JOURNAL OF BACTERIOLOGY, June 1985, p. 1111–1119
0021-9193/85/061111-09$02.00/0

Vol. 162, No. 3

Regulation of Capsular Polysaccharide Synthesis in *Escherichia coli* K-12: Characterization of Three Regulatory Genes

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Received 28 December 1984/Accepted 1 March 1985

The synthesis of the *Escherichia coli* capsular polysaccharide varies with growth medium, temperature of growth, and genetic background. *lac* fusions to genes necessary for capsule synthesis (*cps*) demonstrated that these genes are regulated negatively in vivo by the *lon* gene product. We have now isolated, characterized, and mapped mutations in three new regulatory genes (*rcs*, for regulator of capsule synthesis) that control expression of these same fusions. *rcsA* and *rcsB* are positive regulators of capsule synthesis. *rcsA* is located at min 43 on the *E. coli* map, whereas *rcsB* lies at 47 min. *rcsC*, a negative regulator of capsule synthesis, is located at min 47, close to *rcsB*. All three regulatory mutations are unlinked to either the structural genes *cpsA-F* or *lon*. Mutations in all three *rcs* genes are recessive to the wild type. We postulate that *lon* may regulate capsule synthesis indirectly, by regulating the availability of one of the positive regulators.

The synthesis of the capsular polysaccharide of *Escherichia coli* K-12, colanic acid, can vary over a 100-fold range, depending on environmental conditions and genetic background (20). We have isolated and characterized operon fusions of *lac* with some of the genes necessary for synthesis of capsular polysaccharide, *cpsA-F* (30). The level of β-galactosidase synthesis in these strains reflects the transcriptional expression of the *cps* operons and correlates with the synthesis of colanic acid in cps^+ strains. Mutations in *lon* make *E. coli* cps^+ cells mucoid because of overproduction of capsular polysaccharide. *lon* mutations also increase the synthesis of β-galactosidase in *cps*::*lac* fusion strains, suggesting that *lon* mutations increase capsular polysaccharide synthesis by increasing *cps* gene expression.

The *cps*::*lac* fusions allow a simple screening, with lactose indicator agar, for alterations in genes which regulate the expression of *cps*. We report here the isolation, preliminary characterization, and mapping of three regulatory genes. *rcsA* and *rcsB* act as positive regulators of capsule synthesis, whereas *rcsC* acts as a negative regulator. The existence of these regulatory genes raises the possibility that negative regulation of *cps* transcription by *lon* is indirect. We hypothesize that it may act by controlling the stability of one of the positive regulators.

(A preliminary report on some of this research was given previously [Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, H91, p. 106]. The regulatory locus referred to there as *cpsR* has been renamed *rcsA*.)

MATERIALS AND METHODS

Bacterial strains and strain construction. The bacterial strains used are described in Table 1 or in the tables in which they are used. Bacteriophage are described in Table 2. F′ matings, P1 transduction procedures, and Hfr matings were performed as described by Miller (22).

Isolation of Tn*10* insertions linked to genes of interest and Tn*10* mutagenesis were carried out as described by Kleckner et al. (17) and Silhavy et al. (28). A random set of Tn*10* insertions in the *E. coli* chromosome was isolated by infection of SG20062 (Δ*lac* lon^+) with λ561 (7). P1kc phage grown on the pooled tetracycline-resistant colonies were used to transduce mutant recipients, selecting for tetracycline resistance and screening for the wild-type phenotype. A second round of P1 transduction of the tetracycline-resistant recombinant was used to demonstrate the linkage of Tn*10* with the mutant allele and to isolate Tn*10* linked to either the mutant or the wild-type allele.

To minimize rearrangements of the Mu d*lac* insertion in the *cps*::*lac* fusions, Mu and *bla* sequences in the *cps*::Mu d1 (2) strains were replaced with λ sequences from λ*p*1(209) as described by Komeda and Iino (18). We confirmed that the λ derivatives expressed β-galactosidase at low levels in lon^+ strains and at higher levels in *lon* strains, as the parental Mu d1 fusions did.

ompC mutations were detected by their resistance to phage hy2 (28). Nalidixic acid resistance was tested on LB plates containing 20 μg of nalidixic acid per ml (22). Motility (*fla*) was tested on LB plates containing 0.35% agar rather than the normal 1.5% (29). Methyl methanesulfonate (MMS) sensitivity was tested on LB agar plates containing 0.05% MMS (11).

rcsA, *rcsB*, and *rcsC* were introduced into strains by P1 transduction with closely linked Tn*10* or Tn*5* transposons.

Mutagenesis. Nitrosoguanidine mutagenesis was carried out as described in Silhavy et al. (28). MutD mutagenesis of λ $rcsA^+$ was carried out as described by Enquist and Weisberg (6).

Enzyme assay. β-Galactosidase assays were performed as described by Miller (22). Cells were grown in glucose or fructose minimal M56 medium to 2×10^8 to 5×10^8 cells per ml, toluenized, and assayed by the addition of *o*-nitrophenyl-β-D-galactopyranoside.

Isolation of λ $rcsA^+$ transducing phage. λD69 carrying 5- to 7-kilobase pieces of bacterial DNA from a partial *Sau*3A digest of the Δ*lac* strain SM57 (24) were screened for their ability to complement the *rcsA* mutation in DH40 by examining the color of single plaques of the phage on DH40 on

