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Regulation of the Homologous Two-Component Systems KvgAS and KvhAS in *Klebsiella pneumoniae* CG43

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In *Klebsiella pneumoniae* CG43, deletion of the sensor gene *kvgS* reduced the *kvgAS* expression in M9 medium with 0.2 mM paraquat, 0.2 mM 2,2-dihydropyridyl, or 300 mM NaCl. This result shows an autoregulatory role of KvgS and a stress-responsive expression of the two-component system (2CS). The *kvgS* deletion also appeared to decrease the expression of *kvhAS*, paralogous genes of *kvgAS*. Additionally, measurements of the promoter activity in *kvgA*$^-$ mutant revealed that KvgA is probably an activator for the expression of *kvgAS* and *kvhAS*. The subsequent electrophoretic mobility shift assay, indicating a specific binding of the recombinant KvgA to the putative promoters P_{kvgAS} and P_{kvhAS}, also supported an interacting regulation between the 2CSs. In P_{kvgAS} and P_{kvhAS}, the presence of RpoS binding elements suggested an RpoS-dependent regulation. Nevertheless, the *rpoS* deletion reduced the expression of *kvgA*S but increased that of *kvhAS*. Moreover, the *kvgA* deletion reduced the expression of *katG* and *sodC*. The overexpression of KvhA altered the susceptibility to fosfomycin and an increasing activity of UDP-*N*-acetylglucosamine enolpyruvyl transferase, the target protein of fosfomycin, which suggesting a regulation by KvhA. Taken together, these indicated that the two 2CSs probably belong to different regulatory circuits of the RpoS regulon.

Key words: fosfomycin, *katG*, KvgAS, KvhAS, *rpoS*, *sodC*, two-component system.

Bacterial two-component systems (2CS) consist of a sensor histidine kinase and a response regulator to cope with the capricious environments (*1*). More than ten 2CS genes are generally present in bacteria and it is believed that they form regulatory networks to show dependencies and regulatory hierarchies (*2–4*). For instance, a regulatory cascade from PhoPQ, the virulence-related 2CS of *Salmonella enterica* serovar Typhimurium, to PmrAB, the 2CS responsible for the resistance to antibiotic polymixin B, was demonstrated under the condition of low Mg^{2+}, in which the expression *pmrAB* is controlled by the non-cognate sensor PhoQ (*5*). Furthermore, in *Escherichia coli*, activation of the acid and multi-drug resistance related 2CS, EvgAS, has also been reported to promote the expression of PhoP-activated genes including the *phoPQ* operon (*2*).

RpoS, the stationary-phase sigma factor, is induced to control expression of more than 100 genes or operons to counter different stress conditions (*6–8*). Involvement of the global regulator in 2CS network has been commonly observed in bacteria (*9–11*). For example, the histidine kinase ArcB is able to phosphorylate its cognate regulator ArcA and also the protein RssB, and then the phosphorylated RssB stimulates the proteolysis of RpoS (*11*). In addition, the recent studies of DNA microarray analysis also provided evidence of regulatory interactions that are indicative of cross-regulation or overlapping regulation among the 2CSs EnvZ/OmpR, AtoS/AtoC, and ArcB/RssB and RpoS regulon (*12*, *13*).

We have previously isolated a 2CS operon encoding homologue of *Bordetella pertussis* BvgAS by PCR-subtractive hybridization from a highly virulent strain *Klebsiella pneumoniae* CG43 and the 2CS genes were named *kvgAS*. 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 (*14*). A BLASTP search in *K. pneumoniae* MGH78578 (http://genome.wustl.edu/) revealed highly homologous 2CS genes with amino acid sequence identity of 47.5% to KvgA and 32.8% to KvgS, respectively. The 2CS genes were hence named *kvhAS*, *kvg*AS *h*omologue. In contrast to *kvgAS*, *kvhAS* is present in all the strains collected in the laboratory, as determined by dot-blotting hybridization using *kvhA* as a probe (data not shown). A stress-responsive role of KvgAS has been proposed since *kvgAS* expression was activated in LB medium to which 0.2% paraquat or 0.2 mM 2,2′ dipyridyl was added (*15*). Deletion of *kvgA* or *kvhA* has recently been shown to affect capsulation of the bacteria, thereby reducing the bacterial virulence (submitted to publication). The study demonstrates the regulatory control of the 2CSs and the involvement of RpoS in the expression of *kvgAS* and *kvhAS* in *K. pneumoniae* CG43.

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

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

Strains or plasmids	Descriptions	Reference or source
Strains		
K. pneumoniae		
CG43S3	CG43 Sm^r	(*35*)
CG43S3-Ah01	CG43S3 Δ*kvhA*	(*19*)
CG43S3-Z01	CG43S3 Δ*lacZ*	(*19*)
CG43S3-S01	CG43S3-Z01 Δ*kvgS*	This study
CG43S3-Sh01	CG43S3-Z01 Δ*kvhS*	This study
CG43S3-AZ18	CG43S3-Z01 Δ*kvgA*	(*19*)
CG43S3-AhZ01	CG43S3-Z01 Δ*kvhA*	(*19*)
CG43S3-RpoS01	CG43S3-Z01 Δ*rpoS*	This study
E. coli		
JM109	*recA supE44 endA1 hsdR gyrA96 relA1 thi (lac-proAB)F* [$lacI^q$ *lacZ M15 proAB traD36*]	Laboratory stock
BL21-CodonPlus(DE3)-RIL	*F-ompT hsdS dcm*$^+$ Tet^r *gal* (DE3) *endA* The [*argU ileY leuW* Cam^r]	Laboratory stock
S17-1 λ*pir*	*hsdR recA pro* RP4-2 [Tc::Mu; Km::Tn*7*] (λ*pir*)	(*36*)
Plasmids		
pKAS46	Positive selection suicide vector, *rpsL* Ap^r Km^r	(*36*)
placZ15	A derivative of pYC016, containing a promoterless *lacZ* from K. *pneumoniae* CG43S3 as the reporter, Cm^r	(*19*)
pYC030	1.9-kb fragment, containing *kvgS* with 1.0-kb of an internal *BamHI* deletion, cloned into pKAS46	(*15*)
pSh01	2.3-kb fragment, containing *kvhS* with 1.0-kb of an internal *EcoR* deletion, cloned into pKAS46	This study
pRpoS09	1.6-kb fragment, containing *rpoS* with Tc gene in *BamHI* site, cloned into pKAS46	This study
pkvgA4	Deletion of the receiver domain of *kvgA* gene digested by *ClaI*, and cloned into pET30c, Km^r	(*19*)
pHP4005	A *kvhA* gene containing DNA from *K. pneumoniae* CG43S3 digested with *BamHI* and cloned into pET30c, Km^r	(*19*)
pA16	399-bp *Bam*HI fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	(*19*)
pETm-C	A derivative of pET30C, containing malonate promoter, Km^r	This study
pHAm	A *Bam*HI fragment of pHP4005 carrying entire *kvhA* coding sequence cloned into *Bam*HI site of pETm-c	This study
pA23	360-bp *Bam*HI/*Bgl*II fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	This study
pA26	318-bp *Bam*HI fragment containing the putative *kvgAS* promoter, cloned into BamHI site of placZ15	This study
pA28	196-bp *Bam*HI fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	This study
pA30	52-bp *Bam*HI/*Bgl*II fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	This study
pAh01	516-bp *Bam*HI/*Bgl*II fragment containing the putative *kvhAS* promoter, cloned into *Bam*HI site of placZ15	(*19*)
pAh02	374-bp *Bam*HI/*Bgl*II fragment containing the putative *kvhAS* promoter, cloned into *Bam*HI site of placZ15	This study
pAh03	180-bp *Bam*HI/*Bgl*II fragment containing the putative *kvhAS* promoter, cloned into *Bam*HI site of placZ15	This study
pKatG02	430-bp *Bam*HI/*Bgl*II fragment containing the putative *katG* promoter, cloned into *Bam*HI site of placZ15	This study
pKatE02	815-bp *Bam*HI/*Bgl*II fragment containing the putative *katG* promoter, cloned into *Bam*HI site of placZ15	This study
pSodC02	160-bp *Bam*HI/*Bgl*II fragment containing the putative *sodC* promoter, cloned into *Bam*HI site of placZ15	This study

