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Small untranslated RNAs (sRNAs) perform a variety of important functions in bacterial systems. The 245 nt sRNA of *Escherichia coli* K-12, CsrC, was uncovered using a genetic screen for genes that regulate glycogen biosynthesis. CsrC RNA binds multiple copies of CsrA, a protein that post-transcriptionally regulates central carbon flux, biofilm formation, and motility in *E. coli*. CsrC antagonizes the regulatory effects of CsrA, presumably by sequestering this protein. The discovery of CsrC is intriguing, in that a similar sRNA, CsrB, performs essentially the same function. Both of these sRNAs possess similar imperfect repeat sequences (18 in CsrB, 9 in CsrC), primarily localized in the loops of predicted hairpins, which may serve as CsrA binding elements. Transcription of *csrC* increases as the culture approaches the stationary phase of growth and is activated by CsrA and the response regulator UvrY. Complementation and in vitro transcription-translation experiments reveal that CsrA effects on *csrC* are mediated indirectly, through UvrY. Because CsrB and CsrC antagonize the activity of CsrA and are dependent on CsrA for their synthesis, a *csrB* null mutation causes a modest compensatory increase in CsrC levels and vice versa. An updated model for the signaling circuitry of the Csr system is discussed.

A NOVEL sRNA MEMBER OF THE CARBON

STRORAGE REGULATORY SYSTEM

OF *ESCHERICHIA COLI*

Thomas Weilbacher, B.S.

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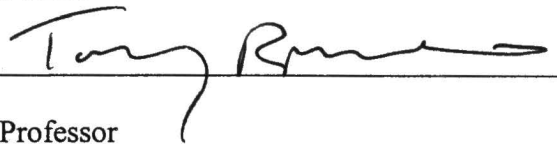

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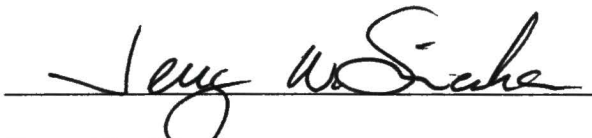

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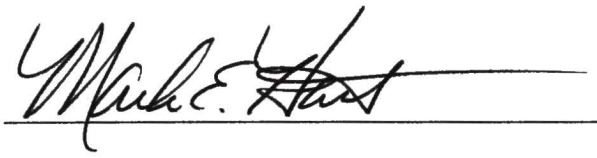

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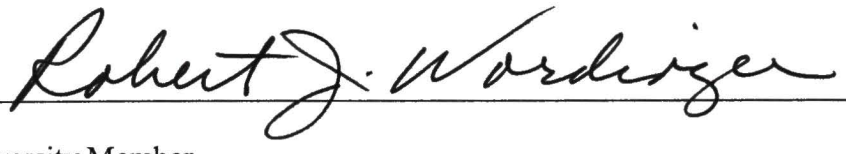

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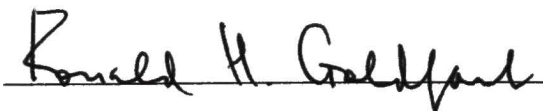
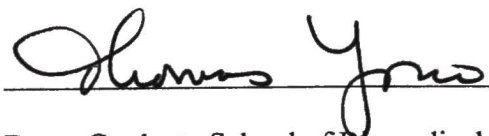


Chair, Department of Molecular Biology & Immunology



Dean, Graduate School of Biomedical Sciences

A NOVEL sRNA MEMBER OF THE CARBON STRORAGE REGULATORY SYSTEM OF *ESCHERICHIA COLI*

THESIS

Presented to the Graduate Council of the

University of North Texas Health Science Center at Fort Worth

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MASTER OF SCIENCE

By

Thomas Weilbacher, B.S.

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ACKNOWLEDGMENTS

I would like to extend special thanks to all those who participated in the evolution of this Master's Thesis. I would like to specifically acknowledge Seshigirao Gudapaty, PhD. for the construction of the *csrC-lacZ* transcriptional gene fusion and innumerable answered questions, Kazushi Suzuki, PhD. for his efforts in S-30 transcription/translation studies, Xin Wang, M.S. for analyses of biofilm formation and motility, Paul Babitzke, PhD. for in vitro analysis of CsrA and CsrC interactions, and Tony Romeo, PhD. for unwavering guidance throughout the degree process.

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INTRODUCTION

Bacterial survival and competition under the feast or famine conditions of the natural environment requires remarkable phenotypic plasticity. In *Escherichia coli*, the transition from exponential to stationary growth phase leads to increased stress resistance, decreased anabolic metabolism, altered cellular and subcellular morphology, and enhanced ability to scavenge nutrients (Hengge-Aronis, 1996, Huisman, 1994). Acquisition of the stationary phase phenotype is brought about through changes in gene expression, which are coordinated by global regulatory systems (Gottesman, 1984, Neidhardt and Savageau, 1996).

The RNA-binding protein CsrA of *E. coli* is the key component of a global regulatory system that represses several stationary phase processes, while it activates certain exponential phase functions (reviewed in Romeo, 1998). Glycogen synthesis and catabolism (Romeo et al., 1993; Yang et al., 1996), gluconeogenesis (Sabnis et al., 1995), and biofilm formation (Jackson et al., 2002) are repressed by CsrA, while glycolysis (Sabnis et al., 1995), motility and flagellum synthesis (Wei et al., 2001), and acetate metabolism (Wei et al., 2000) are activated by this protein. The mechanism by which CsrA represses glycogen metabolism involves the binding of CsrA to the untranslated leader of the *glgCAP* transcript, which blocks translation and causes this transcript to rapidly turnover (Baker et al., 2002; Liu et al., 1995; Liu and Romeo, 1997). Although not as

extensively studied, positive control of *flhDC* expression by CsrA involves a similar post-transcriptional mechanism, whereby CsrA binding to the untranslated leader ultimately stabilizes this mRNA (Wei et al., 2001). Highly conserved CsrA homologues, alternatively designated as RsmA, are found in diverse eubacteria (reviewed in Romeo, 1998), and regulate virulence factors of animal and plant pathogens (e.g. Altier et al., 2000; Chatterjee et al., 1995).

Purification of a his-tagged CsrA protein revealed that it binds to a 366 nt untranslated RNA molecule, CsrB, to form a globular ribonucleoprotein complex containing ~18 CsrA subunits and a single CsrB transcript (Liu et al., 1997). An imperfect repeat sequence (CAGGAUG) that is located primarily in the loops of predicted RNA hairpins may permit CsrA to bind to CsrB. CsrB functions as an antagonist of CsrA, apparently by sequestering this protein (Liu et al., 1997; Gudapaty et al., 2001). CsrA also binds to a hairpin in the untranslated leader of the *glgC* message and to the Shine-Dalgarno sequence of this mRNA, both of which are related in sequence to the repeated elements of CsrB (Baker et al., 2002). CsrA and CsrB levels accumulate as the culture approaches the stationary phase of growth (Gudapaty et al., 2001). Although CsrA binds to CsrB, it does not alter CsrB stability. Instead, CsrA activates *csrB* transcription, providing an autoregulatory mechanism for intracellular CsrA activity (Gudapaty et al., 2001). Activation of *csrB* transcription by CsrA is mediated indirectly, through the BarA/UvrY two-component, signal transduction system (Suzuki et al., in press). Purified UvrY protein was found to stimulate *csrB-lacZ* expression in vitro,

revealing that UvrY resides immediately upstream from *csrB* in the signaling pathway between CsrA and *csrB* (Suzuki et al., in press).

More than a dozen sRNAs have been studied in *E. coli,* and more than 20 additional sRNAs of unknown function have been identified by bioinformatics approaches and comprehensive transcript profiling (Wassarman et al., 2001; Argaman et al., 2001). Many of the known sRNAs regulate translation by base-pairing with complementary segments of mRNAs, with the assistance of Hfq protein (Wassarman et al., 2001). In contrast, CsrB (Liu et al., 1997) and 6S RNA (Wasserman, et al., 2000) regulate gene expression by binding to proteins, CsrA and σ^{70}-RNA polymerase, respectively. In both cases, RNA binding reversibly inhibits the activity of the target protein and results in global changes in gene expression. Of the known sRNAs, only CsrB or its homologues has evolved a mechanism for binding to a large number of subunits of its target protein, providing an efficient means of sequestering CsrA during conditions of nutrient limitation (discussed in Romeo, 1998).

In a prior attempt to identify regulators of the stationary phase synthesis of glycogen, an *E. coli* genomic library in the low copy number plasmid pLG339 was screened for effects on glycogen levels (Romeo et al., 1991). Two clones were found to increase both glycogen levels and the expression of a *glgC'-'lacZ* translational fusion. One of these clones, pMR221, contains the *csrB* gene (unpublished data). The activity of the second clone, pMR2113, was localized to a ~360 bp region that lacks an open reading frame (unpublished data). This

information, and the presence of an apparent Rho-independent terminator sequence located near one end of the functional region, led us to hypothesize that this clone contains a gene that expresses a second regulatory RNA of the Csr system, CsrC. The present study confirms this notion, and reveals that CsrC is similar to CsrB in that it binds to and antagonizes CsrA, possesses several of the imperfect repeat sequences that characterize CsrB RNA, and is transcriptionally activated by CsrA and UvrY.

EXPERIMENTAL PROCEDURES

Strains, plasmids, and phage

Bacterial strains, plasmids and phage used in this study are listed in Table 1.

Media and Growth Conditions

LB medium (Miller, 1972) with 0.2% glucose was used for routine cultures. SOC medium (Miller, 1972) was used for recovery of transformed cells. Kornberg medium (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract containing 0.5% glucose for liquid or 1% glucose for agar) was used for gene fusion assays, northern blot and RNA stabililty studies, and assessment of the glycogen phenotype by iodine staining. Semisolid tryptone medium (pH 7.4) containing 1% tryptone, 0.5% NaCl and 0.35% agar was used for motility studies (Wei et al., 2001). Colonization factor antigen (CFA) medium (pH 7.4) (Evans et al., 1992) contained 1% casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, and 0.0005% $MnCl_2$, and was used to grow cultures for biofilm studies. Antibiotics were added at the following concentrations: chloramphenicol, 20μg/ml; kanamycin, 100 μg/ml; ampicillin, 100 μg/ml; tetracycline, 10μg/ml; rifampicin, 200μg/ml. Liquid cultures were grown at 37°C with rapid shaking, unless otherwise noted.

