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# RmpA2, an Activator of Capsule Biosynthesis in *Klebsiella pneumoniae* CG43, Regulates K2 *cps* Gene Expression at the Transcriptional Level

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**The *rmpA2* gene, which encodes an activator for capsular polysaccharide (CPS) synthesis, was isolated from a 200-kb virulence plasmid of *Klebsiella pneumoniae* CG43. Based on the sequence homology with LuxR at the carboxyl-terminal DNA-binding motif, we hypothesized that RmpA2 exerts its effect by activating the expression of *cps* genes that are responsible for CPS biosynthesis. Two *luxAB* transcriptional fusions, each containing a putative promoter region of the *K. pneumoniae* K2 *cps* genes, were constructed and were found to be activated in the presence of multicopy *rmpA2*. The activation is likely due to direct binding of RmpA2 to the *cps* gene promoter through its C-terminal DNA binding motif. Moreover, the loss of colony mucoidy in a *K. pneumoniae* strain deficient in RcsB, a regulator for *cps* gene expression, could be recovered by complementing the strain with a multicopy plasmid carrying *rmpA2*. The CPS production in Lon protease-deficient *K. pneumoniae* significantly increased, and the effect was accompanied by an increase of RmpA2 stability. The expression of the *rmpA2* gene was negatively autoregulated and could be activated when the organism was grown in M9 minimal medium. An IS*3* element located upstream of the *rmpA2* was required for the full activation of the *rmpA2* promoter. In summary, our results suggest that the enhancement of K2 CPS synthesis in *K. pneumoniae* CG43 by RmpA2 can be attributed to its transcriptional activation of K2 *cps* genes, and the expression level of *rmpA2* is autoregulated and under the control of Lon protease.**

*Klebsiella pneumoniae*, an important nosocomial pathogen, causes suppurative infection, pneumonia, urinary tract infection, and septicemia in humans. Clinically isolated *K. pneumoniae* usually produces large amounts of capsular polysaccharides (CPS) as reflected by the formation of glistening mucoid colonies with viscid consistency (25). The degree of mucoidy has been shown to positively correlate with the establishment of infection (19, 20). Among at least 77 K serotypes distinguished (18), *K. pneumoniae* strains belonging to serotypes K1 and K2 are the most virulent to mice (17). It has been well documented that CPS protects *K. pneumoniae* from complement-mediated serum killing and phagocytosis (6, 11, 30, 37).

Bacterial CPS can be classified into two groups by chemical and physical criteria. Group I polysaccharides contain uronic acid as the acidic component, have high molecular mass, and are coexpressed with specific O polysaccharides. Group II polysaccharides contain a large variety of acidic components and have a relatively low molecular mass (12). *Klebsiella* K2 CPS has been determined as [→)4-Glc-(1→3)-α-Glc-(1→4)-β-Man-(3←1)-α-GlcA)-(1→]$_n$ (34), which is made from a biosynthetic pathway similar to that of the group I CPS in *Escherichia coli* (36). The genomic organization of the chromosomal *cps* (CPS synthesis) region that is responsible for K2 CPS biosynthesis in *K. pneumoniae* Chedid has been determined, and a total of 15 open reading frames organized into two transcriptional units have been identified as indispensable (2) (Fig. 1A).

The regulatory strategy of colanic acid biosynthesis employed by *E. coli* usually serves as a model for that of group I CPS synthesis. It is built around the RcsC/RcsB two-component pair, where RcsC is the transmembrane sensor and RcsB is the response regulator. The active transcriptional regulator that binds upstream of the *cps* gene cluster (9) consists of activated RcsB and an accessory positive regulator, RcsA. The availability of RcsA is normally limited because the RcsA protein is rapidly degraded by the ATP-dependent Lon protease; therefore, mutations at *lon* have been found to lead to the accumulation of colanic acid (9). In *E. coli* K-12, the presence of RcsB is indispensable for CPS biosynthesis, whereas an *rcsA*-negative phenotype can be suppressed by multicopy *rcsB* (4).

*K. pneumoniae* CG43, a clinical isolate of K2 serotype, showed strong virulence in a mouse peritonitis model with a 50% lethal dose ($LD_{50}$) as low as 10 CFU (5). A large plasmid of approximately 200-kb in size was noted to be present in this strain as well as in most, if not all, bacteremic isolates of *K. pneumoniae*. The cure of the 200-kb plasmid from *K. pneumoniae* CG43 resulted in a loss of colony mucoidy and a 1,000-fold decrease in virulence. This plasmid was therefore designated as pLVPK (for large virulence plasmid in *Klebsiella*). Sequence analysis of a pathogenicity island carried by pLVPK has revealed a locus named *rmpA2*, which has been reported to enhance the colony mucoidy of various serotypes of *K. pneumoniae* (34). Introduction of multicopy *rmpA2* could confer on *E. coli* HB101 the ability to produce *Klebsiella* K2 capsule in the presence of K2 *cps* gene cluster, and the transcription of a

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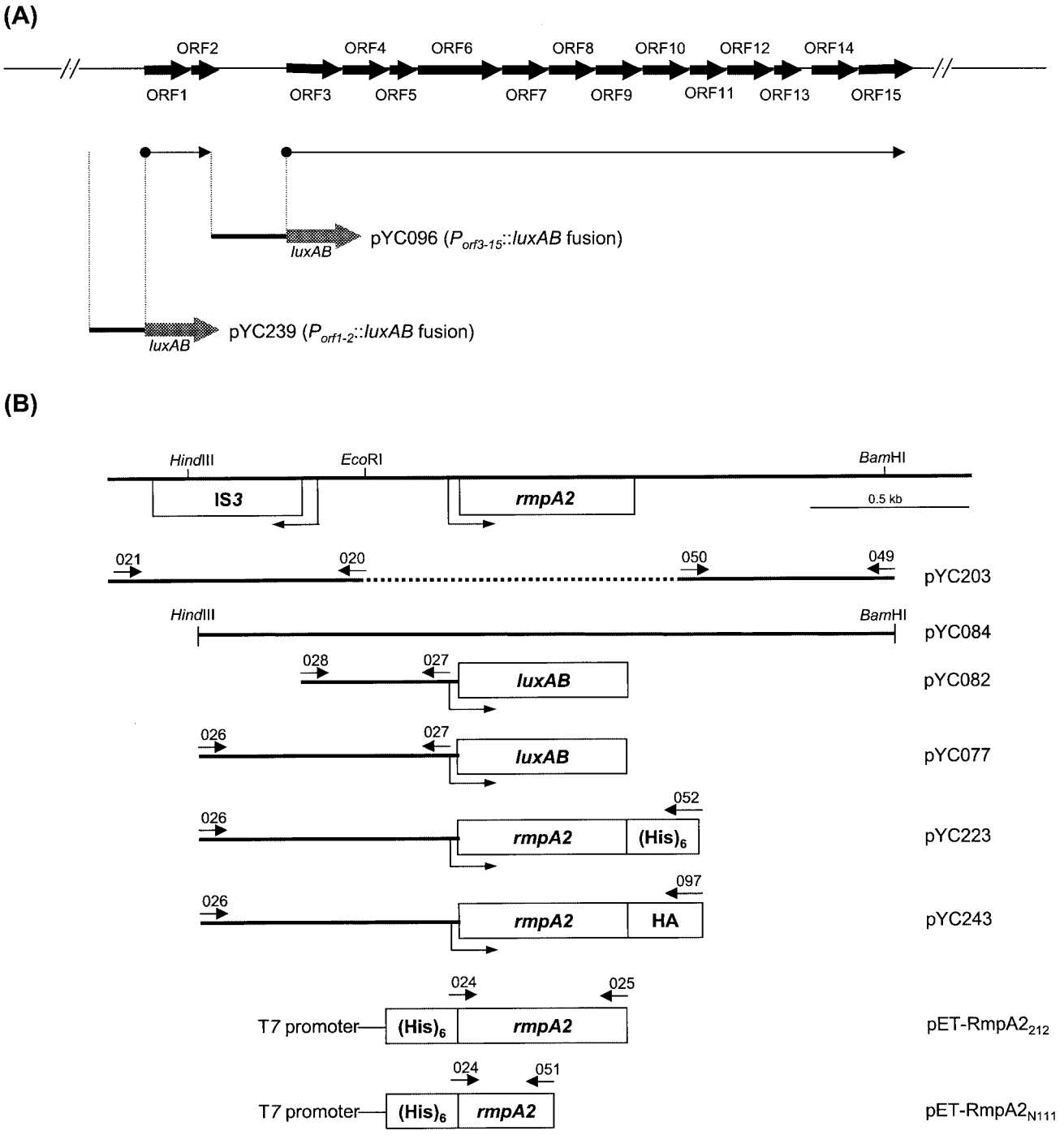


FIG. 1. (A) Organization of the *K. pneumoniae* K2 *cps* gene cluster. The horizontal arrows that begin with a solid circle represent the putative transcriptional units. The putative promoter regions that were cloned into pYC017 as *luxAB* transcriptional fusions are indicated. (B) Physical map of the *rmpA2* gene. The positions of the IS*3* element, the primers used for PCR amplification, and the extents of subclones used in this study are indicated.

long-strand mRNA from K2 *cps* operon was simultaneously increased (1, 33). These findings indicated that RmpA2 functions as a *trans*-acting activator for the CPS biosynthesis. Due to the essential role of CPS in *K. pneumoniae* pathogenesis, it would be important to understand how RmpA2 exerts its activation to the K2 CPS biosynthesis. We report here our results on characterizing RmpA2 as a transcriptional activator for the *Klebsiella* K2 *cps* genes.

