Introduction

Klebsiella pneumoniae is a common nosocomial pathogen, which causes suppurative lesions, septicemia, urinary and respiratory tract infection in immunocompromised patients (15, 29, 55, 77). The wide spread of extended spectrum β–lactamase producing *K. pneumoniae* (ESBLKp) has prompted the search for new drugs to intervene the bacterial infections (100). Recently, development of an antimicrobial approach using two-component systems (2CS) as a drug target to block the signalling pathway has been evaluated (63).

During infection, bacteria f a sensor histidine kinase and a response regulator, acts to r and convert this information into specific transcriptional c (86). After sensing the input signals, the sensor protein catalyzes an autophosphorylation reaction, 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. Phosphorylation of the response regulator would activate the transcription-regulating activity through an appropriate conformational change (69, 97). The number of 2CS varies dramatically in bacteria genomes. The number of 2CS varies dramatically among bacterial genomes. For instance, *Bacillus subtilis* encodes 70 2CS proteins (24), whereas

Helicobacter pylori and *Haemophilus influenzae* contained only 11 and nine 2CS protein-encoding genes, respectively (69). 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 (45, 101). 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 of *pmrAB* is contro ensor PhoQ (85). Furthermore, in *Escherichia coli*, activatio -drug resistance related 2CS, EvgAS, has also been reporte ssion of PhoP-activated genes including the *phoPQ* operon (22).

RpoS, the stationary-phase sigma factor, is induced to control expression of more than 100 genes or operons to counter different stress conditions (31, 49, 95). Involvement of the global regulator in 2CS network has been commonly observed in bacteria (67, 80, 92). 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 (67). In addition, the recent studies which 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 (75, 103).

We have previously isolated a 2CS operon encoding a 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 (54). Downstream to *kvgAS*, a gene encoding KvgA homolog (53.8% similarity), a[illegible]ied namely (51). Interestingly, BLASTP analysis failed to i[illegible] *kvhR* in the *K. pneumoniae* MGH78578 and NTHU-K202[illegible]e.wustl.edu/projects/bacterial/ and http://genome.nhri.org.tw/kp/index.php). A BLASTP search in *K. pneumoniae* MGH78578 and NTHU-K2022 revealed another set of 2CS gene highly homologous to KvgAS with an amino acid sequence identity of 47.5 % to KvgA and 32.8 % to KvgS, respectively. The 2CS genes were hence named *kvhAS* (*kv*gAS *h*omologue). In contrast to *kvgAS*, *kvhAS* is present in all the strains collected in our laboratory, as determined by dot-blotting hybridization using *kvhA* as a probe (50). It has been shown previously in the laboratory that both *kvgAS* and *kvhAS* are organized as an operon using RT-PCR and Southern blot analysis (51). And the addition of paraquat

or 2',2' dipyridyl into *K. pneumoniae* culture activated the expression of *kvgAS* (51). The results strongly suggest that KvgAS is a stress-responsive 2CS, but the role of KvhAS and KvhR have not yet been identified. To gain further understanding of the functional roles of KvgAS, KvhR, and KvhAS, in this dissertation, a *lacZ*-based reporter system including placZ15, a plasmid containing a promoterless *lacZ* reporter, and Z01, a *K. pneumoniae* CG43 *lacZ* deletion mutant, was established. Furthermore, by generation of a series of the response regulator mutants to detect the phenotype, we were able to identify the roles of KvgAS, KvhR, and KvhAS in regulation of the *cps* expression in the bacteria. The regulatory interaction of KvgAS, KvhAS, and RpoS were described in **Part II**).

To date, the complete genomes of many bacteria are publicly available. Based on the well annotated genome databases, it became possible to introduce large-scale technologies to study differential levels of gene expression in bacteria. These methods include microarray DNA chips (20); transposon-based footprinting technique (81); signature tag mutagenesis (STM) (32); comparative genomics (5); mRNA differential display (2); differential fluorescence detection (91); *in vivo* expression technology (IVET) (19); and proteomic study (17, 42). In this dissertation, I have also employed a promoter trapping approach, cDNA substractive hybridization and proteome analysis to compare the differential expression of identify the target

sequences under control by the 2CSs (described in **Part III**).

