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Escherichia coli RcsA, a Positive Activator of Colanic Acid Capsular Polysaccharide Synthesis, Functions To Activate Its Own Expression

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Capsule (*cps*) gene expression in *Escherichia coli* is controlled by a complex network of regulators. Transcription of the *cps* operon is controlled by at least two positive regulators, RcsA and RcsB. We show here that RcsA functions to activate its own expression, as seen by the 100-fold-increased expression of a *rcsA*::*lacZ* transcriptional fusion in strains with high levels of RcsA protein, either due to a mutation in *lon* or due to overexpression of RcsA from a multicopy plasmid. Expression of the *rcsA*::*lacZ* fusion is increased by but not dependent on the presence of RcsB. In addition, the effects of H-NS and RcsB on the expression of *rcsA* are independent of each other. A sequence motif, conserved between the *E. coli cps* promoter and the *Erwinia amylovora ams* promoter and previously shown to be the RcsA-RcsB binding site, was identified in the *rcsA* promoter region and shown to be required for high-level expression of *rcsA*.

Colanic acid capsular polysaccharide (*cps*) gene expression in *Escherichia coli* is governed by a complex network of regulators. At least two pathways which can lead to the activation of *cps* expression have been identified. The first pathway appears to be activated in response to an environmental stimulus, such as osmotic shock (26), which impacts the levels of the membrane-bound protein MdoH, involved in the biosynthesis of membrane-derived oligosaccharides (MDOs) (15). The change in levels of MDOs, in response to changes in the osmolarity of the environment, appears to be the signal (8) that a proposed sensor, RcsC, senses and then relays as an internal signal either directly or indirectly to an activator of *cps* expression, RcsB (10). The second pathway leading to the activation of *cps* expression involves the other *cps* activator, RcsA. RcsA is degraded in a Lon-dependent fashion, with a half-life of approximately 1 min in *lon*$^+$ cells (29). RcsA is the limiting factor for the activation of *cps* expression, and stabilization of RcsA in Δ*lon* cells or overproduction of RcsA from a multicopy plasmid in *lon*$^+$ cells leads to high-level expression of the *cps* operon (29). RcsB apparently is essential for *cps* expression (3). *cps* expression in *rcsB* strains is low, and *cps* expression cannot be activated by RcsA in the absence of RcsB, suggesting an auxiliary role for RcsA in *cps* expression (3, 31). The primary amino acid sequence of RcsA contains a putative helix-turn-helix motif which has been hypothesized to be the DNA binding site of RcsA; however, no in vitro data demonstrating RcsA binding to the *cps* promoter region exists (29). RcsA protein cannot be detected in cells mutant in RcsB and Lon protease activity yet can be detected in *lon rcsB* mutant cells if multiple copies of *rcsA*, controlled by its native promoter, are present in the cell, suggesting that expression of RcsA is not absolutely dependent on RcsB (6). Recently, Sledjeski and Gottesman have identified H-NS, a histone-like protein, as a negative regulator of *rcsA* expression, as well as a small, stable RNA, the product of the *dsrA* gene, which can overcome H-NS silencing when expressed in multicopy (25). Beyond the silencing of *rcsA* expression by H-NS and the multicopy effect of DsrA on H-NS, little is known about the regulation of *rcsA* expression. In this study, we report that RcsA functions to activate its own expression. We have identified a putative RcsA binding site in both the *rcsA* and *cps* promoter regions which appears to be required for high-level expression of both the *rcsA* gene and the *cps* operon.

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MATERIALS AND METHODS

Materials. All restriction enzymes were obtained from New England Biolabs (Beverly, Mass.), chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted, and *Taq* DNA polymerase was purchased from Promega Corp. (Madison, Wis.).

Strains, media, and growth conditions. Strains, plasmids, and phages used in this study are listed in Table 1. Cells were grown at 37°C in Luria-Bertani (LB) broth (21) containing the appropriate antibiotics (ampicillin at 100 μg/ml, kanamycin at 25 μg/ml, tetracycline at 25 μg/ml, and chloramphenicol at 25 μg/ml). LB agar, M63 glucose B1 agar (23), or MacConkey's lactose agar (23) was supplemented with the appropriate antibiotics after autoclaving whenever needed.

P1*vir* and λ lysates, as well as transductions, were prepared as described by Silhavy et al. (23).

Detection of RcsA. Strains were grown in LB broth (containing the appropriate antibiotics) to an optical density at 600 nm of approximately 0.6. One-milliliter samples were removed, washed in 10 mM $MgSO_4$, resuspended in 100 μl sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (21), and boiled for 10 min. Protein concentrations were determined for all samples by the bicinchoninic acid protein assay method (Pierce, Rockford, Ill.). Equal amounts of total cellular protein (30 μg) were fractionated on a 14% Tricine-SDS-polyacrylamide gel (22). Proteins were transferred to a polyvinylidene difluoride membrane (NEF-1000; Dupont) in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer (pH 11) supplemented with 20% methanol (30). After transfer, membranes were briefly air dried and subsequently blocked in Tris-buffered saline (20 mM Tris [pH 7.4], 125 mM NaCl) containing 0.1% Tween 20 and 1% nonfat dry milk (TBSTM). Membranes were incubated in TBSTM with preabsorbed polyclonal antiserum specific to *E. coli* RcsA, washed three times in TBSTM, and incubated with an appropriate dilution of monoclonal goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (American Qualex, La Mirada, Calif.) in TBSTM. After three washes with TBST, immunoreactive proteins were visualized on autoradiographic film (Hyperfilm; Amersham, Arlington Heights, Ill.) by enhanced chemiluminescence (Amersham), according to the manufacturer's instructions.

β-Galactosidase assays. β-Galactosidase activity was assayed as described by Miller (17). Values presented are averages of three independent assays.

