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Characterization of *rcsB* and *rcsC* from *Escherichia coli* O9:K30:H12 and Examination of the Role of the *rcs* Regulatory System in Expression of Group I Capsular Polysaccharides

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In *Escherichia coli* K-12, RcsC and RcsB are thought to act as the sensor and effector components, respectively, of a two-component regulatory system which regulates expression of the slime polysaccharide colanic acid (V. Stout and S. Gottesman, J. Bacteriol. 172:659–669, 1990). Here, we report the cloning and DNA sequence of a 4.3-kb region containing *rcsC* and *rcsB* from *E. coli* O9:K30:H12. This strain does not produce colanic acid but does synthesize a K30 (group I) capsular polysaccharide. The *rcsB* gene from *E. coli* K30 ($rcsB_{K30}$) is identical to the *rcsB* gene from *E. coli* K-12 ($rcsB_{K-12}$). $rcsC_{K30}$ has 16 nucleotide changes, resulting in six amino acid changes in the predicted protein. To examine the function of the *rcs* regulatory system in expression of the K30 capsular polysaccharide, chromosomal insertion mutations were constructed in *E. coli* O9:K30:H12 to independently inactivate $rcsB_{K30}$ and the auxiliary positive regulator $rcsA_{K30}$. Strains with these mutations maintained wild-type levels of K30 capsular polysaccharide expression and still produced a K30 capsule, indicating that the *rcs* system is not essential for expression of low levels of the group I capsular polysaccharide in *lon*$^+$ *E. coli* K30. However, K30 synthesis is increased by introduction of a multicopy plasmid carrying $rcsB_{K30}$. K30 polysaccharide expression is also markedly elevated in an $rcsB_{K30}$-dependent fashion by a mutation in $rcsC_{K30}$, suggesting that the *rcs* system is involved in high levels of synthesis. To determine whether the involvement of the *rcs* system in *E. coli* K30 expression is typical of group I (K antigen) capsules, multicopy $rcsB_{K30}$ was introduced into 22 additional strains with structurally different group I capsules. All showed an increase in mucoid phenotype, and the polysaccharides produced in the presence and absence of multicopy $rcsB_{K30}$ were examined. It is has been suggested that *E. coli* strains with group I capsules can be subdivided based on K antigen structure. For the first time, we show that strains with group I capsules can also be subdivided by the ability to produce colanic acid. Group IA contains capsular polysaccharides (including K30) with repeating-unit structures lacking amino sugars, and expression of group IA capsular polysaccharides is increased by multicopy $rcsB_{K30}$. Group IB capsular polysaccharides all contain amino sugars. In group IB strains, multicopy $rcsB_{K30}$ activates synthesis of colanic acid.

Escherichia coli strains produce a variety of cell surface polysaccharides. Some of these polysaccharides are serotype specific, such as the lipopolysaccharide (LPS) O side chains (O antigen) and the capsular (K antigen) polysaccharides (CPSs). There are approximately 160 different O antigens in *E. coli*, and more than 70 *E. coli* K antigens are recognized (45). The K antigens are classified into one of two groups (designated I and II) by chemical, physical, and genetic criteria (28). Other cell surface polysaccharides, such as colanic acid (M antigen) (37) and enterobacterial common antigen (35), are not serotype specific and are found in many different strains of *E. coli* and in other enteric bacteria. The O and K antigens are thought to act as virulence determinants in pathogenic *E. coli* by providing resistance against complement-mediated serum killing (58) and phagocytosis (26), respectively. In contrast, the function(s) of colanic acid is unclear, since this polymer is generally produced only at low growth temperatures or on nitrogen-limited minimal medium with excess carbon (37). Recent experiments suggest that colanic acid and the group I K30 CPS may provide *E. coli* with some protection against dessication (43).

Colanic acid synthesis is regulated by the *rcs* system in *E. coli* K-12 (reviewed in reference 22). This system has three positive regulatory elements (RcsA, RcsB, and RcsF) and two negative regulators (RcsC and Lon). RcsB and RcsC are proposed to be the effector and sensor, respectively, of a two-component regulatory system (55). The activation of the effector, RcsB, may also involve the product of the recently described *rcsF* gene (20). RcsA is an auxiliary factor which may interact with RcsB to form a heterodimer required for increased transcription of the colanic acid synthesis (*cps*) genes (56). In *E. coli* K-12, RcsA is present in low amounts because it is a substrate for the ATP-dependent Lon protease (56, 59). In contrast to the colanic acid system, relatively little is known of the regulation of group I or group II K antigen CPS.

The *rcs* system is relatively common and regulates expression of structurally distinct polysaccharides in bacteria which occupy diverse ecological niches. *Erwinia* spp. use RcsA to regulate expression of both the species-specific extracellular polysaccharide and a levan polymer (4, 9, 11, 48, 60), and homologs of RcsB-RcsC have been identified (12, 49). RcsA has been identified in two strains of *Klebsiella*

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pneumoniae (1, 39) and was shown to play a role in K antigen expression (39). Although RcsB and RcsC have not been identified in *K. pneumoniae*, *E. coli* RcsB controls the expression of *K. pneumoniae* K2 polysaccharide when K2 biosynthetic genes are cloned in *E. coli* K-12 (62). Recent studies have shown that *rcsA* and *rcsB* also regulate expression of the Vi antigen, a polysaccharide produced by *Citrobacter freundii* and strains of *Salmonella typhi*, *S. paratyphi*, and *S. dublin* (27).

Colanic acid shares features with the group I *E. coli* capsular K antigens, including superficial structural and compositional similarities (18, 33) and the location of the biosynthetic *cps* gene cluster near the *his* genes (36, 53, 61, 66). We have recently shown that *E. coli* K30, a strain with a prototype group I capsule, does not synthesize colanic acid and that the biosynthesis genes for the K30 CPS and colanic acid may be allelic (31). Furthermore, we demonstrated that RcsA from *E. coli* K30 plays a role in regulation of the K30 CPS. Here, we extend these studies by examining the roles of the *rcsB* and *rcsC* genes in expression of the group I CPS from *E. coli* K30. The involvement of the *rcs* system in expression of other *E. coli* group I capsules was also investigated. The *rcs* genes from *E. coli* K-12 and *E. coli* O9:K30 are distinguished by the subscripts K-12 and K30, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* E69 is the type strain for the group I K30 capsular K antigen. *E. coli* CWG44 is a mutant with a wild-type K30 capsule but lacks the serotype O9 LPS O polysaccharide side chains; this strain is described elsewhere as B187 (38). The amount of K30 CPS synthesized by *E. coli* strains E69 and CWG44 is indistinguishable (64). Recombinant plasmids made by using the vectors pVK102 and pGP704 were mobilized by triparental plate matings with the helper plasmid pRK2013 in *E. coli* HB101. pGP704 constructs were made and maintained in *E. coli* SY327 λpir (40). All other plasmids were maintained in *E. coli* JM109. All strains were grown in either LB or M9 minimal medium containing 0.2% D-glucose. Media were supplemented when required with ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (15 μg/ml), amino acids (40 μg/ml), uracil (40 μg/ml), nicotinamide (10 μg/ml), and thiamine-HCl (1 μg/ml). Cultures were routinely grown at 37°C.

