

# Homologous Response Regulators KvgA, KvhA and KvhR Regulate the Synthesis of Capsular Polysaccharide in *Klebsiella pneumoniae* CG43 in a Coordinated Manner

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On the basis of phenotypic analysis, the *Klebsiella pneumoniae* CG43 derived mutants with deletions of the gene encoding respectively the response regulators KvgA, KvhA, and KvhR were classified into two groups. Group I bacteria carrying either *kvgA*<sup>-</sup> or *kvhR*<sup>-</sup> exhibited less mucoidy, lower level of capsular polysaccharide (CPS) synthesis and higher LD<sub>50</sub> than the parental strain. No apparent change of the group II, including *kvhA*<sup>-</sup> and *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutants, was observed. However, the mucoidy of *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant was found to be diminished after introducing into a *kvhA*<sup>-</sup> expressing plasmid. Via promoter-*lacZ* fusion analysis, *kvhA* deletion was found to reduce *kvhR* expression. A regulatory role of KvhA for the expression of *kvhR* was supported further by EMSA showing a specific binding of KvhA to the putative promoter of *kvhR*. The promoter activity measurement and EMSA also revealed that KvgA acted as an autoregulator and an activator for the expression of *kvhAS* and *kvhR*. In addition, deletion of *kvgA* suppressed slightly the promoter activity of the *cps-orf16-17*, and the expression of all three *cps* transcripts *orf1-2*, *orf3-15*, and *orf16-17* were reduced in the *kvhR*<sup>-</sup> mutant. These suggest that the three homologous response regulators interact to control, in coordination, the bacterial *cps* expression.

**Key words:** *cps* expression, homologous response regulator, *Klebsiella pneumoniae* CG43, KvgA, KvhA, KvhR.

Bacterial two-component systems (2CSs), consisting of a sensor histidine kinase and a response regulator, recognize specific signals and convert this information into specific transcriptional or behavioral responses (1). Upon sensing the input signals, the sensor protein catalyzes an autophosphorylation reaction in transmitter domain, which transfers a phosphate from ATP to a conserved histidine residue. The phosphate group is subsequently transferred from the histidine residue to a specific aspartate residue on the receiver domain of the cognate response regulator. The phosphorylated response regulator begins to perform an appropriate regulatory function, possibly by conformational change (2, 3). The number of 2CS varies dramatically among bacterial genomes. For instance, *Bacillus subtilis* encodes 70 2CS proteins (4), whereas *Helicobacter pylori* and *Haemophilus influenzae* contained only 11 and nine 2CS protein-encoding genes, respectively (2). As is widely believed, the 2CS proteins function as components of a signal transduction network, enabling bacteria to respond to complex environmental stimuli. Indeed, the presence of 2CS regulatory circuits in various bacteria has been recently acknowledged (5, 6).

As a common nosocomial pathogen, *Klebsiella pneumoniae* causes suppurative lesions, septicemia, and urinary and respiratory tract infections in immunocompromised patients (7–10). The increasing prevalence of extended

spectrum  $\beta$ -lactamase, which produces *K. pneumoniae* (ESBLKp), prompted the search for new drugs to intervene in the bacterial infections (11). Most recently, the development of an antimicrobial drug that targets bacterial 2CSs has been evaluated (12). In an earlier study, we have isolated 2CS genes by PCR-supported genomic subtractive hybridization from *K. pneumoniae* CG43, a highly virulent clinical isolate of K2 serotype (13). On the basis of the sequence similarity to that of the *Bordetella pertussis* BvgAS, which plays an important role in pathogenesis (14), the *bvgAS*-like genes were named *kvgAS* (15). The analysis using dot-blotting hybridization revealed that *kvgAS* is present in approximately 15% of the laboratory collected clinical isolates, suggesting an accessory role of the 2CS in the bacterial pathogenesis (15). Downstream to *kvgAS*, a gene encoding KvgA homolog (53.8% similarity), namely *kvhR*, was later identified (16). Interestingly, BLASTP analysis was unable to identify either *kvgAS* or *kvhR* in the *K. pneumoniae* MGH78578 genome (<http://genome.wustl.edu/projects/bacterial/>). In stead, *kvgAS*-homologous genes were found and the genes subsequently isolated from *K. pneumoniae* CG43 and designated *kvhAS* as *kvgAS* homolog. In contrast to *kvgAS*, *kvhAS* is present in all the strains collected in the laboratory, as analyzed by dot-blotting hybridization using *kvhA* as a probe (data not shown), suggesting a important role of the 2CS in *K. pneumoniae*. A stress-responsive role of KvgAS has been proposed since *kvgAS* expression was activated in LB medium adding with 0.2% paraquat or 0.2 mM 2,2'-dipyridyl (16). However, functional roles of KvhAS and

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KvhR have not yet identified. In this study, by generation of a series of the response regulator mutants and measurement of their promoter activities, we were able to show an interactive regulation of the 2CSs and also demonstrate their regulatory roles on *cps* gene expression in the bacteria.

#### MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Growth Conditions**—Bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were routinely cultured at 37°C in Luria-Bertani (LB) medium or M9 minimal medium supplemented with appropriate antibiotics.

**Sequence Analysis**—Approximately 2 kb and 3.3 kb DNA, located upstream respectively to *kvgA* and *kvhR*, were subjected to sequence determination. The sequence analysis including Open reading frames (ORFs) identification and annotation were carried out using BLAST (NCBI database). The presence of tRNA sequence was identified by the program tRNAscan-SE (17). The G+C content analysis was performed by the program of GEECEE in EMOBSS.

**Construction of a LacZ Reporter System**—In order to assess each of the promoter activities, a promoter-trap vector with *LacZ* as the reporter, and CG43S3-Z01, derived from *K. pneumoniae* CG43S3 (18) with a deletion of *lacZ* gene, were constructed. Briefly, a promoterless *lacZ* gene was PCR amplified from *K. pneumoniae* CG43S3 with the primer set lac01/lac02 (Table 2) and then inserted into the promoter-trap vector pYC016 (18). The resulting reporter plasmid was designated as placZ15 (Table 1). In addition, two 1 kb DNA fragments flanking the *lacZ* gene were PCR amplified using specific primer sets lac05/lac06 and lac03/lac07 (Table 2). The generated DNA fragments were ligated and subcloned into a suicide vector pKAS46 (19). The resulting plasmid placZ16 was transformed into *Escherichia coli* S17-1 $\lambda$ pir and then mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43S3 by conjugation. A kanamycin resistant transconjugant was initially picked, grown overnight, and then spread onto a LB plate supplemented with 500  $\mu$ g ml<sup>-1</sup> streptomycin. After the occurrence of double crossover, the streptomycin resistant colonies were further ascertained for their susceptibility to kanamycin. The *lacZ* mutation was confirmed by plating the bacteria onto a X-gal containing medium and by Southern hybridization (data not shown), and the mutant was designated as *K. pneumoniae* CG43S3-Z01 (Table 1).

