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Involvement of *rcsB* in *Klebsiella* K2 Capsule Synthesis in *Escherichia coli* K-12

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***Escherichia coli* K-12 harboring a part of the structural genes for the *Klebsiella* K2 capsular polysaccharide (cps_K*) expresses a large amount of K2 capsular polysaccharide as a thick capsule in the presence of plasmids carrying *rmpA* and *rcsB*. We have previously shown that expression of the *Klebsiella* K2 capsule in *E. coli* HB101 harboring cps_K* depends on the presence of *rmpA*, a regulatory gene from a large plasmid of *Klebsiella pneumoniae* Chedid (O1:K2). *E. coli* K-12 JM109, however, produces only a small amount of K2 capsular polysaccharide, even in the presence of plasmids carrying *rmpA* as well as the cps_K* structural genes. Introduction of the *rcsB* gene, a positive regulator of colanic acid capsule synthesis in *E. coli* K-12 which was cloned from HB101 on a plasmid, into JM109 cells carrying cps_K* and *rmpA*, results in the expression of a thick K2 capsule. By Northern (RNA) hybridization analysis, *rcsB* has been found to enhance transcription of a long strand of mRNA (longer than 14 kb) from cps_K*. These *E. coli* transformants which produce a thick K2 capsule also express colanic acid production at high levels. Therefore, *rcsB* can act as a positive regulator of *Klebsiella* K2 capsule production and two capsular polysaccharides can be expressed in *E. coli* simultaneously. With a somewhat different strain background, we have found that both of the colanic acid regulators, *rcsA* and *rcsB*, contribute to the basal level of *Klebsiella* K2 capsule expression but that the presence of multicopy *rcsB* in either an *rcsB* or an *rcsA* mutant of *E. coli* is sufficient to increase the expression of K2 capsular polysaccharide. These results suggest further parallels between the regulation of colanic acid synthesis in *E. coli* and the regulation of *Klebsiella* K2 capsule synthesis.**

Among at least 77 distinct capsular serotypes (17, 20), *Klebsiella pneumoniae* capsular type 2 (K2) strains were most frequently isolated from patients with bacteremia (5, 9, 13). We found that *K. pneumoniae* K2 strains isolated from clinical specimens usually showed strong virulence for mice, with 50% lethal doses as low as 2 CFU when injected intraperitoneally, although there were a small number of encapsulated *K. pneumoniae* K2 strains which were avirulent for mice (16). A loss of or decrease in virulence in association with elimination of the K2 capsule is supportive of the capsule's important role in the virulence of the strains (23). We cloned a part of the *Klebsiella cps* gene cluster (cps_K*), encoding genetic information for *Klebsiella* K2 capsular polysaccharide synthesis, and *rmpA* (18) from the chromosome and a large plasmid of *K. pneumoniae* Chedid, respectively. All of the nonencapsulated mutants of *K. pneumoniae* tested produced K2 capsular polysaccharide when they were transformed by cps_K* alone, whereas *Escherichia coli* HB101 required both cps_K* and *rmpA* for the expression of the thick *Klebsiella* K2 capsule on the surface (3). However, *E. coli* K-12 JM109 did not express a thick K2 capsule when it was transformed with cps_K* and *rmpA*. We looked for a gene from HB101 that has the ability to increase K2 capsule synthesis in JM109. The gene was identified as *rcsB*, a positive regulator for colanic acid capsular polysaccharide in *E. coli* K-12 (11, 25). Here, we present evidence that *rcsB* is also involved in *Klebsiella* K2 capsular polysaccharide production in *E. coli* K-12.

Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown in Luria broth (22) or 2× TY (22). Cells harboring recombinant plasmids were grown on Luria broth agar plates or in medium supplemented with appropriate antibiotics. Tryptone, yeast extract, and agar, the constituents of the media, were purchased from Difco Laboratories, Detroit, Mich. Agarose for electrophoresis and immunodiffusion was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Low-melting-temperature agarose used for the preparation of DNA probes was purchased from International Biotechnologies, Inc., New Haven, Conn. Restriction endonucleases and T4 DNA ligase were purchased from Nippon Gene Co., Ltd., Tokyo, Japan. Ampicillin and kanamycin were purchased from Meiji-Seika Co., Ltd., Tokyo, Japan. ATP and chloramphenicol were from Sigma Chemical Co., St. Louis, Mo. Plasmid DNA was prepared by the alkaline lysis method (22) and was purified by agarose NA (Pharmacia, Uppsala, Sweden) gel electrophoresis followed by electroelution (22). Bacterial chromosomal DNA was extracted by the method of Stauffer et al. (24). For DNA sequencing, the 5-kb *Hin*dIII fragment of *rcsB* clone pCPSX1 was subcloned into pUC118 and pUC119. Deletion mutants were isolated by using a deletion kit (Nippon Gene Co., Ltd.), and DNA sequencing was performed by the dideoxy method (22). Rabbit antiserum against the *Klebsiella* K2 capsular polysaccharide was prepared by using *K. pneumoniae* Chedid (O1:K2) as described previously (10, 17). The antiserum was absorbed with bacterial cells of a nonencapsulated mutant, 8N3 (O1:K2$^-$), isolated from Chedid to avoid cross-reactions due to O antigen and other surface components. Capsular polysaccharides used for double immunodiffusion were prepared by the method of Sugiyama et al. (27). Bacteria (10^9 CFU) were scraped directly from agar plates and suspended in 100 μl of TAE (22) or phosphate-buffered saline (PBS). Double immu-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
K. pneumoniae Chedid (O1:K2)	A laboratory strain	16
E. coli		
HB101	F$^-$ *hsdS20* (r$^-$ m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$	6, 7
JM109	*recA1 supE44 endA1 hsdR gyrA96 relA1 thi-1* Δ*(lac pro)* F′ [*traD36 proA*$^+$*B*$^+$ *lac*I^{q}ZΔM15]	31
SG20250	*cps*$^+$ *rcsB*$^+$ *rcsA*$^+$	S. Gottesman
SG22024	*rcsA*::*kan*	S. Gottesman
SG21028	*rcsB*::Tn*10*	S. Gottesman
Plasmids		
pNM7B06	*cps*$_K$*$^+$ Ap^r Km^r	3
pROJ31	*rmpA*$^+$ Cm^r; vector is pHSG398	This study
pROJ32	*rmpA*$^+$ *rcsB*$^+$ Cm^r; vector is pHSG398	This study
pCPSX11	*rcsB*$^+$ Cm^r; vector is pHSG398	This study
pHSG398	Cm^r	28

[a] Ap, ampicillin; Km, kanamycin; Cm, chloroamphenicol.

nodiffusion was performed as described previously (17). For the fluorescent-antibody staining technique, bacteria cultured in Luria broth at 30°C for 5 h were washed with PBS. A drop of bacterial suspension (10^3 CFU/ml) was fixed to a cover glass by methanol after flame fixation. The fixed cells were treated with 10% non-immunized goat serum for 30 min for blocking. After being washed, the cells were incubated for 30 min with rabbit anti-K2 serum diluted 1:40 with non-immunized goat serum. After being washed, the cells were stained for 30 min with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G serum. The cells were washed and mounted on a glass slide with 80% glycerol containing 40 mM Tris-HCl (pH 8.0), 0.02% NaN_3, and 0.1% *p*-phenylenediamine. The specific fluorescence of the *Klebsiella* K2 capsular polysaccharide was observed with a fluorescence microscope. For analysis of neutral sugar components, capsular polysaccharides extracted as described above and previously (27) were hydrolyzed in 1 N HCl at 100°C for 2 h and neutralized with 1 N NaOH. The samples were analyzed by high-pressure liquid chromatography (HPLC) using LC column ISA-07/S2504 (Shimadzu, Kyoto, Japan).