at 37°C in Luria-Bertani (LB) medium or M9 minimal medium supplemented with appropriate antibiotics.

Construction of kvgS, kvhS, and rpoS Gene–Deletion Mutants—The mutants with specific deletion of either of *kvgS*, *kvhS* or *rpoS* genes were constructed by the allelic exchange strategy. Briefly, two DNA fragments, of approximately 1 kb in size, flanking *kvgS*, *kvhS*, *rpoS* gene were PCR amplified using specific primer sets (Table 2). The generated DNA fragments were ligated and subcloned into pKAS46 and the resulting plasmids, pYC030, pSh01, and pRpoS09 (Table 1), transformed into *E. coli* S17-1λ*pir* and then mobilized to the streptomycin-resistant strain

Table 2. **Primers used in this study.**

Primer no.	Sequence	Enzyme cleaved	Complementary position
rpoS01	5′-ACGATGATTACCTGAGTGCCT-3′		–291 relative to the *rpoS* start codon
rpoS02	5′-TTGAGCGGTGAGAAGATG-3′		+47 relative to the rpoS stop codon
rpoS04	5′-*GGATCC*CTGAGCAAAGCACC-3′	*Bam*HI	+33 of the *rpoS* coding region
rpoS05	5′-*CTAGAT*CTCCTGGGTCACCG-3′	*Bgl*II	–869 relative to the *rpoS* start codon
a09	5′-CATATTGT*GGATCC*TGCTGTTC-3′	*Bam*HI	+22 of the *kvgA* coding region
a14	5′-*GGATCC*TCTACCACCTTAA-3′	*Bam*HI	–399 relative to the *kvgA* start codon
a15	5′-TGCGTT*GGATCC*GTGATTAG-3′	*Bam*HI	–204 relative to the *kvgA* start codon
a17	5′-GGTAACTAAC*GGATCC*ACTC-3′	*Bam*HI	–320 relative to the *kvgA* start codon
a18	5′-*AGATCT*GTTCTGAATTTATTC-3′	*Bgl*II	–361 relative to the *kvgA* start codon
a19	5′-*AGATCT*GGTGGTACCACGATAC-3′	*Bgl*II	–52 relative to the *kvgA* start codon
AS03	5′-TCTTATTTTATCCGTCGT-3′		–1 relative to the *kvhS* start codon
AS04	5′-ATCTGCAGAATATCCCGT-3′		+1534 of the *kvhS* coding region
AS08	5′-GACTTATCGGCAATATTCT-3′		+1942 of the *kvhS* coding region
AS09	5′-GGAAAAAACTGACAAGGATG-3′		+62 relative to the *kvhS* stop codon
AP01	5′-GCTGCTG*AGATCT*GCCGC-3′	*Bgl*II	+99 of the *kvhA* coding region
AP02	5′-GAACGCCGGATCCTACAGC-3′	*Bam*HI	–188 relative to the *kvhA* start codon
A201	5′-*GGATCC*GAAAAAGGATCGTTCA-3′	*Bam*HI	–516 relative to the *kvhA* start codon
A202	5′-*GGATCC*CCAGTACTGTTATTCC-3′	*Bam*HI	–374 relative to the *kvhA* start codon
K01	5′-CGGATCCATTGTTGGATG-3′	*Bam*HI	+36 of the *katG* coding region
K02	5′-CACGCTGAT*AGATCT*GTATTC-3′	*Bgl*II	–422 relative to the *katG* start codon
E01	5′-CGGGTGCTTATC*AGATCT*TAC-3′	*Bgl*II	+18 of the *katE* coding region
E02	5′-CT*GGATCC*GATGTGGATTG	*Bam*HI	–803 relative to the *katG* start codon
D01	5′-GCGAGGGATA*AGATCT*CG-3′	*Bgl*II	+34 of the *sodC* coding region
D02	5′-CAGCAGT*GGATCC*GCATC-3′	*Bam*HI	–121 relative to the *sodC* start codon

K. pneumoniae CG43S3-Z01 by conjugation. A kanamycin resistant transconjugant was initially picked, grown overnight, and then spread onto a LB plate supplemented with 500 μg ml^{-1} streptomycin. After the occurrence of double crossover, the streptomycin resistant colonies were further ascertained for their susceptibility to kanamycin and Southern hybridization. The resulting mutants are *K. pneumoniae* CG43S3-S01 (*kvgS*$^{-}$), -Sh01 (*kvhS*$^{-}$), and -RpoS01 (*rpoS*$^{-}$) (Table 1).

