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Identification of a Second RcsA Protein, a Positive Regulator of Colanic Acid Capsular Polysaccharide Genes, in *Escherichia coli*

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A second form of RcsA, a positive activator of the capsular polysaccharide genes (*cps*), has been identified in *Escherichia coli*. Ferguson plot analysis suggests that the two RcsA proteins differ by size rather than by charge. Both RcsA proteins are expressed from a single *rcsA* gene. Detection of both RcsA proteins in Δ*lon* cells is RcsB dependent.

A complex network of regulatory proteins is involved in the expression of colanic acid capsular polysaccharide in *Escherichia coli*. To date, five regulators have been identified and characterized, namely, RcsA, RcsB, RcsC, RcsF, and Lon protease (9, 13, 15; reviewed in references 11 and 12). The limiting component is RcsA, an unstable protein which is rapidly degraded in *E. coli* wild type for Lon protease activity (13, 22, 24). RcsA, expressed from multicopy plasmids carrying *rcsA*, has a half-life of 3 min in *lon*$^+$ cells and approximately 30 min in Δ*lon* cells (22, 24). RcsA has been identified in *Klebsiella* spp. (1, 16), *Erwinia* spp. (2, 5, 25), and *Salmonella typhi*, (26) and structural evidence suggests it is present in *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Vibrio anginolyticus*, *Xanthomonas campestris*, *Alcaligenes viscolactis*, *Rhizobium leguminosarum*, and *Agrobacterium radiobacter* (6).

Unstable proteins, such as RcsA, are frequently control points in the regulation of cellular responses to specific environmental, metabolic, or developmental signals. Given that RcsA is a highly unstable regulatory protein (22, 24), that its expression appears to be silenced by H-NS, a histone-like protein (21), and that H-NS silencing may be relieved by multiple copies of *dsrA*, a small RNA (21), it would not be surprising to identify additional mechanisms affecting its regulation or its activity.

Detection of two RcsA proteins. Separation of low-molecular-weight (low-MW) proteins (reported sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] MW for RcsA of 27,000) was optimized by replacing the glycine cathode buffer of the Laemmli SDS-PAGE system (14) with Tricine as described by Schagger and von Jagow (20). After fractionation by Tricine-SDS-PAGE, followed by Western blot (immunoblot) analysis (23) with rabbit polyclonal antiserum specific to *E. coli* RcsA (22) and detection by enhanced chemiluminescence, we now detect in Δ*lon* cells two proteins immunologically reactive with RcsA antiserum and which migrate to the distance reported for RcsA (Fig. 1A, lane 2). Neither immunoreactive protein is detected in *lon*$^+$ cells (Fig. 1A, lane 1).

To challenge the possibility that the replacement of glycine by Tricine in the cathode buffer of the Laemmli SDS-PAGE system might contribute to artifactual electrophoretic conditions, we examined the resolving powers of different percentages of acrylamide and of various gel lengths by the traditional Laemmli SDS-PAGE system (glycine cathode buffer). The two RcsA protein bands could be resolved only in a traditional Laemmli SDS-PAGE system with a glycine cathode buffer if a high-percent acrylamide gradient (15 to 20%) was used in a gel runner that spanned at least 8 in. (1 in. = 2.54 cm) (data not shown). This observation suggested that the separation of RcsA into two protein bands by use of a Tricine cathode buffer was not the result of an electrophoretic artifact but rather the result of increased protein separation and resolution by the Tricine system.

Peptide digestion reveals strikingly similar, yet not identical, banding patterns. Peptide mapping by limited proteolysis in SDS-PAGE is a highly reproducible method for examining amino acid sequence homology between two proteins (4). To determine whether the two immunologically related RcsA proteins have peptides in common and therefore have similarity in their primary amino acid sequences, the two RcsA proteins were separated by Tricine-SDS-PAGE and a gel slice containing both RcsA proteins was excised. The gel slice was reelectrophoresed at a right angle (90°) to the original electrophoresis direction while in the presence of α-chymotrypsin and then subjected to Western blot analysis with RcsA antiserum. Figure 2 illustrates that the two proteins exhibit strikingly similar, although not identical, peptide digestion patterns. The RcsA protein that migrates at a higher MW in SDS-PAGE (designated RcsA1) contains one higher-MW peptide fragment not seen in the digestion pattern of the RcsA protein that migrates at the lower MW in SDS-PAGE (designated RcsA2). The digestion pattern for the RcsA2 protein shows one lower-MW fragment not seen in the RcsA1 digestion pattern. All other peptide bands are common to both proteins. This result strongly suggests that the two proteins have extensive homology in their primary amino acid sequences but are not identical proteins.

