsrA, whereas three CsrA targets (*relA*, *rscB*, and *flhD*) were identified and confirmed by REMSA, site-directed mutagenesis, and LacZ reporter gene assays. These findings might partially explain how CsrA positively controls *E. amylovora* virulence by targeting major regulators at the posttranscriptional level.

Keywords: bacterial pathogenesis, CsrA, genomics, posttranscriptional regulation, small noncoding RNAs, RNA-binding protein, T3SS, two-component system

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Erwinia amylovora, a gram-negative bacterium belonging to the Enterobacteriaceae family, is the causal agent of the economically important fire blight disease in apple and pear trees. The bacterium utilizes two pathogenicity factors to cause disease: a *hypersensitive response and pathogenicity* (*hrp*)-type III secretion system (T3SS) and the exopolysaccharide (EPS) amylovan (Khan et al. 2012; Zhao 2014; Zhao et al. 2009). The *hrp*-T3SS translocates effector proteins directly into host cells, thus interfering with host immunity (Büttner 2012; Zhao 2014). The expression of *hrp*-T3SS genes is activated by the master regulator HrpL, an alternative σ factor (McNally et al. 2012; Wei and Beer 1995), where expression of the *hrpL* gene is regulated by another alternative σ factor (RpoN) and other transcription factors, including YhbH, HrpS, integration host factor (IHF), (p)ppGpp, and DksA, all of which are indispensably required for transcription initiation and control of HrpL activity (Ancona et al. 2014; 2015b; Lee and Zhao 2016; Lee et al. 2018). On the other hand, the EPS amylovan plays a major role in biofilm formation and cell survival during late stages of the infection process (Koczan et al. 2009; Nimtz et al. 1996). Genes encoding EPS amylovan biosynthesis components are clustered in the *ams* operon and primarily activated by the Rcs phosphorelay system (Bernhard et al. 1993; Lee and Zhao 2018; Wang et al. 2009, 2012).

The Csr system, which was first reported in *Escherichia coli* as a regulator of glycogen biosynthesis, is one of the major posttranscriptional regulators in bacteria (Vakulskas et al. 2015). It consists of the carbon storage regulator CsrA (or its homologs RsmA and RsmE, repressor of secondary metabolites) and its regulatory noncoding small RNAs (ncsRNAs) (Babitzke and Romeo 2007). CsrA predominantly binds to a GGA motif in a hairpin structure at the 5′ untranslated region of mRNA, leading to RNA stabilization or destabilization, and translation activation or repression (Andrade et al. 2014; Vakulskas et al. 2015; Yakhnin et al. 2013). Recently, changes in RNA secondary structures caused by CsrA binding were also found to affect ρ -dependent termination and riboswitch confirmation, suggesting diverse regulatory action of CsrA at the posttranscriptional level (Figueroa-Bossi et al. 2014; Patterson-Fortin et al. 2013). On the other hand, ncsRNAs such as *csrB* in *Erwinia amylovora*, *rsmB* in *Pectobacterium carotovorum* and *Dickeya dadantii*, and *csrB* and *csrC* in *Escherichia coli* and *Salmonella enterica* contain a number of GGA motifs, which sequester CsrA and inhibit its activity (Babitzke and Romeo 2007). In γ -proteobacteria, expression of ncsRNAs is specifically dependent on the BarA/UvrY two-component system or its homologs such as GrrS/GrrA of *Erwinia amylovora* (Li et al. 2014; Suzuki et al. 2002; Zere et al. 2015). Other regulators such as RNA helicases, IHF, CsrD, (p)ppGpp, and DksA have also been reported to play a role in regulating

ncsRNAs (Edwards et al. 2011; Lee and Zhao 2016; Suzuki et al. 2006; Vakulskas et al. 2014).

Accumulating evidence showed that the Csr system greatly affects virulence gene regulation in diverse pathogenic bacteria. In the genus *Salmonella*, CsrA suppresses the expression of HilD, which acts as a central regulator in the activation of *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 (Martínez et al. 2011). In *Legionella pneumophila*, CsrA negatively regulates the expression of flagellar biosynthesis activator FleQ and quorum-sensing component LqsR. It also directly interacts with transcripts of Dot/Icm type IV secretion system effectors (Sahr et al. 2017). RsmA of *Xanthomonas citri* subsp. *citri* protects the *hrpG* transcript, encoding the master regulator of T3SS, from RNase E cleavage and allows T3SS gene expression (Andrade et al. 2014). In *E. amylovora*, the Csr system is a key virulence regulator activating T3SS, amyloporan production, and motility, while the *grrS/grrA*, *ihf*, and *csrB* mutants exhibited increased expression of those factors (Ancona et al. 2016; Lee and Zhao 2018; Lee et al. 2018; Li et al. 2014). However, target transcripts of CsrA remain unknown.

In *E. amylovora*, the Csr system has been shown to be closely associated with other important virulence regulators. It was reported that expression of *csrB* is under the control of IHF and the Rcs system (Lee and Zhao 2016; Lee et al. 2018). The nucleoid-associated protein IHF is involved in various DNA-dependent processes, including cell division, transcription, and site-specific recombination, via DNA remodeling (Ali Azam et al. 1999; Dillon and Dorman 2010). Transcriptional initiation of *hrpL* also requires IHF to enable interaction between transcription activators such as RpoN and HrpS (Lee and Zhao 2016; Lee et al. 2016). The Rcs system acts as a central two-component system that regulates major virulence factors of *E. amylovora*, including the T3SS, amyloporan production, and motility (Ancona et al. 2015a; Lee et al. 2018; Wang et al. 2012). Furthermore, Lon, an ATP-dependent protease, is also reported to be linked to the Csr system. Absence of Lon resulted in the accumulation of the RcsA and RcsB proteins, which suppress *csrB* expression, while absence of CsrA resulted in differential expression of *lon* at both transcriptional and posttranscriptional levels (Lee et al. 2018). These findings suggest that *E. amylovora*

CsrA could respond to both internal and external stimuli and act as a key component in the regulatory networks that coordinate the expression of different virulence factors.

In this study, we determined the global effect of CsrA and *csrB* at the transcriptional level, and examined the molecular mechanisms of CsrA-dependent virulence regulation in *E. amylovora*. Transcriptomic analysis under the T3SS-inducing condition revealed that mutation of *csrA* resulted in differential expression of hundreds of genes, including those involved in virulence and important physiological processes. A direct physical interaction between CsrA protein and *csrB* ncsRNA was confirmed, and several CsrA target transcripts involved in the regulation of T3SS, amyloporan production, and motility were determined, providing partial explanation of CsrA-dependent virulence regulation in *E. amylovora*.

RESULTS

CsrA acts as a global regulator under the T3SS-inducing condition.