Promoter Activity Measurement—The promoters of *kvgAS*, *kvhAS*, *rpoS*, *katG*, *katE*, and *sodC* were PCR amplified from *K. pneumoniae* CG43S3 by the designed primer sets (Table 2) and subcloned into placZ15. One-tenth overnight culture of the bacteria carrying each of the plasmids were refreshed grown in M9 medium to an optical density at 600 nm (OD_{600}) of about 0.6 to 0.7. The β-galactosidase activity assay was carried out essentially as described (*16*). 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.

Identification of the Transcription Start Site of kvgAS and kvhAS—The start sites of *kvgAS* and *kvhAS* transcripts were mapped by 5′-RACE (5′-Rapid Amplification of cDNA Ends). In brief, total RNA was isolated from mid-exponential phase of *K. pneumoniae* CG43S3 cells (OD_{600} = 0.6–0.8) by extraction with the TRI reagent (Molecular Research Center, Cincinnati, OH, USA). The first strand cDNA synthesis used SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) and the GSPs (antisense gene–specific primers) are GSP-A22 (5′-CATCTGCTGCTTCACCCGTTA-3′), from nucleotides +121 to +101, and GSP-Ah01 (5′-CTGCCGCGACGGTAATACCGT-3′), from nucleotides +88 to +68 downstream of the translation start site (marked as position +1) of *kvgA* and *kvhA*, respectively. The PCR condition was 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C extension for 3 min. Each of the amplicons was then cloned into pCR2.1-TOPO vector (Invitrogen Inc., Madison, WI) confirmed by Southern blotting hybridization, and subject to sequence determination.

Preparation of the Recombinant Proteins KvgA—The coding region of *kvgA* was PCR amplified from *K. pneumoniae* CG43S3 with the specific primers (Table 2), and the PCR products cloned into pUC-T vector (MDBio). The resulting plasmid was designated as pkvgA1. The plasmid pkvgA1 was digested with *Cla*I to remove the receiver domain of KvgA and then the DNA binding domain, $KvgA_t$, was subcloned into the *Sal*I–*Not*I sites of pET30c, which resulted in the expression plasmid pkvgA4. The plasmids pkvgA4 was then transformed into *E. coli* BL21-RIL(DE3). The transformant carrying pkvgA4 was cultured in LB medium to log phase, and expression of the recombinant proteins was induced with 1 mM IPTG for 3 h at 37°C. The overexpressed His-$KvgA_t$ protein formed an inclusion body, but the His-KvhA appeared to be in soluble form. The bacteria carrying pkvgA4 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-$KvgA_t$ protein was refolded through dialysis against a gradient of decreasing concentrations of urea in the reaction buffer (20 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 50 mM KCl, 1 mM $CaCl_2$ and 1 mM dithiothreitol). Finally, the purified His-$KvgA_t$ was concentrated with polyethylene

glycol 20,000 and the concentration of protein was determined by the Bradford assay (*17*).

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 [γ-^{32}P]ATP using T4 polynucleotide kinase. The purified His-KvgA$_t$ or His-KvhA was incubated with the radioactively labeled DNA in a 20 μl solution containing 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 50 mM KCl, 1 mM $CaCl_2$ 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 and pUC19 were used in the competition assay. The samples were then loaded onto a running gel of 5% nondenaturing polyacrylamide in 0.5× 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 either autoradiography or InstantImager™ (Packard Instrument Company).

Antibiotic Susceptibility Assay—Antibiotic susceptibility testing was performed by disk diffusion method. An overnight culture of bacteria was spread onto LB agar, then disks were placed onto the plates and zones of inhibition were measured after 16 h of incubation at 37°C. The antibiotic disks were obtained from Difco (Detroit, Mich), Becton Dickinson (Sparks, Md) and Oxoid Ltd (Basingstoke, Hampshire), and the following concentrations were used: fosfomycin 50 μg; cephalothin 30 μg; piperacillin 100 μg; carbenicillin 100 μg.

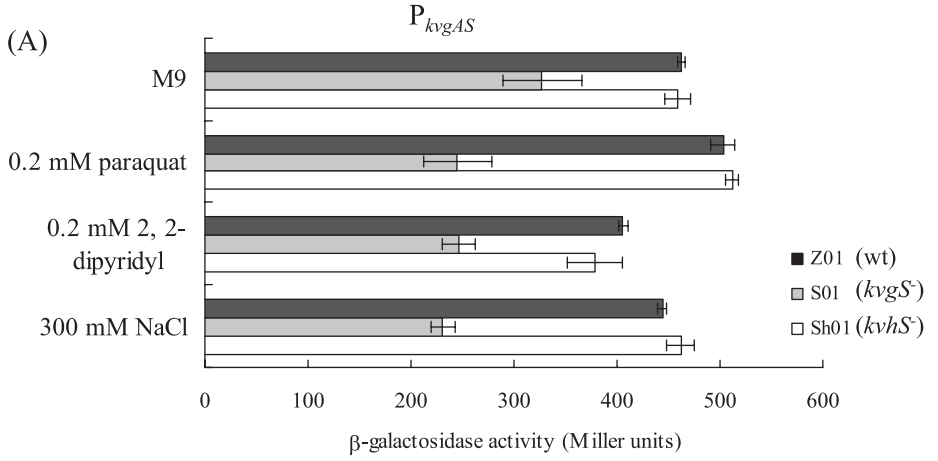
*UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase (MurA) Activity Measurement*—Overnight cultures (4 ml each) of *K. pneumoniae* CG43-S3, *K. pneumoniae* CG43S3 [pAhm] and *K. pneumoniae* CG43S3-Ah01 were harvested, washed twice with ice-cold 50 mM Tris, pH 7.5, and resuspended in 1 ml of 50 mM Tris pH7.5 and 2 mM dithiothreitol (DTT). After disruption by sonication, the cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants were collected for concentration determination using Bio-Rad protein assay (Bio-Rad, Hercules, Calif). As described (*18*), the assay mixture of 50 μl contained 50 mM Tris, pH 7.5, 2 mM DTT, 10 mM UDP-GlcNAc, and 10 μg of the extracted protein. After incubation at 37°C for 15 min, the reaction was started by the addition of 5 μl of 10 mM phosphoenolypyruvate (PEP), and the incubation at 37°C continued for 1 h. Finally, 800 μl color reagent (1% ammonium molybdate, 1 N HCl, 0.15% malachite green) was added to stop the reaction and the release of oethophosphate (P_i) measured by recording the absorbance at 660 nm (OD_{660}). The background reading in the absence of UDP-GlcNAc was used as a blank. 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.

