Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E

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In *Escherichia coli*, the global regulatory protein CsrA (carbon store regulator A) binds to leader segments of target mRNAs, affecting their translation and stability. CsrA activity is regulated by two noncoding RNAs, CsrB and CsrC, which act by sequestering multiple CsrA dimers. Here, we describe a protein (CsrD) that controls the degradation of CsrB/C RNAs. The dramatic stabilization of CsrB/C RNAs in a *csrD* mutant altered the expression of CsrA-controlled genes in a manner predicted from the previously described Csr regulatory circuitry. A deficiency in RNase E, the primary endonuclease involved in mRNA decay, also stabilized CsrB/C, although the half-lives of other RNAs that are substrates for RNase E (*rpsO*, *rpsT*, and RyhB) were unaffected by *csrD*. Analysis of the decay of CsrB RNA, both in vitro and in vivo, suggested that CsrD is not a ribonuclease. Interestingly, the CsrD protein contains GGDEF and EAL domains, yet unlike typical proteins in this large superfamily, its activity in the regulation of CsrB/C decay does not involve cyclic di-GMP metabolism. The two predicted membrane-spanning regions are dispensable for CsrD activity, while HAMP-like, GGDEF, and EAL domains are required. Thus, these studies demonstrate a novel process for the selective targeting of RNA molecules for degradation by RNase E and a novel function for a GGDEF–EAL protein.

[*Keywords*: RNA decay; biofilm formation; Hfq; polynucleotide phosphorylase; degradosome; GGDEF–EAL domain proteins]

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In many species of bacteria, the Csr (carbon storage regulator) and homologous Rsm (repressor of stationary phase metabolites) systems coordinate the expression of diverse genes that facilitate adaptation among major physiological phases of growth; e.g., exponential versus stationary phase, planktonic versus biofilm, and ostensibly acute versus chronic states of infection (Romeo 1998; Wei et al. 2001; Jackson et al. 2002; Goodman et al. 2004; Majdalani et al. 2005). These systems use the RNA-binding protein CsrA (Romeo et al. 1993) to regulate translation and mRNA stability by recognizing specific nucleotide sequences within mRNA leaders. Among the best-studied examples of these are the CsrA-repressed *glgCAP*, *cstA*, and *pgaABCD* mRNAs, which are involved in glycogen metabolism, peptide transport, and biofilm formation, respectively (Liu and Romeo 1997; Baker et al. 2002; X. Wang et al. 2005), as well as the CsrA-activated *flhDC* mRNA, which encodes the master regulator of motility and chemotaxis genes (Wei et al. 2001).

In *Escherichia coli*, CsrA protein activity is regulated by the CsrB and CsrC noncoding RNAs, which contain CsrA recognition sequences (18 and 9, respectively) primarily within the loops of predicted stem-loop structures. Interaction of CsrA with these sites leads to its sequestration (Liu et al. 1997; Weilbacher et al. 2003; Dubey et al. 2005). Thus, this system employs a mechanism distinct from that of other small RNAs such as OxyS and RyhB, which involve RNA–RNA base-pairing (Gottesman 2004). Furthermore, the Csr components in *E. coli* interact within an autoregulatory circuit that provides a homeostatic mechanism for control of CsrA activity (Fig. 1A). In this system, regulation of *csrB/C* tran-

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scription by CsrA requires the two-component signal transduction system (TCS) BarA/UvrY (Gudapaty et al. 2001; Suzuki et al. 2002; Weilbacher et al. 2003). Orthologous TCS regulates host–microbe interactions and quorum sensing (Hammer et al. 2002; Whistler and Ruby 2003; Altier 2005; Lenz et al. 2005), seemingly via Csr homologs.

Although many aspects of this complex regulatory network are now understood, one exception relates to the stability of the CsrB and CsrC RNAs. Since the overall levels of these regulatory transcripts are determined by their relative synthesis and turnover rates, it is important to understand the factors that govern their degradation. In *E. coli*, bulk mRNA decay and many RNA processing reactions involve the essential enzyme RNase E, a single-strand-specific endoribonuclease (Kushner 2002). RNase E contains an N-terminal catalytic domain, an RNA-binding domain, and a C-terminal domain that serves as a scaffold for the association of the 3′- to-5′ exonuclease polynucleotide phosphorylase (PNPase), the glycolytic enzyme enolase, and an RNA helicase (RhlB or CsdA) to form an RNA-degrading complex called the degradosome (Py et al. 1994, 1996; Carpousis 2002; Callaghan et al. 2004; Morita et al. 2004; Prud'homme-Généreux et al. 2004).

RNase E levels are autoregulated by a mechanism involving the degradation of its own transcript (Mudd and Higgins 1993; Jain and Belasco 1995; Diwa et al. 2000; Sousa et al. 2001; Ow et al. 2002). In addition, RNase E catalytic activity can be inhibited by binding to the RraA protein (Lee et al. 2003). Some mRNAs can be selectively targeted for turnover by RNase E by base-pairing with noncoding antisense RNAs, which undergo coincident decay during this process (for reviews, see Majdalani et al. 2005; Storz et al. 2005). The latter reactions require the Sm-like RNA chaperone, Hfq (Massé et al. 2003). It has been suggested recently that Hfq forms a complex with RNase E (Morita et al. 2005). Hfq also interacts with PNPase and poly(A) polymerase I (PAP I) to form a complex that stimulates polyadenylation of mRNAs containing intrinsic transcription terminators (Mohanty et al. 2004). Any or all of these proteins (RNase E,