**Construction of *kvgA*, *kvhA*, and *kvhR* Deletion Mutants**—The mutants with specific deletion of either of *kvgA*, *kvhA*, and *kvhR* genes were also constructed by the allelic exchange strategy described above. The primer sets used for the construction of the deletions are listed in Table 2. The gene-specific deletion mutants derived from *K. pneumoniae* CG43S3-Z01 were generated through homologous recombination and the resulting strains were designated AZ18 (*kvgA*<sup>-</sup>), AhZ01 (*kvhA*<sup>-</sup>) and RZ01 (*kvhR*<sup>-</sup>). For the construction of *kvhR*<sup>-</sup>*kvgA*<sup>-</sup> or *kvhR*<sup>-</sup>*kvhA*<sup>-</sup> double mutant, the pKAS46 derivative containing either a *kvgA* or *kvhA* deletion was delivered respectively from *E. coli* S17-1 $\lambda$ pir into RZ01 by conjugation. The plasmid carrying a *kvhA* deletion was also mobilized from

*E. coli* S17-1 $\lambda$ pir to *K. pneumoniae* AZ18 by conjugation to generate *kvgA*<sup>-</sup>*kvhA*<sup>-</sup>. For *kvhR*<sup>-</sup>*kvgA*<sup>-</sup>*kvhA*<sup>-</sup> triple mutant, the pKAS46 derivative containing *kvgA* deletion was delivered from *E. coli* S17-1 $\lambda$ pir into *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant by conjugation. The selections for the mutants were carried out likewise. The resulting mutants were designated as AAh01 (*kvgA*<sup>-</sup>*kvhA*<sup>-</sup>), AR01 (*kvgA*<sup>-</sup>*kvhR*<sup>-</sup>), AhR01 (*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>) and AAhR01 (*kvgA*<sup>-</sup>*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>). The mutant with deletion of *rcsB*, which has been demonstrated to encode a K2 *cps* activator in *K. pneumoniae* CG43S3 (20), was also generated and named RcsBZ01 (*rcsB*<sup>-</sup>).

**Determination of Promoter Activity**—The putative promoter regions of *kvgAS*, *kvhAS*, *kvhR*, *rcsB*, and the three *cps* transcriptional units (21, 22) were PCR amplified from *K. pneumoniae* CG43S3 by the designed primer sets (Table 2) and subcloned into placZ15 to fuse them with the *lacZ* reporter gene. One-tenth overnight culture of the bacteria carrying each of the plasmids were refreshly grown in M9 medium to an optical density at wavelength of 600 nm (OD<sub>600</sub>) about 0.6 to 0.7. The  $\beta$ -galactosidase activity assay was carried out essentially as described by Miller (23). The data presented were derived from a single experiment which is representative of at least three independent experiments. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

**Preparation of the Recombinant KvgA<sub>t</sub>, KvhA, and KvhR<sub>t</sub>**—The coding region of *kvgA*, *kvhA*, and *kvhR* were PCR amplified from *K. pneumoniae* CG43S3 with the specific primers (Table 2), and the PCR products cloned into pUC-T vector (MDBio). The resulting plasmids were designated as pkvgA1, pHP4004, and pR28, respectively. The plasmid pHP4004 was digested with *Bam*HI and the entire *kvhA* fragment subcloned into pET30c, and the resultant plasmid was named as pHP4005. While overexpression of either *kvgA* or *kvhR* resulted in largely insoluble proteins. In order to resolve the problem, the plasmid pkvgA1 was digested with *Cl*aI to remove the receiver domain. The remaining DNA binding domain of approximately 200-bp, KvgA<sub>t</sub>, was subcloned into the *S*all-*Not*I sites of pET30c, which resulted in the expression plasmid pkvgA4. Likewise, pR28 was digested with *E*coRV and *H*indIII to remove the receiver domain and the remaining DNA binding domain, KvhR<sub>t</sub>, was subcloned into the *E*coRV-*H*indIII sites of pET30a, which resulted in the expression plasmid pR31. The plasmids pkvgA4, pHP4005, and pR31 were then transformed into *E. coli* BL21-RIL. The transformants carrying either pkvgA4, pHP4005, or pR31 were cultured in LB medium to log phase, and expression of either the recombinant His-KvgA<sub>t</sub>, His-KvhA, or His-KvhR<sub>t</sub> protein was induced with 1 mM IPTG for 3 h at 37°C. The overexpressed His-KvgA<sub>t</sub> and His-KvhR<sub>t</sub> protein formed an inclusion body, but the His-KvhA appeared to be in soluble form. The bacteria carrying pkvgA4 and pR31 respectively were lysed by sonication and the pellet was resuspended and denatured with 6 N urea. After purification by affinity chromatography with His-Bind resin (Novagen), the denatured His-KvgA<sub>t</sub> and His-KvhR<sub>t</sub> protein were refolded respectively through dialysis against a gradient of decreasing concentrations of urea in the reaction buffer (20 mM Tris-HCl, pH 8.0, 4 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM CaCl<sub>2</sub>

