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Biosynthesis of *Klebsiella* K2 Capsular Polysaccharide in *Escherichia coli* HB101 Requires the Functions of *rmpA* and the Chromosomal *cps* Gene Cluster of the Virulent Strain *Klebsiella pneumoniae* Chedid (O1:K2)

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The genes determining the biosynthesis of type 2 (K2) capsular polysaccharide [$\xrightarrow[\beta]{3}$Glc$\xrightarrow[\beta]{1,4}$Man($\xleftarrow[\beta]{1,3}$GlcUA)$\xrightarrow[\alpha]{1,4}Glc\xrightarrow{1}$] of *Klebsiella pneumoniae* Chedid (O1:K2), which is highly virulent for mice, were cloned and introduced into *Escherichia coli* HB101 and into four noncapsulated mutants derived from *K. pneumoniae* reference strains of K1, K7, K9, and K28. The recombinant plasmid pCPS7B06 carried 23 kb of a chromosomal DNA fragment of strain Chedid and encoded a part of the *Klebsiella cps* gene cluster. However, pCPS7B06 encoded enough genetic information for the production of *Klebsiella* K2 capsular polysaccharide on the cell surfaces of four noncapsulated mutants of *K. pneumoniae*. On the other hand, both pCPS7B06 and pROJ3 carrying the *rmpA* gene locus derived from a resident large plasmid of Chedid were required for the biosynthesis of *Klebsiella* K2 capsular polysaccharide on the cell surface of *E. coli* HB101. The insertion inactivation analysis using Tn*5* revealed that the *cps* gene cluster occupied more than 15 kb of the chromosome of Chedid. We conclude that *rmpA*, which has been known to enhance the biosynthesis of colanic acid in *E. coli*, is also involved in the biosynthesis of *Klebsiella* capsular polysaccharide in *E. coli* HB101.

Almost all clinical isolates belonging to the family *Enterobacteriaceae* usually form ample and various kinds of polysaccharides on their surface (16, 31). Capsular polysaccharide (K antigen) is one of the major surface polysaccharides (30), including acidic saccharides such as uronic acids (16). Capsular polysaccharides on the surface of bacterial outer membranes have been thought to act as a barrier against antibacterial immunoreactions of the host, such as phagocytosis by polymorphonuclear leukocytes (45) and the action of complement (14). It has been reported that K1 capsular polysaccharide of *Escherichia coli* is relevant to virulence (6, 17). *E. coli* K1 strains tend to be isolated from patients with extraintestinal infections (7, 32, 37). The K1 capsular polysaccharide of *E. coli* is a poorly immunogenic homopolymer of α2-8-linked *N*-acetylneuraminic acid (24).

Klebsiella pneumoniae is one of the species of gram-negative rods (11, 13) which cause opportunistic infections in compromised hosts or patients with immunodeficiency (8, 19). Clinically isolated *Klebsiella* strains usually produce large amounts of capsular polysaccharides. *Klebsiella* capsular polysaccharides have been classified into at least 77 serotypes (22, 27). Previously it was found that strains of *K. pneumoniae* belonging to the K2 capsular type (29, 38) showed strong virulence for mice (3, 21), although some of them showed very weak or no virulence for mice (21). It was also reported that *K. pneumoniae* isolates belonging to the K2 capsular type were isolated in high frequency from patients with bacteremia (9). In *Klebsiella* spp., the production of capsular polysaccharides also seems to play an important role in the establishment of infection (10, 40). It was also reported that the mucoid capsular phenotype mediated by a large plasmid might be responsible for the virulence of *Klebsiella* K2 strains (23). In this paper, we report the cloning and expression of genes in *E. coli* HB101 for the biosynthesis of *Klebsiella* K2 capsular polysaccharide and we present evidence that two independent gene loci, one of which is located in the chromosome and the other of which is located in a resident large plasmid of *K. pneumoniae* K2 Chedid, are necessary for the biosynthesis of *Klebsiella* K2 capsular polysaccharide in *E. coli* HB101.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains used for cloning and analysis of genes involved in *Klebsiella* K2 polysaccharide synthesis are listed in Table 1.