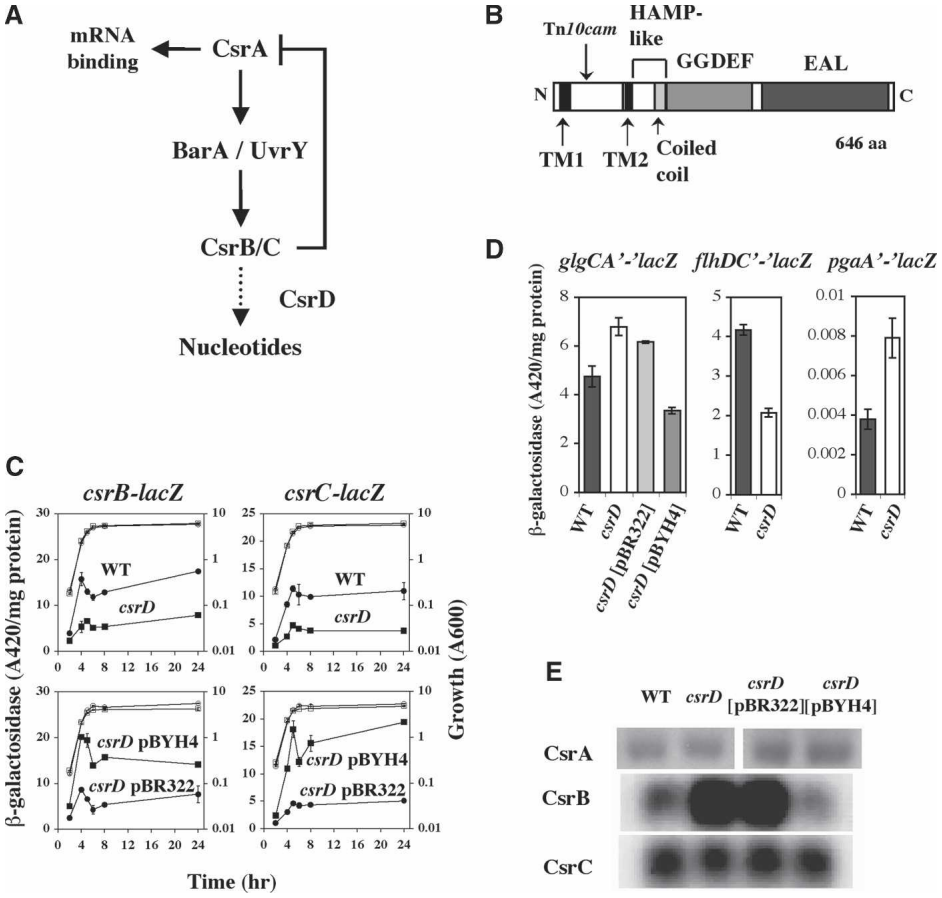

Figure 1. (*A*) Csr regulatory circuitry (Suzuki et al. 2002; Weilbacher et al. 2003), with proposed CsrD function (broken line). (*B*) Domain structure of CsrD with predicted *trans*-membrane regions, HAMP-like domain, GGDEF and EAL domains, and Tn*10 cam* insertion site displayed. (*C*) Effect of *csrD* on β-galactosidase activity expressed from chromosomal *csrB-lacZ* and *csrC-lacZ* fusions. Strains containing *csrB-lacZ* and *csrC-lacZ* were KSB837 and GS1114, respectively. *csrD* strains contained the *csrD*::*cam* insertion. pBYH4 is a clone of *csrD* in pBR322. Closed and open symbols depict activity and growth, respectively. (*D*) Effect of *csrD* on gene expression required for glycogen synthesis (*glgCA-lacZ*), motility (*flhDC-lacZ*), and biofilm formation (*pgaA-lacZ*). (*E*) Effect of *csrD* disruption and complementation (pBYH4) on CsrA protein and CsrB/C RNA levels by Western and Northern analyses, respectively.

PNPase, PAP I, and Hfq) might participate in the turnover of the CsrB and CsrC RNAs.

Although regulation of CsrB and CsrC levels could simply be a function of transcription, a previous study has suggested the presence of at least one additional undefined regulator of *csrB* expression (Suzuki et al. 2002). Our search for this regulator led to the identification and characterization of the CsrD protein, a member of a large family of proteins that contain GGDEF and EAL signaling domains (for reviews, see D'Argenio and Miller 2004; Jenal 2004; Römling et al. 2005). In various species, GGDEF and EAL proteins affect production of exopolysaccharides and surface proteins, and influence adhesion, motility, biofilm formation, and host–pathogen interactions (D'Argenio and Miller 2004; Hisert et al. 2005).

A number of GGDEF and EAL domain proteins are known to synthesize and hydrolyze, respectively, bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), a secondary messenger (e.g., see Hickman et al. 2005; Hisert et al. 2005; Ryjenkov et al. 2005; Schmidt et

Table 1. *Bacterial strains, plasmids, and phages used in this study*

Strain, plasmid, or phage	Description	Source or reference
E. coli K-12 strains[a]		
MG1655	Prototrophic	Michael Cashel
DCMG	MG1655 *csrD*::*cam*	This study
KDMG	MG1655 Δ*csrD*::*kan*	This study
TRMG	MG1655 *csrA*::*kan*	(Romeo et al. 1993)
RGMG	MG1655 Δ*csrB*::*cam*	(Gudapaty et al. 2001)
TWMG	MG1655 Δ*csrC*::*tet*	(Weilbacher et al. 2003)
UYMG	MG1655 *uvrY*::*cam*	(Suzuki et al. 2002)
BAMG	MG1655 *barA*::*kan*	(Suzuki et al. 2002)
CF7789	MG1655 Δ*lacIZ* (*Mlu*I)	Michael Cashel
KSB837	CF7789 Δ(*att-lom*)::*bla* Φ(*csrB-lacZ*)*1*(Hyb)	(Gudapaty et al. 2001)
GS1114	CF7789 Δ(*att-lom*)::*bla* Φ(*csrC-lacZ*)*1*(Hyb)	(Weilbacher et al. 2003)
KSGA18	CF7789 Φ(*glgA*::*lacZ*) (λ*plac*Mu15)	(Gudapaty et al. 2001)
FDCF7789	CF7789 Φ(*flhDC–lacZ*)	(Wei et al. 2001)
XWZ4	CF7789 Δ(*att-lom*)::*bla* Φ(*pgaA–lacZ*)*1*(Hyb)	(X. Wang et al. 2005)
MG1693	*thyA715 rph-1*	*E. coli* Genetic Stock Center
SK5665	*rne-1 thyA715 rph-1*	(Arraiano et al. 1988)
SK7988	Δ*pcnB thyA715 rph-1*	(O'Hara et al. 1995)
SK9971	*rne*Δ*1018*::*bla thyA715 rph-1 recA56 srl-300*::Tn*10* Tc^r/pMOK16 (*rne*Δ*374* Km^r)	(Ow et al. 2000)
SK10019	*pnp*Δ*683*::*str*^{r}/*spc*^{r} *thyA715 rph-1*	(Mohanty and Kushner 2003)
SK10023	*hfq-1 thyA715 rph-1*	(Mohanty et al. 2004)
Plasmids		
pYhdA	*csrD* in TA cloning site of pCR2.1-TOPO	This study
pBYH4	*csrD* in *Eco*RI site of pBR322	This study
pNC-His	*csrD* C-terminal His_6 tag, derived from pBYH4	This study
pR235A	*csrD* R235A C-terminal His_6 tag, derived from pNChis	This study
pHRA	*csrD* HR305AA C-terminal His_6 tag, derived from pNChis	This study
pS307A	*csrD* S307A C-terminal His_6 tag, derived from pNChis	This study
pD308A	*csrD* D308A C-terminal His_6 tag, derived from pNChis	This study
pE430A	*csrD* E430A, derived from pBYH4	This study
pE519Ahis	*csrD* E519A C-terminal His_6 tag, derived from pBYH4	This study
pCRA16	*csrA* in blunt-ended *Vsp*I site of pBR322	(Suzuki et al. 2002)
pBA29	*barA* in blunt-ended *Vsp*I site of pBR322	(Suzuki et al. 2002)
pAdrA7	*adrA* in TA cloning site of pCR2.1-TOPO	This study
pYhjH9	*yhjH* in TA cloning site of pCR2.1-TOPO	This study
pCB44	*csrB* gene in pCR2.1-TOPO	(X. Wang et al. 2005)
pDLE11	pCR2.1-TOPO with disrupted *lacZ* gene	(X. Wang et al. 2005)
pCR2.1-TOPO	TA-cloning vector; Amp^r Kan^r	Invitrogen
pBR322	Cloning vector; Amp^s Tet^r	(Sambrook and Russell 2001)
pKD13	Contains the *kan* gene	(Datsenko and Wanner 2000)
pKD46	For arabinose induction of λRed system	(Datsenko and Wanner 2000)
Bacteriophages		
P1*vir*	Strictly lytic P1	Carol Gross
λNK1324	Contains mini-Tn*10cam* transposon	(Kleckner et al. 1991)

[a]Strain designations containing the prefix DC, KD, TR, RG, TW, UY, or BA indicate that the mutant allele *csrD*::*cam*, Δ*csrD*::*kan*, *csrA*::*kan*, Δ*csrB*::*cam*, Δ*csrC*::*tet*, *uvrY*::*cam*, or *barA*::*kan*, respectively, was introduced by P1*vir* transduction.

al. 2005; Simm et al. 2005; Camilli and Bassler 2006). Amino acid residues required for c-di-GMP synthesis and hydrolysis have been defined by mutagenesis and other approaches (Chan et al. 2004; Kirillina et al. 2004; Paul et al. 2004; Simm et al. 2004; Tischler and Camilli 2004; Christen et al. 2005; Tamayo et al. 2005). In some dual domain proteins, the GGDEF or EAL domain may be inactive or assume an alternate activity (Christen et al. 2005; Schmidt et al. 2005). Although no GGDEF–EAL protein is known to function independently of c-di-GMP, metabolism of this nucleotide signal would seem to be inadequate to account for the sheer abundance of these proteins (e.g., *E. coli* has 19-GGDEF- and 17-EAL-containing proteins). Here we show that the CsrD protein is not involved in c-di-GMP metabolism, but rather appears to target the CsrB and CsrC RNAs for degradation by RNase E.