In a previous study, we showed that CsrA is required for virulence in *E. amylovora* by acting as an essential activator of T3SS, amyloporan production, and motility (Ancona et al. 2016). In order to further understand its global effect, RNA-seq comparing the wild type (WT) and the *csrA* and *csrB* mutants grown under the T3SS-inducing condition was conducted. In total, 13,370,077 to 18,030,556 reads from each biological sample were generated, and the percentage of reads mapped to *E. amylovora* genome ranged from 96 to 98%. The log₂ fold change (FC)/mean of normalized counts plots were generated to visualize expression pattern of individual genes (Supplementary Fig. S1). Principal component analysis (PCA) clearly showed that the first two components explained approximately 82% of the variability in the dataset, and the PCA plot showed a clear separation of the WT from the *csrA* and *csrB* mutants, which were clustered together (Supplementary Fig. S2). Differentially expressed genes (DEGs), which exhibited a |log₂FC| value ≥ 1 and a corrected *P* value < 0.05 between the WT and the mutants, were also functionally categorized based on the clusters of orthologous groups (COGs) in Figure 1.

Fig. 1. Differential gene expression of the **A**, *csrA* and **B**, *csrB* mutants compared with the wild type under the type III secretion system (T3SS)-inducing conditions. Functional classification of differentially expressed genes (DEGs) based on the clusters of orthologous groups (COGs). DEGs were defined as genes with a |log₂ fold change| value ≥ 1 and a corrected *P* value < 0.05 from three independent biological replicates. T6SS = type VI secretion system.

In total, 804 genes, more than 20% of genes in the *E. amylovora* genome, were directly or indirectly affected in the *csrA* mutant, including 317 upregulated genes and 487 downregulated genes (Fig. 1A; Supplementary Table S1). In addition to the functional and regulatory components of T3SS, other virulence genes, including those encoding motility and the type VI secretion system, were rarely expressed in the *csrA* mutant (Table 1), further indicating that CsrA is critical for virulence activation in *E. amylovora*. Moreover, many genes responsible for important cellular processes, including

nucleotide biosynthesis, electron transport chain, and ribosomal biosynthesis, were also significantly downregulated, whereas heat-shock response and sulfur metabolism genes were upregulated in the *csrA* mutant. These dramatic physiological perturbations might explain the nonpathogenic, non-motility, and slow-growth phenotypes in the *csrA* mutant (Ancona et al. 2016). On the other hand, 379 DEGs, including 171 upregulated and 208 downregulated genes, were observed in the *csrB* mutant (Supplementary Table S2). Compared with the *csrA* mutant, DEGs of the *csrB* mutant exhibited relatively

Table 1. Group I differentially expressed genes in *hypersensitive response and pathogenicity (hrp)*-inducing medium^a

Gene ID	Gene description ^b	log ₂ FC Δ <i>csrA</i> /WT	log ₂ FC Δ <i>csrB</i> /WT
EAMY_0491	<i>lidJ</i> , disulphide bond formation protein	-1.31	2.84
EAMY_0492	Phytochelatin synthase	-1.09	3.41
EAMY_0519	<i>hrpK</i> , T3SS, pathogenicity locus protein	-2.39	2.07
EAMY_0520	<i>hsvA</i> , T3SS, Hrp-associated systemic virulence protein	-2.52	3.47
EAMY_0521	<i>hsvB</i> , T3SS, Hrp-associated systemic virulence protein	-1.47	3.40
EAMY_0522	<i>hsvC</i> , T3SS, Hrp-associated systemic virulence protein	-1.10	2.52
EAMY_0524	biphenyl 2,3-dioxygenase	-4.40	1.80
EAMY_0525	<i>hrcU</i> , T3SS, type III secretion protein	-3.17	1.71
EAMY_0526	<i>hrcT</i> , T3SS, type III secretion apparatus protein	-2.66	1.90
EAMY_0527	<i>hrcS</i> , T3SS, type III secretion protein	-4.29	1.89
EAMY_0528	<i>hrcR</i> , T3SS, type III secretion apparatus protein	-3.76	1.93
EAMY_0529	<i>hrcQ</i> , T3SS, type III secretion system apparatus protein	-3.12	1.80
EAMY_0530	<i>hrpP</i> , T3SS, type III secretion protein	-4.35	1.80
EAMY_0531	<i>hrpO</i> , T3SS, type III secretion protein	-5.30	1.81
EAMY_0532	<i>hrcN</i> , T3SS, type III secretion system ATPase	-4.90	1.78
EAMY_0533	<i>hrpQ</i> , T3SS, type III secretion system protein	-5.79	1.83
EAMY_0534	<i>hrcV</i> , T3SS, type III secretion inner-membrane protein	-5.45	1.78
EAMY_0535	<i>hrpJ</i> , T3SS, type III secretion system protein	-5.59	1.73
EAMY_0536	<i>hrpL</i> , T3SS, RNA polymerase σ factor	-5.58	1.68
EAMY_0542	<i>hrpA</i> , T3SS, Hrp pili protein	-6.87	1.72
EAMY_0543	<i>hrpB</i> , T3SS, type III secretion system protein	-6.20	1.67
EAMY_0544	<i>hrcJ</i> , T3SS, type III secretion inner-membrane protein	-5.78	1.66
EAMY_0545	<i>hrpD</i> , T3SS, type III secretion protein	-5.43	1.73
EAMY_0546	<i>hrpE</i> , T3SS, type III secretion apparatus protein	-3.94	1.81
EAMY_0547	<i>hrpF</i> , T3SS, type III secretion protein	-5.67	1.73
EAMY_0548	<i>hrpG</i> , T3SS, type III secretion protein	-5.57	1.73
EAMY_0549	<i>hrcC</i> , T3SS, type III secretion system outer membrane pore	-5.07	1.78
EAMY_0550	<i>hrpT</i> , T3SS, type III secretion lipoprotein	-4.71	1.79
EAMY_0551	<i>hrpV</i> , T3SS, type III secretion protein	-4.75	1.75
EAMY_0552	<i>hrpN</i> , T3SS, harpin protein	-6.50	1.98
EAMY_0553	<i>orfA</i> , T3SS, Tir chaperone family protein	-4.81	1.79
EAMY_0554	<i>orfB</i> , T3SS, avirulence protein	-3.78	1.71
EAMY_0555	<i>orfC</i> , T3SS, HrpW-specific chaperone	-5.28	1.80
EAMY_0556	<i>hrpW</i> , T3SS, harpin protein	-5.82	1.90
EAMY_0557	<i>dspE</i> , T3SS, Hrp secreted pathogenicity-like protein	-5.20	1.58
EAMY_0558	<i>dspF</i> , T3SS, Hrp secreted pathogenicity-like protein	-5.20	1.57
EAMY_0653	<i>eop2</i> , T3SS, type III effector	-3.62	1.85
EAMY_1450	<i>flgN</i> , motility, flagella synthesis protein	-1.77	1.64
EAMY_1451	<i>flgM</i> , motility, negative regulator of flagellin synthesis	-2.16	1.70
EAMY_1462	<i>flgK</i> , motility, flagellar hook-associated protein	-1.83	1.65
EAMY_1463	<i>flgL</i> , motility, flagellar hook-associated protein	-1.92	1.75
EAMY_1498	<i>rcsA</i> , colanic acid capsular biosynthesis activation protein	-1.42	2.73
EAMY_2094	<i>cheW</i> , motility, chemotaxis signal transduction protein	-2.07	1.33
EAMY_2095	<i>cheA</i> , motility, chemotactic sensory histidine kinase	-2.21	1.44
EAMY_2096	<i>motB</i> , motility, flagellar motor protein	-2.25	1.57
EAMY_2097	<i>motA</i> , motility, flagellar motor protein	-2.96	1.46
EAMY_2134	<i>yedO</i> , 1-aminocyclopropane-1-carboxylate deaminase	-1.83	1.48
EAMY_2135	D-cysteine desulphydrase	-1.45	1.41
EAMY_2139	<i>fliA</i> , motility, RNA polymerase σ factor	-5.35	1.09
EAMY_2141	<i>fliC</i> , motility, filament structural protein	-3.22	3.42
EAMY_2142	<i>fliD</i> , motility, flagellar capping protein	-2.71	1.62
EAMY_2143	<i>fliS</i> , motility, flagellin-specific chaperone	-2.72	1.26
EAMY_2144	<i>fliT</i> , motility, flagellar export chaperone	-1.85	1.23
EAMY_2218	<i>yeeF</i> , putrescine transporter	-1.16	1.03
EAMY_2275	<i>aroQ</i> , chorismate mutase	-1.42	1.43
EAMY_2945	Acyltransferase	-1.16	1.79
EAMY_3175	<i>avrRpt2</i> , T3SS, cysteine protease avirulence protein	-1.61	1.54