RESULTS AND DISCUSSION

Deletion of kvgS Affected the Expression of kvgAS and kvhAS—In M9 medium, the activity of the putative promoter P_{kvgAS} of *kvgAS*, containing 399-bp noncoding sequence upstream of *kvgAS* in the *kvgS*$^-$ mutant, CG43S3Z01-S01, was reduced to about 30% below that in wild type CG43S3Z01 (Fig. 1A). Upon the addition of 0.2 mM paraquat, 0.2 mM 2, 2-dipyridyl, or 300 mM NaCl, no apparent change of the P_{kvgAS} activity was found in CG43S3Z01. Whereas, a notable reduction of the P_{kvgAS} activity was observed in the *kvgS*$^-$ mutant under either of the culture conditions. This is consistent with the previous findings (*15*) obtained using luciferase as the reporter, indicating that KvgS likely acts as the sensor protein *via* auto-regulation to encounter with the stress signals. On the other hand, the P_{kvgAS} activity in the *kvhS*$^-$ mutant Sh01 was comparable with that of the parental strain CG43S3Z01 indicating that the deletion of *kvhS* had no effect on the expression of *kvgAS*. As shown in Fig. 1B, deletion of *kvgS* slightly reduced the activity of P_{kvhAS}, the putative promoter of *kvhAS*, in M9 medium implying a positive role of KvgS in regulating the *kvhAS* expression. The *kvhS* deletion did not apparently affect the activity of P_{kvhAS} in the presence of either 0.2 mM paraquat or 0.2 mM 2,2-dipyridyl. However, the P_{kvhAS} activity in M9 that contained 300 mM NaCl was reduced to two-thirds of that measured in M9 (Fig. 1B). Under osmotic stress, deletion of either *kvgS* or *kvhS* appeared to reduce further the P_{kvhAS} activity, indicating cooperative regulation of the two 2CSs is present for modulation of the expression of *kvhAS* upon changes of osmotic potential.

Localization of KvgA Binding Sequences on P_{kvgAS} and P_{kvhAS}—Our previous study has suggested that KvgA positively regulated the expression of *kvgAS* and *kvhAS* (*19*). A series of truncations in pA16 ($P_{kvgAS\text{-}399}$), carrying 399-bp of the *kvgAS* putative promoter containing DNA, and pAh01 ($P_{kvhAS\text{-}516}$) containing 516-bp of the *kvhAS* putative DNA, yielding pA23, pA26, pA28, and pA30, containing the noncoding DNA 360 bp, 318 bp, 196 bp and 52 bp, respectively, upstream of *kvgA*, and pAh02 and pAh03, containing the noncoding DNA 374-bp and 180-bp upstream of *kvhA*, were generated to localize the binding sequence of KvgA on both promoters.

As shown in Fig. 2A, *kvgA* deletion negatively affected not only the activity of pA16, but also that of pA23 and pA26. In the *kvgA*$^-$ mutant AZ18, the pA16 activity appeared to be lower than those of pA23 and pA26, suggesting the presence of two KvgA-regulatory regions for the differential activity. Notably, these promoters retained some activity, implying that, more element(s) are involved in regulation of the *kvgAS* expression. Although the truncation from pA16 to pA28 removes the KvgA binding region, Z01[pA28] exerted a comparable activity with Z01[pA16] suggesting that the deletion alters secondary structure of the DNA leading to a potent promoter. However, the possibility remained to be investigated. The truncation form pA28 to pA30 appeared to diminish dramatically the P_{kvgAS} promoter activity implying that the region that from 52 bp to 196 bp is also important to the expression of *kvgAS*. The following EMSA demonstrated that KvgA$_t$ can bind to the DNA fragments that are contained in pA16, pA23, and pA26 (Fig. 2B), helping to support the above notion that KvgA was probably involved in positive auto-regulation by direct binding to the promoter sequence. When the incubation of pA16 DNA fragment with an increasing amount of His_6-KvgA$_t$ from 0.3 μg to 0.6 μg, different binding complexes, C1 and C2, were found. This also suggests that two KvgA-regulatory elements are contained in pA16. Consistent with the results of



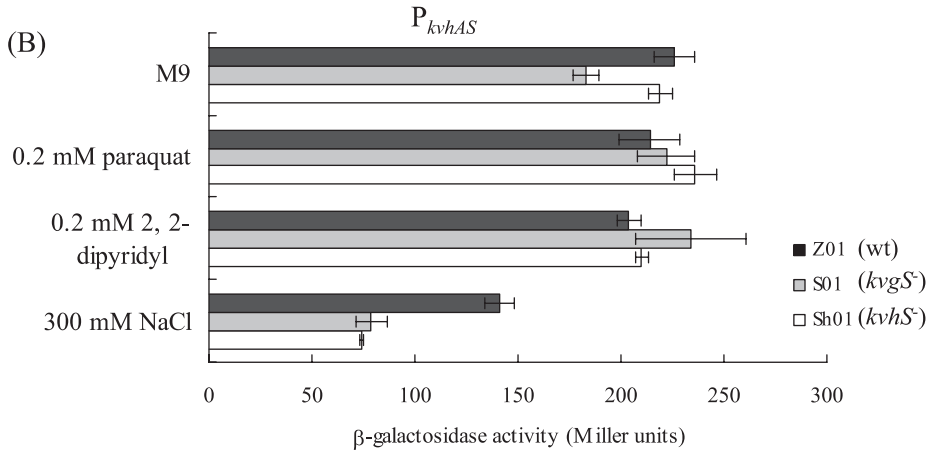


Fig. 1. **Regulation of the *kvgAS* and *kvhAS*.** Activity of the promoters P_{kvgAS} (A) and P_{kvhAS} (B) were examined in wild-type (Z01), *kvgS*⁻ mutant (S01), and *kvhS*⁻ mutant (Sh01). Bacteria were inoculated in M9 medium or the medium supplemented with different reagents at 37°C for 1 h. The β-galactosidase activity was determined.