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Descriptions	Reference or source
<b>Strains</b>		
<i>K. pneumoniae</i>		
CG43S3	CG43 Sm <sup>r</sup>	(18)
CG43S3-Z01	CG43S3 $\Delta$ lacZ	This study
CG43S3-AZ18	CG43S3-Z01 $\Delta$ kvgA	This study
CG43S3-AhZ01	CG43S3-Z01 $\Delta$ kvhA	This study
CG43S3-RZ01	CG43S3-Z01 $\Delta$ kvhR	This study
CG43S3-AAh01	CG43S3-Z01 $\Delta$ kvgA $\Delta$ kvhA	This study
CG43S3-AAR01	CG43S3-Z01 $\Delta$ kvgA $\Delta$ kvhR	This study
CG43S3-AhR01	CG43S3-Z01 $\Delta$ kvhA $\Delta$ kvhR	This study
CG43S3-AAhR01	CG43S3-Z01 $\Delta$ kvgA $\Delta$ kvhA $\Delta$ kvhR	This study
CG43S3-RcsBZ01	CG43S3-Z01 $\Delta$ rcsB	This study
<i>E. coli</i>		
JM109	<i>RecA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i> $\Delta$ [ <i>lac-proAB</i> ]	Laboratory stock
BL21-RIL	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>/r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>]gal dcm</i> [DE3]	Laboratory stock
S 17-1 $\lambda$ <i>pir</i>	<i>hsdR recA pro</i> RP4-2 [Tc::Mu; Km::Tn7] [ <i>λpir</i> ]	(19)
<b>Plasmids</b>		
pKAS46	Positive selection suicide vector, <i>rpsL</i> Ap <sup>r</sup> Km <sup>r</sup>	(19)
pET30a-c	His-tagging protein expression vector, Km <sup>r</sup>	Novagen
pUC-T	TA cloning vector	MDBio
pYC016	Promoter selection vector, LuxAB <sup>+</sup> Cm <sup>r</sup>	(18)
placZ15	A derivative of pYC016, containing <i>K. pneumoniae</i> CG43S3 <i>lacZ</i> as a reporter, Cm <sup>r</sup>	This study
pETm-c	A derivative of pET30C, containing malonate promoter, Km <sup>r</sup>	This study
placZ16	2-kb fragment containing an internal 1.5-kb deletion in <i>lacZ</i> cloned into pKAS46, Ap <sup>r</sup> Km <sup>r</sup>	This study
pA13	2-kb fragment containing an internal 0.6-kb deletion in <i>kvgA</i> cloned into pKAS46, Ap <sup>r</sup> Km <sup>r</sup>	This study
pAhm1	2-kb fragment containing an internal 0.7-kb deletion in <i>kvhA</i> cloned into pKAS46, Ap <sup>r</sup> Km <sup>r</sup>	This study
pR14	pKAS46 carrying a $\Delta$ kvhR fragment	This study
pYC220	2.0-kb fragment containing a 763-bp deletion in <i>rcsB</i> locus cloned into pKAS46	(20)
pRcsB2	The DNA fragment carrying entire <i>rcsB</i> coding sequence cloned into the <i>EcoRV/SalI</i> site of pETm-c	This study
pkvgA1	A fragment of <i>K. pneumoniae</i> CG43S3 <i>kvgA</i> gene generated by PCR, and cloned into pUC-T, Ap <sup>r</sup>	This study
pkvgA4	Deletion of the receiver domain of <i>kvgA</i> gene digested by <i>ClaI</i> , and cloned into pET30c, Km <sup>r</sup>	This study
pHP4004	A fragment of <i>K. pneumoniae</i> CG43S3 <i>kvhA</i> gene generated by PCR, and cloned into pUC-T, Ap <sup>r</sup>	This study
pHP4005	A fragment of <i>K. pneumoniae</i> CG43S3 <i>kvhA</i> gene digested by <i>Bam</i> HI, and cloned into pET30c, Km <sup>r</sup>	This study
pR28	A fragment of <i>K. pneumoniae</i> CG43S3 <i>kvhR</i> gene generated by PCR, and cloned into pUC-T, Ap <sup>r</sup>	This study
pR31	Deletion of the receiver domain of <i>kvhR</i> gene digested by <i>EcoRV/HindIII</i> , and cloned into pET30a, Km <sup>r</sup>	This study
pA16	399-bp <i>Bam</i> HI/ <i>Bgl</i> II fragment containing the putative <i>kvgAS</i> promoter, cloned into <i>Bam</i> HI site of placZ15	This study
pAh01	516-bp <i>Bam</i> HI/ <i>Bgl</i> II fragment containing the putative <i>kvhAS</i> promoter, cloned into <i>Bam</i> HI site of placZ15	This study
pRP05	500-bp <i>Bam</i> HI fragment containing the putative <i>kvhR</i> promoter, cloned into placZ15	This study
pA415	A 1.3 kb <i>Eco</i> RI fragment containing <i>kvhA</i> locus with the putative promoter cloned into pRK415	This study
pAHm	A <i>Bam</i> HI fragment of pHP4005 carrying entire <i>kvhA</i> coding sequence cloned into the <i>Bam</i> HI site of pETm-c	This study
pRC01	A 1.2 kb <i>Bam</i> HI/ <i>Eco</i> RI fragment containing <i>kvhR</i> locus with the putative promoter cloned into pACYC184	This study
pRC02	A 1.2 kb <i>Bam</i> HI/ <i>Eco</i> RI fragment containing <i>kvhR</i> locus with the putative promoter cloned into pRK415	This study
pOrf12	500-bp <i>Bam</i> HI fragment containing the putative <i>orf1-2</i> promoter, cloned into placZ15	This study
pOrf315	900-bp <i>Bam</i> HI fragment containing the putative <i>orf3-15</i> promoter, cloned into placZ15	This study
pOrf1617	300-bp <i>Bam</i> HI fragment containing the putative <i>orf16-17</i> promoter, cloned into placZ15	This study

Table 2. Primers used in this study.

Primer no.	Sequence	Complementary position
lac01	5'-GCGAACGACAAGATCTGACTTA-3'	-24 relative to the <i>lacZ</i> start codon
lac02	5'-ATTATGCCGTTCTAGAGCG-3'	+103 relative to the <i>lacZ</i> stop codon
lac03	5'-TGAAACGCAAGGATCCGAGC-3'	+1444 of the <i>lacZ</i> coding region
lac05	5'-CAGGTGGAGGAGCTCGAAAG-3'	-907 relative to the <i>lacZ</i> start codon
lac06	5'-AAACGGGATCCGCTGGCA-3'	+117 of the <i>lacZ</i> coding region
lac07	5'-GCAGTGGCCTCTAGATCGT-3'	+2498 of the <i>lacZ</i> coding region
a02	5'-CAATATCATAGCCAGCA-3'	+45 relative to the <i>kvgA</i> stop codon
a03	5'-ATTGCTTCACTACCCT-3'	-32 relative to the <i>kvgA</i> start codon
a08	5'-GAGAGCTCGATTATTCATCGA-3'	-834 relative to the <i>kvgA</i> start codon
a09	5'-CATATTGTGGATCCTGCTGTTTC-3'	+22 of the <i>kvgA</i> coding region
a10	5'-CGATGCGGGATCCAATGCCTTTA-3'	+296 of the <i>kvgA</i> coding region
a11	5'-AACAAGATCTAGCTTTTGAT-3'	+699 relative to the <i>kvgA</i> stop codon
a14	5'-ATTTTCAGGATCCACCACCTT-3'	-409 relative to the <i>kvgA</i> start codon
AS02	5'-CAGCCATGCTTTCTCCTT-3'	+156 relative to the <i>kvhA</i> stop codon
AS07	5'-ATCAGGATCCACGCCCC-3'	-18 relative to the <i>kvhA</i> start codon
AS04	5'-ATCTGCAGAATATCCCGT-3'	+1532 of the <i>kvhS</i> coding region
AS12	5'-TCCTGCAATGCTGGAATT-3'	-1245 relative to the <i>kvhA</i> start codon
AS16	5'-GCCCGGGTTATTTTTATC-3'	-52 relative to the <i>kvhA</i> start codon
AS23	5'-CATGGCGGTTTCGCTTAT-3'	-1 relative to the <i>kvhS</i> start codon
A201	5'-GTGAAAAGCTTCGTTC-3'	-516 relative to the <i>kvhA</i> start codon
A203	5'-CAACGACAGCTCTTCCAA-3'	+69 of the <i>kvhA</i> coding region
R01	5'-CTTTTTAAGCTTAAATGA-3'	-469 relative to the <i>kvhR</i> start codon
R02	5'-TTCGGGTACCTTCCATC-3'	+62 relative to the <i>kvhR</i> start codon
R04	5'-AGGCCTTCAATCCCAC-3'	+23 relative to the <i>kvhR</i> stop codon
R07	5'-AGGTTAAGAGCTCCAGGCC-3'	-1097 relative to the <i>kvhR</i> start codon
R09	5'-TGGATCCGTTTGTATGAATGA-3'	+353 of the <i>kvhR</i> coding region
P074	5'-ACTGGATCCACGATCATGGATAAGAT-3'	-724 relative to the <i>orf1</i> start codon
P075	5'-ACTGGATCCTGCGACCGGAATAACC-3'	+42 of the <i>orf1</i> coding region
P040	5'-ACTGGATCCAGGCTGGTAATAGCCATT-3'	-890 relative to the <i>orf3</i> start codon
P041	5'-ACTGGATCCCGCTGTCGTATCTCAATG-3'	+60 of the <i>orf3</i> coding region
P045	5'-GGTGCAGATCTATAAGC-3'	-307 relative to the <i>orf16</i> start codon
P046	5'-ACTGGATCCAGACGGAGGAAGTTC-3'	+89 of the <i>orf16</i> coding region

and 1 mM dithiothreitol). The His-KvhA protein was purified from the soluble fractions of the IPTG-induced bacteria carrying pHP4005. The purified His-KvgA<sub>t</sub>, His-KvhA and His-KvhR<sub>t</sub> were then concentrated with polyethylene glycol 20,000 and the concentration of protein was determined by the Bradford assay (24). Finally, molecular weight and purity of the proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