Identification of the gene from *E. coli* HB101 that has the ability to increase K2 capsule synthesis in *E. coli* JM109. The strategy for cloning the gene from *E. coli* HB101 that has the ability to increase K2 capsule synthesis in *E. coli* JM109 was as described elsewhere (4). By deletion analysis, the functional region was found to be located on the 5-kb *Hin*dIII fragment of pCPSX1 (Fig. 1). This 5-kb fragment was then subcloned into pROJ31, and the resulting recombinant plasmid, which carries both *rmpA* and *rcsB*, was designated pROJ32. When JM109 was transformed with both pROJ32 and pNM7B06 carrying *cps*$_K$*, the transformant produced ample *Klebsiella* K2 capsule, like HB101(pROJ31, pNM7B06) or Chedid. Then the *Eco*RI fragment carrying *rmpA* was removed from pROJ32, and the resulting plasmid carrying only *rcsB* was designated pCPSX11. The restriction maps of pROJ3, pROJ31, pCPSX1, pROJ32, and pCPSX11 are shown in Fig. 1. Plasmid DNAs were prepared from JM109(pROJ31, pCPSX1) and were used to transform *E. coli* HB101. Surprisingly, we have never succeeded in isolating HB101 harboring pCPSX1 (4). However, *E. coli* K-12 JM109 could be transformed by pCPSX1. Thus, JM109 was used for the isolation and amplification of pCPSX1. The DNA sequence of the 1-kb functional region of pCPSX1 was determined. An open reading frame was found, and a DNA sequence completely homologous to that of *rcsB*, which is

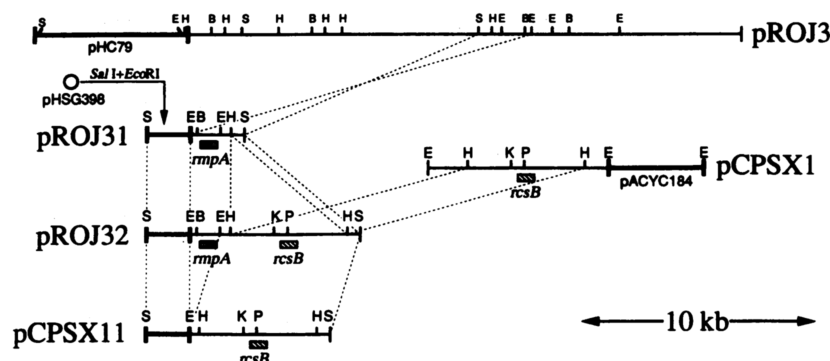

FIG. 1. Restriction maps of pROJ3, pROJ31, pCPSX1, pROJ32, and pCPSX11. The 5-kb *Hin*dIII fragment of pCPSX1 carrying the *rcsB* gene was ligated into the *Hin*dIII site of pROJ31, and then the resulting plasmid was designated pROJ32. The 1.5-kb *Eco*RI fragment on pROJ32 was removed for the deletion of gene *rmpA*, and the resulting plasmid was designated pCPSX11. Abbreviations: E, *Eco*RI; B, *Bam*HI; S, *Sal*I; H, *Hin*dIII; P, *Pst*I; K, *Kpn*I.

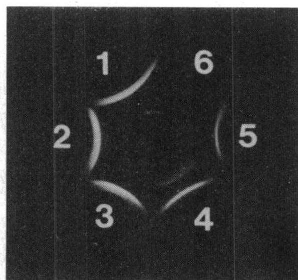

FIG. 2. Double immunodiffusion with rabbit anti-K2 serum (center well) and extracts from transformants of *E. coli* JM109 and *K. pneumoniae* Chedid. Wells: 1, *K. pneumoniae* Chedid; 2, JM109(pNM7B06, pROJ32) harboring cps_K*, *rmpA*, and *rcsB*; 3, JM109(pNM7B06, pROJ31) harboring cps_K* and *rmpA*; 4, JM109(pNM7B06, pCPSX11) harboring cps_K* and *rcsB*; 5, JM109(pNM7B06) harboring cps_K*; 6, JM109.

known as a positive regulatory gene for colanic acid production in *E. coli* (8, 11, 25), was determined.