Media and reagents. Tryptone, yeast extract, and agar were purchased from Difco Laboratories, Detroit, Mich. Bacterial cultures were grown in Luria broth (LB) and LB agar (20). Cells harboring recombinant plasmids were grown in medium supplemented with appropriate antibiotics. An M9 medium agar plate (20) supplemented with 12.5 μg of trimethoprim per ml was used for the conjugational transfer of the plasmid R388rep(ts)::Tn*5* from *E. coli* C600(R388rep (ts)::Tn*5*) to *K. pneumoniae* Chedid. 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 Takara Co., Ltd., Kyoto, Japan. Multiprime DNA labeling kit and [α-^{32}P]dCTP were purchased from Amersham Corp., Amersham, United Kingdom. Ampicillin and kanamycin were purchased from Meiji-Seika Co., Tokyo, Japan. ATP, trimethoprim, and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
K. pneumoniae Chedid	Laboratory strain (O1:K2)	21
K. pneumoniae NM5, NM7, NM10, NM31, NM38, NM29, NM39, NM32, NM37, NM9, NM29, NM2, NM4, NM36, NM1, NM3, NM42, NM11, and NM6	K2-less mutants of Chedid (O1:K2$^-$)	This study
K. pneumoniae Kasuya	Laboratory strain (O3:K1)	
K. pneumoniae NK1004[b]	K1-less mutant of Kasuya (O3:K1$^-$)	This study
K. pneumoniae 4140	K7 reference strain (O1:K7)	18
K. pneumoniae NK7004[b]	K7-less mutant of 4140 (O1:K7$^-$)	This study
K. pneumoniae 56	K9 reference strain (O?:K9)	18
K. pneumoniae NK9007[b]	K9-less mutant of strain 56 (O?:K9$^-$)	This study
K. pneumoniae 5758	K28 reference strain (O2:K28)	26
K. pneumoniae NK28001[b]	K28-less mutant of strain 5758 (O2:K28$^-$)	This study
K. pneumoniae B5055	Reference strain of *K. pneumoniae* K2 (O1:K2)	18
K. pneumoniae K2-112, K2-215, K2-277, K2-324, K2-350, K2-410, and K2-694	Laboratory strains (O1:K2) isolated in Nagoya University Hospital	
E. coli HB101	F$^-$ *hsdS20*(r$^-$ m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	
E. coli C600 (R388rep(ts)::Tn*5*)	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44* F$^-$ λ^- Tp^r Km^r	36
Plasmids		
pNM7B06	Recombinant plasmid which carries 23-kb chromosomal DNA fragment, Ap^r, Km^r	Vector pBR322, this study
pCPS7B06	Recombinant plasmid which carries 23-kb chromosomal DNA fragment, Cp^r, Km^r	Vector pHSG575, this study
pNM6B02	Recombinant plasmid which carries 19-kb chromosomal DNA fragment, Ap^r, Km^r	Vector pBR322, this study
pCPS6B02	Recombinant plasmid which carries 19-kb chromosomal DNA fragment, Cp^r, Km^r	Vector pHSG575, this study
pNM1B01	Recombinant plasmid which carries 15-kb chromosomal DNA fragment, Ap^r, Km^r	Vector pBR322, this study
pROJ3	Cosmid clone which carries 19-kb DNA fragment of resident large plasmid of *K. pneumoniae* Chedid, Ap^r	Vector pHC79, this study
pBRG700	Ap^r, IS*50*$_L$	36

[a] Abbreviations: Ap, ampicillin; Km, kanamycin; Tp, trimethoprim; Cp, chloramphenicol.
[b] Noncapsulated mutants were isolated from their parental strains by nitrosoguanidine treatment.

chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of antiserum. Rabbit antiserum against *Klebsiella* K2 capsular polysaccharide was prepared as described previously (22). The antiserum was absorbed with the bacterial cells of a noncapsulated mutant 8N3 (O1:K2$^-$) isolated from *K. pneumoniae* Chedid (O1:K2) by ultraviolet irradiation for preparation of antisera free from cross-reaction due to O antigen and other cell surface components.

Preparation of DNA. Plasmid DNA was prepared by a rapid alkaline extraction method and was purified by using NA-agarose (Pharmacia, Uppsala, Sweden) gel electrophoresis followed by electroelution. Bacterial chromosomal DNA was extracted as described previously (41).

Isolation of K2 antigen-negative mutants of *K. pneumoniae* K2 Chedid. *E. coli* C600 harboring conjugational transferable plasmid R388rep(ts)::Tn*5* was cultured in LB containing 12.5 μg of trimethoprim per ml to 10^8 CFU/ml at 30°C. *K. pneumoniae* Chedid was also cultured in LB to 10^7 CFU/ml. One milliliter of each bacterial culture was mixed and spotted on an LB agar plate. After incubation for 4 h at 30°C, the culture was replated on M9 medium agar plates supplemented with 12.5 μg of trimethoprim per ml and then incubated at 30°C for 36 h. Some trimethoprim-resistant colonies were isolated and cultured in LB containing 12.5 μg of trimethoprim per ml for 5 h at 30°C and then diluted with LB to 10^5 CFU/ml. Every 200 μl of the strain Chedid (R388rep(ts)::Tn*5*) suspension was plated on an LB agar plate supplemented with 50 μl of kanamycin per ml and then incubated at 42°C for 15 h. Abolition of K2 capsular polysaccharide production in each Tn*5*-inserted noncapsulated mutant of Chedid was judged by a double immunodiffusion test using the anti-K2 rabbit serum. The procedure for the detection of mutants which have only one Tn*5* transposition on the chromosome was as follows. The chromosomal DNAs were prepared from the noncapsulated mutants and were digested with restriction endonucleases *Eco*RI and *Bam*HI. Southern transfer of digested DNAs onto nylon membrane (Hybond-N, Amersham) was done after agarose gel electrophoresis. For probes, the 1,180-bp *Hpa*I-*Pvu*II DNA fragments of IS*50*$_L$ carried by pBRG700 were excised from the low-melting-temperature agarose gel after the electrophoresis. The gel slice containing the DNA fragment was melted, diluted, and labeled with [α-^{32}P]dCTP by using a multiprime labeling kit. The mutants which showed a single hybridization band on the autoradiograms of the *Eco*RI-digested blot and which showed two bands on the autoradiograms of the *Bam*HI-digested blot were estimated to have a single Tn*5* insertion on the chromosome.