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 5.9 kb and 3.3 kb DNA, located upstream respectively to *kvgA* and *kvhR*, were subjected to sequ sequence analysis including open reading frames (ORFs) tation was carried out using BLAST (NCBI database). Th quence was identified by the program tRNAscan-SE (58). lysis 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 (52) 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 (52). 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 (84). The resulting plasmid placZ16 was transformed into *Escherichia coli* S17-1λ*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 μgml^{-1} streptomycin. Afte le crossover, the streptomycin resistant colonies were furthe sceptibility to kanamycin. The *lacZ* mutation was confirmed to a X-gal containing medium and by Southern hybridization. This mutant was designated as *K. pneumoniae* CG43S3-Z01 (Table 1).

Construction of kvgA, *kvhA*, *kvhR, kvgS, kvhS,* and *rpoS deletion mutants*

The mutants with specific deletion of *kvgA*, *kvhA*, *kvhR, kvgS, kvhS,* and *rpoS* 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*$^-$), AhZ01 (*kvhA*$^-$), RZ01 (*kvhR*$^-$), S01 (*kvgS*$^-$), Sh01 (*kvhS*$^-$), and RpoS01 (*rpoS*$^-$). For the construction of *kvhR*$^-$*kvgA*$^-$ or *kvhR*$^-$*kvhA*$^-$ double mutant, the pKAS46 derivative containing either a *kvgA* or *kvhA* deletion was delivered respectively from *E. coli* S17-1 *λpir* into RZ01 by conjugation. The plasmid carrying a *kvhA* deletion was also mobilized from *E. coli* S17-1 *λpir* to *K. pneumoniae* AZ18 by conjugation to generate *kvgA*$^-$*kvhA*$^-$. For *kvhR*$^-$*kvgA*$^-$*kvhA*$^-$ triple mutant, the pKAS46 derivative containing *kvgA* deletion was delivered from *E. coli* S17-1 *λpir* into *kvhA*$^-$*kvhR*$^-$ mutant by conjugation. The selections fo ed out likewise. The resulting mutants were designated as AR01 (*kvgA*$^-$*kvhR*$^-$), AhR01 (*kvhA*$^-$*kvhR*$^-$) and AAhR01 (utant with deletion of *rcsB*, which has been demonstrated to encode a K2 *cps* activator in *K. pneumoniae* CG43S3 (53), was also generated and named RcsBZ01 (*rcsB*$^-$).

Determination of promoter activity

The putative promoter regions of *kvgAS*, *kvhAS*, *kvhR*, *rcsB*, *rpoS, katG, katE, sodC*, and the three *cps* transcriptional units (98, 99) 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_{600}) about 0.6 to 0.7. The β-galactosidase activity assay was carried out essentially as described by Miller (68). 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 [illegible] mapped by 5'-RACE. In brief, total RNA was isolated from r[illegible] *K. pneumoniae* CG43S3 cells (OD_{600}=0.6-0.8) by extractio[illegible] (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 $KvgA_t$, KvhA, and $KvhR_t$

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 int ıltant plasmid was named as pHP4005. While overexpressi *R* resulted in largely insoluble proteins. In order to resolve th kvgA1 was digested with *Cla*I to remove the receiver domaın. The remaınıng DNA binding domain of approximately 200-bp, $KvgA_t$, was subcloned into the *Sal*I-*Not*I sites of pET30c, which resulted in the expression plasmid pkvgA4. Likewise, pR28 was digested with *Eco*RV and *Hin*dIII to remove the receiver domain and the remaining DNA binding domain, $KvhR_t$, was subcloned into the *Eco*RV-*Hin*dIII 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-$KvgA_t$, His-KvhA, or His-$KvhR_t$ protein was induced with 1 mM IPTG for 3 h at 37°C. The overexpressed His-$KvgA_t$ and His-$KvhR_t$ 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-$KvgA_t$ and His-$KvhR_t$ 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 MgC aCl$_2$ and 1 mM dithiothreitol). The His-KvhA protein was pu fractions of the IPTG-induced bacteria carrying pHP4005. Tl is-KvhA and His-$KvhR_t$ were then concentrated with polyethylene glycol 20,000 and the concentration of protein was determined by the Bradford assay (10). 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 [γ-^{32}P]ATP using T4 polynucleotide kinase. The purified His-$KvgA_t$,

His-KvhA, or His-$KvhR_t$ 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 was 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 InstantImager™ (Packard Instrument Company).

