

Posttranscriptional repression of the *cel* gene of the ColE7 operon by the RNA-binding protein CsrA of *Escherichia coli*

Tsung-Yeh Yang¹, Yun-Min Sung¹, Guang-Sheng Lei¹, Tony Romeo² and Kin-Fu Chak^{1,*}

¹Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 11221, Taiwan and

²Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611-0700, USA

Received November 6, 2009; Revised and Accepted March 2, 2010

ABSTRACT

Carbon storage regulator (CsrA) is a eubacterial RNA-binding protein that acts as a global regulator of many functionally diverse chromosomal genes. Here, we reveal that CsrA represses expression from an extrachromosomal element of *Escherichia coli*, the lysis gene (*cel*) of the ColE7 operon (*cea-cei-cel*). This operon and colicin expression are activated upon SOS response. Disruption of *csrA* caused ~5-fold increase of the lysis protein. Gel mobility shift assays established that both the single-stranded loop of the T1 stem-loop distal to *cei*, and the putative CsrA binding site overlapping the Shine–Dalgarno sequence (SD) of the *cel* gene are important for CsrA binding. Substitution mutations at SD relieved CsrA-dependent repression of the *cel* gene *in vivo*. Steady-state levels and half-life of the *cel* mRNA were not affected by CsrA, implying that regulation is mediated at the translational level. Levels of CsrB and CsrC sRNAs, which bind to and antagonize CsrA, were drastically reduced upon induction of the SOS response, while the CsrA protein itself remained unaffected. Thus, CsrA is a *trans*-acting modulator that downregulates the expression of lysis protein, which may confer a survival advantage on colicinogenic *E. coli* under environment stress conditions.

INTRODUCTION

The Csr (carbon storage regulator) global regulatory system of *Escherichia coli* comprises four components. CsrA is a homodimeric RNA-binding protein containing two binding surfaces per dimer. Two non-coding small

RNAs (CsrB and CsrC) antagonize CsrA activity by binding to and sequestering this protein. CsrD is a GGDEF-EAL domain protein that targets CsrB and CsrC for degradation by RNase E (1–4). CsrA was identified as a repressor of glycogen metabolism, and mediates posttranscriptional repression or activation of bacterial gene expression (5–7). The mechanism by which CsrA negatively regulates *glgC*, encoding a glycogen biosynthetic enzyme, has been well documented (8). CsrA binds to the untranslated leader of the *glgCAP* message at two primary sites, one of which overlaps the *glgC* Shine–Dalgarno (SD) sequence and prevents ribosome binding. Binding at the second site, which is found in the loop of an RNA hairpin that lies upstream from the SD sequence, tethers CsrA to the mRNA and facilitates bridging of its remaining RNA binding surface to the SD (9). Translational repression apparently leads to accelerated turnover of *glgCAP* mRNA and decreased expression of the *glgCAP* genes (10). CsrA represses other genes and operons in a similar manner (11,12). CsrA activity is modulated by the action of the small untranslated RNAs, CsrB and CsrC, which contain multiple copies of an imperfectly repetitive sequence element (22 in CsrB and 13 in CsrC) that serves as a CsrA binding site. In this way, CsrB binds to and sequesters ~9 to 10 CsrA dimers, preventing their interaction with mRNA targets (2,3,6). In a variety of species, transcription of Csr RNAs requires a conserved two component signal transduction system, which is known as BarA–UvrY in *E. coli* (6). However, the molecular mechanisms by which environmental stimuli control the Csr global regulatory system remain elusive.

CsrA orthologs are found in many bacteria (13–15) and regulate numerous cellular behaviors (16), including carbon metabolism (8), biofilm formation (17,18), motility (19–22), quorum sensing (23), epithelial cell invasion (24–26) and virulence factor production (19,27–29).

*To whom correspondence should be addressed. Tel: +886 2 2826 7129; Fax: +886 2 2826 4843; Email: kfchak@ym.edu.tw

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Despite its broad regulatory role in bacterial physiology (16,30), only a few direct mRNA targets of CsrA from *E. coli* have been identified (8,11,12,31,32).

Colicins are plasmid-encoded bacteriocins produced by *E. coli*, which are secreted into the environment and exhibit toxicity against *E. coli* and closely related *Enterobacteriaceae* (33). They are produced to gain advantage over the competing bacteria for survival under unfavorable conditions, such as nutrient deficiency and DNA damage (34). The bactericidal activity of Colicin E7 (ColE7) is a nonspecific endonuclease (35), which is encoded by the colicin structural gene *cea* of the ColE7 operon. The ColE7 operon also possesses the immunity (*cei*) and lysis (*cel*) genes, which are responsible for neutralization of colicin toxicity (36), and release of colicin into the extracellular space (37), respectively. Transcription of colicin E7 is regulated by an SOS responsive promoter, which can be induced by ultraviolet (UV) irradiation and DNA-damaging agents such as mitomycin C (38).

Lysis proteins, also referred to bacteriocin release proteins (BRP) or kil proteins, are small lipoproteins of 47 amino acids and synthesized as precursors containing a signal peptide of 19 amino acids at the N-terminus (37). They are synthesized as precursors in the cytoplasm, targeted to the inner membrane by their N-terminal signal sequences, and translocated via the Sec-translocon to the outer leaflet of the inner membrane (39). The precursors are lipid-modified and processed to the mature form on the periplasmic side of the inner membrane. The mature forms of lysis proteins are detached from the inner membrane by the action of an ATP-binding cassette (ABC) transporter LolCDE and a periplasmic carrier chaperone, LolA. Lysis proteins are sequentially transferred from LolA to receptor LolB, which incorporates lipoproteins into the inner leaflet of the outer membrane (40–42). The mature form of lysis protein activates phospholipase A in the outer membrane and consequently increases cell membrane permeability and colicin release (43). Lysis protein is essential for the release of the Col-Im complex from the cell (44,45). Despite the importance of lysis protein in colicin release, overexpression of *cel* gene is lethal to the producing *E. coli* cell (46–48), and should be subject to rigid regulatory control. Thus far, little is known about the fine-tuning of this expression in response to environmental stimuli.

We previously reported that the T1 transcriptional terminator is situated in the *cei*–*cel* intercistronic region (35). Since the *cel* gene is transcribed at lower level than the other two proximal *cea* and *cei* genes, it was proposed that *cel* expression is regulated by the T1 transcriptional terminator (T1 stem–loop structure), resulting in alleviation of the expression below the lethal dose of the *cel* gene (42,49). Nevertheless, unambiguous involvement of regulatory factors in the quantitative control of lysis expression has not yet been established.

Here, we examined the mechanism of CsrA-mediated repression of *cel* expression. Our findings established that CsrA binds directly and specifically to the upstream noncoding segment of the *cel* transcript, which requires sequences at the T1 stem–loop and SD element. CsrA

repressed lysis protein expression by reducing its translational efficiency without affecting the rate of *cel* mRNA decay. Substitution of the first two nucleotides (AC to TT) of the putative CsrA binding site (ACAAGGAGT) overlapping the *cel* SD substantially relieved the CsrA-mediated repression of *cel* gene expression *in vivo*. Intriguingly, the CsrB and CsrC RNA levels were notably decreased under the stress of SOS response, which should increase the intracellular biological activity of CsrA. To our knowledge, this study provides the first evidence for a regulatory role of CsrA in the expression of an extra-chromosomal gene and identifies CsrA as a *trans*-acting factor that specifically modulates *cel* gene expression. A model depicting the way in which the Csr system coordinates lysis of a colicinogenic cell under environmental stress is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5 α served as host for standard cloning procedures. Overnight culture of *E. coli* cells were inoculated at a dilution of 1:100 into 50 ml of Luria-Bertani (LB) medium and grown at 37°C with agitation. Kornberg medium (1.1% K₂HPO₄, 0.85% KH₂PO₄, 0.6% yeast extract containing 0.5% glucose for liquid medium or 1% glucose for agar plates) was used for assessment of intracellular glycogen in colonies by iodine staining (3). Bacteria harboring resistance determinants were grown in the presence of the appropriate antibiotics at the following concentrations: ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml; and chloramphenicol and 50 μ g/ml. For induction of the cloned genes or ColE7 operon, IPTG or mitomycin C (MMC) was added to final concentrations of 1 mM and 0.5 μ g/ml, respectively.