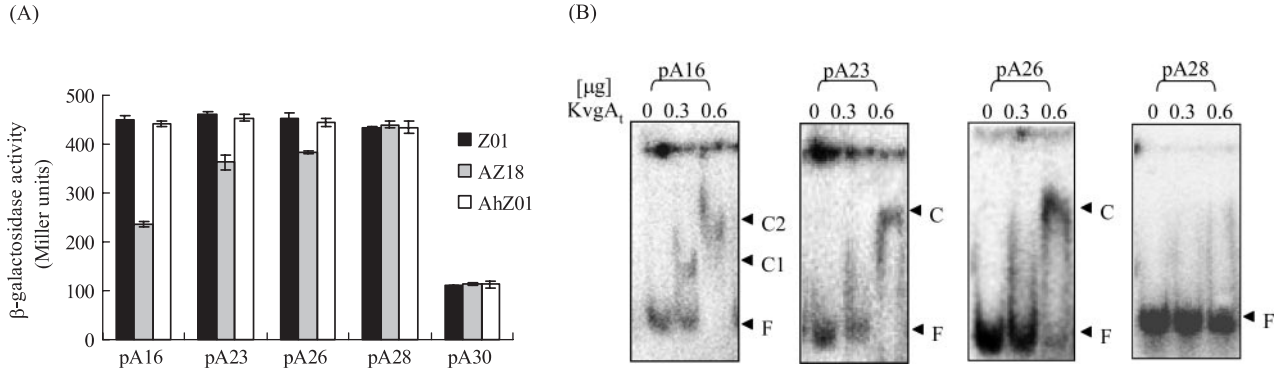


Fig. 2. **Identification of KvgA binding region on P_{kvgAS}.** (A) The promoter activity of *kvgAS* was determined by β-galactosidase activity measurement. The plasmids carrying respectively the P_{kvgAS} regions including pA16, pA23, pA26, pA28 and pA30, were transferred into Z01 (wt), AZ18 (*kvgA*⁻), and AhZ01 (*kvhA*⁻), respectively by conjugation. The cells were grown in M9 medium to an OD_{600} of 0.7, and the β-galactosidase activity was determined. (B) EMSA assessment of the $KvgA_t$ binding activity to P_{kvgAS}. The DNA fragments including pA16, pA23, pA26, and pA28 were used as the binding probes. The amounts of protein used are indicated at the top of each lane. The DNA and protein complexes formed are indicated as C and the free forms are indicated as F.

promoter activity measurement, no DNA-protein complexes could be observed with pA28 DNA.

Interestingly, the activities of pAh02 and pAh03 were lower than that of pAh01 indicating that the truncation from pAh01 to pAh02 probably alters the promoter conformation, affecting the *lacZ* expression. As shown in Fig. 3A, the deletion of *kvgA* appeared to reduce the activity of pAh01, pAh02 and pAh03, suggesting that the 180-bp noncoding sequences of pAh03 contain the KvgA regulatory element. EMSA was performed with the purified $KvgA_t$ protein and the DNA fragments of pAh01, pAh02, and pAh03 to confirm that KvgA indeed binds directly to the

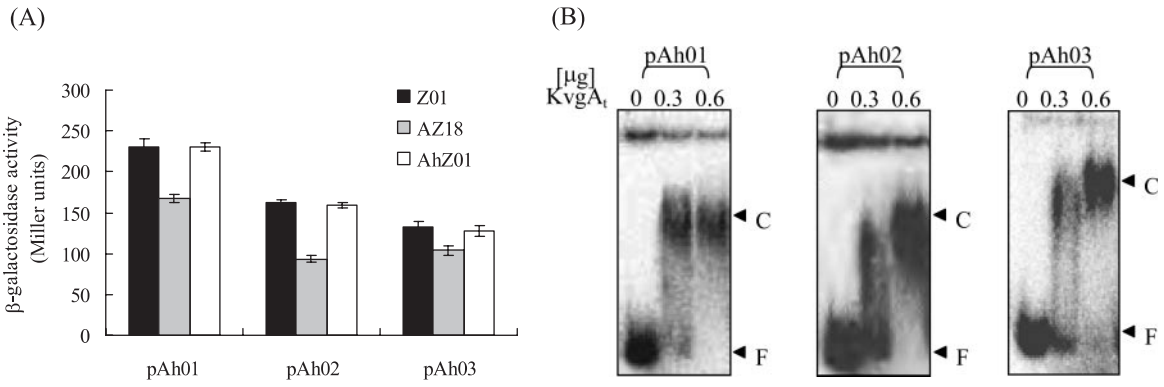


Fig. 3. **Identification of KvgA binding region on P_{kvhAS}.** (A) The promoter activity of *kvhAS* was determined by β-galactosidase activity assay. The plasmids carrying P_{kvhAS} promoter regions, pAh01, pAh02 and pAh03, were transferred into Z01 (wt), AZ18 (*kvgA*$^-$), and AhZ01 (*kvhA*$^-$), respectively by conjugation. The cells were grown in M9 medium to an OD_{600} of 0.7, and the β-galactosidase activity was determined. (B) EMSA assessment of the KvgAt binding activity to the promoter P_{kvhAS}. The DNA fragments including pAh01, pAh02, and pAh03 were used as the binding probes. The amounts of protein used are indicated at the top of each lane. The DNA and protein complexes formed are indicated as C and the free forms are indicated as F.

kvhA promoter. As shown in Fig. 3B, consistent with the measurements of promoter activity, recombinant $KvgA_t$ could bind each of the DNA fragments.

The MEME program (*20*) was then employed to identify a consensus motif between the upstream sequences of *kvgA* and *kvhA* for KvgA binding. However, no conserved sequence could be determined, indicating that searching for more genes under regulation by KvgA are required for a consensus binding element of KvgA.

Both kvgAS and kvhAS Contain Sigma-70 (RpoD) Dependent Promoters—5′ RACE was employed to map the transcriptional start site of *kvgAS* and *kvhAS*. Sequencing of the 5′ RACE products revealed that the transcription start site of *kvgAS* initiated at nucleotide T, 55 nt upstream from the start codon (Fig. 4A), and the initiation site of *kvhAS* was at nucleotide T, 84 nt upstream from the translation start (Fig. 4B). A possible RpoD dependent promoter for *kvgAS* of –10 box (TTTAAA) and –35 box (TTACCC), and for *kvhAS* of –10 box (TGTTAC) and –35 box (TTCCCT) could be identified. The localized KvgA binding region from pA16 to pA26 was found upstream the –35 box of P_{kvgAS}. Whereas, the localized KvgA binding region within pAh03 appeared possibly to overlap with the –10 and –35 box of P_{kvhAS}. Since KvgAS and KvhAS were shown to be stress-related 2CSs, the presence of the RpoS binding sequence (*8*, *21*, *22*) in P_{kvgAS} and P_{kvhAS} was investigated. As shown in Fig. 4A, two close-to-consensus RpoS-dependent sequences could be identified within P_{kvgAS}, 5′-TGACTTATAT-3′ (from –312 to –326) and 5′-TGCATATGCT-3′ (from –229 to –238). Interestingly, the two RpoS-dependent sequences appeared also to be contained within the KvgA binding region, indicating that the possibility of an interacting regulation of KvgA with RpoS to modulate P_{kvgAS} expression. As shown in Fig. 4B, a typical RpoS binding site, 5′-TGCAGATAAT-3′, was found in the sequence of P_{kvhAS}-pAh01 from –239 to –248 but not in the KvgA binding region. The results indicated that the regulations of KvgAS and KvhAS are probably diverse.