Results

Identification of csrD *and determination of its role in the Csr regulatory circuitry*

CsrA indirectly activates *csrB/C* transcription, possibly via effects on BarA sensor-kinase activity. To identify novel regulators of *csrB*, we screened for transposon insertions that alter expression of a *csrB-lacZ* transcriptional fusion in strain KSB837 (Table 1). A mutation that decreased *csrB-lacZ* expression was isolated within a gene, *csrD* (formerly *yhdA*), which is predicted to encode a membrane-bound signaling protein (Fig. 1B) containing GGDEF and EAL domains (http://www.sanger.ac.uk/Software/Pfam). Analysis of the effects of the *csrD* transposon insertion on genes and phenotypes regulated by CsrA—including *csrB*, *csrC* (Fig. 1C), and genes for motility (*flhDC*), biofilm formation (*pgaABCD*), and glycogen biosynthesis (*glgCAP*)—suggested that CsrA activity might be decreased by the *csrD* mutation (Fig. 1D). However, CsrA protein levels and *csrA-lacZ*expression were unaltered in this mutant (Fig. 1E; data not shown, respectively). In addition, expression of *uvrY-lacZ* and *barA-lacZ* fusions showed minimal or no effects of *csrD* (data not shown). Surprisingly, while *csrB-lacZ* and *csrC-lacZ* fusions exhibited decreased expression in the mutant (Fig. 1C), CsrB RNA levels were elevated (2.4-fold) and CsrC levels were essentially unchanged (Fig. 1E). Effects of the *csrD*::*cam* mutation were complemented by the cloned *csrD* gene (pBYH4) (e.g., see Fig. 1C). Furthermore, deletion of the *csrD* coding region in the genome reproduced the mutant phenotype (data not shown), confirming that it is caused by inactivation of *csrD*.

Based on the Csr autoregulatory circuitry (Fig. 1A), the above observations suggested that *csrD* might be required for functional inactivation of CsrB and/or CsrC, since the *csrD* mutation would be predicted to increase CsrA sequestration. To test this hypothesis, we determined the effects of *csrD* on the degradation of CsrB and CsrC RNAs in rifampicin-treated cultures. CsrB and CsrC decay rates were drastically decreased in the *csrD* mutant strain, a phenotype that was complemented by a plasmid carrying the wild-type *csrD* gene (Fig. 2). These dramatic effects of *csrD* on CsrB/C decay were consistently observed in mid-exponential, transition, and early stationary phases of growth under both gluconeogenic (LB medium) and glycolytic (Kornberg medium) conditions (data not shown).

Epistasis experiments were conducted to determine whether the effects of *csrD* on bacterial gene expression were mediated through the Csr regulatory circuitry (Table 2). We observed that regulation of all genes and phenotypes that were tested by *csrD*—including *csrB-lacZ* expression (Table 2, lines 1–4, 15–19), glycogen synthesis (data not shown), and biofilm formation (Table 2, lines 5–8, 11–14)—required functional *csrB/C* and *csrA* genes, respectively. Our model (Fig. 1A) predicts that in the absence of decay, CsrB/C RNAs should accumulate and sequester CsrA, leading to decreased transcription of *csrB/C*. We tested this model by constructing a plasmid from which *csrB* transcription was driven by a heterologous promoter (pCB44), thereby permitting CsrB to be overproduced. This plasmid clone repressed chromosomal *csrB-lacZ* expression, as predicted (Table 2, lines 9–10). Previous analyses revealed that activation of *csrB* expression by CsrA was completely dependent on the response regulator UvrY, but only partly dependent on the sensor-kinase BarA. Furthermore, ectopic expression of *csrA* restored *csrB-lacZ* expression in *csrA* and *barA* mutants, but not in a *uvrY* mutant, and ectopic expression of *barA* was unable to restore a *csrA* mutation (Suzuki et al. 2002). Similarly, ectopic expression of *csrD*

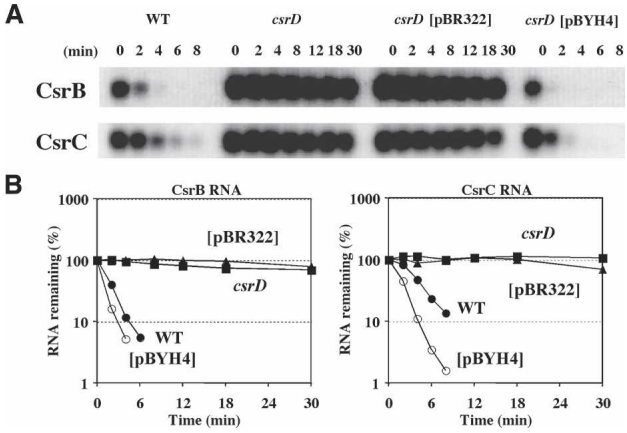

Figure 2. Effect of *csrD* on CsrB and CsrC decay. (*A*) Northern blot of CsrB and CsrC RNAs, following rifampicin addition to 37°C cultures of MG1655 (wild type), or DCMG (*csrD*) with or without plasmids pBR322 and pBYH4 (*csrD*++) at the transition from exponential to stationary phase of growth. RNA was separated by electrophoresis on 1.5% agarose gels containing formaldehyde. (*B*) Quantitative PhosphorImager data obtained from the experiment shown in *A*. The RNA half-lives were determined from the linear portions of the decay curves of *B*. The CsrB half-life in isogenic strains MG1655 (wild type) and *csrD* mutant (*csrD*, *csrD*[pBR322]) and overexpressing (*csrD*[pBYH4]) strains was 1.4, >30, >30, and 0.9 min, respectively. The CsrC half-life in the same strains was 2.2, >30, >30, and 1.1 min, respectively.