**Electrophoretic Mobility Shift Assay (EMSA)**—DNA fragments comprising a series of the putative promoter regions were obtained by PCR amplification with respective primer sets as described in Table 2, and then labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. The purified His-KvgA<sub>t</sub>, His-KvhA, or His-KvhR<sub>t</sub> was incubated with the radioactively labeled DNA in a 20  $\mu$ l solution containing 20 mM Tris-HCl (pH 8.0), 4 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM CaCl<sub>2</sub> and 1 mM dithiothreitol at 37°C for 20 min. Excess amount (approximately 10 times more than the labeled DNA) of each of the unlabeled DNA was used in the competition assay. The samples were then loaded onto a running gel of 5% nondenaturing polyacrylamide in 0.5 $\times$  TBE (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA). Gels were electrophoresed with a 20-mA current at 4°C and detected by InstantImager<sup>TM</sup> (Packard Instrument Company).

**Extraction and Quantification of CPS**—CPS was extracted as described previously (25). Five hundred microliters of bacteria cultured in LB broth overnight were mixed with 100  $\mu$ l of 1% Zwittergent 3-14 detergent (Sigma-Aldrich) in 100 mM citric acid (pH 2.0), and the mixture was incubated at 50°C for 20 min. After centrifugation at 13,500 rpm for 10 min, 250  $\mu$ l of the supernatant was transferred to a new tube, and the CPS was precipitated with 1 ml of absolute ethanol at 4°C for 20 min and then centrifuged at 13,500 rpm for 25 min. The pellet was dried at 37°C and dissolved in 200  $\mu$ l of distilled water, and 1.2 ml of 12.5 mM borax (Sigma-Aldrich) in H<sub>2</sub>SO<sub>4</sub> was added. The mixture was vigorously vortexed, boiled for 5 min, and cooled, and then 20  $\mu$ l of 0.15% (v/v) 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<sup>9</sup> CFU (26).

**Mouse Lethality Assay**—Female BALB/c mice with an average age of four weeks were acclimatized in an animal house for 7 days. The tested bacterial strains were cultured in LB medium at 37°C overnight. Four mice of a group were injected intraperitoneally with bacteria suspended in 0.2 ml of saline in 10-fold steps graded doses. The LD<sub>50</sub>,

based on the number of survivors after 10 days, was calculated and expressed as CFU as described (27).

RESULTS AND DISCUSSION

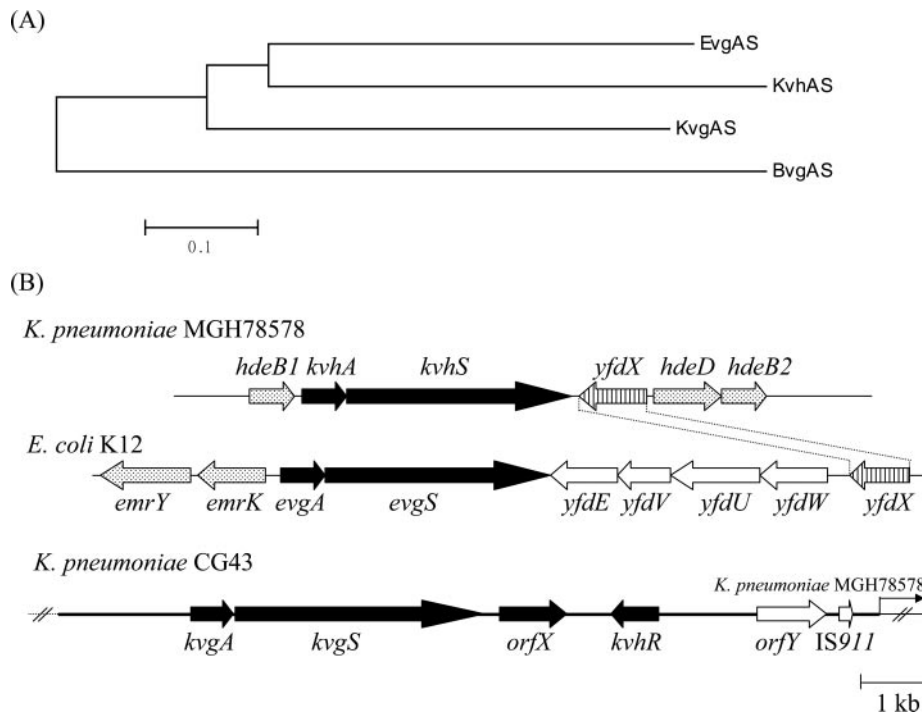
**Sequence Comparisons of KvgA, KvhA, and KvhR**—Increasing studies have acknowledged that, excluding sequences of closely related homologues, the transmitter domain from any two sensors typically share 20% to 50% sequence identity (average sequence identity, 25%). On the other hand, receiver domains from any two response regulators share sequence identity at only 20% to 30% (28–30). Sequence analysis of the receiver domains revealed a 47.9% amino acid identity between the two response regulators KvgA and KvhA, and KvhR shares 43.8% and 46.3% amino acid identity with KvgA and KvhA respectively. The results, together with the high sequence identity of the transmitter domain, which is 45.8%, between KvgS and KvhS strongly suggested that KvgS and KvhS are paralogous sensors, and KvgA, KvhA, and KvhR are paralogous response regulators.

We have previously shown by BLASTX sequence analysis that KvgAS is highly homologous to *B. pertussis* BvgAS (31, 32) and *Escherichia coli* EvgAS (Utsumi *et al.*, 1992). The *bvg* system controls the expression of major virulence factors in *B. pertussis*, such as filamentous haremaggultinin (*fha*), pertactin (*prn*), adenylate cyclase toxin (*cya*), and pertussis toxin (*ptx*) (33). While in *E. coli*, EvgAS has also been shown to be involved in regulating the gene expression of virulence-related property such as multi-drug resistance and acid resistance (34–36). As shown in Fig. 1A, phylogenetic analysis, on the basis of the comparison of overall amino acid sequence of the sensor and response regulator, revealed that KvgAS and BvgAS are relatively distant from the branches of KvhAS and EvgAS, that appeared to be clustered together. This

implies that KvhAS and EvgAS are most likely to be orthologous 2CS.

**Sequence Analysis of the DNA Fragments That Contain kvhAS and kvgAS**—Figure 1B shows a comparative analysis of the genes near *kvhAS* with that of *E. coli* *evgAS* revealing a YfdX homologue (49% amino acid sequence identity), a hypothetical protein for acid resistance in *E. coli* (37). Moreover, flanking both sides of *kvhAS*, homologues of putative acid resistance proteins HdeB and HdeD, which are positively regulated by EvgA (35), including HdeB1 (23% amino acid sequence identity), HdeB2 (38% amino acid sequence identity) and HdeD (26% amino acid sequence identity), were also identified. In analogy to the regulatory role of EvgAS, which modulates expression of the flanking genes, including putative efflux pump *emrKY* (36, 38, 39) and the acid-resisting gene *yfdX* (37, 40), we speculate that KvhAS controls expression of the nearby genes, *hdeB*, *hdeD* and *yfdX*. This possibility remains to be validated.