Identification of a putative Rcs box and construction of *rcsA*::*lacZ* and *cps*::*lacZ* promoter fusions. Alignments of the region upstream of the *rcsA* tran-

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

Strain,[a] plasmid or bacteriophage	Relevant genotype	Construction, source, or reference
Bacterial strains		
DH5α[b]	F⁻ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169*(ϕ*80dlac*Δ(*lacZ*)M15)	Gibco BRL
DDS90	*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *rcsA*$_{90}$::*lacZ*	D. Sledjeski; 19, 24a
JT2029	*proCYA221 zaj-403*::ΔTn*10 rcsA*$_{90}$::*lacZ*	DDS90 + P1 (SG1030)
JT2046	Δ*lon-510 rcsA*$^+$ *rcsB*$^+$ *rcsA*$_{90}$::*lacZ*	JT2029 + P1 (SG4144)
JT2055	Δ*lon-510 rcsA72*::ΔTn*10 rcsB*$^+$ *rcsA*$_{90}$::*lacZ*	JT2046 + P1 (SG23001)
JT2056	Δ*lon-510 rcsA*$^+$ *rcsB62*::ΔKan *rcsA*$_{90}$::*lacZ*	JT2046 + P1 (SG23002)
JT2059	Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::ΔKan *rcsA*$_{90}$::*lacZ*	JT2056 + P1 (SG20806)
JT4000	Δ*lon-510*	SG1030 + P1 (SG4144)
SG1030	F⁻ Δ*lac araD proCYA221 zaj-403*::ΔTn*10*	33
SG4144[c]	N99 Δ*lon-510*	16
SG20250	*lon*$^+$ Δ*lac*	11
SG20250-λi$_{434}$		This study
SG20780	Δ*lon-510 cpsB*$_{10}$::*lacZ*	3
SG20781	*lon*$^+$ *cpsB*$_{10}$::*lacZ*	3
SG20806	*rcsA72*::ΔTn*10*	3
SG21081	*lon*$^+$ *cps*$^+$ *rcsA72*::ΔTn	3
SG23002	*rcsB62*::ΔKan	29
WE10	*hns*::ΔKan *rcsA*$_{90}$::*lacZ*	DDS90 + P1 (DDS1398)
WE12	Δ*lon-510 hns*::Δ*Kan rcsA*$_{90}$::*lacZ*	JT2046 + P1 (DDS1398)
WE20	*hns*::ΔTn*10*	This study
WE28	*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *hns*::ΔTn*10 rcsA*$_{90}$::*lacZ*	DDS90 + P1 (WE20)
WE29	Δ*lon-510 rcsA*$^+$ *rcsB*$^+$ *hns*::ΔTn*10 rcsA*$_{90}$::*lacZ*	JT2046 + P1 (WE20)
WE30	*lon*$^+$ *rcsA*$^+$ *rcsB62*::ΔKan *hns*::ΔTn*10 rcsA*$_{90}$::*lacZ*	JT2058 + P1 (WE20)
WE31	Δ*lon-510 rcsA*$^+$ *rcsB62*::ΔKan *hns*::ΔTn*10 rcsA*$_{90}$::*lacZ*	JT2056 + P1 (WE20)
WE1002	*lon*$^+$ *rcsA*$^+$ *rcsA*$_{109}$::*lacZ*	SG20250 + λRS45-*rcsA*$_{109}$::*lacZ*
WE1003	*lon*$^+$ *rcsA*$^+$ *cps*$_{103}$::*lacZ*	SG20250 + λRS45-*cps*$_{103}$::*lacZ*
WE1004	*lon*$^+$ *rcsA*$^+$ *cps*$_{41}$::*lacZ*	SG20250 + λRS45-*cps*$_{41}$::*lacZ*
WE1012	*lon*$^+$ *proCYA221 zaj-403*::ΔTn*10 rcsA*$_{109}$::*lacZ*	WE1002 + P1 (SG1030)
WE1013	*lon*$^+$ *proCYA221 zaj-403*::ΔTn*10 cps*$_{103}$::*lacZ*	WE1003 + P1 (SG1030)
WE1014	*lon*$^+$ *proCYA221 zaj-403*::ΔTn*10 cps*$_{41}$::*lacZ*	WE1004 + P1 (SG1030)
WE1022	Δ*lon-510 rcsA*$^+$ *rcsA*$_{109}$::*lacZ*	WE1012 + P1 (SG4144)
WE1023	Δ*lon-510 rcsA*$^+$ *cps*$_{103}$::*lacZ*	WE1013 + P1 (SG4144)
WE1024	Δ*lon-510 rcsA*$^+$ *cps*$_{41}$::*lacZ*	WE1014 + P1 (SG4144)
WE1052	Δ*lon-510 rcsA72*::ΔTn*10 rcsA*$_{109}$::*lacZ*	WE1022 + P1 (SG21081)
WE1053	Δ*lon-510 rcsA72*::ΔTn*10 cps*$_{103}$::*lacZ*	WE1023 + P1 (SG21081)
WE1054	Δ*lon-510 rcsA72*::ΔTn*10 cps*$_{41}$::*lacZ*	WE1024 + P1 (SG21081)
WE1062	Δ*lon-510 rcsB62*::Δ*Kan rcsA*$_{109}$::*lacZ*	WE1022 + P1 (SG23002)
WE1063	Δ*lon-510 rcsB62*::Δ*Kan cps*$_{103}$::*lacZ*	WE1023 + P1 (SG23002)
WE1064	Δ*lon-510 rcsB62*::Δ*Kan cps*$_{41}$::*lacZ*	WE1024 + P1 (SG23002)
WE1082	Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan rcsA*$_{109}$::*lacZ*	WE1052 + P1 (SG23002)
WE1083	Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan cps*$_{103}$::*lacZ*	WE1053 + P1 (SG23002)
WE1084	Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan cps*$_{41}$::*lacZ*	WE1054 + P1 (SG23002)
Plasmids		
pATC400	pBR322-*rcsA*$^+$	31
pRS415	*bla*-T1$_4$ *Eco*RI-*Sma*I-*Bam*HI *lacZ*	24
pRS415-cpsBox	pRS415 plus 194-bp *cps* promoter fragment	This study
pRS415-cpsNoBox	pRS415 plus 132-bp *cps* promoter fragment	This study
pRS415-rcsANoBox	pRS415 plus 121-bp *rcsA* promoter fragment	This study
pACYC184-*lon*::ΔKan	pACYC184-*lon* plus 1.5-kb Kan^r cassette from pUC-4K	This study
Bacteriophages		
λRS45	*imm*21 *ind*$^+$ *bla'-lacZ*$_{SC}$	24
λcI⁻ *imm434*	*c*I⁻ *imm*434	N. Trun
λ *imm434*	*imm434*	N. Trun