Table 2. *Epistasis analyses of csrD effects on csrB-lacZ expression and biofilm formation*

Strain	Relevant genotype	*csrB-lacZ*[a]	Biofilm[a]
1. KSB837	wild type	100 ± 3	
2. KDKSB837	*csrD*	31 ± 2	
3. RGTWKSB837	*csrB csrC*	132 ± 0	
4. RGTWKDKSB837	*csrB csrC csrD*	129 ± 1	
5. MG1655	wild type		100 ± 1
6. KDMG1655	*csrD*		672 ± 37
7. RGTWMG1655	*csrB csrC*		18 ± 1
8. RGTWKDMG1655	*csrB csrC csrD*		16 ± 2
9. KSB837[pDLE11]	wild type	100 ± 0	100 ± 6
10. KSB837[pCB44]	*csrB++*	40 ± 1	774 ± 73
11. MG1655[pDLE11]	wild type		100 ± 6
12. MG1655[pYhdA]	*csrD++*		9 ± 1
13. TRMG[pDLE11]	*csrA*		616 ± 55
14. TRMG[pYhdA]	*csrA csrD++*		618 ± 29
15. KSB837[pBR322]	wild type	100 ± 8	
16. DCKSB837[pBR322]	*csrD*	22 ± 1	
17. DCKSB837[pBYH4]	*csrD++*	101 ± 9	
18. TRKSB837[pBR322]	*csrA*	7 ± 1	
19. TRKSB837[pBYH4]	*csrA csrD++*	5 ± 1	
20. BAKSB837[pBR322]	*barA*	11 ± 0	
21. BAKSB837[pBYH4]	*barA csrD++*	103 ± 14	
22. UYKSB837[pBR322]	*uvrY*	5 ± 0	
23. UYKSB837[pBYH4]	*uvrY csrD++*	4 ± 0	
24. DCKSB837[pCRA16]	*csrD csrA++*	101 ± 7	
25. DCKSB837[pBA29]	*csrD barA++*	23 ± 0	

[a]Assays for *csrB-lacZ* expression and biofilm formation were conducted as described in Materials and Methods. Resulting values (percent ± standard deviation) were normalized with respect to those of the isogenic parent strain (wild type) in each data set.

restored *csrB-lacZ* expression in *csrD* and *barA* mutants (Table 2, lines 15–17, 20–21), but not in *csrA* or *uvrY* mutants (Table 2, lines 18–19, 22–23). In addition, *csrB-lacZ* expression in a *csrD* mutant was restored by ectopic expression of *csrA* but not by *barA* (Table 2, cf. lines 16 and 24–25). These studies fully supported the regulatory circuitry depicted in Figure 1A.

CsrD-mediated RNA decay requires RNase E

To determine which enzymes participated in the turnover of CsrB/C RNAs, we measured their half-lives in a series of mutants that affect mRNA turnover. Strikingly, the half-lives of both CsrB and CsrC increased >10-fold in an *rne*-1 mutant at the nonpermissive temperature (Fig. 3A). This immediate (<2 min) cessation of decay and large increase in half-life suggested that RNase E may be directly involved in the decay of both regulatory RNAs. Ectopic expression of *csrD* from a multicopy plasmid did not cause CsrB/C turnover under RNase E nonpermissive conditions (Fig. 3C). Furthermore, CsrB decay over an extended period of time (90 min) was identical in a strain lacking RNase E activity versus one lacking both RNase E and CsrD (Supplementary Fig. S1), confirming that these two factors function within a single decay pathway. Decay of CsrB was also dependent to a lesser extent upon PNPase, a 3′-to-5′ exonuclease that is also a component of the RNase E-based degradosome (Fig. 3B). In fact, CsrB decay intermediates accumulated in the *pnpΔ683* mutant (Figs. 3B, 4A). Interestingly, the half-life of CsrB RNA increased almost threefold in the absence of degradosome assembly in *rneΔ374* (Fig. 3B), but was unaffected by loss of PAP I *(ΔpcnB1)* or Hfq (Fig. 3B). While the decay of the CsrC RNA also required RNase E activity, loss of PNPase activity did not substantially affect the half-life of its products (Fig. 3B). In addition, CsrC half-life decreased modestly in the absence of degradosome assembly (Fig. 3B). Further evidence that the decay of CsrB and CsrC RNAs employed slightly different mechanisms was derived from an analysis of a *csrA csrD* double mutant. This experiment determined if the formation of CsrA/CsrB or CsrA/CsrC complexes required CsrD for their turnover. In the case of CsrB, turnover was still dependent on CsrD, while CsrC decay became CsrD independent (Supplementary Fig. S2). CsrB

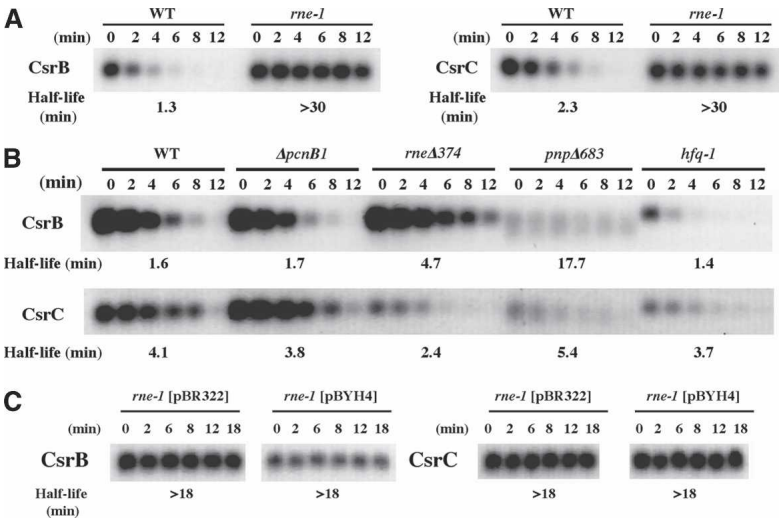

Figure 3. Effects of RNase E, PNPase, Hfq, PAP I, and the RNase E degradosome scaffold on CsrB/C turnover. (*A*) Northern blot of CsrB and CsrC RNAs from MG1693 (wild type) and SK5665 (*rne-1*), following temperature shift of cultures (at transition to stationary phase) from 30°C to 44°C and rifampicin addition, or from (*B*) MG1693 (wild type), SK7988 (Δ*pcnB*), SK9971 (*rne*Δ*374*), SK10019 (*pnp*Δ*683*), and SK10023 (*hfq-1*) grown at 37°C followed by rifampicin addition. (*C*) Northern blot of CsrB and CsrC RNAs following temperature shift of SK5665 (*rne-1*) strains containing pBR322 vector or pBYH4 (*csrD*$^+$). RNA half-lives were determined as in Figure 2.

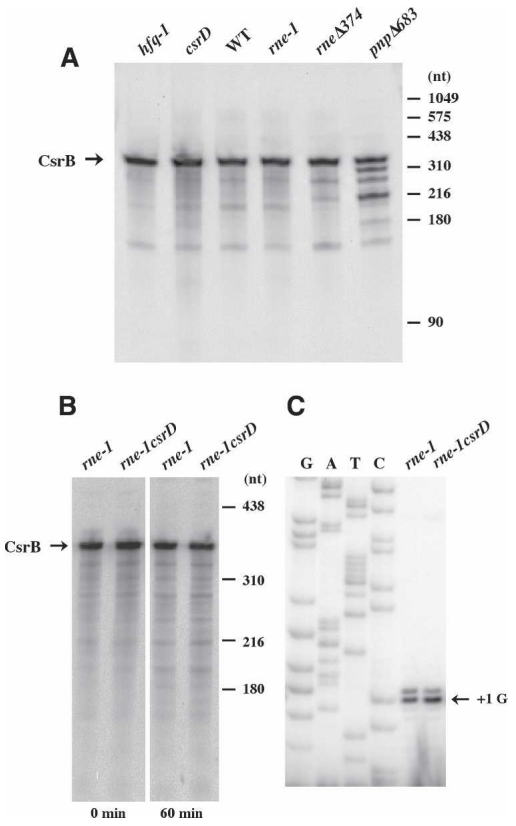

Figure 4. CsrB decay patterns. (*A*) Northern blot of CsrB RNA from MG1693 (wild type), SK5665 (*rne-1*; grown at 30°C), SK9971 (*rne*Δ*374*), SK10019 (*pnp*Δ*683*), SK10023 (*hfq-1*), and DCMG (*csrD*). Steady-state RNA was prepared from cultures harvested at the transition to stationary phase growth. (*B*) Northern blot of CsrB RNA from SK5665 (*rne-1*) and DCSK5665 (*rne-1 csrD*) at $T = 0$ and 60 min following temperature shift from 30°C to 44°C and rifampicin addition. In this experiment, RNA was prepared from cultures harvested 0 and 60 min after temperature shift and rifampicin addition. (*C*) Primer extension analysis of RNA preparations (60 min) shown in *B*. The major 5′ end of CsrB RNA is indicated by +1, and is identical to the CsrB transcription initiation site reported previously (Gudapaty et al. 2001).

and CsrC did not appreciably influence each other's decay (Supplementary Fig. S2).