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

Strain, Plasmid or Phage	Description	Source or Reference
Strains		
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 relA1 endA1gyrA96thi-1*	Ausubel, 1989
BW3414	*ΔlacU169 rpoS(Am)*	B. Wanner
CF7789	MG1655 *ΔlacI-Z(mluI)*	M. Cashel
BW25113	*Δ(araD-araB)567 ΔlacZ478 ,lacIp-4000 rpoS396 rph-1Δ(rhaD-rhaB)568 rrnB-4 hsdR514*	Datsenko, 2000
MG1655	Prototrophic	M. Cashel
TRMG1655	*csrA::kanR*	Romeo, 1993
TWMG1655	*csrC::tetR*	This study
RGTWMG1655	*csrB::camR csrC::tetR*	This study
TRTWMG1655	*csrA::kanR csrC::tetR*	This study
KSGA18	CF7789 *Φ(glgA-lacZ) (λplacMu15)*Kan^R	Gudapaty, 2001
RGKSGA18	KSGA18 *csrB::camR*	Gudapaty, 2001
TWKSGA18	KSGA18 *csrC::tetR*	This study
RGTWKSGA18	KSGA18 *csrB::camR csrC::tetR*	This study
GS1114	CF7787 *Δ(λatt-lom)::bla Φ(csrC-lacZ)1(hyb)* Amp^R Kan^S	This study
TR GS1114	GS1114 *csrA::kanR*	This study
RGGS1114	GS1114 *csrB::camR*	This study
TWRG1113	GS1114 *csrC::tetR*	This study
UVRGS1114	GS1114 *uvrY::camR*	This study

KSB837	CF7787 *Δ(λatt-lom)::blaΦ(csrB-lacZ) 1(hyb)* $Amp^R Kan^S$	Gudapaty, 2001
TRKSB837	KSB837 *csrA::kanR*	Gudapaty, 2001
RGKSB837	KSB837 *csrB::camR*	Gudapaty, 2001
TWKSB837	KSB837 *csrC::tetR*	This study
UVRKSB837	KSB837 *uvrY::camR*	Gudapaty, 2001

Plasmids

pUC18	Cloning Vector, Amp^R	Ausubel, 1989
pMR2113	Clone of *csrC* region in low copy plasmid pLG339, Kan^R	Romeo et al., 1991
pMR2113-D1	*csrC HinDIII* fragment from pMR2113 in the *HinDIII* site of pUC19	This study
pCSRC1	Transcribed region of *csrC* oriented downstream from the *lac* promoter in pUC18	This study
pCSRCRe1	Transcribed region of *csrC* oriented opposite of the *lac* promoter in pUC18	This study
pKD46	Vector containing RED genes, *araBp-gam-bet-exo*	Datsenko, 2000
pBR322	Cloning vector, source of *tetR* marker for *csrC::tetR*	Ausubel, 1989
pCRA16	*csrA* in pBR322, Tet^R	Suzuki, in press
pBR322-UY14	*uvrY* in pBR322, Tet^R	Suzuki, in press
TOPO-XL	Commercial cloning vector, Kan^R	Invitrogen
pSPT18	Transcription vector with SP6 & T7 promoters, Amp^R	Boehringer Mannheim
pSPT18-CsrB	*csrB* cloned into pSPT-18 behind SP6 promoter	Gudapaty, 2001
pSPT18-D1	*csrC* cloned into pSPT-18 behind T7 promoter	This study

Bacteriophage		
P1*vir*	Strictly lytic P1	C. Gross

Molecular biology

Standard procedures were used for plasmid isolation, restriction digests, transformation, transduction, and PCR (Ausubel et al., 1989)

RNA preparation

Total cellular RNA was isolated using the Masterpure™ RNA purification kit (Epicentre), quantified by UV absorbance, and suspended in 70% ethanol at –80°C.

Primer extension

Total RNA was harvested from a culture grown to the transition to stationary phase in Kornberg medium. The oligonucleotide primer D1prB (Table 2) was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci mmol^{-1}; NEN Life Science Products Inc.) according to standard procedures (Ausubel et al., 1989). Approximately 15 ng of labeled primer was annealed to 10 µg of total RNA. cDNA was synthesized using 15 U of ThermoScript RT (Gibco BRL) in a 20 µl reaction mixture, incubated 60 min. at 48°C, and terminated 5 min. at 85°C. RNA was degraded with 2 U RNase H for 20 min. at 37°C. The same labeled primer was used to prepare a DNA sequencing ladder, with pMR2113-D1 as the template, which served as a standard for the reverse transcription product. Products were analyzed on urea-containing sequencing gels (e.g. Preiss and Romeo, 1989).

Table 2. Oligonucleotide primers used in this study[a]

Primer	Sequence (5' to 3')
D1KOF	CTGATGGCGGTTGATTGTTTGTTTAAGCA
	AAGGCGTAAACGCACATTTCCCCGAAAAG
D1KOR	TTATTCAGTATAGATTTGCGGCGGAATCTA
	ACAGAAACAATTCTTGGAGTGGTGAATCCG
D1PrB	CCATTTCCGTTTAATTACGTC
D1Pr1	GTAGCACCCATAGAGCGAG
D1Pr2	GCGGCGGAATCTAACAG
D1SerB	GCTGCGTGAGTTTGAAGATGATG
D1Check1	TGTGCAAATACTGATGGCGG
csrC-UP	GGCGAATATCAGGCGCACTCATCAC
csrC-DN	CTATGGGTGCTACTTTACGCCTTT

[a]Primers were purchased from Integrated DNA Technologies Inc., Coralville, Iowa.

Riboprobe synthesis

The *csrB* riboprobe was produced from plasmid pSPT18-CsrB as described elsewhere (Gudapaty, 2001). The plasmid for the production of *csrC* riboprobe was generated by subcloning a 209 bp NsiI/KpnII fragment from pMR2113-D1 into the multiple cloning site of pSPT18. The resulting plasmid, pSPT18-D1, was used to generate digoxigenin-labeled (DIG) riboprobe, using T7 RNA polymerase and the DIG-RNA Labeling Kit (SP6/T7), according to manufacturers instructions (Boehringer Mannheim, Indianapolis, Ind.) The synthesis reaction was carried out for 2 hrs. at 37°C, followed by 15 min. incubation with 2 µl RNase-free DNaseI. The reaction was subsequently terminated with the addition of 2 µl of 0.2 M EDTA. Probes were stored at – 80°C.

Northern hybridization

Total cellular RNA (5 µg) was separated on formaldehyde agarose (1%) gels, transferred overnight onto positively charged nylon membranes (Boehinger Mannheim) in 20X SSC, and immobilized by baking at 120°C for 30 min (Sambrook et al., 1989). Prehybridization, hybridization to DIG-labeled riboprobes (2 µl probe per 10 ml of prehybridization buffer), and membrane washing were conducted using the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim), according to the manufacturer's instructions, except that the membrane was incubated for 10 hrs. in blocking solution. The

resulting chemiluminescent signals were detected using Kodak X-OMAT-AR film and were quantified by phosphorimaging using a GS-525 Phosphor Imager (Biorad, Hercules, CA) with a chemiluminescent screen. Phosphorimaging data were analyzed using Molecular Analyst (version 2.1.2) software and Microsoft Excel. Loading of RNA was normalized using 23S ribosomal RNA signal. Ribosomal RNA was stained with ethidium bromide, photographed, and digitally analyzed with Molecular Analyst.

Construction of a *csrC* null mutant

A linear PCR product was generated that contained the *tetR* gene from cloning vector pBR322 flanked on either side by 40 nucleotides homologous to the upstream and downstream regions of *csrC*. Primers used in generating the fragment were D1KOF and D1KOR (Table 2). Linear recombination of this fragment into the *E. coli* genome was performed using the protocol described elsewhere (Datsenko, Waner 2000). Cells were plated on Kornberg containing tetracycline. Overnight colonies were chosen for confirmation by PCR and northern blot. PCR was performed using 15μl whole cells harvested from overnight culture. Cells were resuspended in 10 μl deionized water and heated 5 min. at 94°C (www.protocol-online.org). The resulting lysate was used as the DNA template to generate a PCR product using primers D1serB and D1Check1. The identity of the resulting PCR product was confirmed by restriction digestion

with *Eco*RV, *Bam*HI, and *Sal*I. *csrC* strains were designated with the "TW" prefix.

Construction of a minimal *csrC* clone

The transcribed region of *csrC* was amplified by PCR using primers D1Pr1 and D1Pr2 (Table 2). This PCR product was T-A cloned into the TOPO-XL cloning vector (Invitrogen). The *csrC* region was excised from this clone using *Eco*RI and subcloned into the *Eco*RI site of pUC18. Clones containing *csrC* in the forward or reverse orientation with respect to the *lacZ* promoter of pUC18 were designated as pCSRC1 and pCSRCre1, respectively. Plasmid DNA inserts were sequenced at the University of Arizona core facility using primers D1Pr1 and D1Pr2.

Construction of a chromosomal *csrC-lacZ* transcriptional fusion

A 243-bp PCR product was prepared, which contained the 3'-end of the *yihA* gene, the upstream untranscribed region of *csrC,* and the first 4 transcribed nt of *csrC*. The primers csrC-UP and csrC-DN (Table 2) were used for generating this PCR product. The PCR product was gel-purified, blunt-ended with T4 DNA polymerase and cloned into *Sma*I-treated and dephosphorylated pGE593 plasmid. The resulting plasmid, pCCZ1, was partially sequenced and found to be free of PCR-generated mutations. The *csrC-lacZ* fusion in pCCZ1 was moved into the *E. coli* CF7789 chromosome using the λInCh1 system, as described (Boyd et al.,

2000). The resulting strain that was chosen for subsequent studies, GS1114, was Amp^r Kan^s and was no longer temperature sensitive. The presence of the *csrC-lacZ* transcriptional fusion in this strain was confirmed by PCR analysis, as recommended (Boyd et al., 2000).

RNA decay analysis

The transcription inhibitor rifampicin was added to cultures at 2 hrs. post-exponential phase. The culture was harvested at regular intervals thereafter, and total cellular RNA was isolated. CsrC RNA was analyzed by northern blot and phosphorimage analysis (Gudapaty, 2001).

Glycogen, β-galactosidase, total protein, and motility assays

Glycogen accumulation was examined by staining colonies with iodine vapor (Liu et al., 1997). β-galactosidase activity was assayed as described previously (Romeo et al., 1990). Total protein was measured by bicinchonic acid assay using bovine serum albumin as standard (Smith, 1985). Motility was assessed on tryptone semisolid medium as described previously (Wei et al., 2001).