DNA manipulation. A cosmid gene bank was constructed from size-fractionated (20 to 25 kb) chromosomal DNA from *E. coli* E69; the DNA was obtained from partial digests with *Hin*dIII. DNA fragments were ligated to *Hin*dIII-digested pVK102, and the ligation mixtures were packaged into lambda particles. The resulting recombinant phage were transduced into *E. coli* LE392 (*lon*$^+$). Plasmid DNA was purified by an alkaline lysis method, and transformations were performed with $CaCl_2$-treated competent cells (51). Plasmid DNA sequencing was performed by the dideoxy chain termination method (52) with Sequenase version 2.0 (USB). Both strands were sequenced with sequential custom oligonucleotide primers which were obtained from Vetrogen Corp. (London, Ontario, Canada) or synthesized with an Applied Biosystems oligonucleotide synthesizer (model 391-EP). Southern hybridizations were performed by standard protocols (51), with Zeta-Probe membranes (Bio-Rad Laboratories). DNA probes were labeled with [α-^{32}P]dATP with the random-primer DNA-labeling system from Bethesda Research Laboratories. Enzymes were purchased either from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or from Boehringer Mannheim Canada (Laval, Quebec, Canada) and used as recommended by the manufacturer.

Analysis and composition of cell surface polysaccharides. Cell-free and cell-bound polysaccharides were prepared as described previously (31). Briefly, colonies grown on agar plates were scraped into saline and resuspended with a Vortex mixer. The cells were collected by centrifugation. The supernatant containing cell-free polysaccharides was treated with DNase, RNase, and finally proteinase K, dialyzed against water, and lyophilized. Cell-bound polysaccharides were extracted from the cells by a modification (31) of the hot aqueous phenol method originally described by Westphal and Jann (63). The sugar composition of acid-hydrolyzed cell surface polysaccharides was determined by the Dionex high-pressure liquid chromatography (HPLC) procedure, which has been described in detail elsewhere (10). The amount of K30 polysaccharide was determined from the glucuronic acid content (a K30-specific marker) and expressed as micrograms of uronic acid per 10^7 bacterial cells. However, the colorimetric method used previously to assay uronic acid (31) was replaced by quantitation with the Dionex HPLC (10). Calibration was done with D-glucuronolactone as the standard.

Nucleotide sequence accession number. The $rcsB_{K30}$-$rcsC_{K30}$ sequence reported has been entered in GenBank under accession number L11272.

RESULTS

Cloning and characterization of the $rcsB_{K30}$ and $rcsC_{K30}$ genes from *E. coli* K30. In *E. coli* K-12 (LE392), plasmids containing the $rcsA_{K30}$ (31) and $rcsB_{K30}$ genes activated the synthesis of colanic acid. The resulting mucoid colonies were readily identified, and the production of colanic acid was confirmed by polysaccharide composition analysis. Two types of mucoid colonies were identified, with visible differences in the amount of colanic acid synthesized by each type. Plasmids were isolated from each type of colony and used to transform *E. coli* VS20186 and VS20187, which have characterized defects in $rcsA_{K-12}$ and $rcsB_{K-12}$, respectively (Table 2). Plasmids from highly mucoid colonies complemented only *E. coli* VS20186 (32). The properties of these plasmids therefore resembled those of pWQ504 ($rcsA_{K30}^+$) (Table 2), which has been described previously (31). The presence of $rcsA_{K30}$ on these new plasmids was confirmed by physical mapping and Southern hybridization (30). From the restriction enzyme digest profiles, plasmids isolated from the less-mucoid recombinants were all identical, and plasmid pWQ600 was selected for further study.

pWQ600 complemented the mutations in both *E. coli* VS20186 and VS20187 to give mucoid recombinants (Table 2). These complementation results suggested that pWQ600 contained $rcsB_{K30}$, since it has been established with *E. coli* K-12 that multicopy RcsB can overcome the requirement for RcsA and stimulate colanic acid expression in an *rcsA* mutant background. In contrast, multicopy RcsA can not induce a mucoid phenotype in the absence of RcsB (22). Southern hybridization experiments determined that pWQ600 does not carry $rcsA_{K30}$ (32).

The physical map of pWQ600 is shown in Fig. 1. Subclones of pWQ600 were constructed in an attempt to localize the region of the plasmid responsible for the complementation. The derivative pWQ603 (Fig. 1) retained the ability to