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TABLE 1. Bacterial strains

Strain	Relevant genotype[a]	Source or reference
MC4100-derived strains		
SG1039	*ilv*::Tn*5 zaj-403*::Tn*10 proC*	(30)
SG1041	*lon-100*	(30)
SG20001	*proCYA221 zaj-403*::Tn*10 ilv*::Tn*5 galT20001*::Mu d1	(30)
SG20015	*lon-100 ilv*::Tn*5 galT20001*::Mu d1	(30)
SG20061	*ilv*::Tn*5 galT20001*::Mu d1	SG20001 plus P1(MC4100)
SG20062	*ilv*::Tn*5*	(30)
SG20157	*proC zaj-403*::Tn*10 cpsB10*::Mu d1	(30)
SG20160	*cps-153*::Mu d1	(30)
SG20161	*cps-153*::Mu d1 *lon-100*	(30)
SG20177	*lon-100 cps-11*::Mu d1	(30)
SG20180	*cps-11*::Mu d1	(30)
SG20200	*proC zaj-403*::Tn*10 cpsF163*::Mu d1	(30)
SG20250		SG20062 plus P1(SG13082)
SG20303	*cps-11*::Mu d1 *lon-100 ton*	SG20177 plus ϕ80*vir*
SG20304	*lon-100 cps-11*::Mu d1 *ompC*::Tn*10 rcsB42 ton*	SG20303 plus P1(DH72)
SG20329	*ompC*::Tn*5 rcsC137 cps-11*::Mu d1	SG20309 plus P1(SG4124)
SG20505	*cps-153*::*lac*-Mu-*imm*$^{\lambda}$	SG20160 plus λ*p*1(209)
SG20506	*cps-153*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100*	SG20161 plus λ*p*1(209)
SG20508	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$ *proC zaj-403*::Tn*10*	SG20157 plus λ*p*1(209)
SG20511	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$	SG20508 plus P1(SG1041)
SG20513	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$ *lon*-r1	SG20508 plus P1(HR521)
SG20542	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *proC*	SG20200, Tet^{s} λ*p*1(209)
SG20543	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100*	SG20542 plus P1(SG1041)
SG20559	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100 rcsB43 zeh-50*::Tn*10*	DH43 plus P1(*zeh50*::Tn*10*)
SG20581	*cpsB10*::*lac*-Mu-imm$^{\lambda}$ *lon-100*	(30)
SG20582	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$	(30)
SG20589	*cps-153*::*lac*-Mu-*imm*$^{\lambda}$ *rcsC137 ompC*::Tn*10*	EA137 plus P1(TK363)
SG20594	*cps-11*::Mu d1 *lon*-100 *nadA*::Tn*10* [Δ8 *attB.B' bio-936* Δ(*sal-xho*) λ *c*I857ΔH1][b]	SG20177 plus P1 (SG12021)
SG20595	*cps-11*::Mu d1 *lon-100*[b]	SG20594, nad^{+}
SG20597	*cps-11*::Mu d1 *lon-100 rcsB43 zeh-50*::Tn*10*[b]	SG20595 plus P1(SG12013)
SG20598	*cps-11*::Mu d1 *lon-100 rcsA40 zed-14*::Tn*10*[b]	SG20595 plus P1(SG12014)
SG20599	*cps-11*::Mu d1 *nadA*::Tn*10*[b]	SG20180 plus P1(SG12021)
SG20600	*cps-11*::Mu d1[b]	SG20599, nad^{+}
SG20604	*cps-11*::Mu d1 *rcsC137 ompC*::Tn*10*[b]	SG20600 plus P1(SG12019)
SG20618	*cps-11*::Mu d1 *rcsB43 ompC*::Tn*5 lon-100*[b]	SG20597 plus P1(SG4124)
SG20619	*cps-11*::Mu d1 *rcsB43 ompC*::Tn*5 lon-100*[b] *thr*::Tn*10* r^{-} m^{+}	SG20618 plus P1(SG4118)
SG20650	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$ *zed-650*::Tn*10 rcsA**3	SG20511 plus P1(Tn*10* pool)
SG20651	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$ *zei-651*::Tn*10 rcsC2*	SG20511 plus P1(Tn*10* pool)
DH30	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100 rcsB30*	SG20543, NTG[c]
DH32	*rcsB32 cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100*	SG20543, NTG
DH40	*rcsA40 cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100*	SG20543, NTG
DH42	*rcsB42 cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100*	SG20543, NTG
DH43	*rcsB43 cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100*	SG20543, NTG
DH62	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *rcsA40 lon-100 his-2204*::Tn*10*	DH40 plus P1(SG2204)
DH72	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *lon-100 rcsB42 ompC*::Tn*10*	DH42 plus P1(TK363)
EA137	*cps-153*::*lac*-Mu-*imm*$^{\lambda}$ *rcsC137*	SG20505, NTG, 39°C
EA145	*cpsB10*::*lac*-Mu-*imm*$^{\lambda}$ *rcsC145*	SG20511, NTG, 39°C
ATC4000	*cpsF163*::*lac*-Mu-*imm*$^{\lambda}$ *rcsA40 zed-14*::Tn*10*	DH40 plus P1(Tet pool)
ATC5112	*cps-11*::Mu d1 *lon-100 rcsA72*::Δ16Δ17Tn*10*	Torres-Cabassa and Gottesman, in preparation
ATC12014	*thr leu zed-14*::Tn*10*	C600 plus P1 (ATC4000)
Other strains		
AA04	*thr*::Tn*10* r^{-} m^{+}	A. Abeles
C600	F^{-} *thr leu tonA rpsL supE*	NIH[d] strain collection
CS770	Δ*lacU169 araD rpsL recA* TulA *ompC*::Tn*5*	C. Schnaitman
HR521	F′ 42 *lacZ521/lacZ521 lon-r1*	(11)
LE30	F^{-} *mutD5 rpsL azi galU95*	(6)
MSF1334	F′ 1334/*thr leu pro his argF lac gal ara xyl mlt hag recA67*	(29)
NK6970	*nadA*::Tn*10*	N. Kleckner
N38	*thr leu* r^{-} m^{-}	NIH strain collection
N99	F^{-} *galK2*	NIH strain collection
N4156	*polA end thy gyrA* (Nal^{r})	(8)
N6377	r^{-} m^{+} *thr leu pro* Δ8[b]	(13)
SA500	F^{-} *his rpsL*	S. Adhya
Sϕ422	F^{-} *upp-11 udk-2 his metB rpsL*	(12)
SG1053	F′ 150 *his-2204*::Tn*10*/ Δ(*gnd-his*) *trpam* Δ(*edd-eda*)	(30)
SG4044	F^{-} Δ(*gal-pgl*)*324 thi rpsL lon-100*	(10)
SG4118	F^{-} *lon-100* Δ(*gal-pgl*)*324 thr*::Tn*10* r^{-} m^{+} *rpsL*	SG4044 plus P1(AA04)
SG4124	F^{-} *galK2 ompC*::Tn*5*	N99 plus P1(CS770)
SG12007	*thr leu ton lac lon-100*	11

Continued

TABLE 1—*Continued*

Continued

TABLE 1—*Continued*

Strain	Relevant genotype[a]	Source or reference
SG12013	F⁻ *thr leu zeh-50*::Tn*10* *rcsB43 tonA lon-100*	SG12007 plus P1(SG20559)
SG12014	*thr leu tonA zed-14*::Tn*10* *rcsA40 lon-100*	SG12007 plus ATC4000
SG12019	*thr leu tonA ompC*::Tn*10* *rcsC137*	C600 plus P1(SG20589)
SG12020	*thr leu tonA zed-650*::Tn*10* *rcsA*3*	C600 plus P1(SG20650)
SG12021	r⁻ m⁺ *nadA*::Tn*10*[b]	N6377 plus P1(NK6970)
SG13082	F⁻ *his leu*::Tn*10* *lon-100 sulA366 rpsL*	(10)
SM32	F⁻ *his pyrD lon-100 galΔ*	(23)
SM51	F⁻ *his pyrD lon-100 galΔ trp*::Tn*10* *supF*	SM32 plus P1(*trp*::Tn*10* *supF*)
TK363	*lac araD ompC*::Tn*10*	S. Garrett and T. Silhavy