Quantitative biofilm assay

Cultures were grown for 24 hrs at 26°C in microtiter plates, and biofilm formation was monitored using crystal violet staining, as described previously (Jackson et al., 2002). The experiment was conducted twice, with eight replicates

per sample in each trial. The data were analyzed by Tukey Multigroup Analysis (StatView-SAS Institute Inc., Cary, N.C.).

Sequence and Secondary structure analysis of *csrC*

The position of the *csrC* gene on the *E. coli* K-12 genome (Blattner et al., 1997) and *csrC* homologues were identified by BLAST analyses, courtesy of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Secondary structure predictions for CsrC RNA were generated with the MFOLD program, which utilizes an algorithm for minimizing free energy of RNA structures (Zucker et al., 1999, http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). Default parameters (Zucker et al., 1999) were used in all fields for the predictions, and the structure with the lowest predicted free energy is presented herein.

RESULTS

The *csrC* gene specifies a ~245 nucleotide regulatory RNA

The original *csrC* clone, pMR2113, was isolated due to its stimulatory effects on glycogen accumulation, and was shown to activate *glgC'-'lacZ* expression (Romeo et al., 1991). Subclones and deletions from this insert revealed a minimal functional region of 0.36 Kb, which lacked an apparent open reading frame (unpublished data; summarized in Fig. 1). This suggested that pMR2113 might either contain a cis-acting element that sequesters a transcriptional repressor or may express a regulatory RNA. Furthermore, the nucleotide sequence of the functional region contains an apparent Rho-independent transcriptional terminator at one end, consistent with the latter hypothesis (Fig. 1). Northern analysis, using a riboprobe designed to detect transcription from this region, revealed a small RNA which accumulates as the culture approaches the stationary phase of growth (Fig. 2). The levels of this RNA were affected by *csrA*, but not by *rpoS*, which encodes a sigma factor needed for the expression of a variety of stationary phase genes (reviewed in Hengge-Aronis, 1996). Primer extension analysis revealed a single 5'-terminus for this small RNA molecule (Fig. 3). A σ^{70} promoter sequence is located immediately upstream from this site in the *csrC* gene (Fig. 1), strongly suggesting that this 5'-terminus represents the initiation site for *csrC* transcription.

Figure 1. **Sequence of the *csrC* gene.** The *csrC* gene is located in the intergenic region between the open reading frames *yihA* and *yihI,* as shown. The 5'-end of CsrC RNA and the –10 and –35 elements of the apparent promoter are underlined. Repeat sequences representing putative CsrA binding sites are underlined. Inverted repeats of the apparent Rho-independent terminator are indicated by arrows. Asterisks flank a minimal functional region, deduced by subcloning and deletion analysis.

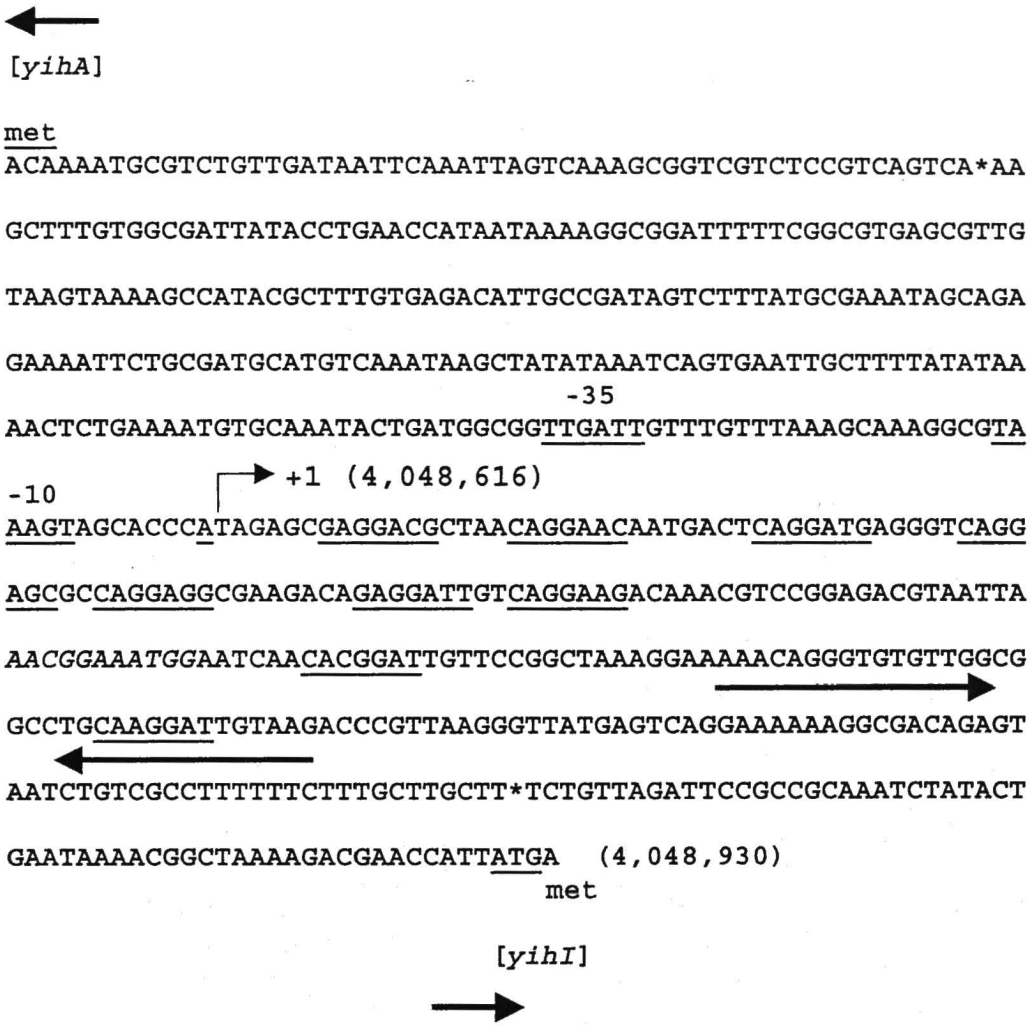

[yihA]
met
ACAAAATGCGTCTGTTGATAATTCAAATTAGTCAAAGCGGTCGTCTCCGTCAGTCA*AA
GCTTTGTGGCGATTATACCTGAACCATAATAAAAGGCGGATTTTTCGGCGTGAGCGTTG
TAAGTAAAAGCCATACGCTTTGTGAGACATTGCCGATAGTCTTTATGCGAAATAGCAGA
GAAAATTCTGCGATGCATGTCAAATAAGCTATATAAATCAGTGAATTGCTTTTATATAA
-35
AACTCTGAAAATGTGCAAATACTGATGGCGGTTGATTGTTTGTTTAAAGCAAAGGCGTA
+1 (4,048,616)
-10
AAGTAGCACCCATAGAGCGAGGACGCTAACAGGAACAATGACTCAGGATGAGGGTCAGG
AGCGCCAGGAGGCGAAGACAGAGGATTGTCAGGAAGACAAACGTCCGGAGACGTAATTA
AACGGAAATGGAATCAACACGGATTGTTCCGGCTAAAGGAAAAACAGGGTGTGTTGGCG
GCCTGCAAGGATTGTAAGACCCGTTAAGGGTTATGAGTCAGGAAAAAAGGCGACAGAGT
AATCTGTCGCCTTTTTTCTTTGCTTGCTT*TCTGTTAGATTCCGCCGCAAATCTATACT
GAATAAAACGGCTAAAAGACGAACCATTATGA (4,048,930)
met
[yihI]

Figure 2. **CsrC RNA during the growth curve.** A DIG-labeled riboprobe, specific for the deduced functional region of *csrC* clone pMR2113, was used for Northern analysis of CsrC RNA harvested throughout the growth curve of wild type strain MG1655, *csrA* strain TRMG1655, and *rpoS* strain BW3414. The positions of RNA standards are marked in panel B. Stationary phase occurred at ~6 hrs.

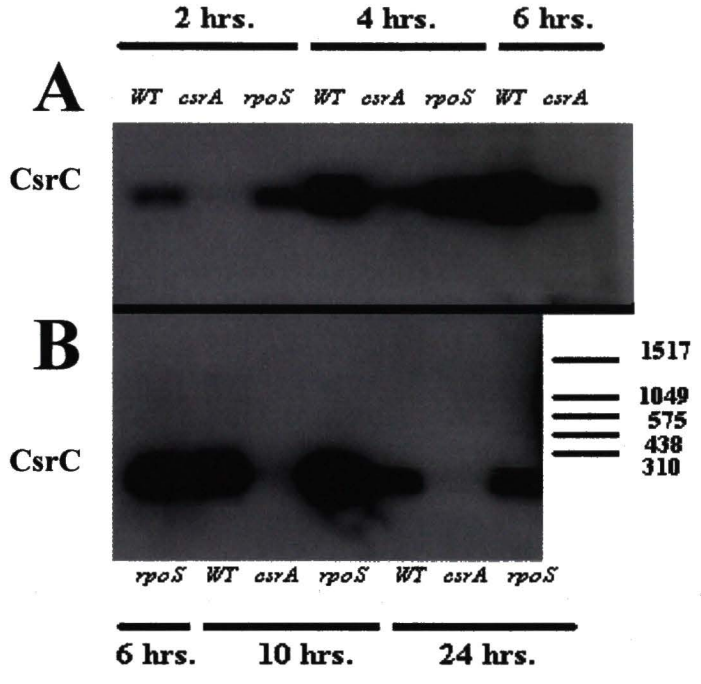
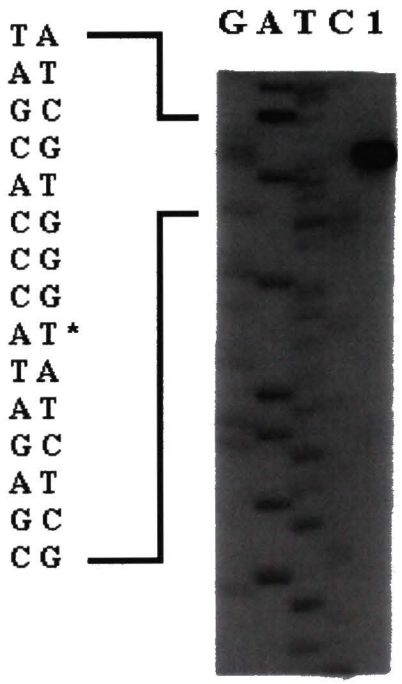

2 hrs.
4 hrs.
6 hrs.
A
WT csrA rpoS WT csrA rpoS WT csrA
CsrC
B
CsrC
1517
1049
575
438
310
rpoS WT csrA rpoS WT csrA rpoS
6 hrs.
10 hrs.
24 hrs.