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
LE392	F⁻ *hsdR514* (r_K^- m_K^-) *supE44 supF58 lacY1* or Δ(*lac-proAB*)*6 galK2 galT22 metB1 trpR55* λ⁻	51
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* λ⁻ Δ(*lac-proAB*) [F′ *traD36 proAB lac*^{q}ZΔM15]	68
HB101	F⁻ *hsdS20* (r_B^- m_B^-) *supE44 recA13 ara-14 proA2 rpsL20* Str^r *xyl-5 mtl-1 supE44 lacY1 galK2* λ⁻	51
SY327 λ*pir*	K-12 λ*pir araD* Δ(*lac-pro*) *argE recA nalA* Rif^r	40
VS20186	MC4100 but *lon-100 rcsA51*::ΔKan	V. Stout
VS20187	MC4100 but *lon-100 rcsB62*::ΔKan	V. Stout
G3404-41	Serotype O8:K8:H⁻	F. Ørskov
2667	Serotype O9:K9:H⁻	B. Jann
2146	Serotype O9:K26:H⁻	B. Jann
E56b	Serotype O8:K27:H⁻	F. Ørskov
K14a	Serotype O9ab:K28:H⁻	F. Ørskov
Bi161-42	Serotype O9:K29:H⁻	F. Ørskov
Su3973-41	Serotype O9:K31:H⁻	F. Ørskov
E75	Serotype O9:K34:H⁻	F. Ørskov
2150	Serotype O9:K37:H⁻	B. Jann
2151	Serotype O9:K39:H9	B. Jann
2775	Serotype O8:K40:H9	B. Jann
2176	Serotype O8:K41:H11	B. Jann
A295b	Serotype O8:K42:H⁻	F. Ørskov
2178	Serotype O8:K43:H11	B. Jann
2179	Serotype O8:K44:H⁻	B. Jann
2167	Serotype O8:K45:H⁻	F. Ørskov
2181	Serotype O8:K46:H4	B. Jann
2182	Serotype O8:K47:H2	B. Jann
2183	Serotype O8:K48:H9	B. Jann
2184	Serotype O8:K49:H21	B. Jann
2185	Serotype O8:K50:H9	B. Jann
N24c	Serotype O9:K55:H⁻	F. Ørskov
D227	Serotype O8:K87:H19	F. Ørskov
E69	Serotype O9:K30:H12, prototroph	I. Ørskov
CWG44	E69 but O⁻:K30:H12 rfb_{O9} *his trp lac* Str^r	38
CWG120	E69 but *rcsA*; pGP704 insertion, Ap^r	This study
CWG121	E69 but *rcsB*; pGP704 insertion, Ap^r	This study
CWG123	CWG44 but *rcsC*; isolated following ethyl methanesulfonate mutagenesis	This study
CWG131	CWG123 but *rcsB*; pGP704 insertion	This study
Plasmids		
pVK102	Cosmid vector, Tc^r Km^r	34
pRK2013	Helper plasmid; RK2 derivative, Km^r Mob$^+$ Tra$^+$ ColE1	14, 19
pUC19	Cloning vector, Ap^r	68
pGP704	pJM703.1 derivative, *oriR6K mobRP4* Ap^r	40
pWQ504	pVK102 derivative containing 5-kb *Sal*I fragment; $RcsA_{K30}^+$ Km^r	31
pWQ600	pVK102 derivative containing 27-kb insert of *E. coli* E69 DNA; $RcsB_{K30}^+$ $RcsC_{K30}^+$ Tc^r	This study
pWQ602	Deletion derivative of pWQ600 containing 20.2-kb insert; $RcsC_{K30}^+$ Tc^r	This study
pWQ603	pUC19 derivative containing 3.7-kb *Sal*I fragment from pWQ600; $RcsB_{K30}^+$ Ap^r	This study
pWQ510	pGP704 containing 287-bp internal *Hin*dII-*Eco*RV fragment from $rcsA_{K30}$, Ap^r	This study
pWQ650	pGP704 containing 288-bp internal *Eco*RV fragment from $rcsB_{K30}$	This study

complement the mutations in *E. coli* VS20186 and VS20187 (Table 2). The presence on pWQ600 and pWQ603 of *rcsB* sequences similar to that in *E. coli* K-12 was rapidly detected by polymerase chain reaction amplification (30), with primers designed from the sequence available for *E. coli* K-12 *rcsB* (55), and confirmed by DNA sequence analysis as described below. Interestingly, removal of the region later identified as $rcsC_{K30}$, in the subclone pWQ603, resulted in a substantial increase in the amount of visible colanic acid produced by *E. coli* K-12 recombinants and a phenotype indistinguishable from that of recombinants with plasmids harboring $rcsA_{K30}$ (30).

The sequence of a 4,295-bp region of pWQ600 containing $rcsB_{K30}$ and $rcsC_{K30}$ was determined and compared with the sequence available for the same region in *E. coli* K-12 (55). The 651-bp open reading frames (ORFs) for the *rcsB* genes were identical in *E. coli* K-12 and K30, and the DNA sequences flanking *rcsB* were also identical for the upstream 480-bp region examined. The DNA upstream of $rcsB_{K-12}$ described by Stout and Gottesman (55) includes a putative RpoN-activated promoter at bp −121 to −133. Recent studies by Gervais et al. (21) also identified a σ^{70} promoter and a LexA binding site upstream (positions −202 to −218) of *rcsB* in *E. coli* K-12. All of these features were found upstream of *rcsB* in *E. coli* K30. In *E. coli* K-12, a further undefined sequence extending approximately 400 bp upstream of *rcsB* is required for the expression of *rcsB* from multicopy plasmids (21). Although this region in *E. coli* K-12 has not been characterized, both pWQ600 and pWQ603

TABLE 2. Complementation of *rcsA* and *rcsB* mutations in *E. coli* K-12 with plasmids containing DNA from *E. coli* O9:K30:H12

Plasmid	Mucoid phenotype[a]	
	E. coli VS20186 (*lon rcsA*)	*E. coli* VS20187 (*lon rcsB*)
None	−	−
pWQ600 (*rcsB*$^+$ *rcsC*$^+$)	+	+
pWQ602 (*rcsC*$^+$)	−	−
pWQ603 (*rcsB*$^+$)	++	++
pWQ504 (*rcsA*$^+$) (control)[b]	++	−

[a] Assessed visually after 18 h of growth on LB at 37°C.
[b] From reference 31.

contain sufficient flanking DNA to carry the corresponding portion of the *E. coli* K30 chromosome.

The *rcsB* and *rcsC* ORFs are separated by 196 bp in *E. coli* K-12 (55). This region is 199 bp long in *E. coli* K30. Conserved repeat sequences, homologous to consensus repetitive extragenic palindromic sequences, are found in both *E. coli* K-12 (55) and *E. coli* K30.

$rcsC_{K30}$ is a 2,802-bp ORF, identical in size to its counterpart in *E. coli* K-12 (55). Sixteen nucleotide changes occurred in the $rcsC_{K30}$ sequence, and once translated, the deduced protein sequence for $RcsC_{K30}$ contained six amino acid changes compared with $RcsC_{K-12}$ (Fig. 2). Four of the changes occur in the terminal 125-amino-acid region; this region has limited homology with the N termini of effector proteins (55).

Construction and characterization of chromosomal *rcsA* and *rcsB* mutations. To directly determine the effects of these positive regulators on K30 synthesis, chromosomal mutations were constructed in $rcsA_{K30}$ and $rcsB_{K30}$. These constructs were made in *E. coli* E69 (*lon*$^+$). Internal fragments from the *rcsA* (pWQ510) and *rcsB* (pWQ650) genes were cloned in the suicide plasmid pGP704 (40) by the strategy shown in Fig. 3. These plasmids were then transferred to *E. coli* E69 by conjugation, and ampicillin-resistant recombinants were selected. Because *E. coli* K30 lacks the *pir* gene product required for pGP704 replication, homologous recombination between genes cloned in pGP704 and the chromosomal copy is required to rescue the plasmid sequences and allow growth on ampicillin. Integration of the plasmid disrupts the chromosomal gene. Four strains each of the putative *rcsA* and *rcsB* mutants were selected randomly for further analysis. The strains proved to be indistinguishable, and only the results for *E. coli* CWG120 ($rcsA_{K30}$) and CWG121 ($rcsB_{K30}$) are presented.