Dot-blotting hybridization using the probe of either *orfX* or *kvhR* gene, which is located downstream of the *kvgAS* operon, shows that only about 70% of the *kvgAS*-carrying isolates also harbored the *orfX* and *kvhR* genes (data not shown), suggesting that the *kvgAS* operon and *kvhR* were not acquired concurrently. Subsequently, 3.3 kb DNA upstream of *kvhR* and 2 kb DNA upstream of *kvgAS* were sequenced (the sequences deposited in the GenBank database under accession number AJ250891) and the sequences analyzed to confirm whether mobile elements are present. As shown in Fig. 1B, only the sequences 3 kb beyond *kvhR* could be identified as the counterpart in the *K. pneumoniae* MGH78578 genome. Analysis of the sequences upstream of *kvhR* identifies an ORF, namely *orfY*, encoding a putative exported lipase and a partial sequence of IS911. Intriguingly, the 177-bp intergenic sequence between *kvgS* and *orfX*, 640-bp intergenic



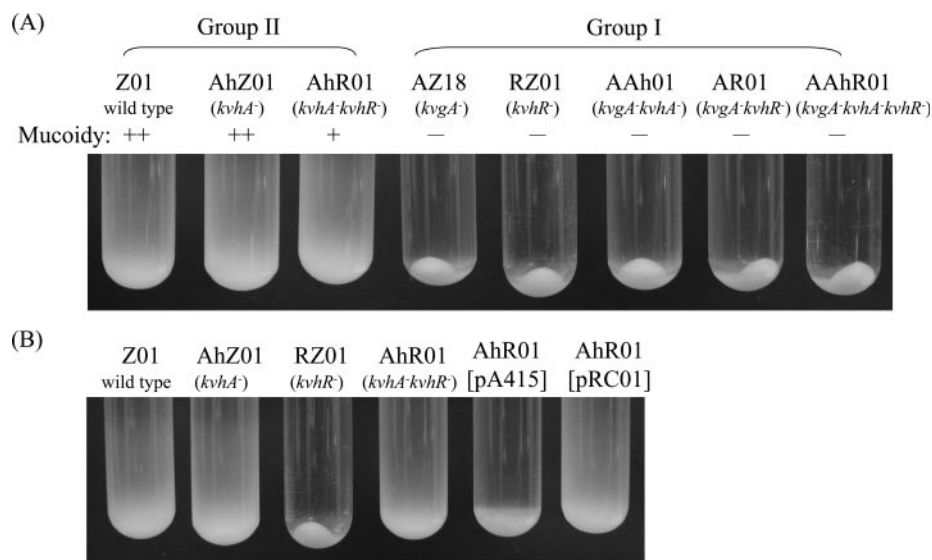
**Fig. 1. Evolutionary relationship of KvhAS, KvgAS, and KvhAS gene clusters.** (A) The phylogenetic tree of KvgAS, KvhAS, EvgAS, and BvgAS. The Neighbor-Joining tree was built by CLUSTAL W 1.81 (52) with the deduced amino acid sequences. The resultant tree was visualized by MEGA2 (53). The black bar represents 10% sequence divergence. (B) Comparison of the respective gene clusters flanking *kvhAS*, *kvgAS*, and *evgAS*. The respective ORFs flanking *kvgAS* in *K. pneumoniae* CG43, *kvhAS* in *K. pneumoniae* MGH78578 and *evgAS* in *E. coli* K12 are shown. The amino acid identities are indicated.

sequence between *orfX* and *kvhR*, and 2 kb sequences upstream of *kvgAS*, revealed neither ORF nor mobile element. The G+C content of the 12 kb DNA, containing *kvgAS-orfX-kvhR* and the flanking sequences, was 43%, which is somewhat lower than that of the *K. pneumoniae* MGH78578 genome (~55%). The lower G+C content of the DNA fragment, which can be identified only in some of the clinical isolates (15), implied that the gene cluster had been recently acquired by horizontal transfer.

**Phenotype Analysis of the Mutants** *kvgA*<sup>-</sup>, *kvhA*<sup>-</sup>, *kvhR*<sup>-</sup>, *kvgA*<sup>-</sup>*kvhA*<sup>-</sup>, *kvgA*<sup>-</sup>*kvhR*<sup>-</sup>, *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> and *kvgA*<sup>-</sup>*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>—The mutants, including AZ18 (*kvgA*<sup>-</sup>), AhZ01 (*kvhA*<sup>-</sup>), RZ01 (*kvhR*<sup>-</sup>), AAh01 (*kvgA*<sup>-</sup>*kvhA*<sup>-</sup>), AR01 (*kvgA*<sup>-</sup>*kvhR*<sup>-</sup>), AhR01 (*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>) and AAhR01 (*kvgA*<sup>-</sup>*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>) displayed a relatively large, glistening colony on LB agar. The morphology was indistinguishable from that of the wild-type strain. Nevertheless, a reduction in the mucoid characteristics was noted when the bacteria cultures were subjected to low-speed centrifugation. The sedimentation test to assess bacterial mucoidy allowed these mutants to be classified into two groups. Group I bacteria, carrying either *kvgA* or *kvhR* mutation, exhibited faster precipitation than that of the parental strain Z01. Group II bacteria, including *kvhA*<sup>-</sup> and *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutants, exhibit precipitation that is similar to that exhibited by the parental strain Z01 (Fig. 2A). As determined by the string test (41), the viscous colony nature of the group I bacteria appeared to be considerably diminished suggesting a reduction of the CPS (Fig. 2A). It is of interest to note that the *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant of group II exhibited a less mucoidy than either wild type or *kvhA*<sup>-</sup> mutant of the same group. While the *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant supplied with the plasmid pRC01, containing a *kvhR* locus, exerted no effect on the

bacterial phenotype indicating that the deleting effect of *kvhR* was suppressed by *kvhA* deletion. On the other hand, transformation of *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> with the plasmid pA415 carrying a *kvhA* locus, converted the phenotype from group II to group I (Fig. 2B). This suggests an upstream regulation of KvhA for a proper expression of *kvhR*.