Plasmid construction

The plasmids used in this study are described in Table 1. Table 2 lists the oligonucleotide primers used. Plasmid pColE7-K317 (pK317) referred in this study, which was different from the wild-type plasmid, was engineered by inserting *bla* gene (β -lactamase producing, ampicillin-resistant) into the BglII-NdeI sites on wild-type ColE7 plasmid. For the complementation experiment in TR1-5 mutant, the *csrA* gene was amplified from *E. coli* MG1655 by PCR using the primers *csrA*ComFor and *csrA*ComRev. The resulting gene fragments were inserted into the BamHI–SphI sites of the plasmid pACYC184, generating plasmid pACYC184-CsrA. For CsrA protein preparation, the DNA fragment encoding the *csrA* gene was amplified from *E. coli* MG1655 using primers *csrA*29For and *csrA*29Rev. The PCR product digested with NdeI and XhoI was ligated into the corresponding cloning sites of plasmid pET29a to generate plasmid pYM24. This plasmid was designed to facilitate the purification of CsrA containing six additional His residues at the C-terminus. Plasmid pYM9820 contains the 134-nt fragment consisting of *glgC* leader and the

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
<i>Escherichia coli</i> strains		
MG1655	F ⁻ λ ⁻	(5)
TR1-5MG1655	MG1655 <i>csrA::kan</i>	(5)
BL21(DE3)	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dem</i> (DE3)	Novagen
DH5α	F ⁻ <i>ψ80dlacZΔM15 Δ(lacZYA-argF) U169 hsdR17 recA1 thi-1 relA1</i>	Laboratory stock
KSB837	CF7789 <i>Δ(λatt-lom)::blaΦ(csrB-lacZ)I</i> (Hyb) Amp ^r	(50)
GS1114	CF7789 <i>Δ(λatt-lom)::blaΦ(csrC-lacZ)I</i> (Hyb) Amp ^r	(3)
Plasmids		
pColE7-K317	pK317 derivative with <i>bla</i> gene inserted in BglII–NdeI sites; Amp ^r	This study
pACYC184	A general cloning vector compatible with ColE1 plasmid; P15A replicon; Tet ^r , Cm ^r	New England Biolabs
pACYC184-CsrA	pACYC184 derivative containing <i>csrA</i> gene (BamHI–SphI sites); Cm ^r	This study
pET29a	IPTG-inducible expression vector; T7 promoter; Kan ^r	Novagen
pYM24	pET29a derivative containing <i>csrA</i> gene on an NdeI–XhoI fragment; Kan ^r	This study
pSPT18	Cloning vector for <i>in vitro</i> transcription with SP6/T7 promoter; Amp ^r	Roche
pYM9820	pSPT18 derivative carrying HindIII/EcoRI fragment of <i>glgC</i> behind SP6 promoter; Amp ^r	This study
pYM9821	pSPT18 derivative carrying HindIII/EcoRI fragment of <i>trpL</i> behind SP6 promoter; Amp ^r	This study
pYM9865	pSPT18 derivative carrying HindIII/EcoRI fragment of <i>cel</i> behind SP6 promoter; Amp ^r	This study
pYM9866	pYM9865 derivative with a GGA to CCC alteration in the loop region of T1 hairpin; Amp ^r	This study
pYM9867	pYM9865 derivative with a GGA to CCC alteration in the <i>cel</i> SD region; Amp ^r	This study
pYM9868	pYM9866 derivative with a GGA to CCC alteration in the <i>cel</i> SD region; Amp ^r	This study
pTY003	pColE7-K317 derivative with a AC to TT alteration upstream of the <i>cel</i> SD region; Amp ^r	This study

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')
Construct generation	
blaFor	TGATAATAATAGATCTTTAGACGTCAGG
blaRev	GGTGCTACACATATGTTGAAGTGGTG
csrAComFor	ATTGCAATAATATAAGGATCCGGCAATGCC
csrAComRev	GTCTCACGCATGCTTAGTGATGGTGATGGTAACTGGACTGCTGGGATTTT
csrA29For	CAAGGAGTTCATATGCTGATTCTG
csrA29Rev	GAGACGCGGA <u>ACTCGAGG</u> TAACTG
RNA gel mobility shift assays	
glgCFor	CCCCAAAGCTTGGAGAGGATAACC
glgCRev	TTTCAAGAATTCCTGGCGCGCCAACAT
trpLFor	TACGATAAGCTTAGCTTAGAAATACAC
trpLRev	AAAGACGAATTCCTGGGTAGAAATAACATAA
celFor	AAAGAAAAGCTTGCTGCTAACGGTAA
celRev	AGCAA <u>GAATTCT</u> GGCTGCAAGAA
Site-directed mutagenesis	
BS1mutFor	GTTTAACCCTGAATGACTGGCATTCTTTCACAACAAGGAGTCGTTATG
BS1mutRev	CATAACGACTCCTTGTGTGAAAGAATGCCAGTCATTAGGGTTAAAC
BS2mutFor	GACTGGCATTCTTTCACAACAACCCGTCGTTATG
BS2mutRev	CCTGTTATTTTTTTCATAACGACGGGTTGTTGTGAAAGAATGCCAGTC
CelmutFor	GGCATTCTTTCACATTAAGGAGTCGTTATGAAAAAATAACAG
CelmutRev	CTGTTATTTTTTTCATAACGACTCCTTAATGTGAAAGAATGCC
<i>In vitro</i> transcription	
pSPT18-SP6	CTGACCATTTCGGGTG
pSPT18Rev	CTCACTATAGGGAGACCG
Real-time PCR	
LysE7RTFor	TCGTTATGAAAAAATAACAGGGA
LysE7RTRev	ACGACGGTGATACTGT
csrBRTFor	ACACTTCTGCAGGACACACCAG
csrBRTRev	TGACCCACCGAATCATCCTGAC
csrCRTFor	ATAGAGCGAGGACGCTAACAG
csrCRTRev	ACACACCCTGTTTTCTTTAGC
rrnHRTFor	AGTCGAACGGTAACAGGAAGA
rrnHRTRev	GCAATATCCCCACTGCTG
lacZRTFor	CACCCGAGTGTGATCATCTG
lacZRTRev	GATACAGCGCGTCGTGATTA

The underlined letters indicate the restriction sites used in the relevant primers.

2-nt mutagenesis in the *cel* leader just upstream of the SD sequence were introduced into pColE7-K317 using the primer pairs CelmutFor/CelmutRev and the QuikChange II protocol (Stratagene), resulting in plasmid pTY003.

Western blotting analysis

Escherichia coli cells were grown under the conditions as previously described until they reached an OD₆₀₀ of 0.6; then MMC was added to a final concentration of 0.5 µg/ml to induce the ColE7 operon. At 0.5, 1 and 1.5 h after MMC induction, volumes equivalent to 0.36 OD₆₀₀ units were harvested by centrifugation at 4°C and 13 500 r.p.m. for 8 min. The cell pellets were resuspended in sample buffer (62.5 mM Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 100°C for 10 min. Lysates equivalent to 0.045 OD₆₀₀ units were subjected to a 15% Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. Rabbit polyclonal antibody against the LysE7 protein was produced in this study. The antigen used to raise the anti-LysE7 serum was a peptide corresponding to amino acid residues 20–47 of the LysE7 protein (Figure 1A). The membranes were probed with a 1:4000 dilution of rabbit anti-LysE7 or a 1:5000 dilution of rabbit anti-His (SC-803; Santa Cruz Biotechnology) primary antibody. For detection of LexA and RecA proteins, blots were probed with a 1:1000 dilution of goat polyclonal LexA-specific antibody (SC-1725; Santa Cruz Biotechnology) and with goat polyclonal RecA-specific antibody at a dilution of 1:2000 (SC-30382; Santa Cruz Biotechnology). For detection of CsrA proteins, blots were probed with a 1:5000 dilution of rabbit antiserum raised against purified CsrA-CsrB complex (50). Immunodetection of GroEL was performed with a 1:80 000 dilution of rabbit anti-GroEL HRP-conjugated antibody (Sigma). The band densities were quantified with ImageQuant software.

Purification of histidine-tagged CsrA (CsrA-His₆)

Culture of *E. coli* BL21 (DE3) harboring pYM24 was the same as described above. The cell pellet was suspended in lysis buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl and 5 mM imidazole (5 ml of buffer per gram of cells). Cells were lysed by French press, followed by centrifugation at 24 000 r.p.m. for 20 min at 4°C. The resulting supernatant was collected and mixed with pre-equilibrated 1.5 ml of Ni-IDA resin (Novagen) for 2.5 h at 4°C. The mixture was then loaded into a column and washed with 50 ml of buffer A (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 100 mM imidazole). CsrA-His₆ was eluted with 9 ml of buffer B (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 1 M imidazole). Column fractions were analyzed by 15% SDS-PAGE and Coomassie Blue staining. The CsrA-His₆ containing fractions were combined and dialyzed against 50 mM NaH₂PO₄ (pH 7.0) and 10% glycerol. The dialysate was loaded onto the SP FF column (GE Healthcare) that was

pre-equilibrated with 50 mM NaH₂PO₄ (pH 7.0) and CsrA-His₆ was eluted from the column between 350 and 700 mM NaCl. The protein-containing fractions were combined and dialyzed against 10 mM Tris-HCl, pH 7.0, 100 mM KCl, 10 mM MgCl₂ and 25% glycerol. The CsrA-His₆ concentration was estimated using Bio-Rad protein assay and BSA (PIERCE) as the standard.

RNA gel mobility shift assay

The *glgC*, *trpL*, *lys* and mutant *lys* runoff transcripts were synthesized from PCR products using SP6 RNA polymerase. Plasmids pYM9820, pYM9821 and pYM9865 to pYM9868 were used as templates for PCR with primers pSPT18-SP6 and pSPT18Rev. The PCR products were gel-purified and used as templates for *in vitro* transcription using Ambion MEGAscript kit. RNA was dephosphorylated with calf intestinal alkaline phosphatase (NEB) and subsequently 5'-end-labeled using [γ -³²P]-ATP and T4 polynucleotide kinase (Promega) following the manufacturer's instructions. The labeled RNAs and unlabelled competitor RNAs were purified using MEGAclean Kit (Ambion), heated to 85°C and then slowly cooled to room temperature. Binding reaction mixtures (10 µl) contained 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 32.5 ng of yeast RNA, 20 mM dithiothreitol, 10% glycerol, 4 U of RNase inhibitor (Ambion), 0.1 mg ml⁻¹ xylene cyanol, 0.1 mg ml⁻¹ bromophenol blue, 5'-end-labeled RNA probes and various concentrations of purified CsrA-His₆ (see figure legends for details). CsrA-His₆ was added last to the binding reaction containing various unlabelled RNA competitors (see figure legends for details) in competition assay. Reaction mixtures were incubated at 37°C for 30 min to allow complex formation. Samples were then fractionated on 5% native polyacrylamide gel and radioactive bands were visualized with a phosphorimager (Molecular Dynamics) after drying the gels.