The mutations in CWG120 and CWG121 were confirmed by Southern hybridization (Fig. 4). Chromosomal DNA from each strain was digested with *Eco*RI and *Sal*I, which cut within pGP704 but not in either of the target genes. Interruption of *rcsA* or *rcsB* is therefore indicated by the introduction of single *Eco*RI and *Sal*I sites into the inactivated gene. As expected, the *rcsA* probe hybridized to single 23-kb *Eco*RI and 10.5-kb *Sal*I fragments in both *E. coli* E69 and CWG121 (*rcsB*). Two fragments each were detected in the *Eco*RI (16 and 10 kb) and *Sal*I (13.5 and 1 kb) digests of *E. coli* CWG120 (*rcsA*). The increase in the total size of the fragments detected by hybridization corresponded to the size of pWQ510 (approximately 4 kb). Similarly, the *rcsB* probe hybridized to single 9-kb *Eco*RI and 4-kb *Sal*I fragments in *E. coli* E69 and CWG120 (*rcsA*). In *E. coli* CWG121, two *Eco*RI (8.5 and 4.5 kb) and two *Sal*I (1 and 7 kb) fragments were detected.

K30 polysaccharide expression in *rcsA* and *rcsB* mutants. Colonies of *E. coli* CWG120 (*rcsA*) and CWG121 (*rcsB*)

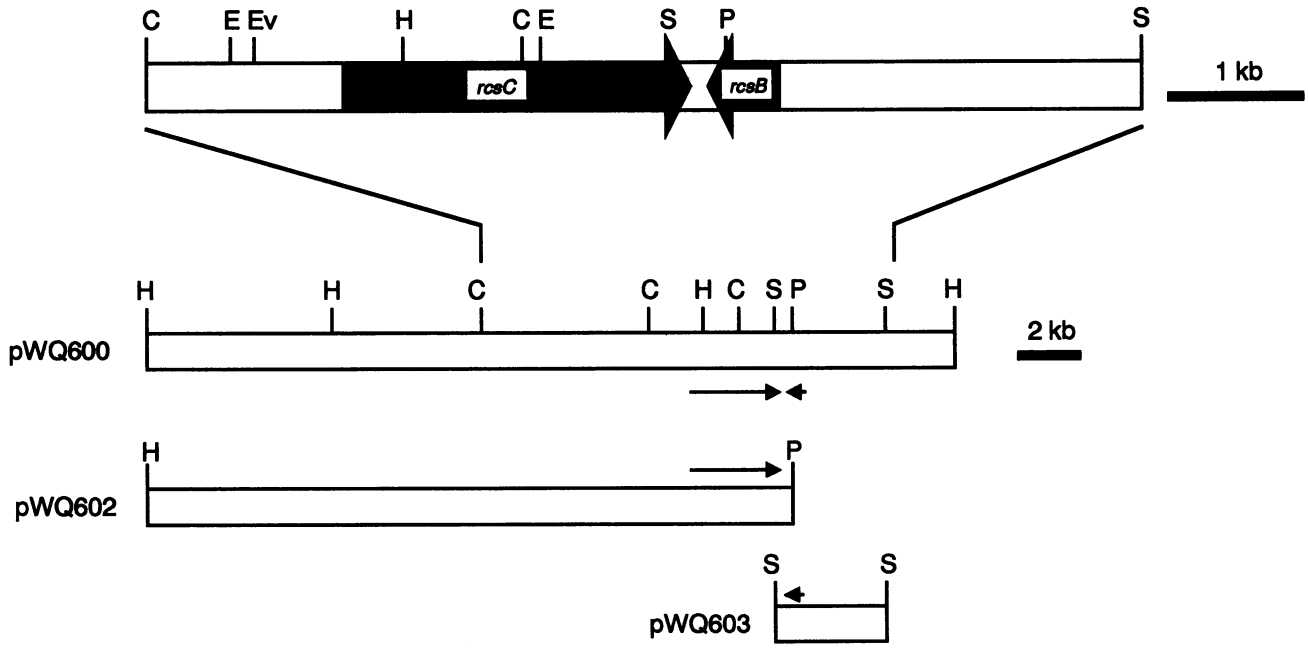

FIG. 1. Physical map of pWQ600 and its derivatives, containing the *rcsB-rcsC* region of the chromosome of *E. coli* O9:K30:H12. pWQ600 contains a 27-kb *Hin*dIII fragment of chromosomal DNA. Subclone pWQ602 is a deletion derivative of pWQ600 and contains *rcsC* and upstream flanking DNA in pVK102. pWQ603 is a pUC19 derivative containing *rcsB* and upstream flanking DNA. The positions and directions of transcription of the *rcsB* and *rcsC* genes were established by DNA sequence analysis. Only insert DNA is shown. The enzymes used were *Cla*I (C), *Eco*RI (E), *Eco*RV (Ev), *Hin*dIII (H), *Pst*I (P), and *Sal*I (S).