Figure 3. **Primer extension analysis of CsrC RNA.** The radiolabeled oligonucleotide primer D1PrB was annealed to total RNA from MG1655, and extended using reverse transcriptase. The single product of this reaction (lane 1) was analyzed with a Sanger sequencing ladder prepared using the same primer and pMR2113-D1.


G A T C 1
T A
A T
G C
C G
A T
C G
C G
C G
A T *
T A
A T
G C
A T
G C
C G

Collectively, northern hybridization, primer extension, and sequence analysis reveal an RNA of ~245 nt in length, which we call CsrC (Fig. 1).

To determine whether CsrC RNA affects glycogen accumulation, the transcribed region of the *csrC* gene, lacking the apparent promoter sequence, was amplified and subcloned into the multiple cloning site of pUC18 in both orientations. Glycogen levels were affected only if *csrC* was downstream from and in the same orientation as the *lac* promoter(data not shown). This revealed that *csrC* must be transcribed to be functional and provided strong genetic evidence that CsrC is a regulatory RNA molecule.

Effects of a *csrC* null mutation on glycogen levels and *glgA'-'lacZ* expression

A *csrC* mutant, TWMG1655 (Table 1), was generated using a linear transformation protocol (Datsenko and Wanner, 2000), wherein the transcribed region of *csrC* was precisely replaced with a *tetR* marker. Glycogen levels in this *csrC* mutant were similar to those of the parent strain (Fig. 4). However, glycogen was observably decreased in a *csrB csrC* double mutant, relative to the wild type strain or to either the *csrB* or *csrC* single mutant (Fig. 4). The effects of *csrC* deletion on expression of a *glgCA'-'lacZ* translational gene fusion were also negligible (Fig. 5). However, *csrC* disruption in a *csrB* mutant strain background decreased the expression of this gene fusion ~50%. Finally, pCSRC1, which expressed *csrC* from the *lac* promoter, stimulated *glgCA* expression by 2- to 3-fold (Fig. 5).

Figure 4. **Effects of *csrC* on glycogen levels.** Iodine levels were assessed by iodine vapor stain of cells grown on Kornberg medium A. Effects of *csrA, csrB, and csrC* mutations of wild type strain MG1655 on glycogen levels. Wild type strain is designated as "WT". Isogenic mutants are identified by deleted gene. *csrC* over-expression from plasmid pCSRC1 is identified as "*csrC*++".

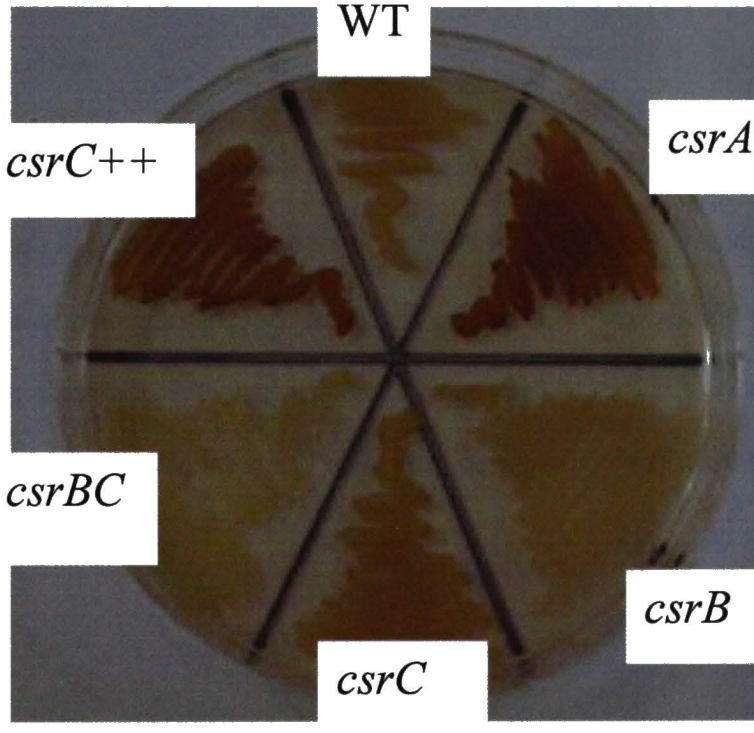
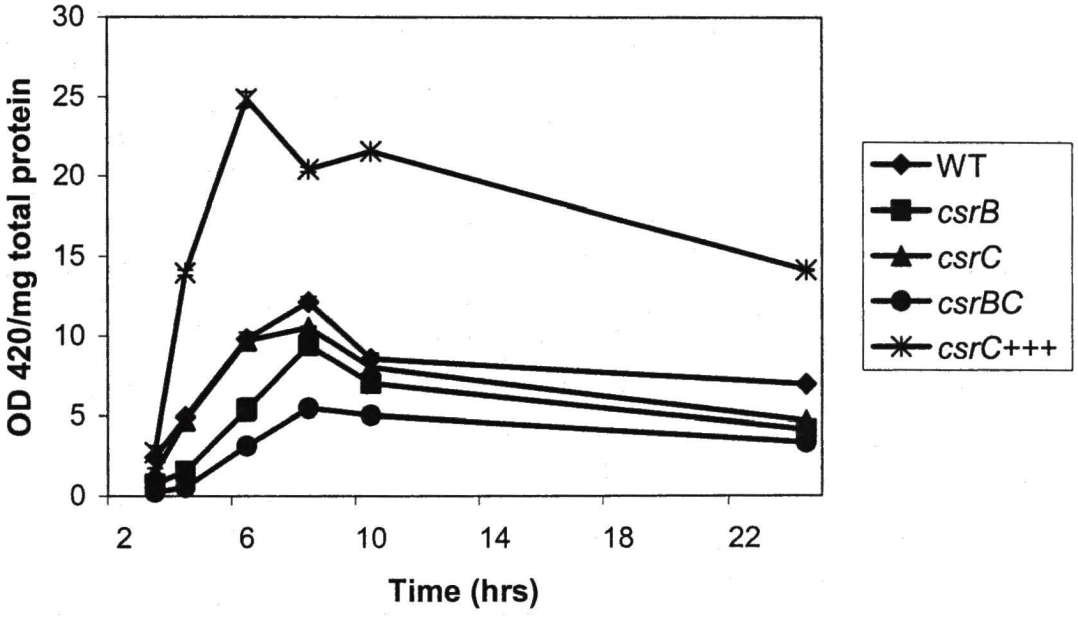

WT
csrA
csrC++
csrBC
csrB
csrC

Figure 5. **Effects of *csrC* on expression of a chromosomal *glgCA'-'lacZ* fusion.** Cultures were grown in Kornberg medium and and assayed for β–galactosidase and total protein as described in Experimental Procedures. This experiment was repeated once with essentially identical results. Strains used were KSGA18 and isogenic mutants as described in table 1. "*csrC*+++" denotes over-expression of *csrC* from plasmid pCSRC1.


30
25
20
15
10
5
0
OD 420/mg total protein
2
6
10
14
18
22
Time (hrs)
WT
csrB
csrC
csrBC
csrC+++

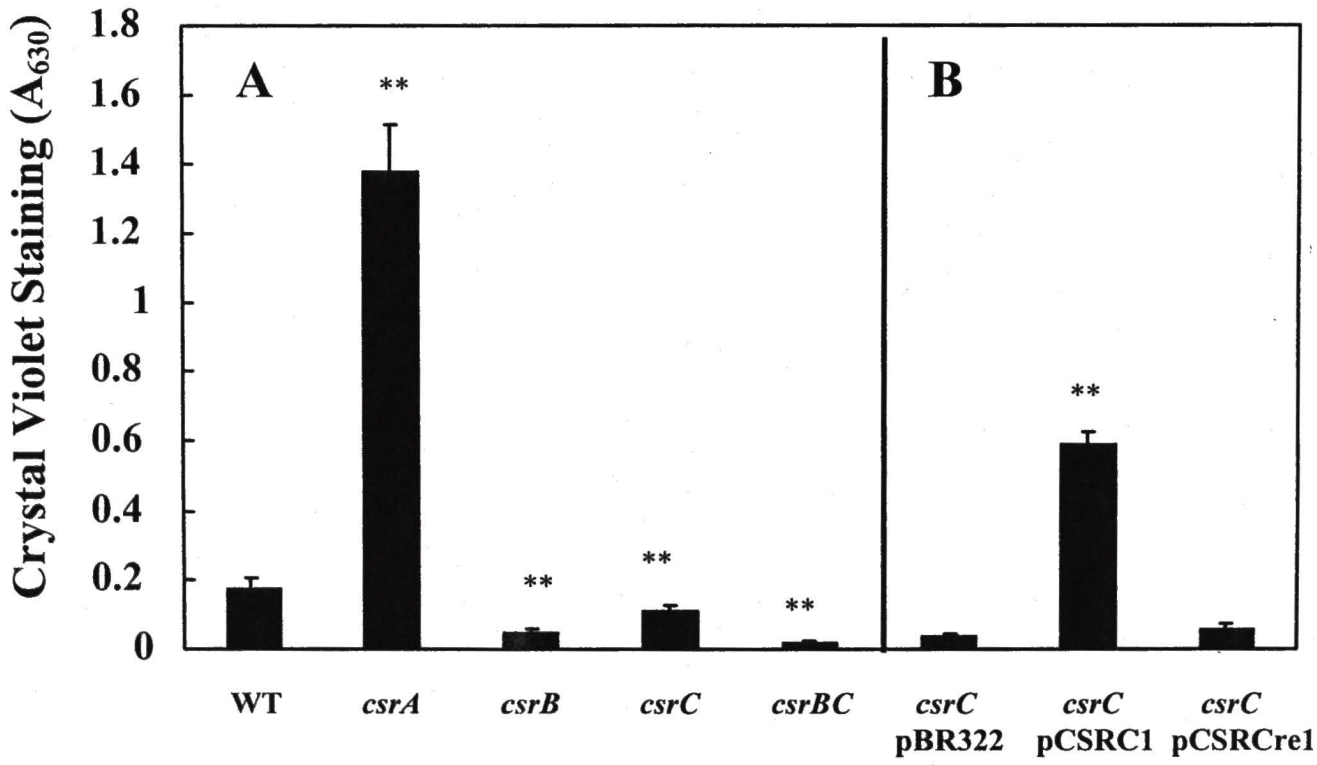
Effects of CsrC on biofilm formation and motility

CsrA is a repressor of biofilm formation, while CsrB activates this process (Jackson et al. 2002). The effect of *csrC* on biofilm formation in static cultures was measured using a microtiter plate assay (Jackson et al., 2002). Disruption of *csrC*

modestly decreased biofilm formation, an effect that was determined to be statistically significant (Fig. 6A). A *csrB csrC* double mutant was severely compromised for biofilm formation and produced <10% of the biofilm of the wild type strain. Increased gene dosage of *csrC* led to several-fold greater accumulation of biofilm (Fig. 6B), an effect that was dependent upon *csrC* transcription. Thus, CsrC RNA activates biofilm formation in *E. coli*, similar to CsrB.