RNA isolation and cDNA synthesis

For total RNA preparation, cells from 500 µl of culture were harvested and frozen in liquid nitrogen. Total RNA was isolated using the illustra RNAspin Mini Isolation Kit (GE Healthcare) and treated with on-column DNase I digestion according to the manufacturer's instructions. The eluted RNA samples were stored at -80°C. Equal amount of total RNA (0.1 µg) was used for cDNA synthesis using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The reverse transcription mix (10 µl) containing 0.1 µg of total RNA, 50 ng of the random hexamer, 1 µl of dNTP mix (10 mM), and DEPC-treated water was incubated at 65°C for 5 min, and then placed on ice for 5 min. To continue the RT reaction, the cDNA synthesis mix (Invitrogen) was added and incubated at 25°C for 10 min, followed by 50°C for 50 min and a 5-min inactivation step at 85°C. To remove RNA from cDNA, 1 µl of *E. coli* RNaseH (2 U/µl) was added and incubated at 37°C for 30 min. To ensure that the RNA preparations were free from DNA contamination, RT reaction was performed with or without reverse transcriptase.

Real-time RT-PCR analysis of steady-state levels of *cel* mRNA and measurement of RNA half-life

Preparation of cell culture, total RNA purification and reverse transcription were the same as described above. The primer pairs *csrBRTFor/csrBRTRev*, *csrCRTFor/csrCRTRev*, *LysE7RTFor/LysE7RTRev* and *lacZRTFor/lacZRTRev* were used for PCR amplification of the *csrB*, *csrC*, *cel* and *lacZ* RT products, respectively. The detailed procedure and precaution of Real-time RT-PCR were carried out using LightCycler Faststart DNA Master^{plus} SYBR Green I kit (Roche) and performed by the LightCycler Carousel-Based System (Roche) as described by the instruction of the manufacturer (Roche). A negative control, replacing cDNA with PCR-grade water, was also included in each PCR run. For normalization of *lacZ*, *cel*, *csrB* and *csrC* RNA levels, real-time RT-PCR were conducted with each sample for 16S rRNA quantification using the primer pairs *rrnHRTFor* and *rrnHRTRev*. The data were analyzed using the $2^{-\Delta\Delta CT}$ method to determine the relative changes in gene expression from real-time PCR experiments (51). The fold change of the target gene (*cel* mRNA), normalized to 16S rRNA and relative to the calibrator (wild-type strain MG1655) was calculated for each time point using $2^{-\Delta\Delta CT}$. The mean and SD values of three independent experiments were determined at each time point.

For *cel* mRNA half-life studies, *csrA* wild-type (MG1655) and mutant TR1-5 strains both harboring pColE7-K317 were grown as described above. After MMC induction of expression of the ColE7 operon for 30 min, bacterial cultures were treated with rifampicin at a final concentration of 250 $\mu\text{g ml}^{-1}$ to inhibit transcription. Cells were harvested at 0, 5, 10, 15, 20 and 30 min following rifampicin addition. Total RNA was purified, and the amount of *cel* mRNA in each sample was determined using real-time RT-PCR as described above. The percentage of RNA remaining at each time-course was determined by calculating the difference in cycle threshold (ΔC_T) compared to the 0-min time point.

Glycogen staining and β -galactosidase assay

Glycogen accumulation was examined by staining colonies with iodine vapor (52). β -Galactosidase activity was measured using the Miller protocol (53).

Sequence alignment

Selected *cei-cel* sequences from several E group Col plasmids were aligned using the program DNAMAN. We aligned the corresponding regions comprising the T1 stem-loop structure and the *cel* SD element (−97 to +43 sequence, +1 being the A nucleotide of the initiation codon of *cel* gene) between several types of ColE plasmids. The following GenBank accession numbers were obtained from the NCBI database for the sequences of plasmid Colicin E2 operon (M29885), plasmid Colicin E3 operon (J01574), plasmid Colicin E6 operon (X15856), *cei* and *cel* gene of plasmid Colicin E7 (X63620), plasmid

Colicin E8 operon (X06119) and plasmid Colicin E9 operon (FJ573246).

RESULTS

Two putative CsrA binding sites within the leader sequence of the *cel* gene

Previous study has defined a conserved high affinity binding sequence for CsrA, RUACARGGAUGU (R = A or G), within which the GGA motif is 100% conserved and is located within the loop of variable short stems (54). In the present study, we analyzed the 5' leader region of *cel* mRNA and found two potential binding sites in this region, one in the loop region of the T1 stem-loop (binding site 1, BS1, Figure 1B), and the other one overlapping the SD sequence of *cel* (binding site 2, BS2, Figure 1B). The sequence alignment with the previously known CsrA binding sites is shown in Figure 1C. The high sequence similarity of these two sites with known CsrA binding sequences and their location at the translation initiation region suggested that they might participate in regulating the expression of the *cel* gene.

CsrA regulates *cel* gene expression

To investigate the effect of CsrA on the expression of *cel* gene, growth curves for both *csrA* wild-type strains MG1655 and their isogenic *csrA* mutant TR1-5MG1655 harboring pColE7-K317 were performed after MMC induction (Figure 2A). We observed that the wild-type strains began to lyse at 1.5 h after MMC induction, while the *csrA* mutant began to lyse 0.5 h earlier than the wild-type strains. The decline in culture turbidity from TR1-5MG1655(pColE7-K317) was more severe compared to that of the MG1655(pColE7-K317) cells. Furthermore, the distinct degree of quasi-lysis did not result from a growth defect of the *csrA* mutant, as there was no significant difference between the growth rates of the wild type and the mutant strains carrying no ColE7 plasmid after MMC induction (data not shown). To further validate the expression of *cel* gene in both bacterial strains, Western blotting analysis with LysE7-specific anti-serum was conducted to quantify the intracellular level of lysis protein in the wild-type and the *csrA* mutant strains (Figure 2B). As expected from the lysis patterns, the *csrA* mutant strain expressed substantially elevated levels of lysis protein as compared to the wild-type strain. Taken together, these results suggested that CsrA may be a negative regulator of the expression of Lys protein.

To further examine the effect of CsrA on the expression of Lys protein, complementation of the *csrA* mutation was accomplished by introducing the low-copy-number vector pACYC184 containing the *csrA* gene into the TR1-5MG1655(pColE7-K317) strain. Interestingly, quasi-lysis did not occur in the complemented *csrA* mutant strain (Figure 2C). Moreover, we observed a continuous rise in culture turbidity of the complemented mutant even at 2 h after MMC induction (Figure 2C). Apparently, ectopic expression of *csrA* from a multicopy