***Klebsiella* K2 capsular polysaccharide synthesis in *E. coli* JM109.** *E. coli* HB101(pNM7B06) did not produce any detectable amount of *Klebsiella* K2 capsular polysaccharide, but *E. coli* JM109(pNM7B06) produced a small amount of K2 capsular polysaccharide (Fig. 2). When we introduced plasmid pCPSX11 carrying *rcsB* into JM109, cells showed a mucoid phenotype because of overproduction of colanic acid. The introduction of this $rcsB^+$ plasmid into JM109 harboring cps_K* increased the amount of K2 capsular polysaccharide to slightly more than that produced in JM109 harboring cps_K* alone (Fig. 2). Neither JM109 harboring cps_K* and *rcsB* nor JM109 harboring cps_K* and *rmpA* produced a thick K2 capsule around the cell surface (Fig. 3), although they produced a detectable amount of K2 capsular polysaccharide (Fig. 2). On the other hand, the introduction of plasmid pROJ32 carrying both *rmpA* and *rcsB* into JM109 harboring cps_K* led to increased synthesis of K2 capsular polysaccharide as a thick K2 capsule around the cell surface similar to the K2 capsule of *K. pneumoniae* Chedid (Fig. 3) or of HB101 harboring cps_K* and *rmpA* (3).

***rcsB* regulates *Klebsiella* K2 capsular polysaccharide synthesis at the transcriptional level.** By Northern (RNA) hybridization, *rcsB* was found to enhance a long strand of mRNA (longer than 14 kb) from cps_K* (Fig. 4), indicating that *rcsB* regulates the expression of K2 capsular polysaccharide at the transcriptional level. Although the *rcsB* gene is intact in both HB101 and JM109 (4), JM109 required additional *rcsB* cloned from HB101 for K2 capsule synthesis, while HB101 did not. Therefore, there must be some difference between HB101 and JM109, for instance, in the level of intracellular active RcsB. A single copy of *rcsB* on the chromosome of *E. coli* is sufficient to positively regulate colanic acid capsule synthesis, but normally it does so at a very low level. However, we had to introduce multicopy *rcsB* with both