Extraction and quantification of CPS was extracted as described [illegible] hundred microliters of bacteria cultured in LB broth overnight were mixed with 100 μl 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 μl 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 μl of distilled water, and 1.2 ml 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% (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 10^9 CFU (8).

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 doses. The LD_{50}, based on the number of survivors after 10 d expressed as CFU as described (78).

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; piperacillin100 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-HCl pH 7.5 and 2 mM DTT. After disruption by sonication, the cell lysates were centrifuged at 13,000 rpm for 10 min at 4 ℃ and the supernatants were collected for concentration determination using Bio-Rad protein assay (Bio-Rad, Hercules, CA). The assay mixture of 50 µl contained 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM UDP-GlcNAc, and 10 µg of t . After incubation at 37℃ for 15 min, the reaction was tion of 5 µl of 10 mM phosphoenolypyruvate (PEP), ℃ 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 inorganic phosphate (Pi) 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.

cDNA substractive hybridization

Total RNA was isolated from mid-exponential phase *K. pneumoniae* CG43 cells (OD_{600} = 0.6~0.8) by extraction with the TRI reagent (Molecular Research Center, Cincinnati, OH). Contaminating DNA was eliminated from the RNA samples with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). Total RNA (1 μg) was reverse-transcribed using SMART PCR cDNA synthesis kit (CLONTECH Laboratories, Inc., Palo Alto, CA) to produce the cDNA pool. The substractive hybridization libraries were generated using the reagents and protocols provided by Clontech (PCR-Select cDNA ı, Palo Alto, CA). Briefly, the two cDNA pools from the wil utant were digested with *Rsa*I and ligated to two different ad (**Appendix I**) were performed by stoichiometrically mixing the cDNAs ın two rounds of controlled hybridizations. The resulting cDNA molecules were then subjected to two rounds of PCR to amplify and enrich the desired differentially expressed sequences. The primers used in the first or primary PCR were based on the adaptor sequence, whereas the forward and reverse primers for the secondary PCR were based on sequences nested into the adaptors. The PCR products were then cloned into pCR 2.1 vector using a TA cloning kit (Invitrogen, San Diego, CA), and then were sequenced.

2-D gel electrophoresis

One-tenth overnight culture of the bacteria were refreshly grown in LB medium to an optical density at wavelength of 600 nm (OD_{600}) about 0.6 to 0.7. The cells were then harvested and washed by double distilled H_2O and dried by freez-drying. Approximately 5~10 mg dried becteria were resolved in 500 μl of lysis buffer (8 M urea, 4% CHAPS, 2% Phamalyte 3-10) and discrupted by sonication. After centrifuged at 13,500 rpm for 10 min to remove the insoluble fraction, concentration of the soluble proteins was determined by the Bradford assay (10). The protein samples (about 400 μg) were ydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer. 0.0 0.2% DTT), and were applied to immobilized pH 3-10 linea Gphor (Amersham Pharmacia iotech, Sweden). After isoelectric focusing, the IPG strips were incubated with 10 ml of equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT) for 15 min, and then transferred to another 10 ml of equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% idoacetamide) for 15 min. The electrophoresis in the second dimension was performed on a 12.5% polyacrylamide SDS gel using a Hoeffer Dalt system (Pharmacia Biotech) and the gel stained with Comassie Blue, and the image obtained using a Pharmacia Biotech image scanner.

In-gel digestion

Stained protein spots were excised from gels and were rinsed with distilled water. The spots were destained with a solution containing 50 mM ammonium bicarbonate and acetonitrile (1:1) for 15 min, and then the gels were transferred into another solution containing 50 mM ammonium bicarbonate and acetonitrile (3:2) for 15 min. After repeating the destain procedure, the protein was incubated in 500 μl acetonitrile to remove the water and dried using a speed vaccum concentrator. After immersed in 50 μl of 25 mM ammonium bicarbonate, the protein was digested with 3–5 μl trypsin (20 μg/l ml) at 37℃ for 12 to 16 s were then recovered using a solution containing 0.1% trif in acetonitrile. The resulting peptide extracts were proces nalysis by core facilities for proteomics and structural biology reaserch in Academia Sinica (http://proteome.sinica.edu.tw/).