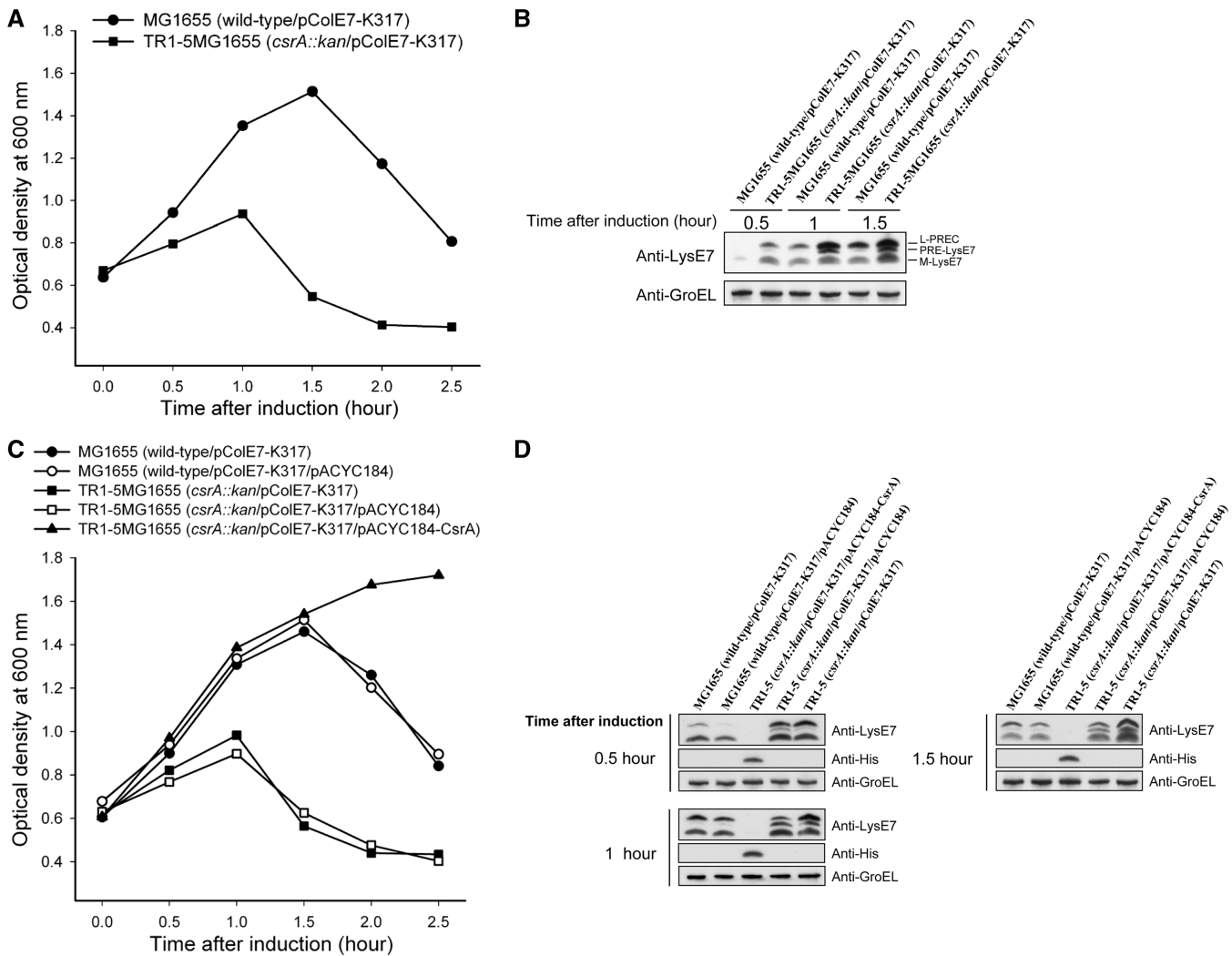


Figure 2. Effects of *csrA* disruption (A and B) and complementation (C and D) on expression of LysE7 protein. (A) Growth curves of MG1655 and *csrA* mutant strain TR1-5MG1655. Cultures of strains harboring pColE7-K317 were grown in LB medium to an OD₆₀₀ of 0.6, and induced with MMC (0.5 μg/ml) at zero time. The OD₆₀₀ of the cultures was measured at various times after induction. Sample symbols: closed circles, MG1655 (wild-type/pColE7-K317); closed squares, TR1-5MG1655 (*csrA::kan*/pColE7-K317). (B) Western blotting analysis of *csrA* effect on LysE7. Strains MG1655(pColE7-K317) and TR1-5MG1655(pColE7-K317) were harvested at the indicated times after MMC (0.5 μg/ml) induction and assayed for intracellular level of LysE7 by Western blotting. Western detection of GroEL served as a control for sample loading. L-PREC, lipid-modified precursor of LysE7; PRE-LysE7, unmodified precursor of LysE7; M-LysE7, mature form of LysE7. (C) Growth curve of complemented *csrA* mutant strain. Cultures of strains harboring pColE7-K317 were grown in LB medium to an OD₆₀₀ of 0.6, and induced with MMC (0.5 μg/ml) at zero time. The OD₆₀₀ of the cultures was measured at various times after induction. Strains MG1655 and TR1-5MG1655 containing pACYC184 were the empty vector controls. Sample symbols: closed circles, MG1655 (wild-type/pColE7-K317); open circles, MG1655 (wild-type/pColE7-K317/pACYC184); closed squares, TR1-5MG1655 (*csrA::kan*/pColE7-K317); open squares, TR1-5MG1655 (*csrA::kan*/pColE7-K317/pACYC184); closed triangles, TR1-5MG1655 (*csrA::kan*/pColE7-K317/pACYC184-CsrA). (D) *In trans* complementation with pACYC184-CsrA resulted in a significant reduction of LysE7 expression. Cells were harvested at the indicated times after MMC (0.5 μg/ml) induction and assayed for intracellular level of LysE7 by Western blotting using anti-LysE7 and recombinant CsrA-His₆ was detected with anti-His antibody. GroEL was employed as sample loading control.

pACYC184-CsrA plasmid caused severe repression of the lysis protein synthesis, making the complemented mutant relatively unresponsive to MMC induction. Western blotting analysis further revealed that the lysis protein was essentially undetectable in the complemented mutant as compared with the *csrA* wild-type and mutant strains containing only the vector pACYC184 (Figure 2D).

CsrA binds directly and specifically to *cel* RNA

The *cel* transcript contains a 61-nt untranslated RNA segment within the *cei-cel* intercistronic region.

To examine the interaction of CsrA with *cel* RNA, gel mobility shift assays were performed with a transcript containing the T1 stem-loop and the SD element of *cel* (nucleotides -97 to +43 relative to the initiation codon of *cel*, Figure 1A). As there were no significant differences in the RNA-binding properties of the native CsrA protein and a C-terminally His-tagged CsrA (CsrA-His₆) (31), the CsrA-His₆ protein was used for these studies. Two distinct shifted complexes were observed. Essentially, the entire amount of starting RNA used was shifted to the first complex when the concentration of CsrA reached

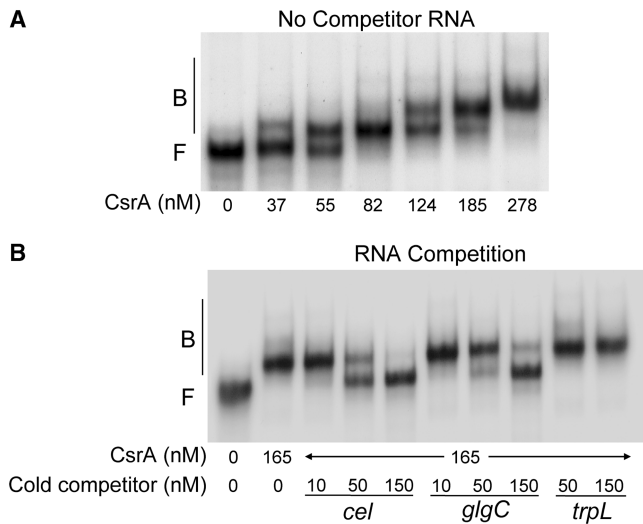


Figure 3. Gel mobility shift analysis of CsrA–*cel* RNA interaction. Gel shift assays were performed in the absence or presence of unlabelled competitor RNA. The positions of bound (B) and free (F) RNA are shown at the left of each panel. (A) Detection of CsrA–*cel* RNA interaction. 5'-end-labeled *cel* RNA (0.5 nM) was incubated with the various concentrations of CsrA indicated at the bottom of each lane. (B) Competition assay for CsrA–*cel* RNA complex formation to verify the binding specificity. Competition reactions used specific (*cel*, *glgC*) and non-specific (*trp* leader from *B. subtilis*) unlabelled competitor RNAs at the concentrations shown.

82 nM (Figure 3A). Moreover, further binding of CsrA to *cel* RNA was also observed as the second distinct shifted complexes at 124–185 nM. Interestingly, we observed that the increase of CsrA concentration to 278 nM resulted in the disappearance of the first complex species at 82 nM and the appearance of the new shifted species (Figure 3A). These gel mobility shift assays raised the possibility that more than one CsrA binding site might be located on the *cel* transcript. In combination with the results of Western blotting, they also suggested that *cel* gene expression is directly regulated by CsrA binding.

To examine the specificity of CsrA–*cel* RNA interaction, competition experiments were conducted with the specific (*glgC* RNA and *cel* RNA) and non-specific (*B. subtilis trp* leader RNA) unlabelled RNA competitors (Figure 3B). Both *cel* and *glgC* transcripts served as effective competitors, while the *B. subtilis trp* leader RNA did not compete with the CsrA–*cel* complex formation. Taken together, these results established that CsrA binds specifically to the *cel* transcript, which may contain two putative CsrA binding sequences that are located within the T1 stem-loop and *cel* SD sequence.

T1 stem-loop and the *cel* SD sequence are required for CsrA binding

We suspected that the CsrA binding site that overlaps the SD sequence of *cel* should be particularly important for regulation of translation. To define the regions required for CsrA binding, transcripts were constructed that contained base substitutions in the T1 stem-loop (BS1), *cel* SD sequence (BS2) and at both sites (BS1–BS2). The importance of a conserved GGA motif for CsrA binding has

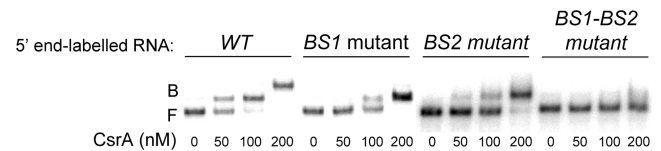


Figure 4. Effect of site-directed mutations of putative CsrA binding sites on CsrA–*cel* RNA interactions. Gel mobility shift assays were performed using wild-type (WT) or mutant (BS1, BS2 and BS1–BS2) 5'-end-labeled *cel* RNA (0.5 nM). The concentration of CsrA was indicated at the bottom of each lane. The positions of bound (B) and free (F) RNA are shown. BS1, *cel* transcript containing the mutations in the loop region of T1 stem-loop; BS2, *cel* transcript containing the mutations in the Shine–Dalgarno sequence; BS1–BS2, *cel* transcript containing the mutations in both GGA motifs.

been previously reported (9,54,55), hence the GGA motifs in BS1 and BS2 were replaced with CCC. Noticeably, computer modeling using MFOLD (56) did not predict any significant unintended structural rearrangements in any of these mutant transcripts. Gel shift patterns of the mutant transcripts were clearly distinct from that of wild-type RNA, and revealed that replacement of the GGA motif with CCC led to an observable reduction of CsrA affinity in every case (Figure 4). CsrA binding to wild-type transcripts was detected as two distinct shifted complexes between 50 and 200 nM CsrA. The complete shift of first complex was observed at 100 nM CsrA, and essentially all of the wild-type RNA was present in the second shifted complex when the concentration of CsrA reached 200 nM. In contrast, under the same conditions, 50 nM CsrA failed to shift the transcripts containing mutations in either BS1 or BS2. Moreover, complete shifting of the first complex was not observed until the CsrA concentration reached 200 nM in BS1 mutant RNA. The gel shift pattern in BS2 mutant was similar to that of the BS1 mutant transcript, resulting in the formation of a single complex. These results indicated that BS1 or the BS2 mutations caused a similar reduction in CsrA affinity. Apparently, substitutions in either T1 stem-loop or the *cel* SD sequence alone left the other site intact and available for interaction with CsrA. Combining these two sets of substitutions in the T1 stem-loop and *cel* SD sequence (BS1–BS2) resulted in a complete loss of CsrA binding (Figure 4), implying that both the loop region of the T1 hairpin and the *cel* SD sequence serve as binding sites for CsrA.