^a Group I genes were downregulated in the *csrA* mutant but upregulated in the *csrB* mutant with $|\log_2$ fold change ($\log_2$ FC)| value ≥ 1 and a corrected *P* value < 0.05 between the wild type (WT) and the mutants.

^b T3SS = type III secretion system.

less fluctuation in transcription levels across the genome, including metabolic and stress responsive genes (Fig. 1B). The expression of genes responsible for all of the major virulence factors, including T3SS, EPS amylovoran, and motility, was significantly increased in the *csrB* mutant. This is consistent with the results from phenotypic data of our previous studies (Ancona et al. 2016; Li et al. 2014).

Though many DEGs in both the *csrA* and *csrB* mutants were annotated as hypothetical proteins, Venn diagrams (Fig. 2A) identified four groups of genes whose expression might be closely associated with the Csr system. Group I included 68 genes that were downregulated in the *csrA* mutant but upregulated in the *csrB* mutant, including mostly T3SS and flagellar genes (Table 1). Expression of the *rcaA* gene also exhibited the same trend, whereas expression of the *rcaB*, *rcaC*, and *rcaD* genes in the Rcs system was not significantly changed in either mutant (Table 1), suggesting that CsrA might positively regulate the Rcs system at the posttranscriptional level. Group II contained 33 genes that were upregulated in the *csrA* mutant but downregulated in the *csrB* mutant, including *rcaV* (LuxR-family transcriptional regulator), *narPQ* (two-component system), *ompT* (outer membrane protease), and *CRISPR* (clustered regularly interspaced short palindromic repeat)-associated (Cas) protein genes (Table 2). Interestingly, the *rcaV* gene was approximately 60-fold upregulated and the most differentially expressed in the *csrA* mutant, whereas it was 3-fold downregulated in the *csrB* mutant.

Groups III and IV included 20 and 97 genes, respectively, that were significantly upregulated or downregulated in both mutants. Posttranslational regulators such as ATP-dependent protease *hslUV* genes; molecular chaperone *groEL* and *hspG* genes; and sulfur transport systems, including *tauABCD* (EAMY_3404 to EAMY_3407) and *ssuEADC* (EAMY_1370 to EAMY_1373) gene clusters, were all upregulated (Supplementary Table S3), whereas genes encoding glycosyl-transferase, lipoproteins, and membrane-associated proteins were all downregulated (Supplementary Table S4). To validate RNA-seq data, nine representative DEGs of the *csrA* or *csrB* mutant were randomly selected, and their expression was determined in the *hrp*-inducing medium (HMM) using real-time quantitative reverse-transcription (qRT)-PCR (Fig. 2B). Although a different magnitude of the fold change was observed in the T3SS genes such as *hrpL* and *hsvA*, all genes tested showed similar trends between qRT-PCR and RNA-seq results, suggesting that our results are reliable and reproducible.

CsrA does not directly interact with *hrpL* and *hrpS* transcripts.

It is assumed that *csrB* ncsRNA acts antagonistically to regulate CsrA in *E. amylovora*; however, their direct interaction was not experimentally confirmed (Ancona et al. 2016). RNA electrophoretic mobility shift assay (REMSA) analysis showed that *csrB* sRNA led to a band shift with as low as 10 nM CsrA protein (Fig. 3A), indicating that binding affinity between *csrB* ncsRNA and CsrA is high. On the other hand, both *hrpL* and *hrpS* are barely expressed in the *csrA* mutant but are significantly upregulated in the *csrB* mutant, suggesting that *hrpL* or *hrpS* transcripts might be the direct targets of CsrA (Ancona et al. 2016; Lee and Zhao 2018). However, REMSA showed no interaction between CsrA protein and both *hrpL* and *hrpS* RNA probes (Fig. 3A), suggesting that CsrA does not directly target *hrpL* and *hrpS* transcripts and might target other regulatory genes involved in the regulation of T3SS gene expression.

CsrA is required for efficient translation of *relA*.

In *E. amylovora*, the nucleotide second messenger (p)ppGpp is a key activator of T3SS gene expression by activating the

RpoN-HrpL alternative σ factor cascade and *hrpS* expression through HrpX/HrpY (Ancona et al. 2016; Lee and Zhao 2018). Because our transcriptomic analysis showed that major T3SS regulators under (p)ppGpp control are differentially expressed in the *csrA* mutant but the transcript levels of (p)ppGpp biosynthesis genes *relA* and *spoT* are not significantly changed (Ancona et al. 2015b; Lee and Zhao 2018), we hypothesized that CsrA might be involved in their regulation at the post-transcriptional level. REMSA showed a direct interaction between CsrA and *relA* and *spoT* RNA probes (Fig. 3), but only expression of the *relA* gene from the translational fusion construct was slightly reduced in the *csrB* mutant (Fig. 4A). Secondary structure analysis showed two hairpin loop structures containing the putative CsrA-binding sites in the *relA* leader sequence (Fig. 4B). To further examine the role of CsrA on the *relA* translation, the putative CsrA-binding site near the putative Shine-Dalgarno sequence was mutated from GGA to GGG to eliminate CsrA-binding effect without affecting RNA secondary structure (Fig. 4B). Translation of the *relA* gene in both the WT and the *csrB* mutant was decreased to approximately 80% after mutation in the GGA motif (Fig. 4C), indicating that CsrA binding contributes to efficient *relA* expression. However, it is still unclear whether *spoT* is also a direct target of CsrA based on our current data.