Cloning of the chromosomal *cps* gene cluster. Chromosomal DNAs from the noncapsulated mutants of *K. pneumoniae* Chedid described above were digested with *Bam*HI and

ligated into the same cloning sites of plasmid vector pBR322. *E. coli* HB101 was transformed with these recombinants, and colonies were isolated on LB agar plates supplemented with 50 μg of kanamycin per ml and the same concentration of ampicillin. Recombinant plasmids which conferred resistance against both kanamycin and ampicillin to HB101 were prepared and digested with restriction enzymes *Eco*RI and *Bam*HI, and digestion patterns for the mapping of restriction sites and for the determination of Tn*5* insertion sites were then compared by electrophoresis. A recombinant plasmid named pCPS7B06, which carries 23 kb of the chromosomal 29-kb *Bam*HI fragment encoding the *cps* gene cluster derived from a noncapsulated mutant of Chedid (NM7), was used for the transformation of the noncapsulated mutants of various strains of *K. pneumoniae*, as described below, and of *E. coli* HB101.

Introduction of the *cps* gene cluster into *K. pneumoniae*. Four noncapsulated mutants, which are listed in Table 1, were isolated from K1, K7, K9, and K28 capsular type reference strains of *K. pneumoniae* by nitrosoguanidine treatment. These four noncapsulated mutants were transformed by pCPS7B06. For increasing transformation efficiency, a competent cell line of *K. pneumoniae* was prepared by using high-Ca^{2+} buffer (100 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.2]) and was then kept at 4°C for 15 h before transformation.

Complementation analysis. NM5 was transformed by pNM1B01 for the complementation of the inactivated gene. *E. coli* HB101(pCPS7B06) was also transformed by pNM6B02 or pNM1B01 for the complementation of the deleted region of plasmid pCPS7B06 carrying a part of the *cps* gene locus of NM7.

Cloning of the *rmpA* gene mediated by a resident large plasmid. The DNA of strain Chedid was partially digested with *Sau*3AI and ligated into the *Bam*HI site of a cosmid vector pHC79, and a gene library was constructed. *E. coli* HB101(pCPS7B06) was transformed by this gene library, and colonies expressing *Klebsiella* K2 capsular polysaccharide as identified by agglutination with anti-K2 rabbit serum were isolated. The DNA sequence of the functional region of pROJ3, designated for the cosmid clone capable of making *E. coli* HB101(pCPS7B06) produce *Klebsiella* K2 capsular polysaccharide, was determined by using the method of Sanger et al. (34).

Double immunodiffusion analysis. Cell extracts for double immunodiffusion analysis were prepared from 1.5 ml of LB culture incubated at 30°C by using two different methods, that of Westphal and Jann (46) and the alkaline-sodium dodecyl sulfate (SDS) lysing method (43). Double immunodiffusion was performed in 1.0% agarose in phosphate-buffered saline (PBS). Halos were observed after an 18 h incubation at 37°C.

Quellung test. Bacteria were cultured on an LB plate at 30°C for 24 h. The quellung test was performed according to the conventional method (2) by using anti-K2-specific polyclonal rabbit serum.

Southern hybridization. Transfer of DNA onto nylon membrane (Hybond-N, Amersham) was achieved by the method of Southern which was outlined by Maniatis et al. (20). For the hybridization probes, mixed probes of the 23-kb and 15-kb DNA fragments carried by pCPS7B06 and pNM1B01, respectively, were used. The 12-kb *Sal*I-*Sal*I fragment of pROJ3 was also used for the hybridization analysis of the large plasmid. DNA fragments were excised from low-melting-temperature agarose gel after electrophoresis. The gel slice was labeled directly with [α-^{32}P]dCTP by using the multiprime labeling kit. Hybridization was performed as described previously (1).

Virulence assay. SMA mice of both sexes weighing 20 to 25 g were obtained from the Institute for Laboratory Animal Research, Nagoya University School of Medicine, Nagoya, Japan. The bacterial strains to be tested were cultured in LB at 30°C for 4 h after inoculation from a plate. Bacteria were washed twice with PBS. Bacteria were diluted with PBS, and the lethality of the organisms for mice was tested by intraperitoneal inoculation. The 50% lethal dose (LD_{50}) of the organisms was calculated from the death rate as described previously (21).