## MATERIALS AND METHODS

**Plasmids, bacterial strains, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli*, *K. pneumoniae* CG43 (5, 22),

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| Strains | | |
| *K. pneumoniae* | | |
| CG43 | Clinical isolates | 22 |
| CG43-17 | CG43 with chromosome Tn*5* insertion, *galU* $Km^r$ | 5 |
| CG43S3 | CG43 $Sm^r$ | This study |
| CG43S3-R2035 | CG43S3 Δ*rmpA2* $Sm^r$ | This study |
| CG43S3-B2202 | CG43S3 Δ*rcsB* $Sm^r$ | This study |
| CG43S3-L2117 | CG43S3 Δ*lon* $Sm^r$ | This study |
| CG43S3-RB01 | CG43S3 Δ*rmpA2* Δ*rcsB* $Sm^r$ | This study |
| CG43S3-RL01 | CG43S3 Δ*rmpA2* Δ*lon* $Sm^r$ | This study |
| *E. coli* | | |
| S17-1 | *hsdR recA pro* RP4-2 (Tc::Mu; Km::Tn*7*) | 28 |
| S17-1λ*pir* | *hsdR recA pro* RP4-2 (Tc::Mu; Km::Tn*7*)(λ *pir*) | 28 |
| NovaBlue(DE3) | *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*$^q$ *Z*ΔM15Tn*10*] (DE3) | Novagen |
| Plasmids | | |
| pET30a | His-tagged protein expression vector, $Km^r$ | Novagen |
| pKAS46 | Suicide vector, *rpsL* $Ap^r$ $Km^r$ | 28 |
| pRK415 | Shuttle vector, *mob*$^+$ $Tc^r$ | 14 |
| pYC017 | Promoter selection vector, $GalU^+$ $LuxAB^+$ $Cm^r$ | This study |
| pYC077 | Fragment containing the 0.9-kb region upstream of *rmpA2* cloned into pYC017 | This study |
| pYC082 | Fragment containing the 0.5-kb region upstream of *rmpA2* cloned into pYC017 | This study |
| pYC084 | 2.1-kb *Hin*dIII/*Bam*HI fragment containing the entire *rmpA2* locus cloned into pRK415 | This study |
| pYC096 | 1.0-kb fragment containing the promoter region of K2 CPS *orf3* through *orf15* cloned into pYC017 | This study |
| pYC203 | 2.0-kb fragment containing a 530-bp deletion in *rmpA2* locus cloned into pKAS46 | This study |
| pYC211 | 1.9-kb fragment containing a 1.3-kb deletion in *lon* locus cloned into pKAS46 | This study |
| pYC220 | 2.0-kb fragment containing a 763-bp deletion in *rcsB* locus cloned into pKAS46 | This study |
| pYC223 | 1.5-kb fragment containing a C-terminally fused His tag with the *rmpA2*-coding region cloned into pRK415 | This study |
| pYC239 | 0.7-kb fragment containing the promoter region of K2 CPS *orf1-orf2* cloned into pYC017 | This study |
| pYC243 | 1.5-kb fragment containing a C-terminally fused HA tag with the *rmpA2*-coding region cloned into pRK415 | This study |
| pET-RmpA2$_{212}$ | 636-bp fragment containing full-length RmpA2 cloned into pET30a | This study |
| pET-RmpA2$_{N111}$ | 333-bp fragment containing the N-half of RmpA2 cloned into pET30a | This study |

and its derivatives were propagated at 37°C in Luria-Bertani (LB) broth. M9 minimal medium was prepared as described previously (26). The density of the bacterial culture was determined by measuring the absorbance of adequate dilutions at an optical density at 600 nm ($OD_{600}$) with a Shimadzu UV-1201 spectrophotometer and expressed as $OD_{600}$ × dilution factor.

**Construction of gene-specific mutants.** *K. pneumoniae* CG43 mutants disrupted specifically at *rmpA2*, *rcsB*, or *lon* genes were constructed by the allelic exchange strategy. The primer sets used for PCR amplification of the DNA fragments that flank the regions to be deleted are listed in Table 2. For homologous recombination, the generated DNA fragments were cloned into pKAS46, and the resulting plasmids were then mobilized to *K. pneumoniae* CG43S3 through conjugation from *E. coli* S17-1λ*pir*. Plasmid pKAS46 (a generous gift of K. Skorupski, University of New Hampshire) is a suicide vector containing the *rpsL* gene, which allows positive selection with streptomycin for the loss of the vector (28). One of the kanamycin-resistant transconjugants was picked, grown overnight, and then spread onto an LB plate supplemented with streptomycin (500 μg/ml). After the occurrence of double crossover, the streptomycin-resistant and kanamycin-sensitive colonies were selected, and the deletions of *rmpA2*, *rcsB*, or *lon* were verified by PCR and by Southern blot analysis with a gene-specific probe. Three gene-specific mutant strains—*K. pneumoniae* R2035 (Δ*rmpA2*), B2202 (Δ*rcsB*), and L2117 (Δ*lon*)—were obtained, and *K. pneumoniae* R2035 was used further to generate double-mutant strains *K. pneumoniae* RB01 (Δ*rmpA2* Δ*rcsB*) and RL01 (Δ*rmpA2* Δ*lon*) (Table 1).

**Construction of *luxAB* transcriptional fusions.** For complementation of the *rmpA2* deletion, a 2.1-kb *Hin*dIII/*Bam*HI fragment that comprises the entire *rmpA2* locus (Fig. 1B) was cloned into a shuttle vector pRK415 (14) to generate pYC084. To construct $P_{cps}$::*luxAB* fusion plasmids compatible with pYC084, the primer sets (Table 2) were designed based upon the published sequence of K2 *cps* genes (GenBank accession no. D21242) and used for PCR amplification of the putative promoter regions. Primer sets used to amplify the upstream region of *rmpA2* gene are also listed in Table 2. These generated promoter fragments were then cloned into pYC017, a derivative of pYC016 (15), which contains a copy of promoterless *luxAB* of *Vibrio fischeri* as a reporter and a control cassette from pKK232-8 that ensures transcriptional fusion and prevents interference from a fortuitous plasmid promoter. The fragments that encode a full-length RmpA2 protein with a C-terminally fused six-His tag and a hemagglutinin (HA) tag driven by its own promoter were also amplified by PCR with respective primer sets 026/052 and 026/097 (Table 2), cloned into pRK415 to generate pYC223 or pYC243. All these constructs were transferred into *K. pneumoniae* CG43S3, and its mutant derivatives through conjugation from *E. coli* S17-1.

**Luciferase activity assay.** The expression of different promoter::*luxAB* fusions was assessed by measuring the luciferase activity as follows. Overnight bacterial cultures were recovered with 1:10 dilution in M9 medium at 25°C for 4.5 h. Five hundred microliters of the recovered culture was mixed with 500 μl of 0.1% (vol/vol) *n*-decyl aldehyde (Sigma-Aldrich, Milwaukee, Wis.). The mixture was made to stand at room temperature for 60 s and then read in full integral, auto ranging mode of a 30 s integration time with a luminometer (TD-20/20; Turner Designs). The data shown was normalized and expressed as relative light units/$OD_{600}$.

**Mouse lethality assay.** Female BALB/c mice with an average weight of 25 g were obtained from the animal center of National Taiwan University and were acclimatized in an animal house for 3 days. The tested bacterial strains were cultured in LB medium at 37°C for overnight. Five mice of a group were injected intraperitoneally with bacteria resuspended in 0.2 ml of saline in 10-fold steps graded doses. The $LD_{50}$s, based on the number of survivors after one week, were calculated by the method of Reed and Muench (24) and expressed as CFU.

**Resistance to serum killing.** One hundred microliters of bacterial suspension in saline was mixed with 100 μl of pooled serum from healthy volunteers, and the mixture was incubated at 37°C for 30 min. The number of viable bacteria in the mixture was then determined by plating.

**Extraction and quantification of CPS.** CPS was extracted by the method described previously (7). Five hundred microliters of bacterial culture was mixed

TABLE 2. Primers used in this study

| Primer no. | Sequence | Enzyme cleaved | Complementary position |
|---|---|---|---|
| 020 | 5′-CTGTGTGATTAAGAATTCATA-3′ | 5′-*Eco*RI | −223 relative to the *rmpA2* start codon |
| 021 | 5′-ATCCTCTAGAGTCGACGCGTT-3′ | 5′-*Xba*I | −1392 relative to the *rmpA2* start codon |
| 024 | 5′-CATGCCATGGGAAAATATATTTAC-3′ | 5′-*Nco*I | +1 of the *rmpA2* coding region |
| 025 | 5′-ATATCTCGAGTTATCTAGGTATTTGATG-3′ | 5′-*Xho*I | +636 of the *rmpA2* coding region |
| 026 | 5′-ACGCGGATCCTAGCTCCACAGGTAAAGT-3′ | 5′-*Bam*HI | −812 relative to the *rmpA2* start codon |
| 027 | 5′-ACGCGGATCCGTCCAGTTAACTGCTTTA-3′ | 5′-*Bam*HI | +96 relative to the *rmpA2* start codon |
| 028 | 5′-ATATGGATCCCACTTAGTCCTGTGTCCA-3′ | 5′-*Bam*HI | −390 relative to the *rmpA2* start codon |
| 040 | 5′-ACTGGATCAGGCCTGGTAATCGCCATT-3′ | 5′-*Bam*HI | −891 relative to the *orf3* start codon |
| 041 | 5′-ACTGGATCCCGCTGTCGTATCTCAATG-3′ | 5′-*Bam*HI | +60 relative to the *orf3* start codon |
| 049 | 5′-GCCGAGCTCTGGACATAGACAGC-3′ | 5′-*Sac*I | +639 relative to the *rmpA2* stop codon |
| 050 | 5′-CCGGAATTCCTAAAGGGTGTGATTATG-3′ | 5′-*Eco*RI | +295 relative to the *rmpA2* start codon |
| 051 | 5′-ATATCTCGAGTTAAATTTCCTTGCATGTTGAC-3′ | 5′-*Hin*dIII | +333 of the *rmpA2* coding region |
| 052 | 5′-CCCAAGCTTCAGTGGTGGTGGTGGTGGGTATTTGATGTGC ACCA-3′ | 5′-*Hin*dIII | +636 of the *rmpA2* coding region |
| 053 | 5′-TGCTCTAGAAGCGACTCATCGATCAG-3′ | 5′-*Xba*I | +999 relative to the *lon* stop codon |
| 054 | 5′-CCCAAGCTTGCGCTGCAGAACGCC-3′ | 5-*Hin*dIII | +61 relative to the *lon* stop codon |
| 055 | 5′-CCCAAGCTTCACGCGCTCCAGGCCATA-3′ | 5-*Hin*dIII | +993 relative to the *lon* start codon |
| 056 | 5′-AGAGAGCTCTATGAATCCTGAGCG-3′ | 5′-*Sac*I | +10 relative to the *lon* start codon |
| 061 | 5′-AGAGAGCTCTGCAGCTGCTCATCAACA-3′ | 5′-*Sac*I | −983 relative to the *rcsB* start codon |
| 062 | 5′-CCCAAGCTTGCGCATCCTTTTCGCGA-3′ | 5′-*Hin*dIII | −10 relative to the *rcsB* start codon |
| 063 | 5′-CCCAAGCTTATTCCCGCCCTTTACGCA-3′ | 5-*Hin*dIII | +47 relative to the *rcsB* stop codon |
| 064 | 5′-TGCTCTAGAGGGGATCCCGGCGAAA-3′ | 5′-*Xba*I | +1018 relative to the *rcsB* stop codon |
| 074 | 5′-ACTGGATCCACGATCATGGATAAGAT-3′ | 5′-*Bam*HI | −723 relative to the *orf1* start codon |
| 075 | 5′-ACTGGATCCTGCGACCGGAATAACC-3′ | 5′-*Bam*HI | +42 relative to the *orf1* start codon |
| 097 | 5′-CCCAAGCTTCTAAGCGTAGTCTGGGACGTCGTATGGGTAGGTATTT GATGTGCACCAA-3′ | 5′-*Hin*dIII | +636 of the *rmpA2* coding region |