Two RcsA proteins differ by size but not charge. Two assumptions are made when SDS-PAGE is used to fractionate proteins: (i) all proteins bind SDS at the same weight ratio (1.4 g of SDS per g of protein), resulting in identical charge-to-mass ratios (17), and (ii) all SDS-protein complexes assume the same conformation, thus the sizes of the complexes vary linearly as a function of MW (18). One or both of these assumptions are violated by proteins that migrate anomalously. Ferguson plots (8), graphing the $\log_{10}$ relative mobility

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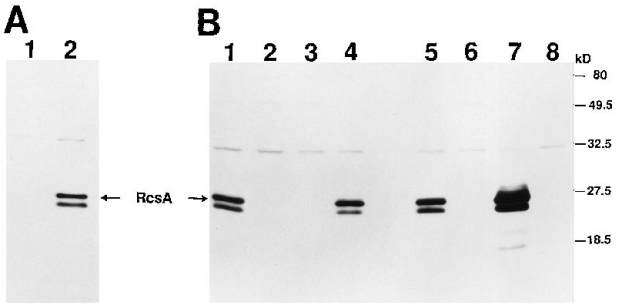

FIG. 1. Detection of RcsA from cells boiled in sample buffer, fractionated on a 14% Tricine-SDS-polyacrylamide gel, analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein, and visualized by enhanced chemiluminescence (A) Lanes: 1, SG20250 *lon*$^+$ *rcsA*$^+$ (20); 2, JT4000 Δ*lon rcsA*$^+$ (this laboratory). (B) Lanes: 1, JT4000 Δ*lon rcsA*$^+$; 2, KD302 (JT4000 *rcsA72*::Tn*10*); 3, KD302 plus pBR322; 4, KD302 plus pATC400(*rcsA*$^+$); 5, SG20780 Δ*lon rcsA*$^+$ *cpsB10*::*lacZ* (3); 6, KD303 (SG20780 *rcsA72*::Tn*10*); 7, KD303 plus pATC400; 8, KD303 plus pBR322. The positions of molecular mass standards are indicated on the right. All strains are MC4100 derived (Δ*lacU169 araD flbB rel*).

($\text{Log}_{10}R_f$) versus gel concentration (%T; grams of acrylamide plus grams of bisacrylamide per 100 ml), were constructed for both RcsA proteins (Fig. 3) with gel concentrations of 6, 8, 10, 12, and 14%T in Tricine-SDS-PAGE gels. The migration distances of each RcsA protein, each molecular weight standard, and a reference marker protein were measured. Relative mobility was calculated by dividing the migration distance of the reference protein band by the migration distance of each RcsA protein and each molecular weight standard. The respective slope, K_R (a measure of the retardation of the protein by the gel), and its ordinate intercept, Y_o (a measure of the mobility of the protein in free solution), were calculated. Anomalous migration can be detected by comparison of the migration patterns of the two proteins relative to each other and by comparison of their ordinate intercepts to those of the MW protein standards. Rodbard et al. classified four sets of separation patterns that can be determined by Ferguson plot analysis depending on whether size and/or charge is the major determinant in the protein's migration in a PAGE gel (19). One of the separation patterns, designated size isomerism, gives rise to the typical migration patterns seen with SDS-denatured proteins: all proteins have very similar charge densities because of the binding of SDS, and subsequently, they have very similar mobilities in free solution and thus migrate strictly according to size. In this case, the intersections of their Ferguson plots will be at the point where the gel concentration (%T) is approximately equal to zero. The results illustrated in Fig. 3 support the conclusion that the two RcsA proteins migrate in SDS-PAGE gels as predicted for typical denatured proteins which differ in size (size isomerism): as gel concentration (%T) increases, the separation distance between the two proteins increases as a result of the higher retardation coefficient of the larger protein. Furthermore, the two proteins exhibit very similar Y_o values, indicating similar relative free mobilities due to identical charge densities. Finally, the MW protein standards and the RcsA protein bands intersect the *y* axis over a narrow range (data not shown). This further supports the assumption that the proteins have bound SDS at the same weight ratios, have assumed the same conformation as other SDS-protein complexes, and are migrating according to size rather than charge differences. The relative difference in size (approximately 1,000 Da) of the two RcsA proteins was calculated from their migration distances relative to migration distances of molecular weight standards on four separate 14% SDS-PAGE gels (data not shown).