RESULTS

Isolation of noncapsulated mutants of *K. pneumoniae* K2 Chedid. Forty-five noncapsulated mutants were isolated from Chedid by Tn*5* insertion inactivation into the chromosomal *cps* gene cluster. Hybridization analysis suggested that 19 of 45 noncapsulated mutants had a single Tn*5* insertion in their chromosome. The production of K2 capsular polysaccharide was tested by double immunodiffusion methods with anti-*Klebsiella* K2 rabbit antiserum, and it was found that none of the mutants except NM9 produced detectable amounts of K2 capsular polysaccharide. Although *K. pneumoniae* NM9 did not show agglutination by anti-*Klebsiella* K2-specific serum, a trace amount of K2 capsular polysaccharide was detected in NM9 by double immunodiffusion analysis when the specimen was prepared by the alkaline-SDS lysing method (43). The parental strain Chedid is highly virulent for mice by intraperitoneal inoculation (LD_{50}, 2 CFU) (21). By contrast, all of these 19 mutants lost their virulence toward mice (LD_{50}, $>10^8$ CFU). The viscid consistency of colonies was abolished concurrently with the loss of K2 capsular polysaccharide production in each mutant.

Cloning of the *Klebsiella cps* gene cluster. The chromosomal DNAs were prepared from the 19 noncapsulated mutants of Chedid described above. Hybridization analysis by using insertion sequence ($IS50_L$) of Tn*5* as probes suggested that every Tn*5* transposed into the same 29-kb *Bam*HI fragment. It was therefore suggested that the *Klebsiella cps* gene cluster existed on this chromosomal 29-kb *Bam*HI fragment. First, *Bam*HI-digested chromosomal DNA fragments of strain Chedid which were longer than 25 kb were purified and a gene library was constructed by using cosmid pHC79. We then tried to isolate the recombinant which carries the entire 29-kb *Bam*HI fragment directly from this gene library. We screened thousands of colonies by a colony hybridization technique using the 15-kb DNA fragment of pNM1B01 as a probe, but in vain. It was later found that the clone which harbored the recombinant plasmid carrying the *Klebsiella cps* gene cluster grew so slowly that it was impossible to distinguish the colonies of the transformants which harbored clones carrying the *cps* gene cluster from surrounding satellite colonies. Therefore, 19 clones carrying a part of the *cps* gene cluster were isolated together with the kanamycin resistance gene of Tn*5* from the above 19 noncapsulated mutants. By comparing endonuclease digestion patterns among the 19 clones, the insertion site of Tn*5* in each noncapsulated mutant was mapped and restriction sites of several endonucleases were determined (Fig. 1a). Although all Tn*5* insertion sites of each noncapsulated mutant were mapped on the same 29-kb *Bam*HI fragment, insertion sites of Tn*5* occupied 15 kb of the 29-kb *Bam*HI fragment (Fig. 1a). Recombinant plasmids obtained are also shown in Fig.