CsrA is required for motility of *E. coli* under a variety of conditions, e.g. tryptone medium (Wei et al. 2001). The effects of *csrC* disruption and increased copy number on motility were examined in tryptone medium. Similar to its isogenic parent, the *csrC* null mutant of MG1655 was fully motile (data not shown). In contrast, increasing the copy number of *csrC* completely inhibited motility if the plasmid-encoded gene could be transcribed (pCSRC1), but had no effect on motility if *csrC* lacked a promoter (pCSRCrel; data not shown).

Figure 6. **Effects of *csr* genes on biofilm formation.** Biofilm formation was assayed by crystal violet staining as described in the experimental procedures. Bars depict the means and the standard errors from two independent experiments with 8 replicates per strain in each experiment. Asterisks denote statistically significant differences between strains ($P<0.01$), as determined by Tukey Multigroup Analysis. Strains used were wild type (WT) strain MG1655 and isogenic mutants identified by deleted gene.


Crystal Violet Staining (A_{630})
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A
B
**
**
**
**
**
WT
csrA
csrB
csrC
csrBC
csrC pBR322
csrC pCSRC1
csrC pCSRCre1

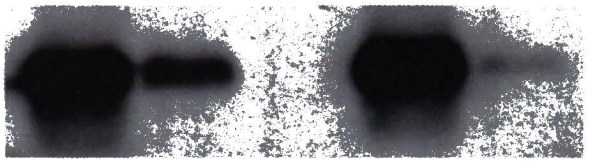
Northern analysis of CsrC regulation

Since CsrC is related to CsrB in its ability to antagonize CsrA, we were interested to determine if the expression of the *csrC* and *csrB* genes also share regulatory features. CsrC and CsrB levels were measured at 2 hours post-exponential phase in a series of isogenic strains varying in *csrA*, *csrB*, *csrC* or *uvrY* (Fig. 7). This experiment showed that the CsrB and CsrC riboprobes were specific, since no signal was detected from the *csrB* mutant with the CsrB probe or from the *csrC* mutant with the CsrC probe. Furthermore, CsrC accumulation was found to depend upon both CsrA and UvrY, although CsrC levels were somewhat less sensitive than those of CsrB (Fig. 7).

Interestingly, a ~20% increase in CsrB levels was noted in cells lacking CsrC RNA (Fig. 7C). Likewise, in the absence of CsrB, CsrC transcript levels were similarly elevated (Fig. 7D). Although these effects were modest, they were reproducible in 2 independent experiments (data not shown). We suspect that the compensatory effects of the two sRNAs result from increased intracellular availability of CsrA in the absence of one or the other of its sRNA antagonists.

Figure 7. **CsrB and CsrC levels in wild type strain MG1655 and isogenic mutants.** A. Northern blot probed for CsrB RNA. B. Northern blot probed for CsrC RNA. C. Phosphoimage analysis of CsrB blot. D. phosphorimage analysis of CsrC blot. The phosphoimage data in C & D show the average and standard deviation of two independent experiments.

A CsrB RNA

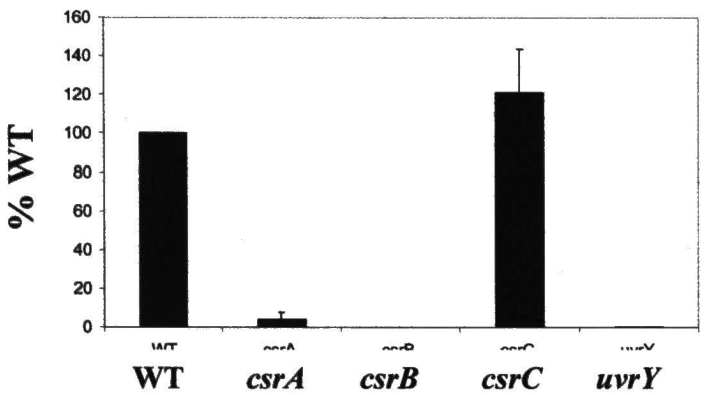


WT *csrA* *csrB* *csrC* *uvrY*

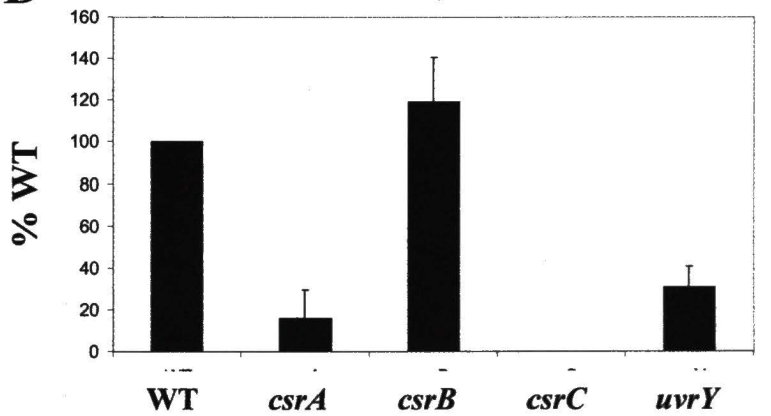
B CsrC RNA

WT *csrA* *csrB* *csrC* *uvrY*

C



D



Stability of CsrC RNA

While CsrA generally activates or inhibits gene expression post-transcriptionally by increasing or decreasing mRNA stability, respectively (Liu et al., 1995; Wei et al., 2001), it regulates CsrB RNA levels by altering *csrB* transcription, and does not affect CsrB RNA stability (Gudapaty et al., 2001). Similarly, the half-life (~2 min.) of CsrC was not altered in a *csrA* mutant (Fig. 8). Because the levels of any RNA molecule are determined by its rates of synthesis and turnover, this result strongly suggested that CsrA affects CsrC synthesis.

Expression of a *csrC-lacZ* transcriptional fusion.

The results of the northern hybridization were confirmed using *csrC-lacZ* and *csrB-lacZ* gene fusions (Fig. 9). The *csrC* and *csrB* (Gudapaty et al., 2001) fusions were designed to contain the upstream sequences and only four base pairs of the RNA templates of these two genes. Thus, transcripts synthesized from these fusions lack CsrA-binding elements. The expression of each gene fusion was activated by *csrA* and by *uvrY*, confirming that the effect of CsrA on *csrC* (*and csrB*) expression was mediated

Figure 8. **CsrC RNA stability in wild type MG1655 and isogenic *csrA* mutant strains.** Cultures were grown to 2 hrs. post-exponential phase, treated with rifampicin, and total cellular RNA was harvested at regular intervals thereafter. CsrC levels were quantified by Northern blot and phosphorimage analysis, as described in Experimental Procedures. This experiment was repeated once with essentially identical results.

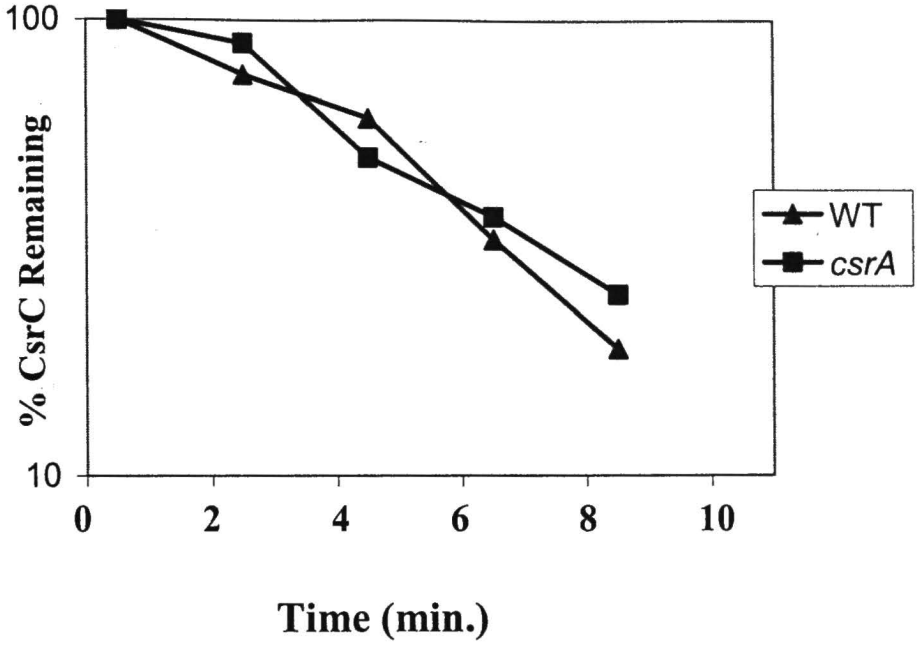
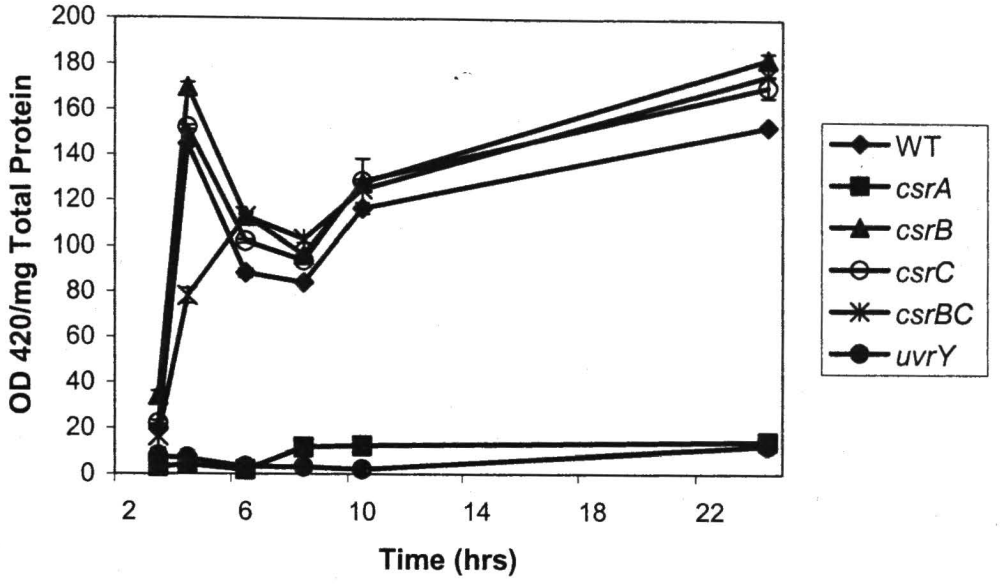
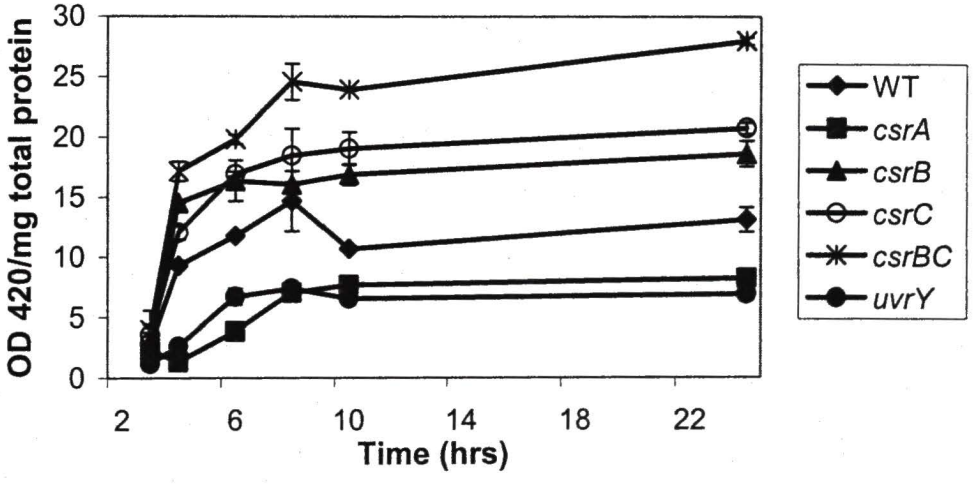