Fig. 2. A, Venn diagrams show overlapping and unique differentially expressed genes (DEGs) between the *csrA* and *csrB* mutants. DEGs were defined as genes with a $|\log_2$ fold change| value ≥ 1 and a corrected P value < 0.05 from three independent biological replicates. **B,** Validation of RNA-seq data. Relative gene expression of the selected genes in the *csrA* and *csrB* mutants compared with the wild type (WT) grown in hypersensitive response and pathogenicity (*hrp*)-inducing medium at 18°C for 6 h. The *rpoD* gene was used as an endogenous control. Values of the relative fold change were the means of three replicates, and values with the same letter in each gene do not differ significantly ($P < 0.05$). Error bars indicate standard deviation.

CsrA positively regulates *rcsB* expression at the posttranscriptional level.

Previous genetic studies and transcriptomic analysis showed increased expression of many Rcs-dependent genes in the *csrB* mutant, including *rcsA*, *hrpS*, and *ams* operon genes; thus, we hypothesized that the activity of the Rcs system might be positively regulated by CsrA (Lee and Zhao 2018; Lee et al. 2018). REMSA analyses showed that CsrA specifically binds to the leader sequence of *rcsB* (Fig. 5A and B; Supplementary Fig. S3). Under the T3SS-inducing condition, transcript level of the *rcsB* gene was not changed in the *csrB* mutant (Lee et al. 2018); however, expression of *rcsB* from the translational fusion construct increased approximately 1.5-fold. No increase was observed for the *rcsD* gene, which is in the same operon as *rcsB* (Fig. 5C). These results suggest that binding of CsrA to the *rcsB* transcript positively regulates *rcsB* expression at the post-transcriptional level.

CsrA positively regulates motility through *flhD* at the posttranscriptional level.

One of the well-studied CsrA target transcript in *Escherichia coli* is *flhD*, encoding the master regulator of flagellar gene expression. It has been shown that CsrA binds to the leader sequence of *flhD* and increases its translation by protecting the transcript from RNase E-mediated cleavage (Wei et al. 2001; Yakhnin et al. 2013). Although the leader sequences of *flhD* in *E. coli* and *Erwinia amylovora* are not homologous, genetic analyses suggested that CsrA positively regulates motility in *E. amylovora*, possibly through *flhD* (Ancona et al. 2016; Lee and Zhao 2016; Li et al. 2014). To determine its underlying mechanism, expression of different classes of flagellar gene using transcriptional fusion constructs in the *csrB* mutant was measured in tryptone broth (TB) (Fig. 6A). Compared with the WT, expression of class II and class III flagellar genes (*fliL*, *fliA*, *flgM*, and *fliD*) increased approximately threefold, whereas expression of a class I flagellar gene (*flhD*) was not considerably changed, suggesting that posttranscriptional regulation might occur in the *flhD* gene. REMSA showed that

Fig. 3. A, Gel shift assays (RNA electrophoretic mobility shift assay) of CsrA interacts with the leader sequences of *csrB*, *hrpL*, *hrpS*, *relA*, and *spoT*. Black arrows at the bottom and top indicate free probe and the protein-RNA complex, respectively. The concentration of protein (in nanomoles) is indicated above each lane. Experiments were repeated three times with similar results. **B**, Competition assays of interaction between CsrA and *relA* and *spoT* RNA probes by adding unlabeled *csrB* RNA. Black arrows at the bottom and top indicate free probe and the protein-RNA complex, respectively. The concentration of protein (in nanomoles) is indicated above each lane. Experiments were repeated three times with similar results.

Table 2. Group II differentially expressed genes in *hypersensitive response and pathogenicity* (*hrp*)-inducing medium^a

Gene ID	Gene description ^b	log ₂ FC Δ <i>csrA</i> /WT	log ₂ FC Δ <i>csrB</i> /WT
EAMY_0054	LuxR-family transcriptional regulator	2.22	-1.47722
EAMY_0249	<i>ompT</i> , outer membrane protease	1.13	-1.01609
EAMY_0441	Creatininase	2.41	-2.6658
EAMY_0442	Dihydroorotate dehydrogenase	2.51	-2.74218
EAMY_0444	<i>mcvE</i> , glutamate-1-semialdehyde aminotransferase	2.84	-2.9406
EAMY_0445	<i>mcvE</i> , β -ketoacyl synthase	2.18	-2.36154
EAMY_0446	<i>irp</i> , Polyketide synthase	2.38	-2.78833
EAMY_0447	<i>sypC</i> , gramicidin S synthetase II	2.50	-2.96328
EAMY_0448	<i>ppsD</i> , polyketide synthase	2.41	-2.81697
EAMY_0449	Polyketide synthase	1.84	-2.13904
EAMY_1738	<i>ynfM</i> , major facilitator superfamily transporter	1.80	-1.03952
EAMY_2423	<i>fabB</i> , 3-oxoacyl-(acyl-carrier-protein) synthase	2.87	-1.15477
EAMY_2475	Hypothetical protein	1.44	-1.81294
EAMY_2506	<i>narQ</i> , two-component system histidine kinase	1.15	-1.44746
EAMY_2552	Major facilitator superfamily transporter	3.62	-1.22834
EAMY_2800	<i>rcsV</i> , LuxR-family transcriptional regulator	5.85	-1.68411
EAMY_2813	<i>ygbF</i> , CRISPR-associated protein Cas2	1.32	-1.70694
EAMY_2814	<i>ygbT</i> , CRISPR-associated protein Cas1	1.29	-2.01252
EAMY_2815	<i>ycgH</i> , Cse3-family CRISPR-associated protein	1.12	-2.10032
EAMY_2816	<i>ycgI</i> , CRISPR-associated protein Cas5	1.21	-2.21313
EAMY_2817	<i>ycgJ</i> , Cse4-family CRISPR-associated protein	1.39	-2.2221
EAMY_2818	<i>ycgK</i> , Cse2-family CRISPR-associated protein	1.61	-2.25029
EAMY_2819	<i>ycgL</i> , Cse1-family CRISPR-associated protein	1.90	-2.20392
EAMY_2820	<i>ycgB</i> , CRISPR-associated helicase Cas3	1.68	-1.68656

^a Group II genes were upregulated in the *csrA* mutant but downregulated in the *csrB* mutant with $|\log_2$ fold change ($\log_2$ FC)| value ≥ 1 and a corrected *P* value < 0.05 between the wild type (WT) and the mutants.