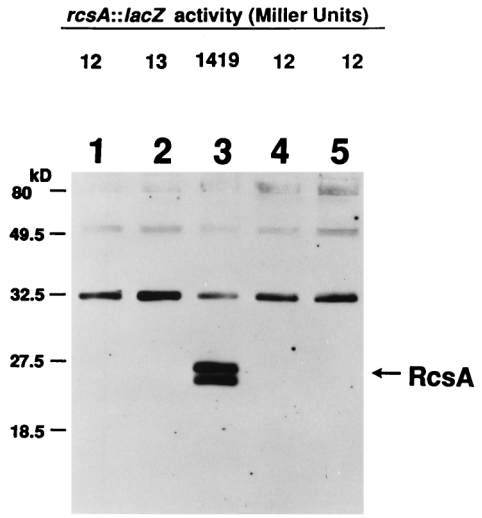
[a] All strains are derived from MC4100 (Δ*lacU169 araD flbB rel*) unless otherwise noted.
[b] Commercial strain.
[c] National Institutes of Health strain.

scriptional start site (25) and the region upstream of the *cps* transcriptional start site (28) were carried out with the Bestfit program of the Genetics Computer Group software package (5). *rcsA* and *cps* promoter fragments, either with or without the putative RcsA box, were amplified by PCR with SG20250 chromosomal DNA as a template and the following primers: rcsANoBoxF, 5′ CCG AAA AAG AAT TCC TAC GA 3′; rcsAR, 5′ GGC GGA CTT AGG ATC CCG TA 3′; cpsBoxF, 5′ CAA CCT AAA GGA ATT CCT AA 3′; cpsNoBoxF, 5′ GCC AAT TAC CGA ATT CTT AT 3′; cpsR, 5′ CCG TCT CAG GAT CCA GTC GT 3′. All forward primers were designed to include an *Eco*RI restriction site, while the reverse primers contained a *Bam*HI restriction site. Reaction mixtures (100 μl) containing 30 ng of DNA template, 0.5 mM concentrations of each primer, 1.5 mM $MgCl_2$, 5% acetamide, 200 mM (each) deoxynucleoside triphos-

phates, and 2.5 U of *Taq* DNA polymerase were incubated for 35 cycles (1 min at 94°C, 1 min at 55°C, and 3 min at 72°C) following a hot-start cycle (5 min at 94°C followed by 2 min at 80°C). Amplification products were digested with *Eco*RI and *Bam*HI, purified with Qiagen (Santa Clarita, Calif.) PCR purification kits, and ligated into pUC18 digested with *Eco*RI and *Bam*HI. Recombinant plasmids were isolated, inserts were verified by restriction digests followed by agarose gel electrophoresis, and the inserts were sequenced on an ABI377 automated sequencer with the M13 forward and reverse primers. (Sequencing reactions were carried out at the Center for Gene Research and Biotechnology, Central Services Laboratory, Oregon State University.) Promoter fragments with the correct sequence were subcloned from pUC18 into pRS415 (24). The resulting *lacZ* operon fusions (pRS415-rcsANoBox, pRS415-cpsBox, and pRS415-cpsNoBox) were crossed onto λRS45 as follows. Fifty microliters of a JT4000 culture, transformed with the recombinant plasmids, was infected with λRS45 at multiplicity of infection of approximately 1.0 and incubated at room temperature for 5 min. Two milliliters of LBMM (LB plus 0.2% maltose–10 mM $MgSO_4$) were added, and the culture was incubated at 37°C until complete lysis of the bacteria was evident. Remaining cells were lysed by the addition of 100 μl of chloroform and brief vortexing, followed by a 5-min centrifugation step. To isolate recombinant phages, 1, 10, and 100 μl of the lysate was mixed with 100 μl of JT4000 culture, 100 μl of 20 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml, and 3 ml of melted top agar (LB broth plus 0.7% agar); the mixture was then plated on LB agar and incubated at 37°C overnight. Blue plaques were picked and purified by preparing a lysate, which was subsequently plated out as described above. Once a pure lysate, resulting from a single blue plaque, was obtained, the lysate was titered and the presence of the correctly sized insert was determined by PCR with the following primers specific to regions upstream (RS45F) and downstream (RS45R) from the promoter regions of the fusions: RS45F, 5′ GGA ATT GGG GAT CGG AAT TC 3′; RS45R, 5′ CGA CGG CCA GTG AAT CCG GT 3′. PCRs were carried out as described above with the following modifications: 10 μl of a 1:100 dilution of the phage lysates (approximately 10^9 PFU/ml) was used as the template, and the annealing temperature was raised to 65°C. Once the inserts were verified, the fusions were introduced into the chromosome of SG20250 at the λ*att* site according to the procedure described by Simons et al. (24). One hundred microliters of a fresh SG20250 overnight culture in LBMM was mixed with 100 μl of phage stock ($>10^6$ PFU/ml) and incubated at room temperature for 20 min; 2 ml of LBMM was added, and the culture was incubated for 2 h at 37°C before dilutions of 10^{-3} to 10^{-8} were plated on LB plus X-Gal (3 ml of a 20-mg/ml stock per liter of agar) plates and incubated overnight at 32°C. Individual blue colonies were purified, and the presence of the correctly sized fusion in the chromosome was verified by PCR with chromosomal DNA (30 ng) from each strain as the template under the PCR conditions described above. The prophage copy number was determined by using the Ter test (18, 24); single-copy lysogens were used for all subsequent experiments.