100
10
% CsrC Remaining
0
2
4
6
8
10
Time (min.)
WT
csrA

Figure 9. **Regulation of *csrB-lacZ* and *csrC-lacZ* expression.** A. Effects of *csrA*, *csrB*, *csrC,* and, *uvrY* isogenic mutations on *csrB-lacZ* expression in strain KSB837. B Effects of *csrA*, *csrB*, *csrC,* and, *uvrY* isogenic mutations on *csrC-lacZ* expression in strain GS1114.

A


OD 420/mg Total Protein
200
180
160
140
120
100
80
60
40
20
0
2
6
10
14
18
22
Time (hrs)
WT
csrA
csrB
csrC
csrBC
uvrY

B

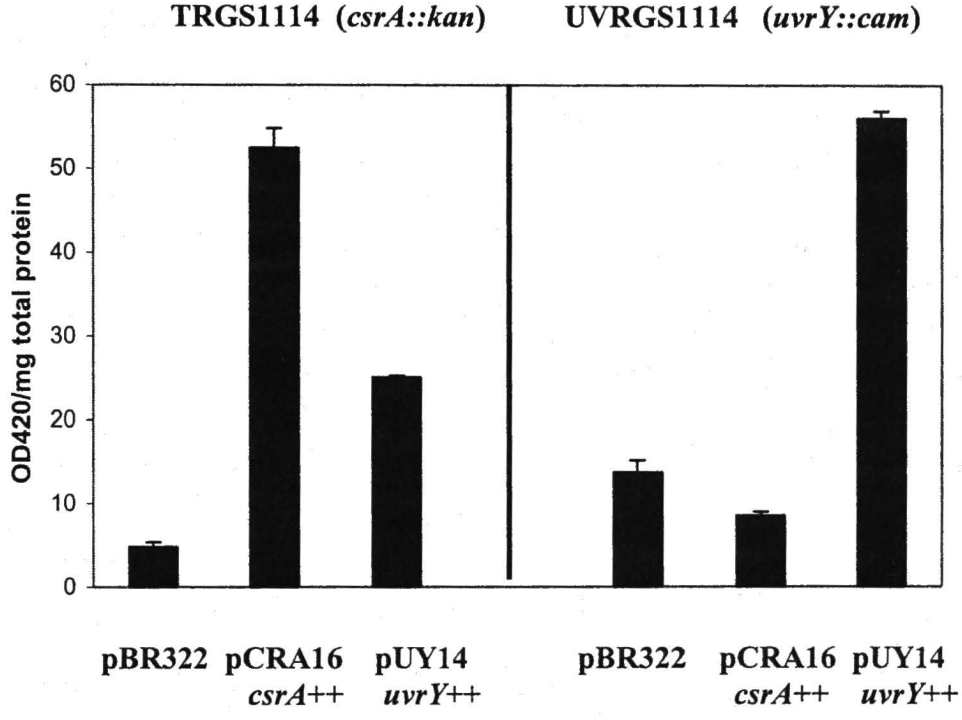

OD 420/mg total protein
30
25
20
15
10
5
0
2
6
10
14
18
22
Time (hrs)
WT
csrA
csrB
csrC
csrBC
uvrY

at the level of transcript initiation. The gene fusion assays also revealed a modest, but reproducible, increase in *csrC-lacZ* expression in a *csrB* mutant, as well as an increase in *csrB-lacZ* expression in the *csrC* mutant. Thus, the compensatory effects of these two RNAs, which were first noted in northern hybridization experiments (Fig. 7), were determined to be mediated at the level of transcription.

Complementation analyses

CsrA activates *csrB* transcription indirectly (Gudapaty et al., 2001). This effect of CsrA on *csrB* transcription was recently shown to be completely dependent upon a functional *uvrY* gene, which encodes a response regulator. Purified UvrY stimulates *csrB* expression in vitro and in vivo (Suzuki et al., in press). Thus, complementation studies were conducted to assess whether the effect of CsrA on *csrC* expression is also UvrY-dependent. Ectopic expression of either *csrA* or *uvrY* from a multicopy plasmid complemented the defect in *csrC-lacZ* expression that was caused by a *csrA* mutation (Fig. 10, panel A). In contrast, only *uvrY* complemented a *uvrY* defect; ectopic expression of *csrA* failed to stimulate *csrC-lacZ* expression in the *uvrY* mutant (Fig. 10, panel B). In conjunction with the results from the northern hybridization, gene fusion assays, and in vitro transciption-translation studies, these experiments demonstrated that activation of *csrC* transcription by CsrA depends on UvrY, and that UvrY is poised immediately upstream from *csrA* in a signaling pathway from CsrA to CsrC.

Figure 10. **Complementation studies:** effects of ectopic expression of *csrA* (pCRA16) and *uvrY* (pUY14) on expression of a *csrC-lacZ* transcriptional fusion in isogenic *csrA* (TRGS1114) or *uvrY* (UVRGS1114) mutants. The vector control in each case was pBR322. Liquid cultures were grown to 2 hrs. post-exponential phase and specific β-galactosidase activity was determined as the average of duplicate samples. Error bars indicate standard deviation. This experiment was repeated in entirety, with essentially identical results.


TRGS1114 (csrA::kan)
UVRGS1114 (uvrY::cam)
OD420/mg total protein
60
50
40
30
20
10
0
pBR322
pCRA16 csrA++
pUY14 uvrY++
pBR322
pCRA16 csrA++
pUY14 uvrY++

Predicted secondary structure of CsrC

A striking feature of CsrB RNA is the observation that 18 conserved sequences are located primarily in the loops of predicted hairpins or other single stranded regions of CsrB (Liu et al., 1997). CsrA:CsrB complex contains ~18 subunits of CsrA (Liu et al., 1997). Furthermore, the repeated sequences of CsrB resemble the high affinity CsrA-binding site of *glgC* mRNA (Baker et al., 2002), suggesting that these sequences are binding sites for CsrA. Not surprisingly, CsrC RNA contains 9 similar repeated sequence elements, which tend to be located in predicted single stranded loops of the molecule (Fig. 12). In addition, the 3'-half of CsrC is predicted to form two large and several small hairpins. The apparent terminator of CsrC RNA may form an unusual 16 bp double-stranded stem, in which the oligo-U sequence of the terminator is base-paired to an oligo-A sequence (Fig. 11).

CsrC homologues

The recent availability of numerous microbial genome sequences offered the opportunity to assess the phylogenetic distribution of *csrC*. Blast analysis revealed highly conserved *csrC* homologues in species representing 3 genera of Enterobacteriaceae (Fig. 12). Homologues were not apparent in species of any other eubacterial families, perhaps because the functionality of CsrC RNA, like that of CsrB, may require only limited sequence conservation (discussed in Romeo, 1998).

Figure 11. **Predicted secondary structure of CsrC RNA.** Secondary structure of CsrC RNA was predicted using MFOLD, as described in the Methods. Imperfect repeat sequences that resemble the CsrB-type repeats are shown in red, predicted base-paring interactions are indicted by dark circles.