with 100 μl of 1% Zwittergent 3-14 detergent (Sigma-Aldrich) in 100 mM citric acid (pH 2.0), and then the mixture was incubated at 50°C for 20 min. After centrifugation, 250 μl of the supernatant was transferred to a new tube, and CPS was precipitated with 1 ml of absolute ethanol. The pellet was then dissolved in 200 μl of distilled water, and a 1,200-μl volume of 12.5 mM borax (Sigma-Aldrich) in $H_2SO_4$ was added. The mixture was vigorously vortexed, boiled for 5 min, and cooled, and then 20 μl of 0.15% 3-hydroxydiphenol (Sigma-Aldrich) was added and the absorbance at 520 nm was measured. The uronic acid content was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed as micrograms per $10^9$ CFU (3).

**RNA dot blotting analysis.** Total RNA was isolated from mid-log-phase *K. pneumoniae* cells ($OD_{600}$ = 0.6 to 0.8) by extraction with the TRI reagent (Molecular Research Center, Cincinnati, Ohio). Contaminating DNA was eliminated from the RNA samples with RQ1 RNase-free DNase (Promega, Madison, Wis.). Probes used in hybridization assay were labeled with fluorescein-11-dUTP by random priming with the *Gene Images* kit (Amersham-Pharmacia, Piscataway, N.J.). Five and ten micrograms of total RNA was transferred onto a Hybond-$N^+$ membrane (Amersham-Pharmacia) by dot blotting, prehybridized for 1 h at 65°C, hybridized overnight at the same temperature, washed, and detected with the CDP-*Star* reagent (Amersham-Pharmacia).

**Expression and purification of recombinant RmpA2.** The coding region of *rmpA2* was amplified with primers 024 and 025 (Table 2), and cloned as an *Nco*I/*Xho*I fragment into pET30a (Novagen, Madison, Wis.). The resulting plasmid pET-$RmpA2_{212}$ allowed in-frame fusion of the full-length *rmpA2* coding region to six histidine codons at the N terminus and transcription from a T7 promoter. A C-terminal truncated form of RmpA2, comprising codons 1 to 111 of *rmpA2*, was amplified with primers 024 and 051 (Table 2), cloned into pET30a, and resulted in plasmid pET-$RmpA2_{N111}$. The overexpressed His-RmpA2 proteins were then purified from the soluble fraction of total cell lysate by affinity chromatography with His-Bind resin (Novagen). The purified His-$RmpA2_{212}$ and His-$RmpA2_{N111}$ were then concentrated and dialyzed against 1× storage buffer (100 mM KCl, 20 mM $MgCl_2$, 10 mM $Na_2HPO_4$ [pH 7.4], 1.8 mM $KH_2PO_4$ [pH 7.4], and 10% glycerol), and the purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

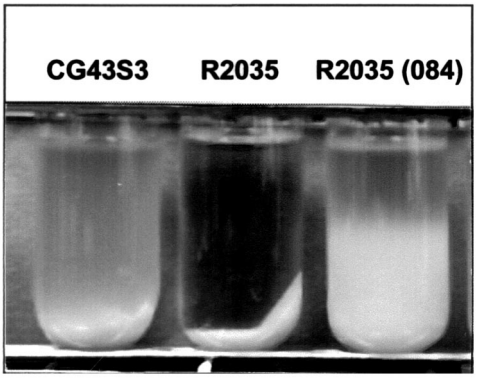


FIG. 2. Comparison of precipitation speeds of *K. pneumoniae* CG43S3, *K. pneumoniae* R2035 (Δ*rmpA2*), and *K. pneumoniae* R2035 [pYC084]. The strains tested were cultured overnight in LB broth at 37°C and subjected to centrifugation at 1,000 ×*g* for 5 min.

**DNA electrophoretic mobility shift assay (EMSA).** DNA fragments comprising the testing promoter regions were obtained by PCR amplification with primer sets 027-028, 040-041, and 074-075 (Table 2), respectively, followed by end labeling with [γ-$^{32}$P]ATP. The purified His-RmpA2 proteins, ranging from 50 ng to 1 μg, were mixed with DNA probes (0.1 ng) in 50-μl reaction mixtures containing 12 mM HEPES (pH 7.4), 100 mM KCl, 20 mM $MgCl_2$, 0.6 mM dithiothreitol, and 5% glycerol. The mixtures were incubated at room temperature for 25 min, mixed with 0.1 volume of DNA loading dye, and then loaded onto 5% nondenaturing polyacrylamide gels containing 5% glycerol in 0.5× TBE buffer (45 mM Tris-HCl [pH 8.0], 45 mM boric acid, 1.0 mM EDTA). Gels were electrophoresed with a 20-mA current at 4°C and dried under a vacuum, and the results were detected by autoradiography.

**Determination of turnover of RmpA2 protein.** Pulse-chase and immunoprecipitation experiments were performed as described previously (32). *K. pneumoniae* cells were grown at 37°C to an $OD_{600}$ of 0.6 and labeled for 2 min with 10 μl of L-[$^{35}$S]methionine (1,000 Ci/mmol; New England Nuclear) per ml in M63 medium (27). The labeled cells were chased with M63 medium containing 0.5% L-methionine, and 1.5 ml of the samples was collected at the time indicated. The cell precipitates were resuspended in 30 μl of 1× lysis buffer (1% SDS, 1 mM EDTA, 10 mM Tris-HCl [pH 7.4]), boiled for 5 min, and diluted 30-fold with 1× immunoprecipitation buffer (1 mM EDTA, 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and Complete protease inhibitor [Roche Molecular Biochemicals, Mannheim, Germany]). Five microliters of anti-His monoclonal antibody (MAb) (Novagen) or anti-HA MAb (Roche Molecular Biochemicals) was added, and the incubation was continued at 4°C overnight. The immunoprecipitates were adsorbed onto protein A-Sepharose (Amersham-Pharmacia) and were washed three times before being resuspended in SDS-PAGE loading buffer and electrophoresed. The amount of RmpA2 protein on the gel was determined with a densitometer (Molecular Dynamics).

TABLE 4. Virulence properties of *K. pneumoniae rmpA2* mutant

| Strain | $LD_{50}$ (CFU) | Survival rate in human serum (%)[a] |
|---|---|---|
| CG43S3 | $4 \times 10^3$ | >90 |
| R2035 | $1 \times 10^5$ | >90 |
| R2035[pYC084] | $8 \times 10^2$ | >90 |
| CG43-17 (control)[b] | $1 \times 10^6$ | 0 |

[a] Percent survival rate in human serum is expressed as 100 × (the number of viable bacteria after treatment/the number of viable bacteria before treatment).
[b] CG43-17, a *galU* mutant strain with defective CPS and lipopolysaccharide (5).