Effects of CsrA on steady-state levels and stability of the *cel* mRNA

To determine whether the elevated LysE7 protein levels in the *csrA* mutant are due to an increase in *cel* mRNA levels, as previously reported for other proteins (6,8,12), we examined the steady-state level of *cel* mRNA in the wild-type and *csrA* mutant strains at 0.5 and 1 h after MMC induction using real-time RT-PCR. Our results clearly indicated that the expression levels of *cel* transcript were not substantially different between the wild-type and mutant strains, as the relative abundance of *cel* mRNA in the TR1-5 *csrA* mutant strain was 1.6-fold and 0.8-fold with respect to the wild-type strain at 0.5 and 1 h after

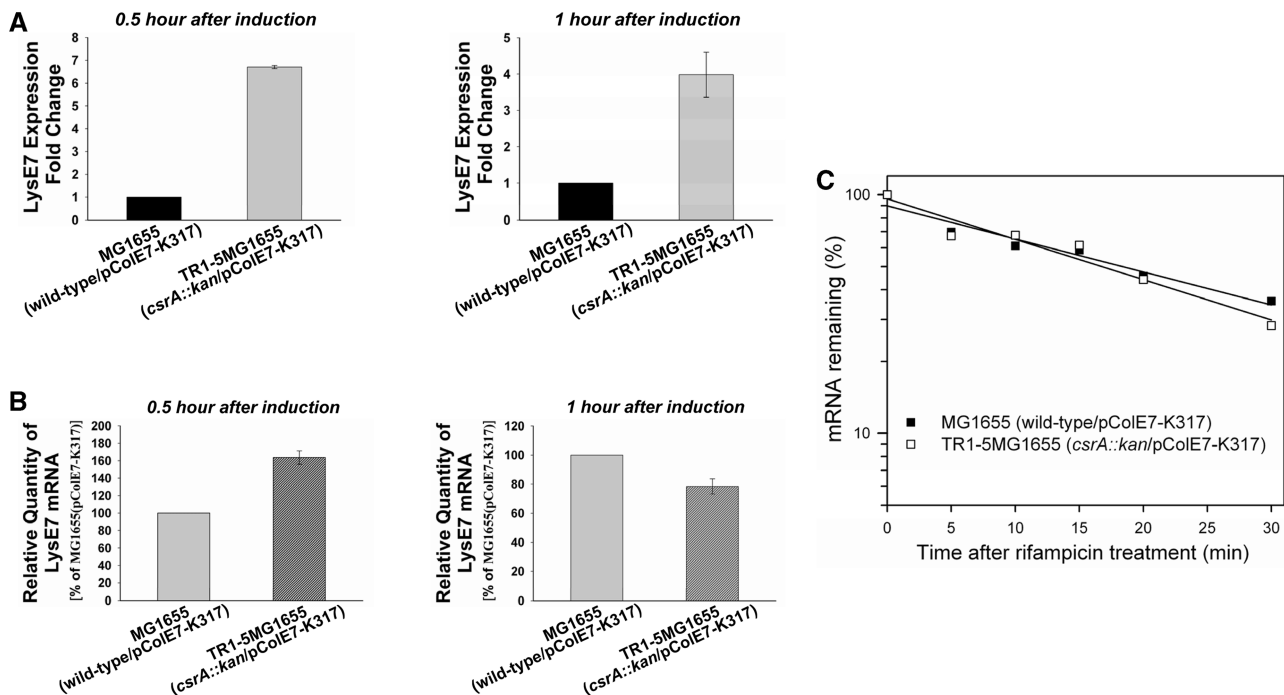


Figure 5. Disruption of *csrA* greatly affects protein levels but not mRNA levels of *cel*. (A) Graph represents densitometric quantification after Western blot analysis (Figure 2B). The amount of LysE7 protein in each sample was quantified by densitometry, normalized against the level of GroEL, and used to calculate expression of LysE7 in TR1-5MG1655 (*csrA::kan*) relative to isogenic wild-type MG1655 cells. Error bars represent mean \pm SD. The experiment was repeated three times with reproducible results. (B) Cells were harvested at 0.5 and 1 h after MMC (0.5 μ g/ml) induction. The steady-state level of *cel* RNA was quantified by real-time RT-PCR and expressed as % of that of MG1655 cells. Relative amounts of *cel* mRNA in each sample normalized to *rrnH* mRNA are presented as the mean \pm SD. All results were confirmed in three independent experiments. (C) Analysis of *cel* mRNA stability in *csrA* wild-type and mutant strains. Parallel cultures of MG1655 and TR1-5MG1655, both containing pColE7-K317, were grown at 37°C in LB medium to an OD₆₀₀ of 0.6, at which time MMC (0.5 μ g/ml) was added to induce the expression of ColE7 operon for 30 min followed by rifampicin (250 μ g/ml) treatment. At time intervals after transcription inhibition with rifampicin, total cellular RNA was isolated from the cultures. The relative levels of *cel* mRNA remaining at the indicated time points were quantified using real-time RT-PCR and normalized to 16S rRNA levels (see 'Materials and Methods' section). The amount of *cel* mRNA in each strain at 0 min with respect to rifampicin addition was set to 100%. The mRNA (%) remaining thereafter was plotted versus time in semi-logarithmic diagrams.

MMC induction, respectively (Figure 5B). Thus, the steady-state level of *cel* mRNA was not concomitant with the \sim 5-fold increase in the LysE7 protein observed in *csrA* mutant strain (Figure 5A), suggesting that CsrA-dependent repression of *cel* occurs mainly or entirely at the level of translation. Calculation of the translational efficiency in MG1655 and TR1-5 strains by dividing the level of LysE7 protein by the *cel* mRNA level for each strain revealed that inactivation of *csrA* caused a \sim 4- to 5-fold increase in *cel* translational efficiency (Table 3).

Chemical half-life determinations revealed that the *cel* mRNA decayed slowly in both wild-type and *csrA* mutant cells, with indistinguishable half-life of 18 ± 1.5 and 19.1 ± 1.9 min, respectively (Figure 5C). Thus, we conclude that CsrA had no significant effect on the stability of the *cel* transcript. Thus, all of our results on the *cel* mRNA and protein imply that CsrA negatively regulates *cel* at the level of translation.

Mutations in the *cel* leader region reduce CsrA-dependent repression

We presumed that the CsrA binding site overlapping the SD element of *cel* (Figure 1B) should be particularly important for translational repression, as binding of CsrA

at this site should interfere with ribosome binding. Thus, substitution mutations were introduced at the CsrA binding site overlapping the SD element of *cel* in pColE7-K317. Noticeably, the ACA and GGA motifs are the most highly conserved nucleotides of the CsrA binding sequence RUACARGGAUG (6,54), and the critical GGA motif in this binding site is a component of the *cel* SD sequence (Figure 1B). In order to avoid any complication, a two-base substitution was only introduced to replace AC in ACAAGGAGT with TT, just upstream from the *cel* SD element (pTY003, Figure 6A), leaving the GGA residues of the SD sequence intact. Then, LysE7 production in *csrA* wild-type cells containing either the parental pColE7-K317 or the pTY003 with MMC treatment was determined by Western blotting. The expression of LysE7 after MMC treatment was significantly increased by the AC \rightarrow TT substitution in MG1655(pTY003) cells (Figure 6B), suggestive of the relief from CsrA-mediated repression in this construct. To determine whether the AC \rightarrow TT alteration itself affects translation efficiency of *cel*, the expression of LysE7 was monitored in the *csrA* mutant strain containing either the pTY003 or the pColE7-K317. The Western blotting analysis revealed that the LysE7 expression in

Table 3. Calculation of the translational efficiency of LysE7

Strain (time after induction)	Relative fold of LysE7 protein ^a	Relative <i>cel</i> mRNA level ^b	Translation efficiency ^c
MG1655 (0.5 h)	1.0	1.0	1.0
TR1-5MG1655 (0.5 h)	6.7 ± 0.07	1.6 ± 0.08	4.1 ± 0.18
MG1655 (1 h)	1.0	1.0	1.0
TR1-5MG1655 (1 h)	4.0 ± 0.62	0.8 ± 0.05	5.0 ± 0.87

Protein and RNA data originate from at least three independent experiments.

The values of MG1655 were given an arbitrary value of 1.0.

^aThe relative LysE7 protein level for MG1655 and TR1-5 was determined by Western blotting analysis and densitometric quantification.

^bThe level of *cel* mRNA was determined by real-time RT-PCR for each strain.

^cTranslation efficiency was calculated by dividing the relative LysE7 protein level by the level of *cel* mRNA.