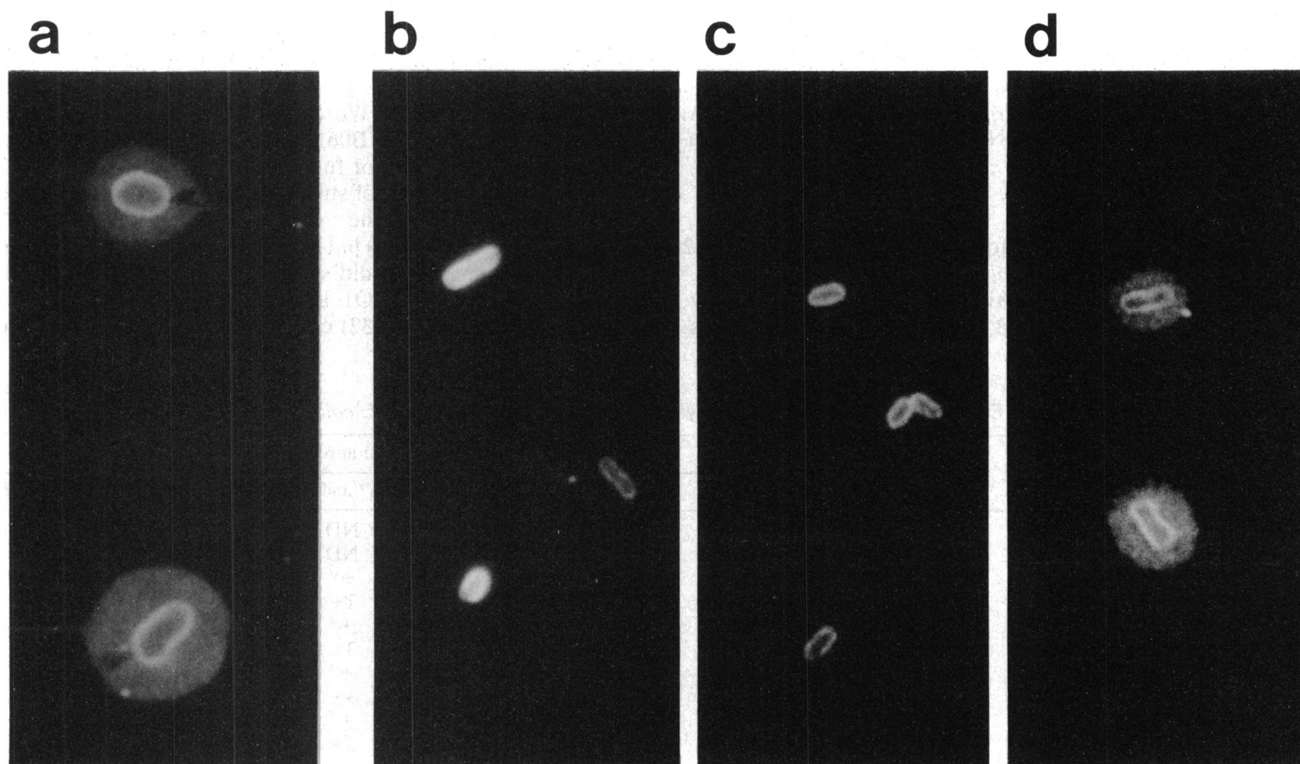

FIG. 3. FITC staining of *K. pneumoniae* Chedid and transformants of *E. coli* JM109. The bacterial cells were stained with FITC-labeled goat anti-rabbit immunoglobulin G serum after treatment with rabbit anti-K2 serum. JM109(pNM7B06, pROJ31) and JM109(pNM7B06, pCPSX11) were exposed to film (Kodak Tri-X 400) four times longer than Chedid and JM109(pNM7B06, pROJ32). Less than 10% of the JM109(pNM7B06, pROJ31) and JM109(pNM7B06 pCPSX11) cells were stained, and the remainders of each were stained too faintly to show up on the photograph. (a) *K. pneumoniae* K2 Chedid; (b) *E. coli* JM109(pNM7B06, pCPSX11) harboring cps_K* and *rcsB*; (c) *E. coli* JM109(pNM7B06, pROJ31) harboring cps_K* and *rmpA*; (d) *E. coli* JM109(pNM7B06, pROJ32) harboring cps_K*, *rmpA*, and *rcsB*. Magnification, ×8,800.