RpoS Controls the Expression of kvgA and kvhA—An *rpoS* deletion mutant, designated as RpoS01 (Table 1), was constructed to investigate whether RpoS affects the expression of *kvgAS* and *kvhAS*. As shown in Fig. 5A, *rpoS* deletion reduced pA16 activity to approximately one-third of that measured in wild type, implying that RpoS positively controlled *kvgAS* expression. While RpoS01 that carried either pA23 or pA26 had a slightly lower level of activity than wild type (Fig. 5A), indicating that the effect of the deletion of *rpoS* on the activity of pA16 differed from that on pA23 or pA26. As shown in Fig. 5A, pA16 contains two potential RpoS regulatory elements but pA23 and pA26 contain only one of the elements, which may be explained by a differential level of regulation by RpoS. Loss of the two RpoS consensus sequences in pA28 appeared to eliminate the σ^S-dependent regulation (Fig. 5A). Consistent with the notion observed in Fig. 2A, pA28 lacking the RpoS consensus sequences retains a comparable activity with that of pA16 in Z01.

Since an RpoS regulatory element was found in the region of P_{kvhAS}, the involvement of RpoS in regulating the expression of *kvhAS* was also investigated. Interestingly, the activity of P_{kvhAS}-pAh01 in RpoS01 increased to approximately double that in the wild type strain (Fig. 5B). Whereas, no apparent change of the activity of either pAh02 or pAh03, in which the RpoS consensus sequence has been deleted. The results indicating that *rpoS* deletion affected the activity of both promoters P_{kvgAS} and P_{kvhAS} suggest that the 2CSs are possible members of the RpoS regulon. To determine if the *rpoS* expression is mutually regulated by either KvgA or KvhA, activity of the *rpoS* promoter, containing 866-bp of the noncoding region upstream of the RpoS start codon, was measured in either wild type strain, *kvgA*$^-$ mutant, or *kvhA*$^-$ mutant. No apparent change of the activity of the *rpoS* promoter indicating that the *rpoS* expression is not regulated by KvgA or KvhA (date not shown).

KvgA Affects the Expression of the Stress Related Genes, katG and sodC—The antioxidant defense genes *katE*, *katG*, and *sodC* have been reported to be components of RpoS regulon (*8*, *23*). As shown in Fig. 6A, deletion of *rpoS* reduced the activities of P_{katE}, P_{katG}, and P_{sodC} in M9 medium. Notably, the deletion of *kvgA* also affected the activities of P_{katG} and P_{sodC}, suggesting that KvgA is positive regulator of the expressions of *katG* and *sodC*. In contrast, the deletion did not apparently affect the