RESULTS

***rcsA* expression increases in Δ*lon* mutant cells.** Dierksen and Trempy demonstrated the absence of RcsA in cells mutant for RcsB and Lon protease (6). This led to the hypothesis that *rcsA* expression might be regulated by other members of the regulatory network controlling *cps* expression, including Lon, RcsB, and RcsA itself. To test this hypothesis, a reporter gene fusion, consisting of the wild-type *rcsA* regulatory region fused to a promoterless *lacZ* gene (designated $rcsA_{90}$::*lacZ* [19, 24a]) was used to assess the expression levels of this fusion in various strain backgrounds. The fusion consists of a 2-kb fragment from pATC352 (31) inserted into the *Eco*RI/*Sma*I sites of the *lacZ* operon fusion vector pRS415 (24). The fusion was subsequently crossed onto bacteriophage λRS45 (24), which was used to lysogenize *E. coli* SG20250, resulting in strain DDS90 (19, 24a). Strain DDS90 is a partial diploid, carrying a wild-type copy of the *rcsA* gene, directing the synthesis of RcsA protein, as well as the $rcsA_{90}$::*lacZ* operon fusion inserted at the λ*att* site, directing the synthesis of β-galactosidase. Levels of RcsA protein and levels of β-galactosidase can be assessed from the same sample by splitting the sample and using one half to determine levels of RcsA protein with a Western blot approach and the other half to determine the activity of the $rcsA_{90}$::*lacZ* operon fusion with a β-galactosidase enzyme assay. This approach allowed the effects of a number of individual mutations and combinations of mutations on the expression level of *rcsA* to be tested and an examination of the levels of RcsA protein present in the cells to be conducted. The data collected from these experiments is shown in Fig. 1. In a wild-type strain (DDS90 lon^+ $rcsA^+$ $rcsB^+$), steady-state levels of RcsA protein are below detection limits, presumably due to Lon-dependent degradation of RcsA, and the activity of the $rcsA_{90}$::*lacZ* fusion is low (Fig. 1, lane 1). No RcsA protein can be detected in a lon^+ strain carrying a mutation in the wild-type copy of the *rcsA* gene (JT2057 *rcsA72*::ΔTn*10*), and the expression level of the $rcsA_{90}$::*lacZ* fusion is similar to that seen in a lon^+ $rcsA^+$ strain (Fig. 1, lane 2). In a Δ*lon* $rcsA^+$ $rcsB^+$ $rcsA_{90}$::*lacZ* strain (JT2046), the steady-state level of RcsA protein is high and, correspondingly, the $rcsA_{90}$::*lacZ* fusion is expressed at a level 100-fold higher than that seen in the isogenic lon^+ strain (Fig. 1, lane 3). These results suggest that a high level of RcsA accompanies high-level *rcsA* expression. In a strain which carries a null mutation in *lon*, as well as a null mutation in the *rcsA* gene (JT2055 *rcsA72*::ΔTn*10*), the expression level of the $rcsA_{90}$::*lacZ* fusion drops back to the level seen in a wild-type strain (Fig. 1, lane 4), thus further supporting the hypothesis that RcsA regulates its expression. Finally, Fig. 1, lane 5, depicts the results obtained with a Δ*lon* $rcsA^+$ *rcsB* $rcsA_{90}$::*lacZ* strain (JT2056). In agreement with previously published data, no RcsA protein was detectable in this strain



lon	+	+	-	-	-
rcsA	+	-	+	-	+
rcsB	+	+	+	+	-

FIG. 1. *rcsA* expression increases in the absence of Lon protease. *rcsA* expression was assessed at the transcriptional level with a $rcsA_{90}$::*lacZ* fusion and at the protein level by Western blotting. β-Galactosidase assays were carried out as described by Miller (17). Specific activity is expressed in Miller units. Values are means of three assays. To assess RcsA protein levels, equal amounts of protein (30 μg) from whole-cell extracts were boiled in sample buffer, fractionated on a 14% Tricine-SDS-polyacrylamide gel, and analyzed by Western blotting with preabsorbed polyclonal anti-RcsA (*E. coli*) antiserum (the preabsorbed antiserum cross-reacts with a 32-kDa protein which is used as an internal control for equal protein loading). Consistent with data published by Dierksen and Trempy (6), two separate forms of RcsA can be detected. Immunoreactive proteins were visualized by enhanced chemiluminescence. Lanes: 1, DDS90 (lon^+ $rcsA^+$ $rcsB^+$); 2, JT2057 (lon^+ *rcsA72*::ΔTn*10* $rcsB^+$); 3, JT2046 (Δ*lon* $rcsA^+$ $rcsB^+$); 4, JT2055 (Δ*lon rcsA72*::ΔTn*10* $rcsB^+$); 5, JT2056 (Δ*lon* $rcsA^+$ *rcsB62*::ΔKan). Genotypes of strains are indicated at the bottom. +, wild-type; −, null mutation. All strains are MC4100 derivatives carrying an $rcsA_{90}$::*lacZ* fusion integrated at the λ*att* site.

TABLE 2. Effects of mutations in *lon*, *rcsB*, and *hns* on expression of the *rcsA*$_{90}$::*lacZ* transcriptional fusion and expression of capsule

Strain[a]	Relevant genotype	β-Galactosidase activity[b]	Mucoidy[c]
DDS90	*lon*$^+$ *rcsB*$^+$ *hns*$^+$	23	−
JT2058	*lon*$^+$ *rcsB hns*$^+$	27	−
WE28	*lon*$^+$ *rcsB*$^+$ *hns*	264	+
WE30	*lon*$^+$ *rcsB hns*	242	−
JT2046	Δ*lon rcsB*$^+$ *hns*$^+$	2,415	+
JT2056	Δ*lon rcsB hns*$^+$	21	−
WE29	Δ*lon rcsB*$^+$ *hns*	2,664	+
WE31	Δ*lon rcsB hns*	197	−

[a] All strains are MC4100 derivatives carrying an *rcsA*$_{90}$::*lacZ* fusion integrated at the λ*att* site.

[b] β-Galactosidase assays were carried out as described by Miller (17). Values (in Miller units) are averages of three independent assays.

[c] Mucoidy was assessed visually. −, nonmucoid; +, mucoid.

(6). Furthermore, *rcsA*$_{90}$::*lacZ* expression is similar to that seen in the wild-type strain (*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$).