^b CRISPR = clustered regularly interspaced short palindromic repeat.

Fig. 4. CsrA is required for efficient translation of *relA*. **A**, LacZ activities of translational fusion constructs carrying upstream sequences of the *relA* and *spoT* genes in the wild type (WT) and the *csrB* mutant grown in hypersensitive response and pathogenicity (*hrp*)-inducing medium (HMM) at 18°C for 6 h. Values of Miller units were the means of four replicates. **B**, Predicted secondary structure of the *relA* leader sequence. The putative CsrA-binding site and the start codon are indicated with the circle and arrow, respectively. **C**, LacZ activities of translational fusion constructs carrying different upstream sequences of the *relA* genes in the WT and the *csrB* mutant grown in HMM at 18°C for 6 h. Values of Miller units were the means of four replicates, and the values with the same letter do not differ significantly ($P < 0.05$). Experiments were repeated three times with similar results.

CsrA directly and specifically binds to *flhD* RNA (Fig. 6B and C). A translational fusion reporter gene assay showed that expression of *flhD* increased approximately twofold in the *csrB* mutant as compared with that of the WT (Fig. 6D), indicating that CsrA positively regulates *flhD* expression at the post-transcriptional level in *E. amylovora*.

To further characterize CsrA-dependent *flhD* upregulation, each of four putative CsrA-binding sites (named GGA1 to GGA4) on the leader sequence of the *flhD* gene was deleted, and differential expression using translational fusion constructs was compared (Fig. 6E). In the WT, *flhD* expression was decreased after deletion of GGA1 and GGA4 whereas, in the *csrB* mutant, *flhD* expression was increased after deletion of GGA2 but decreased after deletion of GGA1, GGA3, and GGA4 (Fig. 6F). These results suggest that CsrA might bind to multiple sites of the *flhD* leader sequence, which leads to an increased translation rate of *flhD* in *E. amylovora*.

DISCUSSION

The RNA-binding protein CsrA is indispensable for virulence by positively regulating major virulence factors in *E. amylovora*, including T3SS and amylovoran (Ancona et al. 2016). In this study, we confirmed that CsrA is a global regulator not only of virulence gene expression but also of diverse

Fig. 5. CsrA positively regulates *rcsB* expression at the posttranslational level. **A**, RNA electrophoretic mobility shift assay for CsrA binding to the leader sequence of *rcsB*. **B**, Competition assay of interaction between CsrA and *rcsB* RNA by adding unlabeled *csrB* RNA. Black arrows at the bottom and top indicate free probe and the protein-RNA complex, respectively. The concentration of protein (in nanomoles) is indicated above each lane. **C**, LacZ activities of translational fusion constructs carrying upstream sequences of the *rcsD* and *rcsB* genes in the wild type (WT) and the *csrB* mutant grown in hypersensitive response and pathogenicity (*hrp*)-inducing medium at 18°C for 6 h. Values of Miller units were the means of four replicates, and the values with the same letter do not differ significantly ($P < 0.05$). Experiments were repeated three times with similar results.

cellular processes required for cell growth and viability. We also demonstrated that CsrA directly binds to *flhD*, *rcsB*, and *relA* transcripts and positively regulates their expression at the posttranscriptional level, thus influencing major virulence factors and contributing to virulence in *E. amylovora*.

The global regulatory roles of CsrA have been studied in several species using deep sequencing. In transcriptomic analysis of *S. typhimurium* and *Pseudomonas aeruginosa*, a mutation in *csrA* led to 375 and 506 DEGs, respectively, compared with the WT, which are approximately 10% of total genes in each species (Burrowes et al. 2006; Lawhon et al. 2003). Recent studies using CsrA-RNA complex immunoprecipitation combined with deep sequencing approach (CLIP-seq) also revealed that, in *S. typhimurium*, *L. pneumophila*, and *Campylobacter jejuni*, CsrA directly interacts with approximately 10% of their total gene transcripts (Dugar et al. 2016; Holmqvist et al. 2016; Sahr et al. 2017). On the other hand, integration of different transcriptomic approaches in *E. coli* revealed that approximately 25 and 12.5% of total gene transcripts were differentially expressed in the *csrA* mutant and directly bound by CsrA in vivo, respectively (Potts et al. 2017). These reports collectively indicate that CsrA acts as a major posttranscriptional regulator in bacteria under different experimental conditions.

In this study, our results showed that approximately 20% of the genes in *E. amylovora* genome were differentially expressed in the absence of CsrA. Unlike other species examined, the DEGs included not only virulence genes but also many physiologically important genes. The *csrA* mutant exhibited a significant downregulation of nucleotide biosynthesis, electron transport chain, and ribosomal biosynthesis, suggesting its incapability to maintain cell integrity. The *csrA* mutant also exhibited a significant upregulation of heat-shock genes. Given that transcription of heat-shock genes is triggered in response to various stress conditions, including temperature variations, toxic chemicals, desiccation, and viral infections, to protect intracellular proteins from denaturation and aggregation (Morimoto 1993), upregulation of these genes in the *csrA* mutant indicates unstable intracellular states. Consistently, it was reported in *Escherichia coli* that cells under heat-shock stress exhibited a downregulation of genes involved in ribosome assembly and energy metabolism (Murata et al. 2011), suggesting that a mutation in the *csrA* gene results in severe cellular stress. These results together showed that gene regulation and cell physiology in *Erwinia amylovora* is highly dependent on CsrA and, thus, its absence causes pleiotropic defects, including slow growth and nonpathogenic phenotypes (Ancona et al. 2016).