PART I

Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in *Klebsiella pneumoniae* CG43 in a coordinated manner

Abstract

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*$^-$ or *kvhR*$^-$ exhibited less mucoidy, lower level of capsular polysaccharide (CPS) synthesis and higher LD_{50} than the parental strain. No apparent change of the group II, including *kvhA*$^-$ and *kvhA*$^-$*kvhR*$^-$ mutants, was observed. However, the mucoidy of *kvhA*$^-$*kvhR*$^-$ mutant was found to be diminished after introducing into a *kvhA*$^-$ expressing plasmid. V analysis, *kvhA* deletion was found to reduce *kvhR* expres f KvhA for the expression of *kvhR* was supported further b cific 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*$^-$ mutant. These suggest that the three homologous response regulators interact to control, in coordination, the bacterial *cps* expression.

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% (13, 87, 94). 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 [illegible] KvhA respectively. The results, together with the high sequen[illegible]itter domain, which is 45.8%, between KvgS and KvhS str[illegible]gS 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 (79, 90) 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*) (18). 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 (46, 62, 72). 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

Fig. 1B shows a comparative analysis of the genes near *kvhAS* with that of *E. coli* *evgAS* revealing a YfdX h acid sequence identity), a hypothetical protein for acid r Moreover, flanking both sides of *kvhAS*, homologues of putat ns HdeB and HdeD, which are positively regulated by EvgA (62), 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* (46, 73, 88) and the acid-resisting gene *yfdX* (61, 103), I 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* may have not been acquired concurrently. Subsequently, 3.3 kb DNA upstream of *kvhR* and 2 kb DNA upstream of *kvgA* 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 *kvh* nely *orfY,* encoding a putative exported lipase and a partial guingly, the 177-bp intergenic sequence between *kvgS* and *or* uence 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 (54), implied that the gene cluster had been recently acquired by horizontal transfer.

Phenotype analysis of the mutants kvgA⁻, kvhA⁻, kvhR⁻, kvgA⁻kvhA⁻, kvgA⁻kvhR⁻, kvhA⁻kvhR⁻ and kvgA⁻kvhA⁻kvhR⁻

The mutants, including AZ18 (*kvgA*⁻), AhZ01 (*kvhA*⁻), RZ01 (*kvhR*⁻), AAh01 (*kvgA*⁻*kvhA*⁻), AR01 (*kvgA*⁻*kvhR*⁻), AhR01 (*kvhA*⁻*kvhR*⁻) and AAhR01 (*kvgA*⁻*kvhA*⁻*kvhR*⁻) 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 mutar :wo groups. Group I bacteria, carrying either *kvgA* or *kvhR* : r precipitation than that of the parental strain Z01. Group I *hA*⁻ and *kvhA*⁻*kvhR*⁻ mutants, exhibit precipitation that is similar to that exhibited by the parental strain Z01 (Fig. 2A). As determined by the string test (25), 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*⁻*kvhR*⁻ mutant of group II exhibited a less mucoidy than either wild type or *kvhA*⁻ mutant of the same group. While the *kvhA*⁻*kvhR*⁻ 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*⁻*kvhR*⁻ 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 (97). The possibility that if the signal i KvhS to the orphan response regulator KvhR, remains to ess, the promoter-*lacZ* fusion constructs of *kvgAS*, *kvhAS*, a o investigate whether the three homologous regulators regulate each other. The β-galactosidase activity of P_{kvgAS} (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_{kvgAS}-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_{kvhAS} (pAh01) and P_{kvhR} (pRP05), which contained respectively a 500-bp noncoding 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 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 [illegible] upstream regulator for *kvhR* expression. The BPROM pro[illegible]rry.com) used to analyze the sequences of the P_{kvgAS}, P_{kvhAS} [illegible]fy 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 $KvgA_t$ 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 the corresponding promoter. Fig. 3A shows that $KvgA_t$ which comprises the DNA binding domain could bind to it own