Two RcsA proteins expressed from a single *rcsA* gene. The observation of two similarly sized RcsA proteins might be due to the presence of a second *rcsA* gene in *E. coli*, each gene encoding a protein of slightly different size. To test for the presence of a second gene, Δ*lon rcsA* (*rcsA72*::Tn*10*) (3) mutant cells were probed with RcsA antiserum in a Western blot. In both Δ*lon rcsA* mutant strains, both RcsA protein bands disappear (Fig. 1B, lanes 2 and 6). Both RcsA protein bands are detected in the Δ*lon rcsA*$^+$ isogenic counterparts (Fig. 1B, lanes 1 and 5). To eliminate the possibility that a polar insertion had disrupted a downstream gene which might code for a second protein, pATC400 (24), a pBR322-based plasmid harboring a 2.4-kb fragment containing the *rcsA*$^+$ gene, was transformed into the two Δ*lon rcsA* (*rcsA72*::Tn*10*) mutant strains. Both RcsA protein bands are detected in the two Δ*lon rcsA* mutant strains harboring pATC400 (Fig. 1B, lanes 4 and 7). The two proteins are not detected in the two Δ*lon rcsA* mutant strains harboring pBR322 (Fig. 1B, lanes 3 and 8). These data provide evidence that the two RcsA proteins are expressed from a single *rcsA* gene. This same result is observed with Δ*lon rcsA72*::Tn*10* strains containing a pBluescript II KS$^-$ (Stratagene) plasmid harboring an 897-bp fragment carrying only the *rcsA*$^+$ gene while lacking the adjacent open reading frame, *dsrA*, which encodes a small RNA implicated in *rcsA* regulation. The presence or absence of *dsrA* did not affect detection of either of the RcsA proteins (data not shown).

One possibility to explain the presence of two RcsA proteins, coded for by a single gene but differing in size by approximately 1,000 Da, would be proteolytic processing. To address this possibility, spectinomycin was added (10) to Δ*lon rcsA*$^+$ cells to halt new protein synthesis. Samples were removed at several time points, fractionated by Tricine-SDS-PAGE, and probed with RcsA antiserum in a Western blot. During the 60-min incubation, there was no evidence of cleavage of a preprotein and a concomitant accumulation of the other protein as would be expected if proteolytic processing were occurring (data not shown). There is some decrease in band intensity over time in both bands as predicted for a protein with a half-life of 30 min in Δ*lon* mutant cells.

Neither RcsA protein is detected in the absence of Lon and RcsB. RcsB and RcsA are proposed to interact to activate *cps* transcription (22). However, nothing was known about this interaction in the absence of Lon nor had previous studies been done with RcsA levels reflective of normal expression conditions, i.e., RcsA expressed from a single chromosomal copy of the *rcsA* gene. As revealed by Western blot analysis, both RcsA proteins are readily detected in Δ*lon rcsB*$^+$ cells (Fig. 4, lane 2), while neither RcsA protein is detected in Δ*lon rcsB* mutant cells (Fig. 4, lane 4).