## RESULTS

**Isolation and characterization of an *rmpA2* deletion mutant.** To assess the role of RmpA2 in the regulation of K2 CPS biosynthesis, a *K. pneumoniae rmpA2* deletion mutant designated R2035 was first generated. The mutant strain displayed a large, glistening colony morphology on LB agar that was indistinguishable from that of wild-type strains. However, the colony mucoidy of R2035 appeared to reduce, as determined by the inability of the colony to form a string using a toothpick. The reduction of mucoidy was also evident when the bacterial cultures were subjected to low-speed centrifugation. As shown in Fig. 2, R2035 cells precipitated much faster than its parental strain CG43S3. Introduction of pYC084 (Fig. 1B) that contains a functional *rmpA2* gene into R2035 restored the colony mucoidy as reflected by both the string formation test and the slower precipitation (Fig. 2). The amount of CPS produced in R2035 was further quantified by measuring the glucuronic acid content, which serves as an indicator of *Klebsiella* K2 CPS (21). As shown in Table 3, R2035 synthesized less K2 CPS than the wild-type strain, and the effect was even more pronounced

TABLE 3. Effects of RmpA2 on CPS synthesis in *K. pneumoniae* CG43 strains with different genetic backgrounds

| Strains | CPS synthesized when grown as indicated[d] | | | | | | Mucoid phenotype[c] |
|---|---|---|---|---|---|---|---|
| | LB incubation | | Minimal medium (0.4% glucose) | | Minimal medium (1% glycerol) | | |
| | Mean quantity ± SD[a] | Fold[b] | Mean quantity ± SD | Fold | Mean quantity ± SD | Fold | |
| CG43S3 | 15.4 ± 0.7 | | 110.4 ± 3.9 | | 51.6 ± 6.3 | | + |
| R2035 | 13.5 ± 0.5 | | 79.1 ± 3.6 | | 48.4 ± 5.1 | | − |
| B2202 | 12.9 ± 0.8 | | 72.2 ± 2.9 | | 33.3 ± 2.8 | | − |
| L2117 | 97.4 ± 2.8 | | 600.6 ± 32.4 | | 232.6 ± 9.7 | | +++ |
| RB01 | 10.4 ± 1.2 | | 62.3 ± 4.7 | | 27.3 ± 5.6 | | − |
| RL01 | 10.9 ± 1.4 | | 60.7 ± 5.3 | | 35.1 ± 6.2 | | − |
| S3[pYC084] | 29.2 ± 2.5 | 1.89 | 166.1 ± 6.2 | 1.50 | 65.3 ± 6.4 | 1.27 | ++ |
| R2035[pYC084] | 30.9 ± 1.8 | 2.29 | 235.3 ± 13.4 | 2.97 | 87.4 ± 8.9 | 1.81 | ++ |
| B2202[pYC084] | 31.4 ± 2.7 | 2.43 | 158.5 ± 8.4 | 2.20 | 61.2 ± 7.7 | 1.84 | ++ |
| L2117[pYC084] | 111.8 ± 6.7 | 1.15 | 814.3 ± 31.5 | 1.36 | 417.3 ± 9.6 | 1.79 | +++ |
| RB01[pYC084] | 30.3 ± 3.2 | 2.91 | 188.5 ± 5.8 | 3.03 | 72.0 ± 3.5 | 2.64 | + |
| RL01[pYC084] | 26.1 ± 2.9 | 2.39 | 268.2 ± 7.5 | 4.42 | 67.8 ± 6.4 | 1.93 | ++ |

[a] Values are the averages of triplicate samples and are given as micrograms of uronic acid per $10^9$ CFU.
[b] Compared with each parental strain without pYC084.
[c] Assessed by string formation test after 48 h of growth on M9-glucose medium. Symbols: −, negative; +, positive; ++, strong; +++, very strong.
[d] The production of polysaccharides on the cell surface of each strain was ascertained as K2 CPS by the Quellung test.

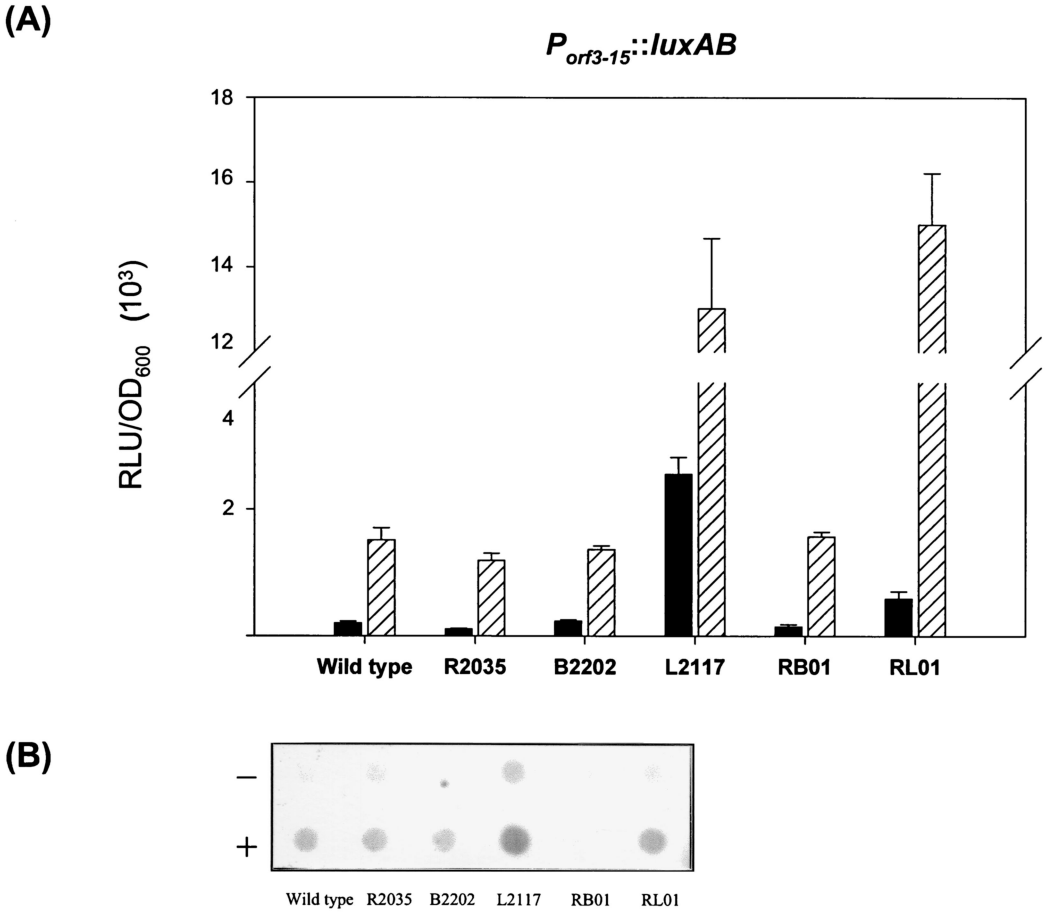


FIG. 3. Expression of K2 *cps* genes in various genetic backgrounds. The luciferase activity of K2 $P_{cps}$::*luxAB* transcriptional fusions in strains CG43S3 (wild type), R2035 (Δ*rmpA2*), B2202 (Δ*rcsB*), L2117 (Δ*lon*), RB01 (Δ*rmpA2* Δ*rcsB*), and RL01 (Δ*rmpA2* Δ*lon*) carrying either the $P_{orf3\text{-}15}$::*luxAB* fusion (A) or the $P_{orf1\text{-}2}$::*luxAB* fusion (C) in the presence (striped bars) or absence (solid bars) of pYC084 was determined (error bars, standard deviations). Ten micrograms of total RNA extracted from different *K. pneumoniae* cells was spotted on a Hybond-N$^+$ membrane and hybridized with probes specific to either *orf3* (B) or *orf1* (D). + and −, presence and absence of pYC084, respectively. RLU, relative light units.

when the cells were grown in M9 medium with 0.4% glucose. The complementation strain R2035[pYC084] produced twice as much CPS as that produced by R2035 (Table 3). Finally, in a mouse peritonitis model, the deletion of *rmpA2* resulted in an increase of $LD_{50}$ by 25-fold, and the reduction in virulence could be restored and even enhanced in strain R2035 complemented with pYC084 (Table 4). However, the *rmpA2* mutant R2035 was as resistant as its parental strain to the human serum killing effect (Table 4).

**Enhancement of K2 *cps* gene expression by *rmpA2*.** In *E. coli* K-12 strains, the amount of colanic acid produced has been reported to be correlated with the transcriptional level of *cps* genes (9). We reasoned that the production of CPS enhanced by *rmpA2* might also have resulted from an elevated transcriptional level of *Klebsiella* K2 *cps* genes. To test the possibility, two *luxAB* reporter fusions, pYC096 ($P_{orf3\text{-}15}$::*luxAB*), which carries the putative promoter region responsible for initiating transcription of the operon containing *orf3* to *orf15*, and pYC239 ($P_{orf1\text{-}2}$::*luxAB*), which comprises the region controlling the expression of *orf1* and *orf2* (Fig. 1A), were generated. The *luxAB* fusion plasmids were then individually transformed into *K. pneumoniae* strains CG43S3 and R2035 in the presence or absence of pYC084. As shown in Fig. 3A and C, the activity of $P_{orf3\text{-}15}$ and $P_{orf1\text{-}2}$ in the wild-type strain CG43S3 was barely detectable and indistinguishable from that in the *rmpA2* deletion strain R2035. However, in the presence of pYC084, expression of the $P_{orf3\text{-}15}$::*luxAB* fusion increased 7-fold in CG43S3 and 11-fold in R2035 (Fig. 3A). A similar enhancement by pYC084 was also observed for $P_{orf1\text{-}2}$, in which RmpA2 performed as a strong activator (Fig. 3C).

The activation was further verified by RNA dot blotting analysis with fluorescein-labeled DNA fragments containing the coding region of *orf3* and *orf1*, respectively, as a probe. As shown in Fig. 3B and D, the amount of both *orf3*- and *orf1*-containing transcripts in the testing strains was increased in the presence of pYC084. Therefore, it can be concluded that the transcriptional level of K2 *cps* genes could be elevated by the multicopy *rmpA2* in a *trans*-acting way.