promoter and that the DNA-protein interaction was specific, as the formation of the His_6-$KvgA_t$ -promoter complex could only be inhibited by the presence of the unlabelled DNA. Furthermore, the two binding complexes, C1 and C2, were observed when the amount of His_6-$KvgA_t$ 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_6-$KvgA_t$ 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 expre ᵢ by direct binding. The assay further established that His_6-K o the [γ-^{32}P]ATP-labeled P_{kvhR} and that the DNA-protein con peted away 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 glistening 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 (76). Like *E. coli* group I CPS biosynthesis, *Klebsiella* K2 *cps* expression is regulated by the 2CS RcsAB at the transcriptional level (43, 59). A CG43S3Z01-derived *rcsB*$^-$ 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 *rcsB*$^-$ 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* tor of 90 to 100 (Table 4). It is most likely that the reduction important virulence factor that affects bacterial resistance to phonuclear cells (30, 33, 43), leads to a decrease of the virulence. The *kvhA* deletion mutant, AhZ01, of group II exhibited unchanged LD_{50} 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*$^-$ mutant (Fig. 2A), the LD_{50} of *kvhA*$^-$*kvhR*$^-$ mutant appeared to be comparable to those of group I bacteria (Table 4). In addition, *kvhA*$^-$*kvhR*$^-$ mutant produced less amount of CPS than either *kvhA*$^-$ mutant or the wild type bacteria. In comparing with the *kvhR* mutant of group I, the *kvhA*$^-$*kvhR*$^-$ mutant produced more CPS, however. 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 $P_{orf1\text{-}2}$, which comprises the non-translated sequence 724-bp upstream of *orf1-2*; $P_{orf3\text{-}15}$, which comprises the non-translated sequence 890-bp upstream of the operon *o* hich comprises the 244-bp non-translated sequence upstr A). These plasmids were then transformed into wild type b ants *kvgA*$^-$, *kvhA*$^-$, *kvhR*$^-$, and *rcsB*$^-$ and also the wild type straın carryıng pAHm, a multicopy plasmid expressing *kvhA*, and the β-galactosidase activities were measured. Fig. 4B(a), (b), and (c) show that the activity of $P_{orf1\text{-}2}$, $P_{orf3\text{-}15}$, and $P_{orf16\text{-}17}$ 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 $P_{orf1\text{-}2}$ was eliminated in the *rcsB* deletion mutant [Fig. 4B(a)], which could be explained by the presence of a typical RcsAB box

5’-TAAGATTATTCTCA-3’ (96) 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 $P_{orf3\text{-}15}$ and $P_{orf16\text{-}17}$, both promoter activities were still affected by *rcsB* mutation. No apparent change for either activity of $P_{orf1\text{-}2}$ or $P_{orf3\text{-}15}$ was observed in the *kvgA* deletion strain. A comparison with the wild-type strain showed that the deletion of *kvgA* reduced $P_{orf16\text{-}17}$ 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 transcrip and ORF17, encoding ManC, GDP-mannose pyrophosphory homannomutase, respectively, have been demonstrated to l nesis of *Klebsiella* K2 sugar nucleotide precursor (4). The questıon 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 $P_{orf1\text{-}2}$, $P_{orf3\text{-}15}$, or $P_{orf16\text{-}17}$ in the *kvhA* deletion strain was indistinguishable from that in the wild type strain Z01. However, in the presence of pHAm, activity of $P_{orf1\text{-}2}$, $P_{orf3\text{-}15}$ and $P_{orf16\text{-}17}$ reduced by approximately 5.5-, 3- and 2.5-fold, which further supported the negative role of KvhA in regulation of the *cps* expression.

Regulation in coordination

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-orf16-17*. 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 sy[illegible]d in *E. coli* CPS synthesis in responding to the environment [illegible]PmrAB and the Rcs regulatory system have been shown to [illegible] *ugd* encoding UDP-glucose dehydrogenase, an enzyme required for the synthesis of polysaccharide on the coordination in *E. coli* (70). While the activity of P_{kvgAS}, P_{kvhAS} and P_{kvhR} were measured, *rcsB* deletion appeared to have no effect on the expression of either *kvgAS, kvhAS,* or *kvhR*. Moreover, no apparent change of P_{rcsB} activity was observed in either of *kvgA*, *kvhA*, and *kvhR* mutants. This suggests an independent regulation of RcsB and the three response regulators on *cps* expression in *K. pneumoniae* CG43 (Fig. 5). I and others have observed that paralogous 2CS proteins may regulate similar functions, probably at different levels (14, 74). By using mutagenesis analysis, promoter activity

measurement and EMSA, I am 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.

PART II

Regulation of the homologous two-component systems KvgAS and KvhAS in *Klebsiella pneumoniae* CG43

Abstract

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 ic binding of the recombinant KvgA to the putative promo also supported an interacting regulation between the 2CSs. ıe presence of RpoS binding elements suggested an RpoS-dependent regulatıon. 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.