**Promoter Activity Measurements of *kvgAS*, *kvhR* and *kvhAS***—The interacting regulation of 2CS network has been reported, which showed that some of the sensor proteins can conditionally transfer the phosphoryl molecules to non-cognate response regulators as well as to their cognate regulators (3). The possibility that if the signal is relayed from KvgS or KvhS to the orphan response regulator KvhR, remains to be examined. Nevertheless, the promoter-*lacZ* fusion constructs of *kvgAS*, *kvhAS*, and *kvhR* were generated to investigate whether the three homologous regulators regulate each other. The β-galactosidase activity of *P*<sub>*kvgAS*</sub> (pA16) measured in wild-type (Z01), *kvgA* mutant (AZ18), *kvhA* mutant (AhZ01) and *kvhR* mutant (RZ01) was found to be higher in M9 minimal medium than in LB (data not shown). Hence, the bacteria were grown in M9 minimal medium to enable the promoter activity to be measured. Table 3 shows that the activity of *P*<sub>*kvgAS*</sub>-pA16, which contains a 399-bp noncoding region of the *kvgA* start codon, in the *kvgA* deletion mutant AZ18, was approximately 50% that of Z01, indicating a positive auto-regulatory role of KvgA. The activity of pA16 measured in AhZ01 and RZ01 were similar, revealing that neither *kvhA* nor *kvhR* deletion affected the expression of *kvgAS*. Interestingly, the activity of *P*<sub>*kvhAS*</sub> (pAh01) and *P*<sub>*kvhR*</sub> (pRP05), contained respectively a 500-bp non-coding region upstream of the start codon of *kvhA* and *kvhR*, were found to be lower in the *kvgA* mutant AZ18, suggesting that KvgA is probably an activator for the



**Fig. 2. Comparison of precipitation speed of the mutants derived from *K. pneumoniae* CG43S3-Z01.** (A) The strains tested were cultured overnight in LB broth at 37°C and subjected to centrifugation at 4,000 rpm (1,500 × *g*) for 3 min. According to collective analysis of both assays, two groups can be identified, in which the group I includes: AZ18 (*kvgA*<sup>-</sup>), RZ01 (*kvhR*<sup>-</sup>), AAh01 (*kvgA*<sup>-</sup>*kvhA*<sup>-</sup>), AR01 (*kvgA*<sup>-</sup>*kvhR*<sup>-</sup>), AAhR01 (*kvgA*<sup>-</sup>*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>), and the group II includes: Z01, AhZ01 (*kvhA*<sup>-</sup>), AhR01

(*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>). Bacterial mucoidy was assessed by string formation test after the bacteria grown on LB plates for 48 h. Symbols: -, negative; +, positive; ++, strong. (B) Effect of the complementation of AhR01 (*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>) with either an intact *kvhA* or *kvhR*. The precipitation speed of the strains was also shown. AhR01 [pA415] was indicated AhR01 (*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>) with an intact *kvhA*. AhR01 [pRC01] was indicated AhR01 (*kvhA*<sup>-</sup>*kvhR*<sup>-</sup>) with an intact *kvhR*.

expression of *kvhAS* and *kvhR*. Although the deletion of *kvhA* or *kvhR* did not affect the expression of  $P_{kvhAS}$ , both mutations appeared to reduce  $P_{kvhR}$  activity, implying that KvhA positively regulates the *kvhR* expression and KvhR is an auto-regulator of its own expression. The finding is consistent with the above-mentioned notion that KvhA is likely an upstream regulator for *kvhR* expression. The BPROM program (<http://www.softberry.com>) used to analyze the sequences of the  $P_{kvgAS}$ ,  $P_{kvhAS}$ , and  $P_{kvhR}$  did not identify any *cis*-element, indicating that more studies are required toward understanding the regulatory mechanisms for the expression of  $P_{kvgAS}$ ,  $P_{kvhAS}$ , and  $P_{kvhR}$  in *K. pneumoniae* CG43.

**EMSA**—Subsequently, EMSA was performed using purified KvgA<sub>t</sub> protein and DNA fragments that contained  $P_{kvgAS}$ ,  $P_{kvhAS}$  and  $P_{kvhR}$ , to verify that KvgA, as a transcriptional activator, indeed binds directly to either of its own promoter  $P_{kvgAS}$ ,  $P_{kvhAS}$  and  $P_{kvhR}$ . Figure 3A shows that KvgA<sub>t</sub> which comprises the DNA binding domain could bind to its own promoter and that the DNA-protein interaction was specific, as the formation of the His<sub>6</sub>-KvgA<sub>t</sub>-promoter complex could only be inhibited by the presence of the unlabelled DNA. Furthermore, the two binding complexes, C1 and C2, observed when the amount of His<sub>6</sub>-KvgA<sub>t</sub> was increased from 0.3 μg to 0.6 μg to bind  $P_{kvgA}$ . This could indicate a higher order complex of the protein either to the same site or to distinct sites. The His<sub>6</sub>-KvgA<sub>t</sub> was shown also to bind  $P_{kvhAS}$  DNA and  $P_{kvhR}$ , and the bindings were demonstrated to be specific since the bindings could only be inhibited by the unlabelled specific DNA (Fig. 3, B and C). The results verified that KvgA positively regulated the expressions of  $P_{kvhAS}$  and  $P_{kvhR}$

by direct binding. The assay further established that His<sub>6</sub>-KvhA bound specifically to the [ $\gamma$ -<sup>32</sup>P]ATP-labeled  $P_{kvhR}$  and that the DNA-protein complex could only be competed in the presence of an excess of unlabelled  $P_{kvhR}$  (Fig. 3D). Finally, as shown in Fig. 3E, specific binding of KvhR to the DNA fragment  $P_{kvhR}$ , containing its own putative promoter, was also demonstrated.

**Deletion of *kvgA* or *kvhR* Affect the CPS Expression**—An extremely thick CPS is characteristic of the genus *Klebsiella*, which provides the bacteria a glistering and mucoid phenotype. Diminished mucoidy of the group I bacteria could be attributed to the reduction of their CPS. The amount of CPS produced in these mutants was determined by measuring the glucuronic acid content, an indicator of *Klebsiella* K2 CPS (42). Like *E. coli* group I CPS biosynthesis, *Klebsiella* K2 *cps* expression is regulated by the 2CS RcsAB at the transcriptional level (43, 44). A CG43S3Z01-derived *rscB*<sup>-</sup> mutant was therefore constructed and the CPS content was also determined and compared. Table 4 reveals that the group I bacteria as well as the *rscB*<sup>-</sup> mutant, synthesized less CPS than the wild-type strain, respectively from 0.51- to 0.68-fold of that of wild type, suggesting a positive regulation by KvgA and KvhR on *cps* expression. In the mouse peritonitis model, the deletion of either *kvgA* or *kvhR* increased LD<sub>50</sub> by a factor of 90 to 100 (Table 4). It is most likely that the

Table 3. Effect of *kvgA*, *kvhA*, and *kvhR* gene deletion on expressions of  $P_{kvgAS}$ ,  $P_{kvhAS}$ , and  $P_{kvhR}$ .

Strains	β-Galactosidase activity (Miller units)		
	[mean ± SD (fold <sup>a</sup> )]		
	pA16 ( $P_{kvgAS}::lacZ$ )	pAh01 ( $P_{kvhAS}::lacZ$ )	pRP05 ( $P_{kvhR}::lacZ$ )
Z01	44 ± 3 (1.00)	226 ± 13 (1.00)	374 ± 6 (1.00)
AZ18 ( <i>kvgA</i> <sup>-</sup> )	232 ± 4 (0.52)	166 ± 8 (0.74)	250 ± 8 (0.67)
AhZ01 ( <i>kvhA</i> <sup>-</sup> )	431 ± 2 (0.96)	228 ± 11 (1.00)	237 ± 5 (0.63)
RZ01 ( <i>kvhR</i> <sup>-</sup> )	438 ± 9 (0.98)	232 ± 6 (1.02)	174 ± 10 (0.47)

<sup>a</sup>Compared with Z01 carrying each the detected plasmid.