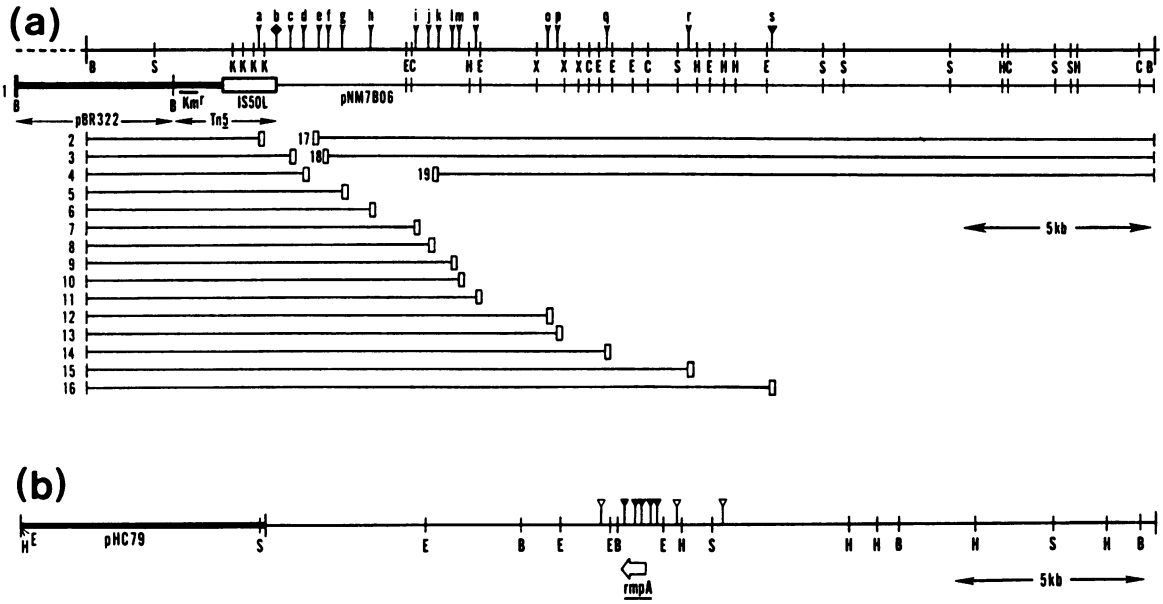

FIG. 1. Map of the *cps* gene cluster and *rmpA*. (a) A linearized restriction map of the chromosomal 29-kb *Bam*HI fragment carrying the *Klebsiella cps* gene cluster is shown with 5-kb indicator below the map. The Tn*5* insertion positions of 19 noncapsulated mutants (| , ♦, ▼) are indicated on the restriction map. The Tn*5* transposition sites in the chromosomes of the two noncapsulated mutants NM7 (♦) and NM6 (▼) are indicated. The insertion positions of Tn*5* in each noncapsulated mutant are shown as follows: a, NM5; b, NM7; c, NM10; d, NM31; e, NM38; f, NM29; g, NM39; h, NM32; i, NM37; j, NM9; k, NM26; l, NM2; m, NM4; n, NM36; o, NM1; p, NM3; q, NM42; r, NM11; s, NM6. A map of pNM7B06 is shown under the restriction map of the chromosomal 29-kb *Bam*HI fragment. Km^r and IS*50*$_L$ indicate the kanamycin resistance gene and the left insertion sequence of Tn*5*, respectively. The horizontal lines below the restriction map of pNM7B06 show the DNA fragments carried by each plasmid as follows: 1, pNM7B06; 2, pNM5B01; 3, pNM10B04; 4, pNM31B02; 5, pNM39B01; 6, pNM32B03; 7, pNM37B01; 8, pNM9B03; 9, pNM2B02; 10, pNM4B02; 11, pNM36B02; 12, pNM1B01; 13, pNM3B01; 14, pNM42B05; 15, pNM11B02; 16, pNM6B02 and pCPS6B02; 17, pNM38B01; 18, pNM29B02; 19, pNM26B05. Oblong box at the end of each horizontal line indicates the vector and a part of Tn*5*. (b) Restriction map of pROJ3 carrying the *rmpA* gene. Sites at which Tn*5* insertions inhibit *Klebsiella* K2 capsular polysaccharide synthesis in HB101(pCPS7B06) (▼) and sites at which insertions showed no change in K2 capsular synthesis (▽) are indicated. Open arrow indicates the position and transcriptional direction of *rmpA*. Thick line shows the position of cosmid vector pHC79. The size of pROJ3 was calculated to be 30.6 kb. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xba*I.

1a. The 23-kb DNA fragment carried by pNM7B06 was transferred into the *Bam*HI site of low-copy-number vector pHSG575 and was designated pCPS7B06.

The restriction map of pROJ3, which carries a 24-kb DNA fragment derived from a resident large plasmid of *K. pneumoniae* Chedid, is shown in Fig. 1b. When HB101 was transformed by pROJ3, it produced a large amount of mucoid substance. By chemical analysis, the mucoid substance was identified as colanic acid (the detailed data will be published elsewhere). According to the DNA sequencing analysis of the functional region of pROJ3, pROJ3 encoded a gene which showed a DNA sequence highly homologous to that of *rmpA* (23).

Expression of K2 capsular polysaccharide on the cell surface of noncapsulated mutants derived from different serotypes of *K. pneumoniae*. Four noncapsulated mutants, NK1004, NK7004, NK9007, and NK28001, derived from K1, K7, K9, and K28 reference strains of *K. pneumoniae*, respectively, were transformed by pCPS7B06. Transformants of these mutants produced a large amount of *Klebsiella* K2 capsular polysaccharide when they were incubated at 30°C (Table 2). The production of K2 capsular polysaccharide was ascertained by the double immunodiffusion test (Fig. 2a) and the quellung test. Although production of K2 capsular polysaccharide on the cell surface of each transformant was definitely observed by the quellung test, the colonies that formed at 30°C appeared less viscous than those of the

TABLE 2. Production of *Klebsiella* K2 capsular polysaccharide

Strain	K2 capsular polysaccharide production
K. pneumoniae	
Chedid (O1:K2)	+
NM5 (O1:K2$^-$)	−
NM5(pNM1B01) (O1:K2)	+
NK1004 (O3:K1$^-$)	−
NK1004(pCPS7B06) (O3:K2:K1$^-$)	+
NK7004 (O1:K7$^-$)	−
NK7004(pCPS7B06) (O1:K2:K7$^-$)	+
NK9007 (K9$^-$)[a]	−
NK9007(pCPS7B06) (K2:K9$^-$)[a]	+
NK28001 (O2:K28$^-$)	−
NK28001(pCPS7B06) (O2:K2:K28$^-$)	+
E. coli	
HB101	−
HB101(pCPS7B06)	−
HB101(pROJ3)[b]	−
HB101(pCPS7B06, pROJ3)	+
HB101(pCPS6B02, pROJ3)	−
HB101(pCPS7B06, pNM6B02)	−
HB101(pCPS7B06, pNM1B01)	−

[a] NK9007 produced unclassifiable O antigen.
[b] *E. coli* HB101(pROJ3) produced a large amount of colanic acid at 30°C.