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. 5A). 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 v (51) obtained using luciferase as the reporter, indicating as the sensor protein via auto-regulation to encounter 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. 6B, 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. 5B). 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}

Previous study in the laboratory has suggested that KvgA positively regulated the expression of *kvgAS* and *kvhAS* (57). A series of truncations in pA16 ($P_{kvgAS-399}$), carrying 399-bp of the *kvgAS* putative promoter containing DNA, and pAh01 ($P_{kvhAS-516}$) containing 516-bp c A, yielding pA23, pA26, pA28, and pA30, containing the n 318-bp, 196-bp and 52-bp, respectively, upstream of *kvg* 03, 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. 6A, *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 from 52-bp to 196-bp is also important for 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. 6B), helping to support the above notion that KvgA was probably involved in positive auto-regulation by direct binding to the promoter sequence. When the A fragment with an increasing amount of His_6-$KvgA_t$ from 0. inding complexes, C1 and C2, were found. This also suggest ory elements are contained in pA16. Consistent with the results of 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. 7A, 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 *kvhA* promoter. As shown in Fig. 7B, consistent with the measurements of promoter activity, recombinant $KvgA_t$ could bind each of the DNA fragments.

The MEME program (7) 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 conta ***endent promoters***

5' RACE was employed to n :art site of *kvgAS* and *kvhAS*. Sequencing of the 5' RACE pr ranscription start site of *kvgAS* initiated at nucleotide T, 55 nt upstream from the start codon (Fig. 8A), and the initiation site of *kvhAS* was at nucleotide T, 84 nt upstream from the translation start (Fig. 8B). 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 (48, 56, 95) in P_{kvgAS} and P_{kvhAS} was investigated. As shown in Fig. 8A, 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. 8B, 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 regula AS are probably diverse.

RpoS controls the expression

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. 9A), 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.9A, 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. 9A). 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 ıs, no apparent change of the activity of either pAh02 or p consensus sequence has been deleted. The results indicati ıffected 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.

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 (27, 95). As shown in Fig. 10A, 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 activity of P_{katE}. Whereas, the deletion of *kvhA* conferred no notable effect on the expression of any of the promoters (Fig. 10A).

EMSA was performed or ein, and the DNA fragment of P_{katG} or P_{sodC}, to determine wh nscriptional factor and directly interacts with the upstream reg nd *sodC*. The EMSA results in Fig. 10B indicated that $KvgA_t$ could specifically bind to the upstream region of *katG* suggesting a transcriptional control on the expression of *katG*. 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 (16, 27, 28, 38, 93, 95). 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 (6). 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 (73). Therefore, I speculate that KvhAS, as an EvgAS ortholog, also plays a role in regulating drug susceptibility. The overexpression plasmids, pHAm and pHAm$_{dHTH}$, carrying the coding sequence of KvhA and the truncated sequence which lacked the HTH domain removed, 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 5, 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.

MurA, a UDP-*N*-acetylglucosamine enolpyruvyl transferase, which catalyzes the first step of peptidoglycan synthesis, transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine (UDP-GlcNAc), has been demonstrated as the target of fosfomycin (44). 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. 11, the MurA activity of *K. pneumoniae* CG43S3 [pHAm] d higher than that of the wild type strain. Moreover, MurA a *vhA* deletion mutant. In *E. coli*, the overexpression of the 32 re d that 13 of them can increase bacterial resistance to β-lactam antibiotics (34). The reported mechanisms of resistance to β-lactam antibiotics include drug detoxification (39), decreased affinity to the target (26), and reduction of the drug permeability (71). The expression of *kvhAS* appeared to respond to an osmotic stress related 2CS (Fig. 5B), 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.

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* reduce S but increased that of *kvhAS*. Deletion of *kvgA* was shown he antioxidant defense genes *katG* and *sodC*. However, the A 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.

PART III

Isolation of the target genes under the control of KvgAS and KvhAS in *K. pneumoniae* CG43

- Isolation of the target genes under control by KvgAS in *K. pneumoniae* CG43 using promoter trapping approach
- Isolation of the KvhA regulated genes using cDNA subtractive hybridization
- Identification of differentially expressed genes between *K. pneumoniae* U9451, U9451-*kvgA*$^-$, U9451-*kvhA*$^-$, and U9451-*kvhR*$^-$ by proteome analysis