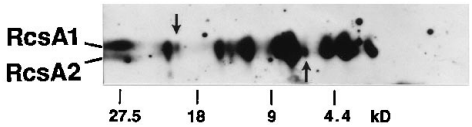

FIG. 2. The two RcsA proteins were fractionated on a 14% Tricine-SDS-polyacrylamide gel, and a band containing both proteins was excised, fractionated in the presence of α-chymotrypsin on a second gel at a right angle (90°) to the first gel, analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein, and visualized by enhanced chemiluminescence. The top bands are peptide fragments from the higher-MW RcsA protein (designated RcsA1). The bottom bands are peptide fragments from the lower-MW RcsA protein (designated RcsA2). Arrows indicate the bands unique to each digest. The positions of molecular mass standards are indicated at the bottom.

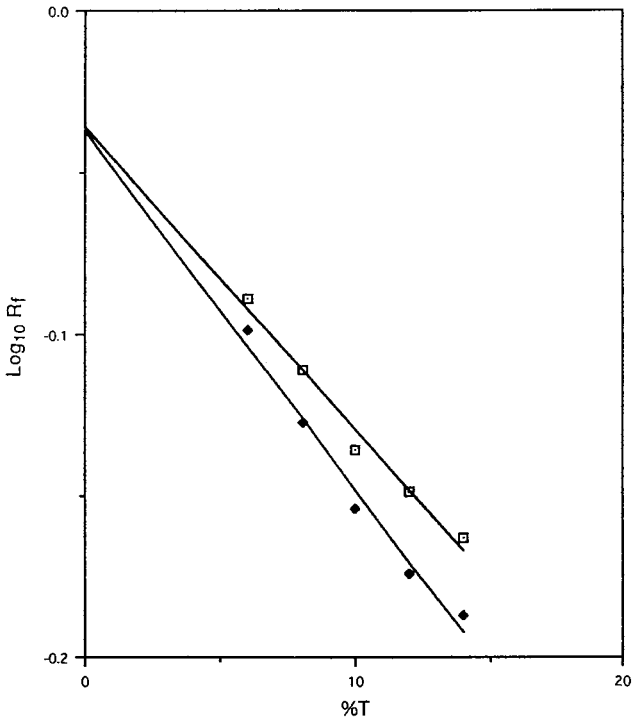

FIG. 3. Ferguson plots of each RcsA protein. Log_{10} of the relative mobility ($Log_{10}R_f$) of each RcsA protein was plotted at 6, 8, 10, 12, and 14%T (gel concentration). The higher-migrating protein on SDS-PAGE is designated RcsA1 (⊡); the lower-migrating form is designated RcsA2 (◆). The calculated values at each gel concentration are shown overlaid with regression lines.

Two possibilities exist to explain the absence of both RcsA proteins in Δ*lon rcsB* mutant cells: (i) RcsB affects RcsA expression or (ii) RcsA, a protein that is stabilized in the absence of Lon, is highly unstable in the absence of both Lon and RcsB. To address whether expression of both RcsA proteins is affected by RcsB, multiple copies of *rcsA* on either pVS103, a pACYC184-based plasmid (gift from V. Stout), or pATC400 (24), a pBR322-based plasmid, were added to Δ*lon rcsB* (*rcsB62*::Kan) (22) mutant cells. If RcsB is required for expression of *rcsA*, RcsA should not be detectable, even when *rcsA* is overexpressed from a multicopy plasmid. As illustrated in Fig. 4 (lanes 9 and 10), both RcsA proteins are readily detected in a Δ*lon rcsB* mutant background. The presence or absence of *dsrA* did not affect these results (data not shown). These results suggest that RcsB is not essential for *rcsA* expression. To address whether RcsB stabilizes both forms of RcsA in cells lacking Lon protease, multiple copies of *rcsB* carried on pJB100 (3), a pBR322-based multicopy plasmid, were added to Δ*lon rcsB* (*rcsB62*::Kan) mutant cells. Both RcsA proteins are readily detected in Δ*lon rcsB* mutant cells carrying pJB100*rcsB*$^+$ (Fig. 4, lane 6). We conclude that RcsB probably is not required for expression of *rcsA* but rather that RcsB appears necessary for protection or stabilization of both RcsA proteins in *lon* mutant cells. Why would RcsB be needed to protect or stabilize RcsA if Lon was absent? An obvious possibility is that an alternate protease, whose substrate specificity overlaps with Lon, is degrading RcsA. In support of this possibility, RcsA is still very unstable in *lon* mutant cells: its half-life is only increased from 3 to 30 min (22, 24). Compared with highly stable proteins, RcsA disappears at a fairly fast rate in *lon* mutant cells, thus implicating an alternate proteolytic mechanism. We determined that Clp protease (reviewed in reference 12) is not involved in the disappearance of RcsA in Δ*lon rcsB* mutant cells (data not shown).