TR1-5MG1655(pTY003) was unaltered with respect to that of TR1-5MG1655(pColE7-K317) (data not shown), indicating that the substitution did not affect basal translation efficiency. Hence, AC to TT substitution at the putative CsrA binding site (ACAAGGAGT) overlapping the *cel* SD caused relief of the CsrA-dependent repression of *cel* expression *in vivo*.

CsrB and CsrC RNA levels are drastically reduced during the SOS response

In *E. coli*, CsrA activity is antagonized by the CsrB and CsrC small RNAs (2,3,50). Expression of the ColE7 operon is regulated by the SOS response (35). In order to investigate whether the SOS response network and Csr global regulatory circuits are interrelated, we examined the CsrA protein level of MG1655 (pColE7-K317) cells treated with or without MMC. In agreement with previous study (50), the Western blot analysis indicated that CsrA levels exhibited modest increase during the culture growth. Nevertheless, the amount of CsrA at all three time points was not significantly altered by MMC induction (Figure 7A). Expression of CsrB and CsrC RNA levels were increased ~3.3-fold and ~2.8-fold, respectively without MMC treatment over time. In contrast, levels of both CsrB and CsrC dropped substantially to <20% relative to the non-treated cells at time zero, within 30 min of MMC treatment (Figure 7B). In addition, the Western blotting analysis revealed that the RecA and LexA, two major governors of SOS response (57), were activated and reduced, respectively, in MMC-treated cells. These results confirmed that the SOS response was activated by MMC treatment (Figure 7A). These experimental results strongly suggested that *csrB* and *csrC* respond negatively to SOS induction via unknown factor(s).

To further determine if the SOS response might contribute to the reduced synthesis of CsrB and CsrC RNA, expression from chromosomal *csrB-lacZ* or *csrC-lacZ* transcriptional fusions was examined in MMC-treated and non-treated cells. Induction of the SOS response does not affect the native *lacZ* expression in MG1655

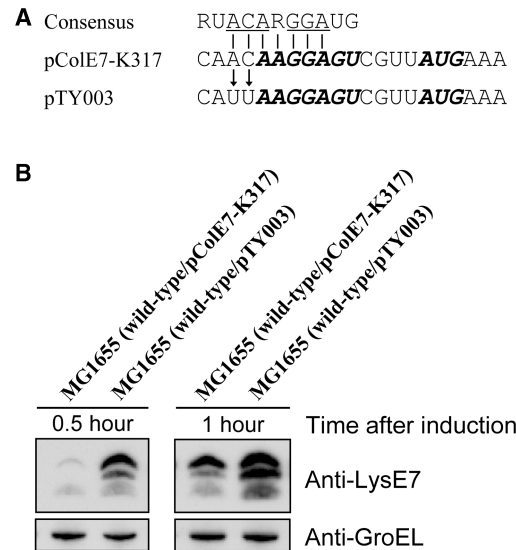


Figure 6. Effect of site-directed mutagenesis of *cel* leader region on expression of LysE7. (A) The CsrA binding consensus sequence is shown above the predicted binding segment in *cel* mRNA; vertical lines identify the nucleotides that match those in the consensus. The highly conserved ACA and GGA binding motifs are underlined. The pColE7-K317 contains the parental sequence. The lower sequence shows the mutated bases in plasmid pTY003. The *cel* ATG translational start codon and its SD element are shown in boldface italic letters. Vertical arrows denote nucleotide substitution introduced into the potential CsrA binding site. (B) Western blotting analysis of *E. coli* MG1655 cells harboring plasmids pColE7-K317 or mutant plasmid pTY003. Whole-cell samples were harvested at 0.5 and 1 h after MMC (0.5 μg/ml) induction and the LysE7 protein levels were determined by Western blotting analysis. GroEL was employed as a loading control. The results were confirmed in three independent experiments.

(pColE7-K317) cells (data not shown). Thus, any observed effect on the expression of *lacZ* reporter driven by *csrB-lacZ/csrC-lacZ* under SOS conditions should be attributable to transcriptional regulation of *csrB/csrC*. The specific β-galactosidase activity from *csrB-lacZ* fusion exhibited a 3-fold increase during the 60-min incubation period, whereas the treatment of MMC resulted in a moderate reduction of *csrB-lacZ* expression (Figure 7C). The *csrB-lacZ* directed β-galactosidase activity in the MMC-treated cells was 1.4-, 1.9- and 2.5-fold lower compared with that in non-treated cells at 30, 45 and 60 min post-treatment, respectively (Figure 7C). As shown in Figure 7C, the specific β-galactosidase activity from *csrC-lacZ* fusion exhibited a 2.8-fold increase in non-treated GS1114 cells during 60 min of incubation period. The effect of SOS response on *csrC-lacZ* expression was similar to those observed in KSB837(*csrB-lacZ*) cells. As a result, the *csrC-lacZ* directed β-galactosidase activity in MMC-incubated cultures was inhibited 1.4-, 1.8- and 2.9-fold relative to the expression in non-treated cells at 30, 45 and 60 min post-treatment, respectively (Figure 7C). It is worth noting that if the synthesized β-galactosidase protein is stable in KSB837(*csrB-lacZ*) and GS1114(*csrC-lacZ*) cells, its activity level may not decrease during downregulation of *csrB/C* expression during SOS conditions. To test this concern, the results

of the reporter assay (Figure 7C) were confirmed by determining the specific *csrB-lacZ* and *csrC-lacZ* transcripts using real-time RT-PCR. In agreement with our prediction and the regulatory pattern obtained by reporter assay (Figure 7C), the *lacZ* mRNA levels in KSB837(*csrB-lacZ*) and GS1114(*csrC-lacZ*) cells were drastically reduced under the stress of SOS response

compared with non-treated cells (Figure 7D). Under culture conditions in the presence of MMC, the level of *csrB-lacZ* transcripts was 3.4-fold and 13.2-fold lower than that in the non-treated cells at 20 and 30 min post-treatment, respectively (Figure 7D). We found that the expression of *csrC-lacZ* transcripts was also downregulated during the SOS response, since the