**Requirement of the C-terminal half of RmpA2 for its *trans*-activating properties.** The *rmpA2* gene contains a poly(G) tract located at position +276 relative to the A residue of the first in frame start codon. The length of the poly(G) tract in the

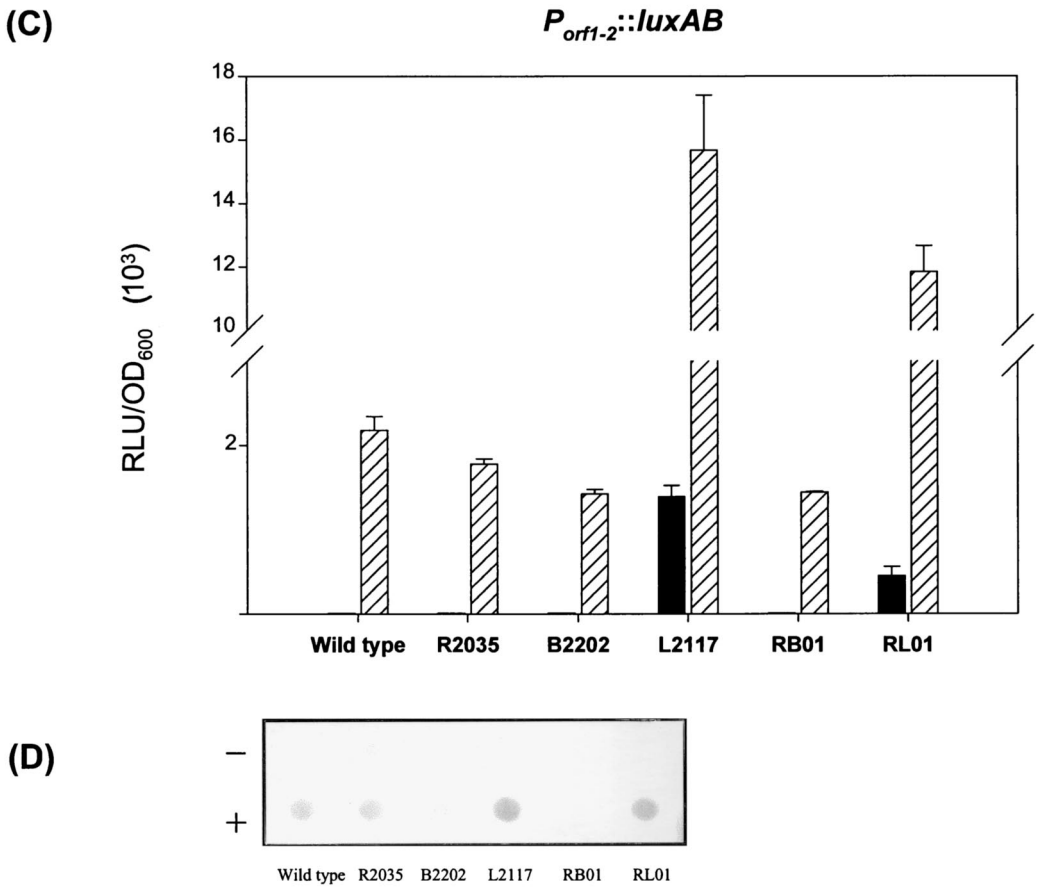


FIG. 3—*Continued.*

*rmpA2* genes has been investigated in a number of clinical isolates of *K. pneumoniae* and was found to be variable, ranging from 9 to 12. Only the 11-G tract allowed *rmpA2* remain in frame to encode a full-length RmpA2 protein. Other G tracts would incur a truncated RmpA2 protein by an amber stop codon in the reading frames. Since truncated forms of RmpA2 are frequently observed in clinical strains, it would be of interest to know whether these gene products retain its *trans*-activating function. Expression constructs for the full-length RmpA2 and a truncated RmpA2 with a tract of 10 G residues were generated and designated pET-RmpA2$_{212}$ and pET-RmpA2$_{N111}$, respectively. These two plasmids were then cotransformed individually with one of the two $P_{cps}$::*luxAB* transcriptional fusions into *E. coli* NovaBlue(DE3). After IPTG (isopropyl-β-D-thiogalactopyranoside) induction, the luciferase activity of each *luxAB* fusion was measured. Consistent with the effects of pYC084, pET-RmpA2$_{212}$ activated the expression of $P_{orf1-2}$::*luxAB* and $P_{orf3-15}$::*luxAB* (Table 5). However, pET-RmpA2$_{N111}$, which produced a truncated form of RmpA2, did not display similar *trans*-activating properties.

**Binding of RmpA2 to its target promoters.** To validate RmpA2 as a transcriptional regulator that interacts with its target promoter directly, DNA EMSA was performed with the purified RmpA2 protein and each of the DNA fragments carrying the promoter region of K2 *cps* genes. As shown in Fig. 4, RmpA2 is capable of binding to the DNA fragments containing $P_{orf3-15}$ and $P_{orf1-2}$, whereas no DNA-protein complexes could be observed with the C-terminal truncated RmpA2. The DNA-protein interaction is specific, and the formation of RmpA2-promoter complex could be inhibited in the presence of specific competitor DNA (Fig. 5A and B).

**Effects of *rmpA2* on K2 CPS biosynthesis in *rcsB* mutant *K. pneumoniae* cells.** Analysis of predicted protein sequence of RmpA2 has shown that it belongs to the UhpA-LuxR family, which also includes RcsA and RcsB (29). To further investigate the relationship between RmpA2 and the Rcs system in CPS biosynthesis, an *rcsB* deletion mutant, B2202 and a double mutant, RB01 (Δ*rmpA2* Δ*rcsB*), were generated in *K. pneumoniae* CG43S3 (Table 1). Similar to the *rmpA2* deletion mutant R2035, strain B2202 lost the colony mucoid characteristic and displayed reduced K2 CPS synthesis (Table 3). In the double-mutant strain RB01, the reduction of K2 CPS was more pronounced (Table 3). The deletion of *rcsB*, however, did not affect the activation of $P_{orf1-2}$ and $P_{orf3-15}$ by RmpA2 (Fig. 3). Complementation of B2202 and RB01with pYC084 not only restored but also enhanced colony mucoidy and CPS production in these strains (Table 3).

**Effects of *rmpA2* on K2 CPS biosynthesis in *lon* mutant *K. pneumoniae* cells.** To investigate the interplay between RmpA2 and Lon protease, a *lon* deletion mutant L2117 and a double-deletion mutant RL01 (Δ*rmpA2* Δ*lon*) were generated. Similar to the *E. coli lon* mutant strains, *K. pneumoniae* L2117 dis-

TABLE 5. Effect of RmpA2 on expression of *luxAB* transcriptional fusions in *E. coli* NovaBlue(DE3)

| Strain | Mean LuxAB activity ± SD (RLU/$OD_{600}$)[a] | | |
|---|---|---|---|
| | $P_{orf3\text{-}15}$::*luxAB* | $P_{orf1\text{-}2}$::*luxAB* | $P_{rmpA2}$::*luxAB* |
| NovaBlue(DE3) | 215 ± 13 | 5 ± 0.74 | 12,638 ± 2,168 |
| NovaBlue(DE3)[pET-$Rmp_{N111}$] | 328 ± 52 | 9 ± 1 | 11,237 ± 1,045 |
| NovaBlue(DE3)[pET-$Rmp_{212}$] | 1,236 ± 83 | 104 ± 2 | 1,813 ± 112 |

[a] Luciferase activity was measured after 8 h of induction with 1 mM IPTG at 25°C. Values are the averages of triplicate samples. RLU, relative light units.

played extremely mucoid colony morphology and produced at least four times as much K2 CPS as the wild-type strain (Table 3). The transcription level and promoter activity of K2 *cps* genes were also found to increase in L2117; however, when the *rmpA2* and *lon* genes were simultaneously deleted, the enhancement of CPS production following *lon* deletion was diminished (Fig. 3 and Table 3). The result suggested that the K2 CPS biosynthesis was negatively regulated by Lon protease and this action was mediated by RmpA2.

**Comparison of RmpA2 stability in wild-type and *lon* mutant *K. pneumoniae* strains.** In *E. coli* K-12 strains, the increase of CPS production in *lon* mutant cells can be explained by the stabilization of RcsA protein (29). Whether the accumulation of CPS in Lon protease-deficient *K. pneumoniae* L2117 is due to stabilization of RmpA2 was investigated. The stability of RmpA2 protein was determined by a pulse-chase analysis with [$^{35}$S]methionine as a label. We have found that the concentration of RmpA2 in *K. pneumoniae* was rather low and was virtually undetectable even in the *lon* deletion strain. To increase the concentration of RmpA2, the low-copy-number plasmid pYC223 or pYC243, which encodes an RmpA2 protein with a C-terminally fused His tag or HA tag, were constructed and subsequently transferred into CG43S3 and L2117. After pulse-chase treatment, the RmpA2 protein was precipitated with anti-His or anti-HA MAb. As shown in Fig. 6, the half-life of either His-RmpA2 or HA-RmpA2 was found to increase from 1 to 2 min in the wild-type *K. pneumoniae* CG43S3 to approximately 10 min in the *lon* mutant strain.

**Autoregulation of *rmpA2*.** As shown in Table 3, the growth of *K. pneumoniae* strains in M9 minimal medium increased the production of CPS. The enhancement might have resulted from an activation of *rmpA2* gene expression. To test the possibility, total RNA was extracted from R2035 [pYC084] grown in LB or under CPS-inducing conditions, with M9 minimal medium supplemented with glycerol or glucose as the sole carbon source. As shown in Fig. 7A, the CPS-inducing conditions increased the amount of *rmpA2* transcripts. Consistent with the result shown in Table 3, the growth in M9 medium–0.4% glucose that induced the highest level of expression of the *rmpA2* gene also led to the highest yield of K2 CPS. To quantitatively analyze the activity of the *rmpA2* promoter, the plasmid pYC082, which comprises the putative control region of *rmpA2*, was generated as a *luxAB* fusion construct (Fig. 1B). At different time points, the expression of $P_{rmpA2}$::*luxAB* was found to be at least twofold more when grown under the CPS-inducing conditions than when grown in LB broth.