[a] Strains derived from MC4100 all contain Δ*lacU169 araD flbB relA*.
[b] Strains contain *att*B.B′*bio*936 Δ(*Sal-Xho*) cI857 ΔH1 prophage.
[c] NTG, Nitrosoguanidine mutagenesis.
[d] NIH, National Institutes of Health.

lactose-tetrazolium indicator plates. The vast majority of plaques were white (Lac⁻) on these plates, but occasional (1 out of 1,000) Lac⁺ (red) plaques were found. After purification, these phage formed stable *rcsA*⁺ lysogens of DH40.

Isolation of λ SV2 *cos rcsB rcsC ompC* cosmid. A library of *E. coli* N38 DNA in λ SV2 *cos* (M. McCormick, G. A. Gaitanaris, B. H. Howard, and M. E. Gottesman, manuscript in preparation) was obtained from Mary McCormick. Partial *Pst*I digestion of *E. coli* N38 DNA was ligated into the *Pst*I site of λSV2 *cos*, a cosmid vector that contains λ replication functions repressible by λ repressor, *cat*, coding for chloramphenicol resistance, the λ packaging site *cos*, and the λ integration site, *att* P.P′ (see Fig. 2). The ligated DNA was packaged in vitro.

Infection of a λ SV2 *cos cat* phage into a strain containing a site for integration, *attB*, the integration function Int, and λ repressor will lead to stable lysogenization of the cosmid into the bacterial chromosome. The necessary site and functions can be provided by a defective heat-inducible prophage carrying *attB*, a short *bio* substitution, and *int* and *xis* (13). To simplify screening for the appropriate cosmid clone, we constructed a restriction-defective strain, SG20619, which carries the defective prophage, *lon-100*, a *cpsB*::*lac* fusion, and an *rcsB42* mutation. Complementation of the *rcsB* mutation converts SG20619 from Lac⁻ to Lac⁺. Similarly, SG20604 *lon*⁺ *rcsC137 cpsB*::*lac* is Lac⁺, but will become Lac⁻ when complemented (see Table 7). These recipient strains were grown at 32°C in TBMM (11) containing biotin (0.0003%), shifted to 40°C for 15 min to induce synthesis of Int and Xis, returned to 32°C, and infected with the in vitro-packaged λSV2 *cos* clones. Cells and cosmid were incubated together for 1 h, and the mixture was spread on LB plates containing chloramphenicol. Chloramphenicol-resistant colonies were screened on MacConkey-lactose plates for complementation of the *rcsB* or *rcsC* defect.

An in vivo packaging method (McCormick et al., manuscript in preparation) was used to obtain lysates from the λSV2 *cos* candidate which complemented the *rcsB* mutation. The lysogen was infected at 32°C with λ *imm*^{434} *rev* p_L. This phage integrates into the host chromosome at the λ reverse site (9), but carries the λ p_L promoter inserted in front of gene *Q*, driving late gene expression. Derepression of the λ p_L promoter thus provides the functions necessary for DNA packaging and phage head and tail assembly. At 40°C, the λSV2 *cos* clone excises from the chromosome and replicates autonomously. The λ *imm*^{434} *rev* p_L phage provides heads and tails that can package the cosmids. The SV2 *cos* λ *rev* lysogen was grown in superbroth (24 g of yeast extract and 12 g of tryptone per liter, 0.5% glycerol, pH 7.6) at 32°C to midlogarithmic growth, transferred to 40°C, and grown for an additional 90 min. At that time, chloroform was added, and the lysate was centrifuged to remove debris. The resulting lysate was capable of transducing the original SG20619 strain to chloramphenicol resistance; all of the chloramphenicol-resistant lysogens became RcsB⁺. This lysate, λ SV2 *cos rcsB 45*, was used to infect a series of strains carrying *lon* and *rcs* regulatory mutations (see Table 7).

Determination of colanic acid. The amount of cellular colanic acid was quantitated by determining the amount of

TABLE 2. Bacteriophage strains

Strain	Relevant genotype	Source or reference
λ55	λ cI857 *b221 rex*::Tn*10* *O*am29	N. Kleckner (17)
λ561	λ cI171::Tn*10* *O*am-29*P*am80 *b221*	N. Kleckner (7)
λhy2	λhPA-2 *imm*λ *vir*	T. Silhavy (28)
λD69	*imm*^{21}	(24)
SB6	*imm*^{21}*int*::*rcsA*⁺	This work
SB13	*imm*^{21} *int*::*rcsA38*	This work
λSV2 *cos*	*attP.P′ cos* λOP	M. McCormick
Blue53	*imm*^{21}cI⁻	NIH[a] phage collection
Blue119	*imm*^{21}cI⁻ *h80*	NIH phage collection
P1kc		NIH phage collection
P1CMclr100	*c*Its100 Cm^r	(25)
Green318	*imm*^{434} *rev* (λpL)BAM	M. E. Gottesman
Green7	*imm*^{434} *int*6	NIH phage collection
Green115	*imm*^{434} *b*538 cI⁻	NIH phage collection
λ*p*1(209)	*imm*λ cI⁺ *trp′BA′*-Δ209-′*lacZYA′*	(18)

[a] NIH, National Institutes of Health.

TABLE 3. Characterization of mutants with increased expression of *cps*::*lac* fusions

Class	Sensitivity to MMS[a]	Complementation by F′ 150[b]	Temp (°C) of selection	Distribution of mutants				Total
				Spontaneous		Nitrosoguanidine		
				cps-153[c]	*cpsB*	*cps-153*	*cpsB*	
I	Sensitive	Mucoid	32	2	4			6
			39	4	6	6	2	18
II	Resistant	Nonmucoid	32	2	1			3
			39	7	4	1	1	13
III	Resistant	Mucoid	32	0	0			0
			39	0	2	6	2	10
SG20505[d]	Resistant	Nonmucoid						
SG20506[e]	Sensitive	Mucoid						

[a] MMS (0.05%) in LB agar.
[b] SG1053 (F′ 150 *his*::Tn*10*/Δ*gnd-his trp*) was crossed with the Lac⁺ mutants; tetracycline-resistant prototrophs were selected on glucose minimal agar containing 15 μg of tetracycline.
[c] *cps-153*, strain SG20505; *cpsB*, strain SG20511. Both parental strains are *lon*⁺.
[d] Lac⁻ phenotype.
[e] SG20506, *cps-153*::*lac lon-100*, Lac⁺ phenotype; the *cpsB10 lon-r1* strain SG20513 had similar properties.

nondialyzable methylpentose, a constituent of the capsular polysaccharide, by a modification of the method of Kang and Markovitz (16).