Summary. This study reveals the in vivo presence of two RcsA proteins differing in size by approximately 1,000 Da. Strains lacking Lon protease activity, either by the deletion of *lon*, by transposon insertion into *lon*, or by point mutation in *lon*, express both RcsA proteins, whereas neither RcsA protein is detected in isogenic *lon*$^+$ strains. Both RcsA proteins are expressed from the same *rcsA* gene, and the presence or absence of *dsrA* did not impact our observations. The *rcsA62* mutation (22), which stabilizes RcsA in the presence of Lon, does not affect the detection of one or the other RcsA protein (data not shown). Additional classes of mutants with *rcsA* point mutations which stabilize RcsA in the presence of Lon have been isolated; these mutant RcsA strains express both RcsA proteins (7). Electrophoretic artifact does not account for the two RcsA proteins. Peptide digestion patterns are almost identical for both proteins, demonstrating that the proteins exhibit a high degree of primary amino acid sequence homology. However, each protein has one peptide band not seen in the other protein. Finally, a construction of Ferguson plots reveals that anomalous migration is not involved and that the two RcsA proteins fractionate by virtue of size and not charge on an SDS-PAGE gel. Taken together, these data argue strongly for the conclusion that RcsA protein is found in vivo as two differently sized proteins.

What would be the advantages of two differently sized RcsA proteins, and how might this impact *cps* expression? One possibility is that binding of one of the RcsA proteins to RcsB leads to a nonfunctional interaction, thus resulting in no *cps* expression. This tactic would ensure that the cell conserves energy during certain favorable growth conditions when capsular polysaccharide is not required. Another possibility is that one of the RcsA proteins titrates Lon protease, thereby permitting the other RcsA protein to remain intact and functional for activation of *cps* expression. This tactic would ensure that high levels of functional RcsA would be available during certain unfavorable growth conditions when high levels of *cps* expression were required. And in this case, the nonfunctional RcsA protein might be a preferred substrate of Lon. The biological implications of two differently sized RcsA proteins are currently under intense scrutiny.

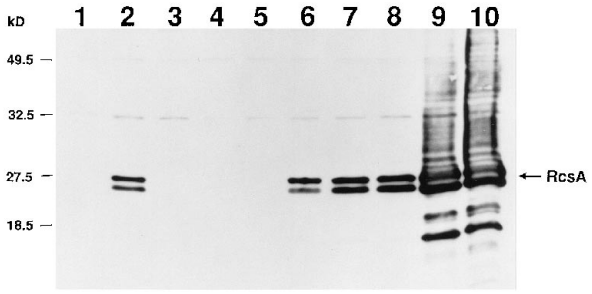

FIG. 4. Detection of RcsA in the presence and absence of RcsB from cells boiled in sample buffer, fractionated on a 14% Tricine-SDS-polyacrylamide gel, analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein, and visualized by enhanced chemiluminescence. Lanes: 1, SG20250 *lon*$^+$ *rcsA*$^+$ *rcsB*$^+$; 2, JT4000 Δ*lon rcsA*$^+$ *rcsB*$^+$; 3, KD317 (SG20250 *rcsB62*::Kan); 4, KD318 (JT4000 *rcsB62*::Kan); 5, KD317 plus pJB100 (p*rcsB*$^+$); 6, KD318 plus pJB100; 7, KD317 plus pVS103 (p*rcsA*$^+$); 8, KD317 plus pATC400 (p*rcsA*$^+$); 9, KD318 plus pVS103; 10, KD318 plus pATC400. The positions of molecular mass standards are indicated on the left. All strains are MC4100 derived (Δ*lacU169 araD flbB rel*).

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