Table 4. Characterization of the *K. pneumoniae* CG43S3-Z01 derived mutants.

Strains	CPS amounts		LD <sub>50</sub> (CFU)
	(mean quantity ± SD <sup>a</sup> )	Fold <sup>b</sup>	
Z01	22.8 ± 3.8	1.00	3 × 10 <sup>3</sup>
RcsBZ01 ( <i>rscB</i> <sup>-</sup> )	11.6 ± 2.8	0.51	ND <sup>c</sup>
AZ18 ( <i>kvgA</i> <sup>-</sup> )	15.7 ± 0.3	0.68	2.75 × 10 <sup>5</sup>
AhZ01 ( <i>kvhA</i> <sup>-</sup> )	24.4 ± 2.4	1.07	3 × 10 <sup>3</sup>
RZ01 ( <i>kvhR</i> <sup>-</sup> )	13.6 ± 1.5	0.59	3 × 10 <sup>5</sup>
AAh01 ( <i>kvgA</i> <sup>-</sup> <i>kvhA</i> <sup>-</sup> )	11.9 ± 2.2	0.52	4 × 10 <sup>5</sup>
AR01 ( <i>kvgA</i> <sup>-</sup> <i>kvhR</i> <sup>-</sup> )	12.9 ± 0.8	0.56	4 × 10 <sup>5</sup>
AhR01 ( <i>kvhA</i> <sup>-</sup> <i>kvhR</i> <sup>-</sup> )	17.6 ± 0.9	0.77	3 × 10 <sup>5</sup>
AAhR01 ( <i>kvgA</i> <sup>-</sup> <i>kvhA</i> <sup>-</sup> <i>kvhR</i> <sup>-</sup> )	15.1 ± 0.3	0.66	4 × 10 <sup>5</sup>

<sup>a</sup>Values are the averages of triplicate samples and are given as micrograms of uronic acid per 10<sup>9</sup> CFU. <sup>b</sup>Compared with Z01. <sup>c</sup>ND means not determined.

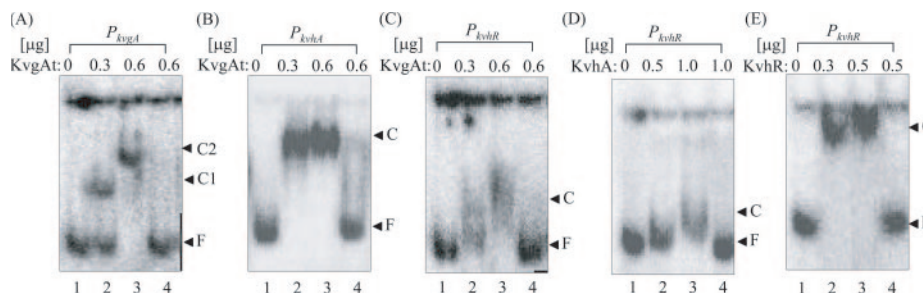


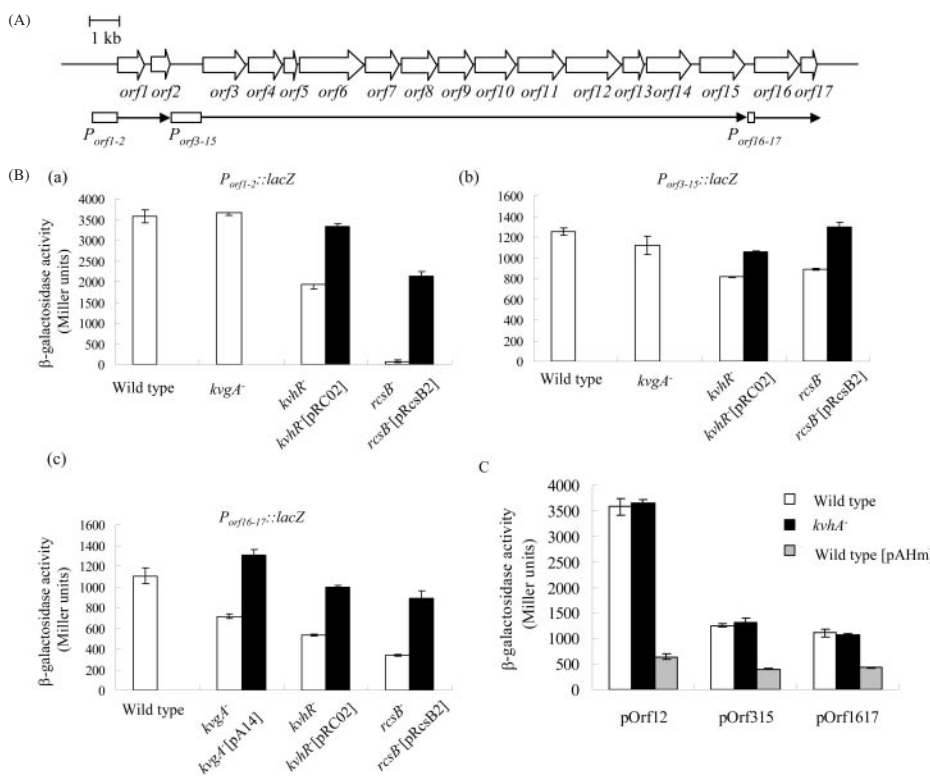
Fig. 3. EMSA assessment of the specific DNA binding activity of KvgA, KvhA, and KvhR. The DNA fragments of the  $P_{kvgAS}$ ,  $P_{kvhAS}$ , and  $P_{kvhR}$  were labeled with [ $\gamma$ -<sup>32</sup>P]ATP and used as probes. The recombinant KvgA<sub>t</sub>, KvhA, and KvhR<sub>t</sub> added to the binding assay mixture. The amounts of protein used are indicated at the top

of each lane (lanes 1 to 3). Specific competition was performed by adding the unlabelled DNA fragments into the mixture (lane 4). The DNA and protein complexes formed are indicated as C and the free forms are indicated as F.

reduction of the bacterial CPS, an important virulence factor that affects bacterial resistance to phagocytosis by polymorphonuclear cells (44–46), leads to decrease of the virulence. The *kvhA* deletion mutant, AhZ01, of group II exhibited unchanged LD<sub>50</sub> and a slight increase of glucuronic acid content in comparing with that of wild type bacteria Z01. Although classified into the same group as the *kvhA*<sup>-</sup> mutant (Fig. 2A), the LD<sub>50</sub> of *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant appeared to be comparable with those of group I bacteria (Table 4). In addition, *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant produced less amount of CPS than either *kvhA*<sup>-</sup> mutant or the wild type bacteria. In comparing with the *kvhR* mutant of group I, however, the *kvhA*<sup>-</sup>*kvhR*<sup>-</sup> mutant produced more CPS. Consistent with the result of string test as shown in Fig. 2A, this suggests a negative role of KvhA on *cps* expression and deletion of *kvhA* released the repression of *cps* expression, and hence more CPS were produced.