Quantitative measurement of nondialyzable methylpentose was done as described by Dische and Shettles (5).

RESULTS

Selection and screening for regulatory mutations that increase expression from *cps*::*lac* fusions. The synthesis of β-galactosidase in *cps*::*lac* fusion strains is very low in *lon*$^+$ cells, but is increased 5- to 50-fold by *lon* mutations (30). As a result, *lon*$^+$ strains that express *lac* solely from a *cps*::*lac* fusion grow poorly on lactose as the sole carbon source. Increased *cps*::*lac* expression can be selected for by requiring growth on lactose minimal agar plates. We isolated both spontaneous and nitrosoguanidine-induced mutations by growth on lactose minimal agar at 32 and 39°C.

lon mutants are sensitive to the SOS-inducing agent MMS (11); 24 of 50 Lac$^+$ isolates were MMS sensitive (Table 3, class I). When a wild-type *cps* region was introduced into the Lac$^+$ mutants on F′ 150, all MMS-sensitive Lac$^+$ mutants became mucoid (Table 3, class I), as do *lon* mutants (Table 3). P1 transduction from 11 class I mutant strains into the *proC cpsB10*::*lac* strain SG20508 demonstrated that both the Lac$^+$ phenotype and MMS sensitivity cotransduced with Pro$^+$ at the frequencies expected for *lon* mutations (15 to 20%) (11); MMS sensitivity was 100% linked to Lac$^+$. Therefore class I mutations are in *lon*.

Of the 50 Lac$^+$ isolates, 16 remained MMS resistant and, on introduction of F′ 150, were nonmucoid (Table 3, class II). These mutations may represent *cis*-acting regulatory mutations or rearrangements in the *cps*::*lac* operon fusions. We have not studied these further.

The remaining 10 Lac$^+$ strains have the phenotypes expected for strains carrying mutations in regulators of capsular polysaccharide synthesis. These Lac$^+$ strains were MMS resistant and therefore *lon*$^+$, yet they became mucoid upon introduction of F′ 150 (Table 3, class III). The mutations therefore increased both *cps*::*lac* expression and capsular polysaccharide synthesis and define *trans*-acting regulatory genes.

Class III mutations were detected only at 39°C. We have observed that *lon* strains show less *cps*::*lac* expression at 39°C than at 30°C (S. Gottesman, unpublished observation), a pattern consistent with the greater synthesis of capsular polysaccharide at low temperature (20). We believe the enrichment for class III mutations at 39°C reflects the detection of fewer *lon* mutations, rather than an actual increase in class III mutations.

Two additional class III mutations were isolated after Tn*10* insertional mutagenesis of SG20511 (*lon*$^+$ *cpsB10*::*lac*; Lac$^-$) (see above). P1 transduction of the Tn*10* from these mutant strains, SG20650 and SG20651, demonstrated that the Tn*10* insertion was closely linked (80 to 90%) to the regulatory mutation. Because the linkage was not 100%, we conclude that the Tn*10* insertions in SG20650 and SG20651 are not in a regulatory gene, but the strains contain regulatory mutations that either arose spontaneously or as a consequence of the nearby Tn*10* transposition event.

Most class III mutations are at one locus. To analyze further the class III mutations, we wished to move them from their original mutagenized background into various *cps*::*lac* fusions. This was accomplished by isolating Tn*10* insertions close to the class III mutations (see above). *zeh-50*::Tn*10* (see below for mapping of this Tn*10* insertion), isolated as linked to the class III mutation in EA137 (*rcsC137*), was linked to five of nine other class III mutations. We tentatively conclude that these mutations lie in the same gene, termed *rcsC* (regulator of capsule synthesis). The class III mutation in SG20651 also mapped in the vicinity of *rcsC*. The mutation in SG20650 was not linked to *rcsC*, but instead was in the vicinity of *rcsA*, and apparently is an *rcsA** mutation (see below). The other four class III mutations unlinked to *zeh-50*::Tn*10* were only weakly Lac$^+$ on lactose indicator agar. We have not characterized these mutants further.

Mutations that decrease expression of *cps*::*lac* fusions, *rcsA* and *rcsB*. We looked for mutations in *trans*-acting positive regulators of the *cps* genes by screening for the simultaneous loss of β-galactosidase expression and mucoidy in SG20543, a *lon cpsF163*::*lac* fusion strain. Because the *cpsF* insertion mutation does not completely abolish capsule synthesis (30), we can screen for both properties simultaneously and easily detect pleiotropic mutations. Independent cultures of SG20543 were treated with nitrosoguanidine and screened on MacConkey-lactose indicator plates for Lac$^-$ nonmucoid colonies. Four independent Lac$^-$ nonmucoid isolates were obtained. None of these mutants was *lon*$^+$, since all remained MMS sensitive.

Three of the four mutations (in DH30, DH32, and DH42) were found to be linked by P1 transduction to *zeh-50*::Tn*10* and therefore are in the region of *rcsC*. These new mutations define *rcsB*, a positive regulatory gene for capsular polysaccharide synthesis.

The fourth mutation, *rcsA40* (in strain DH40), was unlinked to known *rcs* or *cps* loci. A Tn*10* insertion linked to *rcsA* was isolated from the random pool of Tn*10* insertions (see above).