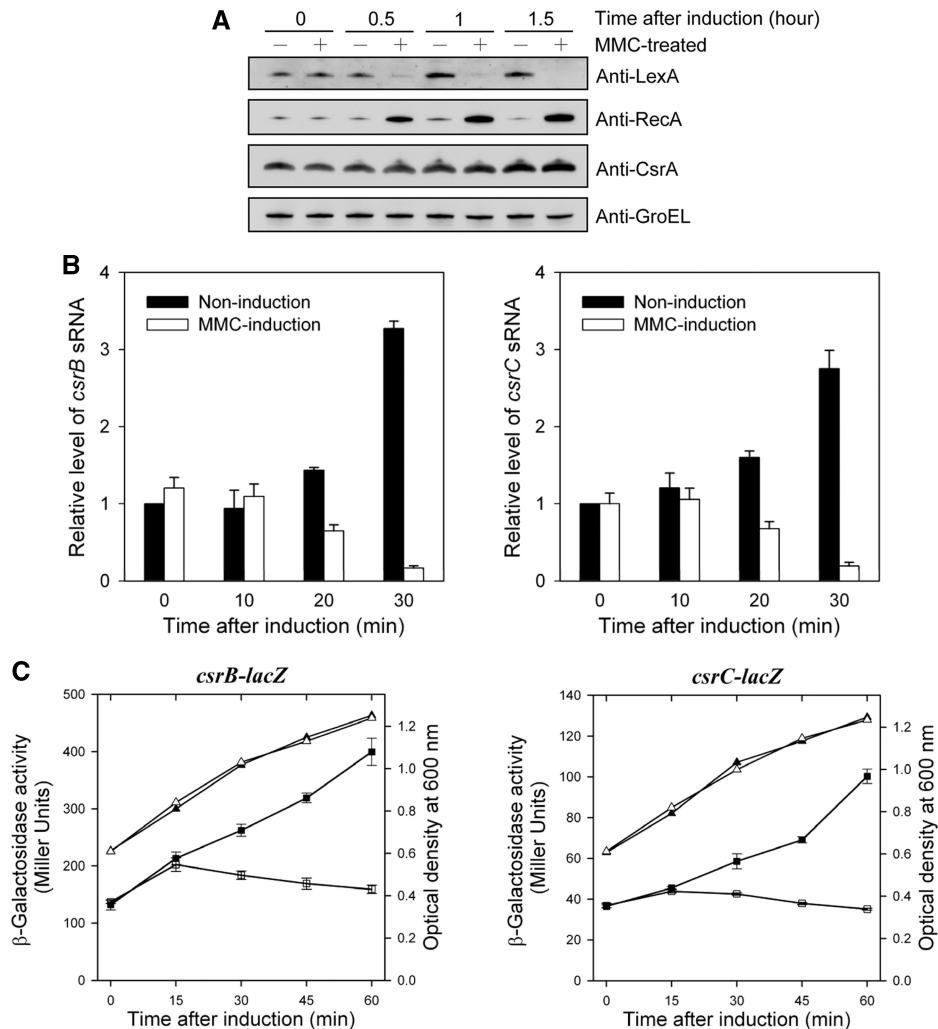


Figure 7. Effect of mitomycin C on expression of carbon storage regulatory system. (A) Western blotting analysis of mitomycin C effect on CsrA expression. MG1655(pColE7-K317) cells were treated with or without MMC (0.5 µg/ml) and harvested at various intervals. The CsrA protein levels were determined by Western blotting analysis. GroEL was employed as loading control. The experiments were conducted in triplicate with essentially the same results. (B) Changes in expression of *csrB* and *csrC* RNAs level in MG1655(pColE7-K317) cells treated with mitomycin C, as measured by real-time RT-PCR. The signals obtained for *csrB* and *csrC* RNAs by real-time RT-PCR were normalized to those of 16S rRNA. In each panel, the mean value of MMC non-treated control cells at time 0 was set to 1. Data are the mean ± SD of measurements from two independent time course experiments performed in triplicate. (C) Effect of mitomycin C on expression of chromosomally encoded *csrB-lacZ* and *csrC-lacZ* transcriptional fusions. Strains containing *csrB-lacZ* and *csrC-lacZ* were KSB837 and GS1114, respectively. KSB837(pColE7-K317) and GS1114(pColE7-K317) cells were incubated in LB medium with shaking, and MMC was added to the culture at a final concentration of 0.5 µg/ml when OD₆₀₀ reached 0.6 (zero time). Cells were harvested at various intervals and assayed for β-galactosidase activity, which is shown as squares. The filled squares represent untreated cultures and open squares indicate the MMC-treated cultures. In each panel, growth curves of the MMC-treated or non-treated cells are depicted by open or filled triangles. β-Galactosidase activities are presented as the mean ± SD from three repeated experiments. (D) Effect of mitomycin C on *csrB* and *csrC* transcription. KSB837(pColE7-K317) and GS1114(pColE7-K317) cells were incubated in LB medium with shaking, and MMC was added to the culture at a final concentration of 0.5 µg/ml when OD₆₀₀ reached 0.6 as the zero time. Cells were harvested at various intervals and the *lacZ* mRNA level was measured by real-time RT-PCR. In each panel, the mean value of MMC non-treated control cells at time 0 was set to 1. Data are the mean ± SD of measurements from two independent time course experiments performed in triplicate. (E) Treatment with mitomycin C decreases glycogen levels in MG1655, but not in its isogenic *csrA* mutant (TR1-5MG1655). To measure glycogen biosynthesis, cultures of MG1655 and isogenic TR1-5MG1655 (*csrA::kan*) were streaked onto Kornberg agar containing 0.5 µg/ml MMC (right) or without MMC (left). The plates were incubated overnight and intracellular glycogen was stained with iodine vapor. The plates with and without MMC were treated with an identical procedure and stained for 45 s.

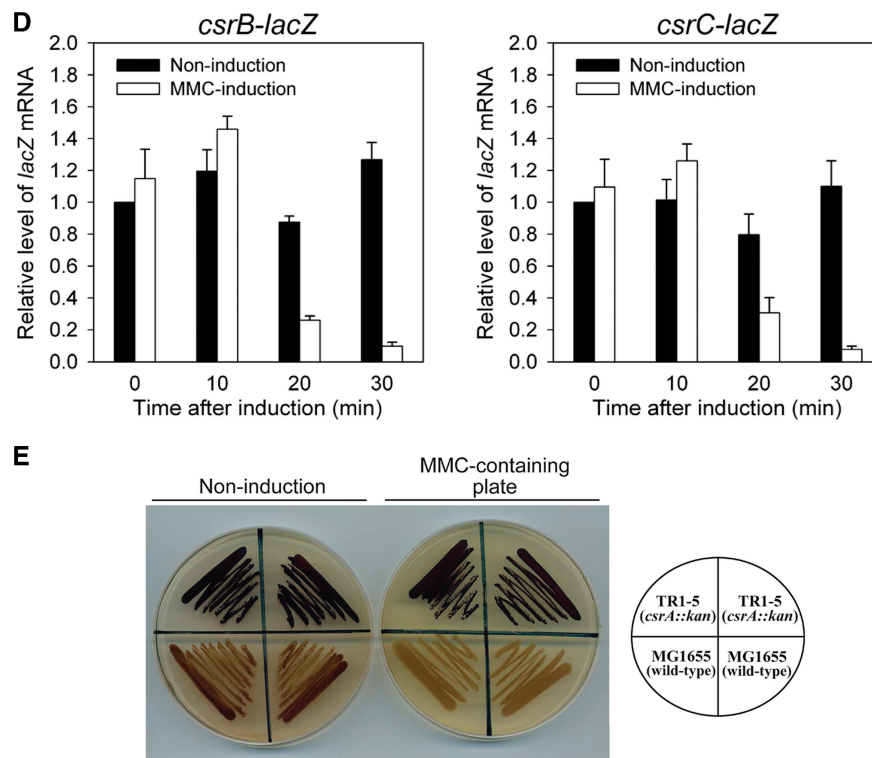


Figure 7. Continued.

relative level of *csrC-lacZ* mRNAs in MMC-incubated cultures was markedly inhibited, 2.5-fold and 13.7-fold, relative to that in non-treated cells at 20 and 30 min post-treatment, respectively (Figure 7D). Taken together, these results suggested that the transcription of *csrB/csrC* was drastically reduced during the SOS response.

The notable reduction of CsrB and CsrC RNA levels, in the absence of detectable changes in CsrA levels, during the SOS response should decrease sequestration of CsrA, which in turn should enhance translational repression of the Lys protein. CsrA negatively regulates the expression of glycogen biosynthetic genes, including *glgC* (8), and overexpression of *csrA* strongly inhibits the synthesis of intracellular glycogen, which can be demonstrated by staining colonies with iodine vapor (5). To obtain further evidence that the SOS response may affect CsrA activity, we examined its effect on glycogen levels. The wild-type MG1655 cells streaked onto Kornberg agar exhibited a medium brown color upon iodine staining (Figure 7E, left bottom panel). When streaked onto MMC-containing agar, however, MG1655 cells yielded colonies that stained a light brown (Figure 7E, right bottom panel). These phenotypes strongly suggested that glycogen synthesis was reduced in MG1655 cells upon SOS response, consistent with a CsrA excess phenotype (5). In a *csrA* mutant background (TR1-5/MG1655) colonies exhibited more intense iodine staining, which was unaltered by the addition of MMC to Kornberg agar (Figure 7E). These findings are consistent with the idea that effects of SOS on glycogen levels are mediated through CsrA, and provide additional support for the

conclusion that intracellular CsrA activity (but not CsrA protein levels) are elevated during the SOS response, which was implied by the CsrA Western blotting and *csrB* and *csrC* gene expression studies.

DISCUSSION

RNA-binding proteins of the CsrA/RsmA family, which act by modulating translation initiation (6,30,55,58), represent an important post-transcriptional regulatory mechanism of prokaryotes. Orthologs of the CsrA family are found in many eubacterial species, in which they control a wide variety of physiological characteristics and cellular processes (59–62). To our knowledge, the present investigation of the involvement of CsrA in the expression of the *ColE7* operon represents the first extrachromosomal regulatory role of CsrA to be established in any species.

Genetic evidence and complementation analyses confirmed that the CsrA protein is indeed a negative regulator of the lysis protein (Figure 2B and D). Several previous studies noted that CsrA controls gene expression in a 1.5- to 10-fold range (5,7,11,12,31), rather than functions as an absolute on-off switch. Of note, we found that CsrA-mediated repression of *cel* expression was ~5-fold (Figure 5A), suggesting that CsrA functions as a modulator to prevent excess synthesis of lysis protein, instead of completely repressing lysis protein. Thus, CsrA may function to calibrate lysis protein levels to accommodate the need for colicin release. The direct involvement of CsrA in modulating the expression of lysis protein was implied by *in vitro* gel mobility shift assay with the CsrA

protein and the *cel* transcripts (Figure 3A and B). Increasing concentrations of CsrA resulted in the sequential appearance of two distinct RNA–protein complexes. While we have not monitored binding stoichiometry of these complexes, this result suggests that two CsrA dimers were bound to each *cel* transcript at the higher protein concentrations. Furthermore, the *cel* transcript used in this study contained two putative CsrA binding sites, located in the loop region of T1 stem–loop structure (BS1) and the *cel* SD sequence (BS2). Both the BS1 and BS2 mutant transcripts gave a single CsrA–RNA complex, which was absent from the BS1–BS2 double-mutant transcript, strongly suggesting that these two RNA sequences serve as CsrA binding sites (Figure 4). Previous studies reported that *glgC* and *pgaA* contain two and six CsrA binding sites, respectively, and that CsrA represses translation initiation in both cases (8,12). Similarly, substitutions designed to disrupt the potential CsrA–BS2 binding site, which overlapped the *cel* SD, relieved CsrA-mediated repression of *cel* gene expression *in vivo* (Figure 6B). Because CsrA did not substantially affect *cel* transcript stability or levels (Figures 5B and C) this implies that binding of CsrA to the *cel* mRNA in the vicinity of the *cel* SD represses translation of the *cel* gene.

The systematic evolution of ligands by exponential enrichment (SELEX) analysis defined a high-affinity consensus binding sequence for CsrA, RUACARGGAUGU, with the underlined residues being 100% conserved (54). The location of conserved primary sequence within the loop of a short hairpin increases the affinity of the CsrA–RNA interaction (30,54), such as in the PA0082 mRNA of *Pseudomonas aeruginosa* (63), in the *hag* transcript of *B. subtilis* (22) and in the *pgaA* transcript of *E. coli* (12). However, CsrA has a lower affinity for *cstA*, in which the GGA motif are not present in stem–loops (11,54). Interestingly, the sequence signature of BS1 and BS2 in the *cel* mRNA showed high similarity to the consensus sequence, as they match the SELEX-derived consensus binding sequence at nine and seven positions, respectively. Furthermore, computer modeling using MFOLD (56) predicted that the GGA motifs of BS1 and BS2 in the *cel* transcript are both situated in the loops of hairpins (Figure 1B). Thus, the presence of GGA residues in the predicted hairpin loop may help to mediate the observed high-affinity interactions between CsrA and the *cel* mRNA.