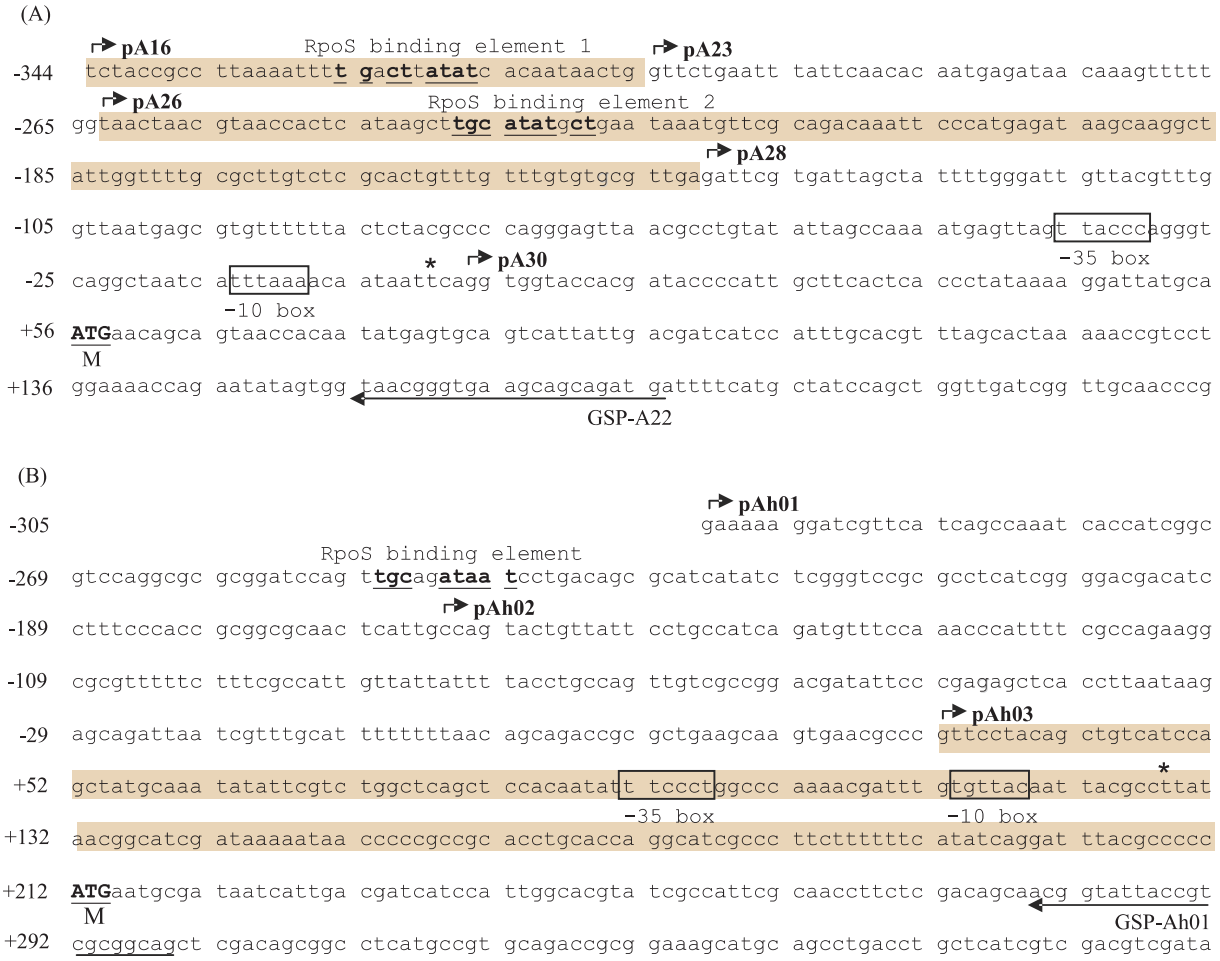


Fig. 4. **Sequence analysis of P_{kvgAS} (A) and P_{kvhAS} (B).** The transcription initiation site (+1) identified is indicated by a star. The predicted –10 and –35 sequences are boxed. The translation start codon (ATG) and the putative RpoS binding element are in boldface and underlined. The shaded sequence contains the regulatory region of KvgA. Each of the promoter constructs is labeled and indicated by vertical arrow. The horizontal arrows indicate the positions and directions of the gene specific primers that were used for PCR amplification in 5′-RACE.

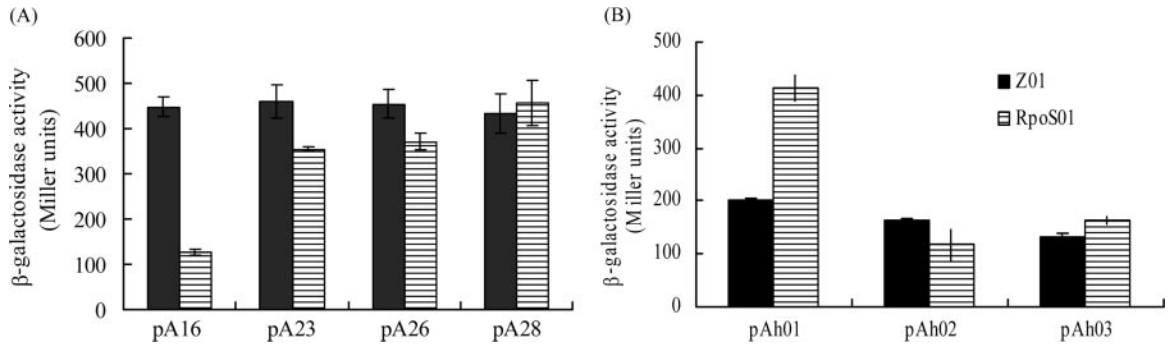


Fig. 5. **Deletion of *rpoS* affects the expression of *kvgAS* and *kvhAS*.** Activities of the promoters of *kvgAS* (A) and *kvhAS* (B) were determined by β-galactosidase activity assay. The plasmids carrying P_{kvgAS} including pA16, pA23, pA26 and pA28, and P_{kvhAS} including pAh01, pAh02 and pAh03, were transferred into Z01 and RpoS01 respectively by conjugation. The cells were grown in M9 medium to an OD_{600} of 0.7, and the β-galactosidase activity was determined.

activity of P_{katE}. Whereas, the deletion of *kvhA* conferred no notable effect on the expression of any of the promoters (Fig. 6A).

EMSA was performed on the purified $KvgA_t$ protein, and the DNA fragment of P_{katG} or P_{sodC}, to determine whether KvgA acts as a transcriptional factor and directly interacts with the upstream regulatory regions of *katG* and *sodC*. The EMSA results in Fig. 6B indicated that $KvgA_t$ could specifically bind to the upstream region of *katG* suggesting a transcriptional control on the expression of *katG*.

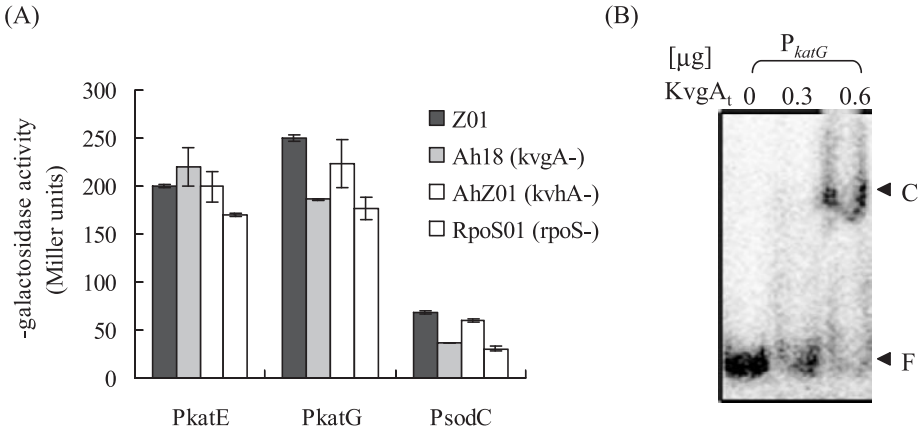


Fig. 6. **(A) Deletion effects of *kvgA*, *kvhA* and *rpoS* on expression *katG*, *katE*, and *sodC*.** The promoter activity of the stress related genes, *katG*, *katE*, and *sodC*, were determined by β-galactosidase activity assay. The plasmids carrying each of the promoters pKatE, pKatG, and pSodC, were transferred into Z01 (wt), AZ18 (*kvgA*$^-$), and AhZ01 (*kvhA*$^-$), and RpoS01 (*rpoS*$^-$) respectively by conjugation. The bacteria of stationary phase cultures were collected and the β-galactosidase activity determined. (B) EMSA of the $KvgA_t$ binding onto P_{katG}. The DNA fragment of the P_{katG} was labeled with [γ-^{32}P]ATP and the recombinant $KvgA_t$, added to the binding assay mixture. The amounts of protein used are indicated at the top of each lane. Specific competition was performed by adding the unlabelled DNA fragments into the mixture (lane 4). The unlabelled pUC19 DNA was also added as a non-specific competitor to the sample in lane 5. The DNA and protein complexes formed are indicated as C and the free forms are indicated as F.

In contrast, no $KvgA_t$-P_{sodC} complex could be detected (data not shown). The result revealed that KvgA regulates the expression by directly binding to the promoter P_{KatG}, whereas, the regulation of P_{SodC} is indirect. Many reports have shown that stress responses in bacteria require various regulators such as OxyR, SoxRS, FNR, and Fur to coordinate expression of the related genes (*8*, *23–27*). Additional regulatory protein(s) is (are) thus likely to be involved in the regulatory network of KvgAS for controlling the expression of *sodC*.