Regulatory mutations affect all fusions similarly. We have described above the isolation of strains with mutations in three regulatory loci, *rcsA*, *rcsB*, and *rcsC*, which affect the synthesis of β-galactosidase from particular *cps*::*lac* fusions. We have transferred these mutations into *cps*$^+$ strains and strains carrying different *cps*::*lac* fusions. β-Galactosidase synthesis from the *cps*::*lac* fusions and colanic acid synthesis behaved in a parallel manner (Table 4). Therefore, *rcsA*, *rcsB*, and *rcsC* are general regulatory loci for *cps*.

rcs regulatory mutations have no discernable effect on the

TABLE 4. Effect of *rcs* regulatory mutations on *cps*::*lac* and *galT*::*lac* expression and colanic acid synthesis

Relevant genotype[a]	Units of β-galactosidase[b]				Colanic acid[d]
	cps-11::*lac*	*cpsB10*::*lac*	*galT*::*lac*[c]		
			No inducer	Inducer	
lon$^+$	4	2	41	307	5
lon-100	40	92	17	190	65
lon$^+$ *rcsC137*	466	452	40	205	141
lon-100 rcsA40	1.5	1.0	27	206	8
lon-100 rcsB42	2.0	1.5	16	281	7

[a] Isogenic sets of strains containing the *rcsC137*, *rcsA40*, or *rcsB42* mutation were constructed by P1 transduction of the parental *lon*$^+$ and *lon-100* strains SG20180, SG20177, SG20582, SG20581, SG20061, SG20015, SG20250, and SG1041 as described in the text.

[b] Cells were grown at 32°C in glucose-M56 medium (except for those with *galT*::*lac* fusions, grown in fructose) and assayed as described in the text.

[c] These strains were grown in fructose-M56 minimal medium with 2.5 mM D-fucose as the inducer. Growth in glucose-M56 gave results similar to those found with fructose without inducer.

[d] Micrograms of nondialyzable methylpentose per milliliter per unit of optical density at 600 nm.

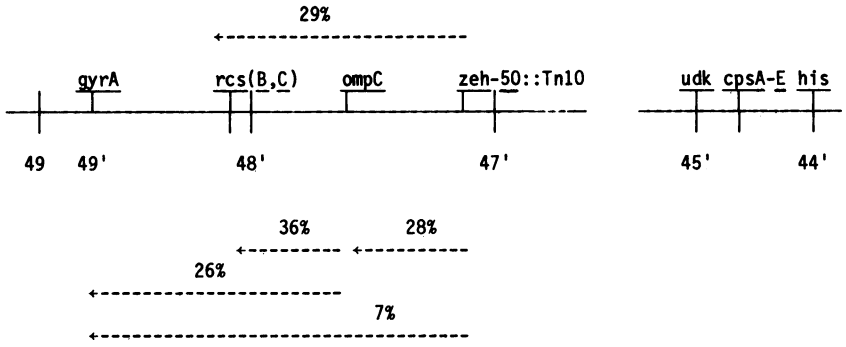

FIG. 1. P1 transductional map of *rcsB* and *rcsC*.

expression of the *galKTE* operon (Table 4); *lon* also has little effect on this operon (30). Therefore, *lon* and *rcs* regulate a common set of operons (the *cps* genes). The *rcs* regulatory mutations do not change *lon* phenotypes other than capsule synthesis. They do not render *lon*$^+$ cells UV sensitive, do not make *lon* cells UV resistant, and do not change the efficiency of plating of λ *O*ts (data not shown) (11). Therefore, our data suggest that *rcsA*, *rcsB*, and *rcsC* do not regulate the synthesis or activity of Lon or of a Lon analog with all Lon activities, but may mediate the *lon* effect on capsule synthesis.

Mapping of *rcsB* and *rcsC*. To map *rcsB* and *rcsC*, we used auxotrophic derivatives of the *rcsB cpsF*::*lac* strain DH42 with Tn*10* in various amino acid biosynthetic genes as recipients in crosses with a variety of Hfr strains. These crosses suggested a location for *rcsB* between *his* (44 min) and the origin of KL16 (61 min), which was confirmed by P1 transductional mapping (Fig. 1). Both *rcsB* and *rcsC* were linked to *ompC*::Tn*10* (47.7 min) (1). *zeh-50*::Tn*10* is also linked to *ompC* (28%) and *gyrA* (48.3 min; 7%), but not to *udk* (45 min; <0.5%) or *cpsB* (45 min; <1%).

Three-factor P1 transductional crosses (Table 5 and Fig. 1) demonstrate that *rcsB* and *rcsC* lie between *gyrA* and *ompC*, at 48 min (1), whereas *zeh-50*::Tn*10* is located counterclockwise to *ompC* at 47.2 min. The order of *rcsB* and *rcsC* cannot be deduced from these experiments; mutations in either gene were 36% linked to *ompC* (Fig. 1).

Schnaitman and McDonald (26) have found that *ompC* is transcribed toward *gyrA*. Our unpublished experiments on the restriction map of the *rcsBC* region (M. Maurizi, P. Trisler, and S. Gottesman, manuscript in preparation) would suggest that *ompC* is transcribed away from *rcsB* and therefore, based on these three-factor crosses, away from *gyrA*. We are investigating this inconsistency, but note that there have been some observations of inversions and rearrangements in this region (C. A. Schnaitman, personal communication). Therefore, the *rcsBC-ompC* region may be inverted in our strains (derived from MC4100) relative to those of Schnaitman and McDonald.

TABLE 5. Three-factor P1 transductional crosses of *rcsB* and *rcsC*

Donor genotype	Recipient genotype[a]	No. of tetracycline-resistant recombinants with deduced genotype[b]			
		gyrA rcs	*gyrA rcs*$^+$	*gyr*$^+$ *rcs*	*gyr*$^+$ *rcs*$^+$
gyrA ompC::Tn*10*	*rcsC145 lon*$^+$	1	14	36	7
	rcsB42 lon-100	0	13	36	9
gyrA zeh-50::Tn*10*	*rcsC145 lon*$^+$	1	3	41	14

[a] Recipient strains carry either *cpsB10*::*lac* or *cpsF163*::*lac* fusions.

[b] Tetracycline-resistant recombinants were selected on LB agar plates containing 15 μg of tetracycline per ml and screened for nalidixic acid sentitivity (*gyrA*) or sensitivity to the hy2 bacteriophage (*ompC*$^-$) as described in the text. *rcsB* and *rcsC* were monitored by color on lactose-MacConkey indicator plates: the *rcsC145 lon*$^+$ *cpsB*::*lac* recipient EA145 is Lac$^+$, but becomes Lac$^-$ when the *rcsC* mutation is replaced by *rcsC*$^+$. DH42, the *rcsB42 lon-100 cpsF163*::*lac* recipient strain, is Lac$^-$, but becomes Lac$^+$ when *rcsB*$^+$ is introduced into the strain.