Sledjeski and Gottesman identified the histone-like protein H-NS as a regulator of *rcsA* expression (25). In light of our observations, it seemed reasonable to further investigate the impact of Lon, RcsB, and H-NS on *rcsA* expression. Levels of the *rcsA*$_{90}$::*lacZ* fusion were measured and a visual assessment of the mucoid phenotype was made in a series of *rcsA*$^+$ strains carrying mutations in *lon*, *rcsB*, and *hns* individually or in combination. The data obtained from these experiments is summarized in Table 2. In a wild-type strain (DDS90 *lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *hns*$^+$), the expression level of the *rcsA*$_{90}$::*lacZ* fusion is low (Table 2, line 1) and the cells are nonmucoid. In a *lon*$^+$ strain carrying a mutation in *rcsB* (JT2058) the expression level of the *rcsA*$_{90}$::*lacZ* fusion remains low and the cells are nonmucoid (Table 2, line 2). Expression of the *rcsA*$_{90}$::*lacZ* fusion increases 10-fold in an *hns* strain (WE28) (Table 2, line 3), and the cells are mucoid. This increase is in agreement with the increase in *rcsA* expression observed by Sledjeski and Gottesman (25) in an *hns* strain. Even though this strain is *lon*$^+$, the mutation in *hns* and the resulting 10-fold increase in *rcsA* expression result in a mucoid phenotype (Table 2, line 3). A strain mutant in both *rcsB* and *hns* (WE30) (Table 2, line 4) still expresses the *rcsA*$_{90}$::*lacZ* fusion at a level 10-fold higher than the DDS90 *lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *hns*$^+$ wild-type strain (Table 2, line 1). This result indicates that the effects of RcsB and H-NS on *rcsA* expression are independent of each other, because a mutation in *rcsB* does not impact the expression of the *rcsA*$_{90}$::*lacZ* fusion in a *lon*$^+$ *hns* strain. Furthermore, this observation indicates that the effect of RcsB on *rcsA* expression is dependent on the presence of RcsA protein. As expected, the WE30 *lon*$^+$ *rcsA*$^+$ *rcsB hns* strain does not produce capsule, presumably due to the absolute requirement of RcsB for capsule gene expression (3). In a strain mutant in Lon protease (JT2046) (Table 2, line 5), expression of the *rcsA*$_{90}$::*lacZ* fusion increases to a level approximately 100-fold higher than that seen in the DDS90 *lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *hns*$^+$ wild-type strain (Table 2, lane 1), and this is consistent with results shown in Fig. 1. In a Δ*lon rcsB* double mutant (JT2056) (Table 2, line 6), expression of the *rcsA*$_{90}$::*lacZ* fusion drops back to the level observed in the DDS90 *lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *hns*$^+$ strain (Table 2, line 1), consistent with previous observations (6) and the results shown in Fig. 1. The JT2056 Δ*lon rcsB* strain (Table 2, line 6) is nonmucoid, in agreement with the observation that RcsB is required for *cps* expression. *rcsA*$_{90}$::*lacZ* expression in a Δ*lon hns rcsB*$^+$ double mutant (WE29) (Table 2, line 7) does not significantly increase compared to the JT2046 Δ*lon hns*$^+$ *rcsB*$^+$ strain (Table 2, line 5). Finally, in a Δ*lon rcsB hns* triple mutant (WE31), expression of the *rcsA*$_{90}$::*lacZ* fusion is increased approximately 10-fold (Table 2, line 8) compared to the isogenic JT2056 *hns*$^+$ strain. Expression of *rcsA*$_{90}$::*lacZ* in the WE31 strain background seems to be representative of the true basal level of *rcsA* expression in the absence of all regulators.

Identification of a putative "Rcs box" in the *rcsA* promoter region. The data obtained from the experiments described above indicate the involvement of RcsA protein in regulating its own expression. Previous work by Stout et al. (29) identified RcsA as a positive activator of *cps* gene expression. If RcsA acts as a transcriptional activator of both *cps* and *rcsA* expression, by specifically interacting with the regulatory regions of these genes, then a prediction can be made that a sequence motif which represents the site of RcsA binding would be identified. In support of this prediction, Kelm et al. (14) have localized a putative RcsA-RcsB binding site to a 40-bp region of the *Erwinia amylovora ams* (amylovoran biosynthesis) regulatory region by demonstrating the binding of *E. amylovora* RcsB and RcsA-RcsB, as well as *E. coli* RcsB and RcsA-RcsB, to a fragment representing the putative *ams* RcsA-RcsB binding site. Neither *E. coli* RcsA nor *E. amylovora* RcsA alone could bind to this region (14). Binding of either *E. coli* RcsA or RcsB has not been demonstrated for the proposed *E. coli cps* RcsA-RcsB binding region. Alignment of the region upstream of the *E. coli rcsA* transcriptional start site (Fig. 2b) and the putative RcsA-RcsB binding sites of the *E. coli cps* operon (Fig. 2a) by using the Bestfit program of the Genetics Computer Group software package (5) identified a 25-bp region of 80% identity between the *rcsA* and *cps* promoter regions which we have termed the "Rcs box" (Fig. 2c). This region lies within the region identified by Kelm et al. (14) as a putative RcsA-RcsB binding site of the *E. amylovora ams* operon (Fig. 2c). Similar to the *E. amylovora ams* region, the putative *E. coli* Rcs box is AT rich (80%), but, in contrast to the *E. amylovora ams* region, the 13 bases at the 3′ end of the box constitute a perfect, for *cps*, and nearly perfect, for *rcsA*, 6-bp inverted repeat separated by a single base (CTTAAT-A-TAATTC). The Rcs box is located 30 bp upstream from the −35 signal of the *cps* promoter (Fig. 2a) and 117 bp upstream from the −35 signal of the *rcsA* promoter (Fig. 2b). The *E. amylovora* RcsA-RcsB binding site identified by Kelm et al. does not have the high degree of homology as do the *E. coli cps* and *rcsA* regions. In particular, the 13-bp region, which is virtually identical between the *E. coli rcsA* and *cps* promoter regions, is not well conserved in the *E. amylovora ams* promoter region. The region of highest conservation between the *cps* and *ams* promoter regions lies upstream of the 13-bp region.

Effects of the Rcs box on expression of *rcsA* and *cps*. To assess the effects of the putative Rcs box on *rcsA* and *cps* expression, a series of strains containing single copies (as determined by the Ter test) of operon fusions of the *rcsA* or *cps* promoter region, with or without the putative Rcs box, to a promoterless *lacZ* gene was constructed as described in Materials and Methods. A prediction can be made that if the putative Rcs box is the site of RcsA binding to the promoter regions of *rcsA* and *cps*, the respective constructs lacking the putative Rcs box should not respond to a mutation in *lon*, since stabilized RcsA would not have a site to bind to in front of the *rcsA* or *cps* promoter. In contrast, the constructs containing the putative Rcs box should respond to a mutation in *lon*, since stabilized RcsA can bind to the putative Rcs box and activate *rcsA* or *cps* expression. β-Galactosidase assays were carried out for all promoter fusion constructs in strains carrying mutations in *lon*, *rcsA*, *rcsB*, and combinations of these mutations. The