Fig. 6. CsrA positively regulates *flhD* expression at the posttranscriptional level. **A**, LacZ activities of transcriptional fusion constructs carrying upstream sequences of five different flagellar genes (*flhD*, *fliL*, *fliA*, *flgM*, and *fliD*) in the wild type (WT) and the *csrB* mutant grown in tryptone broth (TB) at 28°C for 6 h. Values of Miller units were the means of four replicates. **B**, RNA electrophoretic mobility shift assay for CsrA binding to the leader sequence of *flhD*. **C**, Competition assay of interaction between CsrA and *flhD* RNA by adding unlabeled *csrB* RNA. Black arrows at the bottom and top indicate free probe and the protein-RNA complex, respectively. The concentration of protein (nM) is indicated above each lane. **D**, LacZ activities of translational fusion constructs carrying upstream sequences of the *flhD* gene in the WT and the *csrB* mutant grown in TB at 28°C for 6 h. Values of Miller units were the means of four replicates, and the values with the same letter do not differ significantly ($P < 0.05$). Experiments were repeated three times with similar results. **E**, The leader sequence of the *flhD* gene. The transcription start site, four putative CsrA-binding sites, and ATG start codon are underlined. Each GGA motif was deleted using site-directed mutagenesis, and the four different mutated sequences were cloned into pZLac29. Plasmids were designated as pFlhD29-Mut1 to pFlhD29-Mut4 and introduced into the WT and the *csrB* mutant. **F**, LacZ activities of translational fusion constructs carrying different upstream sequences of the *flhD* gene in the WT and the *csrB* mutant grown in TB at 28°C for 6 h. Values of Miller units were the means of four replicates, and the values with the same letter do not differ significantly ($P < 0.05$). Experiments were repeated three times with similar results.

Interestingly, five heat-shock genes (*groE*, *groL*, *hslU*, *hslV*, and *hspG*) were upregulated in both the *csrA* and *csrB* mutants. Because the *csrB* mutant did not exhibit any notable defects in growth and virulence, increased expression of heat-shock genes in the *csrB* mutant appears to be caused by a different source of stress. Extrapolating from the function of heat-shock proteins, one possibility is that heat-shock gene expression might be induced in response to significantly increased levels of T3SS and flagellar proteins in the cell. Transcriptomic analysis in this study was performed when T3SS transcript levels reached a peak in the WT (Lee and Zhao 2018). The *csrB* mutant exhibited up to 11-fold upregulation of T3SS and flagellar proteins, and also up to 6-fold upregulation of proteins for amylovan production, possibly resulting in sudden increased demand for molecular chaperones. Several studies have reported a similar induction pattern of heat-shock genes during overproduction of recombinant proteins in *Escherichia coli* to ensure correct protein folding (Hoffmann and Rinas 2000; Jürgen et al. 2000; Li et al. 2017). Upregulation of heat-shock genes in the *csrB* mutant might be also associated with the function of T3SS or flagellar motor. It was reported in *E. coli* that HtpG is required for the production of virulence-associated secondary metabolites (colibactin and yersiniabactin) and the activity of CRISPR/Cas system (Garcie et al. 2016; Yosef et al. 2011). HtpG of *E. coli* also can directly interact with cytoplasmic flagellar motor components FliN and FliI (Li and Sourjik 2011). Moreover, heat-shock proteins in eukaryotes are widely observed in the assembly process of oligomers and protein complexes (Haslbeck and Vierling 2015), suggesting that bacterial heat-shock proteins might also be capable of promoting the assembly of T3SS or flagellar motor apparatus.

In addition, the *tau* and *ssu* gene clusters, encoding ABC-type sulfur transporters and sulfur metabolism-related products, were also upregulated in both *csrA* and *csrB* mutants. Expression of both gene clusters is reported to be positively regulated by the Cbl transcription factor in *E. coli* (van der Ploeg et al. 1997, 1999). In our transcriptomic data, the *csrA* mutant showed approximately 12-fold increased expression of *cbl* as compared with the WT and, accordingly, showed a significant upregulation of 13 sulfur metabolism genes known to contain Cbl-dependent promoter, including *tauABCD*, *ssuEADC*, *cysK*, *cysHII*, and *sbp* (van der Ploeg et al. 2001). Other sulfur metabolism genes such as *cysP* and *cysCND* were also upregulated in the *csrA* mutant. It has been reported that sulfur metabolism is highly associated with methionine and cysteine biosynthesis as well as oxidative stress response (Gyaneshwar et al. 2005), further suggesting unstable intracellular states in the *csrA* mutant. However, no significant changes in transcript levels of *cbl* and other sulfur metabolism genes were observed in the *csrB* mutant; thus, upregulation of the *tau* and *ssu* genes might be mediated by yet-unknown regulatory factors, which are possibly associated with CsrA.

On the other hand, expression of 33 genes was negatively regulated by CsrA and, of these, the *rscV* gene was the most strongly induced in the absence of CsrA. The *rscV* gene of *Erwinia amylovora* that was first identified can recover EPS production in the *rscA* mutant of *Pantoea stewartii* (Aldridge et al. 1998). However, it was reported that the *rscV* mutant of *E. amylovora* caused no phenotypic differences, and its expression was not induced even under the strong *lac* promoter by unknown mechanism (Aldridge et al. 1998). Our transcriptomic analysis showed that *rscV* expression was highly enhanced in the *csrA* mutant but suppressed in the *csrB* mutant, suggesting that the Csr system might be responsible for the suppression of *rscV* expression under normal conditions. A gene cluster (EAMY_2813 to EAMY_2820), encoding Cas proteins, was also negatively regulated by CsrA. Consistent with its primary role as a defense mechanism against foreign nucleic acids, *cas*

gene expression has been reported to be induced against viral infection and regulated by various transcription factors and signaling molecules (Patterson et al. 2017), whereas a role of the Csr system in the CRISPR/Cas system regulation was not reported. Because the CRISPR/Cas system utilizes guide RNAs to target invading genetic elements, it may also contribute to posttranscriptional regulation through the RNA interference-like system (Bhaya et al. 2011). In *E. amylovora*, studies of the CRISPR/Cas system have been focused on genetic diversity of short DNA repeat sequences from different isolates (McGhee and Sundin 2012; Rezzonico et al. 2011); thus, our knowledge of its function and gene regulation is still incomplete. Further studies are warranted to assess interaction of the Csr system with the CRISPR/Cas system.

The nucleotide second messenger (p)ppGpp is another major global regulatory system in bacteria. Under starvation conditions, bacteria produce high levels of (p)ppGpp, which induces massive transcriptional reprogramming to adjust various stress responses (Dalebroux and Swanson 2012). During early stages of infection, *E. amylovora* undergoes the nutrient-limited conditions. The subsequent accumulation of (p)ppGpp plays an essential role in virulence activation by reducing cell size to increase resistance to abiotic stresses and activating the RpoN-HrpL alternative σ factor cascade for T3SS gene expression (Ancona et al. 2015b; Khakimova et al. 2013). Because the presence of both (p)ppGpp and CsrA is essential for the T3SS, codependence of two global regulators was of particular interest in our studies. In *Escherichia coli*, (p)ppGpp and DksA positively regulate the expression of CsrA and *csrB/csrC* sRNAs, while CsrA suppresses (p)ppGpp accumulation by inhibiting *relA* translation, forming a reciprocal regulatory circuit (Edwards et al. 2011). In contrast, in *Erwinia amylovora*, the absence in *dksA* resulted in approximately twofold decrease in *csrA* transcription but no significant effects by (p)ppGpp were observed on *csrB* expression under the T3SS-inducing condition (unpublished data). *E. amylovora* CsrA is also required for efficient translation of *relA*, although it binds to the region near the ribosome-binding site and the start codon. Similar observations were reported in *L. pneumophila* but the exact molecular mechanisms underlying this auxiliary effect of CsrA binding remain unclear (Sahr et al. 2017). Taken together, CsrA might enable *E. amylovora* to induce higher levels of (p)ppGpp by maintaining a cellular nucleotide pool and also activating efficient *relA* translation.