CsrD is a specificity factor, not a regulator of bulk RNase E activity

Although CsrB/C decay was very defective in the *csrD* mutant (Fig. 2), its growth was unaffected (e.g., see Fig. 1C). Thus, we suspected that CsrD was not required for maintenance of bulk RNase E activity because loss of this enzyme would lead to either a significant growth defect (Ow et al. 2002) or the loss of cell viability. To test this idea directly, we examined the effect of the *csrD*::*cam* allele on the half-lives of three transcripts whose turnover depends on RNase E: the mRNAs *rpsO* and *rpsT*, and the noncoding antisense RNA RyhB. Half-lives of the mRNAs were determined after the addition rifampicin of to cultures, while RyhB transcription was induced by chelation of iron with 2,2′-dipyridyl and inhibited by addition of excess $FeSO_4$, as described (Massé et al. 2003). The decay of these RNAs was unaltered in the *csrD*::*cam* mutant (Supplementary Fig. S3). In addition, Western blot analysis showed that RNase E protein levels were, within experimental error, identical in *csrD* mutant and wild-type control (M. Stead and S.R. Kushner, unpubl.).

CsrD does not appear to be a ribonuclease

The possibility that CsrD might initiate CsrB decay by modifying or cleaving this RNA was suggested by the fact that the bacterial GGDEF domain belongs to the ancestral palm domain family, which includes nucleotidyl transferases and RNA-binding proteins (Pei and Grishin 2001; Li et al. 2002). Thus, the effects of CsrD and other factors on the pattern of CsrB RNA decay products were examined. Steady-state RNA was prepared from wild-type, *csrD*::*cam*, *hfq-1*, *rne-1*, *rne*Δ*374*, and *pnp*Δ*683* strains, separated on 6% urea PAGE gels, and analyzed by Northern blotting (Fig. 4A). In this analysis, the wild type, *csrD*::*cam*, *hfq-1*, and *rne-1* yielded very similar patterns that contained few decay intermediates (Fig. 4A). In contrast, in the *pnp*Δ*683* mutant there were a substantial number of apparent decay intermediates (Fig. 4A). Interestingly, some of these intermediates were observed in the *rne*Δ*374* mutant, where degradosome assembly could not occur.

Subsequently, an experiment was conducted to determine whether CsrD was a nuclease that might facilitate attack by RNase E. *rne-1* and *csrD*::*cam rne-1* mutants were grown into transition phase, at which time rifampicin was added and the strains were transferred to nonpermissive temperature. Immediately, and 60 min thereafter, RNA was isolated and examined by Northern blot analysis. As shown in Figure 4B, there was a single major transcript with the mobility of full-length CsrB and a ladder of minor decay products that were identical in both strains (Fig. 4B). Primer extension analysis revealed identical 5′ ends for CsrB RNAs of both strains (Fig. 4C), which corresponded to the initiating nucleotide of this transcript (Gudapaty et al. 2001). These results indicate that CsrD does not cleave CsrB in order to permit prototypical 5′ end recognition and turnover of this transcript by RNase E (Kushner 2002).

CsrD activity does not involve c-di-GMP synthesis or turnover

CsrD is predicted to be a membrane-bound signaling protein containing GGDEF and EAL domains. Proteins containing GGDEF and EAL domains have been proposed to synthesize and degrade c-di-GMP, respectively (Ryjenkov et al. 2005; Schmidt et al. 2005). Since domain deletions and plasmid complementation tests determined that both of these domains were required for CsrD ac-

tivity (Supplementary Fig. S4), we conducted experiments to assess the possibility that CsrD activity is based on c-di-GMP. Isogenic wild-type, mutant, or *csrD*-overexpressing strains were grown in limiting phosphate and labeled with ^{32}Pi, and guanine nucleotides were extracted and analyzed by 2D-TLC, an approach that is highly sensitive for detection of c-di-GMP (Tischler and Camilli 2004; Hickman et al. 2005). While the other guanine nucleotides were readily identified from these strains, c-di-GMP was not detected (data not shown).

Site-directed mutations in the GGDEF signature sequence have been found to disrupt the function of this domain (García et al. 2004; Kirillina et al. 2004; Paul et al. 2004; Simm et al. 2004). Furthermore, studies of the three-dimensional (3D) structure of PleD (Chan et al. 2004) revealed that the GGDEF motif contains residues involved in substrate binding (G368, G369, and E371) and catalysis (E370), and that residue R300 is also involved in substrate binding. In addition, the GGDEF domain of CsrD contains HRSDF in place of the conserved GG(D/E)EF motif (Chan et al. 2004), suggesting that CsrD might not synthesize c-di-GMP (Fig. 5A). To obtain direct evidence concerning the role of the HRSDF sequence in *csrD* function, single or double amino acid residue changes of HRSD and R235 (corresponding to R300 of PleD) residues were introduced into CsrD by site-directed mutagenesis and tested for their effects on *csrB-lacZ* expression and biofilm formation (Fig. 5A; Supplementary Table S4). None of the residues examined were required for CsrD to regulate these activities.

In the case of the EAL domain, the 3D structure has not been solved and active site residues have not been clearly defined. Nevertheless, single-residue changes in the conserved EAL signature sequence inactivate EAL domain proteins (Kirillina et al. 2004; Simm et al. 2004; Tischler and Camilli 2004; Tamayo et al. 2005). However, E430A substitution in the EML (corresponding to EAL) sequence of CsrD did not affect CsrD activity (Fig. 5B; Supplementary Table S4). Another substitution, E519A, in a highly conserved amino acid residue of EAL proteins, resulted in partial loss of activity (Fig. 5B; Supplementary Table S4). In contrast, YhjH lost all ac-