**Regulation of KvgA, KvhR, and KvhA on *cps* Expression**—In order to validate the role of each of the response regulators on *cps* expression, a series of *lacZ* fusion constructs, containing each of the putative *cps* promoters were generated. These include  $P_{orf1-2}$ , which comprises the non-translated sequence 724-bp upstream of *orf1-2*;  $P_{orf3-15}$ , which comprises the non-translated sequence 890-bp upstream of the operon *orf3-15*, and  $P_{orf16-17}$ , which comprises the 244-bp non-translated sequence upstream of *orf16-17* (Fig. 4A). These plasmids were then transformed into wild type bacteria, the deletion mutants *kvgA*<sup>-</sup>, *kvhA*<sup>-</sup>, *kvhR*<sup>-</sup>, and *rscB*<sup>-</sup> and also the wild type strain carrying a multicopy plasmid expressing with *kvhA*, pAHm, and the  $\beta$ -galactosidase activities were measured. Figure 4B (a, b, and c) shows that the activity of

$P_{orf1-2}$ ,  $P_{orf3-15}$ , and  $P_{orf16-17}$  in the *kvhR* deletion mutant RZ01 were approximately 50% lower than those of Z01, implying a positive regulatory role of KvhR. Transformation of these bacteria with a *kvhR* expressing plasmid pRC02 complemented the deleting effects, which confirmed the positive regulation of KvhR on *cps* expression. The activity of  $P_{orf1-2}$  was eliminated in the *rscB* deletion mutant [Fig. 4B (a)], which could be explained by the presence of a typical RcsAB box 5'-TAAGATTATTCTCA-3' (G) in the region from 168 to 181 nucleotides upstream of K2 *orf1-2*. As shown in Fig. 4B (b and c), despite the lack of a typical RcsAB box in  $P_{orf3-15}$  and  $P_{orf16-17}$ , both promoter activities were still affected by *rscB* mutation. No apparent change for either activity of  $P_{orf1-2}$  or  $P_{orf3-15}$  was observed in the *kvgA* deletion strain. A comparison with the wild-type strain showed that the deletion of *kvgA* reduced  $P_{orf16-17}$  activity by approximately 30% which could also be complemented by supplying the mutant bacteria with a *kvgA* expression plasmid pA14. This reveals that the response regulator KvgA is also involved in the regulation of the expression of transcript *orf16-17*. The ORF16 and ORF17, encoding ManC, GDP-mannose pyrophosphorylase, and ManB, phosphomannomutase, respectively, have been demonstrated to be required in the synthesis of *Klebsiella* K2 sugar nucleotide precursor (48). The question of why the particular step of the CPS biosynthetic pathway in the bacteria involves complex regulation remains to be answered. As shown in Fig. 4C, the activity of either  $P_{orf1-2}$ ,  $P_{orf3-15}$ , or  $P_{orf16-17}$  in the *kvhA* deletion strain was indistinguishable from that in the wild type strain Z01. However, in the presence of pAHm, activity of  $P_{orf1-2}$ ,  $P_{orf3-15}$  and  $P_{orf16-17}$  reduced by approximately 5.5-, 3- and 2.5-fold, which further supported



**Fig. 4. A: Organization of the *K. pneumoniae* K2 *cps* gene cluster.** Putative promoters of the three *cps* transcripts are also indicated. The horizontal arrows that begin with a solid circle represent the putative transcriptional units. **B: Expression of K2 *cps* gene in various genetic backgrounds.** The plasmids carrying  $P_{orf1-2}$  (a),  $P_{orf3-15}$  (b), and  $P_{orf16-17}$  (c) promoter fused with *lacZ* gene and transferred into wild type, *kvgA*<sup>-</sup>, *kvhR*<sup>-</sup>, and *rscB*<sup>-</sup> respectively by conjugation and shown as open bar. The complementation test was performed and shown as black bar. **C: The plasmids, pOrf12, pOrf315, and pOrf1617, were transferred into wild type (open bar), *kvhA*<sup>-</sup> (black bar), and wild type strain carrying pAHm (gray bar).** The *cps*-promoter carrying cells were grown in M9 medium to an OD<sub>600</sub> of 0.7 and the  $\beta$ -galactosidase activities were measured and presented in Miller units as described in "MATERIALS AND METHODS."



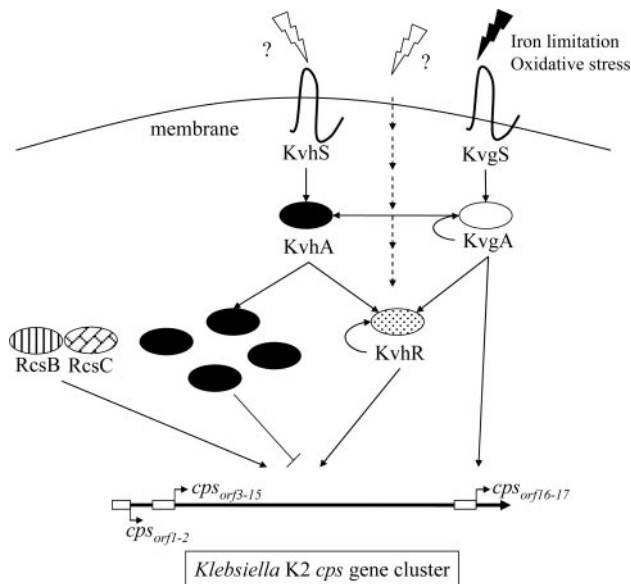


Fig. 5. The model is shown for the regulatory circuit of KvgAS, KvhAS, and KvhR that is responsible for controlling transcriptionally the expression of *Klebsiella* K2 cps gene cluster.

the negative role of KvhA in regulation of the cps expression.

**Regulation in Coordination**—As shown in Fig. 5, a model is proposed for a coordinate regulation of cps expression in the bacteria. Under a stress environment, the response regulator KvgA exhibits an auto-regulatory activity as well as a positive regulation on the expression of kvhAS, kvhR, and cps-orf 16-17. With a relatively low level of promoter activity (Table 4), however, KvhA also affects positively the expression of kvhR. The increasing expression of kvhR hence stimulates the transcription of K2 cps. On the other hand, an overexpression of kvhA under a not yet identified condition, in turn, suppressed the synthesis of K2 CPS at transcriptional level.

A complex 2CS regulatory system has been identified in *E. coli* CPS synthesis in responding to the environmental changes (43). PhoPQ, PmrAB and the Rcs regulatory system have been shown to regulate expression of *ugd* encoding UDP-glucose dehydrogenase, an enzyme required for the synthesis of polysaccharide on the in coordination in *E. coli* (49). While the activity of  $P_{kvgAS}$ ,  $P_{kvhAS}$  and  $P_{kvhR}$  were measured, *rscB* deletion appeared no effect on the expression of either *kvgAS*, *kvhAS*, or *kvhR*. Moreover, no apparent change of  $P_{rscB}$  activity was observed in either of *kvgA*, *kvhA*, and *kvhR* mutants (data not shown). This suggests an independent regulation of RcsB and the three response regulators on cps expression in *K. pneumoniae* CG43 (Fig. 5). We and others have observed that paralogous 2CS proteins may regulate similar functions, probably at different levels (50, 51). By using mutagenesis analysis, promoter activity measurement and EMSA, we are able to demonstrate an interacting regulation among the three paralogous response regulators. In addition, they are all responsible for modulation of the mucoidy and virulence of *K. pneumoniae* CG43, most likely through a transcriptional regulation of the cps expression.

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