The Rcs phosphorelay system is only found in enterobacterial pathogens and is well known as a regulator of EPS production and biofilm formation (Erickson and Detweiler 2006; Ferrières and Clarke 2003; Majdalani and Gottesman 2005). In *E. amylovora*, the Rcs system acts as an essential virulence regulator by activating *hrpS* and amylovan production, and also contributes to the regulation of motility, *csrB* expression, and antibiotic resistance (Ancona et al. 2015a; Ge et al. 2018; Lee et al. 2018; Wang et al. 2009, 2012). Given that RcsB-dependent genes, including *hrpS* and *rscA*, were differentially expressed in the *csrB* mutant without changes in *rscB* transcript level (Lee and Zhao 2018), we assumed that increased activity of CsrA might elevate the activity of the Rcs system. In *E. amylovora*, because *rscB* expression was stable in different conditions, phosphorylation status of RcsB was thought to be a major determinant of the Rcs system activity (Wang et al. 2012). However, our study showed that CsrA can directly interact with the leader sequence of *rscB* and positively regulate its translation, proposing a novel mechanism that affects the Rcs system activity through posttranscriptional regulation.

In *Escherichia coli*, it is well established that CsrA binding of *fliH* inhibits RNase E-mediated cleavage and enhances its translation rate (Wei et al. 2001; Yakhnin et al. 2013). Consistently, CsrA of *Erwinia amylovora* also binds to multiple

sites on the *flhD* transcript, resulting in enhanced translation. This finding reinforces our previous observations that mutant strains with increased CsrA activity such as the *csrB*, *grrS/grrA*, and *ihf* mutants were hypermotile (Ancona et al. 2016; Lee and Zhao 2016; Li et al. 2014). Although it was not detected from the *lacZ* reporter fusion assays under the flagellar gene-inducing condition, transcriptome analysis under the T3SS-inducing condition showed that flagellar class 3 genes (*fliA*, *flgM*, and *fliD*) were approximately threefold upregulated as compared with flagellar class 2 genes (*fliL* and *fliP*) in the *csrB* mutant. This might suggest that CsrA-dependent flagellar gene regulation could also be mediated through the class 3 gene regulators *fliA* and *flgM*, which need further verification.

In summary, our research demonstrated that CsrA is a key component coordinating a number of cellular processes in *E. amylovora*, and its direct targets, including FlhD, RcsB, and RelA, are responsible for the regulation of virulence factors, including T3SS, amyloporan, and motility. However, our understanding of how the Csr system monitors cellular homeostasis is still incomplete. Future direction of this study might include identification of additional CsrA target genes that are associated with the regulation of physiologically important processes.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 3. Luria-Bertani (LB) was used routinely to culture

E. amylovora and *Escherichia coli* strains, and the HMM (1 g of $[\text{NH}_4]_2\text{SO}_4$, 0.246 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g of NaCl, 8.708 g of K_2HPO_4 , and 6.804 g of KH_2PO_4) supplemented with 10 mM galactose as carbon source was used to determine gene expression (Ancona et al. 2014). TB (10 g of tryptone and 5 g of NaCl per 1 liter) was used to measure flagellar gene expression. When required, antibiotics were added at the following concentrations: ampicillin at 100 $\mu\text{g}/\text{ml}$, kanamycin (Km) at 50 $\mu\text{g}/\text{ml}$, chloramphenicol at 10 $\mu\text{g}/\text{ml}$, and gentamicin at 10 $\mu\text{g}/\text{ml}$. Primers used in this study are listed in Supplementary Table S5.

RNA isolation, RNA-seq, and qRT-PCR.

RNA was isolated from cell cultures grown in HMM for 6 h at 18°C using the RNeasy minikit (Qiagen, Hilden, Germany), followed by DNase I treatment using the TURBO DNA-free kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), according to the manufacturer's instructions. The quantity and quality of RNA samples were assessed using either the Nano-Drop ND-100 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, U.S.A.) or Agilent RNA 6000 Nano Chip Bioanalyzer (Agilent, Santa Clara, CA, U.S.A.). For RNA-seq analysis, library construction and sequencing using Illumina HiSeq 2500 (Illumina, San Diego, CA, U.S.A.) were performed on three biological samples for the WT and the mutants by the Keck Center at the University of Illinois at Urbana-Champaign (UIUC). The sequence reads were aligned to the genome of *Erwinia amylovora* CFBP1430 (Smits et al. 2010) using Bowtie

Table 3. Bacterial strains and plasmids used in this study

Strains, plasmids	Description ^a	Reference, source
<i>Erwinia amylovora</i>		
Ea1189	Wild type, isolated from apple	Wang et al. 2009
ΔcsrA	<i>csrA::Cm</i> ; Cm^R -insertional mutant of <i>csrA</i> of Ea1189	Ancona et al. 2016
ΔcsrB	<i>csrB::Cm</i> ; Cm^R -insertional mutant of <i>csrB</i> of Ea1189	Ancona et al. 2016
<i>Escherichia coli</i>		
DH10B	F^- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara leu</i>) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>nupG</i> λ -	Invitrogen
BL21 (DE3)	F^- <i>ompT</i> <i>hsdS_B</i> (r_B^- m_B^-) <i>gal</i> <i>dcm</i> (DE3)	Novagen
XL10-Gold	Tet^R Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA96</i> <i>relA1</i> <i>lac</i> <i>Hte</i>	Stratagene
Plasmids		
pWSK29	Ap^R , cloning vector, low copy number	Wang and Kushner 1991
pET28a	Km^R , T7 expression vector carrying an N-terminal His-Tag/thrombin/T7 Tag configuration plus an optional C-terminal His-Tag sequence	Novagen
pCsrA-His	183-bp DNA fragment containing <i>csrA</i> gene in pET28a	This study
pRS552	Translation fusion vector containing the MCS, <i>EcoRI-SmaI-BamHI-lacZYA'</i>	Simons et al. 1987
pHRP309	Broad-host-range <i>lacZ</i> transcriptional fusion vector, Gm^R	Parales and Harwood
pZLac29	pWSK29 containing the MCS, <i>EcoRI-SmaI-BamHI-lacZ</i>	This study
pFlhD309	626-bp Fragment containing <i>flhD</i> gene (−600+26) in pHRP309	This study
pFliL309	626-bp Fragment containing <i>fliL</i> gene (−600+26) in pHRP309	This study
pFliA309	626-bp Fragment containing <i>fliA</i> gene (−600+26) in pHRP309	This study
pFlgM309	626-bp Fragment containing <i>flgM</i> gene (−600+26) in pHRP309	This study
pFliD309	626-bp Fragment containing <i>fliD</i> gene (−600+26) in pHRP309	This study
pFlhD29	626-bp Fragment containing <i>flhD</i> gene (−600+26) in pZLac29	This study
pFlhD29-Mut1	626-bp Fragment containing <i>flhD</i> gene (−600+26) with a mutation at position −253 to −251 in pZLac29	This study
pFlhD29-Mut2	626-bp Fragment containing <i>flhD</i> gene (−600+26) with a mutation at position −216 to −214 in pZLac29	This study
pFlhD29-Mut3	626-bp Fragment containing <i>flhD</i> gene (−600+26) with a mutation at position −204 to −202 in pZLac29	This study
pFlhD29-Mut4	626-bp Fragment containing <i>flhD</i> gene (−600+26) with a mutation at position −189 to −187 in pZLac29	This study
pRelA29	626-bp Fragment containing <i>relA</i> gene (−600+26) in pZLac29	This study
pRelA29-Mut	626-bp Fragment containing <i>flhD</i> gene (−600+26) with a mutation at position −20 to −18 in pZLac29	This study
pSpoT29	626-bp Fragment containing <i>spoT</i> gene (−600+26) in pZLac29	This study
pRcsD29	626-bp Fragment containing <i>rscD</i> gene (−600+26) in pZLac29	This study
pRcsB29	626-bp Fragment containing <i>rscB</i> gene (−600+26) in pZLac29	This study