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K-12  MFRALALVLWLLIAFSSVFYIVNALHQRESEIRQEFNLSSDQAQRFIQRTSDVMKELKYIAENRLSAENGVLSPRGRETQADVPAFEPLFADSDCSAMSN 100
K30   ....................................................................................................
K-12  TWRGSLESLAWFIGYWRDNFSAAYDLNRVFLIGSDNLCMANFGLRDMPVERDTALKALHERINKYRNAPQDDSGSNLYWISEGPRPGVGYFYALTPVYLA 200
K30   ....................................................................................................
K-12  NRLQALLGVEQTIRMENFFLPGTLPMGVTILDENGHTLISLTGPESKIKGDPRWMQERSWFGYTEGFRELVLKKNLPPSSLSIVYSVPVDKVLERIRMLI 300
K30   ...................................................................................................V.
K-12  LNAILLNVLAGAALFTLARMYERRIFIPAESDALRLEEHEQFNRKIVASAPVGICILRTADGVNILSNELAHTYLNMLTHEDRQRLTQIICGQQVNFVDV 400
K30   ....................................................................................................
K-12  LTSNNTNLQISFVHSRYRNENVAICVLVDVSSRVKMEESLQEMAQAAEQASQSKSMFLATVSHELRTPLYGIIGNLDLLQTKELPKGVDRLVTAMNNSSS 500
K30   ....................................................................................................
K-12  LLLKIISDILDFSKIESEQLKIEPREFSPREVMNHITANYLPLVVRKQLGLYCFIEPDVPVALNGDPMRLQQVISNLLSNAIKFTDTGCIVLHVRADGDY 600
K30   ....................................................................................................
K-12  LSIRVRDTGVGIPAKEVVRLFDPFFQVGTGVQRNFQGTGLGLAICEKLISMMDGDISVDSEPGMGSQFTVRIPLYGAQYPQKKGVEGLSGKRCWLAVRNA 700
K30   ....................................................................................................
K-12  SLCQFLETSLQRSGIVVTTYEGQEPTPEDVLITDEVVSKKWQGRAVVTFCRRHIGIPLEKAPGEWVHSVAAPHELPALLARIYLIEMESDDPANALPSTD 800
K30   ...................................................E................................................
K-12  KAVSDNDDMMILVVDDHPINRRLLADQLGSLGYQCKTANDGVDALNVLSKNHIDIVLSDVNMPNMDGYRLTQRIRQLGLTLPVIGVTANALAEEKQRCLE 900
K30   ...................................................T................................................
K-12  SGMDSCLSKPVTLDVIKQSLTLYAERVRKSRDS                                                              933
K30   ................T..V.....E.
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FIG. 2. Alignment of the RcsC proteins from *E. coli* K-12 and *E. coli* K30. Amino acids in the *E. coli* K30 sequence identical to those in the K-12 sequence are represented by dots.

showed no obvious differences in mucoid phenotype compared with the wild type. All produced K30 antigen, detectable by K30 CPS-specific monoclonal antibodies (25) in agglutination reactions, and all were lysed by K30 CPS-specific bacteriophage (65). In *E. coli* E69, the K30 CPS provides a barrier to exclude O9 LPS-specific bacteriophage (38). This barrier remained in the $rcsA_{K30}$ and $rcsB_{K30}$ mutants. No significant differences were detected in the amount and distribution of K30 polysaccharide synthesized by the wild type and the *rcsA* and *rcsB* mutants, and only the data for CWG121 (*rcsB*) are presented (Table 3). Electron microscopy revealed no obvious changes in the morphology of the capsule in the mutants (32). The capsular phenotype was therefore unaltered by chromosomal mutations in $rcsA_{K30}$ and $rcsB_{K30}$ under laboratory growth conditions (LB medium, 37°C).

Role of *rcs* genes in high-level expression of K30 polysaccharide. In *E. coli* K-12, multicopy *rcsA* elevates expression of colanic acid in an *rcsB*-dependent fashion (22). To determine whether the same requirement holds in K30 CPS expression, plasmids pWQ504 ($rcsA_{K30}^+$), pWQ600 ($rcsC_{K30}^+$ $rcsB_{K30}^+$), and pWQ603 ($rcsB_{K30}^+$) were introduced into *E. coli* strains E69, CWG120, and CWG121 (Table 4). As with *E. coli* K-12, multicopy $rcsA_{K30}$ caused increased mucoid phenotype only in the presence of a functional $rcsB_{K30}$ gene. Multicopy $rcsB_{K30}$ overcame the requirement for $rcsA_{K30}$. Introduction of pWQ600 elevated the level of cell-bound K30 CPS by 8-fold and of the cell-free polymer by 49-fold (Table 3).

We previously reported a highly mucoid *lon* mutant which overproduced K30 polysaccharide (31), indicating that increased levels of K30 polysaccharide can be obtained without introduction of multicopy plasmids. Three other highly mucoid K30 strains were also isolated, and these mutants lack the UV sensitivity and filamentation phenotypes expected of *lon* mutants (37). One mutant, *E. coli* CWG123, was complemented by pWQ602, consistent with an *rcsC* defect. The nature of the other mutations has not been established. *E. coli* CWG123 shows an 11-fold increase in cell-associated K30 CPS and a 21-fold increase in cell-free CPS (Table 3). Introduction of pWQ602 into *E. coli* CWG123 reduced the amounts of both forms of polymer to levels comparable to those in the wild-type strain. Wild-type levels of K30 polysaccharide synthesis were also restored in *E. coli* CWG131 (Table 4). *E. coli* CWG131 was constructed by introducing an *rcsB* defect into *E. coli* CWG123 by the mutagenesis strategy described above (32). The amount of K30 synthesis in CWG131 could be increased by multicopy *rcsB* (pWQ603) but not by *rcsA* (pWQ504). These results show that the elevated production of K30 polysaccharide in the *rcsC* mutant is RcsB dependent and confirm the central role for *rcsB* in high-level expression of K30 CPS.

In all of these experiments, the polysaccharide products were confirmed to be K30 CPS by HPLC analysis.

Effect of multicopy $rcsB_{K30}$ on extracellular polysaccharides produced by other *E. coli* strains with group I capsules. In order to determine whether *rcs* gene products influenced group I capsule expression in other *E. coli* serotypes, pWQ600 was introduced into 22 additional strains by triparental mating experiments. The structures of 15 of these K antigens have been determined (Table 5). Ten of the K antigens (group IA; K26, K27, K28, K30, K31, K34, K37, K39, K42, and K55) have similar structural features. All contain one or more neutral sugars, uronic acid residues, and, in some cases, *O*-acetyl and pyruvate substitutions (Table 5). Five strains (group IB; K8, K9, K40, K44, and K87) produced CPS with amino sugars. pWQ600 increased the mucoid phenotype of all strains examined, with the effect being most evident in strains with group IB capsules. When the sugar composition of the cell-free extracellular polysaccharide fractions was analyzed by HPLC, strains with group IA capsules were found to produce polysaccharides with the same composition in the presence and absence of pWQ600 (Table 6). The composition was that expected of the K