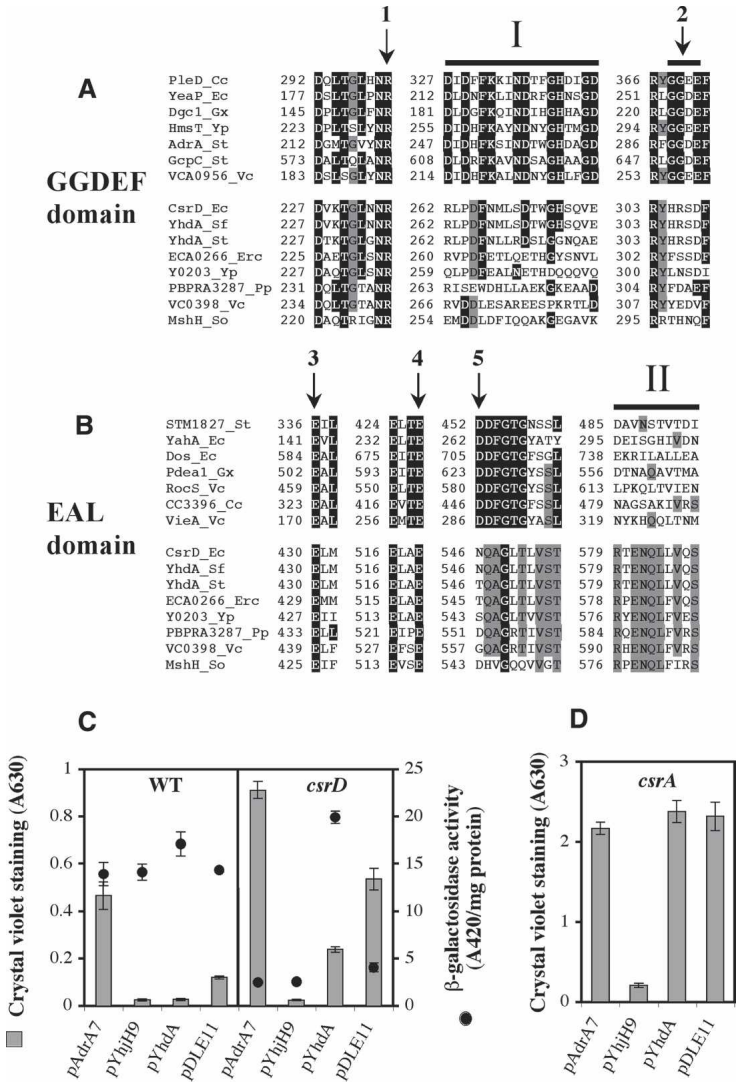

Figure 5. Amino acid sequence comparisons among putative CsrD orthologs and other GGDEF and EAL domain proteins, and effects of c-di-GMP metabolism genes on *csrB-lacZ* expression and biofilm formation. (*A*,*B*) Conserved regions of c-di-GMP metabolizing GGDEF and EAL domain proteins (*upper* sets of sequences) and CsrD orthologs (*lower* sets). Residues identical in >80% of the c-di-GMP metabolizing EAL or GGDEF domains are indicated by a black background. Residues identical in >80% of the CsrD orthologs are indicated by a gray background. The positions shown by arrows (1–5) depict amino acid(s) that have been substituted by alanine or other amino acids in one or more c-di-GMP metabolizing GGDEF or EAL domain proteins or CsrD (see Supplementary Table S4). Region I is highly conserved in c-di-GMP biosynthetic GGDEF domains and region II is one that is highly conserved in the EAL domain of CsrD orthologs, but not in other EAL domain proteins. (Cc) *Caulobacter crescentus*; (Ec) *E. coli*; (Gx) *Gluconacetobacter xylinus*; (Yp) *Yersinia pestis*; (St) *Salmonella enterica* Typhimurium; (Vc) *Vibrio cholerae*; (Sf) *Shigella flexneri*; (Erc) *Erwinia carotovora*; (Pp) *Photobacterium profundum*; (So) *Shewanella oneidensis*. (*C*,*D*) Effects of ectopic expression of *adrA* (pAdrA7), *yhjH* (pYhjH9), and *csrD* (pYhdA) vs. empty vector (pDLE11) on expression of *csrB-lacZ* and biofilm formation in wild type or *csrD* mutant of KSB837 (*C*) and on biofilm formation in a*csrA* mutant of MG1655 (*D*). Strains were cultured in the presence of IPTG (1 mM) to maintain expression of the cloned genes. The vector control was pDLE11 in each case. Crystal violet staining for monitoring biofilm formation and β-galactosidase activity are shown as bars and closed circles, respectively. Values are reported as the average ± standard deviation.

tivity by E136A substitution, which corresponds to E519A of CsrD (Simm et al. 2004). These results suggested that the EAL domain of CsrD activity does not hydrolyze c-di-GMP.

In addition, we determined the effects of genes involved in the synthesis or degradation of c-di-GMP on *csrB-lacZ* expression and biofilm formation. These experiments were based on observations that genes for c-di-GMP-metabolizing GGDEF proteins, as well as EAL proteins, often cross-complement, especially when the heterologous gene is overexpressed (García et al. 2004; Simm et al. 2004, 2005; Tischler and Camilli 2004). In *Salmonella* species, AdrA (GGDEF) and YhjH (EAL) proteins possess diguanylate cyclase and c-di-GMP phosphodiesterase (PDE-A) activity, respectively, through which they activate and repress biofilm formation (Simm et al. 2004). *E. coli* K-12 has closely related orthologs of AdrA and YhjH (75% and 79% identical, respectively), which we expected to possess the same activities. Plasmid clones of these genes were active in vivo. *adrA* stimulated biofilm formation and *yhjH* repressed biofilm formation, via unknown target(s), in both wild-type and *csrD*::*cam* mutant strains. However, unlike *csrD*, neither *adrA* nor *yhjH* affected *csrB-lacZ* expression (Fig. 5C). While CsrD had no effect on biofilm formation in the *csrA* mutant background (Table 2, 11–14), biofilm formation was repressed by *yhjH* in this background (Fig. 5D), indicating that the latter effect is not mediated through the Csr system.

Phylogenetic distribution of csrD

BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) and Clustal X analyses (Thompson et al. 1997) were conducted to assess the phylogenetic distribution of CsrD (data not shown). CsrD orthologs were apparent in the sequenced genomes of Enterobacteriaceae (50%–99% amino acid identity), Vibrionaceae (≥31%), and Shewanellaceae (≥28%) species (Fig. 5). These proposed orthologs were identified based on (1) identical domain structure and high sequence similarity with respect to CsrD, (2) divergence of the GGDEF signature and the conserved EAL and DDFGTG sequences of the EAL domain (Schmidt et al. 2005), and (3) the presence of amino acid sequences that are conserved in the CsrD orthologs (e.g., region II in Fig. 5B) but not in c-di-GMP metabolizing proteins. Site-directed replacement of six residues of the later type in CsrD by alanine revealed a change, L584A, which partially eliminated activity (71% inactivation based on *csrB-lacZ* expression) without affecting protein accumulation (Supplementary Table S5), suggesting an important function for this leucine. The uniform presence of *rne* and *csrA* genes in the genomes of these species (data not shown) is also consistent with a common function for their CsrD-like proteins. While *csrB/C* orthologs are challenging to identify by comparative sequence analyses (Weilbacher et al. 2003), functional homologs of these RNAs are known from other Enterobacteriaceae (Ma et al. 2001; Altier 2005), Vibrionaceae (Lenz et al. 2005), and Pseudomonadaceae (Heurlier et al. 2004).

Discussion

The results presented here demonstrate that in *E. coli* the global regulatory RNAs CsrB and CsrC require a specificity factor, CsrD, for their decay through an RNase E-mediated pathway. To our knowledge, this is the first report of RNA turnover being selectively controlled by a predicted modular signaling protein containing GGDEF and EAL domains. Of equal significance is that the CsrD protein does not function either in the synthesis or degradation of c-di-GMP. It is likely that the CsrD–RNase E-mediated decay pathway operates in many Gram-negative bacteria and broadly influences metabolism, motility/sessility, quorum sensing, host–microbe interactions, and virulence factor expression.