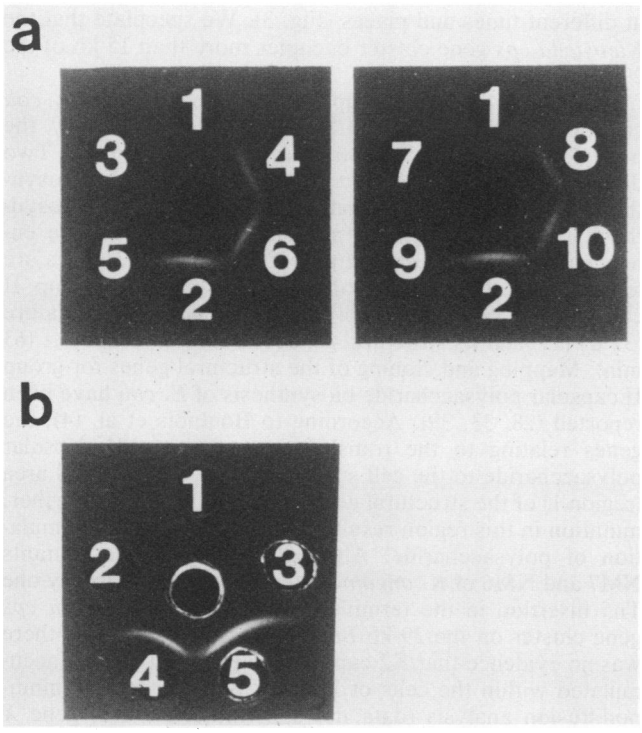

FIG. 2. (a) Double immunodiffusion with anti-K2 rabbit serum (center wells) and extracts (5 μl) from noncapsulated mutants or transformants of four reference strains of *K. pneumoniae* and noncapsulated mutant NM7. Wells: 1, *K. pneumoniae* NM7; 2, *K. pneumoniae* Chedid; 3, *K. pneumoniae* NK1004; 4, *K. pneumoniae* NK1004(pCPS7B06); 5, *K. pneumoniae* NK7004; 6, *K. pneumoniae* NK7004(pCPS7B06); 7, *K. pneumoniae* NK9007; 8, *K. pneumoniae* NK9007(pCPS7B06); 9, *K. pneumoniae* NK28001; 10, *K. pneumoniae* NK28001(pCPS7B06). (b) Double immunodiffusion with anti-K2 rabbit serum (center well) and extracts (5 μl) from transformants of *E. coli* HB101. Wells: 1, *E. coli* HB101; 2, *E. coli* HB101 (pROJ3); 3, *E. coli* HB101(pCPS7B06); 4, *K. pneumoniae* Chedid; 5, *E. coli* HB101(pCPS7B06, pROJ3).

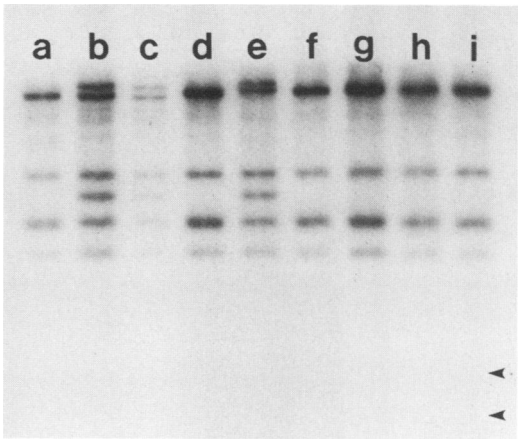

FIG. 3. The hybridization patterns of the 29-kb *Bam*HI fragment to the *cps* gene cluster among various *K. pneumoniae* reference strains (K2:O1). Each chromosomal DNA was digested by *Eco*RI and subjected to agarose gel electrophoresis. Hybridization was achieved after the blotting as described in Materials and Methods. Lanes: a, *K. pneumoniae* K2-112; b, *K. pneumoniae* K2-215; c, *K. pneumoniae* K2-277; d, *K. pneumoniae* K2-324; e, *K. pneumoniae* K2-350; f, *K. pneumoniae* K2-410; g, *K. pneumoniae* K2-694; h, *K. pneumoniae* B5055; i, *K. pneumoniae* Chedid. Small arrows indicate the positions of faint hybridization signals which are visualized after longer exposure. *K. pneumoniae* strains K2-112 to K2-694 were isolated in Nagoya University Hospital 15 years ago. *K. pneumoniae* B5055 was isolated in Europe more than 40 years ago (18). Strain Chedid was isolated in Europe more than 30 years ago.

parental strain Chedid. The expression of K2 capsular polysaccharide on the cell surface of the noncapsulated mutants was less marked when incubated at 37°C than at 30°C. This finding was different from that found with Chedid, in which the expression of K2 capsular polysaccharide occurred intensely at either 30 or 37°C. The virulence for mice of these four noncapsulated mutants of *K. pneumoniae* was not increased by the transformation with pCPS7B06 even if they produced *Klebsiella* K2 capsular polysaccharide.

Complementation analysis. Transformant *K. pneumoniae* NM5(pNM1B01) recovered the ability to produce K2 capsular polysaccharide and its virulence was also recovered to almost the same level as that of the parental strain Chedid (LD_{50}, 3.5 CFU). Although the inactivated gene of pCPS7B06 was complemented by the coexistence of pNM1B01 or pNM6B02, *E. coli* HB101(pCPS7B06, pNM1B01) and HB101(pCPS7B06, pNM6B02) did not produce *Klebsiella* K2 capsular polysaccharide (Table 2).