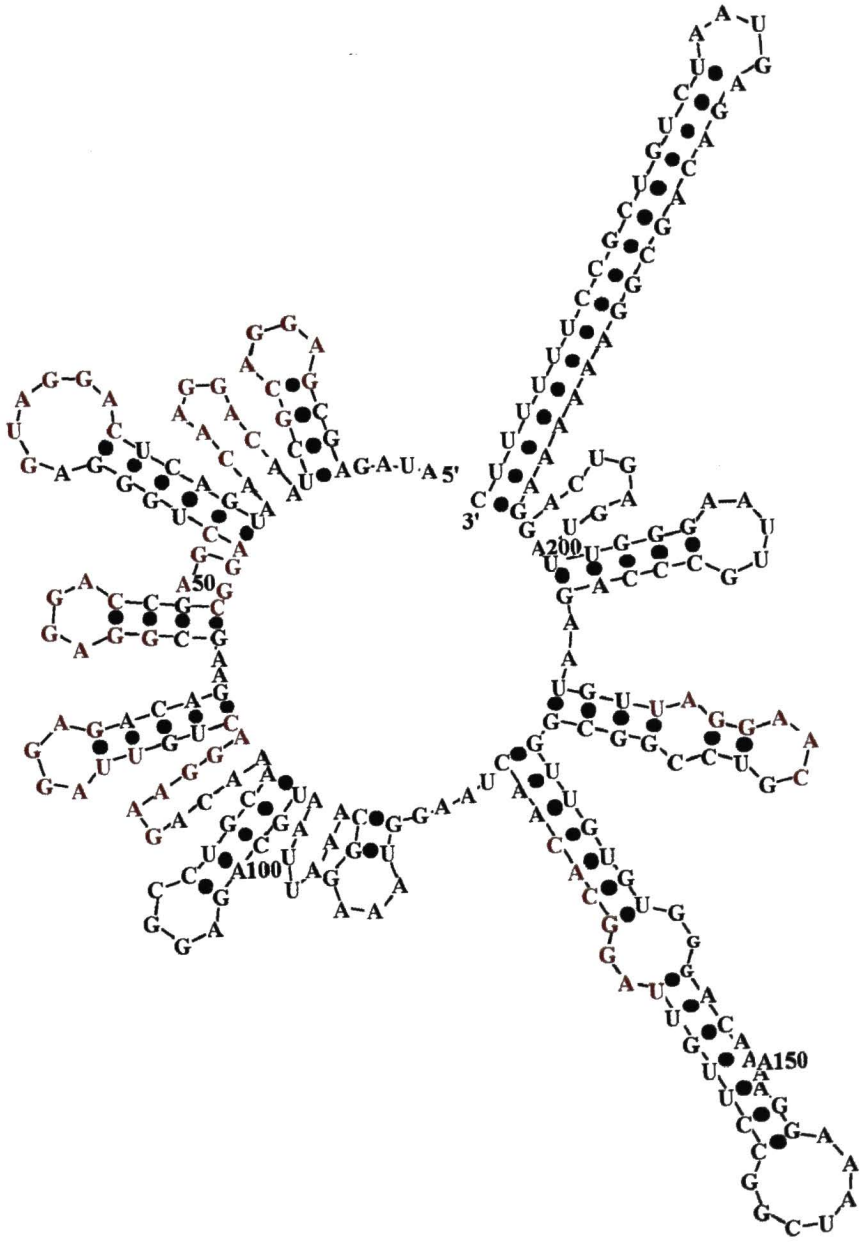
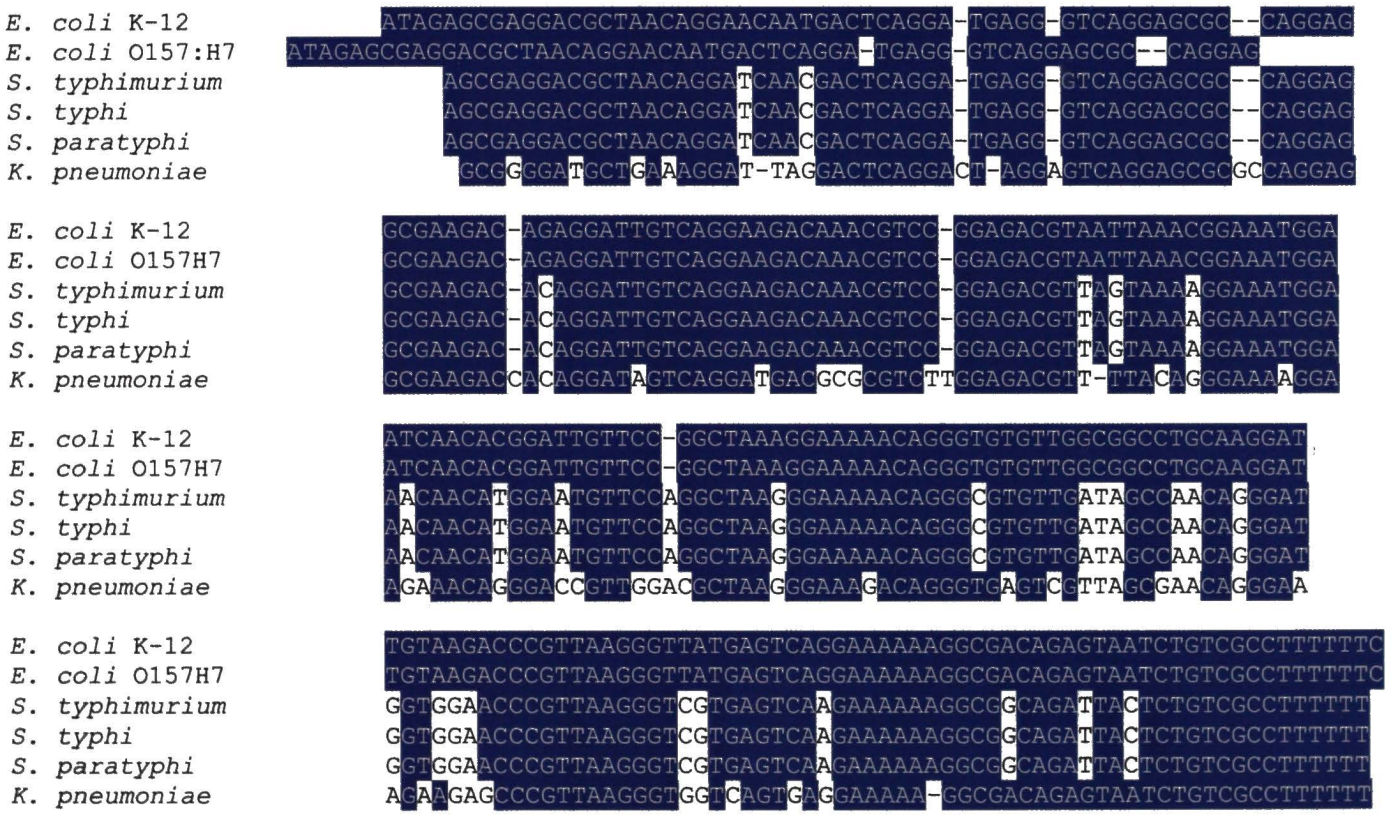

5'
3'
A50
A100
A150
A200

Figure 12. **Homologues of *csrC*.** Homologues of *E. coli* K-12 *csrC* were identified by Blast analysis at NCBI in *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *S. typhi*, *S. paratyphi*, and *Klebsiella pneumoniae*. Residues that are identical to those of *E. coli* K-12 are highlighted.


E. coli K-12
E. coli O157:H7
S. typhimurium
S. typhi
S. paratyphi
K. pneumoniae
E. coli K-12
E. coli O157H7
S. typhimurium
S. typhi
S. paratyphi
K. pneumoniae
E. coli K-12
E. coli O157H7
S. typhimurium
S. typhi
S. paratyphi
K. pneumoniae
E. coli K-12
E. coli O157H7
S. typhimurium
S. typhi
S. paratyphi
K. pneumoniae

DISCUSSION

As our collective knowledge of the many functions of sRNA's grows, it is becoming increasingly clear that their role in bacterial physiology is more extensive than previously suspected. It is a reasonable assertion that the production of small RNA regulators is more metabolically efficient than the translation of protein molecules. Furthermore, the rapid production and relatively short half-life of RNA molecules make them particularly responsive to the changing needs of the cell.

In this study, we present a novel small regulatory RNA, CsrC. With the discovery of CsrC RNA, we add an additional component to the previously characterized Csr system. The RNA binding protein CsrA of *E. coli* post-transcriptionally regulates gene expression by binding to messenger RNA transcripts, leading to stabilization or translational inhibition and turnover. The sRNA molecule, CsrB, apparently exploits the binding specificity of CsrA by possessing 18 repeated sequences for sequestering ~18 CsrA subunits per molecule. We have now found that in addition to CsrB, a second sRNA molecule, CsrC, interacts with CsrA and antagonizes its effects in the cell. Although CsrC is somewhat smaller than CsrB RNA (245 versus 366 nt), both RNAs appear to utilize the same mechanism for antagonizing CsrA activity in the cell.

Through this study we have outlined a number of striking similarities between CsrC and CsrB RNA's. CsrB contains ~18 repeated elements believed to act as binding sites for CsrA. Projected secondary structure models of CsrB demonstrate that these repeated elements are located within stem loop structures. We have identified 9 putative CsrA binding sites on the CsrC RNA. Similarly, these sites also appear to be contained mainly in stem loop structures. Beyond the apparent CsrA binding sites, no sequence homology between CsrB and CsrC has been discernable. We have found that neither *csrC* overexpression nor deletion confers discernable effects on the glycogen phenotype in a *csrA* strain (data not shown). Thus providing strong genetic evidence supporting our hypothesis that observed CsrC effects are mediated through binding and sequestering CsrA. Furthermore, recent studies conducted at Pennsylvania State University have demonstrated direct binding of CsrA to both CsrB and CsrC in vitro (Paul Babitzke, personal correspondence).

CsrC binds and negatively regulates CsrA function, while itself being transcriptionally regulated by CsrA. This type of auto-regulatory relationship has been shown to exist between CsrA and CsrB (Gudapaty, 2001). CsrC is also positively regulated by the response regulator protein UvrY, as is CsrB. Our studies have revealed a regulatory pathway governing the expression of CsrC, in which UvrY regulation lies downstream of that by CsrA. UvrY has been shown to be a direct transcriptional regulator of CsrB (Suzuki, in press), and similar

efforts are currently being undertaken to discern the relationship between UvrY and CsrC.

The addition of CsrC to the growing list of regulatory RNA molecules provides further evidence of the importance of regulatory RNA's in bacterial systems. However, the emergence of this regulator has spawned a number of additional questions. To date, no additional function for either CsrB or CsrC has been determined outside CsrA antagonism. The Csr RNA's apparently exert their observed effects through the same mechanism, and appear to be regulated by the same factors. Furthermore, under the growth conditions used for this study, both CsrB and CsrC levels accumulate as a growing culture enters the stationary growth phase. This apparent lack of functional distinction between the Csr RNA's begs the question of the necessity of a second regulatory RNA in this system.