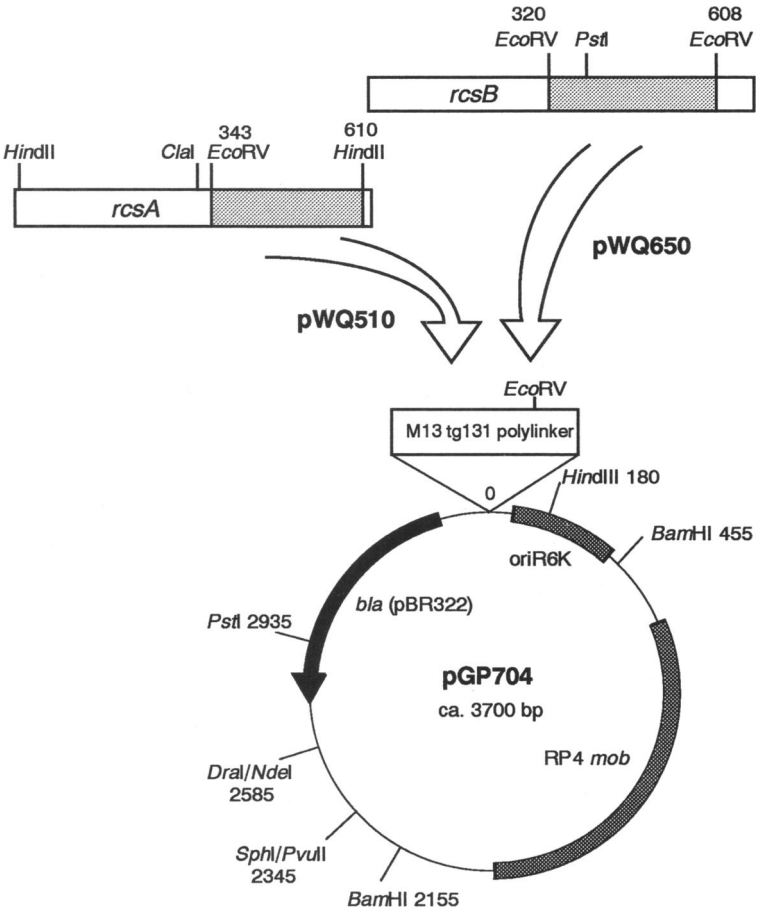

FIG. 3. Strategy for the construction of suicide plasmids pWQ510 and pWQ650. The hatched areas indicate the internal $rcsA_{K30}$ and $rcsB_{K30}$ fragments cloned into pGP704. Plasmid pWQ510 contains the 267-bp *Hin*dII-*Eco*RV fragment from the 3′ end of $rcsA_{K30}$. Plasmid pWQ650 contains a 288-bp *Eco*RV fragment from $rcsB_{K30}$. Both fragments were cloned into the *Eco*RV site of pGP704.

antigen, suggesting that other group IA K antigens (like K30) are regulated by Rcs proteins. In contrast, strains with group IB capsules all produced colanic acid in the presence of pWQ600 (Table 6). No group IB K antigen could be detected in the extracellular polysaccharide fractions from strains containing pWQ600 because of the large amount of colanic acid. However, some K antigen was detectable in a cell-bound polysaccharide preparation (32).

The structures of eight additional group I K antigens (K41, K43, K45, K46, K47, K48, and K50) have not been determined previously. The sugar components of each of these K antigens were tentatively determined by HPLC; detailed compositional analysis was not performed, and the preliminary analysis reported here does not identify *O*-acetyl and pyruvate groups. As indicated in Table 5, six of the unknown structures contained amino sugars, detected as GlcN and GalN in HPLC. K43 lacked amino sugars and is therefore a group IA K antigen. In the presence of pWQ600, the polysaccharide produced by the K43 isolate had a composition identical to that of the K antigen. The remaining six strains all produced colanic acid when pWQ600 was introduced, extending the correlation between the presence of an amino sugar-containing K antigen and ability to produce colanic acid (Table 6).

DISCUSSION

In *E. coli* K-12, the *rcs* gene products interact with the *cps* biosynthetic genes to regulate the production of the slime polysaccharide colanic acid (22). In a previous article, we described the $rcsA_{K30}$ gene from *E. coli* K30 and showed that it is nearly identical to its $rcsA_{K-12}$ counterpart (31). $RcsA_{K30}$ was implicated in the regulation of the group I K30 CPS because multicopy plasmids containing $rcsA_{K30}$ increased the amount of K30 polysaccharide produced. The results presented here show that $rcsA_{K30}$ activation of K30 polysaccharide synthesis is $rcsB_{K30}$ dependent, as is the case in *E. coli* K-12 (22). The $rcsB_{K30}$ gene is identical to the *E. coli* K-12 version, and $rcsC_{K30}$ is highly conserved. From protein sequence homologies with other two-component regulatory systems, $RcsC_{K-12}$ and $RcsB_{K-12}$ were originally proposed to be the sensor-kinase and effector proteins, respectively, of a two-component regulatory system (55). The model for their action was based on similarities to other two-component regulatory proteins and involves autophosphorylation of the sensor (RcsC) in response to environmental stimuli, followed by phosphotransfer from the sensor-kinase to the effector (RcsB) (22).

Recent studies have implicated an additional component (RcsF) in the activation of RcsB, and it has been proposed