Expression of *Klebsiella* K2 capsular polysaccharide on the cell surface of *E. coli* HB101. *E. coli* HB101(pCPS7B06, pROJ3) produced as large an amount of *Klebsiella* K2 capsular polysaccharide as did the parental strain Chedid when transformants were incubated at 30°C (Fig. 2b). The formation of K2 capsular polysaccharide was also confirmed by the quellung test. The colonies of HB101(pCPS7B06, pROJ3) showed the same viscid consistency as did those of Chedid. The expression of K2 capsular polysaccharide on the cell surface of HB101(pCPS7B06, pROJ3) was less marked when it incubated at 37°C than at 30°C. In contrast to *E. coli* HB101(pCPS7B06, pROJ3), neither HB101(pCPS7B06) nor HB101(pROJ3) produced a detectable amount of *Klebsiella* K2 capsular polysaccharide (Fig. 2b). The growth of *E. coli* HB101(pCPS7B06) was significantly suppressed, and the shape of its colonies became slightly rough. *E. coli* HB101(pCPS6B02) and HB101 (pCPS6B02, pROJ3) did not produce K2 capsular polysaccharide (data not shown). These results are summarized in Table 2. Although *E. coli* HB101(pCPS7B06, pROJ3) produced *Klebsiella* K2 capsular polysaccharide, the virulence for mice was not increased (LD_{50}, $>10^8$ CFU).

Southern hybridization analysis. The 29-kb *Bam*HI fragment encoding the *cps* gene cluster hybridized with the chromosomal DNA of Chedid. The hybridization patterns of the 29-kb fragment with the *Eco*RI-digested chromosomal DNAs of various *K. pneumoniae* K2 strains were grouped into two types (Fig. 3). Strains K2-215, K2-277, and K2-350 belonged to the same group, in which six clear *Eco*RI bands were observed. The remaining K2 strains tested, including strain Chedid and the K2 reference strain B5055, belonged to the other group, in which four clear bands were observed. The four clear bands of the latter group corresponded to four of the six clear bands of the former group. On the other hand, the 12-kb DNA fragment of pROJ3 carrying *rmpA* hybridized strongly to the resident large plasmid of Chedid (Fig. 4). Faint hybridization to the chromosomal DNA of Chedid was also observed after longer exposure.

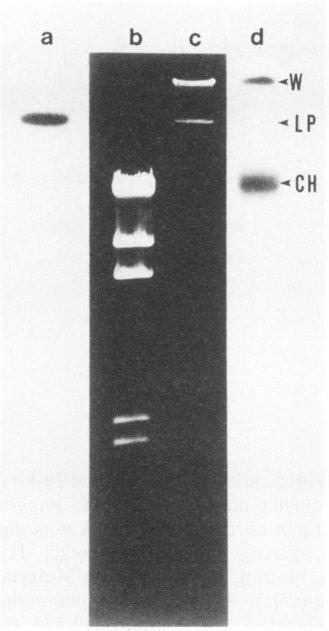

FIG. 4. Southern blot analysis of the genes for production of K2 capsular polysaccharide. Electrophoresis of chromosomal DNA and a large plasmid prepared from strain Chedid by the method of Sasakawa et al. (35) is shown in lane c. Hybridization to the chromosomal DNA by using the 29-kb *Bam*HI fragment as the probe is shown in lane d. The hybridization to the resident large plasmid using the 12-kb *Sal*I-*Sal*I fragment of pROJ3 carrying the *rmpA* gene as the probe is shown in lane a. *Hin*dIII-digested lambda DNA is shown in lane b. Positions of chromosomal DNA and a resident large plasmid are indicated by CH and LP, respectively. The radioactivity indicated by W corresponds to the position of wells.

DISCUSSION

The 23-kb DNA fragment of pCPS7B06 which was cloned from a noncapsulated mutant (NM7) of *K. pneumoniae* Chedid (O1:K2) had a single Tn*5* insertion in the terminal region of the *cps* gene cluster (Fig. 1a). Although pCPS7B06 lacked 6 kb of the left end of the 29-kb *Bam*HI fragment which carried the *cps* gene cluster, pCPS7B06 possessed the ability to produce *Klebsiella* K2 capsular polysaccharide on the cell surfaces of noncapsulated mutants derived from K1, K7, K9, and K28 reference strains of *K. pneumoniae*. These results indicated that, in spite of a deletion of some genes, the 23-kb DNA fragment of pCPS7B06 still encoded enough genetic information for the biosynthesis of *Klebsiella* K2 capsular polysaccharide in *K. pneumoniae*. The results also suggested that an unidentified gene, gene *X*, lost in the chromosome of NM7 is essential for the biosynthesis of K2 capsular polysaccharide, that gene *X* generally exists in various serotypes of *K. pneumoniae*, and that complementation by gene *X* enables pCPS7B06 to reconvert the noncapsulated mutants to the encapsulated forms with the K2 capsular polysaccharide on the cell surface. Our findings suggest that the *Klebsiella cps* gene cluster which we cloned from Chedid corresponds to the structural genes for the biosynthesis of *Klebsiella* K2 capsular polysaccharide. The gene organization of the *Klebsiella cps* gene cluster was well conserved among various *K. pneumoniae* K2 strains, because the hybridization patterns of the 29-kb *Bam*HI fragment with the *Eco*RI-digested chromosomal DNAs were quite similar among the strains, although they were isolated at different times and places (Fig. 3). We speculate that the *Klebsiella cps* gene cluster occupies more than 15 kb of the 29-kb *Bam*HI fragment.