Through the examination of CsrB and CsrC expression, it was found that a deletion mutation of either CSR RNA gene results in a complementary increase in the expression of the counterpart. Thus, in the absence of CsrB, CsrC levels are elevated approximately twenty percent. This data may suggest a possible role for the two CsrB and CsrC in fine-tuned global regulation. CsrA exerts both positive and negative control over various metabolic pathways, ultimately regulating total carbon flux within the cell. Such a central metabolic function must be finely controlled to provide for optimal cell function. The CSR RNA's working in tandem may in fact provide a unique mechanism for the control of CsrA activity.

CsrA positively regulates expression of both *csrB* and *csrC*. In turn, both CsrB and CsrC are negative regulators of CsrA activity. So, fluctuation of a Csr RNA would alter CsrA activity followed by a compensatory shift in RNA expression.

At present it is not clear that the Csr RNA's posses any divergent functions, independent of CsrA regulation. Secondary structure analysis of CsrB shows the presence of putative CsrA binding sites evenly disbursed throughout the molecule. However, the CsrC RNA contains its proposed CsrA binding sites concentrated at the 5' end of the molecule. This may belie an additional function for CsrC. This RNA may in fact bind to an additional protein factor, or participate in a pathway whose mechanism is different from that of CsrA binding. A second possibility exists in differential expression of CsrB and CsrC. Under our laboratory conditions, the Csr RNA's show similar expression profiles. However, conditions may exist in which either CsrB or CsrC is preferentially expressed over the RNA counterpart. Both of these possibilities warrant further analysis.

Recent studies of the E. coli genome have identified several hypothetical small-RNA coding genes. One of the genes identified by genomic analysis and northern blot hybridization was in fact found to correspond to the *csrC* gene (Wasserman, 2001). These findings may provide insight into the heretofore unappreciated importance of this emerging class of RNA molecules. The discovery of CsrC would seem to bolster the assertion that many small non-coding RNA's have yet to be discovered.

REFERENCES

Altier, C., Suyemoto, M., and Lawhon, S. (2000) Regulation of *Salmonella enterica* Serovar Typhimurium Invasion Genes by *csrA*. *American Soc. For Microbiol.* **68:**6790-6797.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E.G., Margalit, H., and Altuvia, S. (2001) Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol.* **11**:941-50.

Ausubel, F.M., Brent, R., Kingsington, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989) Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY.

Baker, C.S., Morozov, I., Suzuki, K., Romeo, T., and Babitzke, P. (2002) CsrA regulates glycogen biosynthesis by preventing translation of *glgC* in *Escherichia coli*. *Mol. Microbiol.* **44:**1599-610.

Blattner, F.R., Plunkett, G. III, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., and Shao, Y. (1997) The complete genome sequence of Escherichia coli K-12. *Science* **277**:1453-1474.

Boyd, D., Weiss, S.D., Chen, J.C., and Beckwith, J. (2000) Towards single copy gene expression systems making gene cloning physiologically

relevant: Lambda InCh, a simple Escherichia coli plasmid-chromosome shuttle system. *J. Bacteriol.* **182**:842-847.

Chatterjee, A., Cui, Y.Y., Liu, Y., Dumenyo, C.K., and Chatterjee, A.K. (1995) Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. carotovora in the absence of starvation/cell density-sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone. *Appl. Environ. Microbiol.* **61**: 1959-1967.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* **97**:6640-6645.

Evans, D. G., Evans, D.J. Jr., and Tjoa, W. (1997) Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. *Infect. Immun.* **18**:330-337.

Gottesman, S. (1984) Bacterial Regulation: global regulatory networks. *Annu. Rev. Genet.* **18:** 415-441

Gudapaty, S., Suzuki, K., Wang, X., Babitzke, P., and Romeo, T. (2001) Regulatory Interactions of Csr Components: the RNA Binding Protein CsrA Activates *csrB* Transcription in *Escherichia coli. J. Bacteriol.* **183**:6017-6027.

Heeb, S., Blumer, C., and Haas, D. (2002) Regulatory RNA as mediator in

GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* **184:**1046-56.

Hengge-Aronis, R. (1996) Regulation of gene expression during entry into stationary phase, in *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, Vol. 1, 2nd edn. Neidhardt, F.C., Curtiss, R. III, Ingraham, J.L., Lin, E.C.C., Low, K. B., Magasanik, B., et al. (eds.). Washington, D.C.: American Society for Microbiology Press, pp. 1497-1512.

Huisman, G.W., and Kolter, R. (1994) Sensing starvation: a homoserine lactone- dependent signaling pathway in *Escherichia coli. Science.* **265:**537-9.

Jackson, D.W., Suzuki, K., Oakford, L., Simecka, J.W., Hart, M.E., and Romeo, T. (2002) Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of *Escherichia coli. J. Bacteriol.* **184:**290-301.

Lease, R., and Belfort, M. (2000) A trans-acting RNA as a control switch in *Escherichia coli*: DsrA modulates function by forming alternative structures. *Proc. Natl. Acad. Sci.* **97**:9919-9924.

Liu, M., and Romeo, T. (1997) The Global Regulator CsrA of *Escherichia coli* is a Specific mRNA-Binding Protein. *J. Bacteriol.* **179:**4639-4642.

Liu, M., Gui G., Wei, B., Preston, J.F., Oakford, L., Yuksel, U., Giedroc, D., and Romeo, T. (1997) The RNA Molecule CsrB Binds to the Global Regulatory Protein CsrA and Antagonizes Its Activity in *Escherichia coli.*

J. Biol. Chem. **272:**17502-17510.

Liu, M., Yang, H., and Romeo, T. (1995) The Product of the Pleiotropic *Escherichia coli* Gene *csrA* Modulates Glycogen Biosynthesis via Effects on mRNA Stability. *J. Bacteriol.* **177:**2663-2672.

Majdalani, N., Cunning, C., Sledjeski, D., Elliot, T., and Gottesman, S. (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc. Natl. Acad. Sci.* **95**:12462-12467.

Miller, J.H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Neidhardt, F., and Savageau, M.A. (1996) Regulation beyond the operon. In: F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, eds. *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C. 1310-1324.

Pernestig, A.K., Melefors, O., and Georgellis, D. (2001) Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli. J. Biol. Chem.* **276:**225-31.

Rivas, E, Klein, R.J., Jones, T.A., and Eddy, S.R. (2001) Computational identification of noncoding RNAs in E. coli by comparative genomics. *Curr Biol.* **11:**1369-73.

Romeo, T. (1998) Global regulation by the small RNA-binding protein CsrA

and the non-coding RNA molecule CsrB. *Mol. Microbiol.* **29:**1321-1330.

Romeo, T., and Preiss, J. (1989) Genetic Regulation of Glycogen Biosynthesis in *Escherichia coli*: In Vitro Effects of Cyclic AMP and Guanisine 5'-Diphosphate3'-Diphosphate and Analysis of In Vivo Transcripts. *J. Bacteriol.* **171:**2773-2782.

Romeo, T., Black, J., and Priess J. (1990) Genetic regulation of glycogen biosynthesis in *Escherichia coli*: in vivo effects of the catabolite repression and stringent response systems in *glg* gene expression. *Curr. Microbiol.* **21:**131-137.

Romeo, T., Gong, M., and Brun-Zinkernagel, A. (1993) Identification and Molecular Characterization of *csrA*, a Pleiotropic Gene from *Escherichia coli* That Affects Glycogen Biosynthesis, Gluconeogenesis, Cell Size, and Surface Properties. *J. Bacteriol.* **175:**4744-4755.

Romeo, T., Moore, J., and Smith, J. (1991) A simple method for cloning genes involved in glucan biosynthesis: isolation of structural and regulatory genes for glycogen synthesis in *Escherichia coli*. *Gene.* **108:**23-29.

Sabnis, N.A., Yang, H., and Romeo, T. (1995) Pleiotropic Regulation of Central Carbohydrate Metabolism in *Escherichia coli* via the gene *csrA*. *J. Biol. Chem.* **270:**29096-29104.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sledjeski, D., Whitman, C., and Zhang, A. (2001) Hfq Is Necessary for Regulation by the Untranslated RNA DsrA. *J. Bacteriol.* **183**:1997-2005.

Smith, P.K., Krohn, R.H., Hermanson, G.T., Mallie, A.K., Gartner, F.H., Provensano, J.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76-85.

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.-K., Melefors, O., Georgellis, D., Babitzke, P., and Romeo, T. Regulatory Circuitry of the CsrA/CsrB and BarA/Uvry systems of *Escherichia coli. J. Bacteriol.* In Press.

Tatarko, M., and Romeo, T. (2001) Disruption of a global regulatory gene to enhance central carbon flux into phenylalanine biosynthesis in *Escherichia coli. Curr Microbiol.* **43:**26-32.

Wassarman, K.M., and Storz, G. (2000) 6S RNA Regulates *E. coli* RNA Polymerase Activity. *Cell.* **101:** 613-623.

Wassarman, K.M., Repoila, F., Rosenow, C., Storz, G., and Gottesman, S. (2001) Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev.* **15:**1637-51.

Wassarman, K.M., Zhang, A., and Storz, G. (1999) Small RNA's in *Escherichia coli. Trends in Microbiol.* **7:**37-44.

Wei, B., Brun-Zinkernagel, A., Simecka, J.W., Prub, B.M., Babitzke, P., and Romeo, T. (2001) Positive regulation of motility and *flhDC* expression by

the RNA-binding protein CsrA of *Escherichia coli*. *Mol. Microbiol.* **40:**245-256.

Wei, B., Shin, S., Laporte, D., Wolfe, A., and Romeo, T. (2000) Global Regulatory Mutations in *csrA* and *rpoS* Cause Severe Central Carbon Stress in *Escherichia coli* in the Presence of Acetate. *J. Bacteriol.* **182:**1632-1640.

Yakhnin, A.V., Trimble, J.J., Chiaro, C.R., and Babitzke, P. (2000) Effects of mutations in the L-tryptophan binding pocket of the Trp RNA-binding attenuation protein of *Bacillus subtilus*. *J. Biol. Chem.* **275:**4519-4524.

Yang, H., Liu, M., and Romeo, T. (1996) Coordinate Regulation of Glycogen Catabolism and Biosynthesis in *Escherichia coli* via the CsrA Gene Product. *J. Bacteriol.* **178:**1012-1017.

Zhang, A., Wassarman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell.* **9:**11-22.

Zuker, M., Mathews, D.H. and Turner, D.H. (1999) Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide. In: Barciszewski J. Clark, B.F.C., eds. RNA Biochemistry and Biotechnology. Dordrecht, Netherlands: Kluwer Academic Publishers. pp. 11-43.