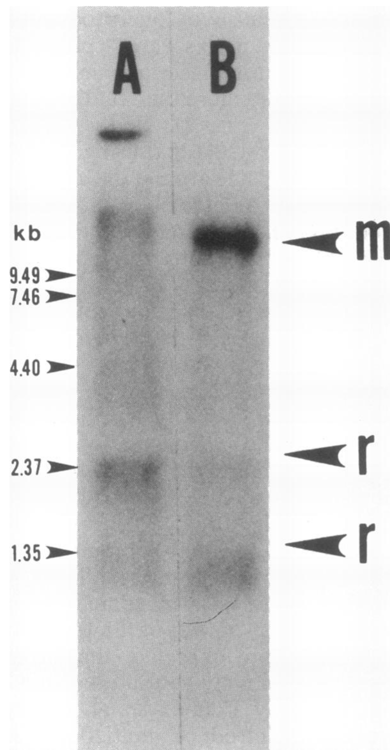

FIG. 4. Northern hybridization analysis of *E. coli* JM109 (pNM7B06) and JM109(pNM7B06, pCPSX11). Preparation of mRNA and blotting were achieved by the method of Kornblum et al. (14). The 23-kb fragment of pNM7B06 carrying a part of the *Klebsiella cps* gene cluster was used as the DNA probe. A long strand of mRNA (>14 kb) was transcribed from cps_K* by using *rcsB*. Hybridization was performed as described previously (2). Lanes: A, JM109(pNM7B06); B, JM109(pNM7B06, pCPSX11). Abbreviations: m, mRNA; r, rRNA.

cps_K* and *rmpA* into JM109 for expression of the thick K2 capsule. The affinity of *E. coli* RcsB for binding to cps_K* may be less than that of *Klebsiella* RcsB (1). Accordingly, larger amounts of *E. coli* RcsB may be required for successful binding to the specific region of cps_K* to elicit a detectable response in *E. coli* JM109. Alternatively, the influences of some other genetic background, for example, the activity of RcsA (15, 26) or Lon protease (29, 30), may be different between *K. pneumoniae* and *E. coli*.

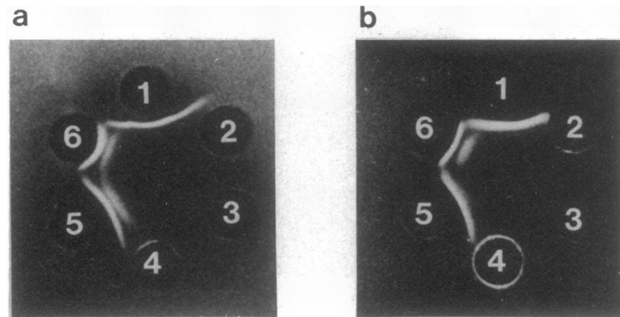

FIG. 5. Double immunodiffusion showing *Klebsiella* K2 capsular polysaccharide production in *E. coli rcsA*::*kan* and *rcsB*::Tn*10* mutants. (a) SG22024, *rcsA*::*kan* mutant; (b) SG21028, *rcsB*::Tn*10* mutant. Wells: 1, Chedid; 2, each strain without any plasmid; 3, each strain with pNM7B06 ($cps_K{}^{*+}$); 4, each strain with pNM7B06 and pROJ31 ($rmpA^+$); 5, each strain with pNM7B06 and pCPSX11 ($rcsB^+$); 6, each strain with pNM7B06 and pROJ32 ($rmpA^+$ and $rcsB^+$); center, rabbit anti-K2 serum.

***E. coli* can express colanic acid and *Klebsiella* K2 capsular polysaccharide simultaneously.** From HPLC analysis of neutral sugar components of capsular polysaccharide produced by JM109(pNM7B06, pROJ32) harboring cps_K*, *rcsB*, and *rmpA*, we found the existence of fucose indicative of synthesis of colanic acid $[\xrightarrow[\beta]{3} \mathrm{Glc} \xrightarrow[\beta]{1,3\ \mathrm{or}\ 4} \mathrm{Fuc}(\xleftarrow[\alpha]{4,1} \mathrm{Gal} \xleftarrow[\beta]{3,1} \mathrm{GlcUA} \xleftarrow[\beta]{4,1} \mathrm{Gal}\beta \rightleftharpoons \mathrm{Pyr}) \xrightarrow[\alpha]{} \mathrm{Fuc(OAc)} \xrightarrow{1}]n$ (19), together with mannose, galactose, and glucose. We could detect fucose in neither JM109 nor JM109(pNM7B06). In contrast, mannose, galactose, and glucose, but not fucose, were detected in the K2 capsular polysaccharide of strain Chedid which was reported previously to have the chemical structure $[\xrightarrow{3}\mathrm{Glc}\xrightarrow[\beta]{1,4}\mathrm{Man}(\xleftarrow[\alpha]{1,3}\mathrm{GlcUA})\xrightarrow[\beta]{1,4}\mathrm{Glc}\xrightarrow[\beta]{1}]n$ (12, 21). Galactose and some of the glucose found in Chedid's capsular polysaccharide preparation might be from O1 lipopolysaccharide. Therefore, JM109(pNM7B06, pROJ32) can simultaneously express the