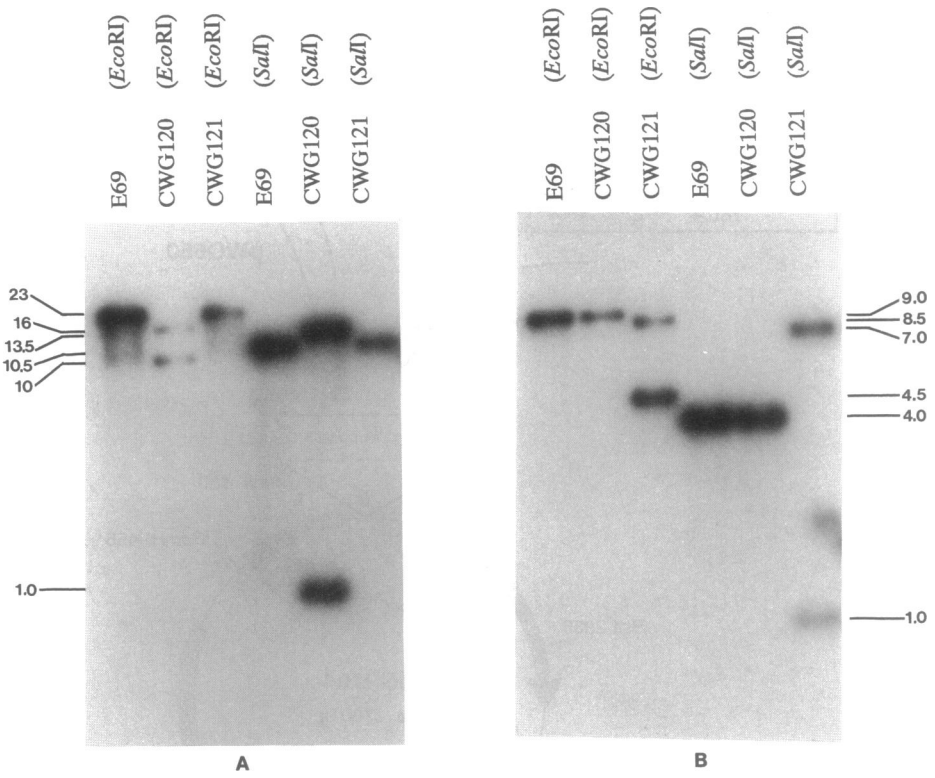

FIG. 4. Confirmation by Southern hybridization of the insertion mutations in $rcsA_{K30}$ (CWG120) and $rcsB_{K30}$ (CWG121). Genomic digests of *E. coli* strains E69, CWG120, and CWG121 were probed with [α-^{32}P]dATP-labeled $rcsA_{K30}$ (A) and $rcsB_{K30}$ (B) probes. The probes consisted of the complete ORFs, isolated without flanking DNA by polymerase chain reaction amplification. The source of the DNA and the restriction enzyme used for each sample are indicated above each lane. The sizes of the fragments (kilobase pairs) are indicated on the outsides of the panels.

that RcsF is the kinase and that the RcsC sensor is involved in dephosphorylation (20, 21). However, phosphorylation of RcsB has not been directly demonstrated. In *E. coli* K-12, RcsB regulates transcription of *cps* (colanic acid synthesis) genes. The near identity of the Rcs proteins from *E. coli* K30 and *E. coli* K-12, together with the ability of the K30 proteins to complement defects in *E. coli* K-12, suggests that the K30 CPS is regulated in a similar fashion. An *E. coli* K30 *rcsC* mutant shows elevated expression of K30 CPS, and the induction is RcsB dependent. Therefore, RcsB plays a central role in induction of high-level CPS synthesis in both *E. coli* K-12 and *E. coli* K30. By analogy with the colanic acid system, the *rcsC* defect in *E. coli* CWG123 most likely affects dephosphorylation of $rcsB_{K30}$, leading to overproduction of K30 CPS.

No colanic acid is produced in *E. coli* K30, and evidence has been presented suggesting that the biosynthetic genes for K30 CPS and colanic acid are allelic (31). Other strains with group I K antigens have not been investigated in such detail, but the analysis presented above suggests that a similar situation exists for other group IA K antigens. However, we cannot rule out the possibility that some strains contain an

TABLE 3. Influence of *rcs* gene products on the amount and distribution of K30 CPS synthesized by *E. coli* strains

Strain	Cell-bound K30 CPS		Cell-free K30 CPS	
	Amt[a]	Fold increase[b]	Amt	Fold increase
E69	0.03		0.03	
CWG121 (*rcsB*)	0.02		0.03	
CWG121(pWQ600)	0.24	8	1.47	49
CWG123 (*rcsC*)	0.34	11	0.63	21
CWG123(pWQ602)	0.05	2	0.01	0

[a] Values are the averages of triplicate samples and are given as micrograms of uronic acid per 10^7 bacteria.
[b] Compared with wild-type strain.

TABLE 4. Ability of multicopy *rcsA* and *rcsB* to stimulate a highly mucoid phenotype by overproduction of K30 polysaccharide in *E. coli* K30 derivatives[a]

Plasmid	Overproduction of K30 CPS				
	E69 (*rcsA*$^+$ *rcsB*$^+$)	CWG120 (*rcsA*)	CWG121 (*rcsB*)	CWG123 (*rcsC*)	CWG131 (*rcsC rcsB*)
None	−	−	−	+	−
pWQ504 (*rcsA*$^+$)[b]	+	+	−	+	−
pWQ600 (*rcsB*$^+$ *rcsC*$^+$)	+	+	+	NT[c]	NT
pWQ603 (*rcsB*$^+$)	+	+	+	+	+
pWQ602 (*rcsC*$^+$)	−	−	−	−	NT

[a] Phenotypes were assessed by colony morphology and visible increase in CPS production after growth on LB for 18 h at 37°C. Each derivative was compared with the parental strain without added plasmid.
[b] Plasmid described in reference 31.
[c] NT, not tested.

TABLE 5. Composition of *E. coli* group I K antigens (CPSs)[a]

K serotype	Group	Amino sugars	Neutral sugars	Uronic acids	Other components	Reference
K8	IB	GlcNAc, GalNAc	Gal	GlcA	*O*-Acetyl	18
K9	IB	GalNAc	Gal		*O*-Acetyl, NeuNAc	17
K26	IA		Rha, Gal	GlcA	Pyruvate	5
K27	IA		Glc, Fuc, Gal	GlcA		29
K28	IA		Glc, Fuc, Gal	GlcA	*O*-Acetyl	2
K30	IA		Man, Gal	GlcA		7
K31	IA		Glc, Gal, Rha	GlcA		18
K34	IA		Gal, Glc	GlcA		16
K37	IA		Gal, Glc	GlcA	Pyruvate	3
K39	IA		Glc, Man, Gal	GlcA		47
K40	IB	GlcNAc		GlcA	L-Serine	13
K41	IB	GlcNAc	Gal, Glc, Man	GalA, GlcA		This study
K42	IA		Gal, Fuc	GalA		42
K43	IA		Fuc, Gal, Man	GalA		This study
K44	IB	GlcNAc, GalNAc	Rha	GlcA		15
K45	IB	GlcNAc	Gal, Glc, Man	GalA		This study
K46	IB	GalNAc, GlcNAc	Gal, Glc, Man			This study
K47	IB	GlcNAc	Gal, Glc, Man			This study
K48	IB	GlcNAc	Gal, Glc, Man, Fuc	GlcA		This study
K49	IB	GlcNAc	Gal, Glc	GlcA	L-Threonine, L-serine	This study
K50	IB	GalNAc, GlcNAc	Gal, Glc, Man			This study
K55	IA		Man	GlcA	Pyruvate, *O*-acetyl	18
K87	IB	FucNAc, GlcNAc	Glc, Gal	GlcA	*O*-Acetyl(?)	57

[a] Abbreviations: GlcA, glucuronic acid; GalA, galacturonic acid; NeuNAc, *N*-acetylneuraminic acid.

inactive colanic acid biosynthesis gene cluster. Until the clusters are cloned and appropriate specific gene probes are available, it is not possible to resolve this question. *E. coli* strains with group IB K antigens are more complicated and have functional clusters for both the K antigen and colanic acid. The chromosomal location has not been determined for any of the group IB K-antigen biosynthesis gene clusters, although they are assumed to map near *his* (28). At 37°C, no detectable colanic acid is produced by *E. coli* strains with group IB K antigens unless multicopy *rcsB* is present. It was not possible to determine whether expression of the group IB CPS was also elevated in response to *rcsB* because of the large amount of colanic acid produced.