An IS*3* element was noted to be located upstream of the *rmpA2* locus and be transcribed in the opposite direction (Fig. 1B). To examine whether the IS*3* element affects the gene expression of *rmpA2*, the plasmid pYC077, which contains the

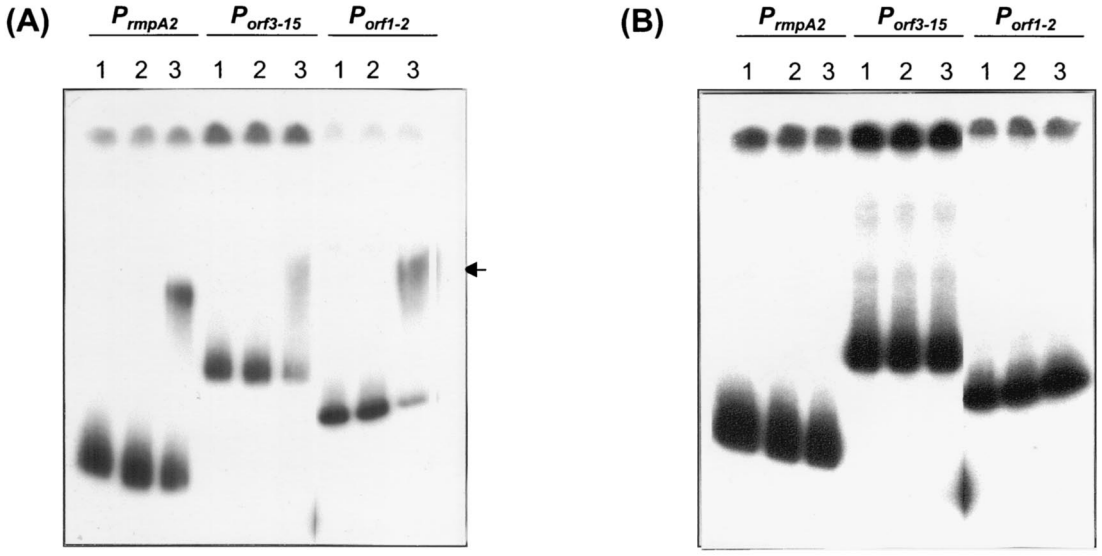


FIG. 4. DNA EMSA of RmpA2 and its target promoters. The $^{32}$P-labeled DNA probes of $P_{rmpA2}$, $P_{orf3\text{-}15}$, or $P_{orf1\text{-}2}$ were incubated with the full-length His-$RmpA2_{212}$ (A) or the C-terminally truncated His-$RmpA2_{N111}$ (B). Lanes 1 to 3 contain RmpA2 amounts of 0, 50 ng, and 500 ng, respectively. The arrow indicates the protein-DNA complex.

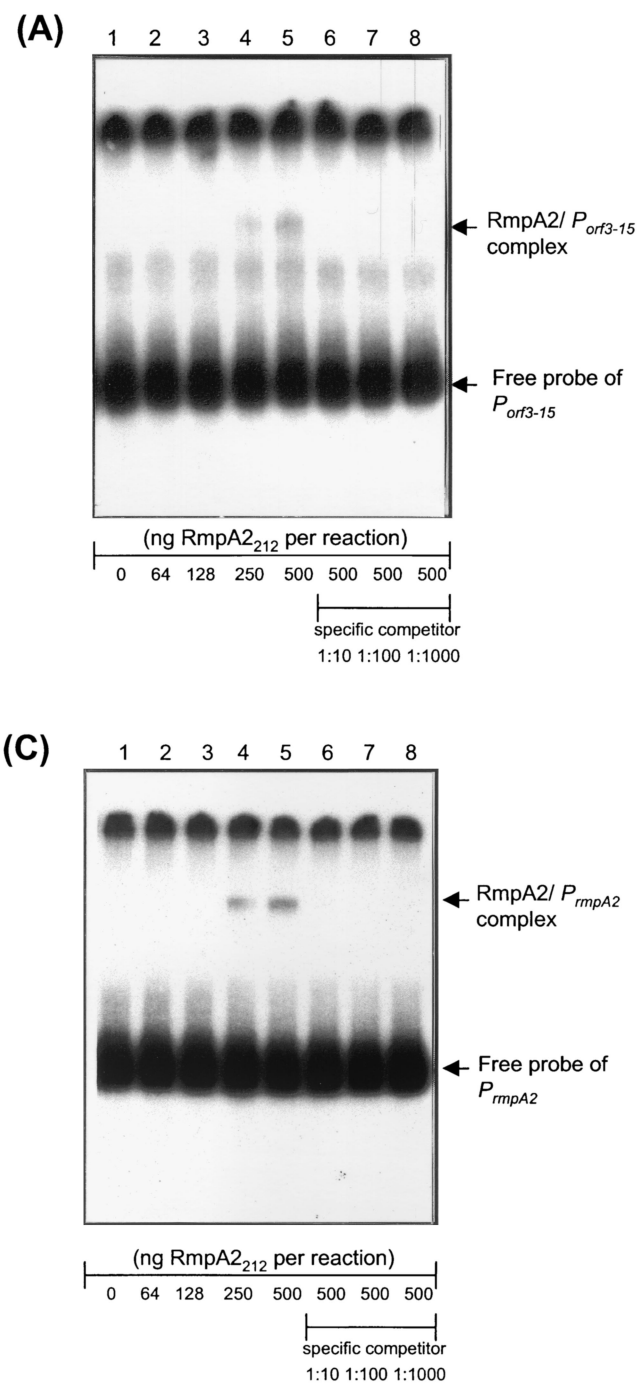


FIG. 5. Binding specificity of RmpA2 to its target promoters. The $^{32}$P-labeled DNA probes of $P_{orf3-15}$ (A), $P_{orf1-2}$ (B), and $P_{rmpA2}$ (C) were incubated with increasing amounts of His-$RmpA2_{212}$ as indicated. Lanes: 1, no protein; 2, 64 ng of protein; 3, 128 ng of protein; 4, 250 ng of protein; 5 to 8, 500 ng of protein. Specific inhibition of binding was investigated by adding the indicated amounts of unlabeled DNA fragment of a target promoter: lane 6, 1 ng; lane 7, 10 ng; lane 8, 100 ng.

upstream region of *rmpA2*, including a 0.4-kb fragment of IS*3*, was generated as a *luxAB* fusion construct (Fig. 1B). As shown in Fig. 8A, the luciferase activity of *K. pneumoniae* CG43S3 [pYC077] was approximately threefold higher than that of CG43S3 [pYC082], in which IS*3* was omitted.

In addition to regulating the target genes, it is not uncommon for a bacterial transcription factor to also control its own expression. As shown in Fig. 8B, in almost all strains tested, the gene expression driven by the *rmpA2* promoter was reduced at least 50% by the presence of multicopy *rmpA2*. In the *lon* mutant strain L2117, the $P_{rmpA2}$::*luxAB* fusion exhibited threefold-lower activity than that in the wild-type strain, presumably due to the increased availability of RmpA2 protein in the *lon* mutant cells. The direct interaction of RmpA2 protein with its own promoter was further verified by EMSA and a specific binding was demonstrated in Fig. 5C.

**Differential regulation of RmpA2-responsive promoters.** As demonstrated above, RmpA2 plays dual functional roles: it activates the K2 *cps* promoters while repressing its own gene expression. To understand how these two types of promoter respond to different concentrations of RmpA2 in cells, their activity was measured over time in an IPTG-inducible RmpA2 expression system in *E. coli* NovaBlue(DE3). As shown in Fig. 9, a relatively small amount of RmpA2 was detected 1 h after the addition of IPTG. At this time, an approximately 20-fold increase in $P_{orf1-2}$ activity was observed, in contrast to nearly no reduction in $P_{rmpA2}$ activity. A more pronounced repressive effect on *rmpA2* promoter was seen 5 h after IPTG induction, presumably due to an accumulation of RmpA2 protein.

## DISCUSSION

The gene *rmpA2* was originally identified as a gene that encodes the ability to enhance CPS synthesis in *K. pneumoniae* (34). Nevertheless, the molecular mechanism underlying the activation exerted by *rmpA2* has been elusive. In this study, we demonstrated that RmpA2 functions as a transcriptional reg-

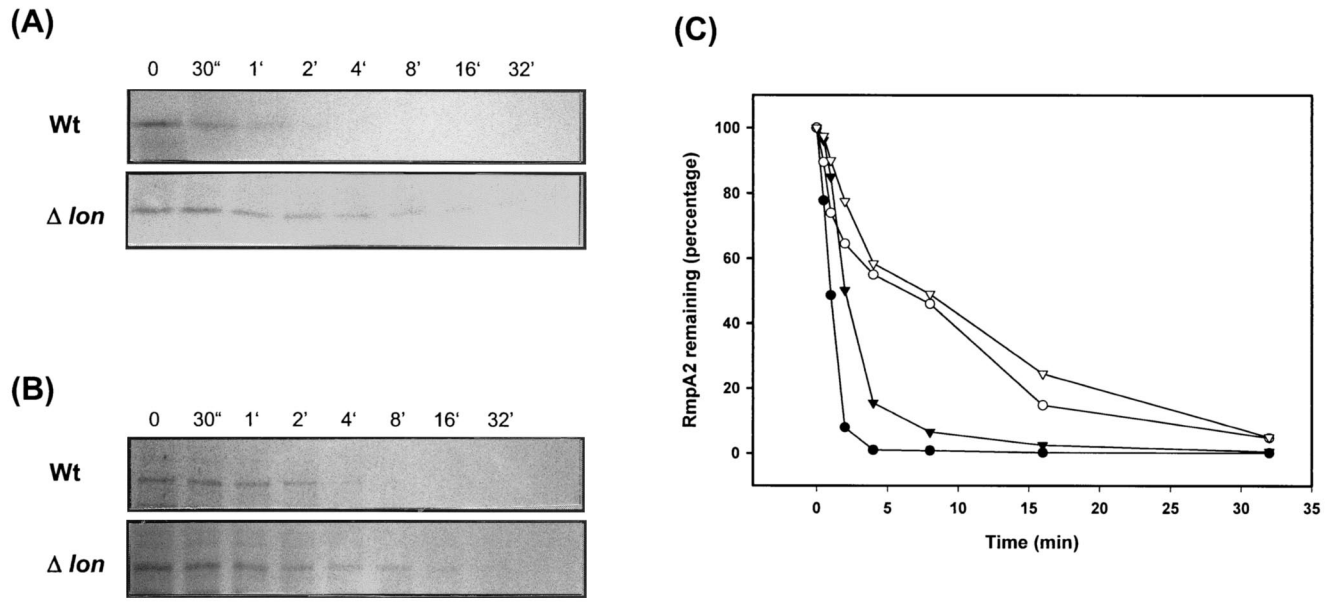


FIG. 6. Stability of RmpA2 protein in *K. pneumoniae*. The bacterial cells were pulse-labeled with [$^{35}$S]methionine and chased at the indicated time points. Tag-fused RmpA2 protein was immunoprecipitated with anti-His MAb or with anti-HA MAb and then subjected to SDS-PAGE. (A) Turnover of His-RmpA2 protein. (B) Turnover of HA-RmpA2 protein in the wild-type strain *K. pneumoniae* CG43S3 (Wt) or in the *lon* mutant strain L2117 (Δ*lon*). (C) Quantification of the autoradiogram shown in panel A of wild-type (solid circles) or *lon* mutant (open circles) cells and of that in panel B of wild-type (solid triangles) or *lon* mutant (open triangles) cells. The quantity of labeled protein at time zero was set at 100%.