TABLE 2. *Klebsiella* K2 capsular polysaccharide synthesis in various *E. coli* strains

E. coli strain	Genotype	Test[a]	Result of gene(s) introduced[b]			
			cps_K*	cps_K* and *rmpA*	cps_K* and *rcsB*	cps_K*, *rmpA*, and *rcsB*
HB101	$rcsB^+$	DB IMM	−	+[c]	ND	ND
		FITC	−	4+	ND	ND
JM109	$rcsA^+$ $rcsB^+$	DB IMM	+[c]	+[c]	+[d]	+[d]
		FITC	1+	1+	1+	4+
SG20250	$rcsA^+$ $rcsB^+$	DB IMM	+[c]	+[c]	+[d]	+[d]
		FITC	2+	2+	3+	4+
SG22024	*rcsA*::*kan*	DB IMM	−	−	+[d]	+[d]
	$rcsB^+$	FITC	−	−	3+	4+
SG21028	*rcsB*::Tn*10*	DB IMM	−	−	+[d]	+[d]
	$rcsA^+$	FITC	−	−	3+	4+

[a] DB IMM, double immunodiffusion analysis; FITC, staining of bacteria by FITC-labeled goat anti-rabbit immunoglobulin G serum after treatment with rabbit anti-K2 capsular polysaccharide serum. By this method, we could observe the morphologies of capsules as well as the amounts of K2 capsular polysaccharide produced by organisms.

[b] The results of double immunodiffusion analysis and FITC were evaluated as follows: −, no precipitation line or staining observed; +, precipitation positive; 1+, less than 10% of the bactera were stained at the surface; 2+, about 40% of the cells were stained at the surface; 3+, about 75% of the cells produced *Klebsiella* K2 capsular polysaccharide around the cell surface but not as the thick capsule; 4+, all cells produced the thick *Klebsiella* K2 capsule. ND, not done.

[c] One precipitation line was observed.

[d] Two precipitation lines were observed.

Klebsiella K2 capsule and the colanic acid capsular polysaccharide. But the amount of colanic acid produced in *E. coli* does not likely represent the thickness of the K2 capsule, since JM109(pNM7B06, pCPSX11) carrying cps_K* and *rcsB*, which produced a large amount of colanic acid (data not shown), did not express a thick K2 capsule, as shown by FITC staining (Fig. 3b).

***Klebsiella* K2 capsular polysaccharide synthesis in *rcsA* and *rcsB* mutants of *E. coli*.** *E. coli* K-12 SG20250 behaved similarly to JM109 in that it produced a thick K2 capsule in the presence of cps_K*, *rmpA*, and *rcsB*. It produced some K2 capsular polysaccharide on the cell surface, but not as a thick capsule, in the presence of cps_K* alone and in the presence of cps_K* and *rcsB* or *rmpA* (data not shown). *E. coli* SG21028 and SG22024, the *rcsB*::Tn*10* and *rcsA*::*kan* mutants, respectively, of SG20250, could not produce K2 capsular polysaccharide at all in the presence of cps_K* alone (Table 2; Fig. 5). Thus, both *rcsA* and *rcsB* are required for the basal level of K2 capsular polysaccharide expression in *E. coli*. Both the *rcsA* and the *rcsB* mutants could produce K2 capsular polysaccharide when multicopy *rcsB* existed, even without *rcsA* (Table 2). We concluded that multicopy *rcsB* could complement *rcsA* and that *rcsB* is crucial for K2 capsular polysaccharide expression in *E. coli*, as it is for colanic acid production.

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