While our studies have not yet clearly defined the mechanism of CsrD action, analysis of decay products (Fig. 4) suggests that CsrD is not a nuclease. In fact, we have isolated a recombinant CsrD protein that lacks the membrane-spanning regions and contains an N-terminal His_6 tag (see pQDY3, Supplementary Fig. S4). This purified protein binds to CsrB and CsrC RNAs with high affinity, but without specificity (Supplementary Fig. S5), and does not degrade CsrB or hydrolyze c-di-GMP (data not shown). These observations are of particular interest since bioinformatics analyses have suggested that the GGDEF domain is homologous to eukaryotic adenylyl cyclases and the palm domains of DNA polymerase β, CCA-adding enzyme, and a number of other proteins that interact with RNA (Pei and Grishin 2001; Li et al. 2002, and references therein).

We therefore hypothesize that CsrD functions by binding to the CsrB and CsrC RNAs, converting them into substrates for RNase E degradation. We predict that the binding of CsrD to CsrB and CsrC may change their structures in such a way as to make them accessible to RNase E. We also hypothesize that CsrD activity is not constant under all conditions, and by modulating CsrB/C decay, it helps to determine when CsrA is active. Consistent with this idea, expression of a chromosomal *csrD-lacZ* translational fusion was modestly repressed (twofold) by CsrA (data not shown). This observation also indicates that CsrD is part of an additional autoregulatory loop within the Csr system. CsrB/C decay rates vary significantly over the course of the growth curve (data not shown), although the precise role of CsrD in this response remains to be determined.

This model predicts that CsrD should be an RNA-binding protein. In fact, in vitro experiments have shown that CsrD binds to both CsrB and CsrC RNAs with high affinity (~25 nM), but that the binding was not specific (Supplementary Fig. S5). Thus, it is possible that there is an additional specificity factor that we have yet to identify. Alternatively, CsrD could be a generalized RNA-binding protein that in vivo is prevented from associating with most RNAs either by proteins (e.g., ribosomes, Hfq) or due to their conformations.

Because the mechanism of action of CsrB/C is fundamentally different from other noncoding regulatory RNAs, it should not be surprising that their decay pathways are distinct. The latter RNAs (with the exception of 6S RNA) (Wassarman and Storz 2000) are antisense RNAs that require Hfq to mediate base-pairing with mRNA targets. Hfq binding typically stabilizes antisense RNAs until they interact with a cognate mRNA, and thereafter targets both RNAs for turnover by RNase E. It has been suggested that this depends on the fact that RNase E and Hfq have similar (AU-rich) target sequences that permit Hfq to protect antisense RNA from RNase E attack until base-pairing has occurred (Massé et al. 2003). Since the decay of RyhB antisense RNA is not affected by CsrD (Supplementary Fig. S4), while CsrB/C degradation is not affected by Hfq (Fig. 3), we hypothesize that CsrD–RNA interactions are necessary for turnover of CsrB/C because they do not contain any obvious RNase E (or Hfq) recognition regions.

Although CsrD is predicted to be anchored in the plasma membrane and it has been suggested that RNase E is associated with the inner membrane (Liou et al. 2001), the presence of the predicted N-terminal membrane anchor was not essential for CsrD activity when the protein was ectopically expressed (Supplementary Fig. S4). Nevertheless, this finding does not exclude a possible role of the membrane anchor in subsubcellular localization or signal sensing. In contrast, the HAMP-like domain (Appleman and Stewart 2003) of CsrD was required for activity. We suspect that this region, including its predicted coiled-coil, may be needed for protein–protein interactions, but have not examined this possibility. Previous proteomic analyses of *E. coli* cytoplasmic membrane proteins did not identify CsrD (YhdA) (Fountoulakis and Gasser 2003), and attempts to prepare clones expressing CsrD fusion proteins for membrane topology analyses failed (Daley et al. 2005). These results may be explained by the fact that *csrD* is expressed at extremely low levels, and the full-length CsrD protein, containing the membrane-spanning regions, causes cell lysis upon overexpression (data not shown). Because *csrB/C* expression is relatively strong and these RNAs are abundant (Gudapaty et al. 2001; Weilbacher et al. 2003), the high rates of turnover mediated by CsrD further imply that this protein is active on these RNAs at substoichiometric concentrations.

Interestingly, the conundrum posed by the abundance of GGDEF–EAL proteins in many species has been partially resolved by this study. Clearly, all of these proteins are not dedicated to c-di-GMP metabolism. Based on criteria defined above, we tentatively identified GGDEF–EAL proteins of other species that are CsrD orthologs (Fig. 5) and likely function in the decay of Csr (Rsm) RNAs. There are also GGDEF and EAL proteins in various species that do not fit our criteria for CsrD orthologs, but nevertheless lack amino acid sequences that should be required for c-di-GMP metabolism (data not shown). Thus, the strategies taken here should be useful for establishing which of these proteins possesses novel mechanisms.

The role of CsrA is not simply to switch genes on or off, but to fine-tune expression; e.g., for governing relative fluxes of competing metabolic pathways (Sabnis et al. 1995; Pernestig et al. 2003). Consequently, CsrA activity is not regulated by covalent modification or small ligand binding, but rather by RNA antagonists whose levels can be rapidly adjusted to offer continuous high-fidelity control of CsrA activity. While CsrB and CsrC RNAs are functionally related and expressed via the same regulatory circuitry, they differ quantitatively in both respects (Suzuki et al. 2002; Weilbacher et al. 2003). Likewise, decay of both CsrB and CsrC utilizes a CsrD–RNase E pathway, but differs in response to ancillary decay factors (PNPase and degradosome assembly) (Fig. 4; data not shown) and CsrA (Supplementary Fig. S2). The presence of orthologs of these RNAs in enteric species suggests that these subtle distinctions, while not fully understood, are likely to be biologically important.

Due to the explosive growth of research on post-transcriptional regulation and RNA decay, common features of eukaryotic and bacterial processes are emerging (e.g., see Gottesman 2005). Antisense RNAs dominate both worlds, no doubt, because base-pairing reactions can mediate highly specific targeting interactions. Csr homologs are not apparent in Archaea or Eukarya. Nevertheless, BC1 RNA of neurons and germ cells is analogous to CsrB/C, in that it binds to and antagonizes RNA-binding proteins involved in translation control and forms ribonucleoprotein complexes (H. Wang et al. 2005, and references therein). Since we have only begun to understand RNA regulatory mechanisms and networks in eukaryotes (Mattick and Makunin 2005), it would not be surprising to find Csr-like regulatory systems in eukaryotes as well.

Materials and methods

Bacterial strains, plasmids, bacteriophage, and growth conditions

All *E. coli* K-12 strains, plasmids, and bacteriophage used in this study are listed in Table 1 or in Supplemental Material, as appropriate. The various mutant alleles of this study were moved among strains by bacteriophage P1*vir* transduction, as described previously (Miller 1972). Luria-Bertani (LB) growth medium (Miller 1972) was used for routine cultures, *flhDC-lacZ* and *pgaA-lacZ* gene expression assays, and biofilm formation assays. Thymine (50 µg/mL) was added to LB medium for growth of strains containing the *thyA715* allele. Kornberg growth medium (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract containing 0.5% glucose for liquid or 1% for solid medium) was used for other gene expression assays, selection of transposon mutants, glycogen phenotype determination, and Western and Northern blot analyses, with the exception of Northern analyses of *thyA715* strains, which used LB plus thymine. The following antibiotics were added, as required, at the indicated concentrations: 20 µg/mL chloramphenicol, 50 µg/mL kanamycin, 100 µg/mL ampicillin, 10 µg/mL streptomycin, 10 µg/mL tetracycline, and 200 µg/mL rifampicin, except that kanamycin was used at 100 µg/mL for the selection of *csrA*::*kan* strains.