ulator that activates the K2 *cps* gene expression and that the concentration of RmpA2 in *K. pneumoniae* cells is governed by autoregulation at transcriptional level as well as by Lon protease posttranslationally.

Despite the fact that deletion of *rmpA2* only results in a slight reduction in CPS production in *K. pneumoniae*, it is apparent that the colony mucoidy is lost in the mutant strain. It has been reported that the colony mucoidy of *K. pneumoniae* is proportional to the complexity of the fibrils surrounding the capsule (34). The loss of mucoidy in the *rmpA2* mutant might be due to a reduction in branching degrees of CPS. Comparative analysis with the group I *cps* region of *E. coli* K30 reveals that the *orf7-15* of the *K. pneumoniae* K2 *cps* operon encodes enzymes responsible for synthesizing the repeating units of the K2 antigen (23). Among them, *orf14* encodes a glycosyltransferase (2) that assembles the K2-specific tetrasaccharide units onto the preformed CPS and is likely the key enzyme in controlling the CPS branching. Therefore, when the expression of *orf14* was elevated in the presence of RmpA2, the enhanced activity of the glycosyltransferase would increase the branching degree of CPS and hence result in a mucoid colony.

Sequence analysis of RmpA2 suggests that it belongs to the UhpA-LuxR family of transcription factors, which also includes RcsA and RcsB of *E. coli* (29). A conserved RcsAB box (35) with a sequence of TAAGATTATTCTCA could be identified in the region 168 to 181 nucleotides upstream of the K2 *orf1* gene. It is not known, however, whether this RcsAB box is critical for RmpA2-mediated gene activation. No obvious similarity could be observed among the other RmpA2-responsive promoters tested here. When the cellular concentration of RmpA2 reached high levels, either by the introduction of a multicopy plasmid or by the increased stability in *lon* mutant cells, the K2 *cps* gene transcription could be activated even in an *rcsB* mutant genetic background. Despite the results which suggest that RmpA2 is capable of exerting the transactivation function independent of RcsB, the possibility that RmpA2 interacts with an additional protein factor such as RcsB to achieve a stronger activation could not be ruled out. Wacharotayankun et al. (34) reported that the central domain of RmpA2 protein displayed on average a 16.5% similarity to that of the NtrC, which activates transcription by the $\sigma^{54}$-holoenzyme. Close examination of the sequence has revealed that the region does not include the major conserved segments that are shared by members of the $\sigma^{54}$ activator family (16) Thus, RmpA2 is likely to exert the effects in a $\sigma^{54}$-independent pathway. Therefore, the detailed mechanisms by which RmpA2 binds and activates its responsive promoters remain to be demonstrated.

It has been reported that *E. coli* K30 strains defective at *rcsA* and *rcsB* remain capable of synthesizing group I CPS (13). We also demonstrated that the basal expression levels of K2 *cps* genes as well as the amount of CPS produced in *K. pneumoniae* were not affected in *rmpA2* mutant and in *rmpA2* and *rcsB* double mutants. The results suggest that neither RmpA2 nor RcsB is essential for basal expression of K2 *cps* genes. Rather, these two factors are required to maintain high expression levels of *cps* genes and hence the production of a thick capsule, which is advantageous for *K. pneumoniae* during infection in humans.

Phase variation due to slip strand DNA synthesis is one of the means of controlling the bacterial gene expression (10). The presence of a poly(G) tract of various lengths in *rmpA2* of

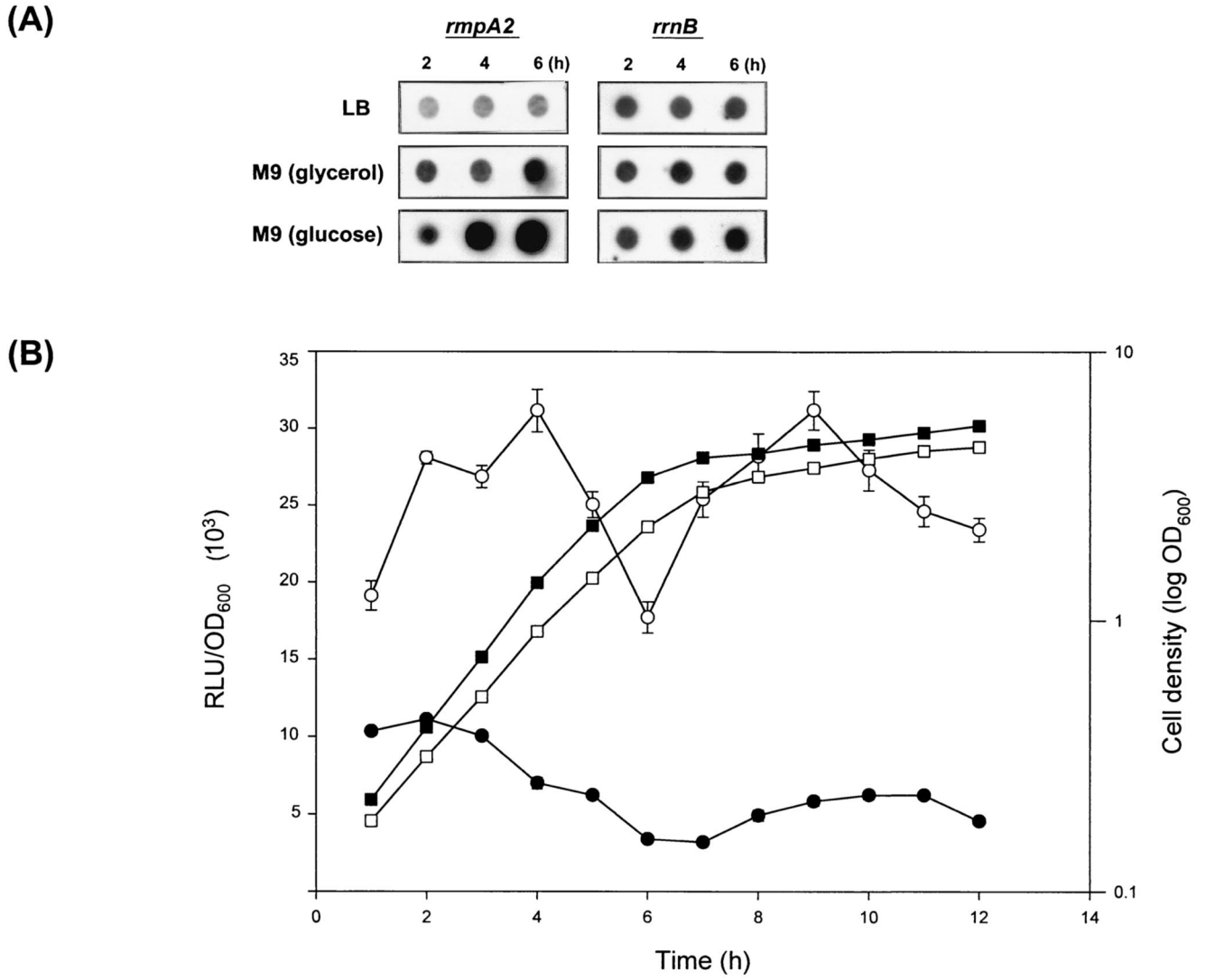


FIG. 7. (A) Dot blot analysis of *rmpA2* transcripts in cells grown under different nutritional conditions. Three different time points—2, 4, and 6 h—were investigated. The *rrnB* gene was an internal control. (B) Time course analysis of $P_{rmpA2}$::*luxAB* expression. *K. pneumoniae* R2035[pYC082] was grown in LB (solid symbols) or M9-glycerol (open symbols). Luciferase activity (circles) and the bacterial cell density (squares) were measured every hour and are represented as the average of triplicate results (error bars, standard deviations). RLU, relative light units.

different *K. pneumoniae* strains indicates that the bacterium might employ this strategy to regulate CPS production. Despite a full-length RmpA2, which could increase the virulence of *K. pneumoniae* CG43 in mice, it is probably not essential for the bacterium to infect immunocompromised patients, since some of the bacteremic isolates of our laboratory collection produce the truncated version of RmpA2. The functional switching on RmpA2 might be useful for the opportunistic *K. pneumoniae* to adjust its metabolic carbon flow upon facing different environments, such as during free-living or parasitic stages.