It was reported that the capsular polysaccharides of *E. coli* can be classified into two groups as determined by the influence of temperature on the capsular formation (25). Two different gene loci of structural genes for capsular biosynthesis have been identified on the chromosome. The biosynthesis of group I capsular polysaccharide tends to be enhanced at a lower temperature and its structural genes are located near the *his* operon (44 min), whereas group II capsular polysaccharide is produced at a higher temperature (37°C) (15) and its structural genes are located near *serA* (63 min). Mapping and cloning of the structural genes for group II capsular polysaccharide biosynthesis of *E. coli* have been reported (28, 33, 39). According to Boulnois et al. (4), the genes relating to the translocation of group II capsular polysaccharide to the cell surface exist in the terminal area (region 1) of the structural gene locus of *E. coli* K1. Further, mutation in this region results in the intracellular accumulation of polysaccharide. Although noncapsulated mutants NM7 and NM6 of *K. pneumoniae* Chedid both had only one Tn*5* insertion in the terminal region of the *Klebsiella cps* gene cluster on the 29-kb *Bam*HI fragment (Fig. 1a), there was no evidence that K2 capsular polysaccharide was accumulated within the cells of NM7 or NM6 in double immunodiffusion analysis (data not shown). Therefore, gene *X* described above does not seem to be the gene relating to the translocation of the capsular polysaccharides but may effect a process conserved among serologically distinct capsular polysaccharides such as the regulation or initiation of polymer synthesis. These results suggest that the gene organization of the *Klebsiella cps* gene cluster might be different from that of the group II capsular polysaccharide of *E. coli* K1. This speculation is also supported by the argument that the way of biosynthesis of *Klebsiella* capsular polysaccharide may be similar to that of group I capsular polysaccharide of *E. coli* (15). We found that the 29-kb *Bam*HI fragment carrying the *cps* gene cluster hybridized with the 43- to 47-min portion of the *E. coli* K-12 chromosome, where the structural genes for the biosynthesis of group I capsular polysaccharide are located (data not shown).

In contrast to the findings with the noncapsulated mutants of *K. pneumoniae*, *E. coli* HB101 required both *rmpA* and the *cps* gene cluster for the production of *Klebsiella* K2 capsular polysaccharide on the cell surface. Recently, genes *rmpA* and *rmpB*, which made *E. coli* mucoid, were identified on a resident large plasmid of a *K. pneumoniae* K2 strain (23). It was reported that *rmpA* enhanced colanic acid production in *E. coli* under the control of *rmpB*, but no enhancement of colanic acid production was observed in *K. pneumoniae* when it was transformed by *rmpA*. It was also suggested that this gene might be relevant to the virulence of *Klebsiella* K2 strains, although the real function of *rmpA* in *K. pneumoniae* was not clarified. The present study demonstrated that *rmpA* also enhances the production of *Klebsiella* K2 capsular polysaccharide as well as colanic acid production in *E. coli* HB101. The other regulatory genes for colanic acid production in *E. coli* were also studied (5, 12, 42, 44, 47). All of these genes, *rcsA*, *rcsB*, *rcsC*, *lon*, and *ops*, were found in the chromosome of *E. coli*. There have been no reports concerning the involvement of genes located on the large plasmid of *E. coli* in the biosynthesis of capsular polysaccharide.

All 19 noncapsulated mutants of strain Chedid lost their virulence for mice concurrently with the loss of K2 capsular

production and viscid consistency of colonies. On the other hand, NM5, a noncapsulated mutant of Chedid, recovered its virulence by transformation with pNM1B01, which complemented the inactivated chromosomal gene in NM5. NM5(pNM1B01) produced a large amount of K2 capsular polysaccharide, and the colonies showed a viscid consistency like that of Chedid at 37°C. The virulence of NM5(pNM1B01) was recovered to almost the same level as that of Chedid (LD_{50}, 3.5 CFU). However, the virulence of noncapsulated mutants of *K. pneumoniae* strains was not increased by transformation with pCPS7B06 when they produced *Klebsiella* K2 capsular polysaccharide. We speculate that the production of K2 capsular polysaccharide on the bacterial cell surface may not be enough for the increase in virulence, and there may be additional important virulence factor(s) which relate closely to the biosynthesis of K2 capsular polysaccharide. The virulence of *E. coli* HB101(pCPS7B06, pROJ3), which is capable of producing *Klebsiella* K2 capsular polysaccharide like that of Chedid, could not be confirmed in the present study. *E. coli* HB101 requires many nutrients for growth owing to several genetic defects and, therefore, it does not seem to be an adequate strain for virulence studies.

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