Mapping of *rcsA*. Preliminary mapping of *rcsA40* was performed by Hfr mating with auxotrophic derivatives of DH40 (*rcsA40 lon cpsF*::*lac*), selecting prototrophic recombinants and screening for the *rcsA*$^+$ phenotype, Lac$^+$. These

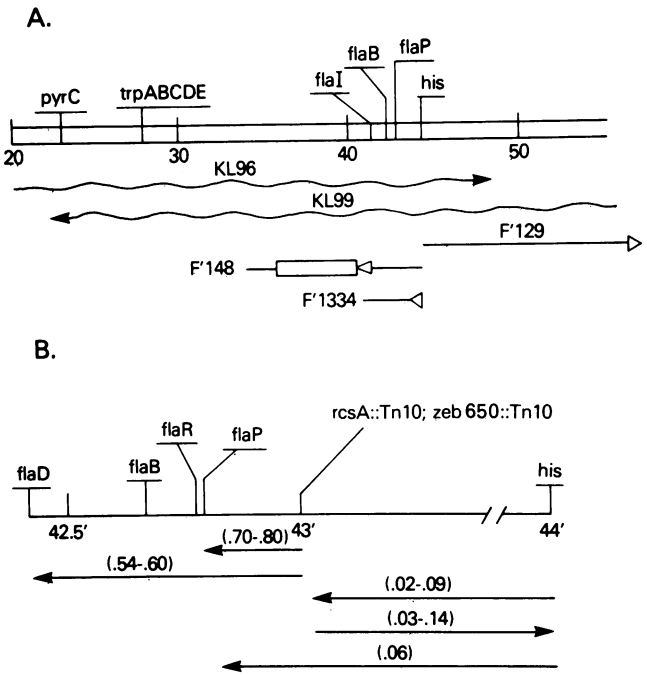

FIG. 2. Mapping of *rcsA*. (A) Locations of markers used to select recombinants are shown on the linear map of the *E. coli* chromosome. Arrows represent various Hfrs (wavy lines) and F′ strains and the chromosomal regions they cover. Arrowheads indicate origin and direction of transfer. Hfrs and F′ strain data were from Low (19), except for F′ 1334 data, which were from Silverman and Simon (29). (B) Placement of *his* and *fla* genes was from Bachmann (1). P1 transduction frequencies are from experiments of Table 6. Arrow ends represent selected marker, and arrowheads represent the marker screened for. Numbers indicate cotransduction frequencies.

TABLE 6. Mapping of Tn*10* insertions in the *rcsA* region by P1 transduction

Donor strain	Donor genotype	Linkage to *rcsA*[a] (%)	Recipient genotype[b]	No. of Tet^r mutants screened	No. with recombinant phenotype (% cotransduction)
SG12020	*zed-650*::Tn*10*	70	*flaP*	50	41 (82)
			flaR	120	76 (63)
			flaB	139	64 (46)
			flaI	88	0 (<1)
			udk	100	0 (<1)
ATC5112	*rcsA72*::Tn*10*	100	*flaP*	133	97 (72.9)

[a] P1 lysates grown on the Tn*10* insertion strains were used to transduce strain DH40 to tetracycline resistance. Colonies were then screened for the Lac$^+$ phenotype. Isolation and characterization of insertions is described elsewhere (Torres-Cabassa and Gottesman, in preparation).

[b] Recipients: YK4144 (*flaP*), YK4150 (*flaR*), YK4165 (*flaB*), YK4181 (*flaI*), all described by Komeda and Iino (18), and SØ422 (*udk*).

crosses suggested a location for *rcsA* in the *his-trp* region. A more precise location was determined by crossing Hfr KL96 (point of origin, 44 min, counterclockwise transfer) carrying a tetracycline-resistant transposon insertion 70% linked to *rcsA* with a *his trp* tetracycline-susceptible recipient (ATC510). Tetracycline resistance was 30% linked to *his* (44 min), but only 3% linked to *trp* (40 min), suggesting that *rcsA* lies counterclockwise to *his*, around min 40 to 43.

The location of the *rcsA40* mutation was further analyzed by complementation tests with F′ *his*$^+$ episomes. With DH62 (*his*::Tn*10* *rcsA40* *lon* *cpsF*::*lac*) as a recipient, His$^+$ merozygotes were selected and screened for *lacZ* expression (Fig. 2). F′ 148 and F′ 1334 complemented *rcsA40*, whereas F′ 129 did not. F′ 148 carrying *zed-3*::Tn*10*, an insertion close to *rcsA*, complemented DH62 (*rcsA* *cpsF163*::*lac* *lon-100*), whereas F′ 148 carrying *rcsA*::Tn*10* did not. These tests place *rcsA* between 42 and 44 min and suggest that *rcsA* is recessive to *rcsA*$^+$.

Additional fine-structure mapping of *rcsA* was accomplished by P1 cotransduction with markers in the *his-fla* region (Table 6). We used a series of Tn*10* insertions located in or near to *rcsA* (Torres-Cabassa and Gottesman, manuscript in preparation). *zed-650*::Tn*10*, 70% linked to *rcsA*, and *rcsA72*::Tn*10* were used as donors for P1 transduction into *fla his* and *udk his* recipients, selecting tetracycline-resistant transductants. Cotransduction frequencies indicated that *zed-650*::Tn*10* and *rcsA72*::Tn*10* are closer to *flaP* (71 to 82% linkage) than to *flaR* (63% linkage), *flaD* (54 to 60% linkage), or *flaB* (46% linkage) (Table 6, Fig. 2). These data are consistent with a location of both Tn*10* insertions clockwise to *flaP*. Cotransduction frequencies of the Tn*10* insertions with *his* (between 2 and 12%; data not shown) and *udk* (<1%), also suggest the map order shown in Fig. 1. Linkages obtained between *his* and *fla* genes were somewhat higher than those previously reported (29).

Although *rcsA* is located near a cluster of *fla* genes, the mutation had no effect on motility. *rcsA40* *zed-4*::Tn*10* (from ATC12014) and *rcsA72*::Tn*10* (from ATC5112) were introduced into the *fla*$^+$ strain N99. All Tet^r transductants were still motile; thus *rcsA* is not necessary for flagellum synthesis.

Complementation studies with *rcsA*, *rcsB*, and *rcsC*. (i) Cloning of *rcsA* on a λ vector. Assuming that *rcsA40* is recessive to the wild-type allele, we screened a λD69 library containing *Sau*3A partial digestion fragments of an *E. coli* Δ*lac* strain by screening for Lac$^+$ plaques on a lawn of DH40 (*rcsA* *lon* *cpsB*::*lac*) on lactose-tetrazolium plates (24). Lac$^+$ plaques were isolated at a frequency of about 1 per 1,000. Single-copy lysogens of the phage purified from these plaques complemented *rcsA* strains, suggesting that the cloned piece carries an intact promoter region.