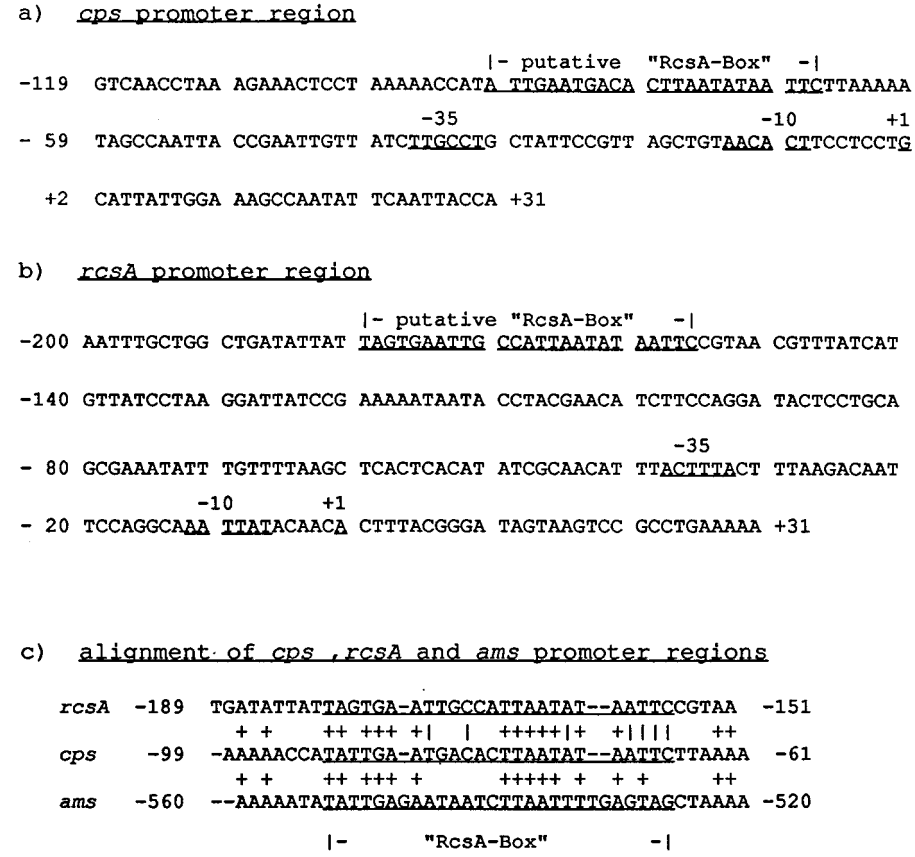


FIG. 2. Identification of a putative Rcs box. *cps* (a) and *rcsA* (b) promoter regions are shown; −10 and −35 regions, the transcriptional start site (+1), and the putative Rcs box are underlined. (c) Clustal multiple sequence alignment of *E. coli cps* and *rcsA* promoter regions and the *E. amylovora ams* promoter region. Residues conserved only between *rcsA* and *cps* are marked by vertical lines; residues conserved between all three promoter regions are marked with plus (+) signs. The putative Rcs box is underlined. Accession numbers: *E. coli cps*, U52666; *E. coli rcsA*, U17137; *E. amylovora ams*, X77921.

results of these experiments are shown in Table 3. As observed before, in a *lon*$^+$ strain the expression level of the *rcsA*$_{90}$::*lacZ* fusion is low (Table 3, column 1, line 1) and the introduction of mutations in *rcsA*, *rcsB*, or *rcsA* and *rcsB* does not affect expression of the *rcsA*$_{90}$::*lacZ* fusion (data not shown). A mutation in *lon* increases the expression level of the *rcsA*$_{90}$::*lacZ* fusion 300-fold (Table 3, column 1, line 2). Introduction of mutations in *rcsA*, *rcsB*, or *rcsA* and *rcsB* in *lon* mutant cells returns the activity of the *rcsA*$_{90}$::*lacZ* fusion to the level seen in a *lon*$^+$ strain (Table 3, column 1, lines 3, 4, and 5). The *rcsA* fusion lacking the putative Rcs box, designated *rcsA*$_{109}$::*lacZ*, expresses levels of β-galactosidase in a *lon*$^+$ strain (Table 3, column 2, line 1) that are similar to levels expressed in *lon*$^+$ strains containing mutations in *rcsA*, *rcsB*, or *rcsA* and *rcsB* (data not shown) and which represent levels previously described as basal for other short *rcsA*::*lacZ* fusions which are nonresponsive to H-NS silencing (25). A Δ*lon* strain carrying the Rcs box-less fusion, *rcsA*$_{109}$::*lacZ* (Table 3, column 2, line 2), does not show the greater-than-100-fold increase in β-galactosidase levels compared to its *lon*$^+$ counterpart and as observed in a Δ*lon* strain carrying the *rcsA*$_{90}$::*lacZ* fusion. These results demonstrate that *rcsA* lacking an Rcs box does not respond to a mutation in *lon*, suggesting that in addition to the loss of H-NS regulation, stabilized RcsA does not have a site to bind to in front of the *rcsA* promoter.

The impact of the Rcs box is further illustrated with the *cps* fusions, where H-NS regulation does not appear to be a factor. Baseline expression of the *cps*$_{103}$::*lacZ* (contains Rcs box) and

TABLE 3. Effects of the putative Rcs box on expression of *rcsA*::*lacZ* and *cps*::*lacZ* fusions

Relevant genotype	β-Galactosidase activity[a]			
	rcsA$_{90}$::*lacZ*[b]	*rcsA*$_{109}$::*lacZ*[c]	*cps*$_{103}$::*lacZ*[b]	*cps*$_{41}$::*lacZ*[c]
lon$^+$ *rcsA*$^+$ *rcsB*$^+$	4	220	7	17
Δ*lon rcsA*$^+$ *rcsB*$^+$	1,189	176	139	20
Δ*lon rcsA rcsB*$^+$	5	219	6	8
Δ*lon rcsA*$^+$ *rcsB*	5	203	3	8
Δ*lon rcsA rcsB*	5	234	12	24

[a] β-Galactosidase assays were carried out as described by Miller (17). Values (in Miller units) are averages of three independent assays. All strains are MC4100 derivatives carrying the described fusion integrated at the λ*att* site.

[b] These fusions contain the putative Rcs box: DDS90 (*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *rcsA*$_{90}$::*lacZ*), WE1003 (*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *cps*$_{103}$::*lacZ*), JT2046 (Δ*lon-510 rcsA*$^+$ *rcsB*$^+$ *rcsA*$_{90}$::*lacZ*), WE1023 (Δ*lon-510 rcsA*$^+$ *rcsB*$^+$ *cps*$_{103}$::*lacZ*), JT2055 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB*$^+$ *rcsA*$_{90}$::*lacZ*), WE1053 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB*$^+$ *cps*$_{103}$::*lacZ*), JT2058 (*lon*$^+$ *rcsA*$^+$ *rcsB62*::Δ*Kan rcsA*$_{90}$::*lacZ*), WE1063 (Δ*lon-510 rcsA*$^+$ *rcsB62*::Δ*Kan cps*$_{103}$::*lacZ*), JT2059 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan rcsA*$_{90}$::*lacZ*), and WE1083 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan cps*$_{103}$::*lacZ*).