^a Cm = chloramphenicol; Cm^R , Tet^R , Ap^R , Km^R , and Gm^R = resistant to chloramphenicol, tetracycline, ampicillin, kanamycin, and gentamicin, respectively.

0.12.7 (Langmead et al. 2009). To perform normalization and statistical analysis on the raw read counts, the R package DESeq2 was used as described previously (Love et al. 2014). DEGs were defined as genes with a $\log_2FC$ value ≥ 1 and a corrected P value < 0.05 from three independent biological replicates. The RNA-seq data files have been submitted to Gene Expression Omnibus at the NCBI with an accession number GSE128088.

For qRT-PCR, reverse transcription was performed using Superscript III reverse transcription (Invitrogen, Carlsbad, CA, U.S.A.), and cDNA samples were mixed with Power SYBR Green PCR master mix (Applied Biosystems, CA, U.S.A.) and appropriate primers. The StepOnePlus Real-Time PCR system (Applied Biosystems) was used for qRT-PCR under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer specificities were assessed using melting curves. The *rpoD* gene was used as an endogenous control to calculate relative quantification ($\Delta\Delta Ct$). The experiment was repeated three times, and statistical analysis was performed using Student's t test with $P < 0.05$.

CsrA protein purification.

The coding sequence of the *csrA* gene was cloned into pET28a expression vector (Novagen, Madison, WI, U.S.A.) and, after confirmation by sequencing at the Keck center of UIUC, final plasmid was introduced into *Escherichia coli* strain BL21 (DE3). Overnight culture of the CsrA-overexpressing strain was inoculated into 500 ml of fresh LB media containing Km at 50 µg/ml. After 2 to 3 h of growth, bacteria culture was treated with 0.1 mM isopropyl- β -D-thiogalactoside to induce protein expression and incubated overnight at 18°C. Cells were harvested, washed with cell wash buffer (50 mM morpholine-propanesulfonic acid [MOPS] and 150 mM NaCl), and resuspended (1:10 ratio [wt/vol]) in cell wash buffer. Cell suspension was treated with lysozyme (Promega Corp., Madison, WI, U.S.A.) at 250 µg/ml for 30 min, cooled on ice for 30 min, and mixed with 1× Halt protease inhibitor (Thermo Fisher Scientific), NaCl (300 mM), and imidazole (60 mM). After sonication, cell lysates were removed by centrifugation at 35,000 × g for 20 min. The supernatant was treated with Ni-NTA agarose resin (Qiagen) at 4°C for 30 min with gentle rocking to collect His-tagged CsrA proteins. Ni-NTA resins were then washed with equilibration or wash buffer (50 mM MOPS, 300 mM NaCl, and 60 mM imidazole), and proteins were eluted with elution buffer (50 mM MOPS, 300 mM NaCl, and 500 mM imidazole). Proteins were dialyzed overnight against buffer containing 20 mM MOPS and 1 mM dithiothreitol, and protein concentration was measured using Invitrogen Qubit protein assay.

REMSA.

Leader sequences of genes of interest were transcribed in vitro using the MEGAscript kit (Thermo Fisher Scientific) and labeled with biotin using the Pierce RNA 3' end biotinylation kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Reaction mixtures were prepared using the Lightshift chemiluminescent RNA EMSA kit (Thermo Fisher Scientific) in volumes of 10 µl, containing 2 nM biotin-labeled target RNA, different amounts of CsrA protein, 1× binding buffer, 5% glycerol, and 0.4 U of RNase inhibitor, incubated at room temperature for 20 min, and mixed with 5× loading buffer. For competition assays, 2 nM unlabeled *csrB* RNA was additionally added to the reaction mixture. CsrA-RNA complexes were separated on a 6% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer (44.5 mM Tris-base, 44.5 mM boric acid, and 1 mM EDTA), followed by UV-light crosslinking to a positively charged nylon

membrane. Chemiluminescent signals were then detected using the ImageQuant LAS 4010 CCD camera (GE Healthcare, Piscataway, NJ, U.S.A.).

β -Galactosidase assay.

A β -galactosidase assay was performed using a microtiter plate, as described previously (Slauch and Silhavy 1991). Cell cultures grown in HMM for 6 h at 18°C were collected and resuspended in Z-buffer (Miller 1972). After measuring optical density at 600 nm (OD_{600}), cell suspensions were treated with 1% sodium dodecyl sulfate and chloroform, then mixed with *o*-nitrophenyl galactoside (ONPG) at 10 mg/ml to initiate the reaction. Units for β -galactosidase assay are defined as (micromoles of ONP formed per minute) $\times 10^6 / (OD_{600} \times \text{milliliter of cell suspension})$ and reported as mean $\pm$ standard deviation. Constructs of transcriptional and translational fusions were generated using vector pHRP309 and pZLac29, respectively, and confirmed by sequencing at the Keck center at UIUC. The vector pZLac29 was generated by transferring multiple cloning sites and *lacZ* reporter genes from pRS552 into pWSK29. The assay was repeated three times, and statistical analysis was performed using Student's t test with $P < 0.05$.

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