λ *rcsA*$^+$ complemented both *flaP* and *flaR* mutations, but not *flaB* or *flaD* mutations, confirming the mapping of *rcsA* close to *flaP*.

We mutagenized λ *rcsA*$^+$ by growing the phage in a *mutD* host and isolated two classes of mutants by the color of plaques on lactose-tetrazolium agar plates. *imm*^{21} λ *rcsA*$^-$ phage that fail to complement the chromosomal *rcsA* mutants form white plaques on DH40 on lactose-tetrazolium agar plates. All such white plaques failed to complement each other in mixed infections. *imm*^{21} λ *rcsA** phage form red (Lac$^+$) plaques on a *lon*$^+$ *cps*::*lac* strain (SG20180) more rapidly than do parental λ *rcsA*$^+$ phage.

Single copy lysogens of these phage in both *lon*$^+$ and *lon* hosts confirm the phenotypes seen in plaque tests (Table 7). *rcsA*$^+$ phage complement *rcsA* mutations, but not *rcsB* or *rcsC* mutations. *rcsA** mutations are dominant to *rcsA*$^+$ and, in fact, lead to increased β-galactosidase synthesis in *lon*$^+$ strains (62 versus 3 U in strain SG20600). *rcsA***62* does not, however, significantly increase β-galactosidase synthesis in an *rcsB* mutant strain (Table 7).

(ii) Cloning of *rcsB* and *rcsC* in a cosmid vector. We have used a cosmid vector that allows the cloning of large (30-kilobase) pieces of DNA and enables cosmid clones to be screened as single copy lysogens (McCormick et al., manuscript in preparation). Figure 3 depicts the screening system we used. *E. coli* bacterial DNA partially digested with *Pst*I was ligated into the *Pst*I site of the cosmid vector λ SV2 *cos*, and the ligated DNA was packaged in vitro. We used this lysate to infect SG20619 (*rcsB* *lon* *cpsB*::*lac*), which contains a heat-inducible defective lambda prophage. By selecting for chloramphenicol-resistant lysogens at 32°C, we isolated approximately 75 independent cosmid clones as lysogens. We directly screened the chloramphenicol-resistant lysogens for *rcsB* activity by color on lactose indicator plates. One cosmid lysogen out of 75 complemented the *rcsB* mutation (Fig. 3). Heat pulse curing of the cosmid reversed the phenotype to Lac$^-$ and simultaneously led to loss of chloramphenicol resistance.

We confirmed that the cosmid clone contained markers in the *rcsBC* region by demonstrating that the λ SV2 *cos45* (*rcsB*$^+$) lysogen complements *ompC*::Tn*5* (data not shown).

From this strain, we induced a lysate of the λ SV2 *rcsB*$^+$ cosmid (see above for in vivo packaging procedure) and used the cosmid lysate to infect isogenic *rcsB* and *rcsC* mutant strains, each carrying the defective lysogenic prophage. The properties of the lysogens (Table 7) confirmed that the λ SV2 cosmid complements the *rcsB* mutation; β-galactosidase synthesis increased more than 100-fold. This increase in *cpsB11*::*lac* expression is specific to *rcsB* mutants; *lon* *rcsA* mutant strains do not return to *rcsA*$^+$ levels of β-galactosidase synthesis upon lysogenization with the λ*SV2-cos-rcsB*$^+$ clone (Table 7).

lon$^+$ *rcsC* mutants, which have high levels of β-galactosidase synthesis, were also complemented by the λ SV2 *rcsB* cosmid (Table 7). Therefore, *rcsC* is carried by the cosmid as well, and *rcsC* mutations are recessive to the wild type. Since both *rcsB* and *rcsC* are recessive to the wild type, we tentatively conclude that they are two separate genes.

TABLE 7. Complementation of *rcsA*, *rcsB*, and *rcsC* mutants

Strain[a]	Genotype[b]	Units of β-galactosidase[c]				
		No phage	λSV2 *rcsB*$^+$*C*$^+$	λ *rcsA*$^+$	λ *rcsA38*	λ *rcsA*62*
SG20600	*lon*$^+$	4	3	3	3	54
SG20595	*lon-100*	97	315	307	152	488
SG20597	*lon-100 rcsB42*	2	315	3	2	17
SG20598	*lon-100 rcsA40*	2.5	0.5	162	1.4	634
SG20604	*lon*$^+$ *rcsC137*	713	2	205	569	227

[a] All strains contain *cps11*::*lac* and *attB.B' bio-936* Δ(*sal-xho*) *c*I857ΔH1.
[b] λ SV2 *rcsBC* lysogens were selected as chloramphenicol-resistant colonies (see the text). *imm*^{21}*rcsA* lysogens were selected with *imm*21*c*I$^-$ and *imm*21*c*I$^-$*h*80.
[c] Cells were grown in glucose-M56 medium at 32°C. Results represent averages of three assays.

DISCUSSION

We have isolated mutations in *trans*-acting regulatory genes that regulate capsular polysaccharide synthesis. Both negative and positive regulators were identified and shown to be distinct from *lon*, another negative regulator of capsular polysaccharide synthesis. Markovitz (20) and his co-workers have shown that colanic acid synthetic enzymes are found at higher levels in *lon* strains, and we have demonstrated that this regulation by *lon* occurs at the level of transcription of *cps* genes (30). The three new regulators, *rcsA*, *rcsB*, and *rcsC*, also regulate transcription of the *cps* genes. They do not affect *lon* phenotypes other than capsule synthesis.

Two negative regulators of capsule synthesis in addition to *lon*, *capS* (near *trp*), and *capT* (unmapped) were described by Markovitz and co-workers (21). *rcsC* (at 48 min) and the dominant *rcsA** mutations (at 43 min) have phenotypes similar to those found for *capS* and *capT*.