[c] These fusions do not contain the putative Rcs box: WE1002 (*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *rcsA*$_{109}$::*lacZ*), WE1004 (*lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ *cps*$_{41}$::*lacZ*), WE1022 (Δ*lon-510 rcsA*$^+$ *rcsB*$^+$ *rcsA*$_{109}$::*lacZ*), WE1024 (Δ*lon-510 rcsA*$^+$ *rcsB*$^+$ *cps*$_{41}$::*lacZ*); WE1052 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB*$^+$ *rcsA*$_{109}$::*lacZ*), WE1054 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB*$^+$ *cps*$_{41}$::*lacZ*), WE1062 (Δ*lon-510 rcsA*$^+$ *rcsB62*::Δ*Kan rcsA*$_{109}$::*lacZ*), WE1064 (Δ*lon-510 rcsA*$^+$ *rcsB62*::Δ*Kan cps*$_{41}$::*lacZ*), WE1082 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan rcsA*$_{109}$::*lacZ*), and WE1084 (Δ*lon-510 rcsA72*::ΔTn*10 rcsB62*::Δ*Kan cps*$_{41}$::*lacZ*).

cps$_{41}$::*lacZ* (lacks Rcs box) fusions is low in a *lon*$^+$ strain (Table 3, columns 3 and 4, line 1). The level seen with both of these fusions is similar to that seen with other wild-type *cps* fusions (i.e. *cpsB*$_{10}$::*lacZ* [33]) when RcsA is degraded in a Lon-dependent fashion. Mutations in *rcsA*, *rcsB*, or both *rcsA* and *rcsB* do not change the expression level of either *cps* fusion in a *lon*$^+$ strain (data not shown). This observation is in agreement with the model for the regulation of *cps* expression, since both RcsA and RcsB are required for optimal *cps* expression. The introduction of a mutation in *lon* leads to a 20-fold increase in *cps*$_{103}$::*lacZ* expression compared to the corresponding *lon*$^+$ strain yet has no impact on *cps*$_{41}$::*lacZ* expression (Table 3, columns 3 and 4, line 2). Furthermore, in comparing the expression of *cps*$_{103}$::*lacZ* to the expression of *cps*$_{41}$::*lacZ*, there is a sevenfold difference of expression in the corresponding Δ*lon* strains (Table 3, columns 3 and 4, line 2), suggesting that regardless of the high levels of RcsA protein in a Δ*lon* strain, the missing putative Rcs box impacts *cps* expression. The expression levels of both *cps* fusions in Δ*lon* strains return to the baseline levels seen in the wild-type strains with the introduction of mutations in *rcsA*, *rcsB*, or *rcsA* and *rcsB*.

DISCUSSION

These studies constitute the first report of an involvement of RcsA protein in regulating its own expression. The observation that RcsA could not be detected in a Δ*lon rcsB* strain (6) and the identification of an H-NS silencing mechanism (25) suggested a multilayer regulatory mechanism for *rcsA* expression. In the course of these studies, it was noted that expression of the *rcsA*$_{90}$::*lacZ* transcriptional fusion used to assess the levels of *rcsA* expression was dramatically increased in strains carrying a mutation in *lon*, and the increased expression of the *rcsA*$_{90}$::*lacZ* fusion was paralleled at the protein level. What might account for the increased *rcsA*$_{90}$::*lacZ* expression in *lon* mutant derivatives of an *rcsA*$_{90}$::*lacZ* diploid strain? In the absence of Lon protease, RcsA protein is stabilized, as reflected by the mucoid phenotype of *lon* mutant cells. If expression of the *rcsA*$_{90}$::*lacZ* fusion is increased in *lon* mutant cells, then a prediction can be made that the stabilized RcsA protein is involved in activating its own expression. Assessment of the levels of *rcsA*$_{90}$::*lacZ* expression in a Δ*lon rcsA* double mutant support this hypothesis: in the absence of Lon and a functional RcsA gene, the activity of the *rcsA*$_{90}$::*lacZ* fusion is low. In contrast, if RcsA is produced from a multicopy plasmid in the presence of Lon, the level of *rcsA*$_{90}$::*lacZ* expression increases (data not shown). Thus, it appears that RcsA is involved in activating its own expression: (i) expression levels of the *rcsA*$_{90}$::*lacZ* fusion increase in response to increased levels of RcsA, and (ii) *rcsA*$_{90}$::*lacZ* expression levels are equally low in *lon*$^+$ *rcsA*$^+$ and Δ*lon rcsA* cells under conditions where no RcsA is present in the cells. In support of these conclusions, two other Lon substrates, CcdA in *E. coli* (20) and σG in *Bacillus subtilis* (3) have been shown to be involved in regulating their own expression. Additionally, Gervais et al. (9) have demonstrated that RcsB is an activator of its own expression; therefore, this mechanism of selfactivation might be a conserved feature of the regulators of *cps* expression in *E. coli*.

The selfactivation of *rcsA* expression in the absence of Lon protease was observed initially in Δ*lon* strains expressing wild-type RcsA protein. If this observation is correct, then presumably an increase in the expression levels of the *rcsA*$_{90}$::*lacZ* fusion should be observed in a *lon*$^+$ strain expressing a mutant RcsA protein (RcsA* [7]) which is stable in the presence of Lon protease. Strains such as these were constructed (7), and indeed, *rcsA*$_{90}$::*lacZ* expression increases in such a strain, indicating that expression of the *rcsA*$_{90}$::*lacZ* fusion is increased whenever the levels of RcsA protein are increased, either through increased synthesis or through increased stability with respect to Lon-dependent degradation (data not shown). Additional support for the hypothesis presented here comes from studies on the Lon-dependent degradation of subunits of the HU protein. Overexpression of either subunit of the HU protein (HUα or HUβ) in *E. coli lon*$^+$ cells induces expression of the *cps* genes (19). This activation of *cps* expression was shown to be due to activation of *rcsA* expression (19). Since individual HU subunits represent substrates for Lon-dependent degradation (2), the increase in *rcsA* expression can be explained with a stabilization of RcsA due to saturation of Lon with either HU subunit, leading to increased *rcsA* expression.