Isolation of transposon mutants and identification of insertion sites

KSB837 (*csrB-lacZ*) strain was infected with λNK1324 containing mini-Tn*10cam* at a multiplicity of infection of 0.15, as described previously (Kleckner et al. 1991). Mutants with altered β-galactosidase activity on Kornberg agar containing X-gal (40 μg/mL) and sodium pyrophosphate (2.5 mM) were isolated and then examined for glycogen accumulation. Mutants with altered *csrB-lacZ* expression and glycogen production were retained. Amplification of chromosomal DNA flanking the transposon insertions by arbitrarily primed PCR and sequencing of the PCR products were conducted as described previously (Wang et al. 2004).

Glycogen, β-galactosidase, motility, and quantitative biofilm assay

Glycogen accumulation was examined by staining colonies with iodine vapor (Liu et al. 1997). β-Galactosidase activity was assayed as described previously (Suzuki et al. 2002). Assays for *flhDC-lacZ* and *pgaA-lacZ* expression were conducted as described (Wei et al. 2001; X. Wang et al. 2005). Single time point β-galactosidase assays were conducted at 6 h of growth. Biofilm formation by cultures at 24 h of growth was assayed by crystal violet staining, as described previously (Jackson et al. 2002).

Cloning of csrD, adrA, and yhjH genes

Plasmid pYhdA encoding the *csrD* gene, including 222 base pairs (bp) upstream of and 266 bp downstream from the *csrD* ORF, was constructed by amplifying the *E. coli csrD* gene with primers yhdAF and yhdAR and ligating it into pCR2.1-TOPO by TA cloning. The orientation of *csrD* was the same as that of the *lac* promoter of the vector, which was confirmed by PCR. The EcoRI fragment of pYhdA was subcloned into the EcoRI site of pBR322 to generate pBYH4. Plasmids pAdrA7 and pYhjH9, which express *adrA* and *yhjH* under control of the *lac* promoter, respectively, were constructed by amplifying the *E. coli adrA* and *yhjH* genes with primer pairs adrA-F/adrA-R and yhjH-F/yhjH-R and ligating them into pCR2.1-TOPO by TA cloning. Each forward primer contains its own ribosome-binding site and ATG start codon. The orientation of each gene was confirmed by PCR. Nucleotide sequences of all plasmid inserts were determined to avoid PCR-mediated mutations. The oligonucleotide primers used in this study are listed in Supplementary Table S2.

Site-directed mutations and domain deletions of csrD

Site-directed mutations and domain deletions of the *csrD* gene were constructed using the plasmids pBYH4 and pNC-His. DNA fragments upstream of and downstream from each mutation/deletion were amplified by PCR using pBYH4 as a template. Primers containing the mutation were complementary in sequence with each other (Supplementary Table S2). The two resulting PCR fragments were annealed together and amplified using the primer pair for the ends of the gene. The final PCR product was digested with appropriate restriction enzymes, cloned into pBYH4 or pNC-His (Supplementary Table S2), and confirmed by nucleotide sequencing. The restriction enzymes and primers are listed in Supplementary Tables S2 and S3.

Construction of csrD-null mutant

The chromosomal *csrD* gene was deleted by targeted gene substitution, as described (Datsenko and Wanner 2000). The *kan* gene was amplified from pKD13 by PCR using primers yhP1 and yhP4, and introduced by electroporation into arabinose-treated BW25113[pKD46]. Transformants were selected on kanamycin, and their insertion sites were confirmed by PCR.

Isolation of total RNA

Bacterial cultures were mixed with 2 vol of RNAprotect Bacterial Reagent (Qiagen) and incubated for 5 min at room temperature. Total cellular RNA was subsequently prepared and treated with DNase I using the MasterPure RNA Purification Kit as recommended (Epicentre).

Northern and Western blotting

Total cellular RNA was separated by electrophoresis on 1.5% agarose gels containing formaldehyde or 6% polyacrylamide gels containing 7 M urea. The RNA in agarose gels was then transferred overnight to positively charged nylon membranes (Roche) by capillary action in 20× SSC. The RNA in polyacrylamide gels was electroblotted onto the same membranes using a Trans-Blot SD semidry transfer cell (Bio-Rad) according to the manufacturer's directions. The blotted membranes were then baked for 30 min at 120°C. DIG-labeled riboprobes were hybridized to RNA on the blots and detected using DIG luminescent detection kit (Roche). Chemiluminescent signals were visualized with ChemiDoc or VersaDoc system (Bio-Rad), and band intensities were quantified using Quantity One software (Bio-Rad). DIG-labeled riboprobes for detection of CsrB, CsrC, RyhB, and *rpsO* and *rpsT* transcripts were synthesized from PCR products containing a T7 promoter using a DIG RNA labeling kit (Roche). The primer pairs (Supplementary Table S2) csrBT7-csrBR, csrCT7-csrCR, ryhBT7-ryhBR, rpsOT7-rpsOR, and rpsTT7-rpsTR were used for synthesis of the templates for *csrB*, *csrC*, *ryhB*, *rpsO*, and *rpsT* DIG-labeled riboprobes, respectively.

Western blotting of CsrA was performed as described (Gudapaty et al. 2001) on cultures at 4 h of growth or as otherwise indicated.

Primer extension of CsrB RNA

Cells were grown in LB supplemented with thymine at 30°C to the transition to stationary phase of the growth and shifted to 44°C, and rifampicin was added to inhibit transcription. Total RNA was prepared 60 min after the addition of rifampicin. Primer PEX1 that anneals at position +12 to +35 relative to the transcription start site of CsrB was 5′-end-labeled with [γ-^{32}P]-ATP (3000 Ci mmol^{-1}, NEN Life Science Products) using T4 polynucleotide kinase (Promega). Unincorporated [γ-^{32}P]-ATP was removed using a MicroSpinTM G-25 Column (Amersham Biosciences). Approximately 3 pmol of labeled primer was added to 5 μg of total RNA. Subsequent cDNA synthesis was performed using the ThermoScriptTM RT–PCR system (Invitrogen). The same labeled primer and pCSRBSF were used to generate a corresponding DNA sequencing ladder using the SequiTherm EXCELTM II DNA Sequencing Kit (Epicentre). The primer extension products were separated alongside the sequencing ladder on an 8% polyacrylamide sequencing gel containing 6 M urea. The gel was dried and subjected to autoradiography using a PhosphorImager (Storm Gel and Blot Imaging system, Amersham Bioscience).

Analysis of guanine nucleotides

Bacterial growth and nucleotide labeling were conducted as described previously, with some modification (Bochner and Ames

1982; Tischler and Camilli 2004). Overnight cultures were grown at 37°C in supplemented MOPS medium (Wanner et al. 1977) and used to inoculate the same medium. These cultures were grown at 37°C with aeration until an OD_{600} = 0.6 was reached. Cells were collected by centrifugation, resuspended in medium plus 100 µCi mL^{-1} ^{32}Pi (PerkinElmer), and incubated for 1–4 h at 37°C to label nucleotides. Following the labeling, nucleotides were extracted and 2D-TLC was conducted as described previously (Tischler and Camilli 2004). The TLC plate was dried and subjected to autoradiography using a PhosphorImager (Storm Gel and Blot Imaging system, Amersham Bioscience).

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