Abstract

The aim of this study is to identify the target genes of the 2CSs KvgAS and KvhAS. The different approaches were adopted. Fristly, the use of a *lacZ*-based promoter trapping system allowed us to isolate four clones of which the expressions are repressed by KvgA. Sequence analysis revealed that all four clones contain unrelated DNA fragments possibly resulted from random ligation. A promoter library containing 500~1000-bp genomic DNA of *K. pneumoniae* CG43 was subsequently constructed in placZ15 to avoid combinations of the unrelated DNA sequences. The library is being screened to search for th :ontrol of KvgAS and KvhAS. Secondly, cDNA substractive was employed to identify the differentially expressed sequ *kvhA* deletion mutant. Four KvhA-activated genes and three repressed genes were obtained. Four KvhA-activated genes encode respectively DucB, CsiD, AraC type DNA-binding domain-containing protein, and hypothetical protein. The three repressed genes code for 6-phosphofructokinase, GabA permease, and membrane-bound lytic murein transglycosylase A, respectively. Finally, via the proteome analysis, several proteins appeared to be induced or repressed in either *kvgA*, *kvhA*, or *kvhR* mutants were identified. They are thioredoxin-dependent hydroperoxide peroxidase, cytochrome C5, YtmB, GreA, OMP decarboxylase, tellurium resistance proteins TerE and TerD, 50S

ribosomal proteins L9 and L10, and YfiD. Nevertheless, how these genes are regulated by these 2CS remains elusive and more experiments need to be carried out for the regulatory role of the 2CSs in *K. pneumoniae* CG43.

Results and discussion

Isolation of the promoters that are repressed by KvgA by using a promoter trapping approach

As shown in Fig. 12, a *lacZ*-based promoter trapping system including placZ15, a plasmid containing a promoterless *lacZ* reporter, and Z01, a *K. pneumoniae* CG43 *lacZ* deletion mutant, was constructed. Subsequently, the promoter library containing a group of 300~500-bp genomic DNA of *K. pneumoniae* CG43 subcloned into placZ15 was obtained to allow the target genes expression monitored by LacZ activity respectively in Z01 and Z01 which can be determined by plating the bacteria on LB aga lue/white selection. While the plasmid conferred Z01 a blue white phenotype implying that the promoter is positively regulated by KvgA; the bacteria having a reverse phenotype indicated that the promoter activity is likely repressed by KvgA.

Through the selection, four clones of which the expressions are repressed by KvgA were obtained and their DNA sequences determined and their promoter activities measured. The respective promoter activities in *kvgA* mutant were found to increase to approximately three to six folds comparing to that of wild type (Table 6). The BLASTN analysis revealed that only one clone carried 120 bp promoter sequences of *murB*, encoding UDP-*N*-acetylenolpyruvylglucosamine reductase in

peptidoglycan biosynthesis (3, 12, 65), other are nonlinear coding sequences indicating that the promoter library is probably over-digested by *Sau*3AI. To avoid obtaining combinations of unrelated DNA sequences, a promoter library containing 500~1000-bp genomic DNA was then constructed. Twenty clones have been arbitrarily selected and the sizes of the inserts confirmed.

Identification of KvhA-regulated genes using cDNA subtractive hybridization analysis

Through the cDNA subtractive hybridization analysis, several DNA amplicons (Fig. 13) that may be regulated by KvgA were isolated. Four KvhA-activated genes and three repressed genes were obtained and the sequences determined. Through the BLASTP analysis, the four KvhA-activated genes encode respectively DucB, CsiD, ArcC type DNA-binding domain-containing protein, and hypothetical protein, and the three KvhA-repressed genes are coding for 6-phosphofructokinase, GabA permease, and membrane-bound lytic mu espectively (Table 7).

DcuB which encodes a catalyzing fumarate/succinate antiport during fumarate respi cuSR two component system (23, 83). In *E. coli*, DcuSR has been identified as a sensory system for fumarate and other C4-dicarboxylates such as succinate, malate and the C4-dicarboxylic amino acid aspartate (41). In the presence of C4-dicarboxylates, *dcuB* is transcriptionally activated by the phosphorylated DcuR (1, 40). If the expression of *ducB* is positively regulated by KvhA and the possibility of a coordinate regulation in controlling *dcuB* expression between KvhAS and DcuRS remain to be investigated.