Overexpression of kvhA Altered the Bacterial Susceptibility to Some Antibiotics—Genes differentially expressed in response to osmotic stress may play a key role in permeability and drug resistance in bacteria (*28*). In *E. coli*, the 2CS EvgAS has been shown to be capable of regulating the expression of a putative efflux pump, *emrKY*, and the overexpression of *evgA* conferred on the *E. coli* a multidrug resistance (*29*). Therefore, we speculate that KvhAS, as an EvgAS ortholog, also plays a role in regulating drug susceptibility of *K. pneumoniae* CG43. The overexpression plasmids, pHAm and $pHAm_{dHTH}$, carrying respectively the entire coding sequence of KvhA and the truncated sequence removal of the DNA binding domain, were generated to demonstrate the possibility. The plasmids were then transformed individually into *K. pneumoniae* CG43S3 and the transformants exposed to various antibiotic disks. As shown in Table 3, the bacteria that harbored pHAm exhibited an increasing susceptibility to fosfomycin but a reduced sensitivity to cephalothin, piperacillin, and carbenicillin. In contrast, the bacteria that harbored $pHAm_{dHTH}$ exerted no apparent change in the drug resistant activity, indicating that KvhA requires the HTH domain to affect the drug susceptibility of the bacteria. Notably, the overexpression of *kvgA* in *K. pneumoniae* CG43 had no effect on the bacterial susceptibility to any of the drugs (data not shown).

MurA, a UDP-*N*-acetylglucosamine enolpyruvyl transferase, which catalyzes the first step of peptidoglycan synthesis, has been demonstrated as the target of fosfomycin (*30*). The overexpression of KvhA probably promoted the synthesis of MurA protein, and in turns, provided more targets to be attacked by fosfomycin leading to an increase of the drug susceptibility. MurA activity in the bacteria was measured to demonstrate whether the overexpression of KvhA increased the expression of MurA. As shown in Fig. 7, the MurA activity of *K. pneumoniae* CG43S3 [pHAm] appeared to be eight-fold higher than that of the wild type strain. Moreover, MurA activity decreased in the *kvhA* deletion mutant. In *E. coli*, the overexpression of the 32 response regulators revealed 13 of them can increase bacterial resistance to β-lactam antibiotics (*31*). The reported mechanisms of resistance to β-lactam antibiotics include drug detoxification (*32*), decreased affinity to the target (*33*), and reduction of the drug permeability (*34*). The expression of *kvhAS* appeared to respond to an osmotic stress related 2CS (Fig. 1B), suggesting the possibility that KvhAS somehow regulates membrane permeability, upon sensing the change of osmolarity, thereby changing the bacterial drug susceptibility. However, the possibility remains to be investigated.

Table 3. **Over-expression of *kvhA* in *K. pneumoniae* CG43S3 affects the drug susceptibility.**

Antibiotics (μg/disk)	Zone (mm)[a]		
	K. pneumoniae CG43S3		
	Host	pHAm	$pHAm_{dHTH}$
Peptidoglycan synthesis inhibitor:			
Fosfomycin 50	22	31	21
β-Lactams (PBP inhibitors):			
Cephalothin 30	21	13	20
Piperacillin 100	25	16	26
Carbenicillin 100	14	7	14

[a]Diameter of zones of inhibition, measured across disks of 6 mm diameter. Antibiotics that did not inhibit growth of the bacterial lawn were assigned a value of 6 mm.

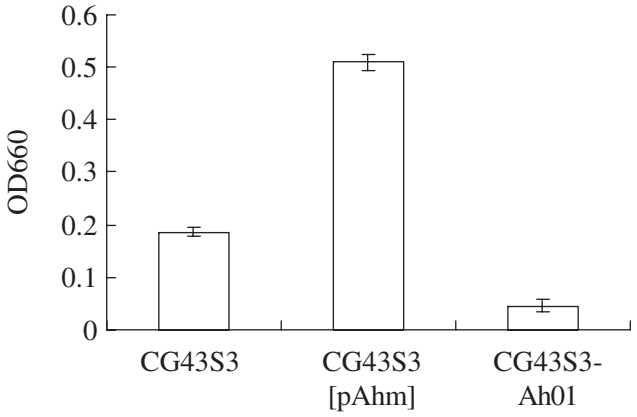


Fig. 7. ***In vitro* MurA activity assay.** The whole cell lysates of *K. pneumoniae* CG43S3, CG43S3 carrying *kvhA* overexpression plasmid [pAhm], and *kvhA* deletion CG43S3-Ah01 were incubated with a mixture containing 10 mM UDP-GlcNAc, 2 mM DTT, and 50 mM Tris-HCl pH 7.5 at 37°C for 15 min. Subsequently, 10 mM PEP was added to start the reaction. After 1 hour of incubation at 37°C, the release of inorganic phosphate was measured by adding the color reagent and the absorbance at OD_{660} was determined (described in "MATERIALS AND METHODS"). Inorganic phosphate release was measured in triplicate.

In conclusion, LacZ was used as the promoter reporter, and the deletion of KvgS is shown not only to affect its own promoter activity, but also the activity of P_{kvhAS}. Subsequent EMSA analysis, indicating a specific binding of the recombinant KvgA to the putative promoters P_{kvgAS} and P_{kvhAS}, also supported an interacting regulation between the two 2CSs. The apparent reduction of P_{kvgAS} activity in M9 supplement with either of paraquat, 2,2-dihydropyridyl, and 300 mM NaCl in *kvgS*$^-$ mutant indicating that KvgAS is most likely a stress responsive 2CS. In the medium with 300 mM NaCl, the expression of *kvhAS* was also reduced to 50% in either *kvgS*$^-$ or *kvhS*$^-$ mutant, suggesting the role of KvhAS in responding to osmotic pressure. Moreover, the deletion of the *rpoS* reduced the expression of *kvgA*S but increased that of *kvhAS*. Deletion of *kvgA* was shown to affect expression of the antioxidant defense genes *katG* and *sodC*. However, the overexpression of KvhA rendered the bacteria more susceptible to fosfomycin but less sensitive to cephalothin, piperacillin, and carbenicillin. These results indicated that the two homologous 2CSs probably belong to different regulatory circuits of the RpoS regulon.

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