In *E. coli* K-12, colanic acid is synthesized at low temperatures (below 30°C) or during growth on nitrogen-poor, carbon-rich media (27). In *E. coli* K-12 strains grown at 32°C, a basal level of β-galactosidase is expressed from *cps*::*lac* fusions, and a basal amount of colanic acid (5 μg of nondialyzable methylpentose per ml of culture) is produced (23). In *E. coli* K-12 grown at 32°C, the effect of *rcsA* and *rcsB* mutations depends to a certain extent on the method of analysis (23). Transcription of *cps*::*lac* fusions is dramatically (>20-fold) reduced if *rcsA* and *rcsB* mutations are introduced into a *lon* background. However, the levels of β-galactosidase activity in *rcsA lon* and *rcsB lon* mutants are reduced by only one- to twofold in comparison to the *lon*$^+$ *rcsA*$^+$ *rcsB*$^+$ control. These small reductions are not correlated with a significant effect on the expression of basal levels of colanic acid if polysaccharide product rather than transcription (β-galactosidase activity) is measured. Synthesis of K30 CPS is not significantly reduced by *rcsA* and *rcsB* mutations in *E. coli* K30 at 37°C. In this respect, the major difference between the colanic acid and K30 systems lies in the amount of "basal" polysaccharide synthesis.

The *rcs* system appears to be more important for high levels of expression. Mutations in *E. coli* K-12 affecting *lon* and *rcsC* (*rcsC137*) increase transcription of two different *cps*::*lacZ* fusions by 10- to 46-fold and 116- to 226-fold, respectively. This equates to 13- and 28-fold increases in colanic acid polysaccharide production in *lon* and *rcsC137* mutants, respectively (23). In *E. coli* K30, *lon* mutations increase cell-free CPS production by 3-fold (31), and as described above, an $rcsC_{K30}$ mutation elevates cell-free K30 CPS levels by 21-fold. Whether the difference between the levels of synthesis in the basal and induced states in the two systems indicates an additional level of regulation or merely reflects slightly altered interactions between the components of similar systems remains to be established.

It is difficult to compare *E. coli* K-12 and *E. coli* K30 directly for a number of reasons. First, 50% of the K30 CPS is surface attached to form a capsular structure in wild-type *E. coli* K30, whereas colanic acid is a slime polysaccharide.

TABLE 6. Effect of multicopy $rcsB_{K30}$ on *E. coli* group I capsule expression[a]

K antigen group	Polysaccharide synthesized		
	Serotypes	Wild type (no plasmid)	Wild type plus pWQ600
IA	K26, K27, K28, K30, K31, K34, K37, K39, K42, K43, K55	K antigen	K antigen
IB	K8, K9, K40, K41, K44, K45, K46, K47, K48, K49, K50, K87	K antigen	Colanic acid plus K antigen

[a] The polysaccharide composition was determined by HPLC analysis.

However, any factor which increases CPS synthesis in *E. coli* K30 causes an altered distribution, resulting in more cell-free polymer. Second, the growth temperatures are different. The K30 capsule is produced at 37°C, and the reason(s) for the lower amounts of colanic acid synthesis in *E. coli* K-12 at 37°C (37) is unclear. Finally, we and others have observed variability in the amounts of colanic acid synthesized in different *E. coli* K-12 strains. The recent report of complex regulation of *rcsB* and its potential interaction with *ftsZ* (21), together with the identification of a new factor (*rcsF*) which also mediates *rcsB*-dependent elevation of colanic acid synthesis (20), suggests that colanic acid synthesis is potentially affected by a variety of factors influenced by genetic background.

It is not established whether environmental conditions exist in vivo which stimulate in wild-type *E. coli* the high levels of polymer synthesis evident in the *lon* and *rcsC* mutants. The environmental cues to which the *rcs* system responds are unclear. There is some evidence that *rcsC* may respond to perturbations in the structure of the outer membrane (46), but these changes are drastic and may not reflect the signal(s) for which *rcsC* is intended. *E. coli* group I CPSs, formerly known as K(A) antigens (45), have been implicated as virulence factors. Group I CPSs are frequently found in enterotoxigenic and enteropathogenic *E. coli* isolates (44), in which they enhance the ability of the bacterium to colonize epithelial surfaces in piglets, lambs, and calves (24, 41, 54). Heavily encapsulated *E. coli* K30 cells have been visualized colonizing the epithelia of calves (8). However, CPS is not involved in adhesion per se, and the presence of K30 CPS may actually hinder the adhesive properties of K99 pili (50).

E. coli group I CPSs are important for evasion of phagocytosis. For example, the group I K29 CPS does not fix complement in the absence of specific antibody and masks underlying structures which do bind complement (26). Colanic acid has no obvious role in pathogenesis, and in most strains grown in the laboratory at 37°C, colanic acid production is minimal (37). In *Erwinia* spp. (4, 9, 11, 12, 48, 49, 60) and *Klebsiella* spp. (1, 39, 62), the CPSs appear to be regulated by homologous *rcs* systems, and the polysaccharides are recognized virulence factors (49, 67). The ability to switch up expression under specific circumstances (e.g., at specific phases during infection) may therefore provide a selective advantage for pathogens. Maintenance of basal levels of expression might confer sufficient protection during adaptation, without a major drain of cellular energy.

The body of accumulated evidence supports the contention that colanic acid belongs with group IA capsules. Common features include similarities in structure (18, 33), chromosomal location (36, 53, 61, 66), and allelism (31) of biosynthetic genes and the involvement of the *rcs* regulatory system. However, there remain several minor distinctions. First, colanic acid synthesis is minimal at 37°C in *E. coli* K-12 and in strains with group II (31) and group IB (this study) capsules; strains with group IA K antigens produce a well-developed capsular structure at 37°C. Second, there remains a difference in the degree of cell association between group IA capsules and colanic acid. Finally, K antigens are serotype specific, and the broad distribution of colanic acid is such that it cannot be considered a serotype-specific polymer.

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