In *E. coli*, *csiD*, an RpoS dependent gene, was identified as a starvation-inducible gene of unknown function (60). Unlike RpoS itself and many RpoS-dependent genes

that are induced in response to a variety of different stress conditions, strongly increased expression of *csiD* was observed only in response to starvation for carbon sources, such as glucose or glycerol. The *csiD* promoter is not only RpoS dependent, but also requires an activator cAMP-CRP (60, 66). Since the expression of *kvgAS* and *kvhAS* have been shown to be RpoS dependent, involvement of KvhAS in the regulation of *csiD* is very likely and awaits to be demonstrated.

6-phosphofructokinase (PFK) is a key enzyme involved in the control of glycolysis, phosphorylating fructose 6-phosphate with ATP to form fructose 1,6-bisphosphate in both pro (35); In *E. coli*, GabP (*gab* permease) is γ-aminobutyric r to catalyse translocation of GABA across the plasma n tive (37) and Gram-positive bacteria (11). GABA is a nonproteın amıno acıd that is present in a large range of organisms including bacteria, yeasts, plants, and animals (9). In plants and bacteria, GABA synthesis and degradation are also associated with biotic and abiotic stresses, including acid conditions (66) and mechanical damage or stimulation (82); Lytic transglycosylases, which catalyse cleavage of the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine, belong to the family of murein hydrolases that are involved in the maintenance of bacterial cell-wall integrity during cell elongation and division (36). It is interesting to note that these genes appear to

encode proteins involved in the metabolism of cell surface. However, how the three genes are regulated by KvhA need to be investigated.

Identification of differentially expressed genes between K. pneumoniae U9451, U9451-kvgA⁻, U9451-kvhA⁻, and U9451-kvhR⁻ by proteome analysis

As shown in Fig. 14, using 2D-PAGE, the majority of the proteins were clustered between pH 4 to 7 and their molecular weights are between 30 to 100 kDa. In order to have better resolution of the proteins, the separation using a pH 4 to 7 IPG strip should be performed. Nevertheless, the protein spots appeared to be induced or repressed were isolated from the gel, subject to trypsin digestion, and the peptides analyzed through MALDI/TOF mass spectroscopy. The obtained peptide profiles were then subject to database searching using Mascot program of ExPASY (http://www.matrixscience.com).

As shown in Table 8, the expression level in wild type strain-U9451 than each of the *kvgA*-, *kvhA*-, and *kvhR*- mutants are thioredoxin-dependent hydroperoxide peroxidase and cytochrome C5. The proteins which have higher expression levels in *kvhA* deletion mutant than in the wild type strain include YtmB, GreA, OMP decarboxylase, tellurium resistance proteins TerE and TerD, 50S ribosomal proteins L9 and L10, and YfiD.

The gene *yfiD* encodes an acetate-induced protein, which responds to decrease of intracellular pH and reduces the accumulation of acidic metabolites (102). Since the genes encoding putative acid resistance proteins HdeB, HdeD, and YfdX were found

flanking both sides of *kvhAS*, the possibility that KvhAS is involved in regulating the bacterial response to acidic stress could be speculated. However, using LacZ as a promoter reporter, no apparent change of *yfiD* promoter activity in *kvhA* mutant could be detected impling an indirect role of KvhA on the expression of *yfiD* (data not shown).

Summary

Taken together, a model is proposed for a regulatory circuit of *kvgAS*, *kvhAS*, and *kvhR* in the bacteria. As shown in Fig. 15, under an oxidative stress environment, RpoS activates the expression of the response regulator KvgA but represses the expression of KvhA.. In addition, KvgA exhibits an auto-regulatory activity as well as a positive regulation on the expression of *kvhAS*, *kvhR, cps-orf16-17*, *sodC,* and *katG*. An approximately 15% prevalence rate of *kvgAS* in of the laboratory collected clinical isolates suggested an accessory role of the 2CS in the bacterial pathogenesis. Interestingly, KvhA also appe  he expression of *kvhR* and the increasing expression of *kvhR* ɔn of K2 *cps*. Nevertheless, the regulation of *cps* expressior ɛ regulators appeared to be independent to the regulation of RcsAB. On the other hand, an overexpression of *kvhA* in turn suppressed the synthesis of K2 CPS but promoted the drug susceptibility. MltA, which is a membrane lytic murein transglycosylase A to responsible for peptidoglycan synthesis, was shown to be positively regulated by KvhA via the cDNA subtractive hybridization analysis. This suggested a role of KvhA in regulating the peptidoglycan synthesis to modulate the bacterial drug susceptibility.

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