The *csrA* mutation did not influence the stability of the *cel* transcript (Figure 5C), but led to a 4- to 5-fold increase in *cel* translational efficiency (Table 3). In most cases, CsrA downregulates its mRNA target levels by binding to the leader region, which blocks translation and also promotes mRNA decay (6,8,11,12). In contrast, CsrA repressed translational efficiency of the *cel* gene without a corresponding alteration in mRNA stability, as observed previously for *hfq* gene expression (31). Because no prior examples such as *cel* have been studied, in which CsrA regulates via an internal mRNA segment, as opposed to the 5'-leader, it is not clear whether decoupled translation control and mRNA stability represents a major theme for this type of regulatory mechanism.

It is notable that the half-life of *cel* transcript (~18 min) is much longer than that of the majority of *E. coli* mRNA, with an average lifetime of 2–4 min (64,65). Perhaps the T2 transcriptional hairpin terminator at 3' terminus of *cel* gene functions as a protective barrier against 3'exonucleolytic attack (66,67). Our unpublished data revealed that mutations which destabilize the T1 stem–loop structure were sufficient to target *cel* mRNA for rapid degradation. Furthermore, these results suggest that the 5'RNA secondary structure must also impede endonucleolytic cleavage, typically considered to be the rate-limiting step in mRNA decay, and stabilize the downstream transcript (68–70). Thus, we propose that both the T1 and T2 secondary structures, proximal and distal to the *cel* gene respectively, provide some degree of protection from nucleolytic attack, resulting in the unusual longevity of the *cel* mRNA.

CsrA indirectly activates the transcription of *csrB* and *csrC* via the BarA/UvrY two-component system, constituting an autoregulatory circuit for CsrA, CsrB and CsrC (3,50,71). Although the key players and cascade of Csr regulatory circuitry are now understood, how Csr system is connected with other global regulatory networks and how it responds to varied environmental stimuli are relatively unexplored. In accordance with the function of CsrB and CsrC in antagonism of CsrA activity, the ratio of CsrA:CsrB/CsrC is important for the regulation of CsrA activity. Here, we reveal the first evidence of a biologically significant interconnected between the SOS response network and the Csr system. Both CsrB and CsrC RNA levels were drastically decreased under the stress of SOS response (Figure 7B). In contrast, the amount of CsrA was not changed (Figure 7A), indicating that the intracellular CsrA activity should be elevated during the SOS response. The results of reporter assays and determination of *csrB-lacZ* and *csrC-lacZ* transcripts level revealed that transcription of *csrB/csrC* was reduced during SOS induction (Figure 7C and D). Furthermore, the effect of SOS response on *csrB/csrC* transcription apparently is mediated indirectly, as no LexA box was found in the upstream promoter region of *csrB* or *csrC*. One possible explanation consistent with our data is that BarA may be the regulatory target of SOS response that affects CsrB/CsrC levels, since *barA* expression was reported to be downregulated by UV damage (72). Further characterization of the regulatory mechanism involved the SOS induction and the transcription factor for *csrB/csrC* is subject of our future investigations.

The sequence alignment of the *cei-cel* intercistronic regions of the E group colicins (E2, E3 and E6 to E9) exhibited a high degree of similarity, almost exceeding 99% (Figure S1). Thus, we hypothesize that the CsrA protein is likely to serve as a regulator of lysis protein expression of the E-group colicins.

Expression of the ColE7 operon must be tightly controlled to safeguard the colicin-producing cells from committing suicide. How the colicinogenic cells achieve the coordinative expression of the polycistronic colicin cluster genes is largely unknown. The overlapping reading frames suggest that translational coupling between the bacteriocin and immunity genes is important

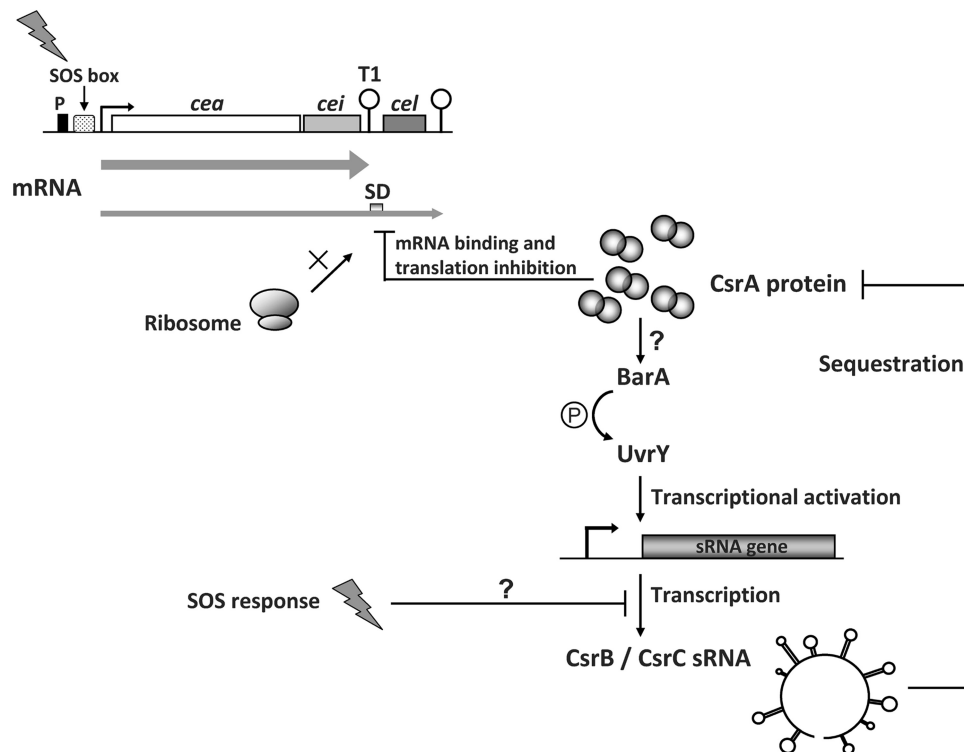


Figure 8. Schematic model of interconnection between the activation of CsrA and the SOS-responsive signaling in the colicinogenic *E. coli*. Two transcripts can be produced from the ColE operon after induction of the SOS responsive promoter. In the presence of T1 stem-loop structure, only a portion of the transcripts read through to the *cel* gene from the SOS promoter, resulting in the production of a long transcript, which consists of the *cea*, *cei* and *cel* genes (35,42,75). In this work, we provide evidence that CsrA RNA-binding protein represses translation of the *cel* mRNA. In addition, sequences in the loop of the T1 stem-loop structure and the *cel* SD sequence are important for CsrA binding. Furthermore, CsrB and CsrC RNA levels are drastically reduced under the stress of SOS response via unknown factors (bottom). BarA-UvrY is a two component signal transduction system and UvrY is the transcriptional activator of the two small RNA (sRNAs), the CsrB and CsrC, which antagonize CsrA by sequestering this protein (3,50,71). Hence, reduced levels of CsrB/C lead to the decreased capacity to sequester CsrA. Thus, during SOS response, more CsrA should be available to negatively regulate translation of the *cel* mRNA (bottom). Note that horizontal arrows represent the transcriptional activity and the thickness of the arrows indicates the degree of the strength of the transcriptional activity. P, promoter; SD, Shine-Dalgarno sequence; T, sequestering or inhibitory effect; X, aborted translation; P, phosphorylation; ⚡, DNA damage induction; ?, an undetermined mechanism.

for the stoichiometric expression of Col-Im complex and safeguard of colicinogenic cells (73,74). Our present studies, together with previous reports (49,75), clearly indicate that the *cel* gene of ColE7 is downregulated at both the transcriptional and the translational levels, and raise the possibility that the expression of *cel* gene may be fine-tuned in response to environmental signals. Previous studies have reported that under the SOS responsive stress, T1 stem-loop structure of the ColE7 operon serves as a transcriptional terminator to reduce read-through from the SOS responsive promoter upstream of the operon (35,42,75). The present study demonstrates that the transcription of *csrB* and *csrC* is decreased remarkably in response to SOS induction via unknown factor(s). Thus, reduction of these two sRNAs should enhance the availability of CsrA to repress translation of the Lys protein (Figure 8). This may help to ensure that the *cel* gene product does not exceed the lethal threshold level (75) and safeguard the colicinogenic cells from suicide incurred by the overexpression of Lys during SOS response. Clearly, additional studies are needed to

provide a detailed understanding of the interrelationship between the Csr regulatory network and the global SOS response system, including the Col operons.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We show deepest gratitude to Dr Gwo-Chyuan Shaw for the generous gift of *Bacillus subtilis*, and Dr Shiau-Ting Hu for sharing laboratory equipment. We are grateful to Dr Ssu-Jean Chang for helpful discussions and suggestions. We thank Dr Chen-Chung Liao for excellent technical assistance in protein purification.

FUNDING

The National Science Council of the Republic of China (NSC 95-2311-B-010 -017 -MY3 to K.-F.C.); National

Institutes of Health (MCB059969 to T.R.); “Aim for Top University Plan” for National Yang-Ming University. Funding for open access charge: The National Science Council of the Republic of China (NSC 95-2311-B-010 - 017 -MY3 to K.-F.C.); “Aim for Top University Plan” for National Yang-Ming University.

Conflict of interest statement. None declared.

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