If RcsA binds to the regulatory region upstream of *rcsA* and *cps*, then a conserved nucleotide sequence should be present. Such a region was identified by comparing the promoter regions identified for both the *rcsA* gene (25) and the *cps* genes (28). The putative Rcs box identified is 25 bp long and is 80% identical between the *rcsA* and the *cps* promoter. Comparing the sequence of the putative Rcs box to the complete genome of *E. coli* with the FASTA search engine did not identify any other putative Rcs box locations on the *E. coli* chromosome. The Rcs box shows the longest stretch of identity on the 3′ side, where a stretch of 12 bp is 100% conserved. This stretch consists of an inverted repeat of 6 bp, which might represent the binding site for RcsA. In the *cps* promoter region, the putative Rcs box is located between positions −91 and −68 with respect to the *cps* transcriptional start site (28), while it is located between positions −180 and −164 with respect to the *rcsA* transcriptional start site (25). The Rcs box identified in the *rcsA* promoter region coincides with a region identified by Kelm et al. as the putative RcsA-RcsB binding site within the promoter regions of the *E. amylovora ams* and the *E. coli cps* operons. RcsB and RcsA-RcsB binding to the *ams* promoter region was demonstrated by Kelm et al. (14); however, direct interaction of RcsB and/or RcsA with either the *E. coli cps* or the *rcsA* promoter region remains to be shown. The region of highest conservation between the *E. coli rcsA* and the *cps* promoter regions is not well conserved in the *E. amylovora ams* promoter region; thus, this region might constitute an RcsA binding site, whereas the region of highest conservation between the *cps* and *ams* promoter regions might represent an RcsB binding site. Binding of RcsA and RcsB to the *cps* promoter remains to be shown, but the observation that RcsB binds to the *ams* promoter constitutes strong indirect evidence for RcsB binding to the *cps* promoter. Furthermore, the sequence motif conserved between the *rcsA* and the *cps* promoter regions strongly suggests a potential binding site for RcsA. Interestingly, there are no sequence motifs resembling the Rcs box present in the *rcsB* promoter region or in the promoter regions of *rcsA* genes identified in other organisms (e.g., *Salmonella typhi*, *E. amylovora*, *Erwinia stewartii*, and *Klebsiella aerogenes*).

The positive effect of RcsB on *rcsA* expression appears to be dependent on the presence of RcsA: overproduction of RcsB from a multicopy plasmid in a *lon*$^+$ strain does not lead to increased *rcsA*$_{90}$::*lacZ* expression, possibly due to the absence of RcsA (data not shown). In support of this, Dierksen and Trempy demonstrated that RcsA protein could not be detected in a *lon rcsB* double mutant strain unless *rcsA* was expressed from a high-copy-number plasmid (6). Therefore, it appears that the RcsB effect on *rcsA* expression can be overcome by excess RcsA, indicating that RcsB functions as an auxilliary factor in *rcsA* expression. This is in contrast to *cps* expression, where multicopy RcsB can overcome the absence of RcsA to

activate *cps* expression. These studies have also demonstrated that the effect of H-NS on *rcsA* expression is independent of RcsB: a mutation in *hns* leads to a 10-fold increase in $rcsA_{90}$::*lacZ* expression in the presence or absence of RcsB.

If both regulatory mechanisms (H-NS silencing and Lon-dependent degradation) for *rcsA* expression are removed, one might expect the expression of *rcsA* to increase continuously. However, *rcsA* expression in a Δ*lon hns* strain does not increase beyond approximately 100-fold above wild-type levels. What factors might explain this observation? If RcsA is involved in activating its own expression, a mechanism might exist to limit *rcsA* expression, thus providing the means to limit expression of *rcsA* to levels adequate under the given circumstances. RcsA has been shown to aggregate into inclusion bodies when present at high levels (12) and thus presumably would not be functionally available beyond a certain level in the cells. RcsA and RcsB are proposed to form heterodimers in order to be functional in the activation of *cps* expression (14, 27). Thus, another possibility is the degradation of free, unpartnered RcsA by alternative proteases with substrate specificities overlapping that of Lon protease. The existence of such proteases was shown by several laboratories (4, 32), and RcsA has been shown to have a half-life of approximately 30 min in a Δ*lon* strain, indicating that RcsA is not completely stable even in the absence of Lon. These observations suggest that alternative proteases which might degrade unpartnered RcsA in the absence of Lon exist. This Lon-independent degradation of RcsA may constitute the limiting factor in the selfactivation of RcsA. Alternatively, high levels of RcsA might be inhibitory to *rcsA* expression, leading to a selflimiting effect of the autoregulation. Such a negative effect on selfactivation has been observed with RcsB (9) and presumably would ensure balanced expression of RcsA.

What might explain the complex regulatory network governing *rcsA* expression and ultimately *cps* expression? The production of the colanic acid capsule in *E. coli* has been implicated in protection from desiccation and osmotic shock (26). An increase in *cps* expression in response to osmotic shock has been shown (26). According to the current model, activation of *cps* expression is accomplished through interactions, either directly or indirectly, between MDOs, RcsC (a sensor), and RcsB (a positive regulator). RcsA, the other known positive regulator, is effectively degraded in a Lon-dependent fashion, and in wild-type cells, RcsA protein would not be available to participate in the activation of *cps* expression. How can a cell accomplish maximal expression of the *cps* genes in the presence of Lon? Many genes under the negative control of H-NS are regulated in response to changes in the environment (1, 34). Changes in the pH or the osmolarity of the medium, cold shock, entry into stationary phase, and other factors have been shown to activate genes silenced by H-NS. The exact mechanism by which H-NS functions in the regulation of many of these genes remains unknown. Increased expression of the *cps* genes has been demonstrated in response to osmotic shock (26), and this increase was shown to be dependent on RcsA, RcsB, RcsC, and MdoH (8, 26). One can envision a mechanism in which the silencing of *rcsA* by H-NS is removed, leading to increased expression of *rcsA*. The level of RcsA in the cell increases, by a selfactivation mechanism, to a point at which the level of RcsA synthesis exceeds the level of Lon-dependent RcsA degradation, allowing for the induction of *cps* expression, *cps* genes are expressed as long as the environmental stimulus persists. Once H-NS silencing is reestablished, Lon can clear RcsA from the system and *cps* expression is turned off. This regulatory pattern is analogous to the regulation of SulA activity, another protein susceptible to Lon-dependent degradation. *sulA* expression is derepressed upon SOS induction, and levels of SulA protein increase to the point where the protein cannot be completely eliminated from the cell. This allows SulA to carry out its function, inhibition of cell division, until *sulA* expression is again repressed; SulA is then cleared in a Lon-dependent fashion from the cell and cell division resumes.

The regulatory mechanism proposed in this study allows for the fine tuning of *cps* expression through two, possibly independent, pathways. A potential stimulus for the induction of *rcsA* expression has not yet been identified, but it can be envisioned that maximum capsule expression might be in response to a stimulus that activates both pathways simultaneously.

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