It has been demonstrated that the *lon* mutations in *E. coli* could elevate the transcription levels of genes responsible for CPS biosynthesis (31). The phenomenon can be explained by the enhanced stability of RcsA, which is a positive regulator of *cps* genes and acts as a substrate for Lon protease. A similar result was observed in the *K. pneumoniae lon* mutant strain L2117, in which the half-life of RmpA2 is increased and is accompanied by an accumulation of K2 CPS. However, other Lon-dependent positive regulators, such as RcsA, may also play a role, since the expression of K2 $P_{cps}$::*luxAB* fusions was still higher in the double-mutant strain RL01 (Δ*rmpA2* Δ*lon*) than that in the wild-type strain. Despite the fact that RmpA2 behaves like RcsA as a Lon-limited regulator, the way they control the expression of their own genes is different. A 100-fold increase in expression of a *rcsA*::*lacZ* transcriptional fusion has been demonstrated in *E. coli* strains with high levels of RcsA protein (8). While RcsA activates its own expression, RmpA2 was found to down-regulate the expression of $P_{rmpA2}$::*luxAB* fusion by acting as a repressor to its own promoter.

If RmpA2 negatively regulates its own gene expression, why

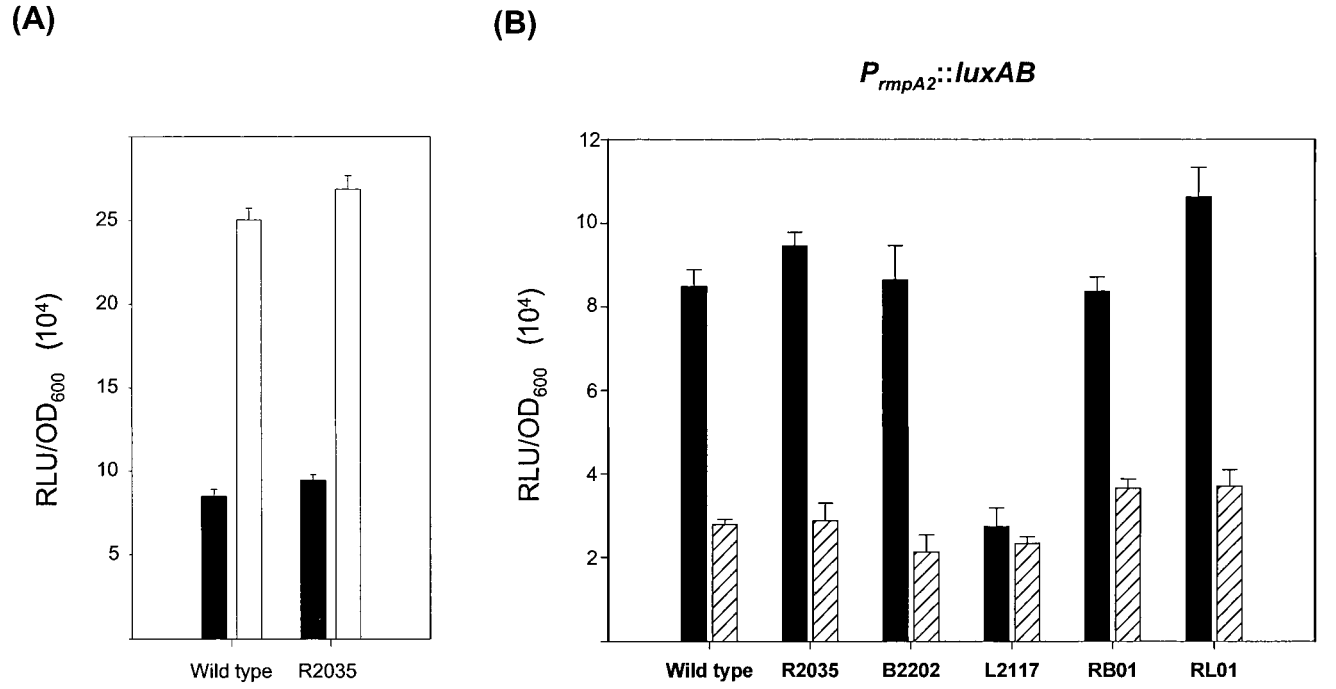


FIG. 8. (A) Comparison of luciferase activity of pYC082 (solid bars) and pYC077 (open bars) in *K. pneumoniae* CG43S3 (wild type) and R2035 (Δ*rmpA2*). (B) Luciferase activity of pYC082 in strains CG43S3, R2035, B2202 (Δ*rcsB*), L2117 (Δ*lon*), RB01 (Δ*rmpA2* Δ*rcsB*), and RL01 (Δ*rmpA2* Δ*lon*) was measured in the presence (striped bars) or absence (solid bars) of pYC084. Error bars, standard deviations; RLU, relative light units.

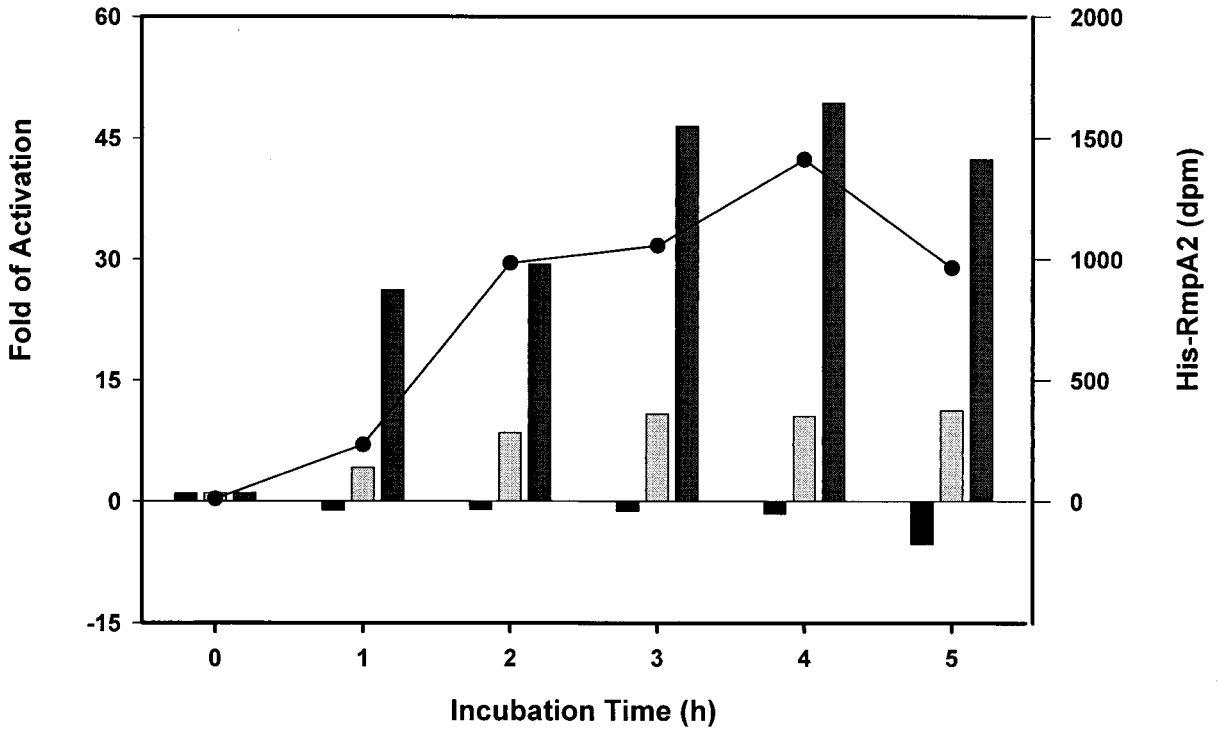


FIG. 9. Response of RmpA2 target promoters. The *luxAB* fusions were cotransformed individually with the RmpA2 expression vector pET-RmpA2$_{212}$ into *E. coli* NovaBlue(DE3). Upon 1 mM IPTG induction, the luciferase activity of $P_{rmpA2}$::*luxAB* (black bars), $P_{orf3\text{-}15}$::*luxAB* (light gray bars), or $P_{orf1\text{-}2}$::*luxAB* (gray bars) was measured every hour and represented as the activation relative to that at time zero. The amount of His-RmpA2 synthesized in *E. coli* NovaBlue(DE3) after IPTG induction was determined by using a His tag-specific MAb followed by counting with a densitometer, and the result is shown on the right-hand axis (closed circles).

is the transcription of K2 *cps* genes increased in *lon* mutant cells, in which more RmpA2 is available? As demonstrated in Fig. 9, before RmpA2 inhibits its own expression, the K2 *cps* promoters could be activated by a relatively small amount of RmpA2. The higher responsiveness of K2 *cps* promoter to RmpA2 is presumably due to a better DNA binding affinity, which is evident by comparing the EMSA results in Fig. 5B with those in Fig. 5C. Therefore, in *lon* mutant *K. pneumoniae* cells, though the amount of stabilized RmpA2 protein increases slightly, it is enough to activate K2 *cps* gene expression; whereas, the repressive effect on *rmpA2* promoter would be seen afterwards when cells accumulate more RmpA2 protein.

Based on these results, the overall regulation scheme exerted by RmpA2 on CPS biosynthesis is proposed as follows. Under normal growth conditions, *K. pneumoniae* synthesizes only a small quantity of RmpA2 protein, which is rapidly eliminated by the Lon protease. Upon encountering certain environmental signals, such as those found in M9 minimal medium, the expression of *rmpA2* gene is activated. In some strains, the *rmpA2* expression could be further enhanced by an upstream IS*3* element, which is likely to be acquired through an in vivo selection for the enhancement of virulence. The increased availability of RmpA2 proteins then binds to its target promoters, including those of *cps* genes, leading to the activation of K2 *cps* gene expression and eventually the accumulation and increase in the mucoidy of K2 capsule. When the RmpA2 protein increases to a threshold level, it negatively autoregulates its own expression to prevent an overwhelming effect on the capsule production.

## ACKNOWLEDGMENTS

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