Lon protein has an ATP-dependent protease activity (3, 4). The loss of this activity in *lon* mutants can explain at least one of the pleiotropic phenotypes of *lon*, sensitivity to DNA damaging agents. It has been shown that an unstable inhibitor of cell division, the product of the *sulA* gene, is synthesized after DNA damage and is stabilized in *lon* mutants (14, 23, 27). The accumulation of SulA in *lon* cells after UV treatment is sufficient to stop cell division, resulting in cell death (15). We postulate that *lon*$^+$ may also inhibit *cps*

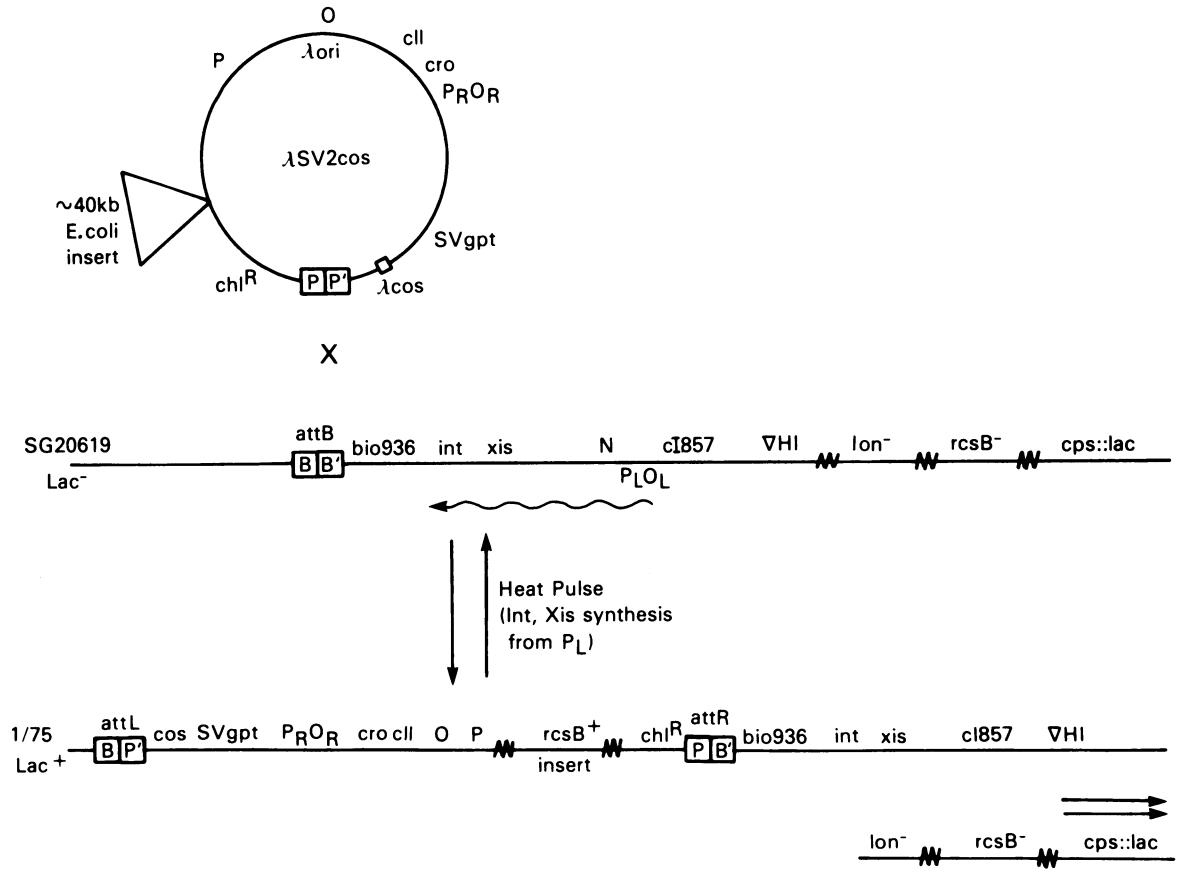

FIG. 3. λ SV2 complementation of *rcsB*. A 15-min heat pulse of SG20619 allows the synthesis of Int and Xis, promoting a site-specific recombination between the λ SV2 *cos* plasmid and the chromosomal deleted prophage. The chloramphenicol resistant (chl^R) lysogen is stable at 30°C, unless Int and Xis are provided.

expression by degrading a positive regulator of capsule synthesis. In the work described here, we have identified two positive regulators, *rcsA* and *rcsB*, which are candidates for such an unstable activator of *cps* expression.

One expected property for a positive regulator subject to proteolysis, which has a recognizable phenotype when proteolysis is decreased (as in a *lon* mutant), is that it should be limiting. Therefore, increasing the amount of this protein in the cell, for instance by increasing the copy number of the gene, may be sufficient to partially mimic the effect of a *lon* (protease deficient) mutation. Silverman and Simon (29) noted that introduction of the F′ 1334 episome into some strains results in mucoidy. We postulate that this results from the increase in gene dosage for the limiting positive regulator *rcsA*. *rcsA* cloned on a multicopy plasmid produces mucoidy in *lon*$^+$ cells (Torres-Cabassa and Gottesman, in preparation). Therefore, RcsA has the genetic properties expected of a limiting, unstable regulator of capsule synthesis, degraded by Lon. We suppose that the dominant *rcsA** mutations may result in overproduction of RcsA, formation of a more stable RcsA, or synthesis of a more active protein.

If Lon protein acts by degrading the RcsA product, how do *rcsB* and *rcsC* act? Preliminary data on multiple regulatory mutant strains suggest that *rcsC* acts before *rcsA* in the regulation of capsule synthesis. *rcsC137 rcsA*::Tn*10* double mutants, either in a *lon*$^+$ or *lon* background, do not synthesize capsular polysaccharide or express β-galactosidase from *cps*::*lac* fusions (Torres-Cabassa and Gottesman, in preparation). Thus, the increase in synthesis in *rcsC* mutants must require *rcsA*. *rcsA** *rcsB* double mutations vary in their effect on *cps*::*lac* fusions, depending on the particular alleles used (Torres-Cabassa and Gottesman, in preparation). Further studies using true null alleles of *rcsB* will be necessary to determine the order of action of *rcsA* and *rcsB* unambiguously.

Although *rcsA*, *rcsB*, and *rcsC* clearly regulate the synthesis of the capsular polysaccharide colanic acid, we have not determined whether they are involved in the regulation of other *E. coli* capsules or have a role in regulation of lipopolysaccharide synthesis, which shares many enzymatic similarities with colanic acid synthesis (20). We also have not yet determined what the normal cellular induction signals might be for capsular polysaccharide synthesis.

ACKNOWLEDGMENTS

rcsA and *rcsB* mutations were first isolated by a student, Dina Hirshfeld, during a summer project. *rcsC* mutations were isolated by another student, Ellen Anderson. We acknowledge their invaluable contribution to this work. We also thank Mary McCormick for the gift of packaged *E. coli* λ SV2 *cos* cosmid. We thank the members of the Prokaryotic Section of the Laboratory of Molecular Biology for suggestions and discussions during the course of this work. In addition, we thank Sankar Adhya, Max Gottesman, and Michael Maurizi for their comments on the manuscript. We are grateful to Annette Kuo for preparation of the manuscript.

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